4.01 Overview and Introduction

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4.01.1 Scope of Volume 4

The present Volume 4 is a continuation of Volume 8 of the first edition,[1] which summarizes progress in chemical ecology. Chemical ecology is an expanding new discipline in the study of chemistry and biology of semiochemicals or biofunctional molecules, which spreads information among individuals.

This volume treats pheromones (Chapters 4.04–4.06), defensive substances and toxins (Chapters 4.08–4.10), antifeedants (Chapters 4.11–4.12), compounds employed in plant-plant and plant-microbe interactions (Chapter 4.13), plant-insect interactions (Chapter 4.14) and microbe-microbe interactions (Chapter 4.07). Hormones of plants (Chapter 4.02) and insects (Chapter 4.03) are also treated in this volume. A unique attempt in the present volume is to regard flavor and fragrance (Chapter 4.15) and taste (Chapter 4.16) as phenomena of human–environmental interactions or human chemical ecology.

Consequently, all organisms including humans will be treated in the light of bioactive natural products. In other words, bioactive natural products related to biological industries including agriculture, forestry, fishery, food industry, cosmetics and personal care, and fermentation industries will be treated in this volume. The treatise, however, will be basic to include chemistry in all aspects of chemical ecology. Many of the applications are treated in Volume 3.

Prior to delving into the details, this overview briefly summarizes the recent progress made as follows: (1) progress in structure elucidation, (2) complexity of the multicomponent pheromone, (3) stereochemical aspects of chemical ecology, (4) dual roles of semiochemicals such as pheromones and kairomones, and (5) new trends in mammalian chemical ecology.

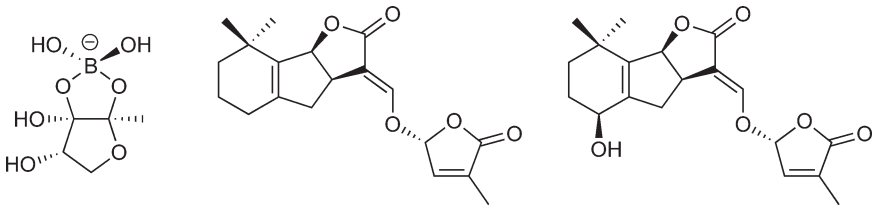
The author originally reviewed three important aspects of semiochemical research that has been discussed in Volume 9. They are as follows: (1) determination of structure including the absolute configuration of bioactive natural products, (2) problems of biological homochirality, and (3) study of structure–activity relationships to invent mimics of bioactive small molecules so as to utilize them as pesticides or medicinals.

Bergström[2,3] recently published two excellent reviews on the birth and growth of chemical ecology as an interdisciplinary field. These two reviews are recommended for those who wish to know the history of chemical ecology over the past 50 years.

4.01.2 Comments on Progress in the Chemistry and Biology of Semiochemicals

4.01.2.1 Progress in Structure Elucidation

Progress in microanalytical methods continuously improves the ability of chemists to clarify the structures of new semiochemicals. In 2002, a new and revolutionary strategy in the structure determination of a biofunctional and small molecule was reported by Hughson and coworkers.[4] It was to determine the structure of a



AI-2 **1** 5-Deoxystrigol **2** Strigol **3**

Figure 1 Structures of bioregulators of microbial or plant origins.

biofunctional molecule even without isolating the compound itself. Autoinducer-2 (AI-2) is a universal signal for interspecies communication (quorum sensing) in bacteria, which allows them to coordinate gene expression. The structure of AI-2 remained elusive until 2002, when the X-ray crystallographic analysis of AI-2 sensor protein (LuxP from bioluminescent marine bacterium *Vibrio harveyi*) in a complex with AI-2 was successfully carried out.[4] LuxP is the primary AI-2 receptor, and recombinant LuxP was overproduced in *Escherichia coli* strain BL21. Eight milligrams of LuxP-AI-2 complex was necessary for the X-ray work. As shown in **Figure 1**, the bound ligand AI-2 was a furanosyl borate diester **1**. The fact that the ligand contains boron could be confirmed by ^{11}B-NMR (nuclear magnetic resonance) analysis of LuxP–AI-2 complex.

This work demonstrates that we can determine the structure of a biofunctional molecule by isolating its complex with a receptor protein, and analyzing the structure of the complex by X-ray. In case a receptor protein is available in a sufficient amount by recombinant technology to make the complex crystalline, Hughson's strategy will be the ideal way to elucidate the structure of a scarce and elusive biofunctional ligand. In the past, isolation of a bioactive but scarce ligand and its structure elucidation was a prerequisite, which then enabled the detection and isolation of its receptor protein. In Hughson's strategy, the receptor protein was the first target, and subsequently, the structure of its ligand was clarified. This is indeed a new method – the so-called 'reversed natural products chemistry.'

The second example of the structure elucidation of a new semiochemical followed the traditional way, but on a very small scale to isolate 18 μg of target molecule **2**, a factor inducing hyphal branching in arbuscular mycorrhizal fungus *Gigaspora margarita*.[5] Arbuscular mycorrhizal fungi form symbiotic associations with the roots of more than 80% of land plants. Host roots release a semiochemical that triggers hyphal branching. In 2005, Akiyama *et al.*[5] isolated 5-deoxystrigol **2** (18 μg) from the root exudates of *Lotus japonica*, and determined its structure by spectral [UV (ultraviolet), IR (infrared), ^{1}H-NMR, EI-MS (mass spectrometry), and CD (circular dichroism)] analysis and synthesis of (±)-**2**. They used activated charcoal to recover **2** from the hydroponic solution of *L. japonica*. 5-Deoxystrigol **2** elicited hyphal branching of *G. margarita* at concentrations ranging from 1 ng to 1 pg per disc. A related compound strigol **3** had been known since 1966 as a germination stimulant for the parasitic witchweed, *Striga lutea*.

Through Akiyama's work, strigolactones such as **2** and **3** were shown to be bioactive in two different phenomena in the plant kingdom, germination of parasitic weeds and hyphal branching in arbuscular mycorrhizal fungi. Perhaps, the parasitic weeds used the plant's signals for symbiosis with fungi to find out their hosts for parasitism.

4.01.2.2 Complexity of the Multicomponent Pheromone

In 1959 when the first pheromone bombykol [(10*E*,12*Z*)-10,12-hexadecadien-1-ol] was identified as the female-produced sex attractant of the silkworm moth *Bombyx mori*, the pheromonal activity was thought to be totally due to that single compound.[6] At present, it is generally believed that a pheromone is composed of many pheromone components (see Chapter 4.04), which resembles the present concept in flavor and fragrance chemistry, that is, to consider a mixture as a whole to be responsible for a particular sense of smell (see Chapter 4.15).

Recently in 2008, Lacey *et al.*[7] reported a typical example showing the complexity of a multicomponent pheromone. A male-produced aggregation pheromone of the cerambycid beetle *Megacyllene caryae* contained as

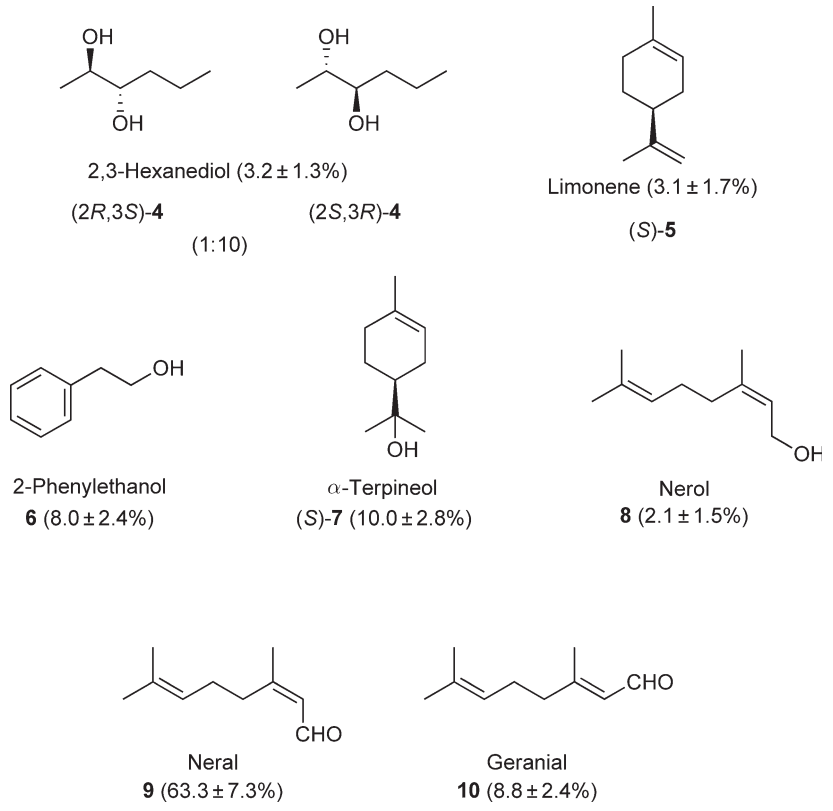


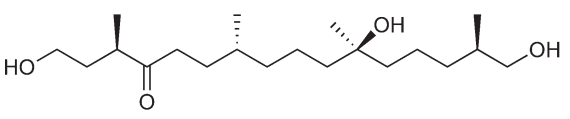
Figure 2 Components of the male-produced aggregation pheromone of the cerambycid beetle *Megacyllene caryae*. Percent compositions are indicated within parentheses.

many as eight male-specific compounds as shown in **Figure 2**. They are (2*R*,3*S*)-2,3-hexanediol **4**, its enantiomer (2*S*,3*R*)-2,3-hexanediol **4**, (*S*)-limonene **5**, 2-phenylethanol **6**, (*S*)-α-terpineol **7**, nerol **8**, neral **9**, and geranial **10**.[7] None of these compounds was attractive as a single component. Both sexes of *M. caryae* were attracted to the complete blend of these eight compounds, but the elimination of any one of them resulted in a decreased trap capture. Blends that were missing such as (2*S*,3*R*)-**4**, (2*R*,3*S*)-**4**, or a mixture of **9** and **10** (1:1) were pheromonally inactive. Modern studies on semiochemicals revealed the importance of a proper mixture to evoke a biological reaction.

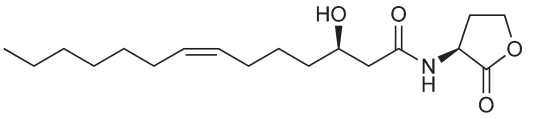
4.01.2.3 Progress in Unraveling the Stereochemical Aspects of Chemical Ecology

The author's overview in Volume 8 of CONAP (first edition) briefly treated the relationship between stereochemistry and pheromone activity among insects.[1] The significance of chirality in pheromone science was also discussed in depth by the author in 2007.[8] Further examples were reported to show the importance of chirality in chemical communications among microorganisms[9,10] and mammals.[11] The microbial cases will be summarized in this section.

Fungi in the genus *Phytophthora* are destructive phytopathogens, and caused the well-known Irish potato famine in 1840s. A factor known as hormone α1 is secreted by the A1 mating type of *Phytophthora nicotianae*, and induces the formation of sexual spores in the A2 mating type. By isolating 1.2 mg of the hormone α1 from 1830 l of culture broth of *P. nicotianae*, its structure was proposed as (15*R*)-**11** shown in **Figure 3**. The configurations at the other three stereogenic centers remained obscure. Interestingly, **11** induces sexual spore formation in the A2 mating types of several other species in the genus *Phytophthora*, and therefore **11** is a universal mating signal in the heterothallic species of *Phytophthora*. In order to determine the absolute configuration of hormone α1, Yajima *et al.*[9] synthesized and bioassayed various stereoisomers of **11**. The only bioactive stereoisomer at a dose of 10 ng was (3*R*,7*R*,11*R*,15*R*)-**11**, which must therefore be the natural hormone α1.



Hormone α1 of *Phytophthora*
(3*R*,7*R*,11*R*,15*R*)-**11**



Small bacteriocin
(2*S*,3′*R*,7′*Z*)-**12**

Figure 3 Structures of microbial semiochemicals.

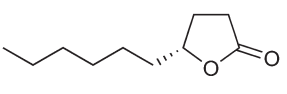
N-Acyl homoserine lactones (AHLs) are important pheromones controlling quorum sensing among Gram-negative bacteria. An AHL called *small* bacteriocin possesses the structure (2*S*,3′*R*,7′*Z*)-**12** as shown in **Figure 3**, and is the quorum-sensing pheromone of a nitrogen fixer *Rhizobium leguminosarum* living in symbiosis with leguminous plants. Yajima's synthesis of **12** and its stereoisomers enabled the examination of the stereochemistry–bioactivity relationships in the case of *small* bacteriocin.[10] The natural (2*S*,3′*R*,7′*Z*)-**12** exhibited the greatest bioactivity, whereas the other three stereoisomers showed 5–500 times weaker bioactivity. Chirality was shown to be also important for chemical communications among bacteria.

4.01.2.4 Dual Roles of Semiochemicals as Pheromones and Kairomones

It has been believed that pheromones act only as pheromones of a certain species. Recent studies have revealed that pheromones act also as kairomones for the predators against the pheromone releasers. Two examples are given below.

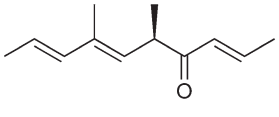
The scarab beetle *Osmoderma eremita* and its larval predator, the click beetle *Elater ferrugineus*, are known as indicators of the species richness of insect fauna of hollow deciduous trees in Northern Europe. (*R*)-4-Decanolide **13** (**Figure 4**) is the male-produced sex pheromone of *O. eremita*.[12] Lactone **13** is also employed by *E. ferrugineus* as a kairomone for the detection of tree hollows containing the larvae of *O. eremita*.[12]

Dunkelblum and coworkers,[13,14] in association with the author's group, studied both the pheromonal and kairomonal activities of three female-produced sex pheromones **14**–**16** of the pine bast scales of *Matsucoccus* species together with their analogues **17** and **18**. The Israeli pine bast scale, *M. josephi*, employs (2*E*,5*R*,6*E*,8*E*)-**14** as its sex pheromone, whereas (3*S*,7*R*,8*E*,10*E*)-**15** is used by *M. feytaudi* and (2*E*,4*E*,6*R*,10*R*)-**16** by *M. matsumurae*. The pheromone **14** was found to be a potent kairomone for *Elatophilus hebraicus* adults, the



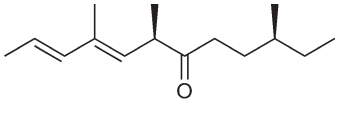
(*R*)-**13**

Pheromone of the scarab beetle *Osmoderma eremita* and kairomone for its predator *Elater ferrugineus*



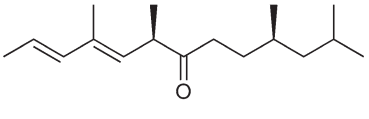
(2*E*,5*R*,6*E*,8*E*)-**14**

Pheromone of the Israeli pine scale *Matsucoccus josephi* and kairomone for its predator *Elatophilus hebraicus*



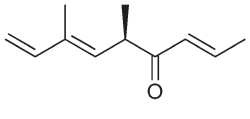
(3*S*,7*R*,8*E*,10*E*)-**15**

Pheromone of the maritime pine scale *M. feytaudi* with kairomonal activities for *E. hebraicus*, *E. crassicornis*, *E. nigricornis*, and *Hemerobius stigma*



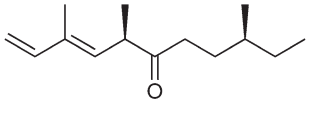
(2*E*,4*E*,6*R*,10*R*)-**16**

Pheromone of the Japanese pine scale *M. matsumurae* with kairomonal activity for *E. hebraicus*



(2*E*,5*R*,6*E*)-**17**

Pheromone mimic for *M. josephi* with no kairomonal activity for *E. hebraicus*



(3*E*,5*R*,9*S*)-**18**

Pheromone mimic for *M. feytaudi* with no kairomonal activity for *E. crassicornis* and *H. stigma*

Figure 4 Structures of insect pheromones and their mimics to illustrate the pheromone–kairomone relationship.

major predatory bug against *M. josephi*. Strangely enough, the Israeli predator *E. hebraicus* was also attracted by **15** and **16**, although both *M. feytaudi* and *M. matsumurae* are absent in Israel. It seems as if the presence of the diene moiety of **14–16** is sufficient to attract the adult *E. hebraicus*. The kairomonal activity of **14–16** is a general phenomenon, and *M. feytaudi* pheromone attracts predators such as *Elatophilus crassicornis* and *Hemerobius stigma* in Portugal and *E. nigricornis* and *H. stigma* in Italy and France.

The pheromone analogues **17** and **18** showed interesting biological properties. The nor-analogue **17** of *M. josephi* pheromone **14** preserved the pheromonal activity but eliminated its kairomonal activity.[13] Similarly, the removal of the terminal methyl group from the diene moiety of **15**, producing the nor-analogue **18**, again preserved its pheromonal activity but eliminated the kairomonal activity.[14] Thus, subtle and designed alterations in the structure of the diene system change the mode of the kairomonal activity markedly. The two mimics **17** and **18** may be useful to capture only the pest pine bast scales without disturbing their predators.

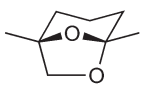
4.01.2.5 New Trends in Mammalian Chemical Ecology

As the science of insect pheromones has fully developed, studies on the pheromones of mammals and aquatic organisms are now gaining popularity among scientists. Two examples involving acetal pheromones in mammals are discussed. As can be seen in the pheromone chapter of Francke and Schulz, acetals have been known originally as aggregation pheromones of bark beetles (Chapter 4.04).

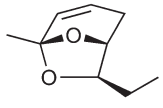
According to Rasmussen's recent study, the male Asian elephants, *Elephas maximus*, release frontalin **19** (**Figure 5**) from the temporal gland on the face during musth, which is an annual period of sexual activity and aggression.[11] The ratio of frontalin enantiomers enables other elephants to distinguish both the maturity of male elephants in musth and the phase of musth. In young males, significantly more (1*R*,5*S*)-(+)-frontalin **19** than (1*S*,5*R*)-(−)-frontalin **19** is released. As the elephant reaches maturity, the ratio becomes almost equal to emit (±)-frontalin. Musth periods get longer as males age. Secretions containing high concentration of frontalin **19** at racemic ratios attracted follicular-phase females, whereas the secretions repulsed males as well as luteal phase and pregnant females. The importance of the enantiomeric composition of frontalin **19** in the behavior of Asian elephants was observed only after the advent of enantioselective (chiral) gas chromatography (GC). It should be noted that bark beetles employ (1*S*,5*R*)-(−)-frontalin **19** as their pheromone component.

Genes in the major histocompatibility complex (MHC), known for their role in immune recognition and transplantation success, are involved in modulating mate choice in mice and perhaps also in humans.[15] Volatile body odors of mice are regulated by MHC genes, and it is these odor differences that underline mate choice and familial recognition. An individual's olfactory identity is coded in part by a pattern of volatile semiochemicals, which is regulated by genes in MHC.[16] In this connection, the effects of mice pheromone components on the attractiveness of a male mouse evolved as an interesting research topic. For this study, previously synthesized mice pheromone components **20** and **21**[17] as well as newly synthesized ones **22** and **23**[18] were employed.

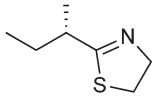
In many wild animals, older males are often preferred by females, because they carry 'good' genes that account for their viability. In the case of mice, *Mus musculus*, higher levels of (1*R*,5*S*,7*R*)-dehydro-*exo*-brevicomin **20**



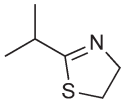
(1*S*,5*R*)-Frontalin **19**



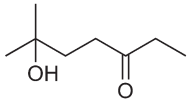
(1*R*,5*S*,7*R*)-Dehydro-*exo*-brevicomin **20**



(*S*)-2-*sec*-Butyl-4,5-dihydrothiazole **21**



2-Isopropyl-4,5-dihydrothiazole **22**



6-Hydroxy-6-methyl-3-heptanone **23**

Figure 5 Structures of pheromone components of male Asian elephants and mice.

(*exo*-brevicomin is a bark beetle pheromone) and (*S*)-2-*sec*-butyl-4,5-dihydrothiazole **21** and 2-isopropyl-4,5-dihydrothiazole **22** were detected in the urine of aged male mice than in normal adult males, whereas a lower level of 6-hydroxy-6-methyl-3-heptanone **23** was observed.[19] When **20**–**22** were added to the urine of normal adult males, their urine showed an enhanced attractiveness against female mice. The addition of **23** had no effect at all. Accordingly, it is established in the case of mice that semiochemicals control the mate selection process.[19] A search to understand the role of semiochemicals in higher animals including humans will continue to be an interesting area of chemical ecology with potential impact on perfume industries.

4.01.3 Future Perspectives in Chemical Ecology

In order to preserve our global ecological system, we require an in-depth knowledge in chemical ecology to understand more about the role of bioactive natural products in the environment. This will enable us to resolve many problems that remain unraveled. Chemical ecology is an interdisciplinary science between chemistry and biology. No one can be an expert in both the areas unless one wants to remain superficial. Thus, this calls us to remember the following words of the Apostle Paul: "The person who thinks he knows something really does not know as he ought to know (1 Corinthians 8:2)." This sentence was also contained in Volume 9 Chapter 5.

Acknowledgments

I wish to thank Dr. T. Tashiro for his help.

Glossary

kairomone A kairomone is a chemical substance produced and released by a living organism that benefits the receiver and disadvantages the donor. The kairomone improves the fitness of the recipient and in this respect differs from an allomone.

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Biographical Sketch

Kenji Mori was born in 1935 and has spent a total of 42 years at the University of Tokyo. He obtained a B.Sc. degree in agricultural chemistry (1957), followed by an M.Sc. in biochemistry (1959), and Ph.D. in organic chemistry (1962). He was then appointed assistant (1962), associate professor (1968), and served as a professor of organic chemistry (1978) at the University of Tokyo until 1995. He is now professor emeritus. Dr. Mori worked for 7 years (1995–2001) as a professor at the Science University of Tokyo. At present, he is a research consultant at RIKEN (Institute of Physical and Chemical Research) and at Toyo Gosei Co., Ltd. He was awarded the Japan Academy Prize (1981), the Silver Medal of the International Society of Chemical Ecology (1996), the American Chemical Society's Ernest Guenther Award in the Chemistry of Natural Products (1999), the Special Prize of the Society of Synthetic Organic Chemistry, Japan (2003), and the Frantisek Sorm Memorial Medal of the Academy of the Czech Republic (2003).

4.02 Plant Hormones

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4.02.1 Introduction

The definition of a plant hormone has not been clearly established, so the compounds classified as plant hormones often vary depending on which definition is considered. In this chapter, auxins, gibberellins (GAs), cytokinins, abscisic acid, brassinosteroids (BRs), jasmonic acid-related compounds, and ethylene are described as established plant hormones, while polyamines and phenolic compounds are not included, according to the definition used in the previous edition of the book *Comprehensive Natural Products Chemistry*.[1] On the other hand, several peptides that have been proven to play a clear physiological role(s) in plant growth and development, similar to the established plant hormones, are referred to in this chapter (see Section 4.02.9), and strigolactone, which was recently claimed to be included in the members of plant hormones,[2,3] is also described in Section 4.02.10. Research on plant hormones can be traced to the comprehensive review and studies published as the 'Power of Movement of Plants'[4] written by Darwin, and published in 1892. However, it was in the 1930s that the active principles later designated plant hormones were characterized, and it was after the 1950s that the term 'plant hormone' became popular.

The word 'hormone' was originally used in a narrow sense for the secretory substances of mammals that were produced in special organs, glands, tissues, or cells, were translocated through veins (or similar tissues) to more or less specific tissues, and exerted some influence on the metabolism in the target tissues, as described in the previous edition of the book *Comprehensive Natural Products Chemistry*.[1] Today, however, the term is universally used for mobile signals of living organisms and is often used with a heading to show the living species, namely animal hormones, plant hormones, insect hormones, etc. The hormones of each living species are often characteristic of that species, reflecting the characteristics of its life phenomena.

The characteristics of plant hormones can be related, at least partially, to the peculiar growth phenomena known as developmental plasticity, where a plant keeps forming new organs and tissues throughout its life cycle. New growth is frequently associated with sites where plant hormones are synthesized and this can be summarized in the following points.

1. The number of known plant hormones is fewer than the number of mammalian hormones. The number of plant hormones has increased since the publication of the previous edition of the book *Comprehensive Natural Products Chemistry* in 1998; BRs are added to the plant hormone group, and the group of jasmonic acid-related compounds, a group of peptides, and strigolactone are also included.
2. Plant hormones appear to be ubiquitous, that is, they are present in all higher plants (and many are also present in lower plants, and even in fungi and bacteria). This universality of plant hormones is remarkable.
3. The effects of plant hormones are quite complex, one plant hormone often having a wide range of effects. However, the various phenomena observed when a plant hormone is applied do not always establish that the plant hormone is directly causing the effect in untreated plants. Some effects of a plant hormone may be direct while others are indirect, with the site of action being quite remote from the location of the end effect. Additionally, there is evidence for rather complex interactions among the various plant hormones.

Although plants do not have a nervous system, they must perceive environmental signals and use them for regulation of growth and organ development. The response systems in plants do not involve a biological architecture similar to a nervous system; nevertheless, they employ small molecule signals with remarkable structural similarities to the neurotransmitters. The basic program for growth and development is recorded in

the genome and modified by environmental signals. This modification by the environment yielding developmental plasticity, as reflected in changes in plant growth and development, is one of the clear differences from mammalian hormones, and plant hormones are considered to act as mediators. Light, temperature, and moisture are particular environmental signals, and these affect the biosynthesis, catabolism, and translocation of plant hormones. Sensitivity to plant hormones is also likely to be affected by environmental conditions. For example, the effect of GA on shoot elongation is greater under a low light intensity than under a high light intensity.

Although progress in plant hormone research had often traditionally trailed that of mammalian hormones, progress during the last decade has been particularly noticeable. The biosynthetic pathways of most plant hormones have been determined, and the genes encoding many of the enzymes that catalyze the biosynthetic steps have been cloned. However, the exact steps in the biosynthesis of auxin (indole-3-acetic acid) still remain obscure in part because of the apparent existence of redundant pathways for its production. The progress in molecular biology has enabled the relationship between the phenomena to be determined, and the responsible genes and the mechanisms regulating their expression have been extensively studied. The microarray technique enables the genes responsive to each plant hormone to be identified and the signal transduction pathways of the plant hormones to be clarified. Recent extensive studies have elucidated significant and clear examples of the cross talk occurring between plant hormones. For example, numerous publications can be found in the literature when it is searched with the keywords cross talk and the name of each plant hormone in PubMed. Some of these interactions are referred to in this chapter.

Among the most noticeable findings from the research on plant hormones, the identification of receptors and receptor genes must be emphasized. In the last decade, since the publication of the previous edition of the book *Comprehensive Natural Products Chemistry*,[5] the receptors and the receptor genes for ethylene, BRs, cytokinins, auxins, GAs, and abscisic acid have been identified, as described in the section for each plant hormone.

The previous edition of the book *Comprehensive Natural Products Chemistry*[5] describes how plant hormones were discovered by a wide range of interesting approaches, and this is introduced in the section for each plant hormone. Progress in molecular biology and the methodology used have introduced new approaches for identifying a new class of plant hormones, as described in Section 8.02.9. Briefly, the complete genomes of model plants such as *Arabidopsis* and rice have been determined and are available in public databases, and the existence of many orphan receptors has been suggested. Surveys of the ligands for such orphan receptors led to the discovery of MCLV3 and tracheary element differentiation inhibitory factor (TDIF (CLE4/44)).[6,7] It would not be an exaggeration that many new plant hormones can be predicted to be discovered through these new approaches.

Since Chailakhyan[8] postulated the 'florigen' concept, the plant hormone that regulates the floral bud induction has attracted the interests of scientists: 'florigen' has, however, remained elusive, although extensive information has been accumulated in the last decade. Four promotive pathways are now proposed for floral initiation based on the studies using *Arabidopsis*: the 'photoperiodic,' 'autonomous,' 'vernalization,' and 'gibberellin' pathways. These promotive pathways all converge on the 'integrator' genes SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) and FLOWERING LOCUS T (FT).[9,10] Review articles on recent progress in flowering are available,[11,12] and the role of GA in flowering is referred to in Section 4.02.3.4.3.

4.02.2 Auxins

4.02.2.1 Introduction

Auxins function as key regulators at the intersection of developmental and environmental events and the response pathways that they trigger. Naturally occurring members of this hormone group include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 4-chloro-indole-3-acetic acid (4-Cl-IAA). Auxin levels vary dramatically within the plant body and throughout the life cycle of the plant, forming complex gradients that appear to be a central component of its regulatory activity for plant development.[13–17] Accordingly, plants have evolved intricate networks with adaptive plasticity as well as genetic and biochemical redundancy to regulate auxin levels in specific tissues in response to changing environmental and developmental conditions.

Our knowledge about auxin has progressed markedly in many areas over the last several years. Accordingly, this chapter will focus primarily on the more recent discoveries and their impact on our current understanding.

In some cases, however, it is critical to place recent work in a proper historical context and we have tried to do this within the confines of the space permitted. Readers are referred to four recent reviews that cover aspects of auxin biology that may prove helpful.[18–21]

4.02.2.2 Chemistry

IAA is a B-excessive hetero-aromatic organic acid, consisting of an indole ring with a weak net positive charge and an acetic acid side chain. The acetic acid–indole bond at the third position of the indole ring in IAA is freely rotating, with the carboxyl group exhibiting a strong negative charge at neutral pH.[22] IAA has a UV absorption spectrum characteristic of substituted indoles, with a strong maximum at 220 nm ($E_{220} = 33\,200$) and a characteristic of three overlapping peaks with maxima at 274, 282, and 288 nm ($E_{282} = 6060$).

Auxins are defined as organic substances that promote cell elongation when applied in low concentrations to plant tissue segments in a bioassay. By this definition, there are several other native auxins that have been reported to occur in plants in addition to the most often studied auxin, IAA. These include the halogen-substituted 4-Cl-IAA,[23] as well as phenylacetic acid and indole-3-butyric acid.[24] All native auxins are found *in planta* as both free acids and conjugated forms through ester or amide linkages. IAA, the auxin most extensively studied, will be the focus of this chapter.

4.02.2.2.1 IAA conjugates in plants

IAA conjugates identified from plants can be esters or acyl anhydrides with simple sugars or cyclitols, esters with larger molecular weight polysaccharides, or polysaccharide moieties of glycoproteins. IAA can also be found conjugated by amide linkage to amino acids such as aspartate or to peptides or proteins.

4.02.2.2.2 IAA peptide conjugates

Several early studies had indicated that protein-like compounds were formed by plants from supplied radioactive IAA, although they were generally not well characterized. Native higher molecular weight amide conjugates isolated from plants include a hydrophobic 3.6 kDa peptide[25] and a 35 kDa protein[26] from bean as well as several proteins from *Arabidopsis*. The gene for the major bean IAA protein (*iap1*) was isolated and cloned.[26] The IAP1 protein was produced in transformed *Arabidopsis* and *Medicago*, but the expressed protein was not modified with IAA in either plant species, indicating selectivity in its use by specific plant species.[27]

There are also immunological data from several other species[26,28] pointing to peptide conjugates, and a class of maize zein proteins was shown to contain an indole-acyl modification.[29] Immunological and analytical studies suggested that these modifications may be a feature common to many plants. For example, previous studies had shown that *Arabidopsis* has both ester- and amide-conjugated IAA;[30] however, in total, the low-molecular-weight conjugates identified did not account for the bulk of the conjugate pool, which could be accounted for by the presence of IAA.[20]

A common feature of these studies was that they were conducted on seed tissues and the IAA-modified proteins had the characteristics of storage proteins or late embryogenesis abundant proteins. Thus, from these results, it was difficult to assign a specific function to the IAA proteins beyond a possible storage role for the phytohormone. More recently, this picture has changed somewhat with the isolation of an IAA protein from rapidly expanding strawberry fruit.[31] Based on sequence homology, this IAA protein appears to be an ATP synthase, the first IAA protein that has an apparent enzymatic function.

4.02.2.2.3 Amino acid conjugates

The formation of IAA conjugates is widely believed to be a means for removal of excess IAA produced during certain times of plant development and also in mutant plants where indolic precursors and IAA metabolites accumulate.[32] In all higher and many lower plants, applied IAA is rapidly conjugated to form IAA–aspartate.[33] The ability of plant tissues to make aspartate conjugates of a variety of active auxins is induced by pretreatment with auxin,[34] and this induction was shown to be blocked by inhibitors of RNA and protein synthesis. After almost 50 years of study, an *in vitro* system from plants was described that accounts for the formation of IAA amide conjugates[35] via a mechanism where the acidic auxin is adenylated followed by acyl transfer to the amino acid. The gene for this reaction had been discovered almost 20 years before, when *GH3* from soybean was shown

to be rapidly induced following auxin treatment.[36] Several IAA–amino acid conjugates (e.g., IAA–aspartate, IAA–glutamate, IAA–alanine) have been identified in untreated plants and plant cell cultures[37,38] and it appears that several of these could be formed by similar mechanisms.

4.02.2.2.4 Amide conjugate hydrolysis

In vitro hydrolysis of amide IAA conjugates was shown with applied conjugates,[39] and a specific conjugate hydrolase was induced in Chinese cabbage with *Plasmodiophora brassicae* infection.[40] Auxin conjugates have also been used as 'slow-release' forms of IAA in plant tissue cultures.[41] A genetic analysis of IAA conjugate hydrolysis in plants identified the gene *ILR1* coding for a hydrolase of IAA conjugates.[42,43] The enzyme hydrolyzed IAA–leucine and IAA–phenylalanine and *ILR1* was shown to be representative of a gene family whose members exhibit varying substrate specificities.[44] *IAR3* hydrolyzed IAA–L-alanine, while *ILL2* was promiscuous in its substrate requirements.[45] Analysis of triple mutants (*ilr1*, *iar3*, and *ill2*) revealed that lateral shoot number, hypocotyl elongation, auxin sensitivity, and IAA levels were altered, suggesting that conjugate hydrolysis is involved in the regulation of such processes *in planta*.[46]

Enzymes from bacterial sources catalyzing the selective hydrolysis of IAA–amino acid conjugates have been described as well. IAA–L-aspartate hydrolase was cloned from *Enterobacter agglomerans*[47] and IAA–L-alanine hydrolase was reported from *Arthrobacter ilicis*.[48] Additional characterization at the level of the gene and analysis of the enzyme protein revealed that the IAA–aspartate hydrolase was related to other bacterial amidohydrolases and with homology to the *ILR1* gene of *Arabidopsis*.[49] Transformation into *Arabidopsis* showed a mild high-auxin phenotype.[30] Subsequent analyses revealed that two zinc-binding histidine residues conserved in bacterial and plant enzymes, His-404 and His-405, were critical for the determination of substrate specificity and activity, respectively.[50]

4.02.2.2.5 Ester conjugates

The ester conjugates of maize endosperm have been extensively studied and reviewed.[33] A higher molecular weight maize ester cellulosic glucan is predominate, with IAA–glucose, IAA–*myo*-inositol, and IAA–inositol glycosides accounting for the remainder. The *in vitro* synthesis of 1-*O*-β-D-IAA–glucose from IAA and UDP-glucose has been described[51–53] and other conjugate-synthesizing reactions of *Zea mays* have also been studied *in vitro*.[54–57] In maize, IAA–glucose is transacylated to *myo*-inositol to form IAA–*myo*-inositol.[55,58] The bond energy of the acyl alkyl acetal bond between IAA and the aldehydic oxygen of glucose is approximately 1.4 kcal above that of the bond between glucose and UDP.[52] Because the formation of IAA–glucose is energetically unfavorable, the conjugate formation reaction is driven by the energetically favored second step of transacylation of the IAA moiety.[52,59] The product of these two steps, IAA–inositol, may then be glycosylated to form IAA–*myo*-inositol–galactoside or IAA–*myo*-inositol–arabinoside by reactions with the appropriate uridine diphosphosugar.[55] The enzyme catalyzing the synthesis of 1-*O*-IAA–glucose, IAA–glucose synthetase, has been purified to homogeneity, characterized, and cloned.[51,52,59] Enzymatic activity and protein levels of IAA–glucose synthase are increased following auxin treatment of maize coleoptiles.[60] Isolation of the *Arabidopsis* gene encoding an IAA–glucose-forming enzyme[61] as well as the availability of the maize gene has resulted in several groups generating transgenic plants with elevated and/or reduced capacity for IAA–glucose formation. The effect of the transgene likely depends on the availability of both excess UDP-glucose and possibly a suitable transacylation receptor in the transformed plant. The use of amino acids or other amines as alternative substrates for transacylations from IAA–glucose or, as recently proposed,[62] IAA–peptides or IAA–proteins could result from such an acyl transfer mechanism. IAA–glucose synthase antisense tomato seedlings showed reduced levels of IAA–glucose and ester IAA pools, but no corresponding alteration in the levels of amide conjugates,[48] suggesting that ester and amide conjugation are not linked via a common acyl transfer intermediate of IAA–glucose, and that amino acids may not, in this instance, serve as suitable indole-acyl acceptors. Unexpectedly, the levels of free IAA either remain unaffected by the level of expression of enzymes for IAA–glucose formation[63] or increase or decrease in parallel with IAA–glucose changes.[61,63]

The cofractionation of IAA–glucose synthetase and two enzymes for IAA–glucose hydrolysis, 1-*O*-IAA–glucose hydrolase and 6-*O*-IAA–glucose hydrolase, suggested that these enzymes exist as a hormone-metabolizing complex.[56,57,64] A bifunctional indole-3-acetyl transferase catalyzes the synthesis and hydrolysis of indole-3-acetyl-*myo*-inositol in the immature endosperm of *Z. mays*.[65]

4.02.2.3 Biosynthesis and Metabolism

It appears that an early evolutionary adaptation in plants was the ability to regulate levels of IAA via the interplay of both tryptophan-dependent and tryptophan-independent pathways (**Figure 1**) for IAA biosynthesis.[66] The use of individual pathways can differ according to a plant's developmental stage and in response to various environmental stimuli.[67–69] Tryptophan-dependent IAA biosynthesis is thought to occur via

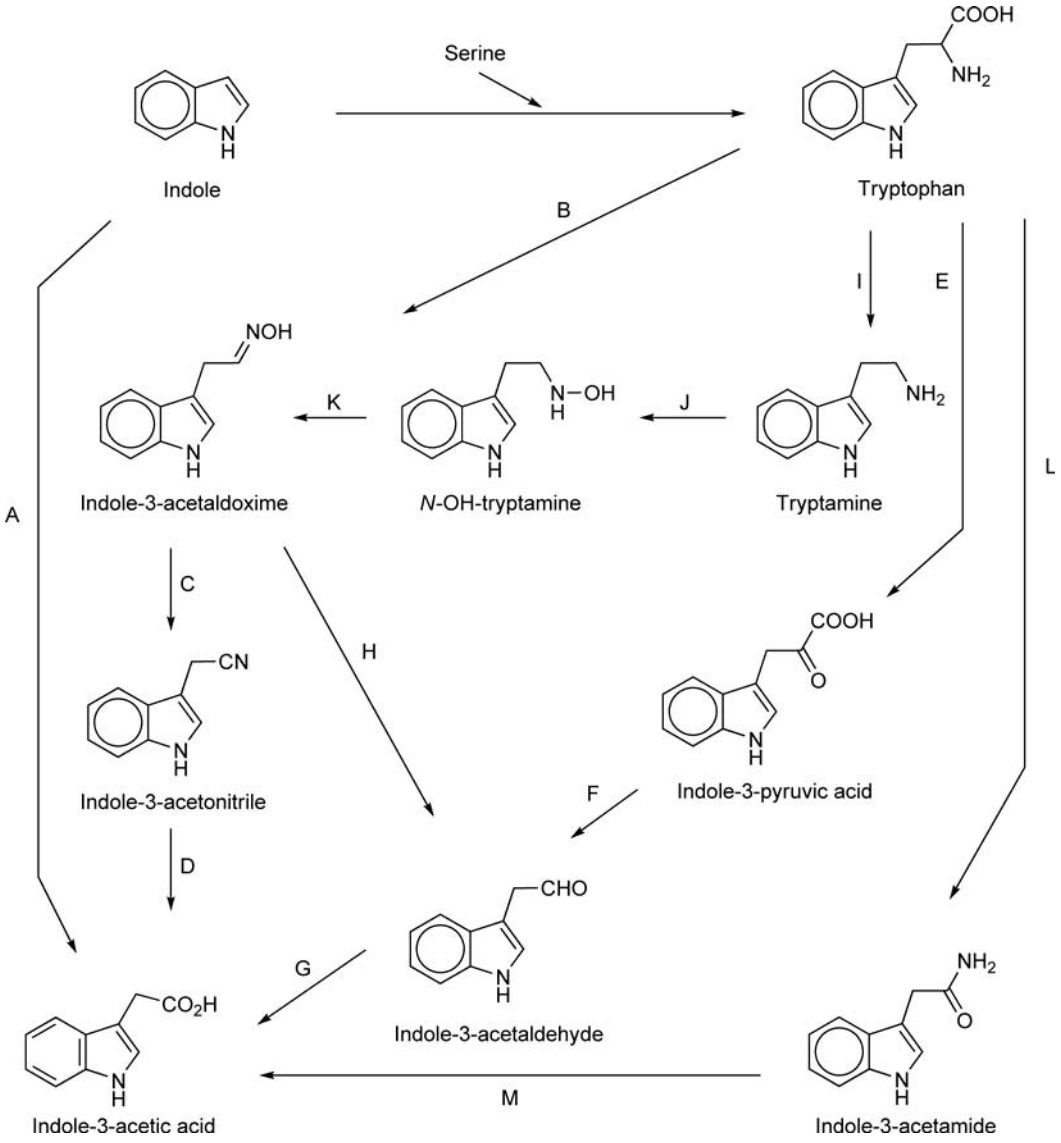


Figure 1 Biosynthetic pathways that have been proposed for the formation of indole-3-acetic acid. The tryptophan-independent pathway, A, is shown from indole, although indole-3-glycerol phosphate and indole are freely interconvertible and the exact branch point has yet to be precisely determined. Reactions B, C, and D (*NIT1–4*, *ZmNit1, ZmNit2*; nitrilases) define the indole-3-acetonitrile pathway. Reaction sequences E, F, and G (*SUR1*; IAAld oxidase) or B, H, and G are characteristic of the IAAld pathway. The indole-3-acetaldoxime pathway would include B (*CYP79B2 CYP79B3*; cytochrome P450s), C, and D or B, H, and G. Although various routes using tryptamine have been proposed over the years, the route defined by *yucca*, I, J, K and either C and D or H and G, has attracted the most attention recently. The indole-3-pyruvate pathway, E, F, and G, has received much attention recently with the description of the *sav3* and *tir2* mutants that appear to block reaction E. The conversion of tryptophan to indole-3-acetamide and its conversion to IAA, reactions L and M, were previously thought to be only microbial, but evidence for plant conversions has also emerged.

deamination and decarboxylation steps without cleavage of the 3″3′ bond.[70] Although a number of individual genes or enzymes have been implicated in the process, the exact reaction sequence, as well as the totality of the genes and enzymes associated, remains largely unknown,[21] and for tryptophan-independent biosynthesis the reaction leading to the addition of the 2-carbon side chain has not been elucidated.[71–73]

4.02.2.3.1 Tryptophan-independent biosynthesis

Since the first studies conducted in the 1940s,[74] the biosynthesis of IAA focused on the degradation of tryptophan,[38] although IAA biosynthesis pathways that did not directly involve tryptophan had been proposed in early studies dating back to the 1950s. However, a series of studies using stable isotopic tracers, backed up by tryptophan biosynthesis mutant plant lines, changed this view.[69] The first studies showed, using metabolic flux analysis, that tryptophan could not be the only precursor of IAA in *Lemna gibba* since the rate of labeling of the IAA pool from tryptophan was too slow to account for the needs of the plant.[75] Computational arguments were soon complemented by genetic analyses when tryptophan biosynthesis mutants became available. The first such mutant, *orange pericarp* (*orp*) of maize[76] – a true tryptophan auxotroph, was examined using the stable isotope approach and D_2O labeling to show IAA was made *de novo* in plants unable to make tryptophan.[32] A detailed labeling study of carrot cells in culture showed that IAA was made *de novo* and was labeled by D_2O and labeled precursors to the same extent with or without 2,4-D (2,4-dichlorophenoxyacetic acid) in the medium. 2,4-D, which induces embryogenesis, changes the labeling of IAA from tryptophan dramatically without changing the efficacy of labeling from indole or D_2O.[67] A series of *Arabidopsis* mutants in specific steps in tryptophan biosynthesis allowed a more detailed analysis than was possible in the maize *orp*.[71,73] The presence of a tryptophan-independent pathway to IAA biosynthesis was confirmed using an *in vitro* system obtained from maize seedlings[72] where indole but not tryptophan yielded IAA.

The choice of biosynthetic pathways has been shown to be controlled both temporally and spatially.[37] Tryptophan-independent biosynthesis appears to be important for providing a morphogenic signal as in later stages of embryogenesis when fine control over low levels of IAA may be critical for the development of a polar axis.[74] Tryptophan-dependent biosynthesis seems to occur when higher levels of IAA are required, as at fertilization and in wounding responses.[78–80] In tomato, the IAA biosynthesis pathway changes during fruit development, switching from the tryptophan-dependent to a tryptophan-independent pathway between the mature-green and red-ripe stages of fruit development.[81] Although it is not known for all plants, at least *L. gibba*, maize, bean, *Arabidopsis*, and carrot use both tryptophan-dependent and tryptophan-independent IAA biosynthesis pathways, confirmed by stable label feeding studies.[67,72,80,82–84] Tryptophan-independent IAA biosynthesis occurs as well in lower land plants such as liverworts, mosses, and ferns, suggesting that it has very early origins.[85]

4.02.2.3.2 Tryptophan-dependent pathways

Biosynthesis of IAA from tryptophan uses the L-form of the amino acid.[75] Some of the enzymes that catalyze the conversion of specific intermediates have been identified, and some of the genes coding for the enzymes have been cloned. Such findings establish that plants are competent to carry out such metabolic conversions; however, the specific involvement of these genes and intermediates requires confirmation, because biochemical studies carried out with applications to tissue segments or with extracts could disrupt tissue and cellular compartmentalization and because enzymes that catalyze the conversion of tryptophan to IAA *in vitro* may never come into contact with the intermediates *in vivo*. Thus, the physiological relevance of some of these pathways remains an open question.[69] An additional concern is that many of the enzymes have wide substrate specificities, so it has been difficult to implicate them solely in IAA biosynthesis. Some of the intermediates and enzymes that have been described to have the competence to carry out these reactions are discussed below.

4.02.2.3.2(i) Indole-3-acetonitrile pathway Indole-3-acetonitrile (IAN) has been found mainly in plants of the Brassicaceae family, including *Arabidopsis thaliana* and *Brassica chinensis*,[86] which produce a large variety of indole compounds in sufficient quantity. IAN may be produced by several routes including the conversion of tryptophan to indole-3-acetaldoxime (IAOx) and to IAN. Interest increased following the description of a family of nitrilases (*NIT1–4*) in *Arabidopsis* with close homology to genes in bacteria.[87,88] There is tissue specificity in expression, and NIT1 and NIT2 are expressed in response to pathogen infection.[87,89] *NIT3* was

shown to be expressed in the vascular tissue of hypocotyls, cotyledons, leaves, and roots, but not in root tips or reproductive structures. *NIT4* expression occurred in seedling root tips, distal tips of young leaves, mature stems, sepals, and the tip and base of siliques. *Arabidopsis* mutants *trp2* and *trp3*, which over-accumulated IAA conjugates,[71] also accumulated IAN 6–11-fold over wild type. When *Arabidopsis* roots were fed [$^2H_5$]tryptophan, both [$^2H_5$]IAN and [$^2H_5$]IAA were extracted from the roots and [$^{13}C_1$]IAN was converted to [$^{13}C_1$]IAA *in vivo*.[90]

Nitrilase enzyme activities have also been found in Gramineae and Musaceae[91] and in tobacco,[92] and homologs of *NIT4* are present in rice.[93] The enzymes encoded by the tobacco genes were not able to hydrolyze exogenous IAN to IAA,[94] but expression of the *Arabidopsis* NIT2 gene in tobacco conferred increased sensitivity to exogenous IAN and IAA. Overexpression of nitrilase genes apparently does not cause phenotypic changes under normal growth conditions.[37] *NIT1* was postulated to encode the predominant nitrilase isozyme based on expression studies, while NIT2–4 were 4–8-fold less abundant.[87] However, the K_m values of NIT1–3 for IAN were an order of magnitude higher than those for their preferred substrate 3-phenyl-propionate.[93] The *nit1* mutant had no dramatic phenotype in the absence of IAN and had levels of free IAA and IAA conjugates similar to wild type. NIT4 did not accept IAN as a substrate.[95] The *nit2* and *nit3* mutants retained sensitivity to applied IAN, which suggests that they do not function in the IAA biosynthesis pathway *in vivo*. Also, the high-IAA phenotype caused by the *Arabidopsis rnt1-1* mutant was not blocked by the *nit1-1* mutant, suggesting that *NIT1* was not involved in IAA biosynthesis in *rnt1-1* mutants.

In maize, two nitrilases, *ZmNIT1* and *ZmNIT2*, are expressed in aleurone/pericarp and at lower levels in the endosperm.[96] In *in vitro* enzyme assays, ZmNIT1 did not convert IAN to IAA, but ZmNIT2 did, and it also released indole-3-acetamide (IAM) as a by-product. ZmNIT2 activity converted several nitriles previously tested as substrates for *Arabidopsis* nitrilases[93,95] and they were converted to their respective acids with greater efficiency than was IAN. The temperature optimum for IAN was also very high (40 °C), as was the apparent K_m of ZmNIT2 (4.1 mmol l^{-1}), and there was no substrate inhibition by IAN in quantities greater than 3 mmol l^{-1}, as had been found in *Arabidopsis*.[93] IAN was present in maize kernels in concentrations ranging from 20 to 350 pmol g^{-1} fresh weight. Given the low substrate levels and high K_m, and the high rate of IAA production in the endosperm (0.23 μg IAA g^{-1} h^{-1}),[97] IAN would likely need to be channeled to obtain the requisite localized concentration.

4.02.2.3.2(ii) Indole-3-acetaldehyde pathway The natural occurrence of indole-3-acetaldehyde (IAAld) in plants was first described in the extracts of cucumber seedlings[98] where the concentration of IAAld was ~0.7 ng g^{-1} fresh weight. Possibly arising via IAOx or indole-3-pyruvate (IPyA), IAAld may act as a convergence point for tryptophan-dependent pathways.[78] In an early study, the capacity to metabolize [$^{14}C_1$]tryptophan via IPyA and IAAld was determined.[99] Two primarily cytoplasmic enzymes were involved and the process was NAD-dependent.

The IAAld pathway has been well characterized in microorganisms[100] where the conversion of IAAld to IAA is catalyzed by IAAld oxidases. Related activities have been characterized in several plants such as potato tubers,[101] oat coleoptiles,[102] cucumber,[98] and pea.[103] Further evidence for the involvement of IAAld oxidases comes from the *Arabidopsis superroot* (*sur1*, allelic to *rooty*, *alf1*, *hls3*, and *ivr*) mutant,[104–106] which accumulated an aldehyde oxidase isozyme specific for IAAld, and had a high-auxin phenotype including increased lateral and adventitious rooting, epinastic cotyledons, and lack of apical hook when grown in darkness.[105] *Sur1* also accumulated free IAA and IAA conjugates.[106,107] The *sur1* mutant phenotype is due to a defect in cysteine-sulfoxide lyase (C-S lyase) that is involved in glucosinolate production from IAOx. As would be expected, the enzymatic activity and transcription of the aldehyde oxidase is elevated in the *rooty* mutant.[105,108]

The *sur2* (*rnt1*) *Arabidopsis* mutant is defective in CYP83B1, a cytochrome P450 monooxygenase that is able to convert IAOx to its *N*-oxide.[109] This is the first committed step in indole-3-methyl glucosinolate biosynthesis, and the mutant had decreased levels of indolic glucosinolates and accumulated high levels of IAAld and IAA. However, little of the IAAld itself was converted to IAA,[110] which seemed to rule out both IAN and IAAld as intermediates in IAA biosynthesis in this case. Indeed, double mutants for *CYP83B1* and *NIT1* had increased IAA production, so this may point to a pathway that does not involve IAN in the IAOx/IAAld pathway, or to another pathway that is in fact also active and compensating.

Aldehyde oxidase purified from maize coleoptiles is a multicomponent enzyme that contains a molybdenum cofactor, nonheme iron, and flavin adenine dinucleotide (FAD) as prosthetic groups.[111] When substrate specificity of the aldehyde oxidase was tested, good activity was detected with IAAld, indole-3-aldehyde, and benzaldehyde among others. The addition of NADP and NADPH did not change the activity. In contrast, in maize endosperm, tryptophan-dependent IAA biosynthesis was dependent on an NADP/NADPH redox system, which may mean that the two tissues of maize are utilizing different pathways or different redox systems for IAA biosynthesis.[112]

The variety of aldehyde oxidases discovered in other plants have similarities to the maize enzyme, but also have some very important differences. Enzymes contained in a cell wall fraction from barley seedlings were able to oxidize IAAld to form IAA at a pH optimum of 7 and K_m of 5 $\mu mol\,l^{-1}$, which was very similar to the enzyme found in maize.[113] Two aldehyde oxidases from potato have also been identified;[101] they had a similar pH optimum (between 7 and 8), but preferred aliphatic aldehydes to aromatic aldehydes. Although oat and cucumber aldehyde oxidases have been shown to oxidize IAAld to produce IAA,[102,114] the oat enzyme had a lower pH optimum and higher K_m than the maize enzyme, and the cucumber enzyme was inhibited by synthetic auxin and activated by 2-mercaptoethanol, which was not true for the maize enzyme. The difference in the enzymes makes it difficult to envision a common evolutionary origin for the IAAld pathway in plants if these particular enzymes are involved in each case.

Enzymes that can catalyze the formation of IAAld from *N*-hydroxyl tryptamine or IAOx, two most likely precursors of IAAld, have not been unequivocally identified.[78] An IAA-forming activity in maize coleoptile extracts was partially purified using chromatography and ammonium sulfate fractionation, and the IAAld oxidase activity copurified with the IAA-forming activity.[115] However, the relative rates of IAA-forming activity to IAAld activity decreased through purification, casting doubt on the role of IAAld in IAA biosynthesis.

4.02.2.3.2(iii) Indole-3-acetaldoxime pathway IAOx is thought to be important for IAA biosynthesis in plants of the Brassicacae family that make indolic glucosinolate secondary metabolites. IAOx is a logical precursor of both IAAld and IAN, and has been proposed as the branch point between indole-3-methyl glucosinolate production and IAA biosynthesis. Recently, the cytochrome P450 *CYP83B1* that encodes the enzyme that N-hydroxylates indole-3-acetaldoxime to the corresponding *aci*-nitro compound 1-*aci*-nitro-indolyl-ethane[116,117] was identified. The *aci*-nitro compound reacts nonenzymatically with thiol compounds to produce an *N*-alkyl-thiohydroximate adduct, the first committed step in glucosinolate production. CYP83B1 had a high affinity for IAOx, with a K_m of 3 $\mu mol\,l^{-1}$, and could also bind tryptamine. IAOx had the ability to displace tryptamine in the enzyme's active site. The low K_m would prevent the accumulation of IAOx. Expression of *CYP83B1* was global; however, it was preferentially expressed in roots, and was induced by wounding or by dehydration. This enzyme has also been suggested to be a regulator of auxin production in *Arabidopsis*, and auxin response elements are found in the promoter region.[116] Knockout mutants lacking the enzyme had phenotypes that included severe apical dominance and overexpression lines exhibited decreased apical dominance. The null mutation of *CYP83B1*, also known as *rnt1-1*, is allelic to *sur2*, a mutant known to accumulate elevated levels of free IAA.[109] *rnt1-1* plants exhibited increased hypocotyl length, epinastic cotyledons, exfoliation of the hypocotyls, adventitious root formation from the hypocotyl, and enhanced secondary root and root hair formation.[117] Wild-type *Arabidopsis* seedlings grown on media supplemented with IAN phenocopied *rnt1-1* seedlings with adventitious lateral root primordia on the hypocotyls and epinastic cotyledons.[118]

Microsomal membrane fractions from cabbage, pea, and maize can convert tryptophan to IAOx, and recently, the enzymes that complete the conversion of tryptophan to IAOx *in vitro* have been characterized.[119] The enzymes in *Arabidopsis* have been identified as the cytochrome P450s *CYP79B2* and *CYP79B3*.[120] Cytochrome P450 monooxygenases are heme-thiolate, membrane-localized enzymes involved in the oxidative metabolism of lipophilic substrates, and are involved in the biosynthesis of phenylprenoids, alkaloids, terpenoids, glucosinolates, cyanogenic glucosides, phytoalexins, and plant hormones.[121] Plants overexpressing *CYP79B2* contained elevated levels of free auxin and displayed auxin overproduction phenotypes such as long hypocotyls and epinastic cotyledons. Overexpression of *CYP79B2* also increased expression of *AUX/IAA* genes, SAURS, and GH3. Expression profiles of *CYP79B2* were similar to that of *yucca*, an auxin overproduction

mutant discussed below. Interestingly, the *CYP79B2* overexpression lines also contained elevated levels of IAN, pointing to IAN being derived from IAOx produced by cytochrome P450s. Although single knockout mutants *cyp79B2* or *cyp79B3* had no profound phenotype, *cyp79B2* and *cyp79B3* double mutants contained reduced IAA levels, showed growth defects such as shorter petioles and smaller leaves, which was consistent with partial auxin deficiency, and contained reduced levels of IAN.[119] The *CYP79B2* overexpression line had increased sensitivity to 5-methyltryptophan,[120] and the double mutant had even higher sensitivity to this toxic tryptophan analog. The lack of orthologs in rice[119] suggests that this pathway may not be well represented in monocots.

4.02.2.3.2(iv) Tryptamine pathway Tryptamine is another potential intermediate of IAA biosynthesis pathway.[122] Recently, *yucca*, an activation-tagged mutant of *Arabidopsis*, was shown to overexpress a flavin monooxygenase catalyzing the N-hydroxylation of tryptamine to form *N*-hydroxyl-tryptamine.[123] The dominant *yucca* line exhibited an elevated auxin level phenotype: epinastic cotyledons and elongated petioles when grown in white light, and short hypocotyls and lack of apical hook when grown in dark. Mature leaves were narrower and epinastic with long blades and petioles, and adults also had increased apical dominance. Free IAA levels were 50% higher than in wild type. The *yucca* mutant could proliferate and differentiate in cell culture on auxin-free medium. Overexpression of the bacterial IAA conjugation gene *iaaL* in the *yucca* plants[124] suppressed the mutant phenotype.[123]

There are 11 members of the YUCCA family in *Arabidopsis*. The roles of *YUC1* and its closest homologs *YUC2*, *YUC4*, and *YUC6* were studied by overexpression, and all yielded phenotypes suggestive of auxin overproduction.[125] The expression patterns of the genes differed, with *YUC1* and *YUC4* showing similar expression in apical meristems and young floral primordial, but each also expressed elsewhere. *YUC2* and *YUC6* were expressed at a low level in the inflorescence apex and were more widely expressed in young flower buds, petals, stamens and gynoecium of young flowers (*YUC2*), and in stamens and pollen (*YUC6*). Single gene mutants did not display obvious developmental defects, while double mutants produced mixed results, with *yuc1yuc4* and *yuc2yuc6* showing growth and developmental defects and *yuc1yuc4* and *yuc2yuc6* having reduced fertility or complete sterility. The triple mutants showed similar floral development defects as the double mutants and the quadruple mutants showed even stronger phenotypes. The triple mutants had reduced venation in flowers and reduced vasculature in leaves, and the quadruple mutants had even more pronounced phenotypes.[125] The disruption of these genes also led to decreased expression of the auxin reporter DR5-GUS[126] in the cells where the *YUCCA* genes are normally expressed, such as young leaves. Auxin application to mutant plants did not complement *yucca* phenotypes but when transformed with *iaaM*[127,128] (under the *YUC6* promoter), floral reproductive organ development was partially rescued, as well as fertility, in double and triple mutants.[125]

A homolog of *YUCCA*, *floozy*,[129] was found in petunia as a flower mutant, and overexpression lines contained increased IAA levels and had high-auxin phenotypes. Seven *YUCCA*-like genes were identified in rice[130] and segregated into three protein clusters, similar to those in *Arabidopsis*. Expression analysis of the genes showed that they were differentially expressed, with *OsYUCCA*s 1, 5, and 6 preferentially expressed at the tip of rice coleoptiles. Rice calli overexpressing *OsYUCCA1* had a decreased growth rate and lower regeneration frequency, and leaf growth and root elongation were also inhibited. *OsYUCCA1* protein sequence is closest to *Arabidopsis YUCCA*s 1, 4, 10, and 11, of which 1 and 4 appear to be involved in *Arabidopsis* IAA biosynthesis.[125] In rice, decreasing *YUCCA* expression using antisense caused a dramatic phenotype,[130] while in *Arabidopsis*, multiple genes needed to be knocked out in order to get a significant phenotype. Combinations of knockouts from different clusters seem to be the most detrimental in *Arabidopsis*.[125] Recently, a YUC/FZY homolog, ToFZY, was described from tomato and converted tryptamine to *N*-hydroxyl-tryptamine *in vitro*.[131]

Tryptamine has been identified as a native compound in tomato,[132] and the gene encoding tryptophan decarboxylase has been isolated from *Catharanthus roseus*.[133] Plants grown on deuterium oxide incorporated more label into tryptamine than IAA, which was consistent with the result expected for a precursor of IAA. IAOx may be a YUCCA pathway intermediate for IAA biosynthesis in *A. thaliana*, and perhaps in rice and maize as well; however, no enzyme has yet been identified for the conversion of *N*-hydroxyl tryptamine to IAOx. Because tryptamine is not a compound universally present in plants[69] and deuterium oxide labeling ruled out tryptamine as an intermediate in tomato,[132] the pathway would have to be species-specific.

4.02.2.3.2(v) Indole-3-pyruvic acid pathway IPyA is present as a natural component in tomato, and in deuterium oxide-labeling studies, incorporated more label than IAA,[132] which one would expect from an upstream precursor. IPyA has also been found in *Arabidopsis* seedlings[134] and pea roots.[135] Although there are several possible reactions leading to the formation of IPyA by oxidative deamination, recent evidence points to a specific aminotransferase that is likely responsible for this reaction, and suggests that an aminotransferase annotated as an allinase is involved.[136,137] One mutant line, *sav3*, was isolated as a shade avoidance deficient mutant,[136] and proved to be allelic to a gene called *TIR2*. Knockout mutants of *TIR2* showed reduced levels of IAA, decreased hypocotyl elongation, and reduced lateral root formation.

In some microorganisms, IPyA is a known precursor of IAA.[138] The bacterial tryptophan aminotransferase, which catalyzes the rate-limiting step in the reaction from tryptophan to IPyA, has a low substrate specificity and high K_m for tryptophan. Bacterial indolepyruvate decarboxylase (IPDC), taking part in the second step in the reaction sequence, has a low K_m for its substrate ($K_m = 15\ \mu mol\ l^{-1}$). The IPDC activity in plant preparations is difficult to assay because of the instability of IPyA.[139] While no specific *IPDC* genes have been isolated from plants thus far, it is possible that the product of one of the five candidate pyruvate decarboxylase genes performs this conversion in *Arabidopsis*.[140]

4.02.2.3.2(vi) Indole-3-acetamide pathway The involvement of IAM in IAA biosynthesis was proposed based on its involvement in the bacterial pathway in *Pseudomonas savastanoi*[141] and in crown gall tissues.[100] The bacterial pathway occurs in two steps utilizing tryptophan monooxygenase and IAM hydrolase. The catalytic domain sequence of the bacterial acylamidohydrolase was used to identify an *Arabidopsis* amidase (*AMI1*).[142] The gene was shown to be part of an *Arabidopsis* gene family (*AMI1–4*) and AMI1 message and protein are found in leaves. IAM has been found as a naturally occurring compound in *Arabidopsis*,[143] and it appeared to be a by-product in the conversion of IAN to IAA by the nitrilase genes *NIT1–3*. The occurrence of IAM is widespread, however, being found in mung bean seeds,[144] orange fruits,[145] clubroots of Chinese cabbage,[146] wounded potato tubers,[147] and aseptically grown cherry seedlings.[148] In some cases, IAM was attributed to microbial activity.

When the IAA biosynthesis gene *IaaM* from *Agrobacterium* is introduced into plants defective in IAA biosynthesis, IAA is produced,[127,128] which may point to the involvement of this intermediate in some plants and demonstrates the ability of the plants to hydrolyze IAM to IAA. IAM has also been found in etiolated seedlings of aseptically grown squash seedlings.[149] The hydrolase was partially purified from roots. However, the bacterial hydrolase had a higher affinity for IAM than the squash root enzyme. IAM hydrolase activity has also been found in cultivated and wild rice.[150] The enzyme in cultivated rice hydrolyzed IAM, 1-naphthaleneacetamide, and IAA–ester conjugates.[151] However, feeding labeled tryptophan to rice yielded labeled IAA, but not labeled IAM.[150]

4.02.2.3.2(vii) IAA synthase An activity associated with a protein fraction described as an 'enzyme complex' was isolated from *Arabidopsis* and proposed to act as a channel system that could catalyze the entire pathway of biosynthesis of IAA from tryptophan.[152] The complex was 160–180 kDa, soluble, and required no additional cofactors. There was also evidence for a nitrilase-like protein in the complex, which suggested that the reaction proceeded through IAN. When an excess of [$^2H_5$]tryptophan ($10\ mmol\ l^{-1}$) was fed to 0.2 mg of the protein fraction containing the complex and incubated at high temperatures (30 °C), IAA was produced. Labeled IAN, IAOx, and IAAld were not detected in the reaction mixture, and did not interfere with the conversion of tryptophan to IAA. However, addition of an excess of unlabeled intermediates did dilute the isotopic abundance of [^2H] in the IAA recovered, probably due to the conversion of unlabeled IAN or IAAld to IAA. Labeled IAN or IAAld was not tested to determine which was being converted to IAA in this case. The high temperature optimum for this complex (40 °C) makes it an unlikely candidate as an *in vivo* IAA biosynthesis pathway in *Arabidopsis* and no genetic evidence exists for this complex.

Preliminary data presented in a short review[153] suggested that IAA biosynthesis in maize endosperm is due to a protein complex of 180 kDa, which was partially purified by gel filtration chromatography. It was found to have a specific activity of $32\ pmol\ mg^{-1}\ min^{-1}$, which was a 64-fold enrichment over the crude endosperm extract. No indolic metabolites were detected that could function as biosynthetic intermediates, but further

purification attempts resulted in loss of activity perhaps pointing to an unstable complex. It remains to be seen if this complex is able to be further purified, and, if so, if it is involved in IAA biosynthesis.

4.02.2.3.3 IAA degradation

Initially, IAA catabolism was thought to occur primarily through the action of IAA oxidase, a companion activity to most plant peroxidases.[69] This idea has been extensively reviewed[38,69] and it seems likely that the contribution of the decarboxylation pathway to IAA degradation was significantly overestimated in studies of IAA oxidation in homogenates or with cut tissue pieces. We now know that the general process of ring oxidation followed by glycosylation appears to a general theme (**Figure 2**), although variable in its specific details in different species.[38,154] In maize, oxidation of IAA *in vivo* yields oxindole-3-acetic acid (oxIAA).[155,156]

The discovery that the IAA conjugate IAA–aspartate can either be hydrolyzed to yield free IAA[39] or serve as an entry point into IAA catabolism[157] placed new emphasis on understanding the biochemistry of this branch-point compound.[154] IAA–aspartate and oxIAA–aspartate were the major metabolites present after feeding IAA to *Populus tremula*.[158] At least three different pathways for the direct oxidation of this IAA conjugate, and likely IAA–glutamate as well, are known.[154] Thus, specific IAA conjugates have a significant role in IAA degradation, and these findings demonstrate that the processes of conjugation and degradation have aspects in common. Recently, several new oxidative metabolites from *Arabidopsis* have been described. 6-OH-IAA conjugates with phenylalanine, valine, and oxIAA–1-*O*-glucose.[159] The same group also reported on a novel series of compounds in rice, the *N*-*β*-D-glucopyranosyl derivative and their aspartate and glutamate conjugates.[160] These are very exciting findings, but exactly where these new compounds fit in the overall scheme for auxin metabolism will need to await further analysis of their flux as well as the determination of their metabolic fate.

4.02.2.4 Perception and Signaling

As mentioned above, auxin induces a multitude of effects modulating plant development. It has always been a central question in plant biology how this simple molecule can regulate such a dazzling variety of diverse responses, and in recent years we have seen tremendous progress in our understanding of the mechanism of auxin signal transduction. While a model for auxin-induced transcriptional changes with the ubiquitin–proteasome system as its key player had been known for some time, the major breakthrough was the unraveling of the perception mechanism[161,162] and with it the identification of a family of auxin receptors[163] revealing a potentially complete, and surprisingly simple, signaling pathway from perception to transcriptional response. However, the historical path to the discovery of the current model took a number of turns to finally arrive at one of the components that was among the first to be identified.

4.02.2.4.1 Transcriptional responses to auxin stimuli

Early molecular approaches to elucidate auxin action revealed that the hormone induces a number of rapid transcriptional changes in several gene families. Three of these – SMALL AUXIN-UP RNAs (*SAUR*s), *GH3*s, and AUXIN/INDOLE-3-ACETIC ACID genes (*Aux/IAA*s) – have been most thoroughly characterized. Levels of early transcripts are dramatically induced only minutes after auxin application,[164–166] suggesting that regulation of transcription would be a major function of the yet-to-be-identified receptor. Conserved auxin-responsible elements (AuxREs) in the promoters of several auxin-inducible genes[167] led to the identification of proteins that mediate their transcription. AUXIN RESPONSE FACTORs (ARFs) interact with the AuxRE and, thus, facilitate auxin-responsive gene expression.[168,169] The ARF family comprises 23 members, which are characterized by an N-terminal DNA-binding domain (DBD), a long middle region, and domains III and IV near the C-terminus. While the DBD binds to the AuxRE, domains III and IV function as dimerization domains. In addition to homodimerizing and heterodimerizing with other ARF family members, domains III and IV also enable interaction with the 29-member family of AUX/IAA proteins.[170] *Aux/IAA* genes encode nuclear proteins with similar domains (domains III and IV).[171] They do not seem to bind DNA directly but instead act as transcriptional repressors through domain I when interacting with ARFs.[172] Aux/IAAs are extremely unstable proteins with half-lives starting in the minutes range, which can be further reduced by auxin or dramatically increased by the application of proteasome inhibitors.[173,174]

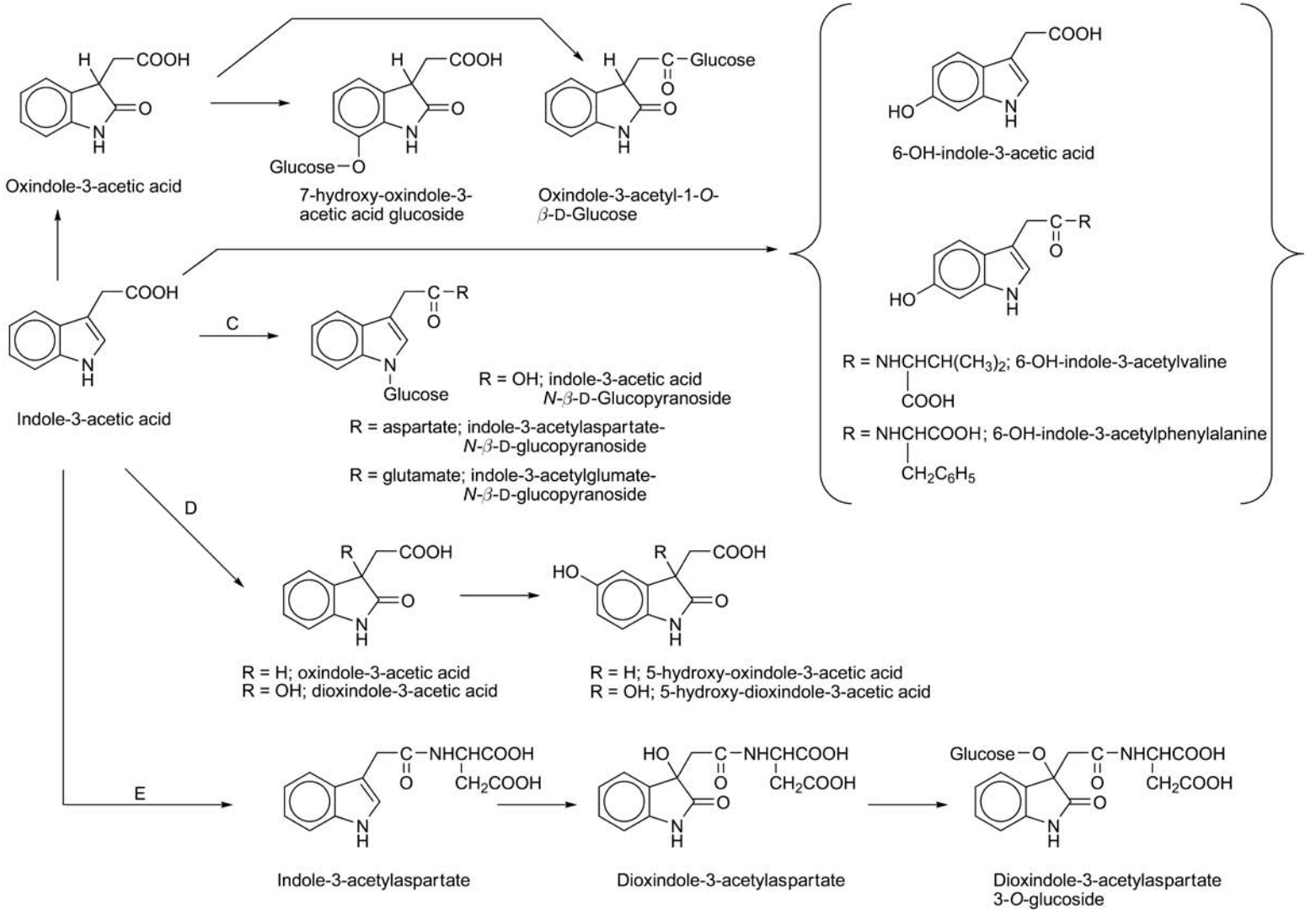


Figure 2 Summary of the major oxidative routes for IAA catabolism. These include oxidation at the 2-position to form oxIAA and its metabolites (A and D), the recently described oxidations at indole-6 (B) and the formation of *N*-glucosides (C), and, finally, the conjugation to IAA–aspartate and its subsequent oxidation (E).

4.02.2.4.2 Regulation of auxin response by the ubiquitin pathway

Forward genetic screens initiated in the 1980s resulted in the identification of a number of auxin-resistant (*axr*) mutants that shed new light on the regulation of auxin response. The first gene isolated was AUXIN RESISTANT1 (*AXR1*), which encoded a protein related to ubiquitin-activating E1 enzymes,[175] indicating the role of proteasomal degradation in auxin signaling. This was in agreement with the short half-lives of the abovementioned Aux/IAA proteins and fit well with the identification of the dominant *axr3* and *axr2* gain-of-function mutations in Aux/IAA family members.[176,177] Further confirmation of the key role of the ubiquitin pathway was provided by the isolation of TRANSPORT INHIBITOR RESPONSE1 (*TIR1*).[178] *TIR1* was initially identified in a screen for mutants resistant to polar auxin transport inhibitors,[179] but a number of response-related phenotypes suggested a role in auxin signaling. *TIR1* encodes an F-box protein. F-box proteins are the target recruiting subunits of SCF-type E3 ubiquitin ligases. SCF complexes are composed of Skp1, cullin, an F-box protein, and a small RING protein RING-BOX1 (Rbx1). The cullin subunit acts as a scaffold, binding Skp1 at its N-terminus and Rbx1 at its C-terminus. Skp1 functions as an adaptor connecting the F-box protein to the rest of the complex. While the F-box domain binds to the Skp1 subunit, TIR1 also contains a C-terminal leucine-rich repeat (LRR) as the protein–protein interaction domain for target recruitment. Therefore, SCF^{TIR1}-mediated ubiquitination was established as the central player in auxin signaling.[180] In the following years, mutants in each of the core SCF subunits were identified and shown to have defects in auxin response.[181,182] And indeed, TIR1 directly interacts with the degron domain II of Aux/IAA proteins in a cell-free system.[183] Remarkably, auxin promotes this interaction. Taken together, genetic approaches suggested a model with auxin promoting Aux/IAA ubiquitination by SCF^{TIR1} resulting in derepression of ARF-mediated transcription of auxin-inducible genes.

4.02.2.4.3 Auxin receptors

Amid an ever clearer picture of auxin action, the auxin receptor remained a conspicuous omission from signaling models. Early biochemical efforts to isolate auxin receptors identified AUXIN-BINDING PROTEIN1 (ABP1).[184,185] However, the majority of ABP1 localizes to the endoplasmic reticulum where the pH is too high for auxin binding. Furthermore, embryo-lethal null mutants and the lack of weak mutant alleles complicated detailed analysis.

The finding that auxin increases the otherwise low affinity of TIR1 toward its substrate AUX/IAA has suggested that TIR1 may also function as an auxin receptor.[161,162] Furthermore, the close homologs AUXIN SIGNALING F-BOX PROTEIN1–3 (AFB1–3) perform the same receptor function as TIR1.[163] The molecular mechanism behind this affinity enforcement was described by elucidating the crystal structure of TIR1 in complex with several auxins and an AXR2 peptide containing the domain II degron sequence.[186] It was found that the AUX/IAA peptide and auxin bind to the same pocket within the LRRs with inositol hexakisphosphate acting as a putative cofactor. The exceptionally long intra-repeat loops of LRRs 2, 12, and 14 are crucial for the formation of the auxin–substrate binding pocket with a pivotal role for loop 2. While auxin binds to a hydrophobic cavity at the base of the pocket, the AUX/IAA peptide docks on top of the auxin molecule. Thereby, auxin enlarges the binding surface and stabilizes the interaction of AUX/IAA with TIR1.[186]

Ten years after the initial identification of the *tir1-1* mutant, its central role in the auxin signaling pathway has been revealed and with this the identification of a novel class of intracellular receptors. However, several early auxin responses occur too rapidly after auxin treatment to be caused by transcriptional changes and *de novo* protein synthesis. This, together with the fact that *tir1/afb1–3* higher order mutants still seem to possess auxin responses,[163] points to further auxin signaling routes waiting to be discovered.

4.02.3 Gibberellins

4.02.3.1 Introduction

GAs are a family of plant hormones comprising more than 135 members, and belong to a class of diterpenoids carrying a basic skeleton that is called *ent*-gibberellane (**Figure 3**). Each different GA that has been found to be naturally occurring and whose structure has been chemically characterized is allocated an A-number, according

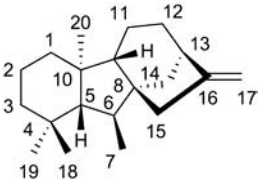


Figure 3 *ent*-Gibberellane skeleton and numbering.

to the proposal by MacMillan and Takahashi to adopt chronological numbering system,[187] and this A-number allocation system has now been managed by Hedden and Kamiya.

The history of gibberellin has been well described in the previous edition of the book *Comprehensive Natural Products Chemistry*,[188] in other books, in a review,[189–191] and on the website http://www.plant-hormones.info/gibberellinhistory.htm and so is briefly reviewed in this chapter.

Only specific GAs carrying a characteristic structural property had been considered to be recognized by a receptor(s) and to show biological activity, although each GA demonstrates diverse biological activities depending on the structure and assay used.[192–194] This has been well shown by the extensive studies using biosynthetic mutants[195–197] and was conclusively confirmed in 2005 by examining the affinity of several GAs for the receptor protein GID1.[198] GID1 was named after the gene responsible for the rice dwarf mutant, *gibberellin insensitive dwarf 1*.

The biosynthesis of GAs was extensively surveyed during the 1960–70s by feeding experiments using *Gibberella fujikuroi* and radioactive precursors.[199,200] The studies on the biosynthesis of GAs in plants were led by Graebe and coworkers.[201–203] Recent research progress in the biosynthesis of GAs is described in Section 4.02.3.4.

The signal transduction of GAs has been studied by analyzing the mutants[204–210] insensitive or constitutively sensitive to GAs as introduced in Section 4.02.3.6. The identification of a GA receptor had been attempted without promising results until 2005. Physiological and cell biological studies using aleurone cells have shown that GAs to be perceived on the outer surface of the cell membrane.[211–213] Finally, Matsuoka's group and Yamaguchi's group collaborated and succeeded in identifying the cytosolic GA receptor, GID1, in 2005.[198] As GID1 plays a decisive role in both the shoot elongation of rice and *Arabidopsis* and α-amylase induction in rice,[198] it remains uncertain whether another GA receptor is located in the cell membrane.

Some gibberellins and related compounds show the antheridiogen activity, which induces the male sex organ and suppresses the formation of the female organ in schizaeaceous ferns. Antheridic acid[214,215] and GA_{104}[216,217] have been isolated and characterized as the principal antheridiogens in *Anemia phyllitidis*, *A. hirsute*, *A. rotundifolia*, *A. flexuosa*, and *A. mexicana*. GA_{73} methyl ester[218] has been identified as an antheridiogen from *Lygodium japonicum*, *L. circinnatum*, and *L. flexuosum*, and GA_9 methyl ester from *L. japonicum* and *L. circinnatum*. The fern antheridiogens have been fully reviewed in the literature,[219] so the details are not described in this chapter.

4.02.3.2 Chemistry

4.02.3.2.1 Fungal gibberellins

After the pathogenetic study on Bakanae disease (foolish rice disease) by Hori[220] and Kurosawa,[221] Yabuta and Sumiki[222] succeeded in 1938 in purifying chemical substances inducing etiolation of the rice seedling, which is a symptom of Bakanae disease, from a culture broth of *G. fujikuroi* and named the active principle gibberellin. The ICI group[223] and the NRRL group[224] followed this work to isolate the active substances. The active substance of Yabuta and Sumiki was later proved to be a mixture of gibberellins GA_1, GA_2, and GA_3 by comparing the samples of the three groups; the ICI group isolated GA_3 (gibberellic acid) and the NRRL group isolated a mixture of GA_1 and GA_3. Many GAs have been isolated from the culture broth of *G. fujikuroi*.

After 1979, some other fungi were found to produce GAs, and it was after 1988 that some bacteria were also found to produce GAs. The fungi and bacteria that were proved to produce GAs are listed in the literature[225]

and information on the GAs in microorganisms is also available on the websites http://www.plant-hormones.info/occurrence_of_gas_in_fungi.htm and http://www.plant-hormones.info/gasinbacteriai.htm.

4.02.3.2.2 Plant gibberellins

4.02.3.2.2(i) Free gibberellins Mitchell *et al.*[226] first reported in 1951 the occurrence of substances that showed gibberellin-like activity in immature beans, and MacMillan and Suter[227] succeeded in the isolation of GA_1, GA_5, GA_6, and GA_8 from immature seeds of the scarlet bean, *Phaseolus coccineus* (*P. multiflorus*), in 1958. This was the first isolation of gibberellin from a plant. West and Phinney[228] also showed the occurrence of GA-like substances in immature seeds of *Phaseolus vulgaris* in 1959. Kawarada and Sumiki[229] reported the isolation of GA_1 from water sprouts of the mandarin orange tree, *Citrus unshu*, in 1959. This was the first report on the isolation of GA from a vegetative tissue. The most notable GA isolated from a vegetative tissue was GA_{19},[230] which is an important biosynthetic intermediate.

As many as 135 GAs had been identified from many plant species by July 2007. It is now considered that all plant species produce GAs in nature, although the number of plant species from which GAs have been conclusively identified is still limited. Some mutants lacking the GA biosynthetic enzyme(s) cannot produce physiologically active GAs and show a dwarf phenotype. The plants from which the identification of GAs has been reported and the information on plant GAs are reviewed in the literature[225] and are available on the website http://www.plant-hormones.info/occurrence_of_gas_in_plants.htm. The publications reporting the characterization of GAs after GA_{118} are cited in the literature.[231–234]

4.02.3.2.2(ii) Gibberellin conjugates In addition to free gibberellins, several gibberellin conjugates have been isolated and characterized. Most of the conjugates are gibberellin glucosyl ethers (GA-GEts). The 3-*O*-β-D-glucosyl ether of GA_3 (GA_3-GEt) was the first to be isolated[235] and characterized, and GA_8-GEt was reported[236] at almost the same time. GA_{26}-GEt, GA_{27}-GEt, GA_{29}-GEt,[237] GA_{35}-GEt,[238] GA_1-GEt,[239] and the 17-*O*-β-D-glucosyl ethers of 16α,17-dihydroxy-16,17-dihydro GA_4 and 16α,17-dihydroxy-16,17-dihydro GA_7 followed.[240] GAs-GEt were often identified as metabolites of exogenously fed GAs,[241,242] and are considered to be inactive catabolites, the formation of GAs-GEt contributing to the homeostasis of the active GA level[242] as well as to other inactivation processes like the oxidation at the C2 position.

Other conjugates are the glucosyl esters of GAs (GA-GEs). GA_1-GEs, GA_4-GEs, GA_{37}-GEs, and GA_{38}-GEs were isolated from mature seeds of *P. vulgaris*,[243] and GA_5-GEs and GA_{44}-GEs from immature seeds of *Pharbitis purpurea*.[244] The physiological roles of GAs-GEs remain unknown, because there is the possibility that they could be easily hydrolyzed to give free Gas, which are active or can be converted to the active type.[245]

Gibberethion is a unique conjugate formed by the coupling between 3-oxo-GA_3 and 2-oxo-thiopropionate, and is found only in immature seeds of *Pharbitis nil*, and possibly in another inactive metabolite of GA_3.[246]

Some reviews on gibberellin conjugates are listed in the literature.[247,248]

4.02.3.2.3 Isolation and characterization

Efficient purification performed by high-performance liquid chromatography (HPLC) and high-sensitivity gas chromatography-mass spectrometry (GC-MS) using a capillary column dramatically improved the purification and identification procedure for GAs as in other natural products. The improved sensitivity in instrumental analyses also requires a correspondingly smaller quantity of GAs for characterization. The purification procedure for GAs has become simpler than that in earlier decades.

Immunoaffinity[249–250] chromatography is expected to be a convenient and powerful technique for purifying GAs, although it has not yet become popular because of the tedium of preparing useful antibodies. The single-chain Fv (scFv) technique, however, enables easier preparation of anti-GA-scFv on a large scale by using transformed microorganisms,[251] and this will facilitate the use of immunoaffinity for purifying GAs.

The accumulation of GC-MS data enables known GAs to be identified or the structure of unknown GAs to be speculated with a picogram level. The structure of a new GA was recently speculated from its GC-MS data and then confirmed by comparing the GC-MS data with those of candidates that were chemically prepared.[231–234]

4.02.3.2.3(i) Solvent fractionation Plant materials are usually extracted by homogenizing in methanol or 50–80% aqueous acetone and then filtering. After removing the organic solvent of the extract *in vacuo*, the aqueous residue is submitted to solvent fractionation, affording an acidic ethyl acetate-soluble (AE) fraction, a neutral ethyl acetate-soluble (NE) fraction, an acidic 1-butanol-soluble (AB) fraction, and a neutral 1-butanol-soluble (NB) fraction. Most of the free GAs are partitioned into the AE fraction, some of the polar GAs such as GA_{28} and GA_{32} into the AB fraction, the glucosyl ethers of GAs (GA-GEts) into the AB fraction, and the glucosyl esters of GAs (GA-GEs) into the NE and/or the NB fractions. Today, HPLC is used for the purification of GAs from plant material. Details of column chromatography for large-scale purification can be found elsewhere.[252,253]

4.02.3.2.3(ii) Solid-phase extraction and immunoaffinity purification Instead of solvent–solvent partitioning, solid-phase extraction, using a small disposable column or a small cartridge prepacked with chemically bonded octadecylsilanized silica (ODS) or with a chemically bonded anion-exchange group such as dimethylaminoalkyl group, is often used as a prepurification tool for instrumental analyses. Solid-phase extraction using an ODS cartridge has been used to separate GAs and their glucosyl conjugates from the less-polar kaurenoid precursors by eluting the cartridge with an increasing concentration of methanol or acetonitrile in water. Immunoaffinity purification using the immobilized anti-GA antibody is also effective.[249–250]

4.02.3.2.4 Qualitative and quantitative analysis

4.02.3.2.4(i) Gas chromatography-mass spectrometry GC-MS is the most powerful technique to identify and quantify GAs in very small quantities at the picogram levels. GAs are identified by comparing the full-scan spectrum and Kovats' retention index of a sample with the reference data, which are available on the website http://www.plant-hormones.info/ga1info.htm. HPLC is frequently used as a purification method before conducting a GC-MS analysis of GAs. Reversed-phase HPLC with an ODS column is most commonly used, and an ion-exchange column is also sometimes used.

Gas chromatography-selected ion monitoring (GC-SIM) is often used for the quantification of GAs by GC-MS, an internal standard labeled with stable isotopes (usually with deuterium) being used as the most reliable and sensitive method. A mass chromatogram reconstructed from full-scan GC-MS is also used for semiquantification. The amounts of GAs are determined by measuring the peak areas of ions characteristic for each GA and comparing these areas to those of authentic samples. When an internal standard labeled with a stable isotope is used, the ratio of the area of an ion peak characteristic for a sample and the area of the corresponding peak of the labeled internal standard is used for quantification. The analysis of GAs by GC-MS has been discussed in numerous publications and review articles.[254–256]

4.02.3.2.4(ii) Liquid chromatography-mass spectrometry Liquid chromatography-mass spectrometry (LC-MS) analysis of GAs has also been reported. LC-MS is as sensitive as GC-MS, having the advantage that both GAs and GA conjugates can be analyzed without derivatization,[257–259] which is essential for GC-MS analysis. The disadvantage of LC-MS is the tendency for fragmentation to be hardly apparent and that the ionization efficiency is often affected by contaminants.

4.02.3.2.4(iii) Immunoassay Immunoassay using an antibody of high specificity is also a very powerful tool to quantify GAs. Although it cannot exclude ambiguity due to the cross-reactivity of the antibody used, it has the advantage of analysis of samples without an extensive prepurification. When combined with suitable HPLC prepurification, it gives fairly reliable results.[260]

4.02.3.3 Biosynthesis and Metabolism

4.02.3.3.1 Biosynthesis in plants

The biosynthesis pathway of GAs in plants has long been studied since their discovery as endogenous growth regulators. There have been two key approaches to unravel the biosynthetic routes and identify the genes encoding the biosynthetic enzymes. First, a cell-free system prepared from immature seeds of dicotyledonous species such as *Marah macrocarpus* and *Cucurbita maxima* allowed researchers to purify and characterize the GA

biosynthetic enzymes, as these species are rich sources of these enzymes.[261] Second, dwarf mutants defective in GA biosynthesis capability have been beneficial to determine the biologically active forms and to identify the GA biosynthetic genes. More recently, the availability of genomics tools in model plant species has accelerated our understanding of the GA pathway and its regulation. An outline of GA biosynthesis and the deactivation pathways in plants and fungi is presented here, with emphasis on recent findings related to these pathways. There are numerous recent reviews that summarize the identification of individual biosynthetic and deactivation genes and their regulation.[262–266]

GAs are synthesized from geranylgeranyl diphosphate (GGDP), a common C_{20} precursor for diterpenoids (**Figure 4**). Three different classes of enzymes are required for the biosynthesis of bioactive GAs from GGDP in plants: terpene cyclases, cytochrome P450 monooxygenases (P450s), and 2-oxoglutarate-dependent dioxygenases (2ODDs). Recent work using isotope-labeled precursors has shown that the methylerythritol phosphate (MEP) pathway in the plastid provides the majority of isoprene units to GAs in *Arabidopsis* seedlings, although there is a minor contribution from the cytosolic mevalonate (MVA) pathway.[267] The relative contributions of the MEP and MVA pathways to GA biosynthesis may vary among tissue types and also depend on the growth conditions.

The cyclization of GGDP to the hydrocarbon intermediate *ent*-kaurene requires two enzymes: *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) (**Figure 4**). There is some experimental evidence to indicate that both CPS and KS are located in the plastids.[268,269] *ent*-Kaurene is then converted to GA_{12} by two P450s. *ent*-Kaurene oxidase (KO; CYP701A) catalyzes a consecutive three-step oxygenation reaction at C-19 to produce *ent*-kaurenoic acid, which is subsequently converted to GA_{12} by another P450, *ent*-kaurenoic acid oxidase (KAO; CYP88A[270]). Experiments using fusion enzymes with green fluorescent protein (GFP) have suggested that KO is located in the outer membrane of the plastid, whereas KAO is present in the endoplasmic reticulum.[269]

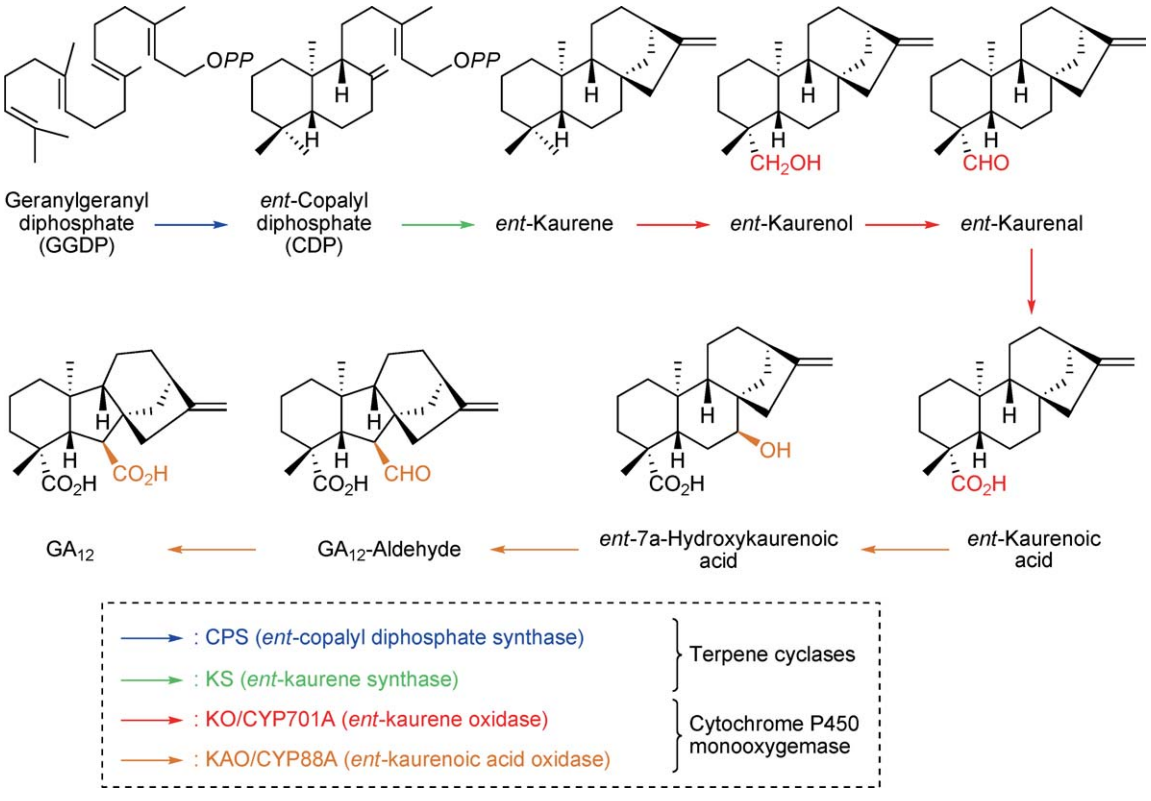


Figure 4 Early part of the GA biosynthesis pathway in plants.

GA_{12} is converted to GA_4, a biologically active form, through oxidation at C-20 and C-3 positions by GA 20-oxidase and GA 3-oxidase, respectively, both of which are soluble 2ODDs (**Figure 5**). Although the subcellular location of these 2ODDs has not been experimentally demonstrated, they are thought to be cytosolic enzymes because of the lack of any apparent targeting sequence. GA_{12} is also a substrate for GA 13-oxidase for the production of GA_{53}, which acts as a precursor of GA_1 in the 13-hydroxylated pathway. Since no 13-oxidase gene has been identified, the nature of this enzyme and the biological relevance of 13-hydroxylation are still unclear.

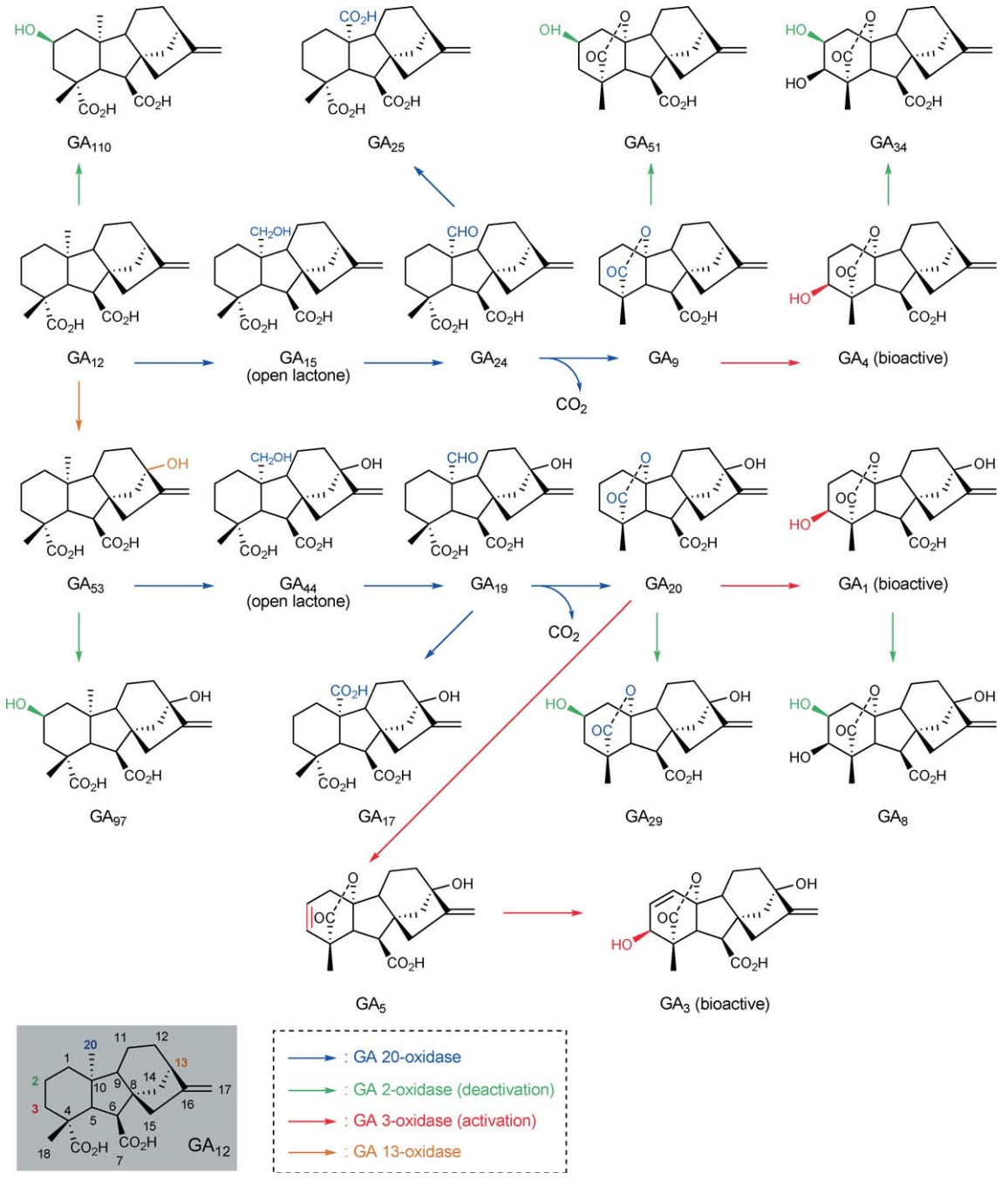


Figure 5 Conversion of GA_{12} into various GAs in plants.

4.02.3.3.2 Deactivation in plants

Deactivation mechanisms are important for effective regulation of the concentrations of bioactive hormones in plants. GAs are metabolically deactivated in several different ways. The best-characterized deactivation reaction has been 2β-hydroxylation by GA 2-oxidases, a class of 2ODDs (**Figure 5**). Conventional GA 2-oxidases use C_{19}-GAs as substrates, including bioactive GAs and their immediate precursors (GA_9 and GA_{20}).[271] A new type of GA 2-oxidase that accepts only C_{20}-GAs has recently been reported.[272,273] These GA 2-oxidases are responsible for the production of 2β-hydroxylated GAs such as GA_{97} and GA_{110} and are likely to play a role in depleting the pool of GAs that are to be converted to bioactive forms.

Another deactivation mechanism has recently been found in rice from studies on the recessive tall mutant *elongated uppermost internode* (*eui*).[274] EUI is a P450 designated as CYP714D1[270] and epoxidizes the 16,17-double bond of non-13-hydroxylated GAs (**Figure 6**). There is an accumulation of 16α,17-dihydrodiols, hydrated products of the 16,17-epoxides either *in planta* or during purification, in transgenic rice plants that overexpress the *EUI* gene. Thus, the discovery of this enzyme explains the occurrence of GA 16,17-dihydrodiols in many plant species.[274] More recently, *S*-adenosine-L-methionine-dependent methylation of the C-6 carboxyl group of GAs by methyltransferases, GAMT1 and GAMT2, has been shown to constitute an additional deactivation mechanism in *Arabidopsis* (**Figure 6**).[257] GAMT1 and GAMT2 utilize a variety of GAs, including bioactive GA_1 and GA_4, as substrates *in vitro*, and produce the corresponding methyl esters. It has yet to be investigated whether methylation of GAs is a common deactivation reaction in other plant species. The formation of GA conjugates such as glucosyl ethers and esters of GAs may also serve as deactivation reactions.[275] Identification of glycosyl transferase(s) responsible for GA conjugations will be helpful to better understand their role in plants.

The identification of the majority of GA biosynthetic/deactivating enzymes has provided a clearer view of the mechanism by which a large variety of GAs are produced in plants. First, only six enzymes are required for the 12-step conversions of GGDP to GA_4, because of the multifunctionality of several enzymes on the pathway. Second, many GA-modifying enzymes, including the 2ODDs, 16,17-epoxidase (CYP714D1), and GAMTs, accept multiple GAs as substrates. The promiscuous nature of these enzymes should contribute to the production of diverse GAs by a relatively small number of enzymes.

4.02.3.3.3 Regulation of gibberellin levels in plants

Both GA biosynthesis and deactivation are regulated by a range of endogenous and environmental signals, consistent with the role of GAs as the key regulators of growth and development. There is accumulating evidence that such signals affect the GA content, at least in part, by altering the transcript abundance of the biosynthetic and deactivating genes. In some cases, transcription factors that directly regulate the GA biosynthetic or deactivating genes have been identified.[276–279] Some plant hormones, including GA itself, act as endogenous regulators of GA concentrations in plants. For example, plants maintain the homeostasis of the bioactive GA levels through feedback and feed-forward regulation of the biosynthetic and deactivating genes.[262,280,281] Auxin,

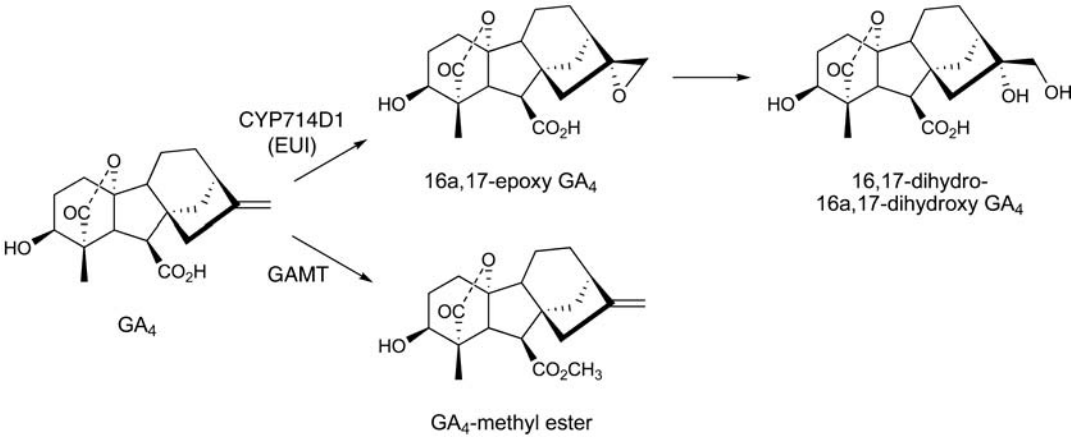


Figure 6 Deactivation by oxidation and methylation.

another class of growth hormones, positively regulates GA biosynthesis in the stem tissues of pea and barley.[282,283] Evidence has been provided that the GA biosynthetic and/or deactivating pathways are regulated by environmental signals such as light and temperature in various developmental processes.[284–289] These results support the idea that GAs act as key regulators of the environmental responses in plants.

4.02.3.3.4 Biosynthesis in fungi

GAs were first isolated as secondary metabolites of the rice pathogen *G. fujikuroi*. The occurrence of GAs in some other fungi and bacteria has also been reported.[219] Recent identification of the genes encoding the GA biosynthetic enzymes from *G. fujikuroi* and a species of *Phaeosphaeria* has revealed remarkable differences in the GA biosynthetic enzymes between plants and fungi.

In plants, two separate terpene cyclases (CPS and KS) are involved in the synthesis of *ent*-kaurene from GGDP (**Figure 4**), while these two reactions are catalyzed by a single bifunctional enzyme (CPS/KS) in fungi (**Figure 6**).[290–292] In *G. fujikuroi*, the multifunctional P450s, P450-4 and P450-1, respectively, play a similar role to KOs (CYP701As) and KAOs (CYP88As) in plants.[293] However, despite their similar catalytic activities, the fungal P450s are distantly related to the plant enzymes in their amino acid sequence. Of particular note is that P450-1 has 3β-hydroxylase activity in addition to KAO activity and produces GA_{14} (**Figure 7**). Thus,

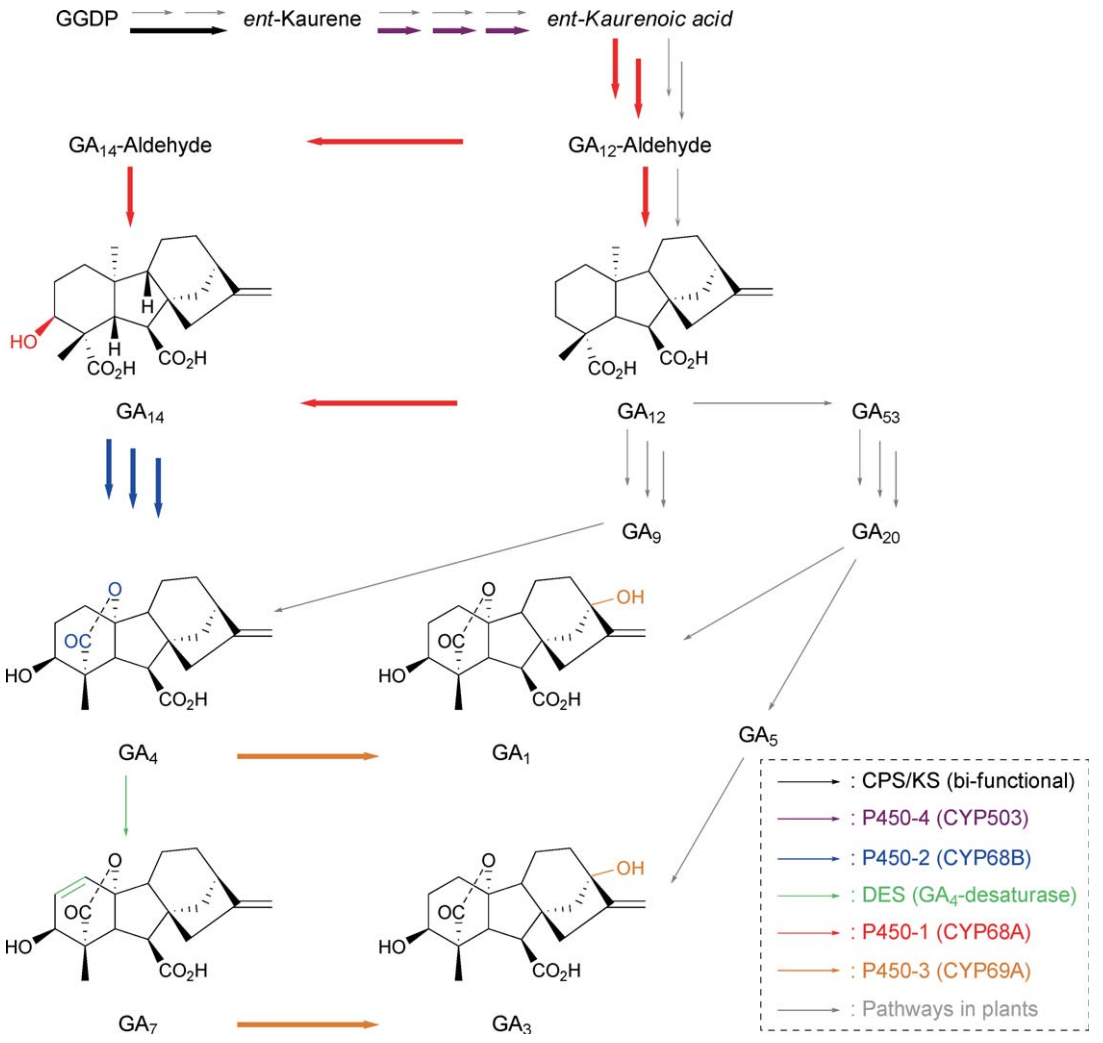


Figure 7 Comparison of the GA biosynthesis pathways in *Gibberella fujikuroi* and plants.

3β-hydroxylation is catalyzed by a P450 at an early stage of the pathway in *G. fujikuroi*, in contrast to plant GA 3-oxidases, which are soluble 2ODDs and catalyze 3β-hydroxylation at the final stage to produce bioactive GAs (**Figure 5**). GA_{14} is converted to GA_4 by another P450, P450-2.[294] Thus, GA 20-oxidation in *G. fujikuroi* is also catalyzed by a P450, unlike the case with plant GA 20-oxidases. GA_4 is then converted to GA_7 by GA_4-desaturase and finally to GA_3 by P450-3 through 13-hydroxylation (**Figure 7**).[295] Interestingly, these GA biosynthetic genes are clustered on a particular chromosome in fungi, whereas they are randomly located on chromosomes in plants.[296,297] Taken together, these substantial differences suggest that the complex GA biosynthetic pathways in plants and fungi have evolved independently.

4.02.3.4 Biological Activities

More than 135 free GAs have been characterized from the plant kingdom, including higher and lower plants, fungi, and bacteria. GAs show biological activity in many aspects of plant growth, shoot elongation and α-amylase induction being commonly used for the evaluation of the biological activity of each GA. Among the free GAs, only a limited number of GAs are biologically active *per se*. GAs that show biological activities in bioassays are those that can be perceived by receptor(s) or those that can be converted to biologically active GAs (see Section 4.02.3.3.1). GAs that are physiologically active in shoot elongation and GAs that are active in α-amylase induction in aleurone cells of cereal seeds carry characteristic and common structural functionalities.

In general, judged from the affinity to the GA receptor (GID1)[198,298] and the activities in bioassays, the following structural characteristics are required for GAs to be active: carrying (1) a 3β-hydroxy group, (2) a free carboxyl group on C-6, and (3) a γ-lactone from C-4 to C-10 (a δ-lactone from C-4 to C-20 instead of γ-lactone reduces the activity, but still results in some affinity to the receptor and activity). The introduction of a hydroxyl group at C-2 or C-17 makes GAs inactive.

Variation in the biological activity of each GA results from the affinity of each GA to a receptor and the susceptibility of each GA to deactivation enzymes such as GA 2-oxidases in the assay plants. In rice shoot elongation assay, GA_1 and GA_4 show no apparent difference in their biological activity, while the affinity of GA_4 to the GA receptor, GID1, is as high as 5 times that of GA_1,[198] suggesting that exogenously applied GA_4 is more easily deactivated than GA_1 before reaching the receptor.

Table 1 shows the relative biological activity of some GAs toward rice shoot elongation, and their relative affinity to OsGID1, the GA receptor in rice.

4.02.3.4.1 Effects on shoot elongation

Shoot elongation is the most apparent effect of GAs, and is mainly caused by cell expansion in the axis and partly by cell division. This effect is prominent with the exogenous application of GAs to young seedlings, the response of maturing plants to GAs generally being low or negligible. The assay systems have been developed

Table 1 Affinity of GAs to OsGID1 and biological activity on rice seedlings

	Relative affinity to OsGID1[194]	*Relative activity on shoot elongation (rice: Tang-ginbozu)*[188]
GA_1	5	High
GA_3	5	Very high
GA_4	100	Moderate
16,17-dihydro GA_4	20	Moderate[a]
3-*epi*-GA_4	<0.1	Not tested
GA_4 methyl ester	0.6	Very low[a]
GA_9	0.1	Moderate
GA_{35}	2	Moderate
GA_{37}	1	Moderate
GA_{51}	<0.1	Inactive

[a] Not listed in Murofushi *et al.*[188]

according to the shoot elongation effects, and dwarf mutants lacking a specific enzyme catalyzing a certain step in the biosynthesis of physiologically active GAs help to enhance the assay sensitivity and indicate the GA species in the assay mixture. Dwarf maize (*Z. mays*) *d1* and *d5* and dwarf rice (*Oryza sativa* L.) cv. Tan-ginbozu and cv. Waito-C have been widely used as assay plants.[192–194] The cucumber hypocotyls assay has also been frequently used.[193–194] Dwarf mutants of *A. thaliana* lacking an enzyme catalyzing each step of GA biosynthesis are also now available.[195,204]

4.02.3.4.2 Effects on enzyme activities and inductions

4.02.3.4.2(i) Effects on enzymes in aleurone cells of cereal seeds

GAs increase the activity of α-amylase in cereal seeds such as barley and rice by enhancing the transcription of some of the α-amylase genes in the aleurone cells.[299] These aleurone cells do not have the set of enzymes for GA biosynthesis, and cannot convert precursor GAs to physiologically active GAs. Only physiologically active GAs show activity in this assay system, while their biosynthetic precursors do not.[193] The activities of protease, β-glucanase, ribonuclease, catalase, peroxidase, citrate synthase, isocitrate lyase, nuclease, and so on in the cereal aleurone cells are also increased by GA application.[299–302] The embryo-less half-seed of barley does not induce α-amylase without an exogenous GA application, while the other half with the embryo does. Based on the foregoing observations and on the expression patterns of the genes for GA biosynthetic enzymes and α-amylase genes, the physiological role of GA in the cereal seeds has been speculated to be as follows.[299] GAs secreted from the developing seed embryo move to the aleurone layer and stimulate the *de novo* synthesis of α-amylase and proteases. These enzymes then move out of the aleurone cells into the endosperm to hydrolyze starch and storage proteins respectively, resulting in the production of sugars and amino acids, which are finally used for further development of the seed embryo.

4.02.3.4.2(ii) Effects on enzymes in vegetative tissues

Microarray technique and proteomics analyses of wild plants and their GA-deficient mutants and the analyses of GA-treated and GA-untreated plants enable to profile the proteins that are regulated by GAs in vegetative tissues. GIP in petunia,[303] carboxypeptidase (PsCP) in pea,[304] and xyloglucan-endotransferase (XET), pyruvate dehydrogenase, fructose-biphosphate aldolase, and so on in rice are detected as the GA-regulated proteins.[300–302]

4.02.3.4.3 Effects on flowering

A GA treatment induces flower formation in some species of long-day plants under short-day conditions,[308,309] while the flower induction in short-day plants is not generally affected by the GA treatment. Based on the studies using *Arabidopsis*, four promotive routes have been proposed for the floral initiation. These are the photoperiodic, autonomous, vernalization, and gibberellin pathways, all of which converge on the 'integrator' genes SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) and FLOWERING LOCUS T (FT), and recent studies have highlighted a role for the product of FT as a component of the floral stimulus or 'florigen'.[9,10] The classic florigen concept[8] can be explained by *FTmRNA* moving through phloem from the leaf to shoot apex, this movement being responsible for long-day-induced flowering in *Arabidopsis*. The flowering of *Arabidopsis* under short-day conditions is induced by the increase of GA_4 in the shoot apex; GA_4 regulates the transcription of both LFY and SOC1.[310]

Another example of GAs regulating flowering has been reported for *Lolium temulentum*.[311] GA_5 and GA_6 are more active in the induction of flowering in *L. temulentum* than GA_1 and GA_4, which are more active in shoot elongation of the same plant. The GA-dependent degradation of DELLA protein is suggested to be one of the factors for the floral initiation under long-day condition in *L. perenne*.[312]

4.02.3.4.4 Other effects

GAs are also active in breaking the dormancy of tubers or seeds.[313] GAs can replace the stimuli in the induction of seed germination in light- or cold-requiring seeds of *Lactuca*, *Nicotiana*, *Perilla*, and so on. The increase of physiologically active GAs through the induction of 3β-hydroxylase by red light has been shown to be a key point in the germination of photoblastic lettuce seeds.[314]

Parthenocarpy is also induced by GAs in plant species such as grape, apple, peach, pear, tomato, and almond.[301,315,316]

4.02.3.5 Perception and Signaling

4.02.3.5.1 Overview of GA signaling in plants

The GA receptors have recently been identified;[198,298] this has enabled the molecular mechanisms of GA perception and signal transduction in plant growth, development, and environmental response to be elucidated. Combining this with all the other information, we can present the GA signal cascade as follows.

When GA binds to its receptor GID1, the GID1–GA complex has affinity for key regulators called DELLA proteins that usually suppress GA signaling. After the binding of these DELLA proteins with the GID1–GA complex, the (GID1–GA)-decorated DELLA protein is recruited by a specific F-box protein of the SCF complex into the 26S proteasome after being tagged with polymerized ubiquitins, to be degraded there. Finally, as the repression of GA signaling by the DELLA protein begins to erode, the signaling begins to pass toward the expressional regulation of various GA-responsive genes (**Figure 8**).

4.02.3.5.2 GID1, the cytosolic gibberellin receptor

It was believed that GA molecules are able to permeate the plasma membrane of plant cells by passive diffusion, because of their hydrophobic nature under the neutral pH condition. Some researchers therefore expected that GA perception would occur in a similar fashion to that of mammalian steroid hormones. In fact, some results have suggested that GA perception occurred in the nucleus; the composition of RNA in nuclei isolated from pea[317] or cucumber[318] seedlings changed when GA was applied. Many attempts were made to identify those proteins that showed a high affinity and a high specificity toward GA from GA-responsive plant materials, one being from elongating seedlings and another from the aleurones of cereal seeds. UK researchers led this field

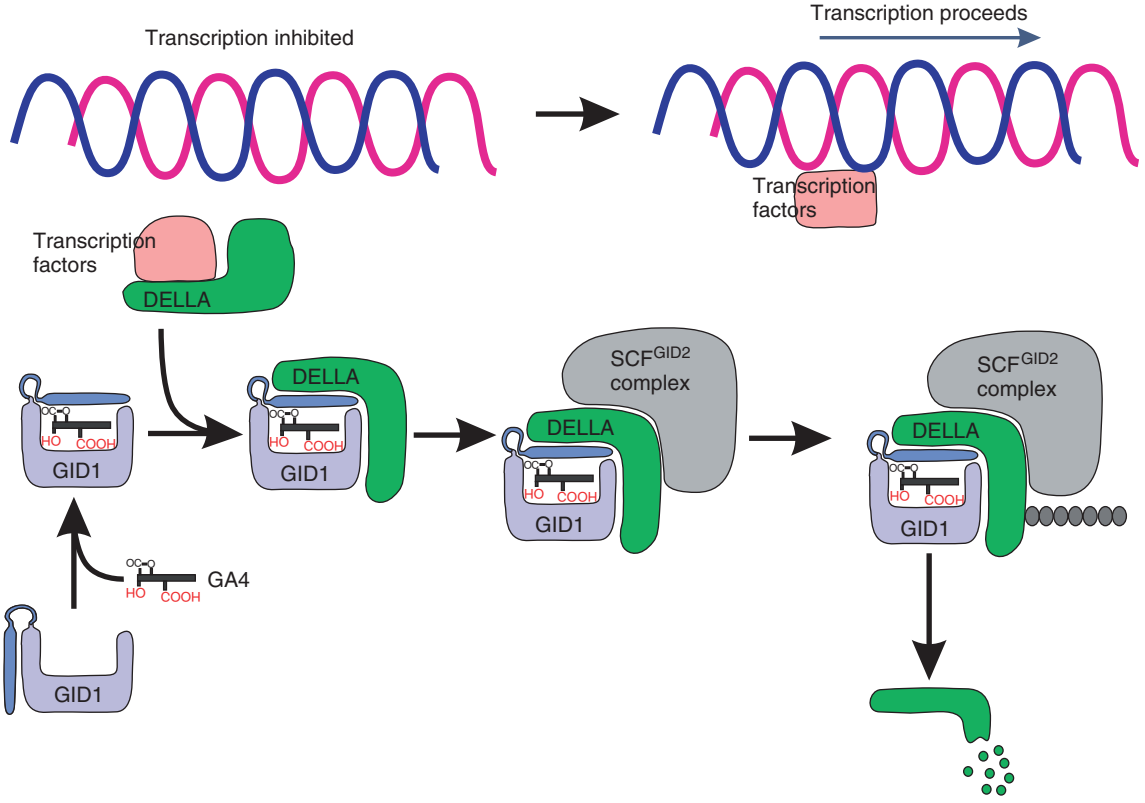


Figure 8 Perception of GA by GID1 and the degradation of DELLA protein. An active GA is perceived by GID1 in the nucleus. Once GA binds to the cavity of GID1, the N-terminal peptide of GID1, which plays the role of a lid, covers GA. DELLA protein, which holds transcription factor(s) to suppress gene transcription, binds to GA–GID1 complex and releases the transcription factor(s). The released transcription factor(s) activates gene transcription. The GA–GID1–DELLA complex forms another complex with SCF(GID2) and DELLA protein in the complex is ubiquitinated and degraded by 226S proteasome.

and showed that GA-inducible α-amylase could be induced, particularly in the cereal aleurone, even by the applications of membrane-impermeable GA derivatives.[211] These findings strongly imbued researchers with the notion that GA perception should occur on the outer surface of the plasma membrane of cereal aleurone cells. The cell-biological experiments by Gilroy and Jones also supported this idea;[213] they showed that microinjected GA failed to induce α-amylase in aleurone protoplasts, but that the incubation of aleurone protoplasts in a GA solution induced α-amylase. The receptor candidates have been biochemically characterized, although none have yet been isolated.[319–321]

The big breakthrough came from the isolation of the rice recessive mutant (*GIBBERELLIN INSENSITIVED WARF*1 *gid1*), which showed a typical GA-insensitive phenotype.[198] A double mutant of *gid1* with *slr1*, which carries a mutation in the key regulator gene for GA signaling, *SLR1* (details are described in Section 4.02.3.5.3), exhibited the *slr1* phenotype and a GA treatment did not reduce the SLR1 level in *gid1* plants, indicating that *SLR1* was epistatic to *GID1*.[198] Although the GA-insensitive phenotype of *gid1* is similar to that of *gid2* mutants, which also carry a mutation in another regulatory gene for GA signaling, *GID2* (described in Section 4.02.3.5.4), there are some differences between the two mutants: the dwarfed level of *gid1* was much stronger than that of *gid2* and the SLR1 level in *gid1* was much lower than that in *gid2*. In general, the phenotypes of *gid1* resembled a GA-deficient rice mutant *eps*, suggesting that GID1 functioned upstream of SLR1 in the GA signaling pathway.[198] The results of biochemical analyses showed that the recombinant GID1 bound to a radio-labeled GA_4 derivative with a dissociation constant (K_d) of 1.4×10^{-6} mol l^{-1}. Its binding characteristics fulfilled the criteria required for a receptor: (1) reversibility, (2) saturability, (3) high affinity to biologically active GAs, and (4) reasonable specificity, that is, a specific preference for active GAs. GID1 was an unknown protein with similarity to a hormone-sensitive lipase (HSL) family.[322] However, it was predicted *in silico* that GID1 should lose its lipase activity because the amino acid histidine, which is one of the HSL catalytic triad, was replaced by valine. In fact, the recombinant GID1 showed no enzymatic activity with artificial substrates for HSL. In addition, GID1 was mainly detected in nuclei and its localization was not changed by the application of GA.[198] Analyses of some *gid1* mutants and of point-mutated recombinant GID1s elucidated that most areas of this receptor molecule were influenced by the binding with GA.[323]

Three receptor genes (*AtGID1a*, *b*, and *c*) exist in *Arabidopsis*, and they have been identified by their structural similarity to rice *GID1* (**Figure 9**).[298] Their products showed ligand specificities that were similar to those of rice GID1. Among the three products, AtGID1b showed stronger pH dependence and 4 times higher affinity to

```
OsGID1   ---MAGSDEVNRNECKTVVPLHTWVLISNFKLSYNILRRADGTFERDLGEYLDRRVPANARPLEGVSSFDHIIDQSVGLE
AtGID1a  ---MAASDEVNLIESRTVVPLNTWVLISNFKVAYNILRRPDGTFNRHLAEYLDRKVTANANPVDGVFSFDVLIDRRINLL
AtGID1b  ---MAGGNEVNLNECKRIVPLNTWVLISNFKLAYKVLRRPDGSFNRDLAEFLDRKVPANSFPLDGVFSFDHVDS-TTNLL
AtGID1c  ---MAGSEEVNLIESKTVVPLNTWVLISNFKLAYNLLRRPDGTFNRHLAEFLDRKVPANANPVNGVFSFDVIIDRQTNLL

OsGID1   VRIYRAAAEGDAEEGAAAVTRPILEFLTDAPAAEPFPVIIFFHGGSFVHSSASSTIYDSLCRRFVKLSKGVVVSVNYRRA
AtGID1a  SRVYRPAYADQEQPPSILDLEKPVDG-------DIVPVILFFHGGSFAHSSANSAIYDTLCRRLVGLCKCVVVSVNYRRA
AtGID1b  TRIYQPASLLHQTRHGTLELTKPLSTT------EIVPVLIFFHGGSFTHSSANSAIYDTFCRRLVTICGVVVVSVDYRRS
AtGID1c  SRVYRPADAG--TSPSITDLQNPVDG-------EIVPVIVFFHGGSFAHSSANSAIYDTLCRRLVGLCGAVVVSVNYRRA

OsGID1   PEHRYPCAYDDGWTALKWVMSQ--------PFMRSGGDAQARVFLSGDSSGGNIAHHVAVRAADEG--------VKVCGN
AtGID1a  PENPYPCAYDDGWIALNWVNSR--------SWLKSKKDSKVHIFLAGDSSGGNIAHNVALRAGESG--------IDVLGN
AtGID1b  PEHRYPCAYDDGWNALNWVKSR--------VWLQSGKDSNVYVYLAGDSSGGNIAHNVAVRATNEG--------VKVLGN
AtGID1c  PENRYPCAYDDGWAVLKWVNSS--------SWLRSKKDSKVRIFLAGDSSGGNIVHNVAVRAVESR--------IDVLGN

OsGID1   ILLNAMFGGTERTESERRLDGKYFVTLQ--DRDWYWKAYLPEDADRDHPACNPFGPNGRRLGGLPFAKSLIIVSGLDLTC
AtGID1a  ILLNPMFGGNERTESEKSLDGKYFVTVR--DRDWYWKAFLPEGEDREHPACNPFSPRGKSLEGVSFPKSLVVVAGLDLIR
AtGID1b  ILLHPMFGGQERTQSEKTLDGKYFVTIQ--DRDWYWRAYLPEGEDRDHPACNPFGPRGQSLKGVNFPKSLVVVAGLDLVQ
AtGID1c  ILLNPMFGGTERTESEKRLDGKYFVTVR--DRDWYWRAFLPEGEDREHPACSPFGPRSKSLEGLSFPKSLVVVAGLDLIQ

OsGID1   DRQLAYADALREDG-HHVKVVQCENATVGFYLLPNT----VHYHEVMEEISDFLNANLYY----------
AtGID1a  DWQLAYAEGLKKAG-QEVKLMHLEKATVGFYLLPNN----NHFHNVMDEISAFVNAEC-------------
AtGID1b  DWQLAYVDGLKKTG-LEVNLLYLKQATIGFYFLPNN----DHFHCLMEELNKFVHSIEDSQSKSSPVLLTP
AtGID1c  DWQLKYAEGLKKAG-QEVKLLYLEQATIGFYLLPNN----NHFHTVMDEIAAFVNAECQ----------
```

Figure 9 Deduced amino acid sequences of GID1s. The two shaded amino acid residues are essential for maintaining the GA-binding activity of OsGID1, and these are substituted by other residues in the gid1-1 or gid1-2 rice mutant; arrows show the three catalytic centers (Ser (S), Asp (D), and His (H)) for hormone-sensitive lipase.

GA ($K_d = 4.8 \times 10^{-7}$ mol l^{-1}) than the other two. The expression of each in rice *gid1* mutants rescued the GA-insensitive dwarf phenotype, demonstrating that AtGID1s function as GA receptors in rice.[298] Multiple loss-of-function mutants of the three AtGID1s have recently shown severely dwarfed and GA-insensitive phenotypes resembling the rice *gid1* mutant.[324–326]

Chandler *et al.*[327] showed that *Gse1*, the gene responsible for GA-insensitive mutation (*gse1*) of barley (*Hordeum vulgare* L.), is an ortholog of *GID1*.

The crystallographic analyses of GA_4–OsGID1 complex and the complex of GA_1–AtGID1–DELLA fragment (the N-terminal peptide of GAI) have been reported.[328,329] These two crystallographic analyses explain the formation of the GA–GID1–DELLA protein complex. As expected from the amino acid sequence similarity to HSLs, GID1 has a cavity that corresponds to a substrate-binding site in HSL, and an active GA binds to the cavity through the interaction of C6-carboxyl group and 3β-hydroxyl group with GID1. After GA is held in the cavity, the N-terminal peptide of GID1, which plays the role of a lid, covers and holds the GA, and this is followed by the binding with DELLA protein (**Figure 8**).[328,329]

4.02.3.5.3 DELLA protein, a key regulator of GA signaling

The DELLA proteins constitute a small group as part of the GRAS family, which was named after the highly conserved motif detected near the C-terminus of the three proteins, GAI, RGA, and SCR, in *Arabidopsis*.[330] The wheat *DELLA* gene, *Rht*, is a well-known contributor to the famous 'green revolution'. The DELLA proteins play a negative but important role in GA signaling. Their gain-of-function mutations like *Arabidopsis gai*,[331] wheat *Rht*,[332] maize *D8*,[332] and rice *SLR1*[333,334] have shown dwarfed phenotypes and a reduced responsiveness to GA. Moreover, sequence analysis of their alleles has revealed that an in-frame deletion occurred in the N-terminal DELLA motif or its neighboring TVHYNP motif. On the other hand, its loss-of-function mutations caused a GA-constitutive response (a slender phenotype) and the induction of GA-inducible α-amylase even at low GA concentrations.[333–335] These results suggest that the N-terminal region around the DELLA motif functions in the perception of the GA signal, and that its C-terminal region is concerned with the suppression of GA signaling. The activity or stability of the DELLA proteins is thought to be regulated by *O*-GlcNAc modification by SPINDLY, another negative regulator of GA signaling, or by phosphorylation by unknown kinases.[209] Unlike the DELLA protein in rice or barley mutant plants, the *Arabidopsis* loss-of-function mutants have shown no clear phenotypes,[336,337] which was probably caused by functional redundancy between the five DELLA proteins, GAI, RGA, RGL1, RGL2, and RGL3, in *Arabidopsis*.[331,336,338–340] However, the analysis of their multiple loss-of-function mutants has shown that only a part of the five *Arabidopsis* DELLA proteins tended to have specific roles in a specific organ or at a specific developmental stage: that is, the loss of function of both RGA and GAI induced a clear phenotype of constitutive GA signaling in stem elongation and early flowering time, while it did not show a clear effect on either germination or flower development, suggesting that other DELLA proteins might have some decisive functions.[338,341,342] It has also been reported that RGL1 and RGL2 were functional in seed germination,[339,340,343] and that RGL2 was the predominant repressor of seed germination, while GAI, RGA, and RGL1 were functional enhancers of RGL2,[344] and that RGA and RGL2 acted dominantly, but that Rhat GL1 did only to a minor extent, in floral development.[343,345,346]

A recent major discovery in GA signaling has been that the GA receptor, GID1, bound directly to the rice DELLA, SLR1, in a GA-dependent manner,[198] indicating that the GA–GID1 complex transduced the GA signal to the DELLA protein through their direct interaction. The GA-binding activity of GID1 was enhanced *in vitro* in the presence of SLR1. This means that SLR1 stabilized the interaction between GID1 and GA: in other words, the GID1–GA complex bound to SLR1, making the GID1–GA interaction even tighter, and resulting in more likely transmission of the GA signal through the formation of GID1–GA–SLR1 complex. A domain analysis has revealed that the DELLA and TVHYNP motifs located in the N-terminal region of the SLR1 were essential for its GA-dependent interaction with GID1.[323–325] Moreover, it has been experimentally shown that this GA-dependent GID1–SLR1 interaction occurred in the nuclei of plant cells.[323] Three GID1s and five DELLA proteins exist in *Arabidopsis*, so 15 GID1–DELLA combinations are possible. A yeast two-hybrid analysis has confirmed that the GA-dependent interaction of GID1–DELLA occurred in all combinations. This suggests that other mechanisms besides the preferences for the GID1–DELLA interaction exist to explain why the five *Arabidopsis* DELLA proteins are mostly redundant in their functions, despite their differential involvement in GA-dependent events being partially required, as already mentioned above.

4.02.3.5.4 F-box protein, recruiter of DELLA for its degradation

The DELLA proteins are subjected to GA-dependent proteolysis via the ubiquitin–proteasome pathway. The degradation of the protein by 26S proteasome-mediated proteolysis was first suggested by the observation that the level of the barley DELLA protein, SLN1, increases when inhibitors of 26S proteasome existed.[347] Later, the *F-box* genes for GA signaling, rice *GID2* and *Arabidopsis SLY1*, were cloned,[348,349] and their loss-of-function mutations, which resulted in a GA-insensitive phenotype, were found. F-box proteins are a component of the SCF complex, which is named after its three subunits, Skp1, Cullin, and F-box. The SCF complex catalyzes the tagging of the target protein with ubiquitins, prior to its sorting to the proteasome.[350] F-box proteins, in general, have a protein–protein interaction domain at the C-terminus. Although both GID2 and SLY1 do not have this domain, their C-terminal region is highly conserved and important, because the deletion of this site causes a loss of function.[348,349]

Some results support the notion that the targets of SCF^{GID2} or of SCF^{SLY1} are DELLA proteins, because a high accumulation of SLR1 or RGA has been observed in *gid2* or *sly1* mutant plants. In addition, the rice *gid2 slr1* and *Arabidopsis sly1 rga* double mutants have respectively shown the *slr1* and *rga* phenotypes, suggesting that the GA-insensitive phenotype of *gid2* or *sly1* depended on the function of SLR1 or RGA.[348,351] Finally, SLY1 interacts directly with RGA or GAI via their C-terminal GRAS domains *in vivo* and *in vitro*,[351] and the product of a gain-of-function allele of *SLY1*, gar2, showed a higher affinity to DELLA proteins than normal SLY1.

4.02.4 Cytokinins

4.02.4.1 Introduction

About 50 years ago, a chemical substance that strongly stimulates cell proliferation in tobacco tissue culture was first purified and crystallized by Skoog and his collaborators from autoclaved herring sperm DNA extracts.[352,353] The compound stimulating plant cell growth, N^6-furfurylaminopurine, was named kinetin (**Figure 10**), although it has never been found in living plants. A naturally occurring kinetin-like substance was first isolated from immature maize endosperm and named zeatin (*trans*-zeatin (tZ); **Figure 10**).[354] Usage of the term cytokinin for kinetin-like compounds was proposed by Skoog's group,[355] defining cytokinin as 'a generic name for substances which promote cell division and exert other growth regulatory functions in the same manner as kinetin'.[356] Since then, various species of cytokinins that fit the definition have been isolated from plants, fungi, bacteria, and algae. Natural cytokinins are currently defined to be a group of phytohormones that contain a common adenine moiety with an N^6-conjugated side chain and play critical roles not only in cell division but also in many aspects of plant growth and development.

Shortly after the discovery of cytokinins, degradation of tRNA was proposed to be the major source of the hormone because prenylated adenines were identified in the hydrolysates of tRNAs.[357–359] Identification of the reaction catalyzed by adenosine phosphate-isopentenyltransferase (IPT) using dimethylallyl diphosphate (DMAPP) and AMP as substrates in *Dictyostelium discoideum*, a slime mold,[360] changed the concept of the cytokinin biosynthetic pathway. In addition, since the publication of the previous edition of the book *Comprehensive Natural Products Chemistry*,[361] remarkable progress in cytokinin research has revealed basic schemes of cytokinin metabolism and signal transduction and their roles in plant growth and development at the molecular level. This section outlines recent research progress toward understanding cytokinin structural diversity, metabolic pathways, signal transduction, and biological actions.

4.02.4.2 Chemistry

4.02.4.2.1 Structures

Natural cytokinins are N^6-substituted adenine derivatives. The N^6 side chain is either isoprenoid or aromatic (**Figure 10**), with the former occurring in greater abundance than the latter. The prenyl side chains vary in the presence or absence of a hydroxyl group and the stereoisomeric position. Common active isoprenoid cytokinins are isopentenyladenine (iP; N^6-(Δ^2-isopentenyl)adenine), *trans*-zeatin (tZ; N^6-(4-hydroxyisopentenyl)adenine; (*E*)-2-methyl-4-(1*H*-purin-6-ylamino)but-2-en-1-ol), *cis*-zeatin (cZ; (*Z*)-2-methyl-4-(1*H*-purin-6-ylamino)but-

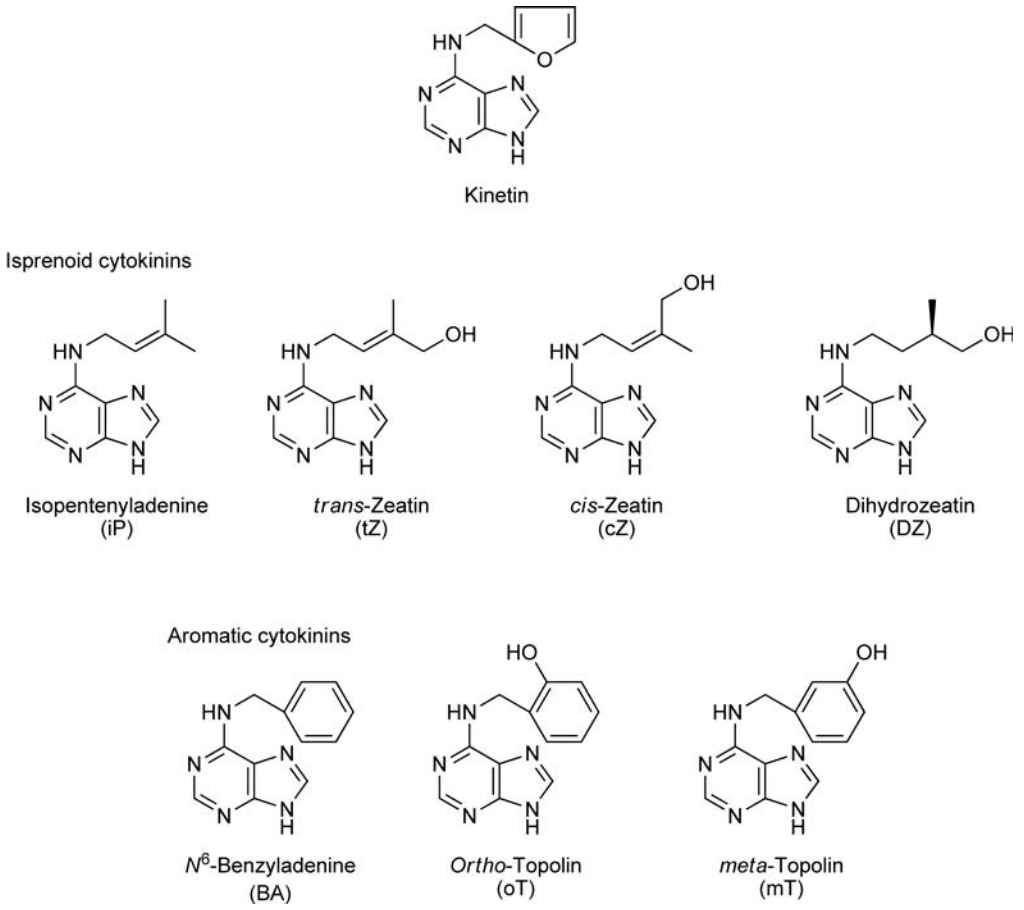


Figure 10 Structures of representative cytokinin species. Trivial names and commonly used abbreviations (parentheses) are shown.

2-en-1-ol), and dihydrozeatin (DZ; N^6-(4-hydroxyisopentanyl)adenine; 2-methyl-4-(1*H*-purin-6-ylamino)butan-1-ol) (**Figure 10**).[362,363]

Natural cytokinins are also present as the corresponding nucleosides, nucleotides, and other glycosides or amino acid conjugates in plants (**Figure 11**). Generally, such conjugations greatly reduce the biological activity but increase the biological stability. The nucleoside and nucleotide, especially the latter, occur abundantly in various plant species rather than the biologically active nucleobase. Cytokinin nucleosides, especially tZ riboside (tZR), are the major cytokinin components of xylem sap in various plants.[364–368] On the other hand, iP riboside (iPR) and iP nucleotide are found in leaf phloem exudate in *A. thaliana*;[369] in addition, cZ-type species (cZ and cZ conjugates) are also found.[370] Glycosylation of cytokinin occurs at the *N3*, *N7*, or *N9* position of the purine moiety as *N*-glucosides (**Figure 11**). In tZ, DZ, and cZ, glycosylation can also occur at the end hydroxyl group of the side chain as *O*-glucosides or *O*-xylosides (**Figure 11**). Abundance of the four cytokinins depends on plant species, tissue type, and developmental stage. iP- and tZ-type cytokinins are the major forms in *Arabidopsis* and many other plant species, whereas cZ-type cytokinins are abundantly found in maize,[371] rice,[372] and chickpea.[373] DZ-type cytokinins are generally less abundant in plants.

Amino acid conjugates of cytokinin have also been isolated from some organisms.[374] Alanine conjugated to tZ at the *N9* position of the adenine moiety, (*S*)-2-amino-3-(((*E*)-4-hydroxy-3-methylbut-2-enylamino)purin-9-yl)propanoic acid (**Figure 12**), was found in lupin seedlings and named lupinic acid.[375] Alanine conjugated to N^6-benzyladenine (BA; *N*-benzyl-1*H*-purin-6-amine) at the same position was also identified in bean seedlings.[376] Lupinic acid shows increased biological stability and less biological activity in comparison to the

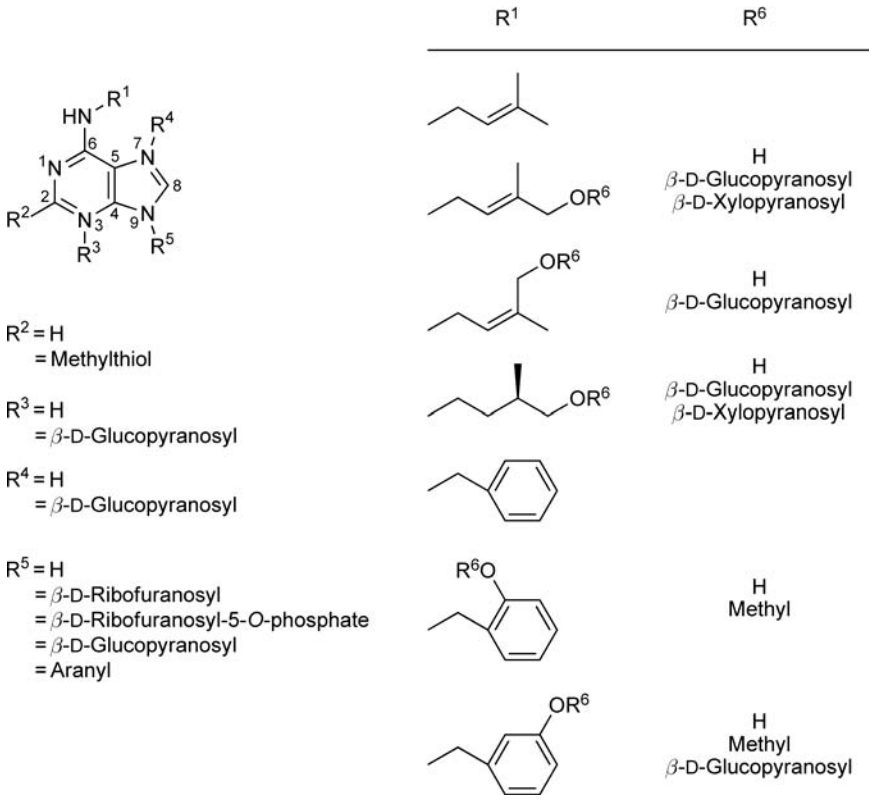


Figure 11 Structures of naturally occurring cytokinin derivatives conjugated with sugars and amino acids.

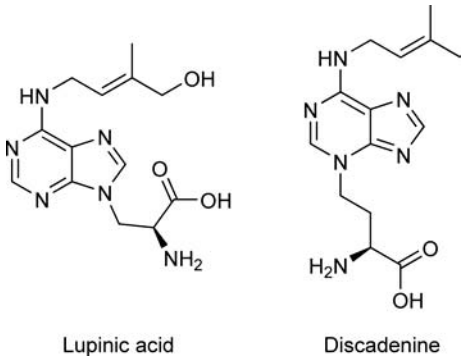


Figure 12 Structures of lupinic acid and discadenine.

corresponding bases in bioassays,[377] suggesting that alanine conjugates of cytokinin may be storage forms. To date, enzymes catalyzing the removal of amino acids from cytokinins have not been identified. In *D. discoideum*, 3-(3-amino-3-carboxypropyl)-N^6-(Δ^2-isopentenyl)-adenine, called discadenine (**Figure 12**), has been isolated and characterized as a spore germination inhibitor.[378]

Aromatic cytokinins are found in poplar,[379–381] anise,[382] tomato,[383] *Cocos nucifera*,[384] and *Arabidopsis*.[381] In addition to BA, aromatic cytokinins are found as *ortho*-topolins, *meta*-topolins, hydroxylated BAs, *ortho*-methoxytopolins, and *meta*-methoxytopolins (**Figure 11**).[380,381] Other derivatives have been isolated as *N3*-, *N7*-, and *N9*-glucosides and *N9*-alanine on the adenine ring,[385] and nucleosides and a glucoside linked to the ribosyl moiety.[386] In spite of the several reports of their occurrence, it is not clear whether the aromatic species are common in plants.

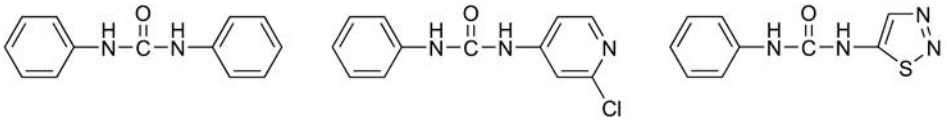


Figure 13 Synthetic phenylurea-type cytokinins.

Phenylurea-related compounds (**Figure 13**) also have cytokinin activity although there is no evidence for the natural occurrence of this class of compounds, strongly suggesting that they are artificial cytokinins. *N*,*N*′-diphenylurea was first reported as a novel type of cytokinin from the liquid endosperm of coconut, but it was later found to be a contaminant from prior chemical analyses. To date, several analogs such as *N*-phenyl-*N*′-(2-chloro-4-pyridyl)urea and thidiazuron[387] have been developed.[388] These compounds are highly stable and have high cytokinin activity.[388]

4.02.4.3 Biosynthesis and Metabolism

4.02.4.3.1 Two pathways for isoprenoid cytokinin biosynthesis

The biosynthetic pathway producing isoprenoid cytokinins has been identified, whereas that of aromatic cytokinins is poorly characterized.[363,385] Two distinct pathways for isoprenoid cytokinin biosynthesis have been described, and each pathway employs a different type of isopentenyltransferase at the initial step. The major pathway in higher plants, which is catalyzed by IPT, is conjugation of adenine nucleotide and DMAPP or 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (HMBDP) (**Figure 14**). In the less frequently used pathway, cytokinins are formed by degradation of prenylated tRNAs. The initial prenylation reaction of tRNA is catalyzed by tRNA-isopentenyltransferase (tRNA-IPT) (**Figure 14**).

4.02.4.3.2 Cytokinin biosynthesis in microorganisms

4.02.4.3.2(i) Biosynthesis in Dictyostelium discoideum Cytokinin biosynthesis through a reaction catalyzed by IPT was first characterized in *D. discoideum* and the reaction product was discadenine (3-(3-amino-3-carboxypropyl)-N^6-(Δ^2-isopentenyl)-adenine).[378] In the biosynthesis of discadenine, iP nucleotide is first synthesized from DMAPP and AMP by IPT.[360] After the formation of iP from iP nucleotide, a methylthiol moiety is conjugated at the *N3*-position of adenine with *S*-adenosyl-L-methionine by discadenine synthetase.[389] *Dictyostelium* IPT utilizes AMP or ADP as a prenyl acceptor, but not ATP or cyclic AMP.[360,390] It is interesting that discadenine has cytokinin activity in tobacco callus growth assay, although the effect is weaker than that of kinetin.[391]

4.02.4.3.2(ii) Biosynthesis in Agrobacterium tumefaciens Cytokinin is also synthesized by phytopathogenic bacteria, such as *Agrobacterium tumefaciens* and *P. savastanoi*, and is a key factor in the formation of plant tumors.[392] The *A. tumefaciens* Ti plasmids commonly contain an IPT gene, designated as *Tmr*, on the T-region that is integrated into the nuclear genome of host plants after infection. Nopaline-forming Ti plasmids contain another IPT gene, *Tzs*, in the *vir* region.[393–395] *In vitro*, both Tmr and Tzs utilize DMAPP and HMBDP as a prenyl-donor substrate for the production of iP nucleotide and tZ nucleotide, respectively (**Figure 14**).[396,397] In contrast, crown galls and transgenic plants expressing *Tmr* most exclusively contain tZ-type cytokinins.[396,398–400] It has been demonstrated that Tmr protein is targeted to host plastids, where it utilizes HMBDP to produce the tZ-type cytokinins.[396] Another IPT, Tzs, is expected to function in bacterial cells and to play a role in the promotion of T-DNA transfer.[394,395]

4.02.4.3.3 Cytokinin biosynthesis in higher plants

Plant *IPT* genes have been identified from *Arabidopsis*,[401–403] petunia,[404] hop,[405] and rice[406] and constitute a small gene family. In contrast to *Agrobacterium* IPT, biochemical analysis revealed that plant IPT predominantly utilizes ATP and ADP rather than AMP as a prenyl acceptor[401,405,406] and DMAPP as the donor[396] (**Figure 14**).

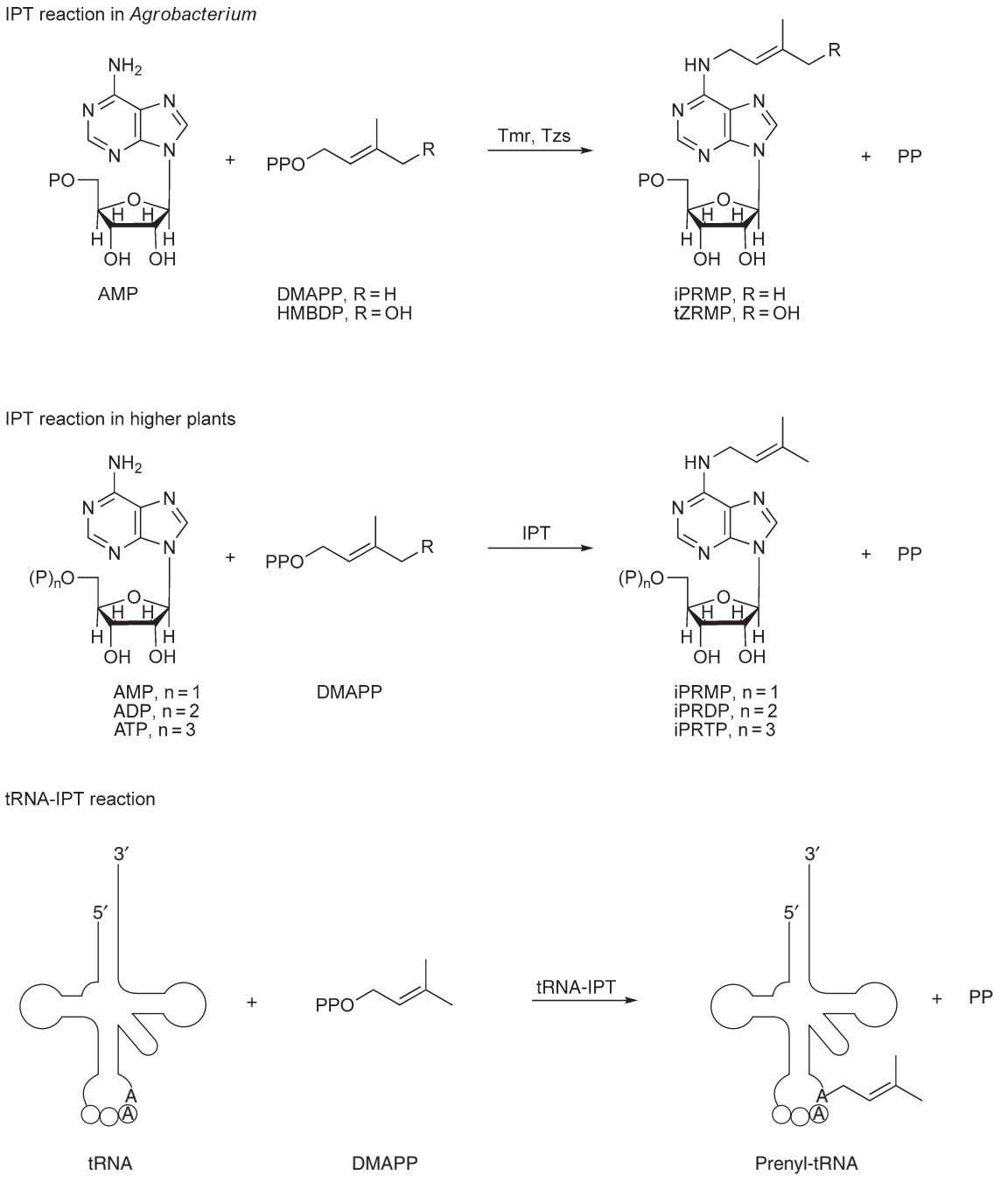


Figure 14 IPT and tRNA-IPT reaction for primary cytokinin biosynthesis. Circles and A in tRNA show anticodon and adenosine, respectively. iPRMP, iP riboside 5′-monophosphate; iPRDP, iP riboside 5′-diphosphate; iPRTP, iP riboside 5′-triphosphate; tZRMP, tZ riboside 5′-monophosphate; PP, diphosphoric acid.

Arabidopsis and rice have seven (*AtIPT1* and *AtIPT3–8*)[401,402] and eight (*OsIPT1–8*) *IPT* genes.[406] Each member of the *Arabidopsis* and rice *IPT* gene family has a unique spatial expression pattern and different sensitivity to plant hormones and nitrogen nutrition, suggesting the existence of functional differences among each member of the plant *IPT* gene family.[406–408] The multiple mutants of *AtIPT*s show severely decreased levels of iP- and tZ-type cytokinins, suggesting that plant IPTs are responsible for the bulk of iP- and tZ-type cytokinin synthesis.[409]

4.02.4.3.4 Cytokinin production by degradation of tRNA

Not only plants but also other organisms, such as mammals, yeasts, and bacteria, contain prenylated adenine in a subset of tRNA species.[410] The modification occurs at the 3′-adenosine adjacent to anticodons starting with adenine. It is thought that the presence of the prenyl group affects the stability of the mRNA–ribosome complex during translation. Degradation of tRNA molecules releases cytokinin, and several cytokinin species derived from tRNA degradation have been reported, such as iPR, methylthio-iPR, *cis*-zeatin riboside (cZR), tZR, and *cis*- and *trans*-methylthio-ZRs.[410] The first step of modification is catalyzed by tRNA-IPT (**Figure 14**). *Arabidopsis* has two *tRNA-IPT* genes, *AtIPT2* and *AtIPT9*,[401,402] and rice also has two homologous genes to *tRNA-IPT* (*OsIPT9* and *OsIPT10*).[406] In *Arabidopsis*, *AtIPT2* has been shown to complement a yeast mutant, *mod5*, deficient in yeast *tRNA-IPT*.[411] The *atipt2 atipt9* double mutant lacked tRNA with isopentenyl and *cis*-hydroxylated side chains, and also cZ-type cytokinins,[409] strongly suggesting that cZ-type cytokinins originate from tRNA degradation in *Arabidopsis*. On the other hand, the level of cZR in tRNA of the *atipt2* mutant was less compared to wild type, whereas the iPR level in tRNA was unchanged.[409] This observation implies that AtIPT2 might be able to transfer a *cis*-hydroxylated isopentenyl side chain to tRNA, although the predicted precursor, *cis*-hydroxyl dimethylallyl diphosphate (4-hydroxy-3-methyl-2-(*Z*)-butenyl diphosphate), has not been reported in any organism.

4.02.4.3.5 IPT *in* Physcomitrella patens

A moss, *Physcomitrella patens*, has at least one *tRNA-IPT* gene, *PpIPT1*, which complements a *tRNA-IPT*-deficient mutant in yeast; however, the *Physcomitrella* genome does not contain canonical *IPT* genes.[412] These results suggest that cytokinins in *P. patens* are produced mainly through the tRNA degradation pathway. Study of *Physcomitrella* will shed light again on tRNA-derived cytokinin synthesis and will yield important clues about the molecular evolution of cytokinin metabolism.

4.02.4.3.6 Metabolic origins of isoprenoid side chains

DMAPP, a prenyl-donor substrate in cytokinin biosynthesis, is an intermediate of both the methylerythritol phosphate (MEP) and mevalonate (MVA) pathways, whereas HMBDP, another substrate of *Agrobacterium* IPTs, is synthesized via only the MEP pathway. In general, the MEP pathway is found in bacteria and plastids, and the MVA pathway is found in the cytosol of eukaryotes.[413–415]

In *Arabidopsis*, AtIPT1, AtIPT3, AtIPT5, and AtIPT8 localize in the plastids[416] and the relative expression levels of *AtIPT3* and *AtIPT5* are higher than those of other *IPT*s.[407,408] A selective labeling experiment revealed that, in *Arabidopsis* seedlings, side chains of iP- and tZ-type cytokinins originate predominantly from the MEP pathway (**Figure 15**).[416] In contrast, AtIPT4 and AtIPT7 localize in the cytosol and mitochondria, respectively.[416] Application of lovastatin, an inhibitor of the MVA pathway, leads to a decrease in tZ-type cytokinin accumulation in tobacco BY-2 cell cultures, suggesting that the MVA pathway is also the predominant origin of tZ-type cytokinins at some specific developmental stages and conditions.[417,418] The selective labeling experiment showed that a majority of cZ-type cytokinin side chains are derived from the MVA pathway, although significant amounts of MEP-derived side chains were also observed[416] (**Figure 15**).

4.02.4.3.7 Activation step of cytokinin biosynthesis

The initial product of *de novo* cytokinin biosynthesis is cytokinin nucleotide. To become biologically active, the nucleotide has to be converted to a nucleobase. The reactions of cytokinin biosynthesis pathway are partly shared with a purine metabolic pathway, called the salvage pathway.[362,363] Exogenously applied cytokinin nucleobases are rapidly metabolized into the corresponding nucleoside and nucleotide.[419] Some of the enzymes in the purine salvage pathway have been characterized in *Arabidopsis* and tobacco. At least three members of the adenine phosphoribosyltransferase (APT) gene family in *Arabidopsis* catalyze the conversion from cytokinin nucleobase to the corresponding nucleotide as well as adenine.[419–421] In tobacco, ADK2S, an isoenzyme of adenosine kinases (ADKs), showed a higher efficiency for phosphorylation of cytokinin nucleosides *in vitro* than the other three ADK isoenzymes.[422] Adenosine phosphorylase has also been reported to play a role in the conversion of cytokinin nucleobase to its corresponding nucleoside.[423]

There are two pathways for producing active cytokinin species from the nucleotide: the two-step pathway and the direct pathway (**Figures 15** and **16**). In the two-step pathway, cytokinin nucleotides are converted to

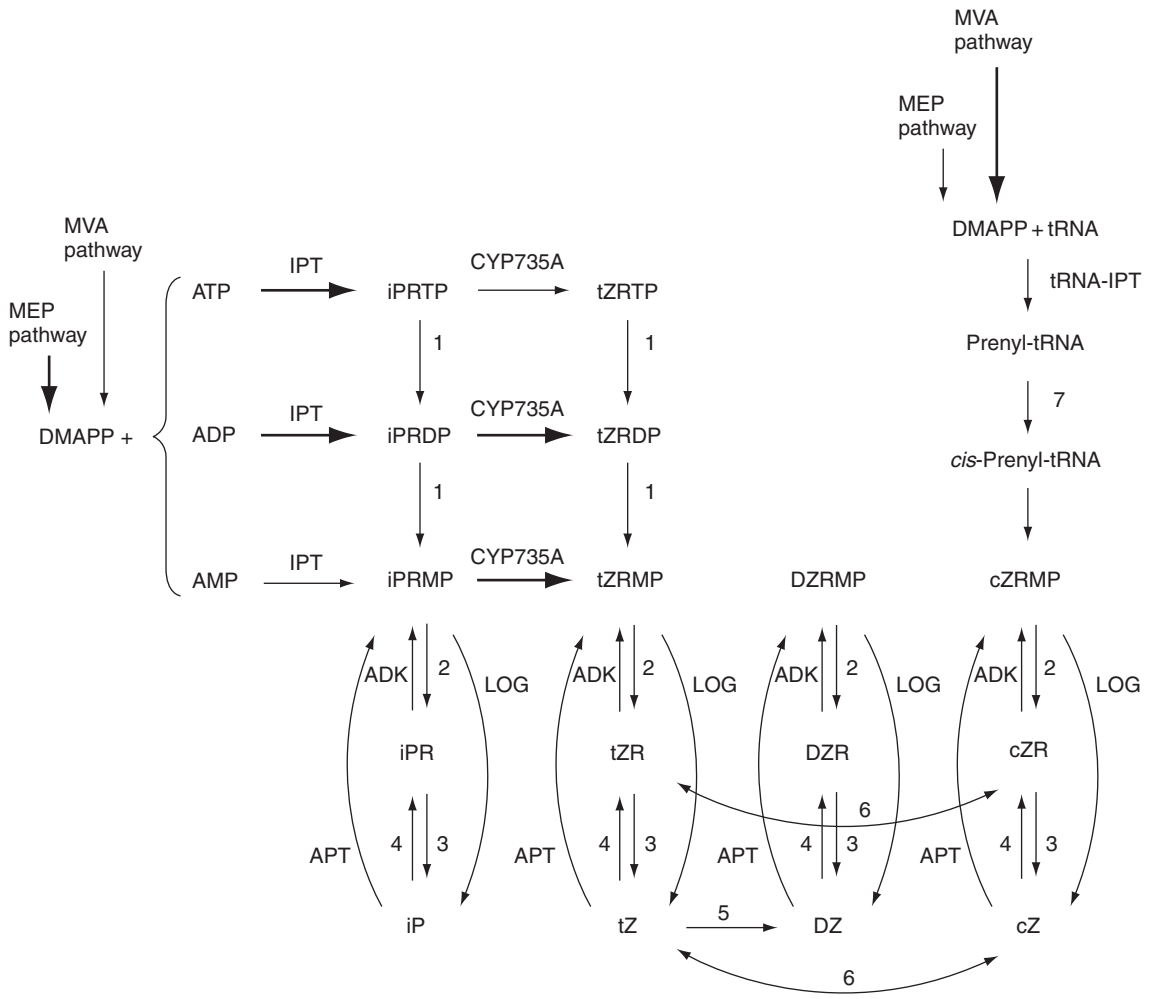


Figure 15 Current model of isoprenoid cytokinin biosynthesis pathway in higher plants. tZRDP, tZ riboside 5′-diphosphate; tZRTP, tZ riboside 5′-triphosphate; DZRMP, DZ riboside 5′-monophosphate; cZRMP, cZ riboside 5′-monophosphate; 1, phosphatase; 2, adenosine monophosphate 5-nucleotidase; 3, adenosine nucleosidase; 4, adenosine phosphorylase; 5, zeatin reductase; 6, zeatin *cis–trans* isomerase; 7, *cis*-hydrolase. The thick arrows show the main pathways.

their corresponding nucleobases by nucleotidase and nucleosidase.[362,363] These two enzymes were partially purified from wheat germ,[424,425] although the responsible genes have not been identified. The two enzymes use not only cytokinin nucleotide or nucleoside, but also AMP or adenosine with a higher affinity.[424,425] The direct pathway mediates the release of cytokinin nucleobase from the corresponding nucleotide in a one-step reaction (**Figure 16**). This enzyme, cytokinin riboside 5′-monophosphate phosphoribohydrolase, called LOG, was discovered through the analysis of rice *lonely guy* (*log*) mutants, which have defects in the maintenance of shoot meristems.[426] LOG catalyzes the formation of only cytokinin nucleotides (monophosphate) and not AMP, suggesting that LOG is a specific enzyme for cytokinin metabolism.[426] At present, it is not clear whether there is a functional differentiation between these two activating pathways or not.

4.02.4.3.8 Modification of isoprenoid side chain

4.02.4.3.8(i)* trans-*Hydroxylation of iP nucleotide for tZ biosynthesis In higher plants, the major pathway for tZ-type cytokinin synthesis is *trans*-hydroxylation of iP-type cytokinins.[396] Microsomal fractions prepared from cauliflower had an activity of *trans*-hydroxylation of iP and iPR, resulting in tZ and tZR,

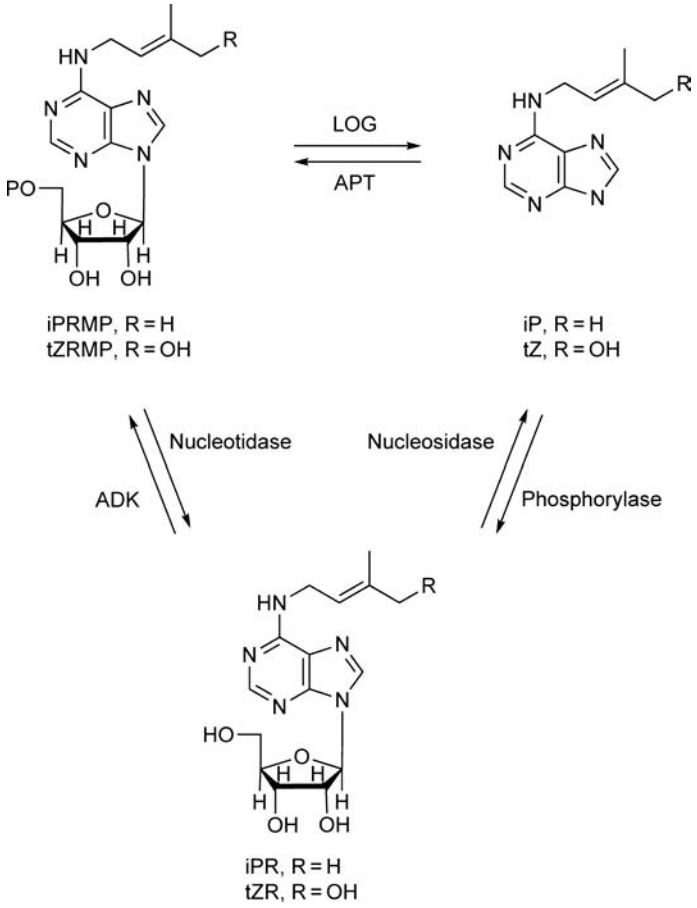


Figure 16 Current model of cytokinin activation in higher plants.

respectively, in the presence of NADPH.[427] In *Arabidopsis*, genes for CYP735A1 and CYP735A2, cytochrome P450 monooxygenases that catalyze *trans*-hydroxylation of iP-type cytokinins, were identified.[428] Contrary to the report from cauliflower microsomes, the CYP735As use iP nucleotides but not iP and iPR as substrates, and are preferential to the mono- or diphosphate rather than the triphosphate (**Figures 15** and **17**).[428]

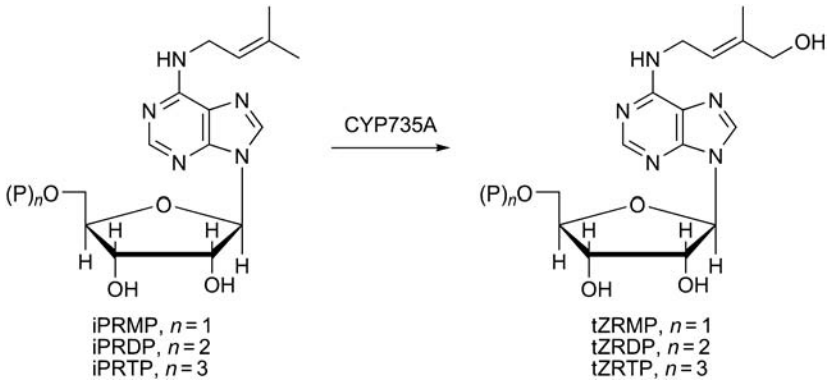


Figure 17 Hydroxylation of cytokinin in *Arabidopsis*.

4.02.4.3.8(ii) Dihydrozeatin formation The double bond of the tZ side chain could be enzymatically reduced by zeatin reductase, an enzyme characterized from immature seeds of *P. vulgaris*, resulting in DZ.[429] Zeatin reductase uses tZ as a substrate, but not cZ, tZR, iP, or tZ *O*-glucoside, and requires NADPH as a cofactor (**Figures 15** and **18**).[429] The responsible genes for this reaction have not yet been identified.

4.02.4.3.8(iii) Zeatin cis–trans *isomerase* In addition to degradation of tRNA, cZ-type cytokinins could be formed by isomerization of tZ, catalyzed by zeatin *cis–trans* isomerase. The isomerase was partially purified from immature seeds of *P. vulgaris*, and it catalyzes isomerization of zeatins in both directions in favor of cZ to tZ and also utilizes cZR and tZR as substrates (**Figures 15** and **19**).[430] In an *Arabidopsis* mutant that is deficient in the expression of four *AtIPT*s, the accumulation of tZ-type cytokinins was greatly reduced compared with that of the cZ-type cytokinins.[409] This result suggests that the *cis–trans* isomerization is a minor pathway for production of tZ-type cytokinins, at least in *Arabidopsis*.

4.02.4.3.8(iv) O-glucosylation and O-xylosylation tZ, cZ, and DZ and the corresponding nucleotides and nucleosides could be modified by O-glucosylation or O-xylosylation at the hydroxyl group of the side chain (**Figure 20**).[374] Genes encoding the zeatin *O*-glucosyltransferase (ZOG) and *O*-xylosyltransferase (ZOX) have been cloned and characterized in *Phaseolus lunatus* and *P. vulgaris*, respectively.[431,432] The ZOG, partially purified from immature embryos of *P. lunatus*, uses tZ as a substrate, but not cZ, DZ, or tZR.[433] Recombinant ZOG from *P. lunatus* uses tZ as a substrate more effectively than DZ.[432] In contrast, recombinant ZOX uses tZ and DZ as substrates, but not cZ and tZR.[431] In maize, the substrate preference of the enzyme is different from that of the *Phaseolus* enzymes; cisZOG1 and cisZOG2 predominantly utilize cZ over tZ (**Figure 20**).[371,434,435] The *ortho*- and *meta*-topolins are also substrates of ZOG1 and cisZOG1.[435] Although the role of *O*-glucosides in metabolic homeostasis is not fully understood, these modified cytokinins might be stable storage forms of the hormone because a maize β-glucosidase, Zm60.1, is reported to release active cytokinins from *O*-glucosides as well as *N3*-glucosides.[436]

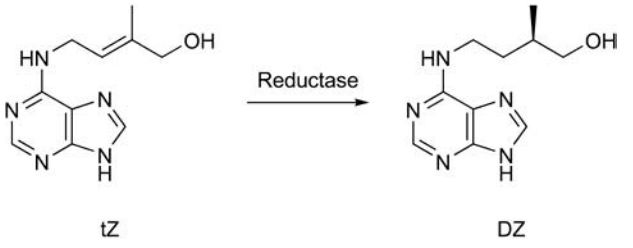


Figure 18 Reduction of cytokinin in *Phaseolus vulgaris*.

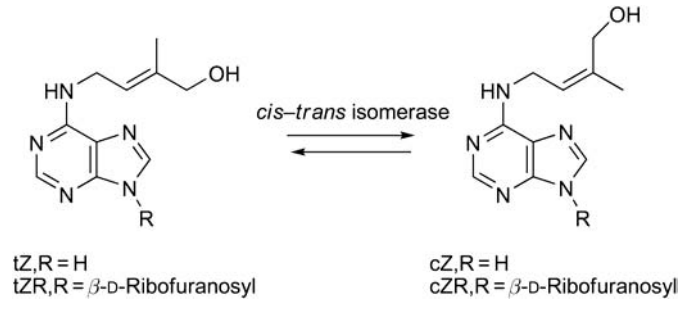


Figure 19 *cis–trans* isomerization of cytokinin in *Phaseolus vulgaris*.

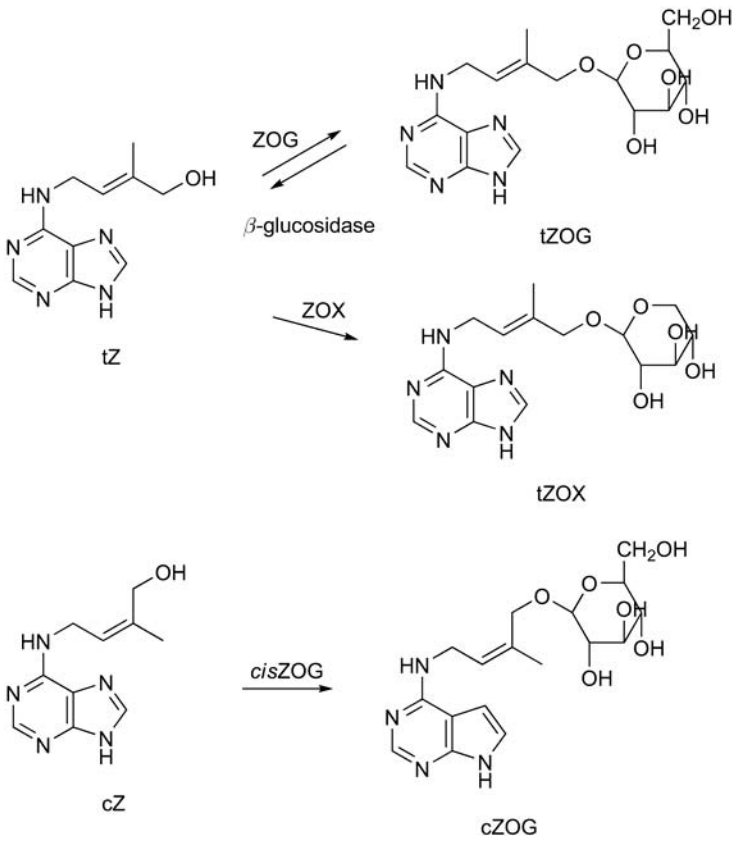


Figure 20 Current model of cytokinin O-glycosylation in higher plants. tZOG, tZ *O*-glucoside; tZOX, tZ *O*-xyloside; cZOG, cZ *O*-glucoside. ZOG and ZOX also utilize DZ for production of DZ *O*-glucoside (DZOG) and DZ *O*-xyloside (DZOX), respectively.

4.02.4.3.9 Modification of adenine moiety

In contrast to O-glycosylation, N-glucosylation at the N7 and N9 positions is thought to be an irreversible reaction.[436] In *Arabidopsis*, cytokinin *N*-glucosyltransferases, UGT76C1 and UGT76C2, conjugate glucose to all cytokinin nucleobases at the N7 or N9 position of the adenine moiety (**Figure 21**).[437] These enzymes could also conjugate glucose at the N7 position of tZ *O*-glucoside.[437] Enzymes catalyzing N-glucosylation at the N3 position have not yet been identified. Alanyltransferase, which converts zeatin to its alanyl derivative, lupinic acid (3-(N^6-(4-hydroxyisopentenyl)adeninyl)-L-alanine), was partially purified from lupin seeds but further characterization has not been done.[438]

4.02.4.3.10 Degradation of cytokinins

In addition to biosynthesis and conjugation to other molecules, degradation is an important step that controls active cytokinin levels. Cytokinin oxidase/dehydrogenase (CKX) mediates irreversible cytokinin degradation (**Figure 22**). CKX utilizes cytokinins with an unsaturated isoprenoid side chain such as iP and tZ and their nucleosides, nucleotides, and *N9*-glucosides with different affinities.[439–443] Biochemical and sequence analysis revealed that CKX is a flavoprotein, a member of an FAD-dependent oxidoreductase superfamily.[444] CKX reacts more efficiently as a dehydrogenase with an electron donor other than oxygen.[440,445]

In *Arabidopsis*, seven *CKX* genes, from *AtCKX1* to *AtCKX7*, have been identified and characterized.[443,446] Among *AtCKX*s, there are significant differences in the gene expression pattern, subcellular localization, pH optimum, and substrate specificity.[443,446] These results might be a reflection of functional differentiation among AtCKXs. Overexpression of each CKX results in a decrease in cytokinin content in tobacco[447] and

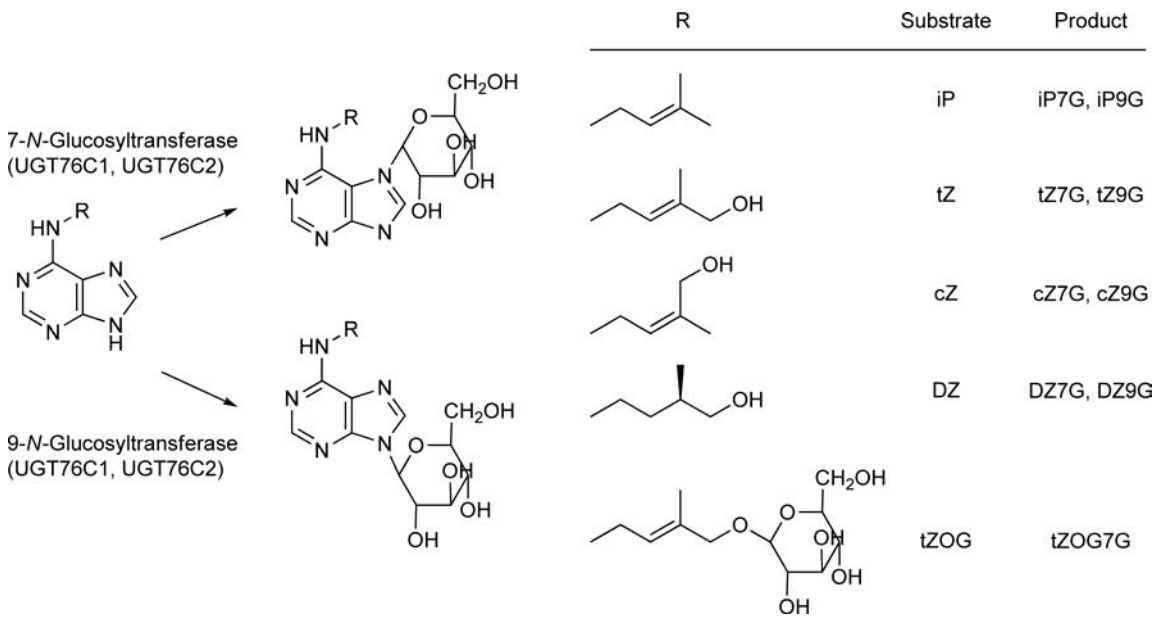


Figure 21 N-glycosylation of cytokinin in *Arabidopsis*. iP7G, iP *N7*-glucoside; iP9G, iP *N9*-glucoside; tZ7G, tZ *N7*-glucoside; tZ9G, tZ *N9*-glucoside; cZ7G, cZ *N7*-glucoside; cZ9G, cZ *N9*-glucoside; DZ7G, DZ *N7*-glucoside; DZ9G, DZ *N9*-glucoside; tZOG7G, tZOG *N7*-glucoside.

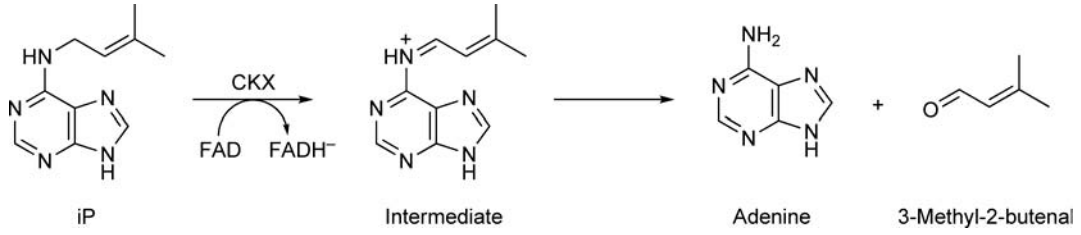


Figure 22 Current model of cytokinin degradation in higher plants. CKX utilizes also iPR, iPRMP, iP9G, tZ, tZR, and tZ9G in *Arabidopsis*. The second step of the reaction is likely nonenzymatic.

Arabidopsis,[446] whereas reduction of *OsCKX2* by natural variation results in increases in cytokinin levels in rice,[448] indicating the central role of CKX in the control of cytokinin levels in plants.

4.02.4.4 Translocation

Recent studies on purine and nucleoside transporters suggest that these proteins may function in cytokinin transport. By using a heterologous expression system in budding yeast, Arabidopsis thaliana purine permease 1 (AtPUP1) was characterized as a purine permease that imports adenine, cytosine, hypoxanthine, caffeine, and cytokinin nucleobases.[449] The expression of *AtPUP1* was observed in hydathodes and stigma, suggesting a role in the retrieval of purines and cytokinins from xylem sap to prevent loss during guttation.[450] On the other hand, adenine transport by AtPUP2 in the same system was strongly inhibited by iP and BA,[450] suggesting involvement in cytokinin nucleobase transport. Expression of *AtPUP2* in leaf phloem implies a potential role in phloem loading and transport of adenine and cytokinins.[450]

Equilibrative-type nucleoside transporters (ENTs) were also characterized in rice[451] and *Arabidopsis*[370] in reference to cytokinin nucleoside transport using the yeast system. One of the four rice ENT gene products, OsENT2, mediates the uptake of cytokinin nucleoside as well as that of adenosine[451] with higher affinity to iPR

than tZR. In *Arabidopsis*, AtENT6 and AtENT8 can potentially mediate the uptake of cytokinin nucleoside.[370,452] AtENT6 also prefers iPR over tZR.[370] However, these results were obtained by *in vitro* studies using the heterologous yeast expression system. Further characterization using loss-of-function mutants should provide definitive evidence for the physiological role of the cytokinin transport candidates.

4.02.4.5 Biological Activities

4.02.4.5.1 Delay of leaf senescence

Leaf senescence is characterized by loss of chlorophyll, leaf yellowing, degradation of proteins, membrane lipids, and RNA, and recycling to young tissues.[453] Delay of leaf senescence by exogenous application of cytokinins has been confirmed by numerous studies,[454] suggesting that cytokinins are key components in the regulation of leaf senescence.

Various studies have been designed to modify cytokinin metabolism to elucidate the role of cytokinin in the regulation of leaf senescence. In tobacco, introduction of an *Agrobacterium* cytokinin biosynthesis gene, *IPT*, under the control of a senescence-associated gene promoter results in suppression of leaf senescence.[455] Similar effects are obtained in lettuce,[456] petunia,[457] tomato,[458] and broccoli,[459] but not in maize.[460] Transgenic petunia overexpressing its own *IPT* gene also results in delay of leaf senescence,[404] whereas early leaf senescence was not observed in cytokinin-deficient tobacco and *Arabidopsis* overexpressing *CKX*.[446,447] Since the CKX-overexpressing plants drastically enhanced root growth and reduced shoot growth,[446] it is possible that alteration in sink–source relations might also interfere with the normal mechanism of senescence.

By means of genetic approaches, an *Arabidopsis* cytokinin receptor, *Arabidopsis* histidine kinase 3 (AHK3; see Section 4.02.4.6.2), was found to play a major role in the control of leaf senescence.[461,462] Analyses of the effect of exogenous cytokinin on chlorophyll retention by detached leaves of cytokinin receptor mutants showed that *AHK3* plays an important role in mediating cytokinin-dependent chlorophyll retention,[462] although cytokinin receptor mutants showed no phenotype in early leaf senescence similar to the result described above for the *CKX*-overexpressing plants.[462] A low cytokinin content or reduced cytokinin signaling may not be a triggering factor for the onset of the senescence process. The molecular analyses of a gain-of-function mutant of *AHK3*, which exhibits a delay in leaf senescence, revealed that AHK3 plays a major role in cytokinin-mediated control of leaf longevity.[461]

4.02.4.5.2 Control of the cell cycle

Several studies have implicated a role for cytokinins in the regulation of both the G_1–S cycle[418,463] and the G_2–M phase transition[417,464] of the cell cycle. Cytokinin activates *Arabidopsis* cell division through induction of D-type cyclin, CycD3, at the G_1–S cell cycle phase transition.[463,465] A sharp increase in the levels of cytokinins was reported in tobacco cell cultures at the G_2–M phase.[417] Roots of multiple mutants of cytokinin receptor genes show delay of the transition in G_2–M phase.[466]

4.02.4.5.3 Control of shoot meristem activity

Overexpression of *CKX* leads to reduced meristem activity in *Arabidopsis* shoots.[438] Moreover, multiple mutants of *Arabidopsis* genes related to cytokinin biosynthesis and signaling show a reduction in meristem activity in shoots.[409,466–468] Recent research reveals that modifying cytokinin metabolism improves crop yields. In rice, reduced expression of a *CKX*, *OsCKX2*, results in increased levels of cytokinins in inflorescence meristems and an increased number of reproductive organs, resulting in enhanced grain yield.[448] Conversely, loss of function of a gene for the cytokinin-activating enzyme, LOG, which is expressed in shoot apical meristems, leads to premature termination of cell division in rice shoot meristems.[426] This result suggests that meristem cells autonomously produce active cytokinins for continuous cell division.

In shoot apical meristems, cytokinin and cytokinin-related regulatory networks are necessary for maintenance of stem cells.[469,470] One of the network components is a transcription factor, Class I KNOTTED1-like homeobox (KNOXI), that determines the identity of the shoot apical meristem, and it positively regulates *IPT* genes in *Arabidopsis* and rice, but negatively regulates gibberellin biosynthesis.[406,471,472] Another component of this network is a homeobox protein, WUSCHEL (WUS), that directly and

negatively regulates a subset of genes related to cytokinin signaling, type-A *Arabidopsis* response regulators (ARRs; see Section 4.02.4.6.4) in *Arabidopsis*.[473]

4.02.4.5.4 Control of root meristem activity

Similar to the patterns of activity in the shoot meristem, multiple mutants of *Arabidopsis* genes related to cytokinin signaling display reduced cell division activity in the root meristem,[466–468] whereas the phenotype is not observed in adventitious roots.[468,474] The reduced cell division activity in primary roots of these mutants might be a side effect of a structural defect in roots and/or caused by a distinct signaling network for activation of cell division between primary and adventitious roots. Transgenic plants with reduced cytokinin levels or signaling lead to increases in root meristem size and activity.[409,446,475] These results lead to the assumption that cytokinins might have two opposing effects on root growth, one inhibitory and another stimulatory; a small reduction in cytokinin level or signaling activity would result in increased activity of the root meristem, but a reduction beyond a critical threshold would result in decreased activity.[476] Exogenous application of cytokinin and molecular analyses using cytokinin metabolism and signaling mutants revealed that cytokinins regulate root meristem size in the elongation zone by controlling cell differentiation.[475] This regulation appears to be mediated by *AHK3* and type-B ARRs, *ARR1* and *ARR12*.[475]

4.02.4.5.5 Regulation of vascular development

Cytokinins have a crucial role in the continuous division of both procambial cells and vascular cambium cells, processes that provide precursor cells for xylem and phloem.[477,478] Mutation of cytokinin receptor gene, *ARABIDOPSIS HISTIDINE KINASE 4/CYTOKININ RESPONSE 1/WOODEN LEG* (*AHK4/CRE1/WOL*), leads to reductions in cell files and differentiation of all procambial cells into protoxylem.[479] A similar phenotype is also observed in multiple mutants of *Arabidopsis* genes related to cytokinin signaling,[468,474,480] indicating that genes involved in cytokinin signaling are important regulators of vascular development.

The double mutant of *wol* and *fass*, a mutation resulting in supernumerary cell layers, shows an increase in the number of vascular cell layers with phloem markers.[471] Furthermore, exogenous application of cytokinin and postembryonic expression of *CKX* under the *AHK4/CRE1/WOL* promoter show phenocopy of the *wol* mutant.[473] These results indicate that cytokinin signaling through a phosphorelay-mediated pathway is required to promote procambial cell division and is also required to promote and maintain cell identities other than protoxylem. This signaling is negatively regulated by pseudo-histidine phosphotransfer protein (pseudo-HPt; see Section 4.02.4.6.3) called AHP6.[481] In the shoots of cytokinin-deficient plants that are overexpressing cytokinin oxidase, the number of both xylem and phloem cells is reduced, indicating that the changes are the result of an overall reduction of cambial activity without affecting cell differentiation specifically.[446,447] This result supports a role for cytokinins in the promotion and maintenance of cambial cell division in addition to procambial cell division.

4.02.4.5.6 Release of buds from apical dominance

Auxin, cytokinin, and other unknown substances are thought to regulate apical dominance.[482] Application of cytokinin or overexpression of the *IPT* gene shows a phenotype with a reduction in apical dominance.[404,483,484] *Arabidopsis* mutants with increased levels of cytokinin also show reduced apical dominance.[485–487] These results suggest that cytokinins play a role in the promotion of bud outgrowth.

Studies using pea have suggested that decapitation leads to increased levels of endogenous cytokinins in the stem and/or xylem sap[488,489] and increased delivery of cytokinins to axillary buds.[490,491] Decapitation of pea leads to increased cytokinin levels in axillary buds following an induction of *IPT* genes in the stem.[491] These lines of evidence suggest that cytokinins synthesized in the stem are transported to the axillary buds after decapitation, resulting in promotion of bud outgrowth. Increases in cytokinin levels and pea *IPT* expression after stem decapitation are partially repressed by auxin application.[489,491] It is not clear whether an increased cytokinin level in buds occurs prior to bud outgrowth, or is the cause of bud outgrowth.

4.02.4.5.7 Regulation of root formation

Plant hormones[492,493] and various nutrients[494] regulate the formation of postembryonic roots. It is well known that cytokinin application inhibits the formation of lateral and adventitious roots, whereas auxin promotes root

formations in various plants.[492] Transgenic tobacco[447] and *Arabidopsis*[446] plants with a reduced cytokinin content due to the overexpression of a *CKX* showed an increase in lateral and adventitious root number. In *Arabidopsis*, *ahk2 ahk3* double knockout mutant showed enhanced lateral root formation.[462] Multiple mutants of *Arabidopsis* genes related to cytokinin signaling showed reduced sensitivity of lateral root formation to cytokinin.[468,495] These results indicate that cytokinins, through the signaling pathway in phosphorelay system, negatively regulate lateral root formation.

Lateral roots in *Arabidopsis* originate from pericycle cells.[493] A critical event in lateral root formation is reentry of differentiated pericycle cells into the cell cycle and initiation of the root developmental program. Paradoxically, cytokinins inhibit the initiation of lateral roots by blocking the pericycle founder cells cycling at the G_2-to-M transition phase,[496] although the hormone is usually considered to be a positive regulator of the cell cycle.[463,464] Cytokinins might have two opposing effects on cell cycling at this phase, and lateral root formation is associated with spatial and temporal regulation of the cytokinin level and/or signaling.[476] With exogenous application of cytokinin, there is a significant difference in growth between primary and lateral roots.[496]

A root-derived suppressor negatively regulating the formation of roots has been hypothesized.[497] This proposed mechanism, termed 'root apical dominance,' is analogous to the means for apical dominance in shoots. The cytokinin, tZR, in root xylem sap is hypothesized to play the role of the main suppressor, and tZR transported from roots to shoots via the transpiration stream negatively regulates the formation of adventitious roots.[365]

4.02.4.5.8 Regulation of nodule formation

Cytokinins play an important role in the nodule development of legumes. Transfer of cytokinin production allows normally nonsymbiotic bacteria to activate nodule formation in alfalfa,[498] suggesting that increased levels of cytokinin could induce nodule formation. Moreover, overexpression of *CKX* results in decreased numbers of nodules in *Lotus japonicus*,[499] and suppression of cytokinin receptor expression by RNA interference reduces nodulation in *Medicago truncatula*.[500] Furthermore, genetic approaches using legumes revealed that cytokinin alone is sufficient to activate nodule formation. Loss-of-function mutations in the *Lhk1* (*Lotus histidine kinase* 1) gene, a cytokinin receptor of *L. japonicus*, abolish nodule primordium formation, but do not affect bacterial invasion of the root.[501] Conversely, a gain-of-function mutation in *Lhk1* results in spontaneous formation of nodules in the absence of rhizobial bacteria.[502] These observations suggest that activation of Nod factor signaling at the epidermis in legumes leads to increased production of cytokinins, which are then transported to the root cortex and perceived by LHK1. This signaling would lead to initiation of cell division, resulting in the formation of nodule primordia.

4.02.4.5.9 Interactions between macronutrients and cytokinins

A close correlation between nitrogen nutrition and cytokinin content has been noted, and nitrate application induces rapid accumulation of cytokinins in the roots of barley,[503] maize,[504] and *Arabidopsis*.[408,505] In *Arabidopsis* roots, application of nitrate initiates tZ synthesis through induction of cytokinin biosynthesis enzymes, *IPT3*[408] and *CYP735A2*.[428] This production of tZR plays a role in sending a long-distance signal from roots to shoots via xylem vessels. Application of nitrate and cytokinin induces expression of variable genes involved in metabolism and developmental programs, suggesting that cytokinins are secondary messengers in the regulation of these genes.[506] Alternatively, cytokinin application represses expression of some macronutrient transporters for ions such as nitrate, ammonium, sulfate, and phosphate.[506] In addition, some genes of nitrate transporters are induced by nitrate, whereas genes of other macronutrient transporters such as ammonium, sulfate, and phosphate are induced by substrate starvation.[506] Repression of sulfate transporters, *SULTR1;1* and *SULTR1;2*, has been reported to be mediated by the cytokinin receptor, AHK4/CRE1/WOL,[507] and the phosphate transporter, *PHT1;1*, is reported to be repressed by AHK3 and AHK4/CRE1/WOL.[508] These results imply that cytokinins might act as messengers to communicate whether the nitrogen supply is sufficient to upregulate the uptake systems for other macronutrients.

4.02.4.6 Perception and Signaling

4.02.4.6.1 Cytokinin-binding proteins

To understand the mechanism of cytokinin perception, many investigators have searched for cytokinin-binding proteins (CBPs);[509] however, the biological function of CBPs as cytokinin receptors remains to be confirmed. *S*-adenosyl-L-homocysteine hydrolase,[510] endochitinase,[511] and osmotin-like protein[511] were isolated as CBPs by purification of proteins having cytokinin-binding ability. Fujimoto *et al.*[512] isolated a CBP from mung bean by affinity chromatography using phenylurea as a probe. This protein, known as *Vigna radiata* cytokinin-specific binding protein (VrCSBP), has sequence similarity to plant pathogenesis-related class 10 (PR-10) proteins. VrCSBP was crystallized in a complex with tZ and the binding mode of VrCsBP to tZ was determined;[513] however, the biological importance of VrCsBP as a receptor has not been elucidated. Thus, clear proof for the function of soluble CBPs is still lacking.

4.02.4.6.2 Cytokinin receptor as a histidine protein kinase (His-kinase)

Advances in molecular genetics of model plants, especially *Arabidopsis*, have brought rapid progress in understanding the mechanisms of cytokinin signal transduction. The first candidate gene for a membrane-bound cytokinin receptor was *CYTOKININ INSENSITIVE 1* (*CKI1*).[514] Overexpression of *CKI1* showed typical phenotypes of cytokinin response including shoot formation from calli independent of exogenous cytokinins.[514] The *CKI1* encodes a protein containing a putative input domain with two membrane-spanning regions, a His-kinase domain, and a receiver domain. This report first implied that cytokinin signaling in plants is perceived and transduced by a two-component system, a mechanism prevalent in bacteria with phosphotransfer between His and Asp residues. However, no data further supporting that CKI1 is directly involved in cytokinin perception have been provided. The His-kinase activity of CKI1 is constitutively active when the gene is expressed in *Escherichia coli* despite the absence of exogenous cytokinins.[515] Knockout mutants of *CKI1* show a lethal phenotype in female gametophytes, demonstrating the importance of this enzyme in plant development.[516]

AHK4/CRE1/WOL, another His-kinase of *Arabidopsis*, was also identified as a cytokinin receptor.[517–519] The *ahk4/cre1/wol* mutant conferred resistance to exogenous cytokinins.[517,519] Introduction of *AHK4/CRE1/WOL* into a His-kinase mutant of budding yeast,[517] fission yeast,[518] or *E. coli*[518] complemented the mutation in a cytokinin-dependent manner. Furthermore, the AHK4/CRE1/WOL protein, expressed in fission yeast or *E. coli*, binds to iP with high affinity: $K_d = 4.5 \times 10^{-9}$ mol l^{-1} [515] and 2.5×10^{-9} mol l^{-1},[520] respectively. These lines of evidence prove that AHK4/CRE1/WOL functions as a cytokinin receptor. The extracellular domain at the N-terminal region of AHK4/CRE1/WOL is designated as cyclase/histidine kinase-associated sensing extracellular (CHASE) domain, which is found in prokaryotes, lower eukaryotes, and plants.[521] Evolutionary proteomics analyses using the CHASE domain proposed five amino acids to be involved in cytokinin binding.[522] AHK4/CRE1/WOL binds not only the nucleobase of isoprenoid cytokinins such as iP and tZ, but also BA[515,520] and diphenylurea.[515]

Two other His-kinases, AHK2 and AHK3, were also identified as cytokinin receptors in *Arabidopsis* (**Figure 23**).[515] AHK3 and AHK4/CRE1/WOL differ in the ligand specificity: AHK3 has a lower affinity to iP, but a higher affinity to DZ, than AHK4/CRE1/WOL.[523] In other species, maize His-kinase ZmHK1, ZmHK2, and ZmHK3a[524] and rice His-kinase OsHK1–4[525] have been shown to function as cytokinin receptors.

The *AHK4/CRE1/WOL* gene was actually first identified as a causal gene of the *wol* mutant, which confers a reduced number of root vascular cells;[479] however, single mutants of *AHK2* or *AHK3* and the double mutant of these receptors did not affect root morphology.[466,467] Only AHK4/CRE1/WOL has strong phosphatase activity in the phosphorelay network,[526] and *AHK4/CRE1/WOL* is expressed more strongly than *AHK2* or *AHK3* in the root procambium, which forms the primary xylem in vascular tissues.[479] The differences in expression patterns and biochemical nature might lead to the different root phenotypes of the mutants.

The *ahk2 ahk3 ahk4/cre1/wol* triple mutant displays a more severe phenotype in roots and shoots than any of the other double mutant combinations, indicating that three cytokinin receptors have overlapping functions.[462,466,467] Although the triple mutant shows no cytokinin responses, this plant can germinate and produce the basic plant organs,[466] suggesting that either cytokinin is not required for gametogenesis and embryogenesis, or there might be another cytokinin receptor for the early stages of plant development.

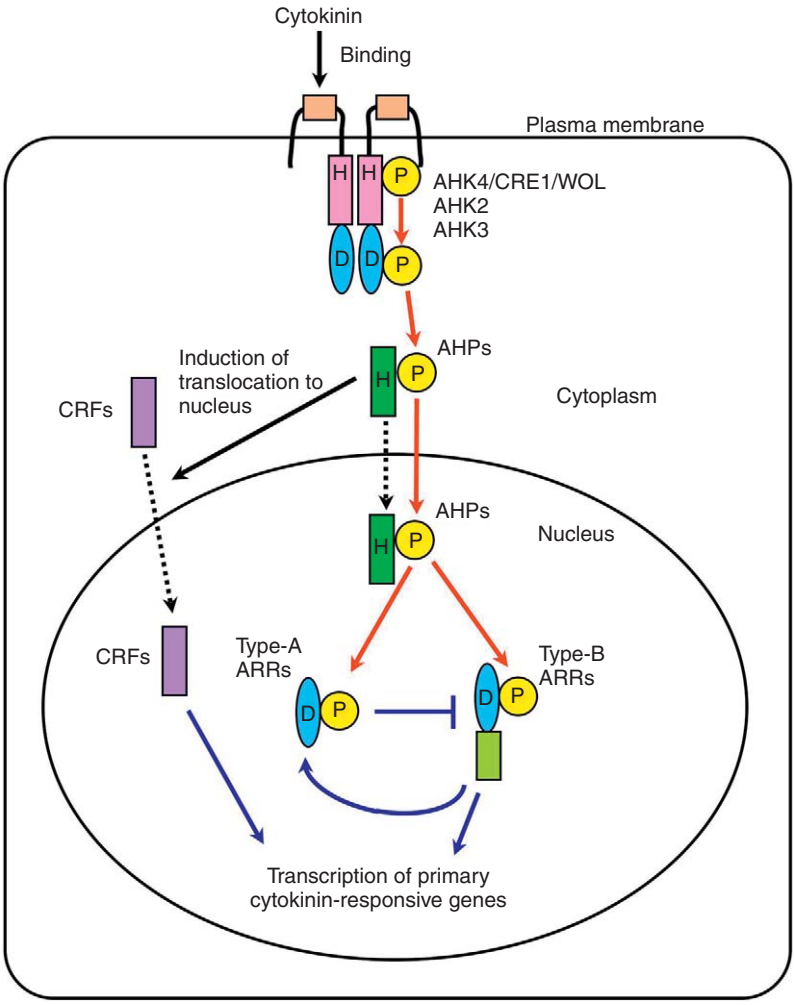


Figure 23 Regulatory network of growth and development mediated by cytokinin. Arrows and blunted lines indicate positive and inhibitory interactions, respectively; solid lines show direct molecular interactions; and dashed lines show interactions that are indirect. H, His residues; D, Asp residues; encircled P, phosphoryl group.

4.02.4.6.3 Phosphotransfer proteins

On ligand binding, the receptors are autophosphorylated at the conserved His residue within the receptor's kinase domain. The phosphoryl group is first transferred from His to a conserved Asp residue in the receptor's receiver domain,[526,527] and then, the phosphoryl group is transferred to a His phosphotransfer protein (HPt) that shuttles between the nucleus and cytosol (**Figure 23**).[526–528] In *Arabidopsis*, five *Arabidopsis* HPt (AHP) genes from a family of six AHP-related proteins, AHP1–5, were demonstrated to be genuine phosphotransfer proteins by functional complementation of a yeast mutant and by *in vitro* assays for phosphate perception and transfer abilities.[529–531] Overexpression of *AHP2* in *Arabidopsis* results in a slight hypersensitivity to exogenous cytokinin treatment.[532] Multiple variant of *ahp*s (e.g., *ahp1 ahp2 ahp3 ahp4 ahp5*) shows reduced cytokinin sensitivity although single or double *ahp* mutants are indistinguishable from wild type in cytokinin response assays, suggesting that the AHPs have a positive and redundant function in cytokinin signal transduction.[468] On the other hand, the His residue is not conserved in AHP6.[481] AHP6 is predicted to be a negative regulator of cytokinin signaling, likely via a dominant negative mechanism.[481]

4.02.4.6.4 Response regulators

In the two-component system, the final acceptor of the phosphoryl group is a response regulator, whose activity is switched by the phosphorylation state of the conserved Asp residue. Plant response regulator genes were first identified as cytokinin-inducible genes in *Arabidopsis*[533] and maize.[504] Twenty-four *Arabidopsis* response regulator (ARR) genes are found in the *Arabidopsis* genome and are basically divided into two classes, the A and B types, based on their structure and expression properties.[534,535]

Type-A ARRs (ARR3–9, ARR15–17) have a conserved receiver domain and a short C-terminus and their transcription is rapidly increased in response to exogenous cytokinin.[534,536–538] The phosphorylation of the receiver domain is required for type-A ARRs function.[539] Overexpression of *ARR4–7* in *Arabidopsis* protoplasts inhibits the expression of the luciferase gene controlled by the *ARR6* promoter.[526] Overexpression of *ARR15* decreases sensitivity to cytokinin treatment.[540] In addition, multiple type-A *arr* loss-of-function mutants are hypersensitive to cytokinin in various assays, although their single mutants have no detectable change in cytokinin responsiveness.[495] These results strongly suggest that type-A ARRs are negative regulators of cytokinin responses, and that there are highly overlapping functions among members of this gene family in *Arabidopsis*. This negative regulation of type-A response regulator is likely conserved in higher plants.[541] In maize, a single type-A mutation (*abphyl1*) causes an alteration of leaf phyllotaxy, suggesting a difference in the level of genetic redundancy among species.[542]

Conversely, the 11 type-B ARRs (ARR1, ARR2, ARR10–14, ARR18–21) have a C-terminal output domain that has a DNA-binding and transcriptional activation domain. Expressions of type-B ARRs are not appreciably affected by cytokinin application.[543] ARR1, ARR2, and ARR10 activate the transcription of a subset of type-A *ARRs*.[526] Overexpression of truncated forms of *ARR1*, which lack the receiver domain, causes an increase in cytokinin sensitivity, suggesting that the receiver domain negatively regulates the output function of these proteins in the absence of cytokinin.[544] Although single loss-of-function mutants such as *arr1*, *arr10*, and *arr12* do not confer visible phenotypes, the *arr1 arr10 arr12* triple mutant displays almost complete insensitivity to cytokinin.[545] These results indicate that there is functional overlap among type-B ARRs and that they act as positive regulators of cytokinin signal transduction.

There are three additional ARRs (ARR22–24) in the *Arabidopsis* genome. Overexpression of *ARR22* shows a similar phenotype to that of the *wol* mutant, a loss-of-function mutant of a cytokinin receptor, suggesting that ARR22 might be an element in the cytokinin-responsive phosphorelay signal transduction pathway.[537]

4.02.4.6.5 Branch of the two-component pathway

Several members of *Arabidopsis* APETALA2 (AP2) transcription factors, cytokinin response factors (CRFs), are transcriptionally upregulated by cytokinin.[546] The CRF proteins rapidly accumulate in the nucleus in response to cytokinin, and this translocation depends on AHKs and AHPs, but is independent of ARRs (**Figure 23**).[546] Comparison of the cytokinin response in type-B *arr* mutants and *crf* mutants revealed substantial overlap among the genes regulated by these two families of transcription factors.[546] These results suggest that CRFs function in tandem with type-B ARRs as a branch of an evolutionarily ancient two-component system for cytokinin response.

4.02.5 Abscisic Acid

Since the publication of the previous edition of the book *Comprehensive Natural Products Chemistry* in 1999,[547] research on abscisic acid (ABA, **1**) has progressed especially concerning its biosynthesis, catabolism, and signal transduction using molecular biology techniques. In this chapter, descriptions of these subjects have been extensively revised and the chemistry of ABA is described in brief although the essential information has been retained.

4.02.5.1 Introduction

ABA was discovered by three independent lines of research.[548] Carns and coworkers found that an auxin inhibitor, 'abscisin,' was involved in the abscission of immature cotton fruits. However, Ohkuma and other

members of Addicott's group failed to isolate the inhibitor from the hexane extracts of cotton burs, and later isolated a new inhibitor, 'abscisin II,' that showed a high activity in the abscission test from the acetone extracts of immature cotton fruits. The basic structure of 'abscisin II' was reported by Ohkuma *et al.*[549] in 1965, and Cornforth *et al.*[550] soon confirmed its structure by synthesizing it. Van Steveninck suggested that an auxin inhibitor that was produced in the basal immature seeds promoted the abortion of the apical flowers of inflorescences in yellow lupine. This inhibitor was identified as the same compound as 'abscisin II' by Koshimizu *et al.*[551] in 1966. Phillips and Wareing suggested that the dormancy of terminal buds of sycamore maple was regulated by a growth inhibitor, 'dormin,' that was formed in the leaves. The structure of 'dormin' was found to be the same as 'abscisin II' by Cornforth *et al.*[552] in 1965. The two names 'abscisin II' and 'dormin' were unified to 'abscisic acid' and the abbreviation 'ABA' was agreed at the Sixth International Congress on the Plant Growth Substances in 1967.[553]

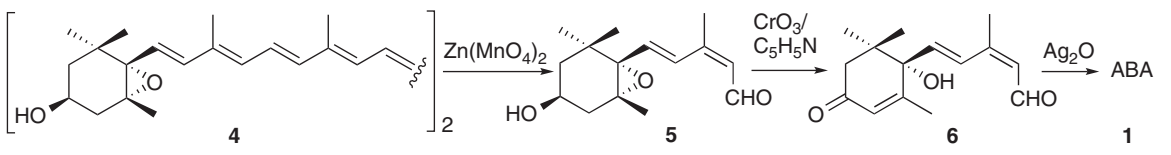
ABA has an asymmetric carbon at the C-1′, and natural ABA is a (+)-isomer. Cornforth *et al.*[554] applied Mills's rule to 1′,4′-*cis*-diol (**2**) and 1′,4′-*trans*-diol (**3**) of ABA converted from (+)-ABA, and proposed that the absolute configuration of (+)-ABA is *R*. (The absolute configuration proposed by Cornforth *et al.* in 1967 was *S* according to the old definition of *R*/*S*, but since the definition of *R*/*S* was changed in 1966,[555] the new definition is used to avoid confusion.) Doubt was cast on the absolute configuration by Taylor and Burden[556] in 1972, who showed a conversion of violaxanthin (**4**) to (+)-ABA via xanthoxin (**5**) and ABA-aldehyde (**6**) as shown in **Scheme 1**. They suggested that the configuration of the C-1′ of (+)-ABA is *S* since the stereochemistry of C-6 of violaxanthin had been elucidated by X-ray analysis to be *S*. The absolute configuration of the C-1′ of (+)-ABA was finally concluded to be *S* by various methods.[557–560]

ABA occurs in all higher plants, ferns, mosses, liverworts, and algae. In early studies, ABA was not detected in liverworts, and Pryce[561] had proposed that lunularic acid, which is a derivative of stilbene, plays an ABA-like physiological role in liverworts. However, subsequent work using a GC-SIM analysis showed that two species of liverworts contained ABA.[562] As a result of the universal distribution in the plant kingdom and many physiological studies, ABA has been classified as a plant hormone.[548,563] Interestingly, ABA occurs in other organisms in addition to plants. More than 20 species of phytopathogenic fungi including *Cercospora* and *Botrytis* are known to produce ABA in culture media.[564,565] A strain of *Botrytis cinerea* that does not produce ABA is virulent to tomato plants,[566] but ABA may increase susceptibility of tomato to *B. cinerea* by suppressing salicylic acid-dependent signaling mechanisms.[567] There is still controversy on the pathological function of ABA in fungi. Natural ABA produced by fermentation of *B. cinerea* is available at a low price, so the chemical synthesis of ABA itself is not practical at present. It was reported by an Italian research group that ABA occurred in marine sponges and hydroids, and that ABA was an endogenous cytokine in human granulocytes with cyclic ADP-ribose as a second messenger.[568] The presence of ABA in mammalian brains was also reported,[569] suggesting that ABA may be a hormone in mammals; however, the occurrence of ABA in marine sponges, hydroids, human granulocytes, and mammalian brains has not been elucidated, and more experiments are needed to establish ABA as a real universal hormone.

4.02.5.2 Chemistry

4.02.5.2.1 Isolation and analysis

ABA is extracted from plants and other sources usually by methanol. Methanolysis of glucosyl ester of ABA sometimes occurs to give its methyl ester as an artifact,[570] and acetone is used for extraction to avoid the methanolysis. The methyl ester of ABA is not always an artifact. Its occurrence in stigma of tobacco has been confirmed by extraction using acetone.[571] Ethyl acetate is used to extract ABA and its natural catabolites,



Scheme 1

phaseic acid (**7**), and dihydrophaseic acid (**8**) and its epimer (**9**), from an aqueous solution after concentration of a methanol solution. ABA is a weak acid with a pK_a of 4.7, and the partition coefficient between ethyl acetate and water at pH 2.5 is 10.[572] Glucosyl ester (**10**) and other conjugated forms of ABA and the catabolites are extracted from the aqueous solution by *n*-butyl alcohol saturated with water.

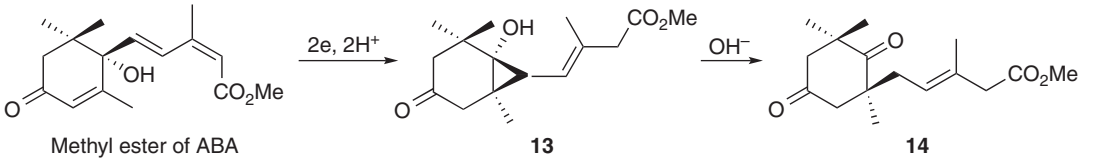
Isolation and chromatographic analysis of ABA are according to a method for a weak acid. Column packings suitable for a rough separation of ABA are active charcoal, silica gel, and others. ABA is observed as a dark spot on a thin-layer plate of silica gel containing fluorescent dye under UV irradiation at 254 nm. The colorless spot of ABA on the plate becomes bright yellow after spraying with a 5% solution of sulfuric acid in ethanol followed by heating, and the spot shows yellowish green fluorescence under UV irradiation at 365 nm.[573] Octadecyl silica (ODS) gel columns are often used in HPLC analysis of ABA.[572] The detection limit of ABA at 260 nm is about 1 ng. The methyl ester of ABA is detected by GC using OV-1, OV-17, SE-30, and XE-60 columns.[574] A GC analysis with an electron capture detector (ECD) is one of the most convenient methods to detect ABA with high selectivity and sensitivity. The detection limit of the methyl ester of ABA by ECD is 1–10 pg.[572] The optical resolution of (±)-ABA, (±)-phaseic acid, and methyl ester of (±)-dihydrophaseic acid is achieved by HPLC with columns packed with chiral materials.[575–577]

ABA is detected and quantified by enzyme immunoassay with a monoclonal antibody.[578] The detection limit of immunoassay with a monoclonal antibody is about 0.02 pmol; however, enzyme immunoassay cannot exclude cross-reactivity with unknown substances, so GC- or LC-SIM analysis using the stable isotope dilution method is recommended for correct quantitation. As internal standards labeled with the stable isotope, deuterated derivatives of ABA such as [3′,5′,5′,7′,7′,7′-$^2H_6$]ABA (ABA-d_6)[579] and [7′,7′,7′-$^2H_3$] derivatives of phaseic and dihydrophaseic acids[580] are used.

4.02.5.2.2 Physicochemical properties

The melting points of (+)-ABA and its racemate are 160–161[581] and 188–190 °C,[550] respectively. ABA is converted to a γ-lactone (**11**) under a strong acidic condition.[582] The 1′-hydroxyl group is resistant to acetylation, and the six-membered ring aromatizes with migration of the methyl groups upon treatment with acetic anhydride and *p*-toluenesulfonic acid to give an aromatic derivative (**12**).[583] Hydrogen and oxygen atoms of ABA can be exchanged with hydrogen and oxygen atoms from a medium. Hydrogen atoms at C-3′, C-5′, and C-7′ are easily exchanged with deuterium of deuterated water under alkaline conditions via an enol form to give ABA-d_6.[579] The order of ease of exchange is 5′-$H_{pro\text{-}S}$, 5′-$H_{pro\text{-}R}$, and 3′-H and 7′-H. Hydrogen atoms at the C-2 and C-6 are also exchangeable in an alkaline solution although it takes longer than 2 months.[584] 4′-Carbonyl oxygen is exchanged with an oxygen of water under acidic and alkaline conditions.[585] ABA has electrophilicity derived from the side chain and the enone group. The methyl ester of ABA shows two reduction voltages, E_p −1.3 and −1.75 V, because conjugation of the side chain and the enone groups is precluded by the 1′-hydroxyl group.[586] The methyl ester of ABA is irreversibly reduced with two electrons at a cathode to give a major product, the structure of which was revised to **13**.[587] This product spontaneously isomerizes to compound **14** under alkaline conditions as shown in **Scheme 2**.

In the infrared (IR) spectrum, the methyl ester of ABA shows absorption bands at 3610 cm^{-1} (the 1′-hydroxyl group), 1667 cm^{-1} (the 4′-carbonyl group), and 1710, 1637, and 1604 cm^{-1} (the side chain).[588] The methyl ester of phaseic acid shows an absorption band at 1723 cm^{-1} for the 4′-carbonyl group. The methyl ester of dihydrophaseic acid shows an absorption band at 3552 cm^{-1}, which is assigned to the 4′α-hydroxyl group since its strength does not decrease even in a dilute solution due to a hydrogen bonding between the 4′-alcoholic hydrogen and the 8′-ethereal oxygen.[589] This assignment means that the C-4′ of dihydrophaseic acid is *S* and that the C-4′ of *epi*-dihydrophaseic acid is *R*.



Methyl ester of ABA **13** **14**

Scheme 2

ABA absorbs ultraviolet with maxima at 240 nm (ε 2.1×10^4, a shoulder peak), 260 nm (ε 2.6×10^4), and 320 nm (ε 50) in an acidic methanol solution.[590] Irradiation with UV with a wavelength shorter than 305 nm isomerizes the 2-(*Z*)-double bond to (*E*) to give an equilibrium mixture of ABA and its 2-(*E*)-isomer with a ratio of 1:1, and also causes decomposition of ABA to unidentified compounds by the excitation of the π–π^* transition of the side chain and the enone groups. ABA has a strong optical activity, and its specific optical rotation is +430° in an acidic methanol solution.[591] In the optical rotatory dispersion (ORD)[591] and the circular dichroism (CD) spectra,[558] ABA shows a positive Cotton effect from 300 to 200 nm. Phaseic acid and dihydrophaseic acid and its epimer, which did not have the enone group, show a small specific optical rotation with a minus value and also a negative plain curve in the ORD.

The ^{1}H NMR signals of ABA are summarized in **Table 2**. In the ^{1}H NMR spectrum of ABA, the chemical shift δ 7.77 ppm of the 4-H is lower than that of usual protons bound to double bonds. This down field shift is explained by the deshielding effect of the 1-carboxyl group. The same proton of 2-(*E*)-ABA is observed at δ 6.5 ppm. A W-coupling is observed between the 5′-H at a lower field than the other 5′-H and the 8′-H, and between the 5′-H at a higher field than the other 5′-H and the 3′-H. Since a half-chair conformation with the pseudoaxial side chain (**HC$_1$**) can allow such relationship (**Figure 24**), the signal can be assigned at a higher field to the 5′-H$_{pro\text{-}R}$ having an equatorial orientation, and at a lower field to the 5′-H$_{pro\text{-}S}$ having an axial orientation.[579] However, strictly speaking, the NMR signals of ABA at room temperature are the average of the signals of different conformers since the ring of ABA is not constrained. The representative conformations of ABA are **HC$_1$** and a half-chair with the pseudoequatorial side chain (**HC$_2$**) as shown in **Figure 25**, and other conformations, the sofas, 1,3-diplanars, and boats, are probably transient, short-lived conformations in the course of inversion between **HC$_1$** and **HC$_2$**.[592] The conformation analyses by a low-temperature ^{1}H NMR revealed that the ^{1}H signals of ABA separated into two sets, **HC$_1$** and **HC$_2$**, below 250 K, and that the ratio of the two sets was 99.4:0.6 at 185 K.[593,594] At 185 K, the free-energy barrier and the free-energy difference for the ring inversion between **HC$_1$** and **HC$_2$** are 11 and 1.4 kcal mol^{-1}, respectively. A crystal of ABA has a conformer different from that in solutions. The X-ray analysis of a crystal of ABA showed that the ring adopts a slightly distorted sofa, similar to the sofa that has the nondistorted enone and the pseudoaxial side chain.[595,596]

The ^{1}H NMR spectrum of phaseic acid shows two W-couplings between the 5′-H$_{pro\text{-}S}$ and the 8′-H$_{pro\text{-}R}$, and between the 5′-H$_{pro\text{-}R}$ and the 3′-H$_{pro\text{-}S}$ because the conformation of the ring is fixed to a chair with the side chain axial (**Figure 24**).[597] Eu(hfc)$_3$ separates the signals of the 7′- or 9′-H of methyl esters of (+)- and (−)-phaseic acids.[598] In the ^{1}H NMR spectrum of *epi*-dihydrophaseic acid, the signal of the 4′-hydroxyl proton is

Table 2 Assignment of ^{1}H and ^{13}C NMR signals of ABA

^{1}H NMR (300 MHz, CD_3OD)				*^{13}C NMR (24 MHz, $CDCl_3$)*	
δ (ppm)	*Multiplicity*	*J (Hz)*	*Assignment*	*δ (ppm)*	*Assignment*
1.03	s		9′-H	19.1	C-7′
1.06	s	[a]	8′-H	21.4	C-6
1.93	d	1.3	7′-H	23.1	C-8′
2.03	d	1.3	6-H	24.3	C-9′
2.18	dd	16.9 and 0.9	5′-H$_{pro\text{-}R}$	41.7	C-6′
2.53	d	16.9	5′- H$_{pro\text{-}S}$	49.7	C-5′
5.74	br s		2-H	79.9	C-1′
5.92	m		3′-H	118.1	C-2
6.23	dd	16.2 and 0.4	5-H	127.0	C-3′
7.77	dd	16.2 and 0.6	4-H	128.3	C-4
				136.8	C-5
				151.4	C-3
				163.0	C-2′
				170.9	C-1
				198.3	C-4′

[a] The coupling constant between 8′-H and 5′-H is very small.

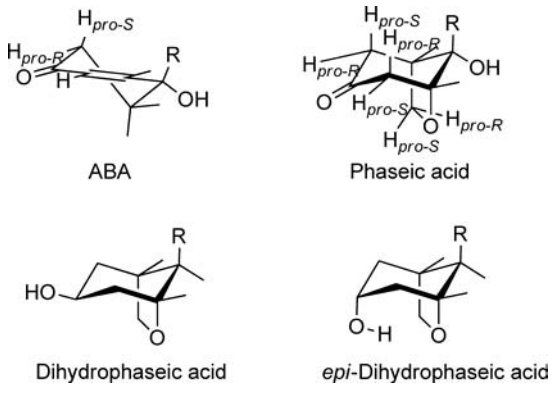


Figure 24 Steric structure of ABA with a preferred conformation and its catabolites (R = side chain).

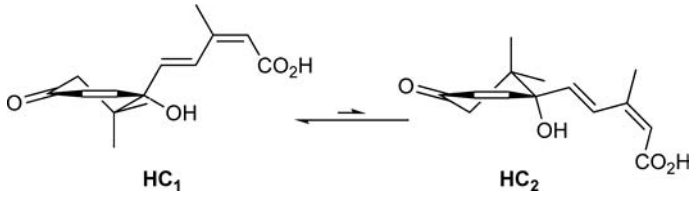
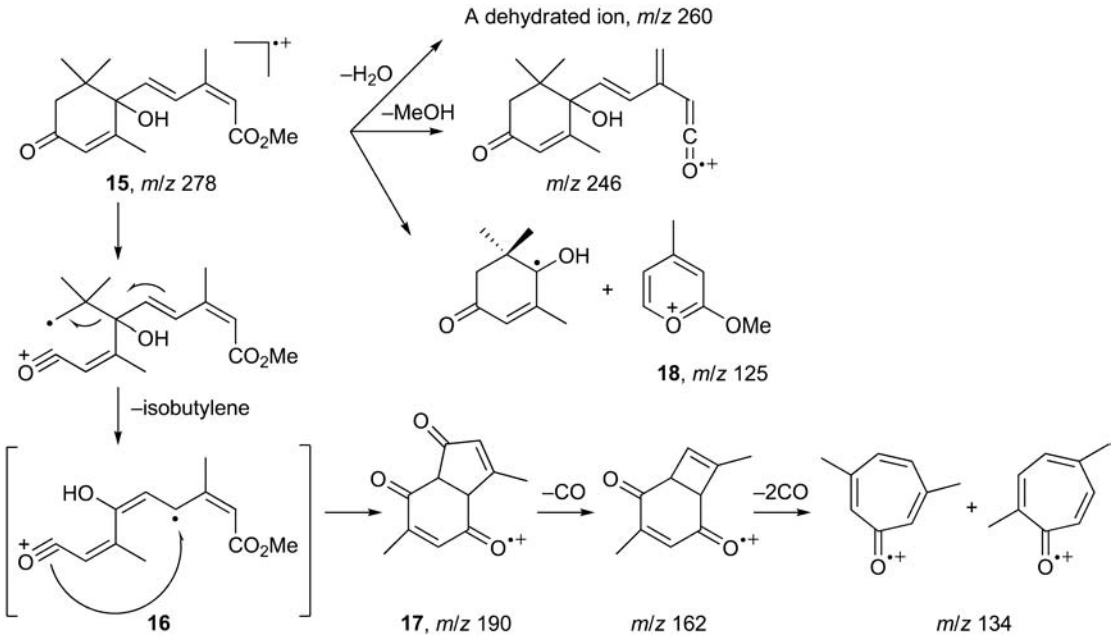


Figure 25 Two stable conformations of the cyclohexanone ring in ABA (**HC_1**, the half-chair conformation with the pseudoaxial side chain; **HC_2**, the half-chair conformation with the pseudoequatorial side chain).

observed as a doublet by coupling with the 4′-H, meaning that the 4′-hydroxyl group is relatively fixed by a hydrogen bonding with the 8′-oxygen (**Figure 24**).[589] The 8′-$H_{pro\text{-}S}$ is shifted to a lower field by the 4′-hydroxyl group close to the 8′-$H_{pro\text{-}S}$. The 4′-hydroxyl proton of dihydrophaseic acid does not show the deshielding effect on the 8′-$H_{pro\text{-}S}$. The ^{13}C NMR signals of ABA,[579,599] assignment of which was confirmed by HMQC and HMBC spectra, are summarized in **Table 2**.

In the electron impact (EI) mass spectrum, the methyl ester of ABA gives a molecular ion at m/z 278, a dehydrated ion at m/z 260, and major fragment ions at m/z 246 $[M\text{-methanol}]^+$, 190, 162, 134, and 125. The major fragmentation pathway of methyl ester of ABA, as shown in **Scheme 3**, was proposed by Gray *et al.*[585] with labeled methyl esters of ABA. The cleavage of the bond between the C-4′and C-5′ of an ion radical (**15**) followed by the elimination of isobutylene through a retro Diels–Alder reaction gives an intermediate ion radical (**16**). The ion radical is cyclized, and a methanol is eliminated to give a bicyclic ion radical (**17**) at m/z 190, which is a base peak in the spectrum. By further elimination of the carbon monoxide, hydrogen radical, and methyl radical, other fragment ions are formed. An ion (**18**) at m/z 125 with a second intensity is derived from the side chain. The strong ions at m/z 190 and 125 are selected as characteristic ions of the methyl ester of ABA for detection and quantitation by GC-SIM. The formation mechanism of the dehydrated ion at m/z 260 had been unknown, but it was found that 66% of the dehydrated ion was formed by elimination of the 1′-hydroxyl group and the residual 34% by elimination of the oxygen atom at the 4′-carbonyl group.[600] ABA methyl esters labeled with stable isotopes revealed that the 1′-hydroxyl group was eliminated mainly with a hydrogen atom at C-4, and that 4′-carbonyl oxygen was eliminated mainly with hydrogen atoms at the 1′-hydroxyl group and at C-4. Involvement of a hydrogen atom at a double bond and of the 4′-carbonyl oxygen in dehydration would be an interesting case of fragmentation.

Negative chemical ionization (NCI) mass spectrometry detects ABA with a high sensitivity since the negative molecular ion $[M]^-$ of methyl ester of ABA is more stable than the positive molecular ion $[M]^+$ due to the high electrophilicity of ABA. The NCI mass spectrum shows $[M]^-$ at m/z 278 as a base peak, and other fragment ions at m/z 310, 260, 245, 141, and 152.[601] The combination of SIM with NCI gives highly selective and sensitive detection of ABA; the lowest detection limit is 0.3 pg, which is 200 times lower than that



Scheme 3

of EI-SIM.[602] The detection limit can be decreased to 20 fg by using the pentafluorobenzyl ester of ABA, which has higher electrophilicity than the methyl ester.[603] This is the lowest detection limit among the several detection methods of ABA.

4.02.5.2.3 Structure–activity relationship

4.02.5.2.3(i) Functional groups essential for the activities Many analogs of ABA have been synthesized to investigate the structure–activity relationship and to develop a highly active analog for practical use in agriculture. The analogs have revealed the essential groups for the activities of ABA although a practical analog for agriculture has not been realized.

The 2-(*Z*)-pentadienoic acid moiety seems to be essential for the activity since modification of the side chain decreases the activity of ABA. 2-(*E*)-ABA is inactive,[604] and the elongation and the shortening of the length of the side chain decrease the activity.[605,606] An analog (**19**) where the geometry of the C-2 is fixed to (*Z*) by introduction of a benzene ring shows low activity probably due to the bulkiness of the benzene ring.[607] An acetylenic acid (**20**) having a triple bond at the C-4 shows high activity,[608] suggesting that the recognition of this part by receptors is not strict. Another analog (**21**) having a phenyl group at the C-1 shows weak activity.[609] 1-Aldehyde[610] and 1-alcohol[608] of ABA are active derivatives and they may be converted to ABA by oxidation in plants. The methyl ester of ABA is active in long-term assays[551] and is not active in the stomatal assay,[611] suggesting that the activity of the ester in long-term assays is expressed after hydrolysis. 1-Azido-ABA, which was synthesized for photoaffinity labeling, is unstable, and 10% as active as ABA.[612] The biological activity of 6-nor-ABA is much lower than that of ABA, indicating that the C-6 methyl group is necessary for activity.[613] 6-Hydroxy-ABA has been reported along with an enantioselective synthesis of ABA, but its activity is unknown.[614]

The 1′-hydroxyl group is not essential for the activity of ABA. (+)-1′-Deoxy-ABA (**22**) retains activity although it is lower than that of ABA.[615] The biological activities of (+)-1′-deoxy-1′-fluoro-ABA (**23**) are 0.1–0.05 those of (+)-ABA, and almost equal to those of (+)-1′-deoxy-ABA.[615] The property of a monofluoro group is similar to that of oxygen of a hydroxyl group except for lack of the ability to donate hydrogen. Thus the 1′-hydroxyl group of (+)-ABA interacts with binding proteins as an uncharged hydrogen-bonding donor. 1′-*O*-Methyl-ABA (**24**) shows weak activities, indicating that the hydroxyl group should be free.[616] This is coincident with the result with (+)-1′-deoxy-1′-fluoro-ABA.

The activities of (+)-7′- and 8′-nor-ABAs and of (+)-8′,9′-dinor-ABA have shown that the C-7′ is critical for the activity while C-8′ and C-9′ are not.[617] The C-7′ must interact with binding sites. The activities of the analogs alkylated at the C-7′, C-8′, and C-9′ have shown that the decrease in activity by alkylating the C-8′ of (+)-ABA is smaller than that by alkylating the C-7′ and C-9′ of (+)-ABA, indicating that the activity is relatively unaffected by the bulky group at the C-8′ of (+)-ABA.[618] The space around the C-8′ in (+)-ABA does not seem to be involved in the interaction with binding sites for exhibiting activity. The C-8′ might be essential only for metabolic inactivation by hydroxylation. 8′,8′,8′-Trifluoro-ABA (**25**) is the most active derivative of ABA, and shows long-lasting activity by resisting enzymatic hydroxylation at C-8′ for catabolism.[619,620] 8′-Methylene and 8′-methylidyne derivatives of ABA (**26** and **27**, respectively) show higher activity than ABA,[621] and it has been suggested that the latter derivative resists the 8′-hydroxylation.[622] 9′-Propargyl-ABA (**28**) competitively inhibits 8′-hydroxylase activity, but its activity is similar to that of ABA.[623] 7′-Difluoro-ABA (**29**) is as active as ABA.[624]

3′-Thioether of ABA (**30**) for affinity chromatography,[625] 3′-fluoro-ABA (**31**),[626] and 3′-azido-ABA (**32**) as a photoaffinity reagent[627] retain activity, showing that ABA is tolerant of the 3′-modification. 3′-Fluoro-ABA is metabolized to 3′-fluoro-8′-hydroxy-ABA, which is more stable than 8′-hydroxy-ABA. The 4′-carbonyl group is probably not essential since 4′-deoxy-ABA[628] and 1′,4′-diols of ABA (**2**) and (**3**)[629] are active. However, the receptors for ABA cannot perceive a bulky group at the C-4′ since 4′-*p*-aminobenzoyl hydrazone of ABA lacks the activity.[630] An anthracenone analog of ABA (**33**) shows one-third activity of ABA.[631] The activities of other analogs have been summarized in the earlier works.[548,594] Identification of an ABA receptor and the binding analysis are necessary to design a highly active and simple analog of ABA that could be used for agriculture.

4.02.5.2.3(ii) Active conformation ABA can adopt many conformations in solutions as described above. Considering the low barrier to interconversion and the thermodynamic stabilization in binding to the active site of the receptor, in addition to the half-chairs the short-lived forms can be the active conformation of ABA. The active conformation has been investigated by the activities of the analogs, which prefer different conformations.[594] The (1′*S*,2′*S*)-2′,3′-dihydro-ABA (**34**) is active, whereas (1′*S*,2′*R*)-2′,3′-dihydro-ABA (**35**) is inactive.[632] The two dihydro-ABAs may adopt a chair form with axial and equatorial side chains, respectively, due to the steric repulsion between the 1,3-diaxial methyl groups, 7′ and 9′, and 7′ and 8′, respectively. This example suggests that the active conformation has an axial side chain, rather than an equatorial one. This hypothesis has been further examined by the cyclopropane analogs synthesized by Todoroki *et al.*[592] 5′β,9′-Cyclo-ABA (**36**) in which the 6′β-substituent is constrained essentially to the axial-like orientation between axial and bisectional orientation shows no activity, while 5′α,8′-cyclo-ABA (**37**) and an achiral analog (**38**) with no axial-like substituent at C-6′β exhibit activity equivalent to ABA. The activities of the analogs suggest that the active conformation of ABA is not the half-chair $\mathbf{HC_2}$ with the pseudoequatorial side chain and the pseudoaxial methyl at C-6′β, but is close to the other half-chair $\mathbf{HC_1}$ with the pseudoaxial side chain and the pseudoequatorial methyl at C-6′β. The computer-aided and ^{1}H NMR analyses indicate that the bonding between the C-1 and C-2 can rotate freely, but the rotation angles of the bondings between the C-3 and C-4 and between the C-5 and C-1′ are 20 and 180°, respectively.[633] However, the active conformation of the side chain is unclear.

Unnatural (−)-ABA shows one-half to one-third of the activity of (+)-ABA in many bioassays,[634] and this small difference in activity between the enantiomers has been explained by the pseudosymmetry of the molecule, which is derived from the 2,6,6-trimethyl-cyclohex-2-en-4-one.[635] **Figure 26** shows the steric structures of (+)- and (−)-ABAs with the preferable conformation, a half-chair with the pseudoaxial side chain, viewed from the carbonyl group at C-4′. In the molecule of (−)-ABA, the C-7′ corresponds to the C-9′ of (+)-ABA, the C-9′ corresponds to the C-7′ of (+)-ABA, and the C-8′ occupies the space facing the *re*-face of the C-2′ in (+)-ABA, whereas a methyl group corresponding to the C-8′ of (+)-ABA is absent. This hypothesis has been supported by the achiral analog (**38**), which shows activity intermediate between (+)- and (−)-ABAs.[592] The activity of (−)-ABA is low in the assay of stomatal closure,[618] which suggests that the receptor of stomata is more specific to (+)-ABA than to (−)-ABA.

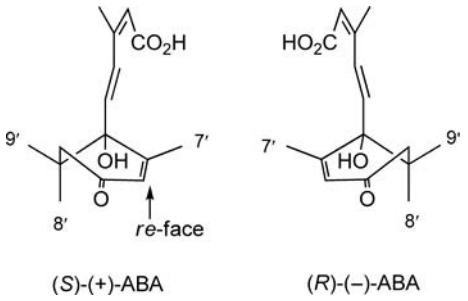


Figure 26 Pseudosymmetry of ABA.

4.02.5.3 Biosynthesis and Metabolism

4.02.5.3.1 Biosynthesis in plants

ABA has a sesquiterpene skeleton consisting of three isoprene units, which suggests that ABA is directly synthesized from mevalonic acid via a biosynthetic pathway of sesquiterpenes; however, the incorporation ratios of labeled mevalonic acid into ABA by plants were low,[636,637] and the chemical conversion of violaxanthin to (+)-ABA by Taylor and Burden[556] revealed that ABA might be indirectly biosynthesized via carotenoids. In the 1990s, Arigoni *et al.* and Rohmer[415] found that isopentenyl diphosphate (IDP) was biosynthesized not only from mevalonic acid but also from 2-*C*-methyl-D-erythritol-4-phosphate (MEP), derived from glyceraldehyde-3-phosphate and pyruvic acid. Furthermore, it was proven that carotenoids, phytol, and plastoquinones, which were biosynthesized in chloroplasts, were products of the MEP pathway.[638] If plant ABA is biosynthesized via carotenoids, ABA should not be a product of the mevalonic acid pathway, but a product of the MEP pathway. Recent studies have solved this question and confirmed that ABA is biosynthesized via carotenoids derived from the MEP pathway in plants.[639]

A convenient method to discriminate between the MEP and mevalonic acid pathways is to label the terpenoids with ^{13}C of [1-^{13}C]-D-glucose. If ABA is biosynthesized by the MEP pathway or the mevalonic acid pathway, ABA will be labeled at C-1, C-5, C-6, C-4′, C-7′, and C-9′, or at C-2, C-4, C-6, C-1′, C-3′, C-5′, C-7′, C-8′, and C-9′, respectively (**Figure 27**). ABA biosynthesized from [1-^{13}C]-D-glucose by the tulip tree showed labeled carbons at C-1, C-5, C-6, C-4′, C-7′, and C-9′.[640] This finding clearly confirmed that ABA was biosynthesized by the MEP pathway.

The carotenoid pathway (**Scheme 4**) was investigated using ABA-deficient mutants lacking enzymes involved in carotenoid biosynthesis.[641] ABA-deficient *aba* mutants of *Arabidopsis* showed a low content of violaxanthin (**4**) and 9′-(*Z*)-neoxanthin (**39**), and a high content of zeaxanthin (**40**), suggesting that zeaxanthin was involved in ABA biosynthesis.[642,643] ABA-deficient *aba2* mutant of tobacco was generated by the transpozon-tagging technique, and the aba2 protein expressed in *E. coli* showed enzymatic activity converting zeaxanthin to antheraxanthin (**41**) and violaxanthin.[644] This meant that the aba2 protein was zeaxanthin epoxidase. The zeaxanthin epoxidase gene of tobacco is expressed at a high level in leaves, but at a low level in roots and seeds.[645] Overexpression of zeaxanthin epoxidase mRNA delays seed germination.[646] Introduction of its antisense mRNA hastened seed germination, but did not affect the tolerance against water stress because the transgenic plant still contained small amounts of violaxanthin and 9′-*Z*-neoxanthin, which may be enough to supply ABA.[647] The neoxanthin synthase gene was identified in tomato[648] and potato.[649] Neoxanthin synthase opens the epoxy ring of violaxanthin and takes a proton from C-7 to form allene compounds.

Violaxanthin and neoxanthin (**42**) have *E* double bonds at C-9 and C-9′, and these *E* bonds should isomerize to *Z* double bonds to form the *Z* double bond at C-2 of ABA. The isomerase of the double bond seems to be essential, but has not been found so far.

The occurrence of xanthophyll dioxygenase, which releases xanthoxin from xanthophyll, was found using the ABA-deficient mutant *vp14* of *Z. mays*.[650] Xanthophyll dioxygenase cleaves the double bond between C-11 (C-11′) and C-12 (C-12′) of 9-*Z*-violaxanthin (**43**) and 9′-*Z*-neoxanthin to give xanthoxin (**5**).[651] The mRNA of xanthoxin dioxygenase in the leaves of *P. vulgaris* increased upon water stress and decreased upon

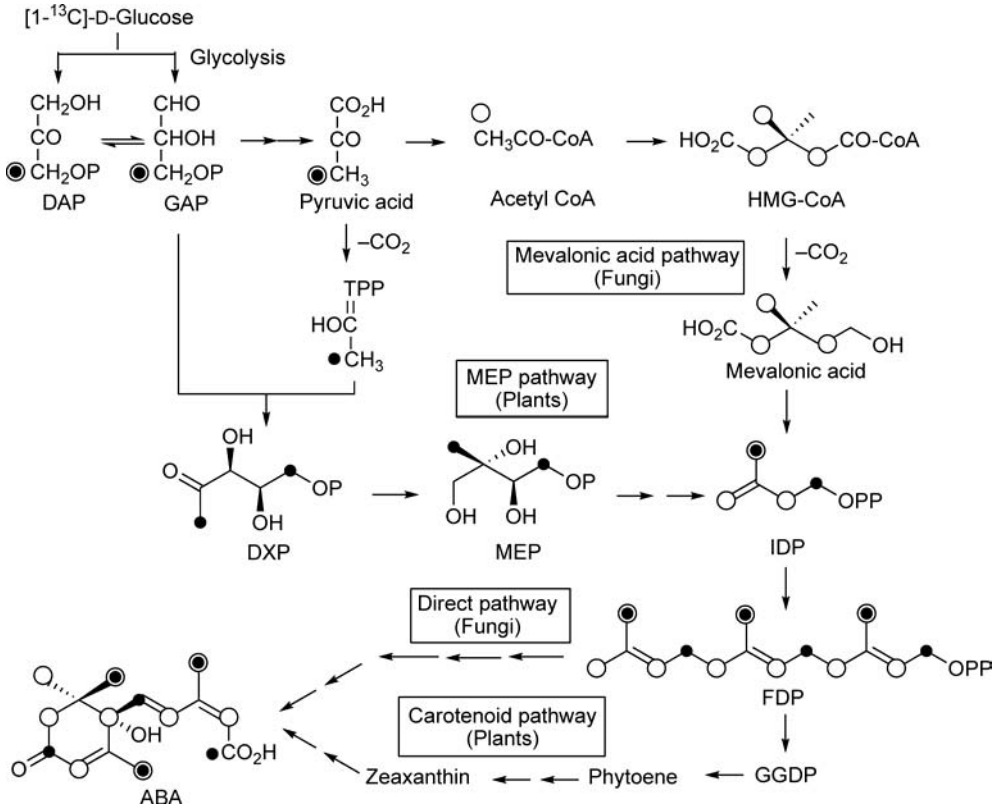
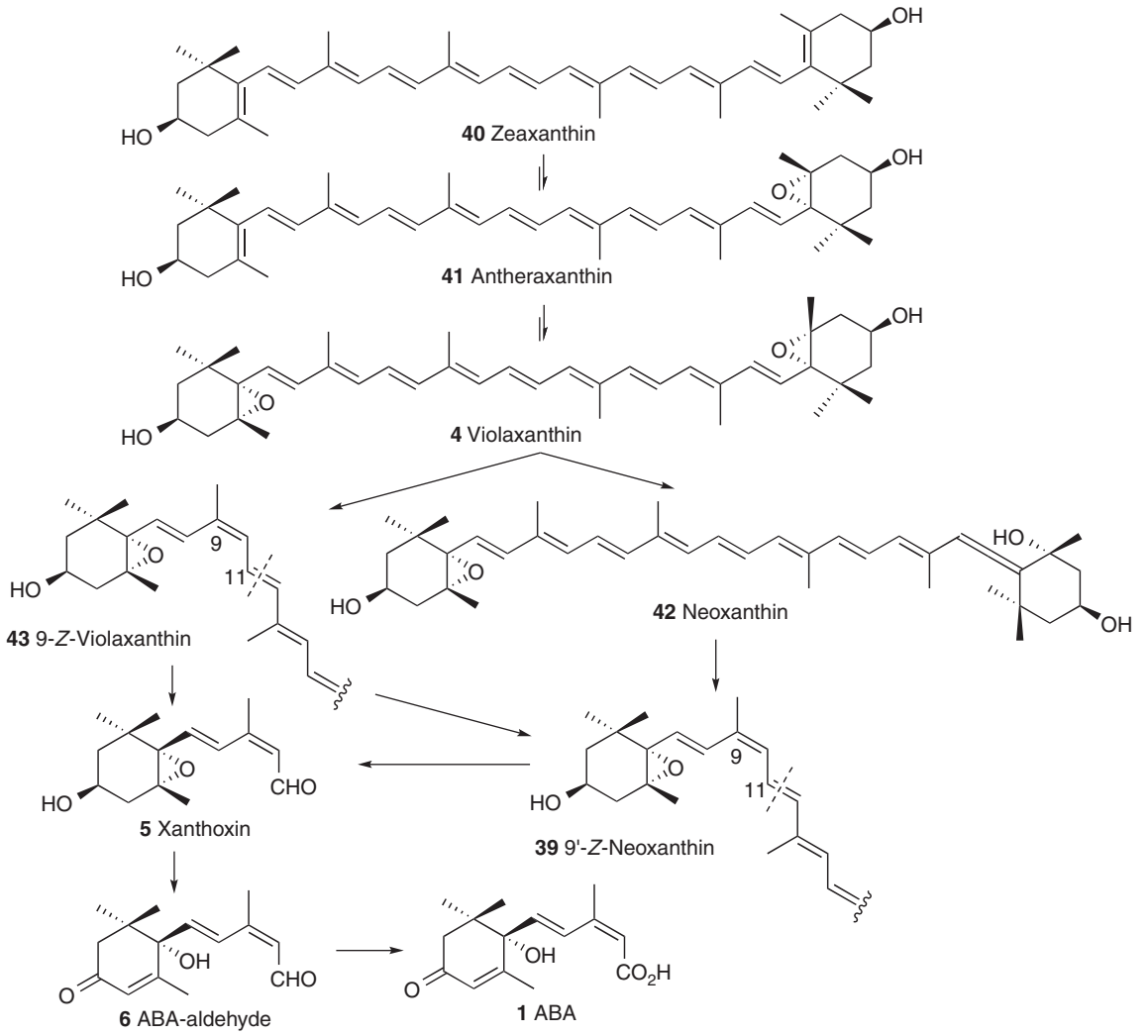


Figure 27 Four possible pathways for ABA biosynthesis. Open and closed circles show the ^{13}C label from [1-^{13}C]-D-glucose in the mevaloic acid pathway and the MEP pathway, respectively. DAP, dihydroxyacetone phosphate; DXP, 1-deoxy-xylulose-5-phosphate; FDP, farnesyl diphosphate; GAP, glyceraldehyde-3-phosphate; GGDP, geranylgeranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IDP, isopentenyl diphosphate; MEP, 2-*C*-methyl-D-erythritol-4-phosphate.

watering,[652] suggesting that xanthophyll dioxygenase is a rate-limiting enzyme for ABA biosynthesis. The ABA-deficient mutant *notabilis* of tomato has a frame shift mutation of xanthophyll dioxygenase.[653] Transgenic plants, which overexpressed xanthophyll dioxygenase, showed higher ABA content than the wild type, and decreased stomatal aperture and increased seed dormancy.[654,655] An inhibitor of xanthophyll dioxygenase, abamine (**44**), has been developed, and it inhibits dioxygenase at K_i 38.8 μmol l^{-1} to decrease the content of ABA in *Arabidopsis*.[656] Such an inhibitor could be used as a herbicide since it decreases the tolerance of plants to water stress.

The genes of the biosynthetic enzymes from zeaxanthin to xanthoxin encode plastid-targeted proteins, suggesting that biosynthesis from IDP to xanthoxin progresses in the plastid. In contrast, biosynthesis from xanthoxin to ABA progresses in the cytosol, meaning that xanthoxin must be transported from the plastid to cytosol, although the transportation process is unknown.

Xanthoxin is endogenous in many plants, and is metabolized to ABA in plant tissues and cell-free systems.[657,658] Although the higher content of 2-(*E*)-xanthoxin than that of xanthoxin casts doubt on the hypothesis,[659] it has been shown that xanthoxin is very unstable and easily isomerizes to 2-(*E*)-xanthoxin during extraction.[660] The exact quantitation revealed that the major xanthoxin is the 2-(*Z*) isomer, and the 2-(*E*) isomer was not detected in tomato. Conversion of xanthoxin to ABA involves six theoretical reactions: oxidation of the aldehyde group at C-1 to the carboxyl group, oxidation of the hydroxyl group at C-4′ to the carbonyl group, abstraction of a proton at C-3′ and cleavage of the epoxide ring followed by formation of a double bond at C-2′ and a hydroxyl group at C-1′. Oxidation at C-4′ of xanthoxin spontaneously gives ABA-aldehyde (**6**) via an intermediate with a carbonyl group at C-4′.[661] Studies on feeding experiments[662] and



Scheme 4

ABA-deficient mutants[663] have confirmed that ABA-aldehyde is the immediate precursor of ABA. *Arabidopsis* has four isozyme genes of aldehyde oxidase, and an AAO3 protein, which is one of the isozymes catalyzing the conversion reaction of ABA-aldehyde to ABA.[664] AAO3 could be the ABA-aldehyde oxidase gene.

At present, there is no doubt about the biosynthesis of ABA by the carotenoid pathway via the MEP pathway in plants. If ABA is biosynthesized by the direct pathway, the first cyclized intermediate will be ionylideneethanol, having a hydroxyl group at C-1 derived from farnesol; however, ionylideneethanol has not been found in plants, so the direct pathway is not involved in ABA biosynthesis. The mevalonic acid pathway may also be excluded because almost no ^{13}C label was incorporated into the carbons, which would be labeled in the mevalonic acid pathway in a feeding experiment with [1-^{13}C]-D-glucose.[640]

4.02.5.3.2 Biosynthesis in fungi

In contrast to plants, it has been shown that fungi biosynthesize ABA via direct pathways from IDP supplied by the mevalonic acid pathway. *B. cinerea* and *Cercospora pini-densiflorae* fed with [1-^{13}C]-D-glucose biosynthesized ABA, C-2, C-4, C-6, C-1′, C-3′, C-5′, C-7′, C-8′, and C-9′ of which were labeled with ^{13}C, being coincident with the biosynthesis of IDP by the mevalonic acid pathway (**Figure 27**).[640] Fungi produce ABA-related compounds, which are converted to ABA in feeding experiments. 1′,4′-*trans*-Diol (**3**) of ABA was found

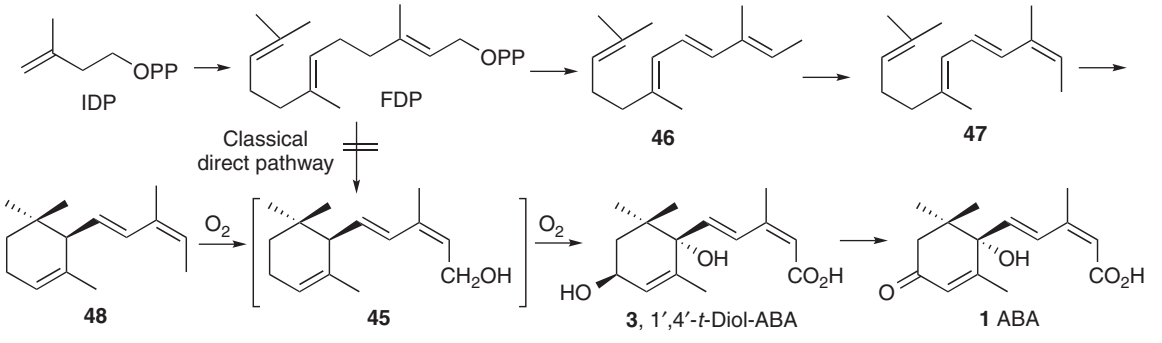
in the culture broth of *B. cinerea*[665] and *C. pini-densiflorae*,[666] and it has been suggested that it is a precursor of ABA in these fungi.[566] *Cercospora cruenta* produces 4′-hydroxy-γ-ionylideneacetic acid and 1′,4′-dihydroxy-γ-ionylideneacetic acid,[667] and converts these γ-acids to ABA.[668] This fungus also produces small amounts of some carotenoids, but does not produce xanthophylls.[669] *Cercospora rosicola* produces α-ionylideneethanol, α-ionylideneacetic acid, 4′-hydroxy-α-ionyldeneacetic acid, and 1′-deoxy-ABA, and these compounds also are converted to ABA by this fungus.[670] These findings suggested that fungal ABA was biosynthesized via the direct pathway, but intermediates between farnesyl diphosphate (FDP) and 2*Z*,4*E*-α-ionylideneethanol (**45**) had not been found for the direct pathway. The precise analysis of metabolites from *B. cinerea* showed that the fungus produced 2*E*,4*E*,6*E*-allofarnesene (**46**), 2*Z*,4*E*,6*E*-allofarnesene (**47**), and 2*Z*,4*E*-α-ionylideneethane (**48**).[671] The feeding experiment with ^{13}C-labeled compounds revealed that **46** was converted to **48** via **47**, and further, **48** was metabolized to ABA via 1′,4′-*t*-diol-ABA (**3**). *B. cinerea* cultured under an $^{18}O_2$ atmosphere incorporated ^{18}O into all oxygen atoms at C-1, C-1, C-1′, and C-4′ of ABA, although the ^{18}O label at C-4′ had easily been lost by exchange with ^{16}O from water during culture. This finding meant that *B. cinerea* reduced FDP to allofarnesene, and subsequently isomerized and cyclized allofarensene to form (**48**), and oxidized (**48**) with molecular oxygen to give ABA as shown in **Scheme 5**. Oxidation of (**48**) seems to be catalyzed by a cytochrome P450 enzyme, and the *bcaba1* gene encoding P450 monooxygenase of *B. cinerea* was identified as the ABA biosynthetic gene,[672] supporting the direct pathway via ionylideneethane in *B. cinerea*. *C. cruenta* also produced allofarnesenes and 2*Z*,4*E*-γ-ionylideneethane, and converted these compounds to ABA via 2*Z*,4*E*-1′,4′-dihydroxy-γ-ionyldeneacetic acid.[669] The direct pathway via ionylideneethane seems to be common among ABA-producing fungi.

It had been suggested that plant genes for ABA biosynthesis might be transferred into phytopathogenic fungi in a process of coevolution between host plants and parasites; however, these fungi would biosynthesize ABA via the direct pathway from IDP supplied by the mevalonic acid pathway, so we conclude that the fungi obtained the genes for ABA biosynthesis independently of the plant genes.

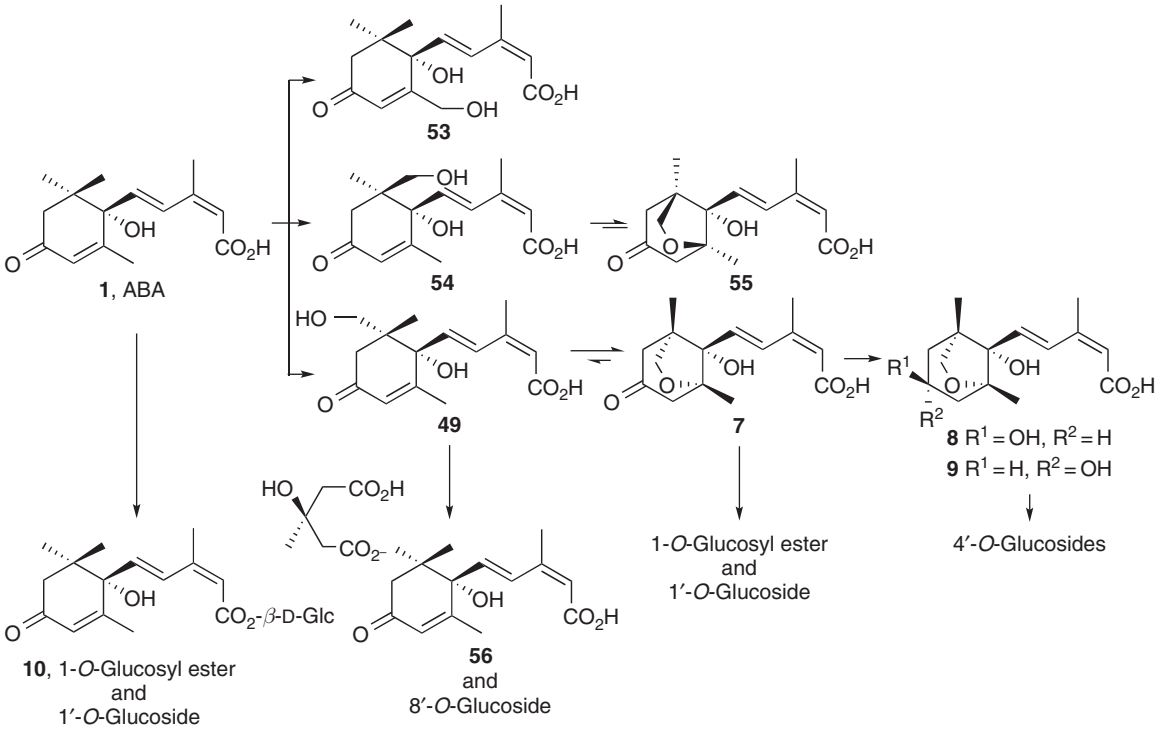
4.02.5.3.3 Metabolism

ABA is inactivated through the catabolic pathway after expression of its physiological function. The endogenous level of ABA in plants is regulated not only by its biosynthesis but also by its catabolism. The catabolic inactivation of (+)-ABA in plants is classified into the modification of the ring moiety and the conjugation with hydrophilic compounds as summarized in **Scheme 6**.[639] The former pathway intrinsically inactivates ABA by oxidation and reduction reactions, while the latter pathway increases the hydrophilicity of free catabolites by conjugation with polar compounds for transportation from cytosol to vacuoles and other organs.

4.02.5.3.3(i) Modification of the ring moiety ABA has three ring-modification pathways, 7′-, 8′-, and 9′-hydroxylation pathways, but the 8′-hydroxylation pathway is the major and common in many plants. In the 8′-hydroxylation pathway, ABA is hydroxylated at C-8′ to give 8′-hydroxy-ABA (**49**). ABA 8′-hydroxylase of *Arabidopsis* is encoded by CYP707A, which is a cytochrome P450 gene.[673,674] Genes related to CYP707A exist in rice, tomato, maize, and other plants. ABA 8′-hydroxylase is a monooxygenase that requires molecular oxygen



Scheme 5



Scheme 6

and NADPH as essential factors, and the reaction is inhibited by carbon monoxide. For substrate recognition, ABA 8′-hydroxylase strictly requires C-8′ and C-9′, but does not require C-6 and 1′-hydroxyl group.[613] ABA 8′-hydroxylase is induced by ABA itself and water stress probably due to maintaining the endogenous level of ABA constant. Expression of *OsABA8ox1* gene, which encodes ABA 8′-hydroxylase in rice, is induced by submergence.[675] Inhibition of ABA 8′-hydroxylase activity could increase the resistance of plants to water stress by suppressing the decrease in the ABA level. Screening of triazole compounds for inhibitors of ABA 8′-hydroxylase showed that uniconazole-P (**50**),[676] an inhibitor of gibberellin biosynthesis, and (+)-diniconazole (**51**),[677] a fungicide, were effective inhibitors. Uniconazole-P inhibits ABA 8′-hydroxylase at K_i 8.0 nmol l^{-1}, and protects *Arabidopsis* seedlings from water stress through increases in the ABA level. A specific inhibitor of ABA 8′-hydroxylase may become a new agrochemical to protect plants from environmental stresses.[678] Analogs resistant to 8′-hydroxylation such as 8′,8′,8′-trifluoro-ABA (**25**) could also be useful for protecting plants from environmental stress through the long-lasting effect of ABA; however, there is no analog that is practically used in agriculture due to the high cost of its chemical synthesis.

8′-Hydroxy-ABA is extremely labile, isomerizing spontaneously to phaseic acid (**7**). This isomerization is an intramolecular Michael addition: the 8′-hydroxyl group attacks C-2′ from the α-face to form an enol intermediate (**52**), which is converted to phaseic acid. Involvement of an enzyme in this isomerization has been suggested by analyses of the catabolites of ABA-d_6[597] and 3′-fluoro-ABA.[626] Isomerization is an equilibrium reaction, and the ratio of phaseic acid and 8′-hydroxy-ABA is 98:2 in a solution of pH 3 at 25 °C, showing that the difference in free energy between the two isomers is 2.3 kcal mol^{-1}.[679]

Phaseic acid is further converted to dihydrophaseic acid (**8**) and *epi*-dihydrophaseic acid (**9**) in plants. The endogenous level of *epi*-dihydrophaseic acid in plants is about 10% of the level of dihydrophaseic acid. *epi*-Dihydrophaseic acid is not an artifact that is formed during extraction since *epi*-dihydrophaseic acid converted from [4′-^{18}O]-ABA by bean seedlings retains the label at C-4′, as does dihydrophaseic acid.[574] Enzymatic activity reducing the 4′-carbonyl group of phaseic acid has been found in a soluble fraction from liquid sperm of Eastern wild cucumber.[680] Dihydrophaseic acid might be further catabolized in plants since the presence of 5′-hydroxydihydrophaseic acid has been suggested in ferns.[681] However, the catabolism of the ring moiety after

dihydrophaseic acid has not been confirmed. It has been reported that ABA was metabolized to 8′-hydroxyphaseic acid and 8′-hydroxydihydrophaseic acid via 8′-hydroxy-ABA in maize cell cultures,[682] but this catabolic pathway seems to be characteristic to the cell cultures.

The catabolic inactivation of ABA would be achieved in a stepwise manner. The biological activity of 8′-hydroxy-ABA cannot be tested due to its instability, but several studies have suggested that the activity of 8′-hydroxy-ABA is about 20% of that of ABA.[626,683,684] The activity of phaseic acid is low, about 5% of that of ABA, in many biological assays,[548] and may be contributed by 8′-hydroxy-ABA present in a sample of phaseic acid. Dihydrophaseic acid and its epimer are almost inactive in assays tested, meaning that the catabolic inactivation is completed in the step reducing phaseic acid to dihydrophaseic acid and its epimer.

7′-Hydroxy-ABA (**53**) has been found in the leaves of *Vicia faba*[685] and other plants. 9′-Hydroxy-ABA (**54**) has been found in *Brassica napus* siliques along with its isomerized catabolite neophaseic acid (**55**),[686] which had been formerly known as *epi*-phaseic acid.[687] The occurrence of 7′- and 9′- hydroxy-ABAs is restricted to several plants at present, suggesting that these hydroxy-ABAs are minor catabolites of ABA. ABA 7′- and 9′-hydroxylases would be different from ABA 8′-hydroxylase since 7′- and 9′-hydroxy-ABAs are not detected in the products of ABA 8′-hydroxylase reaction.

4.02.5.3.3(ii) Conjugation ABA is conjugated with β-D-glucose at the 1-carboxyl and 1′-hydroxyl groups to form the glucosyl ester and the glucoside, respectively. The glucosyl ester is a major conjugate of ABA, and is found in many plants.[688] The ABA glucosyltransferase of adzuki bean, which synthesizes the glucosyl ester from ABA and UDP-glucose, is encoded by the *AOG* gene.[689] AOG recombinant protein shows broad substrate specificity, but cannot catalyze the glucosylation of phaseic acid. The optimum pH of ABA glucosyltransferase from *Macleaya microcarpa* is 5.0,[690] suggesting that the glucosylation occurs in the vacuole. The major role of conjugation would be excretion of ABA by compartmentation into the vacuole. It has been suggested that the conjugate of ABA does not subsequently release ABA.[691] Thus plants exposed to water deficiency supply ABA by *de novo* synthesis. The 1′-*O*-glucoside exists in tomato plants and apple seeds.[692] The 1′-*O*-glucoside not only decomposes to methyl ester of ABA and glucose upon treatment with diazomethane but also isomerizes spontaneously to the glucosyl ester in an acidic methanol solution, probably due to the steric repulsion. A bound form of ABA that releases ABA and its methyl ester upon mild acid hydrolysis has been isolated from peas and barley.[693] This 'adduct' might be a conjugate of the 4′-en-4′-ol form of ABA.

Labile 8′-hydroxy-ABA is conjugated with 3-hydroxy-3-methylglutaric acid to form a stable ester (**56**),[694] which seems to be a characteristic catabolite of *Robinia pseudacacia*. The absolute configuration of 3-hydroxy-3-methylglutaryl group has been revised to *S*, meaning that the ester is formed by acylation of 8′-hydroxy-ABA with 3-hydroxy-3-methylglutaryl CoA.[695] 8′-*O*-β-D-Glucoside of 8′-hydroxy-ABA has been found in avocado seeds.[696] The 1′-*O*-β-D-glucoside of phaseic acid has been found in apple seeds and tomato leaves fed with (±)-[2-^{14}C]-ABA.[697] Exogenous phaseic acid is converted to its 1-*O*-β-D-glucosyl ester.[698] The glucoside is an endogenous catabolite in plants. The 4′-*O*-β-D-glucoside of dihydrophaseic acid has been found in avocado fruits[699] and has been isolated from tomato seedlings fed with (±)-[2-^{14}C]-ABA.[700] The 4′-*O*-β-D-glucoside of *epi*-dihydrophaseic acid has also been found in avocado seeds.[696]

4.02.5.4 Biological Activities

ABA regulates many physiological processes including germination, growth, flowering, seed maturation, senescence, and adaptation to various environmental stresses such as water deficiency, low temperature, and freezing.[563,701] Exogenous ABA shows unique activities on plants, which reflect its physiological role as a hormone.

4.02.5.4.1 Stomatal closure

One characteristic activity of ABA is its effect on stomata, which protects plants from water stress. At 10^{-7} mol l^{-1}, ABA given through a transpiration stream from cut ends of shoots causes stomatal closure.[702] The activity is more effective in epidermal strips floated on a buffer solution than in shoots. At 10^{-10} mol l^{-1}, ABA closes stomata in epidermal strips at pH 5.5.[703] The activity at pH 6.8 is 10^3 times lower than that at pH 5.5, suggesting that the active form of ABA is not a dissociated acid but an undissociated acid. The simulation study

on the redistribution of ABA in plant tissues after pH changes of apoplasts and synplasts has suggested that ABA accumulates in guard cell walls according to the anion-trap mechanism for weak acids. Hartung and Slovik[704] have proposed that ABA may be an ideal 'stress messenger' for stomata.

A candidate for the ABA receptor in guard cells has been found, as described later. ABA activates phospholipase C after binding to the receptor, and then the concentration of cytosolic Ca^{2+} ion is elevated by stimulation of the production of inositol 1,4,5-trisphosphate and cyclic ADP-ribose in guard cells preceding stomatal closure.[705] Elevation of Ca^{2+} concentration activates the anion channel in the plasma membrane resulting in the promotion of efflux of the K^+ ion from cytosol. ABA also inhibits the influx of ABA into guard cells. The decrease in the concentration of cytosolic K^+ induces guard cell turgor and, consequently, the stomata close. ABA induces the production of hydrogen peroxide (H_2O_2) in the leaves of maize plants exposed to water stress, suggesting that H_2O_2 acts as a second messenger of ABA.[706]

4.02.5.4.2 Inhibition of seed germination and other activities

ABA inhibits germination of many seeds at 10^{-5}–10^{-7} mol l^{-1}. The suppression of α-amylase induction would be a cause of the inhibition by ABA in starch seeds. The induction of α-amylase by gibberellin in barley half-seeds without embryos is inhibited by ABA at 10^{-7} mol l^{-1}.[707] Barley seeds have two isozymes of α-amylase, and ABA suppresses biosynthesis of the isozyme with a higher isoelectric point than that of the other isozyme.[708] The suppression is antagonized by gibberellin. Germination of photoblastic lettuce seeds is regulated by endogenous levels of ABA and gibberellin.[709] In lipid seeds, the target of ABA seems to be different from that in starch seeds since ABA inhibits the increase of isocitrate lyase activity, which catalyzes β-oxidation of fatty acids during germination of bean seeds.[710] Gibberellin antagonizes the inhibitory effect of ABA on the increase of isocitrate lyase activity. It has been suggested that the inhibitory effect of ABA on the α-amylase biosynthesis is mediated by Ca^{2+} ion since ABA suppresses the increase of the cytosolic levels of Ca^{2+} ion by gibberellin.[711] Cutler *et al.*[712] screened mutants of *Arabidopsis* that showed an enhanced response to exogenous ABA in the seed germination tests, and found that the responsive gene encodes the β-subunit of a protein farnesyl transferase. Farnesyl transferase may act as a negative regulator of ABA in the signal transduction.

ABA inhibits the growth of seedlings and hypocotyls of many plants at 10^{-5}–10^{-7} mol l^{-1}. In contrast to the inhibitory effect, ABA promotes the growth of tomato and bean shoots at low concentrations,[713] and increases the volume of wheat protoplasts in the presence of Ca^{2+}.[714] ABA also has both effects on root growth. The growth of root tips of pea is promoted at a low concentration of ABA, and is inhibited at a high concentration of ABA.[715] Such properties of ABA suggest that growth at certain stages is regulated by endogenous levels of ABA, that is, ABA is not only a brake but also an accelerator.

Diverse effects of exogenous ABA on organs and tissues have been known.[716] ABA fed to one side of root tips caused curvature of roots, suggesting involvement of ABA in gravitropism of the root. Since ABA induces the morphogenesis of leaves of water plants from a submersed type to a floating type, ABA induced by water stress might act as a morphogen in some water plants. ABA generally inhibits induction of flower buds although flowering of some short-day plants is promoted by ABA. ABA may be involved in the storage of starch and lipids during the development of embryos. Application of ABA before exposure to low temperature confers cold or freezing tolerance on plants.

4.02.5.4.3 Receptors of ABA and ABA-related genes and proteins

ABA binds to its receptor to trigger the transduction of a signal for expression of physiological effects. Several proteins with the properties of the ABA receptor have been reported so far, suggesting that ABA has multi-receptors.[717,718] Syntaxin is an ABA-binding protein of tobacco, and its expression at the plasma membrane is important for the function.[719] The ABAP1 protein is an ABA-binding protein of barley aleurone layers, and binding of ABA to the ABAP1 protein is suppressed by H_2O_2.[720] This suppression might be a feedback response since ABA stimulates production of H_2O_2. The gene of the 42 kDa protein of broad bean, which is bound to ABA, encodes the H subunit of the Mg-chelatase involved in both chlorophyll biosynthesis and plastid-to-nucleus signaling.[721] In *Arabidopsis*, leucine-rich repeat receptor-like kinase1 has been identified as an AB-binding protein bound to membrane.[722] An RNA-binding protein involved in the signal transduction of flowering was reported as a receptor of ABA in a research paper;[723] however, this paper was retracted by the authors because of errors in the calculations.[724] A G protein-coupled receptor GCR2 has been identified as another receptor of

ABA of *Arabidopsis*.[725] However, other groups have argued that GCR2 is not a receptor of ABA but a plant homolog of bacterial lanthionine synthetases.[726,727] Further study is needed to find a real receptor of ABA.

In recent years, ABA-responsive genes have been intensively investigated using the microarray technology. More than 300 genes of *Arabidopsis* were identified as those that respond to ABA.[728–730] However, the number of gene products the physiological function of which was characterized has been limited to about 50.[731] The typical proteins induced by ABA are dehydrin and Lea protein.[732] Dehydrin is expressed during late embryogenesis of corn seeds before the beginning of desiccation. Dehydrin protects embryos from desiccation since it apparently retains water molecules in the domain containing lysine and serine residues. Lea proteins are involved in the desiccation of cotton seeds. Expression of some kinase and phosphatase involved in the signal transduction is also induced or repressed by ABA. The *Rab*16 gene of rice is induced by ABA and osmotic stress. Its expression is regulated by a *cis* element, and the binding protein of the element was found to be a bZip protein. These properties of proteins induced by ABA suggest that the proteins are involved in adaptation to stress.

4.02.6 Brassinosteroids

4.02.6.1 Introduction

Brassinosteroids (BRs) are steroidal plant hormones involved in a variety of physiological events of plants.[733–744] In Japan, BRs were first investigated as auxin-like principles that induce rice lamina bending. In 1968, Marumo *et al.*[745] reported the partial purification of these compounds from leaves of *Distylium racemosum*. However, no structural information was obtained, because analytical techniques at that time were not developed enough. In the United States, BRs were investigated as growth-promoting factors contained in rape pollen that induce growth promotion of bean seedlings. In 1970, Mitchell *et al.*[746] reported the isolation of the active principles named brassins. However, a minor constituent contained in brassins turned out to be responsible for growth promotion, and, in 1979, this principle was isolated as crystals and named brassinolide by Grove *et al.*[747] This compound was determined to be a steroid by X-ray crystallography (**Figure 28**). In 1982, castasterone was isolated by Yokota *et al.*[748] from insect galls of chestnut and the structure indicated that castasterone is the direct

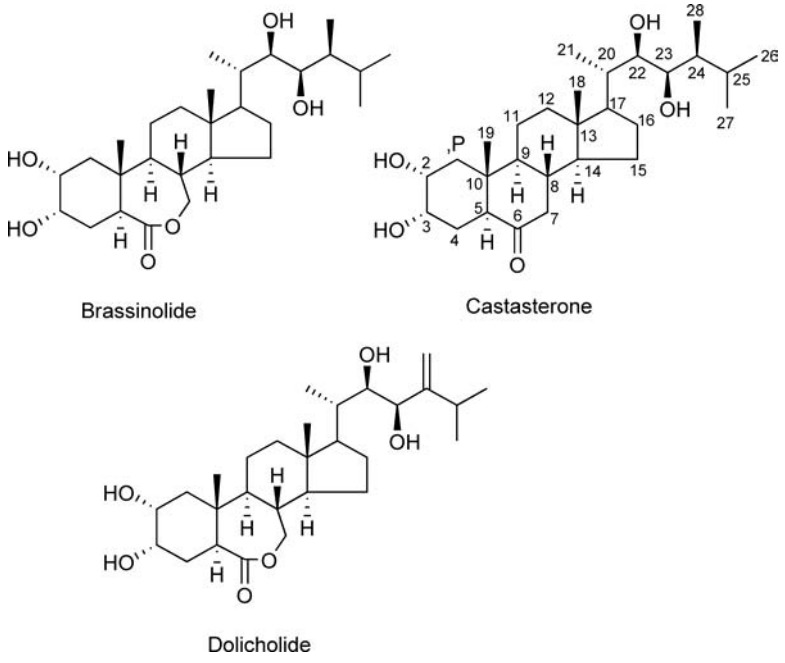


Figure 28 Structures of three brassinosteroids first isolated from plants. Numbers of carbon atoms are shown in the structure of castasterone.

precursor of brassinolide (**Figure 28**). In the same year, Yokota *et al.*[749] isolated dolicholide from immature seeds of *Dolichos lablab* and determined its structure to be $\Delta^{24(28)}$-brassinolide (**Figure 28**). Later on, various analogs related to these steroids were isolated from various plant sources and became collectively known as BRs.[733]

4.02.6.2 Chemistry

4.02.6.2.1 Distribution

Brassinosteroids have been identified from a wide range of higher plants including angiosperms and gymnosperms.[735,739] Brassinosteroids have also been identified from lower plants such as a pteridophyte (*Equisetum arvense*), a liverwort (*Marchantia polymorpha*),[750] and an alga (*Hydrodictyon reticulatum*). At present, no fungus has been found to produce BRs. Brassinosteroids have been detected in various plant organs including stem, leaf, pollen, and seed.[735,739] Especially, pollen/anther and seeds are rich sources of BRs.

4.02.6.2.2 Structure

To date, over 40 BRs have been isolated from natural sources. They have structural differences in the A ring, B ring, or side chain, arising from modifications through biosynthesis and metabolism (**Figure 29**).[734,735] BRs are defined as steroids that have an oxygen moiety at C-2 and additional ones at one or more of the C-3, C-6, C-22, and C-23 carbon atoms.[741] Depending on the alkyl groups at C-24 in the side chain, BRs are divided into C_{27} (no alkyl), C_{28} (α- or β-methyl), or C_{29} (α-ethyl). Among them, the most abundant in plants are C_{28} BRs such as castasterone and brassinolide, followed by C_{27} BRs.[738] Here, it should be noted that, in the steroid side chain, groups in front of the plane are α-oriented while those in back are β-oriented.[751] The occurrence of such BRs with different carbon numbers suggests that BRs are synthesized from various plant sterols with different alkyl groups at C-24.[734,738] **Figure 30** shows such plant sterols having C_{27} (cholesterol), C_{28} (campesterol, 22-dihydrobrassicasterol, and 24-methylenecholesterol), and C_{29} (sitosterol, isofucosterol, and 25-methylcampesterol).

4.02.6.2.3 Analysis

In order to analyze endogenous BRs from plant tissues in which the levels of BRs are usually lower than few micrograms per kilogram fresh weight, BRs are first enriched by partitioning between *n*-hexane and 80% methanol. Brassinosteroids partitioned into the latter are further purified by chromatography on silica gel, Sephadex LH-20, or charcoal, which allows purification of BRs as a group.[739] Respective BRs are frequently separated by HPLC using ODS supports.[739] The rice lamina inclination bioassay has been frequently used to guide purification and isolation of BRs from various plants.[752] Immunoassay for castasterone and brassinolide has been developed, although this has only been used for partially purified extracts of stems and seeds of *P. vulgaris*.[753]

GC-MS with or without SIM is the most reliable identification tool of BRs.[739] Prior to GC-MS analysis, vicinal hydroxyls in BRs are derivatized to methaneboronates, while isolated hydroxyl groups are converted to trimethylsilyl ethers (**Figure 31**). Fragment ions derived from the side chains have been used as diagnostic ions for mass spectrometric detection of BRs (**Figure 31**). Mass spectrometric data on methaneboronate derivatives of BRs have been reviewed.[754] For quantitation of the endogenous levels of BRs, deuterated BRs have been frequently used as internal standards by adding to plant extracts.[739]

HPLC equipped with a fluorimetric detector can be used to detect BRs at subnanogram amounts when derivatized to fluorescent boronates such as dansylaminophenylboronates and phenanthreneboronate, while ferroceneboronates can be monitored by an electrochemical detector.[755] LC/MS methods for the determination of BRs as their boronates have been developed using atmospheric pressure chemical ionization and electron spray ionization (ESI).[756] Fast atom bombardment has been used in LC/MS analysis of acyl-conjugated teasterones from lily pollen.[757] Recently, microanalysis of plant hormones has been developed by means of LC-ESI-MS/MS where various plant hormones including castasterone and brassinolide were subjected to analysis without any derivatization.[758] ^{1}H NMR and ^{13}C NMR data are also available.[759,760]

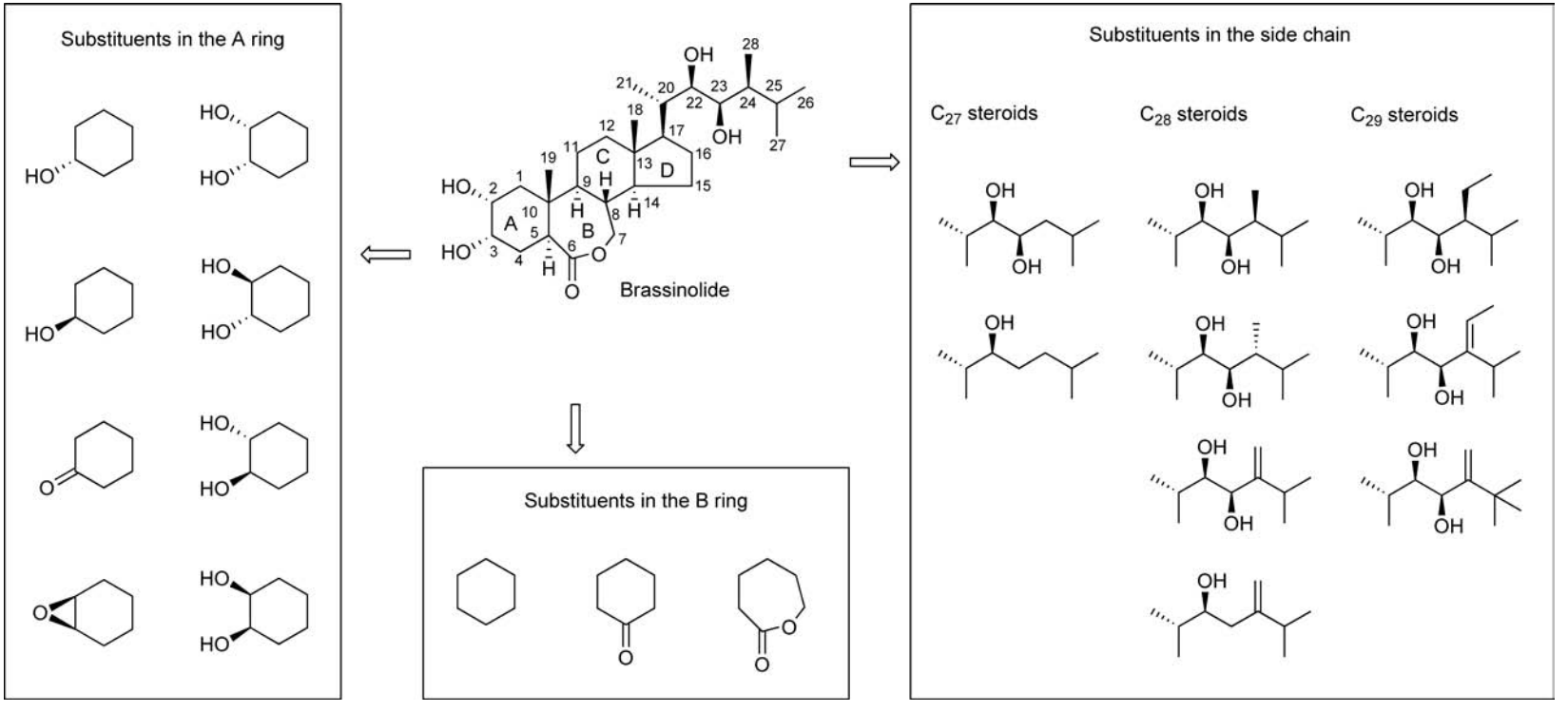


Figure 29 Structural varieties of naturally occurring brassinosteroids.

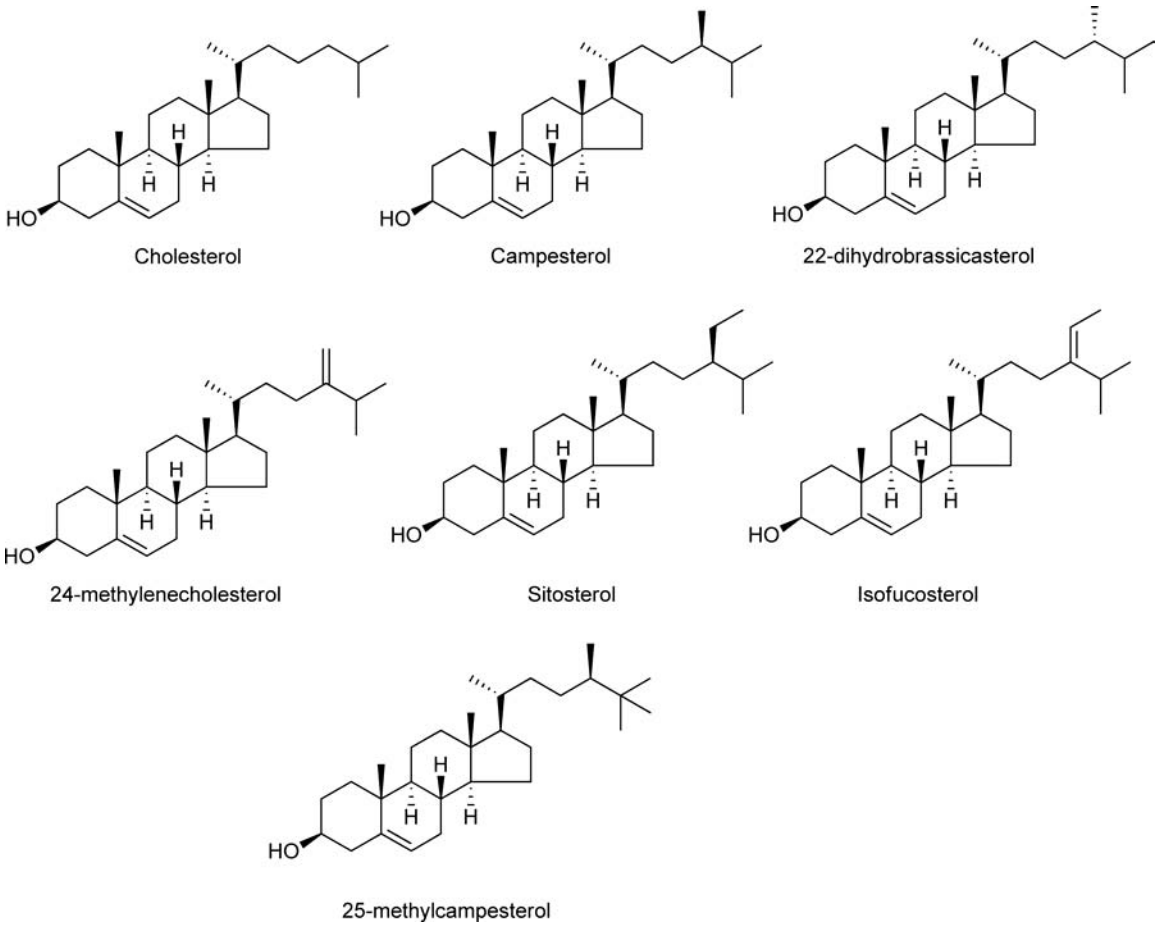


Figure 30 Various plant sterols that may be converted into brassinosteroids.

4.02.6.2.4 Synthesis

Various synthetic approaches of BRs have been reported.[740,743,761–764] Among natural BRs, syntheses of brassinolide and castasterone have been investigated by many workers. Methods of introduction of α-oriented vicinal glycol in the A ring and a lactone or ketone group in the B ring have been established. In many cases, steroid rings were synthesized using a C-22 aldehyde obtained from stigmasterol. Construction of the side chain containing four contiguous chiral centers has been extensively studied, but is still attracting synthetic chemists. Efficient syntheses of brassinolide have been reported.[763,764] Along with brassinolide, 24-epibrassinolide and 28-homobrassinolide are often used for large-scale application studies and these can now be effectively synthesized from plant sterols such as ergosterol, brassicasterol, and stigmasterol.[763,764] Nonsteroidal mimetics of brassinolide were synthesized.[765]

4.02.6.2.5 Structure–activity relationship

Structure–activity relationships have already been reviewed.[737,738,766] The structure–activity relationships of BRs have been investigated using bean, rice, tomato, and radish with the first two being frequently used.[766] The bean bioassay to examine the elongation of the second internodes, used in the early stage of researches, is very sensitive to structural changes.[767] For example, loss of the methyl group at C24 nearly abolishes the biological activity. Brassinolide is 100-fold more active than castasterone although both are known to be biologically active on their own. The overall structural requirements of BRs with *trans*-A/B ring system are 2α,3α-vicinal diol in A ring, 7-oxa-6-one (lactone) in B ring, as well as 22*R*,23*R*- or 22*S*,23*S*-diol and 24-methyl in the side chain.

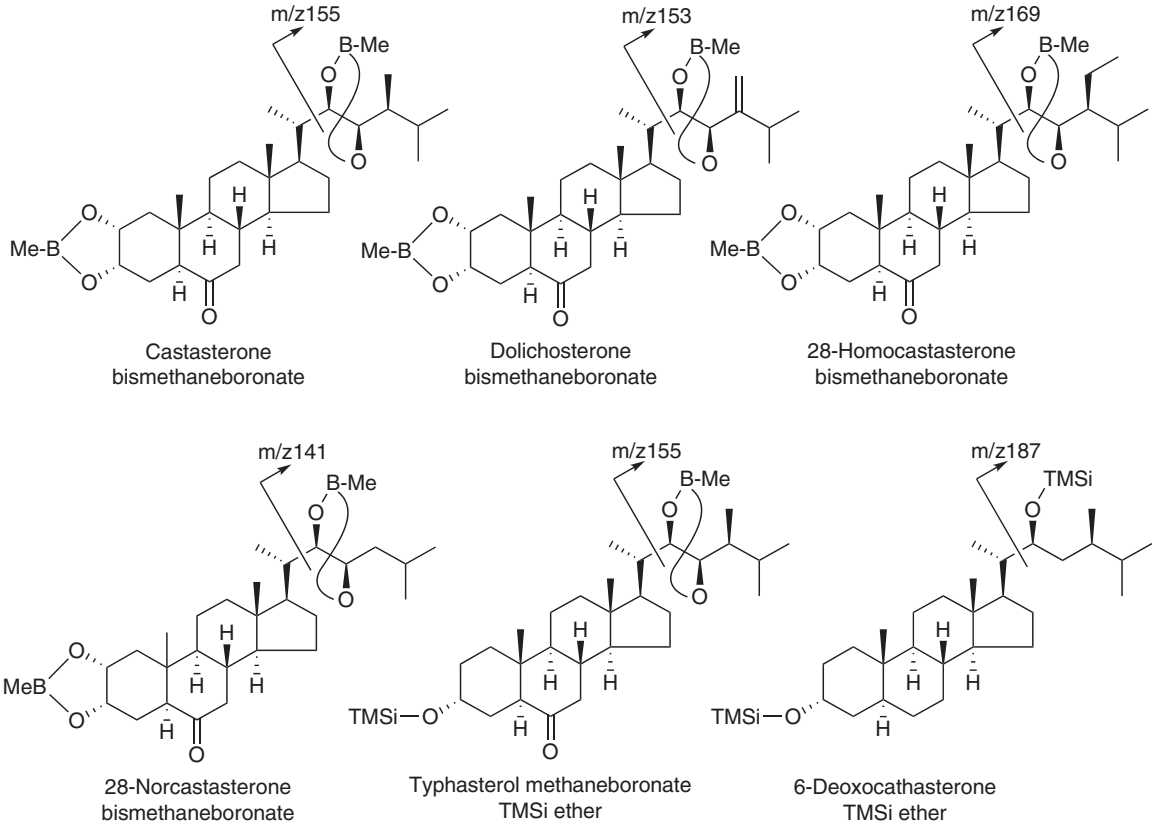


Figure 31 Derivatives used for GC-MS analysis of brassinosteroids and their diagnostic ions for the side chain structures.

The rice bioassay to examine the promotive effect of BRs on the lamina inclination has been extensively utilized.[768,769] This bioassay is affected to a lesser extent by structural variations than the bean test is. For example, castasterone is fourfold less active than brassinolide, this being in good correlation with their binding abilities to the receptor BRI1. Favorable functionalities are 2α,3α-vicinal diol or 3α,4α-vicinal diol in A ring, 7-oxa-6-one or 6-ketone in B ring, as well as methyl, ethyl, methylene, or ethylidene at C-24, or an additional methyl group at C-25 (terminal tertiary butyl group) in the side chain.[766] Among naturally occurring BRs, brassinolide is the most biologically active except that 25-methylbrassinolide found only in *Phaseolus* seeds shows higher activity.[770] The effects of substituents at C-14, C-25, C-26, and C-28 were further examined, revealing that introduction of functional groups such as hydroxyl and halogen into these carbons reduced the biological activity[771–773] although the presence of a methoxy group at C-25 increased the biological activity.[771] The requirement of the *trans*-A/B ring system for the biological activity was rigorously confirmed by a new synthetic approach.[774,775]

4.02.6.3 Biosynthesis and Metabolism

Brassinolide and castasterone are biologically active C_{28} BRs widely distributed in plants.[734,735,738] Available evidence shows that the biosynthesis of BRs takes place in every organ or tissue and BRs are rarely transported after their synthesis. It seems that BRs may be synthesized close to the site of action. Biosynthetic pathways of BRs hitherto demonstrated to be operating in plants are shown in **Figure 30**. The first report on the biosynthesis of BRs was made on the conversion of brassinolide from castasterone in *C. roseus* cells.[776] The biosynthesis of castasterone has also been studied using cultured crown gall cells of *C. roseus* and/or *Arabidopsis* seedlings to reveal biosynthetic pathways from campesterol via campestanol.[777,778] Such campestanol-dependent synthesis of castasterone was demonstrated to comprise the early C-6 oxidation pathway and the late C-6 oxidation pathway. Later, a branch without the involvement of campestanol was found indicating that campesterol is directly oxidized at C-22.[779] Recent studies indicated that this branch is linked to the later part of the late C-6 oxidation pathway, constituting campestanol-independent pathway believed to be a major biosynthetic pathway of C_{28} BRs.[780]

A number of genes and enzymes responsible for the biosynthesis and metabolism of BRs were isolated by analyzing a variety of mutants including *Arabidopsis*, pea, tomato, and rice.[781,782] It has been shown that genetic modulation of BR biosynthesis and perception can give rise to profitable effects on the production of crop such as barley[783] and rice.[784,785]

Triazole inhibitors of BR biosynthesis such as brassinazole and Brz22012 (**Figure 32**) have been developed and used to analyze the physiological function and signal transduction of BRs.[786]

4.02.6.3.1 Biosynthesis of castasterone

4.02.6.3.1(i) Campestanol-dependent pathway This pathway, demonstrated using cultured cells of *C. roseus* crown gall, starts with hydrogenation of campestanol, followed by either early C-6 oxidation or late C-6 oxidation to yield castasterone.[777,778]

In the early C-6 oxidation pathway, campestanol is first oxidized at C-6 to yield 6-oxocampestanol, which is then hydroxylated at C-22 to give cathasterone (**Figure 33**). Further hydroxylation at C-23 yields teasterone, which is converted to typhasterol through successive oxidation and reduction of 3-hydroxyl group.

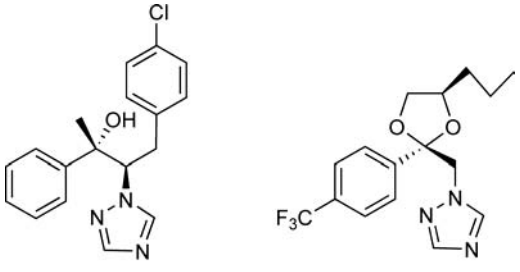


Figure 32 Structures of triazole inhibitors of brassinosteroid biosynthesis.

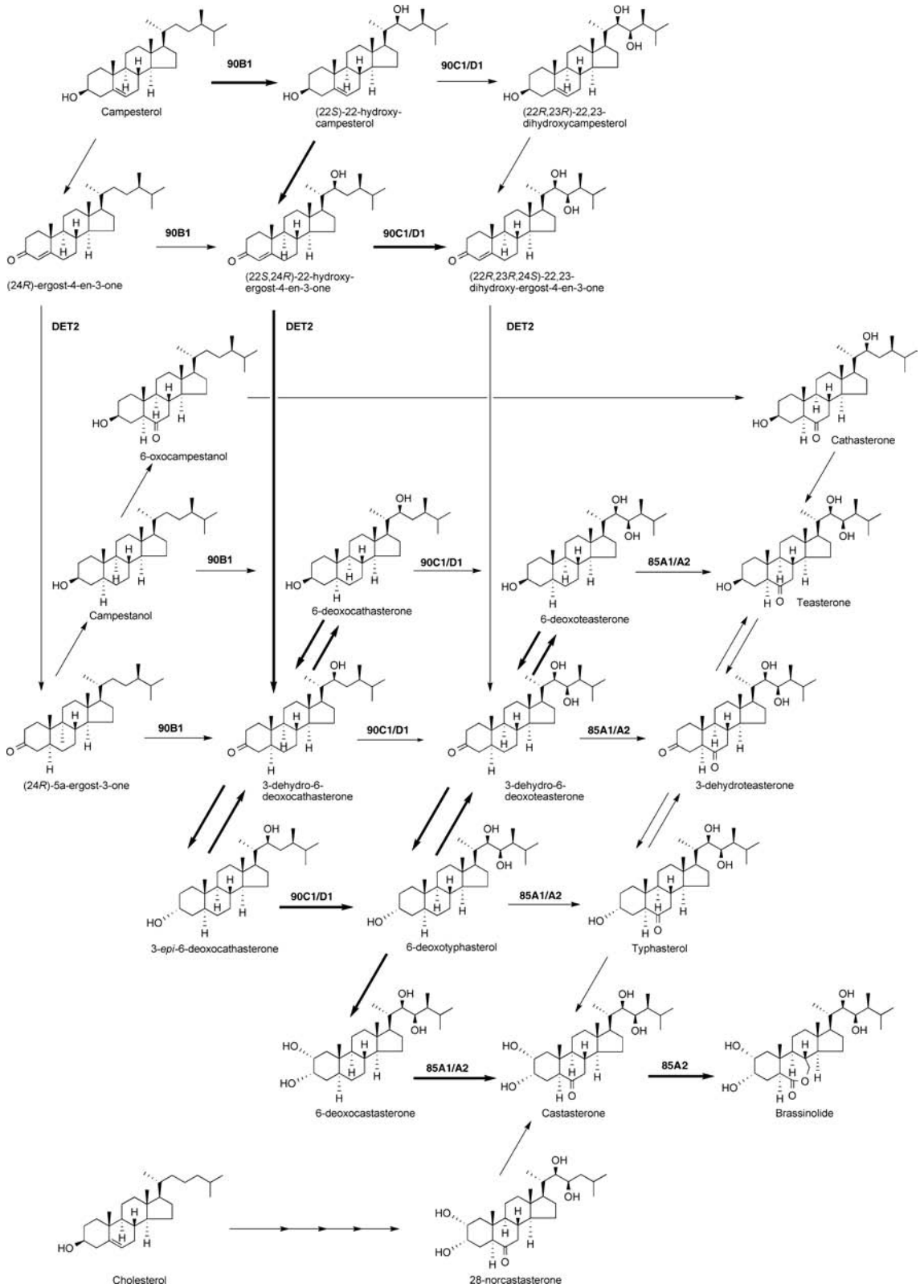


Figure 33 Biosynthetic pathways of brassinosteroids. The larger the arrows, the more principal the routes. *Arabidopsis* enzymes involved in the biosynthetic reactions are indicated in bold letters.

Typhasterol is converted to castasterone by hydroxylation at C-2. The early C-6 oxidation pathway was first thought to be the major biosynthetic pathway, because BRs having a 6-oxo group elicit higher biological activities than 6-deoxo BRs. However, the occurrence of cathasterone was found only in *C. roseus* cells.[787] Furthermore, recent biochemical studies showed that 6-oxocampestanol was not the substrate for steroid 22-hydroxylases of *Arabidopsis* (CYP90B1)[788] and tomato (CYP724B2 and CYP90B3),[789] which belong to cytochrome P450 monooxygenases. Therefore, it seems that the early C-6 oxidation pathway occurs only in limited biological systems.

In the late C-6 oxidation pathway, campestanol is first hydroxylated at C-22 to give 6-deoxocathasterone, which is further hydroxylated at C-23 to yield 6-deoxoteasterone (**Figure 33**). Successive oxidation and reduction of the 3-hydroxyl group yield 6-deoxotyphasterol. 6-Deoxotyphasterol is then hydroxylated at C-2 to yield 6-deoxocastasterone, which is finally oxidized at C-6 by the CYP85A family enzymes to give castasterone.[790] It was found that C-6 oxidation occurs also at the stages of 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, and 6-deoxotyphasterol.[791,792] Brassinosteroids lacking a 6-oxo group, which is involved in the late C-6 oxidation pathway, are all far more abundant in most plants than those having a 6-oxo group. Therefore, it has been believed for years that the late C-6 oxidation pathway is predominant in the plant kingdom.[779] However, it recently turned out that this may not be the case as described below.

4.02.6.3.1(ii) Campestanol-independent pathway Recent biochemical evidence revealed that campestanol-independent pathway without intermediary of campestanol is the main biosynthesis stream of BR (**Figure 33**). In 2002, Fujioka *et al.*[779] demonstrated a biosynthetic branch named the early C-22 oxidation pathway, where campesterol is first hydroxylated at C-22 to give rise to (22*S*)-22-hydroxylcampesterol. Use of recombinant *Arabidopsis* CYP90B1,[788] tomato CYP724B2, and tomato CYP90B3,[789] which are steroid C-22 hydroxylases, revealed that campesterol is a far more favorable substrate than campestanol, indicating that the early C-22 oxidation pathway is a predominant biosynthetic pathway of BRs. The resulting (22*S*)-22-hydroxycampesterol is oxidized into (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one, where the identity of the enzyme involved remains controversial.[780] Succeeding hydrogenation of (22*S*,24*R*)-22-hydroxy-ergost-4-en-3-one is effected by DET2 (*Arabidopsis* steroid 5α-reductase) to give 3-dehydro-6-deoxocathasterone, namely (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one. 3-Dehydro-6-deoxocathasterone is converted to either 6-deoxocathasterone or 3-*epi*-6-deoxocathasterone. These conversions deemed reversible so that 6-deoxocathasterone and 3-*epi*-6-deoxocathasterone are interconvertible via 3-dehydro-6-deoxocathasterone. (22*S*,24*R*)-22-Hydroxy-ergost-4-en-3-one, 3-dehydro-6-deoxocathasterone, and 3-*epi*-6-deoxocathasterone are favorable substrates of CYP90C1 and CYP90D1 (*Arabidopsis* steroid 23-hydroxylases).[780] So, it is most likely that, *in planta*, these three steroids are hydroxylated at C-23 by CYP90C1 or CYP90D1 to give (22*R*,23*R*,24*S*)-22,23-dihydroxy-ergost-4-en-3-one, 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol, respectively, linking the early C-22 hydroxylation pathway to the late C-6 oxidation pathway. Although (22*S*,24*R*)-22-hydroxy-ergost-4-en-3-one and its metabolite (22*R*,23*R*,24*S*)-22,23-dihydroxy-ergost-4-en-3-one were not detectable in *Arabidopsis* plants, these compounds were assumed to be efficiently converted to downstream metabolites.[780] 6-Deoxocathasterone was a poor substrate of CYP90C1 and CYP90D1. However, the levels of 6-deoxocathasterone in the shoots of many plants are very high,[793] suggesting that it may be utilized to control the endogenous levels of 3-dehydro-6-deoxocathasterone and 3-*epi*-6-deoxocathasterone. This idea is supported by the recent finding that 6-deoxocathasterone functions as a storage form in pea seeds.[794]

4.02.6.3.1(iii) Biosynthesis from 28-norcastasterone Surprisingly, tomato enzyme preparations were also found to convert 28-norcastasterone, a C_{27} BR, to castasterone by methylation (**Figure 33**).[795] This pathway seems to be important, because 28-norcastasterone is widely distributed in plants.[738] On the other hand, it was assumed in the same enzyme system that 28-norcastasterone is synthesized from cholesterol via cholestanol through late C-6 oxidation.[795,796] However, it may be expected that cholestanol-independent pathway should be operating *in planta*, because, as discussed above, castasterone has been demonstrated to be synthesized through campestanol-independent pathway. It has also been found in some plants that deuterium-labeled castasterone was demethylated to give 28-norcastasterone.[797] However, this reaction seems to be an artifact due to an isotopic effect of the deuterium atoms attached to C-28, because in liverwort and tomato, C-28

demethylation occurred in deuterium-labeled castasterone and brassinolide but not in nonlabeled castasterone and brassinolide.[795,798]

4.02.6.3.1(iv) Other biosynthetic pathways

2,3-Epoxybrassinosteroids were demonstrated to be synthesized from either teasterone or typhasterol and further converted to castasterone in seedlings of *Secale cereale*.[799,800] It has not been known if this pathway is operating in other plants.

4.02.6.3.2 Biosynthesis of brassinolide from castasterone

The Baeyer–Villiger oxidation of castasterone to brassinolide has been demonstrated in *C. roseus* crown gall cells in 1990.[776] However, the enzyme involved in this reaction had not been identified until 2005 when steroid 6-oxidases, CYP85A2 of *Arabidopsis* and CYP85A3 of tomato, were demonstrated to be responsible for this reaction by heterologously expressing in yeast (**Figure 33**).[801,802]

4.02.6.3.3 Regulation of biosynthesis

The biosynthesis of endogenous BRs is controlled so as to regulate the proper growth rate of plants. Microarray analysis indicated that exogenous brassinolide suppresses the expression of *Arabidopsis* cytochrome P450 genes such as CPD/CYP90A1, DWF4/CYP90B1, ROT3/CYP90C, CYP90D, and BR6ox/CYP85A1, suggesting that P450-mediated BR synthesis reactions are all controlled by active BRs.[803] The BR receptor mutants such as *Arabidopsis bri1*, pea *lka*, and tomato *cu-3* have defects in the BR receptors and, due to the loss of BRs signaling, are forced to accumulate high levels of the active BRs, castasterone and/or brassinolide.[777] Thus, feedback controls of the expression of P450 genes by active BRs seem to play a central role in regulating the BR levels in tissues. The *Arabidopsis* genes *DWF7*, *DWF1*, and *DET2* are implicated in sterol synthesis, and mutations of these genes result in dwarfism due to the deficit of BRs. However, these genes are not regulated by BR-based feedback control.[803]

4.02.6.3.4 Metabolism and its regulation

Biologically active BRs such as brassinolide and castasterone, as well as their biosynthetic precursors, are metabolized in various manners so as to control the levels of biologically active BRs. Metabolic reactions seen in earlier reviews[738,777,778] include (1) epimerization of the 2- and 3-hydroxyls followed by glucosylation or esterification, (2) hydroxylation of C-20 and successive side chain cleavage, (3) glucosylation of C-23 hydroxyl group, and (4) hydroxylation of C-25 or C-26 followed by glucosylation. Recently, removal of C-26 methyl group was found to occur when brassinolide and castasterone were applied to liverwort cells[798] and tomato cell-free preparations,[795] respectively. It seems that, prior to such demethylation, hydroxylation should occur at C-26. Enzymes responsible for hydroxylation of C-26 were identified with the CYP734A subfamily including CYP734A7 in tomato[804] and CYP734A1/BAS1 (formerly CYP72B1) in *Arabidopsis*.[805,806] In *Arabidopsis*, another C-26 hydroxylase was isolated as CYP72C1/CHI2.[807–809] The levels of *CYP734A1* and *CYP72C1* transcripts are reduced by light irradiation, while the application of exogenous brassinolide elevates the transcript level of *CYP734A1*,[803] but does not affect that of *CYP72C1*. Glucosylation of 23-hydroxyl group was found to be catalyzed by UGT73C5, a UDP-glucosyltransferase.[810] Two sulfotransferases of BRs, AtST4a and AtST1, have been characterized from *Arabidopsis*.[811] AtST4a was suggested to be involved in the regulation of biologically active BRs, while AtST1, with a strong preference for 24-epicathasterone, was regarded as a more general detoxication enzyme.

4.02.6.4 Physiology and Signal Transduction

4.02.6.4.1 Physiology

Brassinosteroids are known to be involved in stem elongation, pollen tube growth, leaf bending, leaf unrolling, inhibition of root growth, lateral root formation, proton pump activation, acceleration of 1-aminocyclopropane-1-carboxylic acid production, increase of transverse-oriented microtubules, xylogenesis, and cotton fiber synthesis.[736,739,744] Furthermore, BRs have been known to increase the yield of crops[739,740,761,812] although their effects have been affected largely by environmental conditions or places (or countries) where the

experiments were done.[812] Brassinosteroids can also alleviate or protect injuries caused by various stresses including chilling, heat, salt, nutrition, and disease.[739,740,761,812]

Earlier, BRs were regarded as mimicry substances of plant hormones. However, various dwarf mutants with defective biosynthesis or signal transduction of BRs were isolated and their shortened cells were elongated by exogenous BR but never by auxin and gibberellin, revealing that BR is a prerequisite for cell elongation[734] and that co-occurrence of BR, auxin, and gibberellin is required for normal cell elongation. Distinct synergism has been observed between BR and auxin in the elongation of pea and adzuki bean stems, bending of rice lamina,[736] and gravitropism of maize root.[813] In accord with this, BR promotes the expression of auxin-related genes, especially early auxin-inducible genes.[814,815] In addition, BR promotes the expression of cell wall synthesis genes and affects the expression of P450 genes related to BR biosynthesis and metabolism.[803]

The dwarf mutants of *Arabidopsis* show, when grown in the dark, many of the characteristics of light-grown plants, including cotyledon expansion, primary leaf initiation, anthocyanin accumulation, and depression of light-regulated gene expression. Involvement of BR in de-etiolation has been discussed.[816]

4.02.6.4.2 Signal transduction

Various reviews on the BR receptor and signal transduction have appeared.[744,816–819] The (*BRASSINOSTEROID-INSENSITIVE 1 BRI1*) gene encoding a BR receptor has been cloned by analyzing the BR-insensitive *Arabidopsis* mutant *bri1*. The receptor BRI1 is a plasma membrane-localized leucine-rich repeat receptor-like kinase (LRR-RLK). The extracellular domain of BRI1 is mainly composed of 25 LRRs interrupted by a 70-amino acid island domain between the 21st and the 22nd LRR. The extracellular domain is connected through a single-pass transmembrane domain to the cytoplasmic domain where a typical Ser/Thr kinase core is localized (**Figure 34**). The *BRI1* orthologs such as *CU3* in tomato, *LKA* in pea, *OsBRI1* in rice, and *UZU* in barley have been cloned by analyzing BR-insensitive dwarf mutants. The *BRI1*-like genes, *BRL1* and *BRL3*, having high similarities with *BRI1*, have been cloned from *Arabidopsis*. While *BRI1* is ubiquitously expressed, *BRL1* and *BRL3* are predominantly expressed in the vascular tissues (**Figure 34**).[820] Mutation of *BRL1* increases phloem and reduces xylem differentiation compared with the wild type. It is most likely that *BRL1* and *BRL3* play distinct and overlapping roles with BRI1 in vascular differentiation and growth promoted by BR.

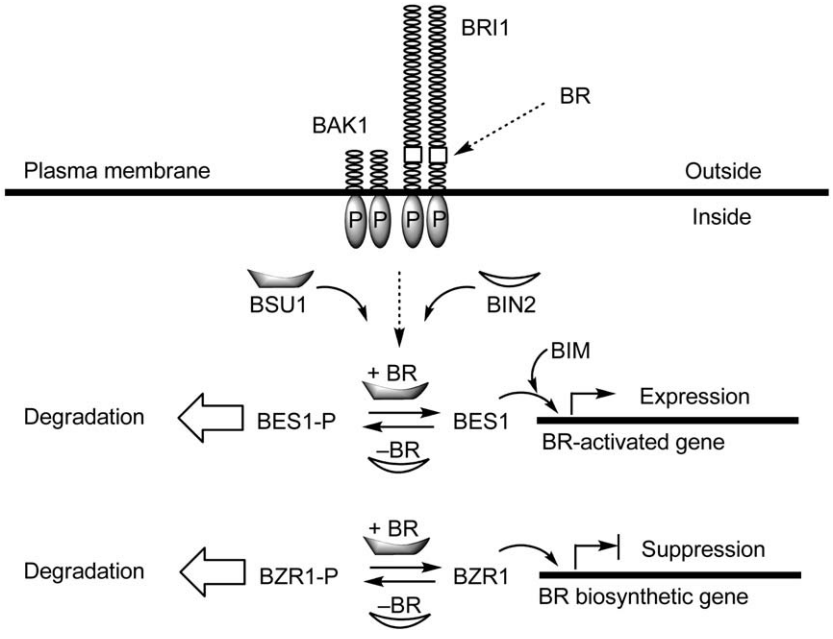


Figure 34 Structures of brassinosteroid receptors and current model for brassinosteroid signaling.

Extracellular BR binds to the domain consisting of the 70-amino acid island and the 22nd LRR. Such BR binding leads to homodimerization of BRI1 along with heterodimerization of BRI1 to BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (**Figure 34**). Like BRI1, BAK1 is also an LRR-RLK although containing only five LRRs and no BR-binding domain. Transphosphorylation between BRI1 and BAK1 seems to activate BRI1. The phosphorylation of Ser/Thr residues in the catalytic domain of BRI1 activates Ser/Thr kinases, leading to downstream intracellular signaling.

Inside the nucleus, two transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) are capable of directly binding to some BR-responsive genes (**Figure 34**). The binding of BES1 to the CANNTG motif (E-box) of the *SAUR-AC1* promoter, synergistically enhanced by three BES1-interacting Myc-like proteins (BIMs), activates the gene expression, while the binding of BZR1 to the CGTG(T/C)G motif (BR-responsive element (BRRE)) in the promoters of the BR biosynthesis genes, *CPD* and *DWF4*, feedback-suppresses the gene expression (**Figure 34**). In the absence of BR, both BES1 and BZR1 are phosphorylated by BIN2 followed by proteasome-mediated degradation, blocking BR signaling. In the presence of BR, both BES1 and BZR1 are activated by dephosphorylation due to BSU1, regulating the expression of many BR-responsive genes. Rapid progress in this area should be anticipated to increase our accurate understanding of the BR signaling processes.

4.02.7 Jasmonates and Oxylipins

4.02.7.1 Introduction

Jasmonic acid (JA) and methyl jasmonate (MeJA) (collectively termed JAs) are known to be ubiquitous plant signaling compounds. In 1962, MeJA was primarily isolated from the essential oil of *Jasminum grandiflorum*.[821] Because of its aromatic odor, this compound is known to be useful in cosmetics and perfume industries. In spite of its usefulness in the industries, physiological activities of JAs in plants have long been undefined. Cucurbic acid, a closely related compound of JA, was first identified as a growth inhibitor from seeds of *Cucurbita pepo* L.[822] Then, MeJA was isolated from different plant species as an active substance to promote senescence.[823] At the same time, JA was also identified from several plants as a growth inhibitor of rice seedlings.[824,825] After these early findings on jasmonate function by Japanese researchers, Vick and Zimmerman[826] established its biosynthetic pathway from linolenic acid, a major polyunsaturated fatty acid in plant membranes. As for the physiological function, a derivative of JA, tuberonic acid, was identified as a tuber-inducing substance in potato at the end of the 1980s.[827]

In 1987, Weidhase *et al.*[828] found that accumulation of several proteins was induced by JA treatment. Interestingly, this dynamic change of protein pattern involves degradation of Rubisco protein in agreement with the activity to promote senescence.[829] Furthermore, in 1990, Farmer and Ryan[830] showed that airborne MeJA could induce wound-responsive proteinase inhibitor in tomato. Separately, Gundlach *et al.*[831] found that JA induces accumulation of secondary metabolites and *de novo* transcription of defense-related genes, such as phenylalanine ammonia lyase, in plant cell cultures. Importantly, they also revealed that the endogenous level of JA is raised in some cultured plant cells after treatment with a yeast elicitor. These results demonstrate the integral role of JA in a general signal transduction system regulating inducible defense responses in plants.

As described above, the roles of JAs in the response to stress such as wounding and diseases were of major interest for many researchers in the early 1990s. In 1994, however, positive action of this compound in developmental processes was first proposed by Turner's group[832] using a coronatine, a bacterial toxin, insensitive mutant that also lacks sensitivity to JAs. The mutant has a defect in fertility, and thus a role for JAs in flower development was first suggested. Separately, McConn and Browse[833] isolated a JA biosynthesis mutant that totally lacked biosynthesis of trienoic fatty acids, precursors for JA synthesis.[833] This mutant also had a defect in fertility and the phenotype was complemented with the treatment of JA. After these findings, several mutants for JA biosynthesis were identified, and some of these mutants have defects in both accumulation of JA and fertility.[834–836]

Studies on JA signaling were substantially advanced with a series of research on the COI1, Coronatine Insensitive 1. This protein has an F-box motif, which has been originally found in a component of the ubiquitin–proteasome system.[837] Another well-known mutant of jasmonate function is *jar1*, which is impaired

in the formation of an amino acid derivative of JA.[838] Although the *jar1* mutant is significantly impaired in JA-mediated root growth inhibition, this mutant is not male sterile. These two mutants have been utilized as effective tools for investigating JA-mediated signaling in plants. The finding of these mutants raises important questions that remain to be clarified: What is the role of the COI1 protein? How is JA signal transduced? How is the amino acid conjugate involved in JA signaling? Very recent work on JAZ family, COI1 target proteins, shed light on these key issues in JA signaling.[839–840] Research advances in chemistry, biology, and signaling mechanism for JAs made in this decade are overviewed in this chapter. Because of space limitation, many articles are not cited here. The readers are recommended to refer other well-documented reviews that cover biology and signaling mechanism of these molecules in more detail.[841]

4.02.7.2 Chemistry

4.02.7.2.1 Chemical structure

Most of the major metabolites of linolenic acid, listed in **Figure 35**, contain the cyclopentane (or -ene) ring and the two side chains arrayed in the *cis* fashion on the ring. Some of the metabolites, however, take the *trans* arrangement. Although isomerization to the *trans* isomer during isolation/purification is a likely process, a

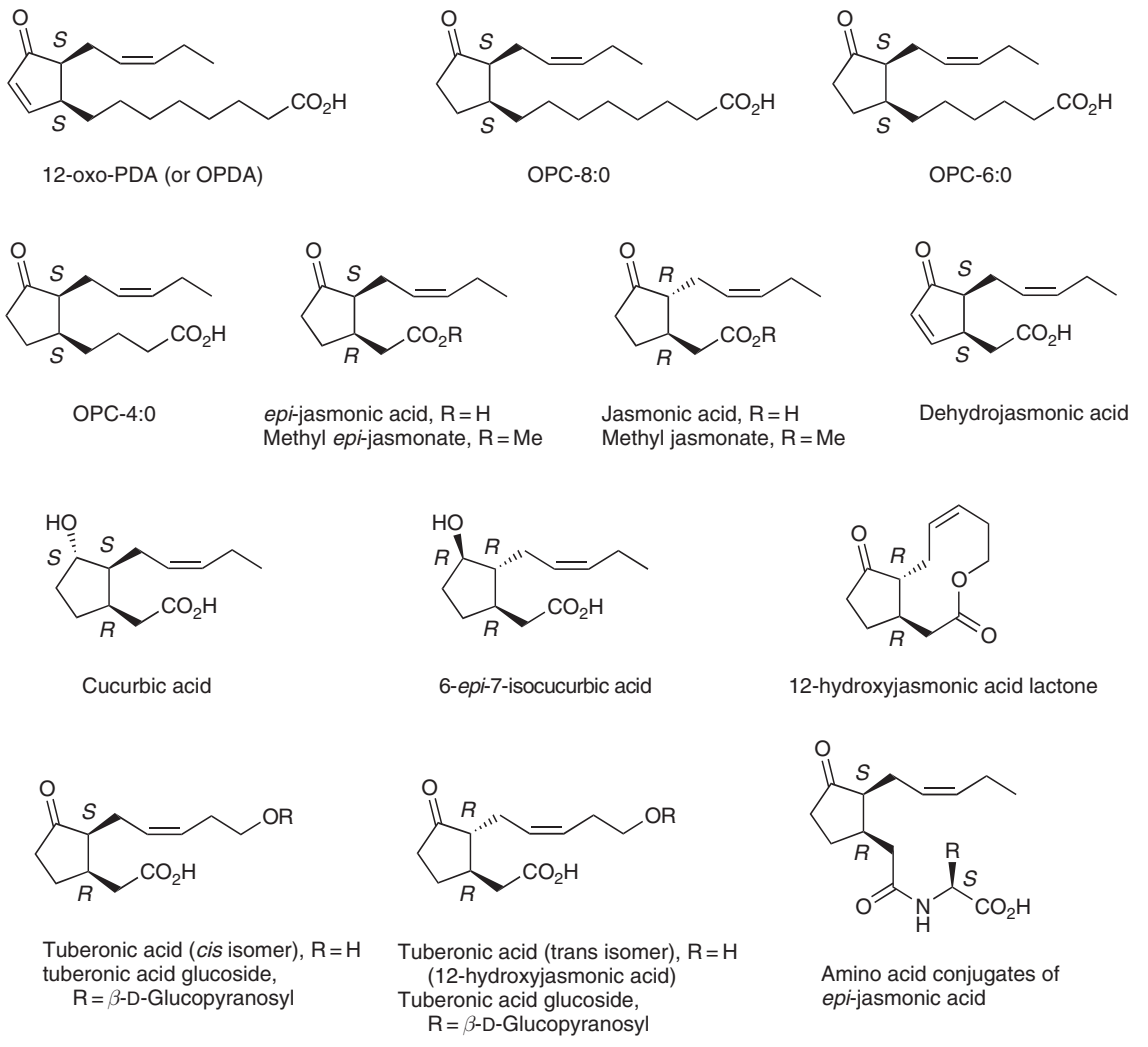


Figure 35 Structures of the linolenic acid metabolites with the notation of the absolute configurations.

pathway directly producing the *trans* compounds is also possible. In addition, several products containing the hydroxyl group on the (2*Z*)-pentenyl chain have been isolated.

The chiral centers are indicated in the structures in **Figure 35**. The chirality at the carbon possessing the $(CH_2)_nCO_2H$ is dependent on the length of CH_2 and the local structures. The *R* configuration is defined to the compounds possessing the CH_2CO_2R and the saturated cyclopentane ring, while the *S* configuration is defined to others and dehydrojasmonic acid possessing the cyclopentene ring.

4.02.7.2.2 Chemical stability

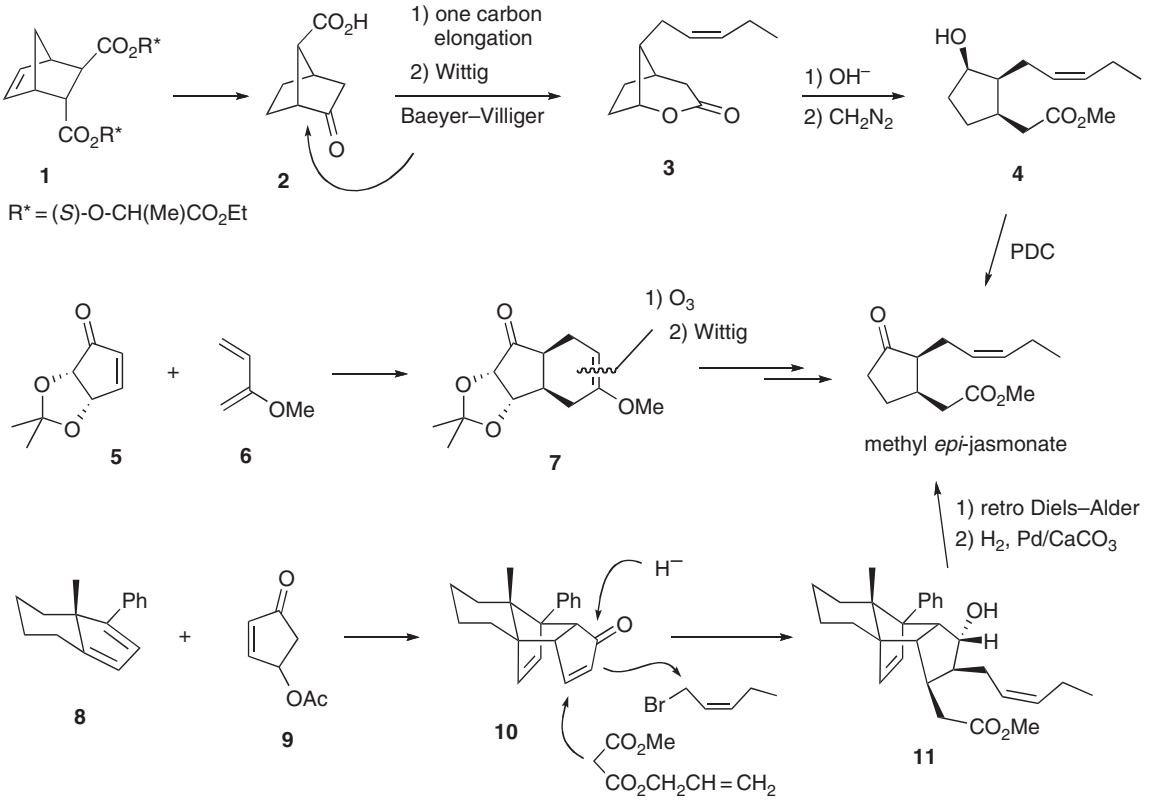
Since the pentenyl chain is located at the α-position of the carbonyl group except for cucurbic acid, the α-position is susceptible to epimerization to the more stable *trans* isomer under basic and acidic conditions. The equilibrium ratio of methyl *epi*-jasmonate (*cis* isomer) and jasmonate (*trans* isomer) calculated taking into account the Boltzmann distribution of the conformers is 4:96 at 25 °C,[842] which is well consistent with the observed ratio of 5:95.[843] In an alkaline solution, the *cis* to *trans* isomerization takes place quickly at room temperature. On the other hand, the isomerization under weakly acidic conditions is rather slow. The autoisomerization by the acidic part in the acid metabolites is negligible in a neutral solution. For example, no isomerization of 12-oxo-phytodienoic acid (OPDA) was observed by ^{1}H NMR spectroscopy in untreated $CDCl_3$ (containing a trace amount of DCl produced by decomposition of $CDCl_3$) and in DCl-free $CDCl_3$ at room temperature for 26 days.[844,845] A similar stability was confirmed for OPC-8:0. Tuberonic acid in CD_3OD (MeOH-d_4) was stable at room temperature for 21 days.[846] However, the isomerization took place with a catalytic HCl in MeOH at room temperature for 2 h. The isomers can be detected conveniently by ^{1}H NMR spectroscopy (*cis* isomer, δ 2.8–2.9; *trans* isomer, δ 2.6–2.8).

4.02.7.2.3 Chemical synthesis of the metabolites on the linolenic acid cascade

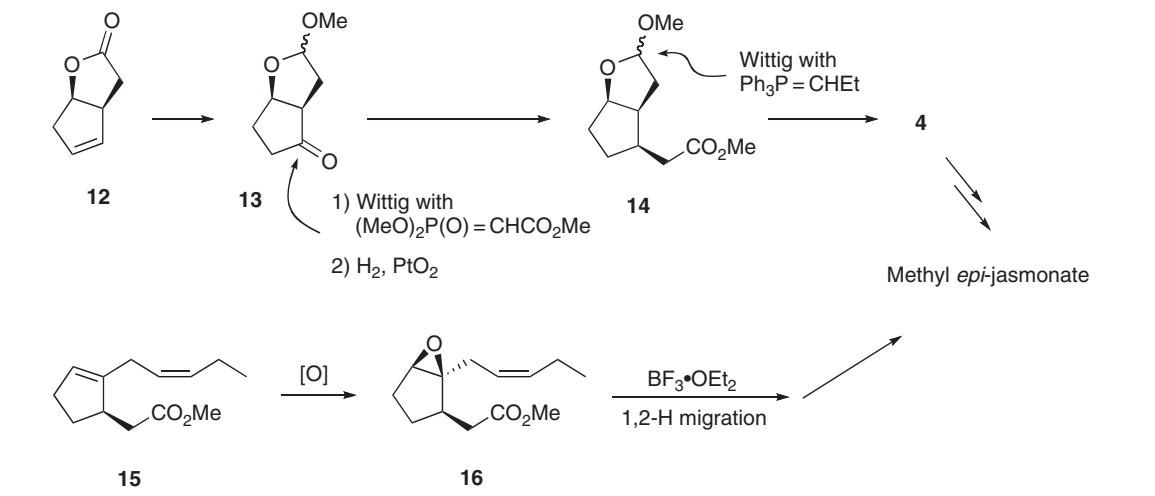
Since the metabolites are stable under neutral to slightly acidic conditions, oxidation of cyclopentanols (or cyclopentenols) has been used in the last step of the syntheses. For example, oxidation of methyl cucurbate and/or the epimeric alcohol of methyl cucurbate with PDC in CH_2Cl_2,[847] H_2CrO_4 at 0 °C for 7 min,[848] and with Dess–Martin periodinane at room temperature for 30 min[849] afforded methyl *epi*-jasmonate without epimerization, while oxidation with $Na_2Cr_2O_7/H_2SO_4$ overnight[850] and with PCC/NaOAc for 19 h[851] resulted in epimerization. *epi*-Jasmonic acid, OPDA, and OPC-8:0 have been synthesized from the corresponding diols by Jones oxidation.[844,845,852]

4.02.7.2.3(i) Synthesis of methyl epi*-jasmonate* The syntheses of *epi*-jasmonate through Diels–Alder reaction are summarized in **Scheme 7**. Diels–Alder adduct **1** from the fumarate of (*S*)-ethyl lactate and cyclopentadiene was transformed to the bicyclic carboxylic acid **2**, which possesses the handholds for stereoselective construction of the side chains.[847] After one carbon elongation, Wittig reaction and Baeyer–Villiger oxidation produced lactone **3**, which was converted to alcohol **4**. Finally, PDC oxidation of the alcohol afforded methyl *epi*-jasmonate without epimerization. Oxidative cleavage of the Diels–Alder adduct from 2-cyclopenten-1-one and butadiene followed by Wittig reaction was the key conversion of the previous racemic synthesis of methyl *epi*-jasmonate (see Tanaka *et al.*;[853] cf. Roth *et al.*[854]). Later, the chiral version of this approach was realized using the chiral cyclopentenone **5** and diene **6**.[855] Diels–Alder reaction between the optically active cyclopentadiene **8** and cyclopentenone **9** followed by elimination of AcO from the adduct afforded enone **10**.[851] Installation of the two side chains took place stereoselectively on the less-crowded side of the olefinic face to produce **11**. To prevent the unwanted epimerization during the subsequent retro Diels–Alder reaction, the ketone group was reduced and submitted to the retro reaction to furnish the cyclopentenol, which upon hydrogenation with $Pd/CaCO_3$ followed by oxidation of the alcohol furnished methyl *epi*-jasmonate. The same strategy was previously used in the synthesis of OPDA, in which retro Diels–Alder reaction of the ketone (**29** in **Scheme 11**) was successfully carried out at room temperature with $EtAlCl_2$.[856]

The stereoselective constructions of the second side chain on the cyclopentane ring are shown in **Scheme 8**. Ketone **13** derived from optically active lactone **12** was converted to ester **14** via Wittig reaction with $(MeO)_2P(=O)CH_2CO_2Me/NaH$ followed by hydrogenation of the resulting olefin from the convex face.[848] Stereoselective epoxidation of olefin **15** with CF_3CO_3H and the subsequent 1,2-hydride migration in the epoxide with $BF_3{\bullet}OEt_2$ produced *epi*-jasmonate without epimerization.[857]

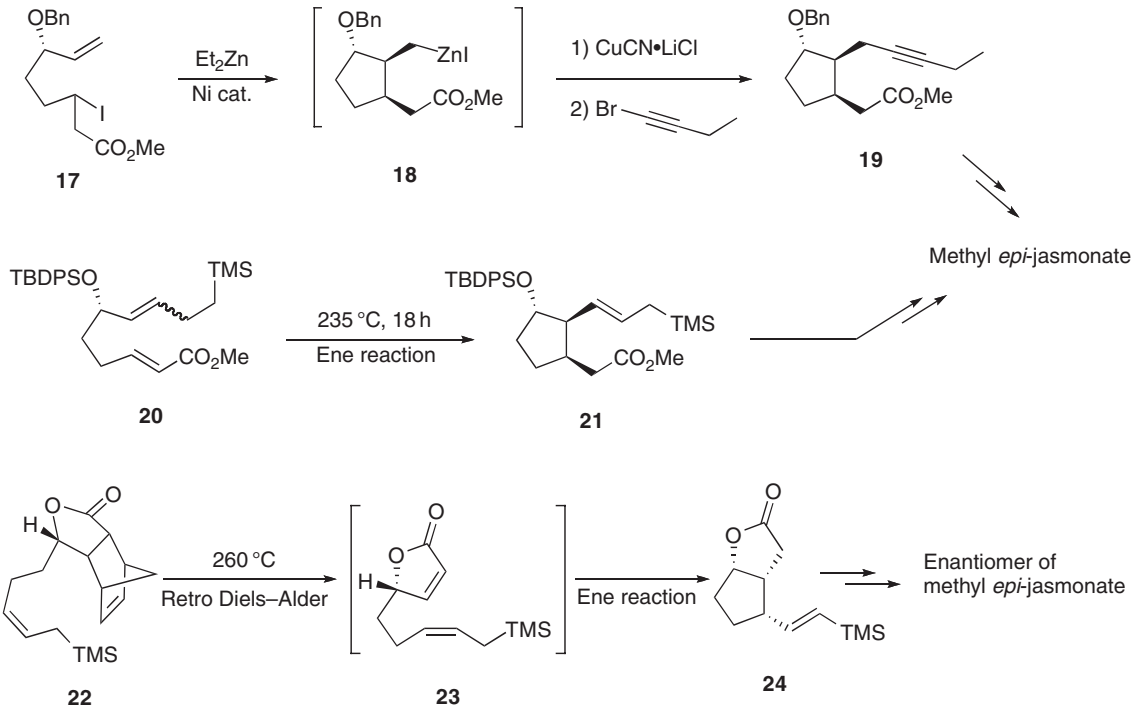


Scheme 7 Synthesis of methyl *epi*-jasmonates using Diels–Alder reaction.



Scheme 8 Miscellaneous syntheses of methyl *epi*-jasmonate.

Formation of the cyclopentane ring and concomitant arrangement of the two side chains in the *cis* fashion is another efficient approach to methyl *epi*-jasmonate as delineated in **Scheme 9**. In the upper sequence, nickel-catalyzed carbozincation of **17** with Et_2Zn produced organozinc **18**, which was converted to a copper reagent for coupling with 1-bromoacetylene to produce **19** efficiently.[849] The ene reaction of **20** and **23** has afforded **21** and **24**, respectively.[858,859] In the latter case, **23** was generated *in situ* by retro Diels–Alder reaction of **22**.

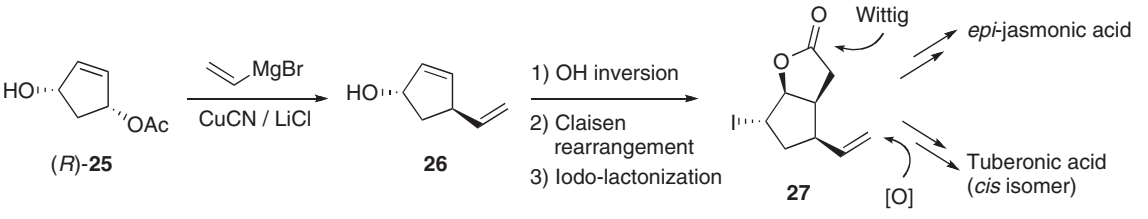


Scheme 9 Construction of the cyclopentane ring and the *cis* array of the side chains leading to the formation of methyl *epi*-jasmonate.

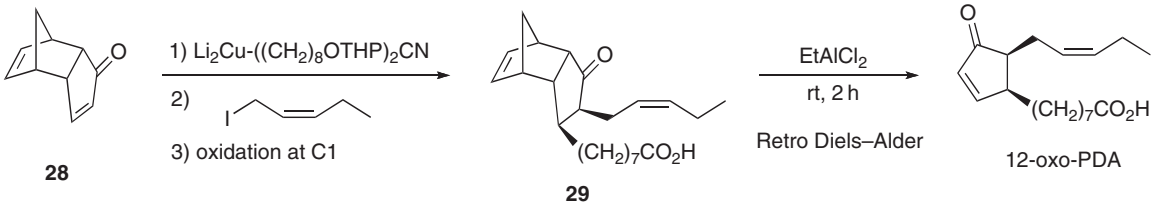
4.02.7.2.3(ii) Synthesis of* epi-*jasmonic acid Although *epi*-jasmonic acid is the key intermediate in the metabolic pathway leading to methyl *epi*-jasmonate and amino acid conjugates, synthesis of the acid in an optically active form was only orally communicated.[852] As shown in **Scheme 10**, the vinyl group was installed regioselectively on the ring of acetate (*R*)-**25** as a latent CH_2CO_2H via the copper-assisted reaction[860–862] with CH_2=CHMgBr in the presence of LiCl. The resulting alcohol **26** was transformed to lactone **27**, which produced the acid through Wittig reaction. Similarly, tuberonic acid (*cis* isomer) was synthesized.[846]

4.02.7.2.3(iii) Synthesis of OPDA, OPC-8:0, and related compounds In contrast to the high temperatures for retro Diels–Alder reaction, $EtAlCl_2$ promotes the reaction to be proceeded at room temperature. This finding was applied successfully to **29** to produce OPDA without epimerization (**Scheme 11**).[856]

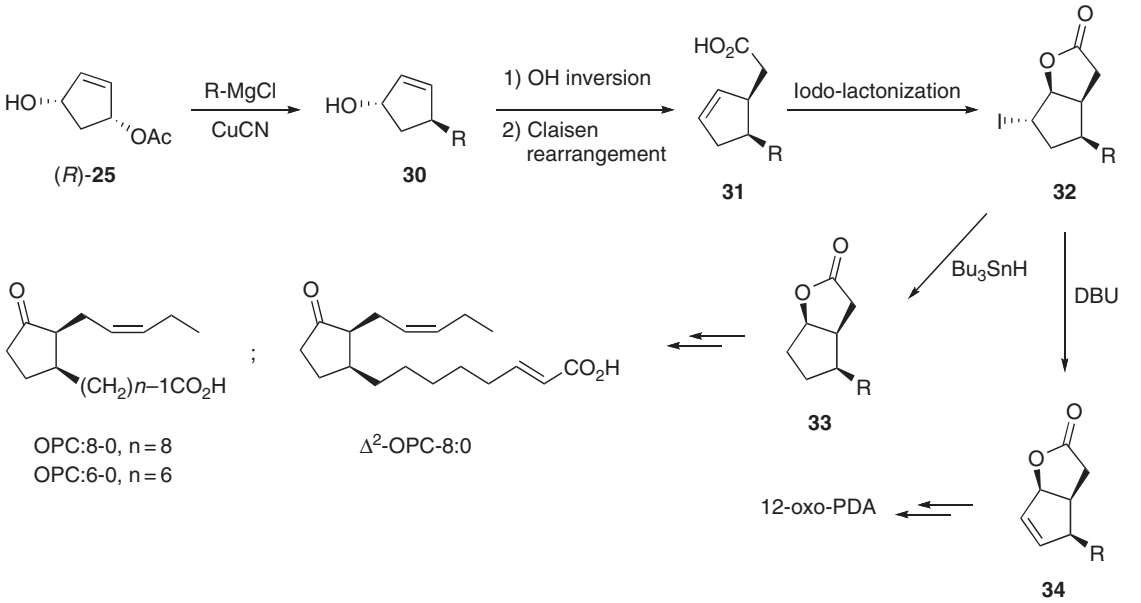
Regioselective allylic substitution of (*R*)-**25** giving the S_N2-type product **30**, established with $R_2Cu(CN)(MgCl)_2$ derived from RMgCl and CuCN,[860–862] has been applied to the synthesis of OPDA and OPC-8:0 as summarized in **Scheme 12**.[844,845] Later, Δ^2-OPC-8:0 and OPC-6:0 were synthesized as well.[863] It should be noted that the high regioselectivity in the copper-catalyzed reaction used in the first step is attainable only with RMgCl (not RMgBr) and CuCN in THF.



Scheme 10 Synthesis of *epi*-jasmonic acid starting with (*R*)-**25**.



Scheme 11 Retro Diels–Alder approach to 12-oxo-PDA.

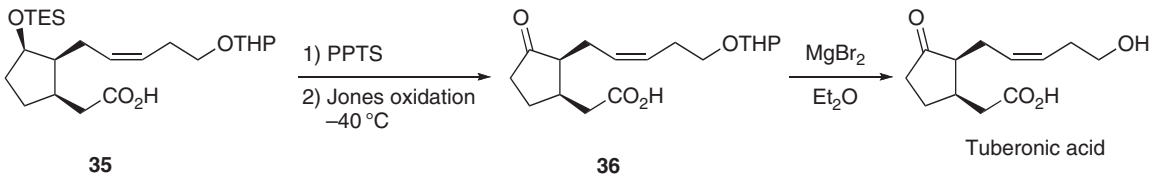


Scheme 12 Synthetic method using (*R*)-25 leading to linolenic acid metabolites.

Allylic substitution was utilized in another synthesis of OPDA.[864] Elongation of the CH_2CO_2H chain of the jasmonate to $(CH_2)_7CO_2H$ was reported.[865]

4.02.7.2.3(iv) Synthesis of tuberonic acid, 12-hydroxyjasmonic acid, and β-glucopyranosyl derivatives The presence of the hydroxyl group at the end of the pentenyl group severely limits reagents and reaction conditions to be used at the final stage. The method shown in **Scheme 8** (the upper sequence) was applied successfully to the synthesis of the methyl esters of tuberonic acid and its glucopyranosyl derivative.[866] The *trans* isomer of the latter was obtained by epimerization (NaOMe/MeOH, room temperature, 2 h) of the tetraacetate derivative. However, hydrolysis without epimerization is uncertain.

Recently, the protected tuberonic acid was synthesized by using the method shown in **Scheme 10** and deprotection was studied with success to afford tuberonic acid for the first time (**Scheme 13**).[846] Through the synthesis it was revealed that tuberonic acid and its derivative are liable to epimerization more easily than *epi*-jasmonic acid. In addition, exposure of tuberonic acid to NaOH in aqueous MeOH produced 12-hydroxyjasmonic acid.



Scheme 13 Synthesis of tuberonic acid.

4.02.7.3 Biosynthesis and Metabolism

4.02.7.3.1 Biosynthetic pathway in plants

JAs are derived from linolenic acid via an octadecanoid pathway consisting of several enzymatic steps (**Figure 36**). Multiple compartments in plant cells participate in JA synthesis. The early steps of this pathway occur in chloroplasts, where linolenic acid is converted to OPDA by means of the three enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC).[867–869] Linolenic acid is oxygenated by 13-LOX producing a peroxidized fatty acid 13-hydroperoxylinolenic acid. The product is subsequently metabolized by AOS to an unstable compound allene oxide. Allene oxide is sequentially converted by AOC to produce OPDA. An alternative pathway from another trienoic fatty acid, hexadecatrienoic acid (16:3), is present in chloroplasts.[870] In this pathway, dinor OPDA is produced instead of OPDA. OPDA and dinor OPDA are transported into the peroxisome. An ABC transporter involved in this transport was identified in

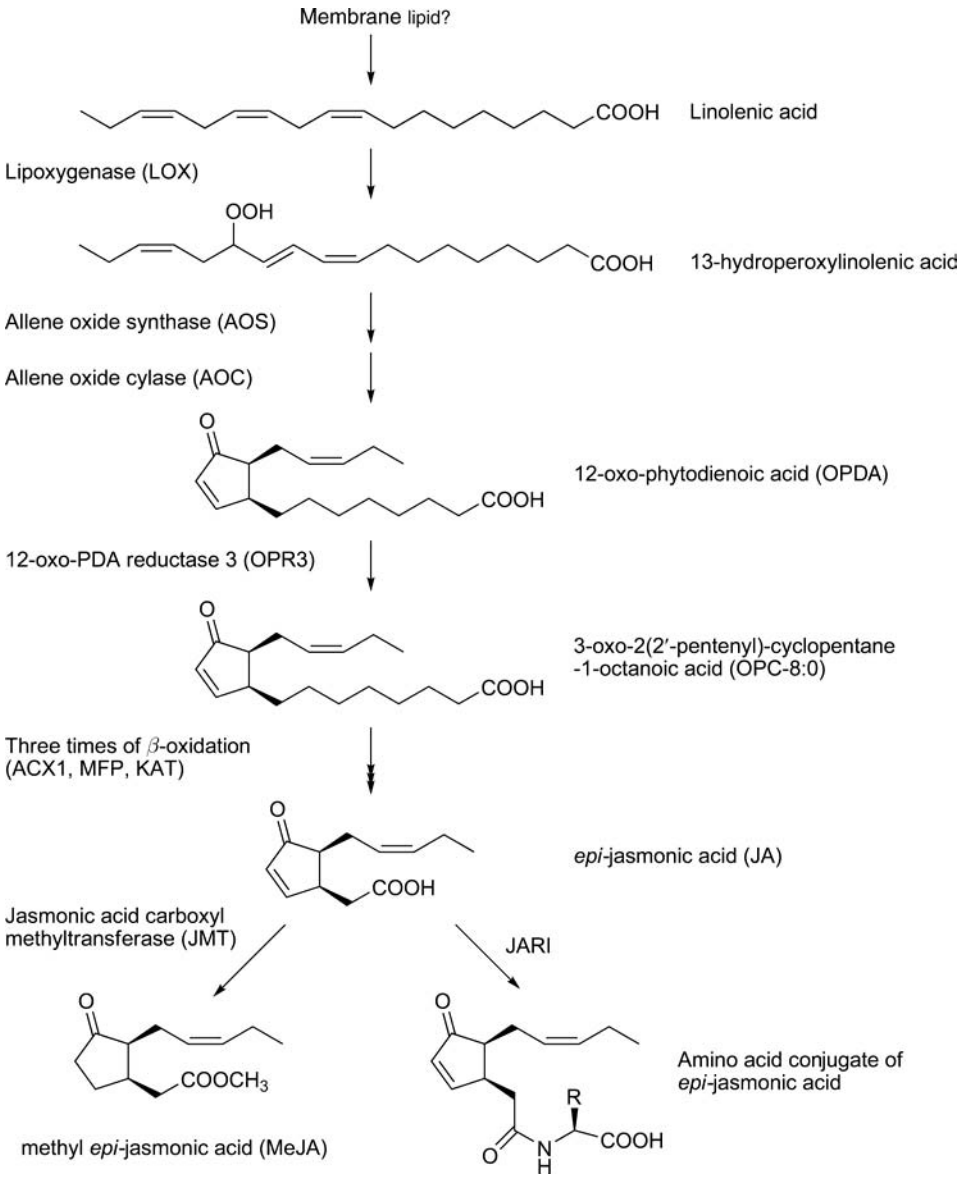


Figure 36 Biosynthetic pathway of jasmonates.

Arabidopsis.[871] However, it is still uncertain how OPDA is transported into the peroxisome by the transporters. Transported OPDA is reduced by OPDA reductase (OPR3) in its double bond of the cyclopentenone ring. The product OPC8:0 is converted into OPC8:0-CoA,[872] and then processed by three rounds of β-oxidation generating JA (OPC6:0 produced from dinor OPDA undergoes two rounds of β-oxidation).[870,873,874]

As for the modifications for JA, conjugation of isoleucine to JA is known to be catalyzed by JAR1 (jasmonate resistant 1).[838] The gene for JAR1 has been identified as a site of mutation for JA-mediated inhibition of root elongation.[875] Methylation of JA is catalyzed by a jasmonic acid methyltransferase. The corresponding gene was isolated from *Arabidopsis*.[876]

The release of the substrate fatty acids (18:3 and 16:3) from membrane has long been believed to be a primary step of JA biosynthesis. *Arabidopsis dad1* mutant shows delayed anther dehiscence and shorter filament length.[877] Since the mutant is deficient in JA and a corresponding gene encodes a phospholipase A1 that preferentially catalyzes the liberation of fatty acid from the *sn*-1 position of glycerophospholipids, this protein DAD1 is thought to be involved in the supply of linolenic acid from membrane in flowers. Indeed, DAD1 is located in plastids where JA synthesis occurs. A recent work, however, suggested that conversion of the trienoic fatty acids to OPDA and dinor OPDA might occur in membrane-bound form.[878] Stelmach *et al.*[879] first found that OPDA was attached in a glycerol backbone of a chloroplast galactolipid MGDG (MGDG-O) in wounded leaves. Subsequently, various types of oxylipin-containing galactolipids (arabidopsides) were identified.[880–882] Buseman *et al.*[878] systematically identified the oxylipin-containing galactolipids in wounded plants. They found that OPDA was esterified in both *sn*-1 and *sn*-2 positions of the glycerol backbone. In contrast, dinor OPDA was esterified only in *sn*-2 position, reflecting the fact that the substrate 16:3 is a fatty acid solely esterified in the *sn*-2 position of glycerol moiety. Since the esterified positions of these oxylipin-containing galactolipids are basically the same as the positions where trienoic fatty acids, the oxylipin precursor, are attached, they assumed that these oxylipins are mainly produced as membrane-attached forms. Although DAD1 participates in JA biosynthesis in flowers, it should be clarified whether the enzyme catalyzes the release of linolenic acid or OPDA attached to the membrane *in vivo*.

4.02.7.3.2 Lipid-derived reactive electrophile species and nonenzymatically formed oxylipins

In addition to the enzymatically produced oxylipins, various types of nonenzymatically formed oxylipins were found in plant tissues. Phytoprostanes are a large family derived from nonenzymatic oxidation of 18:3 or 18:2 fatty acids.[883] These molecules are significantly accumulated in wounded and diseased plants. Even in healthy plants, the levels of some phytoprostanes exceed those of OPDA. Some of the phytoprostanes show strong activity to induce phytoalexin accumulation and gene expression.[884] Oxidative modification of unsaturated fatty acids upon various stresses produces another type of oxylipins, RES (reactive electrophile species).[885] The RES has α, β-unsaturated carbonyl groups that possess high reactivity to proteins and other lower molecular weight compounds. Two particularly active RES, small vinyl ketones and 2(*E*)-alkenals, were shown to induce gene expression in plant tissues.

4.02.7.4 Physiology

4.02.7.4.1 Responses to biotic and abiotic stresses

JAs are known to show a broad array of function in plants. There have been numerous physiological analyses of the function of JAs. The role of JAs in the response to biotic stresses, such as insect or fungal attack,[886,887] and abiotic stresses, such as mechanical wounding,[888–890] has been well documented. Indeed, exogenous application of JAs induces the expression of a large number of stress-responsive genes.[890–892] Several reports have shown that a defect in JA biosynthesis or some component of the JA signaling is accompanied by an impairment in JRG (jasmonate-responsive gene) expression,[886,893,894] resulting in increased sensitivity to stresses such as pathogen infection or herbivore attack. Levels of endogenous JAs increase after treatment with elicitors and following wounding.[895] Transgenic plants containing higher levels of MeJA than wild type cause constitutive expression of JRGs and exhibit elevated resistance to virulent pathogen *B. cineria*.[876] Thus, JRG expression appears to be induced under these stress conditions and may contribute to stress tolerance in plants. JAs or some components derived from JA biosynthetic pathway are proposed to participate not only in local response of gene expression

but also in systemic induction in herbivore attack. In tomato, grafting studies with *coi1* mutant revealed that signal perception is required for recognition of the transmissible signal in distal responding leaves.[896] Conversely, experiments with several JA biosynthetic mutants indicated that the production of the graft-transmissible signal requires JA synthesis in wounded tissues. Particularly, the necessity of peroxisomal β-oxidation for production of the systemic wound signal is proved with *acx1* mutant defective in an acyl-CoA oxidase (ACX1A) that catalyzes the first step of the β-oxidation in the octadecanoid pathway.[897] This result indicates that a downstream component in JA biosynthesis after β-oxidation is a systemic signal in wound stress response in tomato.

When plants undergo various stresses, certain secondary metabolites, including defense compounds, accumulate. Several secondary metabolites such as terpenoid indole alkaloids, indole glucosinolate, nicotine alkaloids, and polyamines are known to accumulate through the induction of biosynthetic genes by jasmonates.[898–900] MeJA also induces genes involved in the formation of tryptophan derivatives, terpenoid indole alkaloids.[901] These compounds are known to be involved in defense response to pathogen attack as phytoalexins.

Another interesting feature of JAs in stress responses is their role in oxidative stresses. In particular, JAs have been shown to have some role in ozone stress response.[902] Accumulation of JA also occurs upon ozone exposure in plants.[903] Moreover, *jar1* mutant shows impaired ozone response.[904] Sasaki-Sekimoto *et al.*[892] revealed that JA induces gene expression related to antioxidant biosynthesis such as ascorbic acid and glutathione biosyntheses, and thereby provides oxidative stress tolerance. In this report, they performed a comprehensive analysis of jasmonate-regulated metabolic pathways using cDNA macroarrays. This approach identified nine metabolic pathways belonging to two functionally related groups: (1) ascorbate and glutathione metabolic pathways, which are important in defense responses to oxidative stress, and (2) biosynthetic enzymes for indole glucosinolate, which is a defense compound in the Brassicaceae family. In jasmonate-deficient *Arabidopsis opr3* mutants, the induction of antioxidant genes was abolished. Compared with the wild type, *opr3* mutants were more sensitive to O_3 exposure. They suggest that the coordinated activation of the metabolic pathways mediated by jasmonates provides resistance to environmental stresses.

4.02.7.4.2 Stress response in the absence of jasmonic acid

Among the various oxylipins having biological activity, JA and MeJA are the best characterized. As described above, β-oxidation-derived signal has a major role in wounding response in tomato.[897] However, *opr3* mutant, which lacks these two oxylipins, is known to be resistant to the dipteran *Bradysia impatiens*, as are wild-type plants.[905] This suggests that OPDA present in the *opr3* mutant may be the active signaling molecule and have a role in the induction of defense response genes. Moreover, investigators have shown that OPDA upregulates not only COI1-dependent genes induced by JA, but also several COI1-independent genes that do not respond to JA. They also suggested that OPDA may function cooperatively with JAs to regulate the expression of defense response genes. Taki *et al.*[906] analyzed OPDA-dependent gene expression by comparing responses to OPDA and JAs using DNA microarrays covering 80% of the *A. thaliana* genome. They identified a group of genes designated 'ORGs,' which responded to OPDA but not to JAs. OPDA treatment of *coi1* mutants demonstrated that ORG expression is independent of the COI1-mediated JA signaling pathway. Using the OPDA and JA biosynthetic mutants, *aos* and *opr3*, they showed that the response of the ORG expression to wounding is impaired in *aos* but not in *opr3* (see **Figure 36**). Thus, OPDA is a lipid signal mediator *in vivo*, regulating ORGs that function in wounding response.

4.02.7.4.3 Effect of jasmonates on developmental processes

JAs are known to inhibit plant growth. This effect has been reported by Yamane *et al.*[824,825] in the early 1980s and frequently utilized for screening of their signaling mutants. *Jar1* and *jin1* have been isolated primarily as mutants in which JA-dependent root growth inhibition is reduced.[875,907] *Coi1* also shows similar phenotype. By contrast, mutants that exhibit constitutive expression of JA-responsive genes (cf. *cev1*) show reduced root growth similar to JA-treated plants.[908] The mutants generally have elevated levels of JAs.

JAs also play important roles in flower development.[832,833,837] Particularly, the requirement of JA biosynthesis for anther development was clearly shown by JA biosynthesis mutants.[834–836] AOS and OPR3, two enzymes required for JA biosynthesis, are encoded as a single gene in *Arabidopsis* genome. An *opr3* mutant in *Arabidopsis*,

which could not produce JA, exhibits delayed anther dehiscence, resulting in male sterility.[834,835] Studies with this mutant showed that JA, and not its precursors, is the active signaling molecule that regulates anther development, since the application of JA, but not OPDA, restored fertility.[835] As for the signaling components, *Arabidopsis coi1* also shows male sterility.[832] However, a tomato mutant for COI1 homolog has a crucial defect in female organs, and results in female sterility. This evidence shows that JA function in regeneration step is diverse in plant species.[909]

Tuber formation is also known to be induced by a JA derivative called tuberonic acid.[827] Tuberonic acid has been primarily found in potato as a tuber-inducing substance, and named as tuberonic acid. This compound is also shown as a substrate of a sulfotransferase in *Arabidopsis*.[910]

Promotion of senescence by JAs has also been reported in the early days of jasmonate study.[823] Gene expression for the enzyme chlorophyllase, which is involved in the early step of chlorophyll degradation, is known to be strongly induced by exogenous treatment of MeJA.[911] This enzyme might be a major player in JA-mediated senescence. JA levels are known to be higher in senescing leaves than in nonsenescing leaves.[912] Arabidopside A also shows senescence-promoting activity.[913] This compound shows much stronger activity than JAs.

4.02.7.4.4 OPDA on developmental processes

OPDA was shown to promote tendril coiling response in *Bryonia*.[914] This compound was also shown to induce stomatal opening.[915] The stomatal opening activity of OPDA is higher than that of other phytohormones that induce stomatal opening.

4.02.7.5 Signaling

Among several components that are identified in signaling via JAs, COI1 is regarded as a central component. It has an F-box motif that shows similarity to proteins involved in specifically interacting proteins for its proteolytic removal.[837,916] A gene for COI1 was first identified from a mutant insensitive to coronatine.[832] Coronatine is a bacterial toxin produced by *Pseudomonas syringae*. Since the toxin has a structural similarity to JAs, a mutant for coi1 is insensitive to both coronatine and JA. COI1 is thought to be involved in ubiquitination and subsequent degradation of proteins that repress JA signaling.

ORCA is one of the well-investigated downstream transcription factor in JA signaling.[899] This factor was identified as a key component in controlling alkaloid biosynthesis in periwinkle. Ethylene response factor 1 (ERF1) was identified as a master switch in the cross talk between ethylene and JA signaling.[917] Another major component in JA signaling is MYC2, a regulator in JA-mediated gene expression.[918,919] There are two branches in JA signaling pathway that are antagonistically regulated by MYC2 and ERF1. MYC2 positively regulates expression of wound-inducible genes, and by contrast, it negatively regulates pathogen-responsive genes. For ERF1, the effect is vice versa. This antagonistic action of MYC2 and ERF1 may cause the independence between wound signaling and pathogen-defense signaling in *Arabidopsis*. Memelink *et al.*[900] found *Arabidopsis* homologs of ORCAs that are initially identified in *C. roseus* cell suspension cultures. Among them, ORA47 is a COI1-dependent positive regulator of JA biosynthesis.

Although the target(s) of COI1 has long been unknown, the proteins were identified by two independent approaches. Chini *et al.*[839] found the COI1 target, JAZ family proteins, from the analysis of a jasmonate-insensitive mutant, *jai3-1*. They showed that *jai3-1* is a dominant mutant that lacks the C-terminal end of the encoded protein. Since this mutant protein is resistant to proteolysis by 26S proteasome, it may block the COI1 function and thus exhibit insensitivity to JAs. Moreover, they proved that one of the JAZ proteins binds to COI1 *in vivo*. Another group, Thines *et al.*,[840] globally analyzed JA-mediated gene expression in stamen. Among the genes they identified, they noticed that many of the genes belong to the same gene family homologous to ZIM1, a protein family having a zinc finger DNA-binding motif. However, none of the JAZ proteins has the DNA-binding motif. Importantly, they showed binding of the JAZ1 protein to COI1 *in vitro* only in the presence of isoleucine conjugate of JA. This result strongly suggests that COI1 or JAZ1 (or both) is a receptor of isoleucine conjugate of JA (**Figure 37**). How does JAZ family control JA function? A gene corresponding to JAZ10 (JAS1) produces two different transcripts.[920] One of the transcripts encodes a truncated protein that lacks the C-terminus of another gene product. This truncated protein may behave as an effective repressor in JA signaling

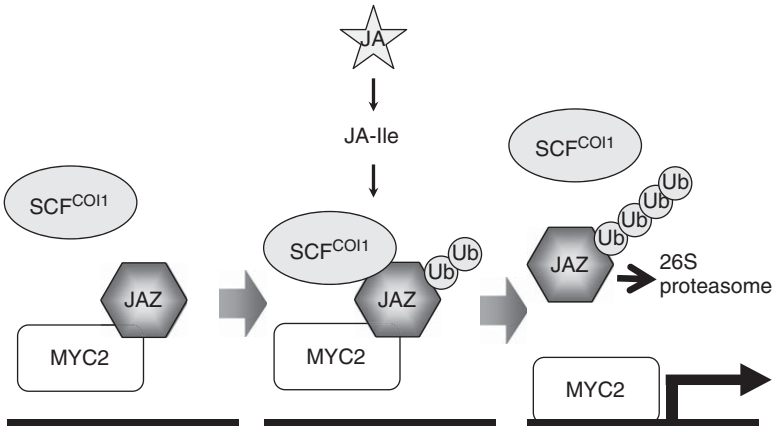


Figure 37 Model for jasmonate signaling.

in vivo. Although the receptor(s) for the JA signaling is still unidentified, studies on JA signaling will be greatly accelerated with these recent findings of JAZ family proteins.

4.02.8 Ethylene

4.02.8.1 Introduction

Ethylene is a simple gaseous molecule that plays important roles in plant growth and development, including seed germination, leaf senescence, fruit ripening, abscission, and response to abiotic and biotic stresses.[921] Ethylene is among the best-known plant hormones because of the extensive biochemical, molecular, and genetic studies conducted on its biochemistry, signal transduction, and regulation. In this chapter, we will focus on the progress made in the last decade.

4.02.8.2 Chemistry

In 1967, Burg and Burg[922] examined various analogs of ethylene for biological activity to suppress elongation of pea stems. No analogs of ethylene-like biological activity have been found as naturally occurring substances so far.

Silver ions[923] and 2,5-norbornadiene (NBD)[924] are well known as effective inhibitors of ethylene action. Silver, applied in the form of thiosulfate (silver thiosulfate (STS)), is a very effective inhibitor of ethylene action. It has been used with much success on cut flowers and potted plants. But as it is a heavy metal it cannot be used on food and feed, and is harmful to environment. As NBD requires continuous exposure and a high concentration, and has a strong odor, it also cannot be used on cut flowers and food. Furthermore, both compounds are toxic for plant growth in high concentration.

In the late 1990s, Sisler and Serek[925] discovered 1-methylcylopropene (1-MCP) as the new inhibitor of ethylene action. Now 1-MCP is the most useful compound among recently developed inhibitors of ethylene responses. It is stable and active at a considerably lower concentration ($0.5\ \mathrm{nl\,l^{-1}}$). It binds almost irreversibly to the receptors. Therefore, the effect continues until the receptors replace *de novo* synthesized ones. Now it is available commercially.

4.02.8.3 Biosynthesis

Ethylene is produced not only by higher plants but also by microorganisms. Microorganisms produce ethylene by two different pathways: 2-oxoglutarate-dependent pathway[926] and 2-oxo-4-methylthiobutyrate-dependent pathway.[927] On the other hand, higher plants produce ethylene by ACC (1-aminocyclopropane-1-carboxylic

acid) pathway, which includes L-methionine, *S*-adenosylmethionine (AdoMet), and ACC as intermediates.[928] These pathways have been established.

4.02.8.3.1 Transcriptional regulation of ACC synthase

In the ethylene biosynthesis pathway, the step in which AdoMet is converted to ACC by ACC synthases (ACSs) is the rate-limiting one. ACS consists of a multigene family,[929] and different genes show distinct patterns of expression during growth and development, and in response to various external stimuli. Increases in ACS activity in response to auxin and wounding are inhibited by inhibitors of protein synthesis and RNA synthesis, suggesting that synthesis of ACS is transcriptionally regulated. In all tissues examined, mRNA is not present at a detectable level in tissue producing no ethylene. Northern blot analyses show that the genes of ACS isozymes are differentially regulated by different stimuli. The tomato ACS family consists of at least eight genes, and among them five genes (LeASC1A, LeACS2, LeACS3, LeASC4, and LeACS6) are well studied with respect to transcriptional regulation, especially in tomato fruit development.[930] LeACS2 and LeACS4 are the primary ACS genes expressed during tomato fruit ripening, and furthermore LeASC2 is remarkably induced by wounding, but not LeACS4. LeACS4 is repressed by wounding and is expressed only during ripening. In addition, LeACS2 is expressed either on or off the vein. LeACS4 is expressed only on the vein, not off the vein. Therefore, in the transgenic tomato, in which LeACS2 is repressed by antisense gene, detached fruits do not ripen. While attached fruits ripen due to expression of LeACS4 although LeASC2 is not expressed. LeACS6 is the only ACS gene expressed in mature-green fruit, but it is not expressed during ripening.[930] LeACS1A is expressed transiently during the breaker stage, but it is not expressed after the breaker stage.[930] LeASC2, but not others, is autocatalytically induced by ethylene. Expression of LeACS6 is repressed by ethylene. In addition, LeACS1A and LeACS6 are induced by only touch in seedling, leaf, and mature-green fruit.[931] The expressions of LeACS1A and LeACS6 increased within 10 min after touch, and mRNAs for both genes disappeared by 2 h. Thus, their expressions are transient. These results propose the question whether the stimuli to induction of LeACS1A and LeACS6 during fruit ripening are developmental or external cues. LeACS2 expression induced by wounding in excised tissues is enhanced by auxin. Expression of LeACS3, but not LeACS2, is rapidly induced in intact seedlings sprayed with auxin, indicating that LeACS3 is undoubtedly an auxin-inducible gene.

4.02.8.3.2 Posttranslational regulation of ACC synthase

ACS is regulated not only at the transcriptional level but also at the posttranslational level. Tatsuki and Mori[932] first reported that LeACS2 is phosphorylated at Ser-460 by a CDPK and the phosphorylation is involved in protein turnover, stability, but not enzymatic activity. ACSs consist of seven conserved sequences and the C-terminal region is not conserved. However, the phosphorylation site (RLSF/L) is found in the C-terminal region and is conserved in many ACSs. In addition, the phosphorylation site of mitogen-activated protein (MAP) kinase is also found in the C-terminal region.[933] ACS proteins can be classified into three groups based on their C-terminal sequences: (1) type 1 proteins have extended C-termini containing three conserved Ser residues that are targets for phosphorylation by MAP kinase and a conserved Ser residue that is a target for phosphorylation by CDPK; (2) type 2 proteins have shorter C-termini containing only the CDPK site; and (3) type 3 proteins have short C-termini that lack both phosphorylation sites. The results of treatments of protein kinase/phosphatase inhibitors showed that the half-life of phosphorylated LeACS2 was longer than that of nonphosphorylated LeACS2. These results suggest that phosphorylation/dephosphorylation regulates the turnover of LeACS2 protein in the cell and that dephosphorylation of LeACS2 causes degradation. Regulation of ACS stability is also under the control of MAP kinase cascade.[933] To examine the role of MPK6, which is a homolog of SIPK (stress-induced MAP kinase) in *Arabidopsis*, in ethylene responses, the activated form of NtMEK2 was expressed in wild-type and *mpk6* mutant *Arabidopsis* plants under the control of a DEX-inducible promoter. DEX application increased the amount of ethylene produced and ACS activity in wild-type plants, but not in *mpk6* plants, indicating that MPK6 is required for NtMEK2-induced ethylene biosynthesis. MPK6 was shown to phosphorylate ACS2 and ACS6 *in vitro*, and transgenic plants, in which a phosphomimic-activated mutant ACS6 was expressed, showed increased ethylene production. These results indicate that MPK6 phosphorylates ACS proteins, thereby decreasing their turnover and increasing ethylene biosynthesis after pathogen stress.

On the other hand, ethylene-overproducing *eto* mutants in *Arabidopsis* provide very important information on the mechanism underlying ACS stability.[934] The *eto* mutants produce 10–40 times more ethylene in the dark than wild-type seedlings and show a triple response morphology despite the absence of exogenous application of ethylene.[935] The *eto2* and *eto3* mutants are dominant mutations in the C-terminus of ACS5[936] and ACS9,[937] respectively. The *eto2* mutation, a gain-of-function allele of the *ACS5* gene, is the result of a single base-pair insertion that is predicted to disrupt the C-terminal 12 amino acids. This region just includes the putative phosphorylation site of CDPK. The *eto2* mutation does not increase the steady-state level of *ACS5* mRNA but increases the stability of the ACS5 protein. In a similar manner, the *eto3* mutation, a gain-of function allele of the *ACS9* gene, is the result of single amino acid change, V457D, at the C-terminus of ACS9, which is in the neighborhood of the phosphorylation site. This single amino acid mutation gets a negative charge in the region, suggesting that this mutation mimics the phosphorylation. The *eto1* mutant is a recessive mutation that elevates basal ethylene biosynthesis, especially in etiolated seedlings.[935] Cloning of *ETO1* revealed that it encodes a BTB (Broad-complex, Tramtrack, Bric-à-brac) domain containing protein, a type of protein that has been shown to link CUL3-based ubiquitin ligase to substrate protein.[938] ETO1 also contains six predicted tetratricopeptide repeat motifs, which are involved in diverse protein–protein interactions. Analysis of ETO1 reveals that it acts as a substrate-specific adaptor protein for ACS5 and other ACS isozymes, especially type 2 ACSs.[939] The BTB domain of ETO1 interacts with CUL3/E3 ligase and the TPR domain of ETO1 interacts with the C-terminus of ACS protein to bring the substrate into contact with the E2 enzyme. The ligase then ubiquitinates the substrate ACS, thus targeting the protein for degradation by 26S proteasome.

The proposed model is that phosphorylation of type 1 and type 2 ACS proteins blocks the ability of ETO1/EOL (ETO1 homolog ETO1-LIKE) proteins to bind, thus inhibiting the ubiquitination of these ACS proteins and thus their degradation by 26S proteasome.

4.02.8.4 Perception and Signaling

The molecular analyses of ethylene signal transduction began with genetic screening of mutants based on the triple response phenotype of etiolated *Arabidopsis* seedlings after treatment with ethylene.[940] Initial studies revealed a linear framework for the ethylene signal transduction pathway, leading from ethylene perception at the membrane to transcriptional activation in the nucleus. That is, five ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2)[941–944] relay the ethylene signal to the CTR1 protein kinase, which is a negative regulator of ethylene responses. CTR1 is similar to the Raf family of MAPKKKs,[945] and is therefore presumed to be the first component of an MAP kinase cascade. EIN2, which acts downstream of CTR1, has an integral membrane domain with similarity to the Nramp family of metal ion transporters,[946] but its biochemical function is unknown. Downstream of EIN2 is EIN3,[947] which is a transcription factor that promotes transcription of *ERF1*. It also encodes a transcription factor that binds to the GCC-box promoter element to activate transcription of specific ethylene response genes. Each component will be reviewed below.

4.02.8.4.1 Ethylene receptors

Ethylene receptors are similar to histidine protein kinase receptors of the bacterial two-component system. This finding is the first to show that eukaryotes utilize bacterial two-component signaling system as a signal transduction pathway.[941] The ethylene receptors can be divided into two subfamilies. Subfamily I receptors (ETR1 and ERS1) have three transmembrane domains in the N-terminus and a conserved histidine kinase domain in the C-terminus, while subfamily II receptors (ETR2, ERS2, and EIN4) have four transmembrane domains in the N-terminus but the histidine kinase domain is not conserved in the C-terminus. Furthermore, a genetic study reveals that the receptors do not need the histidine kinase activity for signal transduction.[948]

The ethylene receptors localize to the endoplasmic reticulum (ER) membrane and the N-terminus is on the luminal side and the C-terminus is on the cytosolic side of the ER membrane.[949] The transmembrane domains are responsible for formation of disulfide-linked dimerization[950] and bind to ethylene with a copper cofactor that is provided by the RNA1 (Responsive to Antagonist 1),[951] a putative copper transporter similar to human Menkes Wilson disease protein.

The first mutant *etr1-1* isolated in the ethylene receptor genes was dominant gain-of-function mutation conferring ethylene insensitivity.[952] Single receptor loss-of-function mutants essentially show no phenotype

owing to a high degree of functional redundancy among the receptors, but null mutant combinations exhibit varying degrees of constitutive ethylene responses, demonstrating that the ethylene receptors are negative regulators of ethylene responses.[953]

The degradation of ethylene receptors plays a pivotal role in enhancing sensitivity. Tomato LeETR4 and LeETR6 receptors are degraded rapidly by 26S proteasome on ethylene binding.[954] The degradation of these receptors results in early fruit ripening, demonstrating how receptor levels can control the sensitization of plant tissues to ethylene. This degradation helps to explain why the dramatic increase in ethylene receptor mRNA during ripening does not result in the inhibition of ripening. Similarly, the binding of ethylene to *Arabidopsis* ETR2 receptor leads to degradation by 26S proteasome.[955]

4.02.8.4.2 RTE1/GR

Arabidopsis Reversion-to-Ethylene Sensitivity 1 (*RTE1*) was identified on the basis of loss-of-function *rte1* mutants that suppress the ethylene-insensitive mutant *etr1-2*.[956] *rte1* mutant shows hypersensitivity to ethylene, similar to the *etr1* null mutant. *RTE1* encodes a novel integral membrane protein involved in regulating ETR1 function. RTE1 is highly conserved in plants, but its molecular function is unknown. The RTE1 protein colocalizes with ETR1 in the Golgi and ER.[957] While tomato dominant *Green-ripe* (*Gr*) mutant exhibits inhibition of fruit ripening and floral senescence, inhibition of hypocotyl elongation and petiole epinasty remains normal. Cloning reveals that GR is a tomato homolog of RTE1.[958] The Gr mutant carries a deletion in the GR promoter region that causes ectopic overexpression of GR, which leads to ripening inhibition, demonstrating that RTE1/GR represses ethylene response.

4.02.8.4.3 CTR1

Genetic epistatic analyses reveal that CTR1 acts downstream of the ethylene receptors in the ethylene signal pathway.[945] Loss-of-function *ctr1* (*constitutive triple response1*) mutants exhibit pleiotropic constitutive ethylene response, and thus CTR1 is thought to be a negative regulator of ethylene responses.[945] The CTR1 protein has a kinase domain and high sequence similarity to the Raf family of MAPKKKs, suggesting that CTR1 may act in an MAPK phosphorylation cascade. However, other than the sequence similarity of CTR1 to MAPKKK, there is no conclusive evidence to support an MAPK cascade operating in the ethylene signal transduction so far. CTR1 protein interacts with ethylene receptors physically, and thus is localized in the ER membrane although it has no obvious transmembrane domain or membrane attachment motifs.[959] The current belief is that, in the absence of ethylene, the active receptors can interact with and recruit CTR1 to the ER membrane, which in turn activates CTR1 and shuts down the ethylene pathway.

4.02.8.4.4 EIN2

Genetic epistatic analyses reveal that EIN2 acts downstream of CTR1.[946] EIN2 functions as a pivotal positive regulator of the ethylene signal transduction, because loss-of-function *ein2* mutations result in complete insensitivity to ethylene in all ethylene-related responses examined. The N-terminal portion of EIN2 protein consists of 12 putative membrane-spanning domains, which are similar to the Nramp family of metal ion carriers. But no ion carrier activity has been demonstrated so far, and thus biochemical functions are still unknown. The subcellular localization of EIN2 is also unknown. Overexpression of the C-terminus of EIN2 can constitutively activate a subset of ethylene response phenotypes, and ethylene-inducible transcripts are increased. However, it cannot restore ethylene sensitivity in *ein2* null mutants. These results suggest that the N-terminus of EIN2 represents an input domain in sensing upstream signaling, while the C-terminus represents an output domain interacting with downstream components.

4.02.8.4.5 EIN3

Genetic epistatic analyses reveal that EIN3 acts downstream of EIN2.[960] EIN3 is a plant-specific transcription factor involved in ethylene-regulated gene expression. EIN3-related proteins consist of a multigene family in *Arabidopsis*, including EIN3, EIN3-like (EIL1), EIL2, EIL3, EIL4, and EIL5. Among these, EIN3 and EIL1 are the most closely related homologs. Genetic studies presume that EIN3 and EIL1 are the major transcription factors in mediating ethylene responses, while EIL2–5 might regulate ethylene responses in specific tissues or certain developmental stages, or instead function in ethylene-unrelated pathway.

Based on biochemical studies, EIN3 and EIL1 can directly bind to the promoter of *ERF1* (*ethylene response factor 1*), which belongs to the EREBP (ethylene response element binding protein)[961] family of AP2 type transcription factors. ERF1 in turn binds to a *cis*-acting sequence known as the GCC-box located in the promoters of secondary target genes, such as those encoding basic chitinase and defensin PDF1.2. In addition, four other transcription factors, EDF1–4 (ethylene-responsive DNA-binding factors), could also be potential target genes of EIN3, because their mRNA levels are rapidly increased by ethylene treatment, and their knockout mutants show partial ethylene insensitivity.[962] Taken together, a transcriptional cascade from EIN3 and EIL1 to ERF1 and EDF1–4 is involved in the ethylene response pathway.

The stability of EIN3 is controlled through two MAPK phosphorylation sites, one required for stabilization of EIN3 and the other involved in the degradation of EIN3.[963] Two MAPKs, MPK3 and MPK6, phosphorylate EIN3 on Thr-174 to stabilize EIN3. MPK3/MPK6 can be activated by MAPKK, MKK9. That is, MKK9 cascade promotes EIN3 stability. On the other hand, unidentified MAPK pathway mediated by CTR1 phosphorylates EIN3 at Thr-592 to degrade EIN3. But whether CTR1 has direct control of an MAPK cascade is still unknown. In summary, both the inhibition of CTR1 and the activation of MKK9 are required for ethylene signaling specificity.

Another mode of regulation of EIN3 is reported. In the absence of ethylene perception, two F-box proteins, EBF1 and EBF2, in an SCF E3 ligase complex, target EIN3 and EIL1 for degradation via the 26S proteasome.[964,965] EBF1 degrades EIN3 and EIL1 before ethylene signaling, whereas EBF2 plays a major role in degrading EIN3 and EIL1 after ethylene responses have been activated. The distinct but overlapping roles of EBF1 and EBF2 provide fine-tuned posttranslational regulation of EIN3 and EIL1, which may be essential for rapid responses to environmental stresses.[966]

Furthermore, *EBF1* and *EBF2* mRNAs are regulated by *EIN5*, which acts downstream of *CTR1* and encodes the 5′→3′ exoribonuclease XRN4.[967] EIN5/XRN4 is localized in the cytoplasm and function in mRNA and rRNA degradation. However, EIN5/XRN4 does not appear to directly degrade *EBF1* and *EBF2* mRNAs, because the half-life of *EBF1*/*EBF2* mRNAs in the *ein5* mutant background is the same as that in the wild type. Therefore, the accumulation of *EBF1* and *EBF2* mRNAs may be owing to increased transcription with *EIN5*/*XRN4* promoting a repressor of *EBF1* and *EBF2* transcription.[968]

4.02.9 Peptide Hormones in Plants

4.02.9.1 Introduction

While many peptides have been reported as signal compounds in mammals and microorganisms, the first peptide signal compound, systemin, in higher plants was reported in 1991. In the last 15 years, several plant peptides have been discovered, and their number is expected to increase even further.[969] Some of these peptide signal compounds have been shown to distribute among various plant species and some are species-specific, unlike conventional plant hormones. Thus, the conventional definition of 'plant hormone' is not applicable to these peptides; however, the term 'peptide hormone' has recently been adapted to describe this category of compounds and is used here. The main peptide hormones described here were discovered as regulatory substances controlling physiological phenomena in plants. Because most of these peptides were identified using biochemical approaches, the chemical structures of the peptide hormones have also been elucidated. Additionally, a number of plant peptides have been identified based on mutations in genes that showed typical phenotypes. However, the chemical structures of the biologically active peptides encoded by these genes remain mostly unconfirmed. In this chapter, peptide hormones with known chemical structures are discussed first, followed by a brief discussion of those with unknown chemical structures. Because of the limited space, each peptide is described in this chapter including its background, history, chemistry, and other things different from other plant hormones.

4.02.9.2 Systemins

Because plants cannot escape from insect injury, many plants protect themselves by producing defense proteins. Protease inhibitors, which protect plants by inhibiting the feed digestion of insects, are well-studied defense proteins. These protease inhibitors are induced in leaves distant from points of injury, indicating that signal substances are involved in systemic resistance induction in plants.

a. TomSys: AVQSKPPSKRDPPKMQTD

b. TobSysI: RGANLPP*P*SP*ASSP*P*SKE, TobSysII: NRKPLSP*P*SP*KPADGQRP

c. TomHypSysI: RTP*YKTP*P*P*P*TSSSP*THQ, TomHypSysII: GRHDYVASP*P*P*P*KPQDEQRQ, TomHypSysIII: GRHDSVLPP*P*SP*KTD

d. RALF:ATKKYISYGALQKNSVPCSRRGASYYNCKPGAQANPYSRGCAITRCRS

e. AtPep: ATKVKAKQRGKEKVSSGRPGQHN

Figure 38 Amino acid sequences of systemines (a–c), RALF (d), and AtPep (e). P*, hydroxyprolines with glycoside chain.

In 1991, Pearce *et al.*[970] isolated a peptide from wounded tomato leaves, and designated this peptide systemin (tomato systemin (TomSys)). TomSys shows protease inhibitor-inducing activity and comprises 18 amino acid residues (**Figure 38**(**a**)) . The TomSys precursor gene encodes 200 amino acid residues, including the TomSys sequence in the C-terminal region.[971] Homologous genes to the systemin precursor are present in other Solanaceae plants, such as potatoes and sweet peppers. A tomato strain in which systemin production is suppressed was created using antisense DNA, and the strain was damaged by a type of moth larva to which wild tomato plants are usually resistant.[972]

A protein with high binding activity to TomSys (SR160) was detected in tomato cell membranes. SR160 was found to include a leucine-rich repeat receptor-like kinase (LRR-RLK), and was highly homologous to brassinolide receptors (BRI1) in *Arabidopsis*.[973] SR160 was later reported to be identical to the brassinolide receptor in tomatoes (tBRI1).[974] In a tBRI1 mutant strain, it was found that all leaves, except for apical buds, were insensitive to TomSys. When SR160/tBRI1 was overexpressed in tobacco cells, TomSys photoaffinity labels were specifically observed.[975] These results suggest that the same receptor is utilized by both brassinolide and systemin, but recently tBRI is reported to be not essential for wound signaling.[976]

Although tobacco belongs to Solanaceae and shows systemic resistance against wounding, there is no ortholog of the TomSys precursor. When TomSys is added to liquid culture of tomato cells, the culture solution is alkalinized. Two active peptides were isolated by application of this assay to tobacco,[977] and because these peptides also exhibit protease inhibitor-inducing activity, they were designated tobacco systemin I and II (TobSys I and TobSys II, respectively). Although both comprise 18 amino acid residues like TomSys, the sequences are completely different, and furthermore, these peptides contain hydroxyprolines to which carbohydrate chains are attached (**Figure 38(b)**). The TobSys precursor gene encodes 165 amino acid residues, including TobSys I sequence in the N-terminal region and TobSys II sequence in the C-terminal region. Three types of hydroxyproline-rich glycopeptides, which are also encoded in the same gene, have been isolated from tomatoes (**Figure 38(c)**).[978] These peptides, TomHypSys I, TomHypSys II, and TomHypSys III, comprise 20, 18, and 15 amino acid residues, respectively, and their precursors are synthesized in phloem parenchyma cells in response to wounding.

Using an alkalinization assay, a peptide called rapid alkalinization factor (RALF) was obtained from tobacco leaves (**Figure 38(d)**).[979] A gene coding a secretory peptide homologous to the C-terminal region of RALF was discovered in *Arabidopsis*, and it was designated RALF-like (RALFL). To date, 34 such genes have been identified, and their expression has been observed in various tissues; however, the physiological functions of the gene products remain unknown.

4.02.9.3 *At*Pep1

*At*Pep1, which was isolated from *Arabidopsis* using alkalinization assay, activates genes specifically for defense against pathogens.[980] *At*Pep1 is a 23-amino acid peptide (**Figure 38(e)**) and is encoded at the C-terminus of the 92-amino acid precursor protein *At*proPep1, which is inducible by wounding, MeJA, and ethylene. This peptide activates transcription of defensin, the production of H_2O_2, and the expression of its own precursor gene *PROPEP1*. Six paralogs of *PROPEP1* are present in *Arabidopsis*, and orthologs are found in many plants, including both dicots and monocots. Transgenic plants, which constitutively overexpress *PROPEP1*, exhibit increased root development and enhanced resistance toward the root pathogen *Pythium irregulare*. The receptor

of *At*Pep1 was isolated from *Arabidopsis* suspension-cultured cells using ^{125}I-labeled azido-Cys-*At*Pep1 photoaffinity labeling.[981] The triptic fragments of purified protein were identified as being derived from At1g73080, which encodes the precursor of *At*Pep1 receptor PEPR1. PEPR1 is a 1124-amino acid protein that includes a signal peptide, 26 LRR motifs, a transmembrane region, and a kinase domain.

4.02.9.4 Phytosulfokine

In the case of a single-cell or a cell aggregate culture from a small number of cells, growth is usually slow. In order to overcome this problem, a method known as nurse culture is used. In this method, the nurse cell, which has a high proliferation ability at high density, is placed near the target cells, thus promoting the growth of the target cells. A positive correlation between initial cell density and initial cell growth rate has been noted for liquid culture, and cell growth in low cell density culture is promoted by the addition of conditioned medium (CM), which is supernatant from cells that are actively growing. Such phenomena can be explained by the hypothesis that individual cells secrete certain levels of growth factor(s), and as the concentration of growth factor increases proportionally to the cell density, cells start to divide when the concentration reaches a threshold level.

In 1996, Matsubayashi and Sakagami[982] successfully isolated a peptide growth factor from *Asparagus* CM, and successfully elucidated the chemical structure. The peptide was designated phytosulfokine (PSK), which has five amino acid residues including two sulfated tyrosine residues (**Figure 39**(**a**)). PSK promotes cell division at 1 nmol l^{-1}, and the three amino acid residues at the N-terminus are essential for expression of biological activities, for which the sulfate groups are also required. PSK was also isolated from CM of monocots, rice, and maize, as well as dicot plant, *Zinnia elegans*, and showed mitogenic activity to these cells.[983,984] The *Z. elegans* cell culture system has been known to form tracheary elements depending on the density of cultured cells. PSK was also shown to exhibit this activity and promote embryogenesis in carrots.[984,985]

The PSK precursor gene was first cloned from a rice Oc strain and was shown to produce the highest levels of PSK (*OsPSK*).[986] This gene (*OsPSK*) encodes 89 amino acids, and the 22 amino acid residues of the N-terminal region constitute a signal peptide that is frequently observed in secretory proteins, while the PSK sequence is located in the C-terminal region. It was later revealed that there are five types of PSK precursor genes in *Arabidopsis* (*AtPSK1–5*).[987] Genes encoding prepro-PSK are widely distributed in the plant kingdom.[988]

A solubilized membrane protein with a molecular weight of 120 kDa was purified from the membrane fraction of cultured carrot cells using the PSK derivative with a lysine residue in the C-terminal region in an affinity column.[989] This protein was finally shown to belong to the LRR-RLK family. This receptor kinase has 21 LRRs in its extracellular region, an island domain, which has a pattern similar to brassinolide receptor (BRI1), a transmembrane domain, and serine/threonine kinase in the intracellular domain. When this protein is overexpressed in cultured carrot cells, the number of binding sites for PSK is significantly increased, and callus growth is promoted in the presence of PSK. Recently, the PSK-binding site was determined to be the island domain of the receptor.[990] Loss-of-function and gain-of-function mutations of the PSK receptor gene have affected cell longevity and the potential for growth without morphological change in the *Arabidopsis* plant.[991]

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a. PSK : Y(SO3H)IY(SO3H)TQ

                    C1        C2        C3                C4C5          C6C7  C8
b. SCR6    :NLKKNCVGKTRLPGPCGDSGASS-CRDLYNQTEKTMPVS-CRC---VPTGR-CFCSL-CK
c. SP11-8  :NLMKRCTRGFRKLGKCTTLEEEK-CKTLYPRGQ-------CTCSDSKMNTHSCDCKS-C

d. MCLV3 : RTVP*SGP*DPLHH
e.  TDIF  : HEVP*SGP*NPISN
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Figure 39 Amino acid sequences of PSK (a), SCR (b), SP11 (c), MCLV3 (d), and TDIF (e). Y(SO3H), sulfated tyrosine residue; **C1–C8**, conserved cysteine (b and c); P*, hydroxyproline (d and e).

Although PSK was discovered as a growth factor in cell culture system, these findings indicate the range of physiological roles of PSK in plants.

4.02.9.5 Regulatory Factors for Self-Incompatibility

Self-incompatibility[992] is found in various plants, but is most extensively studied in *Brassica* plants. The S multiple allele system determines self-incompatibility of Brassicaceae plants; incompatibility occurs when the phenotype of the pollen S gene matches that of the stigma S gene. A distinct characteristic of this incompatibility is that the recognition reaction occurs on the surface of the pollen and stigma. When the stigma recognizes the pollen as self-pollen, water absorption, and germination and elongation of pollen tubes are inhibited. In order to elucidate the molecular mechanism of this phenomenon, it is necessary to clarify the factors in both the stigma and the pollen that are encoded in the S locus. In the stigma, a gene was found to encode a protein that contains the extracellular region (S domain), transmembrane region, and serine–threonine-type kinase region. This protein, S receptor kinase (SRK), is involved in transmitting signals into cells by binding the extracellular S domain to the pollen factor, which activates the kinase.

Research groups in the United States and Japan independently succeeded in elucidating the pollen factor. The American group obtained the gene for S locus cysteine-rich (*SCR*), which encodes a cysteine-rich anther-specific protein.[993] Introduction of this gene into a plant from a different strain produced pollen with a phenotype that had incompatibility altered to that of the introduced gene. Thus, the peptide encoded by *SCR* is the pollen factor. The Japanese group also identified genes that are specifically expressed in the anther based on *S* locus analysis. It was demonstrated that *SP11* gene product inhibits water absorption of compatible pollen on stigma.[994] Because SCR and SP11 are essentially identical, this factor is referred to as SCR/SP11 in this chapter.

SCR/SP11 is a cysteine-rich peptide with a total length of 74–83 amino acids (**Figure 39(b)**). It has a signal sequence comprising approximately 24 amino acid residues in the N-terminus. Takayama *et al.*[995] successfully obtained an active form of SP11 peptide by synthesizing a peptide without the signal peptide region and cross-linking eight cysteine residues (C1–C8, C2–C5, C3–C6, and C5–C7). This peptide was shown to bind only to the stigma membrane fraction with the same S genotype, and it specifically induces phosphorylation of the corresponding SRK.

An *SCRL* (*SCR-related*) gene family has been discovered in *Arabidopsis*.[996] This family comprises 28 genes encoding 4.4–9.5 kDa basic and hydrophilic peptides that include a signal peptide region and eight highly conserved cysteine residues. It is known that some *SCRL*s are expressed in a variety of regions in plant bodies; however, their functions have not yet been elucidated.

4.02.9.6 MCLV3 and CLE Peptides

CLVATA (*CLV1*, *2*, and *3*) genes were cloned from *A. thaliana* as genes involved in a mutant with meristem enlargement.[997] CLV1 is a receptor-like protein including LRR and serine–threonine kinase regions. CLV2, which has LRR but not the kinase region, is believed to form a heterodimer with CLV1. On the other hand, *CLV3* encodes 96 amino acids that include a signal peptide region comprising 18 amino acid residues in the N-terminus. The phenotypes of *clv1* and *clv3* mutants are similar; thus, CLV3 appears to share a signal transduction pathway with CLV1. In other words, CLV3 is thought to be a peptide ligand that binds to the CLV1 receptor kinase. The signal of CLV1 receptor kinase inhibits expression of the transcription factor *WUS*. While abnormal division of stem cells occurs upon overexpression of *WUS*, differentiation of stem cells is promoted upon inhibition of this expression. On the other hand, *WUS* maintains growth balance of apical meristems by promoting the expression of *CLV3*, which results in the formation of a feedback loop. The gene for embryo-surrounding region (ESR) protein in maize, which is expressed in part of the endosperm, encodes a secretory polypeptide. Although CLV3 and ESR are significantly different as a whole, high homology is observed in a 14-amino acid sequence in the C-terminal region.[998] This gene family was designated *CLAVATA3/ESR related* (*CLE*). In *Arabidopsis*, many *CLE*s, in contrast to *CLV3*, are expressed in most tissues.

Analysis was performed on slices from a callus produced from *CLV3*-overexpressing *Arabidopsis*, by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS), and it showed a

specific ion composition compared with a control prepared from a wild-type callus.[7] The molecular weights of peptide and fragment ions obtained by MS/MS analysis suggested that the mature CLV3 peptide (MCLV3) is a dodecapeptide with two hydroxylated prolines (**Figure 39(c)**). This structure was confirmed by comparison with synthetic peptides. The application of MCLV3 to wild-type *Arabidopsis* showed typical biological activities expected from a plant in which *CLV3* is overexpressed.

Xylogen, a unique arabinogalactan protein, was identified as a mediator of vascular development by xylogenic culture system using *Zinnia* mesophyll cells.[989] During isolation of xylogen, TDIF was identified. TDIF was purified and its structure was determined as a dodecapeptide with two hydroxyprolines (**Figure 39(e)**).[6] The amino acid sequence of TDIF is identical to that of the C-terminal region of CLE41/44. There are 31 CLE genes including CLV3 in *Arabidopsis* and they encode 26 kinds of dodecapeptide sequences. All peptides were synthesized and bioassayed with the suppression of tracheary element differentiation and root growth. Only TDIF (CLE41/44) and CLE42 peptides suppressed the tracheary element differentiation and did not inhibit root growth. Most of the CLE peptides, as well as MCLV3, inhibited root growth but some peptides did not show any activity in either assay. These results indicate that CLE peptides may have various physiological roles in plants in addition to those of MCLV3 and TDIF. Because hydroxylation of proline residues did not affect the biological activities of MCLV3 and TDIF, it may serve other functions. The identification of mature CLV3 peptide helps to explain the direct interaction between ligand and receptor, CLV1 and/or CLV1/CLV2. Although receptors of TDIF and other CLE peptides remain unknown, LRR-RKs are putative candidates for this role.

4.02.9.7 Other Peptides

As mentioned at the beginning of this chapter, a number of studies on genes that are supposed to encode peptides have been reported. Here, representative examples are briefly described.

In *POLARIS* (*PLS*) mutant, the length of the main root is shorter and a decrease in branch veins among rosette leaf veins is also observed.[1000] Such phenotypes may arise from changes in sensitivity to hormones in the mutant, as it is assumed that the *PLS* gene is required for the maintenance of homeostasis in the cytokinin–auxin system.

The *Arabidopsis* inflorescence deficient in abscission (*ida*) was discovered from a mutant in which flowers do not detach.[1001] The *IDA* gene encodes a peptide comprising 77 amino acid residues, including a signal sequence. A highly conserved sequence in the C-terminal region of the peptide encoded by five paralogs in *Arabidopsis* suggests that this region is the mature peptide hormone. The *HAESA* gene, which encodes LRR-RLK in *Arabidopsis*, is expressed in the flower detachment site.[1001] When the expression of this gene is inhibited, flower detachment is repressed. Based on comparisons of these phenotypes, HAESA may be a receptor for the peptide encoded by the *IDA*.

The *ROT4* (*ROTUNDIFOLIA4*) gene was discovered when screening for mutations related to leaf shape by activation tagging.[1002] A *rot4-1D* mutant plant has short and round leaves, and a short flower and flower stalk. The *DEL1* (*DEVIL1*) gene was identified at the same time as *ROT4*, and the phenotype of *dvl1-1D* mutant is similar to that of *rot4-1D* mutant.[1003,1004] *ROT4* and *Del1* encode peptides comprising 53- and 51- amino acid residues, respectively. According to the *Arabidopsis* gene database, *ROT4* and *DEL1* belong to a family of 23 genes. It was confirmed that other members of this gene family have functions similar to those of *ROT4* and *DEL1* in overexpression experiments.

4.02.9.8 Remarks

The number of reports suggesting that peptides in plants play an important role as hormone-like molecules has been increasing in recent years. Most of the biologically active peptides are biosynthesized from precursors by processing and/or specific modification, as was the case with PSK and CLE peptides. In order to develop peptide hormone research, it is necessary to accurately determine the chemical structures of mature peptides. Once the chemical structures of the peptides are determined, it is expected that research will further expand, focusing on biosynthesis, receptors, and signal transduction.

By acquiring entire gene sequences of *A. thaliana*, plant research has also arrived at the so-called postgenome era. It is thought that the genes of *A. thaliana* encode many secretory peptides and more than 400 receptor-like proteins.[1005,1006] While most of the receptors are orphan receptors, whose ligands are unknown, it is possible that a large number of ligands for such orphan receptors are peptide hormones, the number of which will continue to increase as discoveries continue.

4.02.10 Strigolactones

4.02.10.1 Introduction

4.02.10.1.1 Strigolactones as rhizosphere signaling molecules

Root parasitic plants of the *Striga*, *Orobanche*, and *Alectra* genera (Orobanchaceae) cause serious losses in crop yields in many parts of the world. Control of these parasitic plants is extremely difficult, because they produce a large number of seeds that remain viable in the soil for many years until they detect germination stimulants released from host roots. A pure crystalline germination stimulant of *Striga* seeds was first isolated in 1966 from cotton root exudates by Cook *et al.*[1007] This stimulant, termed strigol, was determined to be a terpenoid lactone carrying a unique four-ring structure[1008] (**Figure 40**). Following this discovery, several strigol-related compounds have been identified as germination stimulants of parasite seeds in root exudates of various plant species. A group of these terpenoid lactones were named 'strigolactones'.[1009–1011]

Until recently, the biological function of strigolactones for the host plants was unknown. In 2005, Akiyama *et al.*[1012] found that strigolactones are plant-derived signals that induce hyphal branching of arbuscular mycorrhizal fungi (AM fungi), a phenomenon that is observed at the initial stage of colonization. Since AM fungi facilitate the uptake of water and mineral nutrients (such as phosphate and nitrate) by the host plants, it is considered that plants have evolved to produce strigolactones in order to enable AM fungi to colonize their roots.[1010,1011,1013] Parasitic plants are thought to abuse these chemical signals to recognize the vicinity of a potential host plant (**Figure 41**).

An extensive survey of strigolactones revealed their occurrence in root exudates of a wide variety of plant species,[1010] including nonhosts of AM fungi (e.g., Brassicaceae, of which *Arabidopsis* is a member).[1014,1015] This suggests that strigolactones may have as yet unidentified biological role(s) in plants. In 2008, two research groups reported that strigolactones were not merely communication chemicals in the rhizosphere but endogenous hormones that regulate shoot branching in plants (**Figure 41**).[2,3]

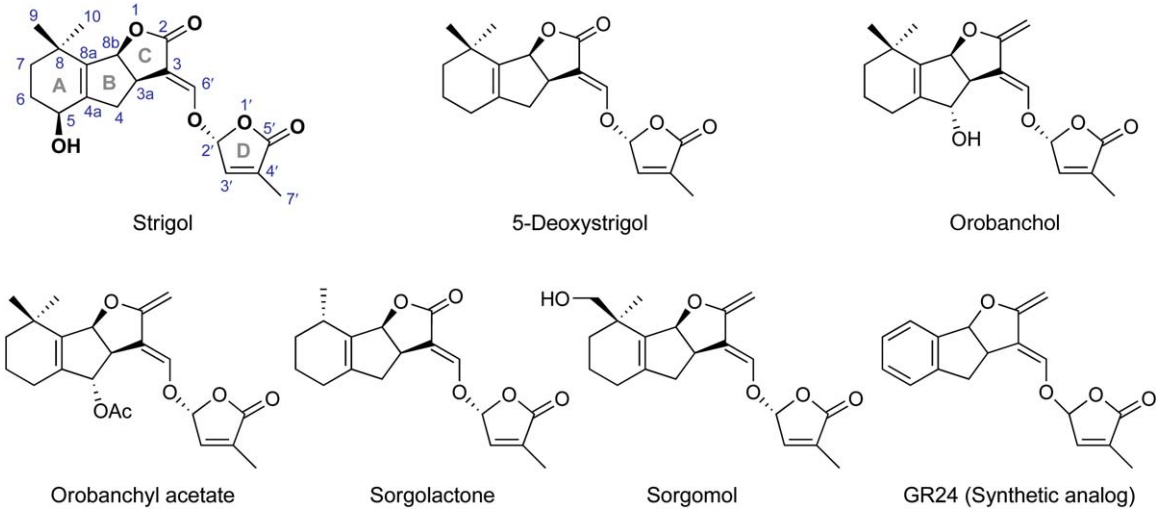

Figure 40 Naturally occurring strigolactones and a synthetic strigolactone analog, GR24.

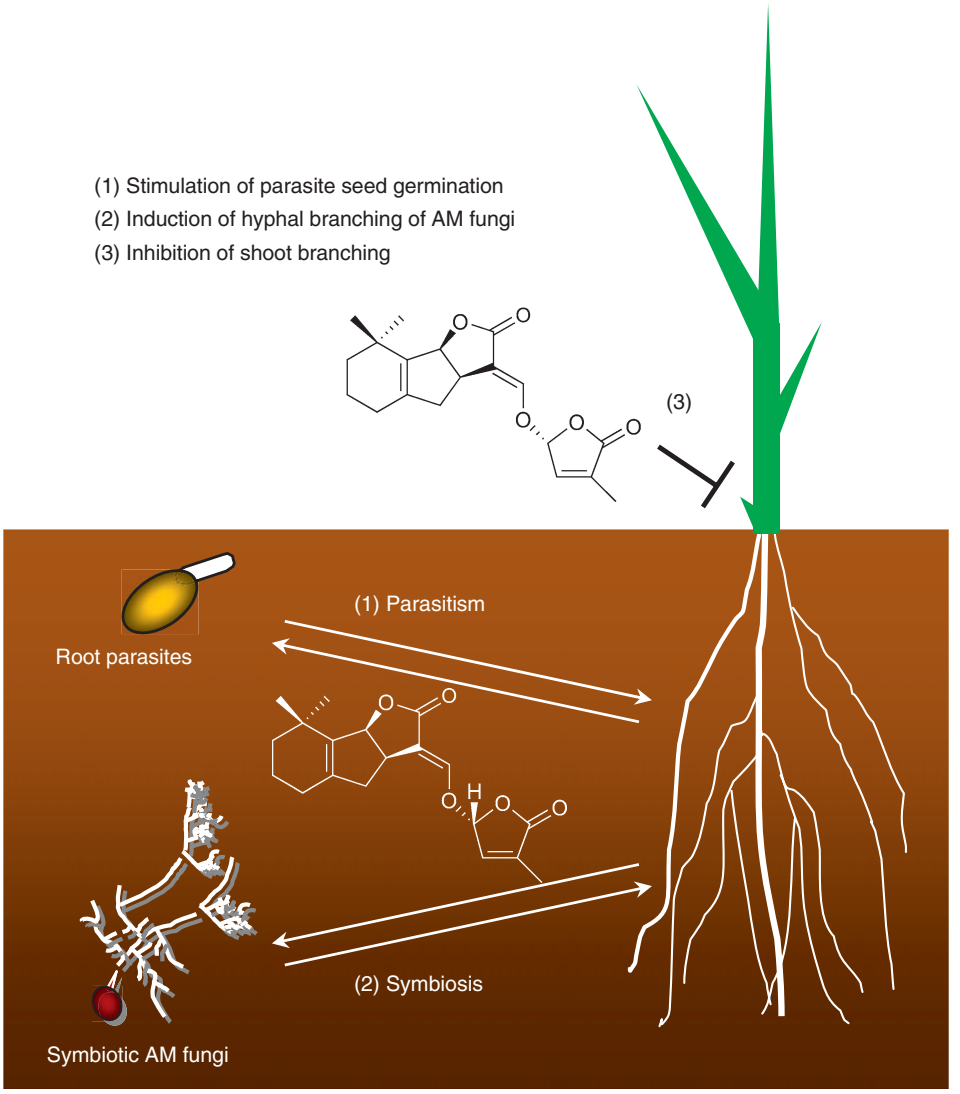

Figure 41 Biological functions of strigolactones.

4.02.10.1.2 A novel shoot branching inhibitor suggested by genetic studies

In many plant species, only a small proportion of axillary buds grow out to form branches, with both the timing and the extent of bud activation being tightly regulated by endogenous and environmental factors. The participation of two plant hormones, auxin and cytokinin, in the regulation of axillary bud activity has long been known. Besides these two classical hormones, studies with enhanced shoot branching mutants suggested the involvement of a novel hormone-like signal in inhibiting axillary bud outgrowth. These mutants include *ramosus* (*rms*) of *Pisum sativum* (pea),[1016–1019] *decreased apical dominance* (*dad*) of *Petunia hybrida* (petunia),[1020,1021] *more axillary growth* (*max*) of *Arabidopsis*,[1022–1026] and particular *dwarf* (*d*) mutants of *O. sativa* (rice).[1027–1029] Grafting experiments suggested that some of these mutations affect genes required for the synthesis of a graft-transmissible mobile signal, while others are required for the perception or transduction of the signal.[1030]

This idea was supported by the molecular identification of the *Arabidopsis MAX* genes and several *RMS*, *DAD*, and *D* loci in pea, petunia, and rice, respectively.[1030] *MAX3*, *RMS5*, and *D17* encode carotenoid cleavage dioxygenase 7 (CCD7),[1019,1025,1028] while *MAX4*, *RMS1*, *D10*, and *DAD1* encode another subclass of CCDs designated CCD8[1020,1023,1029] (**Figure 42**). *MAX1* encodes a protein in the cytochrome P450 monooxygenase

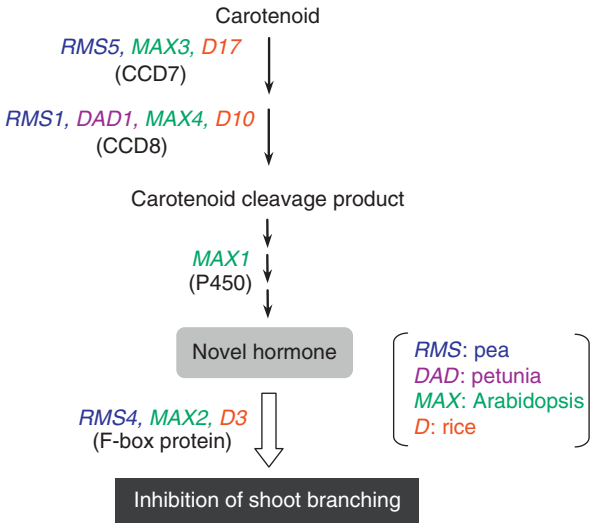

Figure 42 A hypothetical pathway for the novel branching inhibitor. Carotenoid cleavage dioxygenases (CCDs) and a cytochrome P450 monooxygenase (P450) are required for the synthesis of this novel hormone, whereas an F-box protein is probably involved in the perception or transduction of the hormonal signal.

superfamily,[1026] the members of which are typically involved in the metabolism of lipophilic small molecules. All these findings are consistent with the idea that MAX1, MAX3, and MAX4 participate in the biosynthesis of a carotenoid-derived compound (**Figure 42**). MAX2, RMS4, and D3 are orthologous members of the F-box protein family, which are known to function as the substrate recognition subunit of SCF ubiquitin E3 ligase for proteasome-mediated proteolysis.[1019,1022,1027] Some members of this protein family include TIR1, GID2/SLY1, and COI1, all of which are components of the perception or signal transduction of a hormone (e.g., see Section 4.02.2 for auxin), suggesting that *max2*, *rms4*, and *d3* mutants are defective in the response to a novel hormone (**Figure 42**).

4.02.10.2 Chemistry

Following the isolation of strigol from cotton root exudates, diverse strigol-related compounds (strigolactones) with modifications on ring A and/or ring B were isolated in root exudates of various plants as germination stimulants of parasite seeds or hyphal branching inducers of AM fungi.[1010,1011,1013,1031] Strigolactones commonly possess a characteristic four-ring structure, two of them being lactones linked via an enol ether bridge (**Figure 40**). Structure–activity studies using synthetic analogs of strigol have shown that the germination-stimulating activity of strigolactones resides in the C/D ring portion of the molecule, including the enol ether bridge.[1009,1010] Strigol, 5-deoxystrigol, sorgolactone, and GR24 (a synthetic analog) exhibited biological activity in inducing hyphal branching of AM fungi.[1012] Strigol, 5-deoxystrigol, and GR24 have been shown to inhibit shoot branching in rice and GR24 in pea and *Arabidopsis*.[3] Detailed structural requirements of strigolactones for the last two biological activities have yet to be determined.

4.02.10.3 Biological Activity

As described above, strigolactones are released from roots and act as communication chemicals with root parasite seeds and symbiotic AM fungi (**Figure 41**). More recent investigations have shown that strigolactones play a role in inhibiting shoot branching through the proposed genetic pathway[2,3] (**Figure 42**). Two lines of evidence support this idea. First, strigolactone levels in root and root exudates (determined by LC-MS) are significantly decreased in the *ccd7* and *ccd8* mutants of pea and rice. Second, axillary bud outgrowth in *ccd7* and *ccd8* mutants of pea, rice, and *Arabidopsis* is inhibited by exogenous

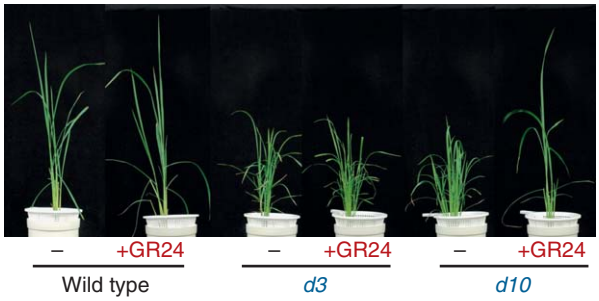

Figure 43 The branching (tillering) and semi-dwarf phenotypes of the rice *d10* mutant are complemented by exogenous GR24, a synthetic strigolactone analog (supplied at 1 μmol l^{-1} in the hydroponic culture media). In contrast, *d3* mutant plants are insensitive to GR24.

strigolactone. For the rice *ccd8/d10* mutant, it was shown that the branching phenotype as well as the plant height was nearly fully complemented by including GR24 in culture media (**Figure 43**). In contrast to the *ccd7* and *ccd8* mutants, pea and rice branching mutants characterized as lacking a response to the branching inhibition signal (*rms4*, *max2*, and *d3*; **Figure 42**) are insensitive to exogenous GR24, and are not deficient in strigolactones. These results indicate that the inhibitory effect of strigolactones on shoot branching is specific to the proposed RMS/D pathway. It is not known whether strigolactone itself is an active form of this hormone class, because applied strigolactone (analog) might be metabolized in plants and converted to an active compound. Together, current data indicate that strigolactones act as a new class of branch-inhibiting hormones or their biosynthetic precursors.[2,3]

4.02.10.4 Biosynthesis and Its Regulation

Until recently, the biosynthetic origin of strigolactones was unknown. In recent studies using chemical inhibitors and mutants of *Z. mays* (maize) and *Solanum lycopersicum* (tomato), strigolactones were shown to be derived from carotenoids.[1032,1033] This is consistent with the discovery that carotenoid cleavage dioxygenases, CCD7 and CCD8, participate in strigolactone biosynthesis.[2,3] Recombinant CCD7 and CCD8 proteins from *Arabidopsis* and rice sequentially cleaved β-carotene, forming a C_{18} apocarotenoid *in vitro* and in bacteria cells[1034,1035] (**Figure 44**). However, CCD7 could also use other carotenoid substrates, and the exact catalytic reactions by these CCDs *in planta* have not been conclusive.[1025,1036] It is crucial to determine whether any apocarotenoid produced by recombinant CCD7 and CCD8 *in vitro* is converted to strigolactones *in planta*. MAX1 is a cytochrome P450 monooxygenase (CYP711A1), which potentially catalyzes a later step of strigolactone biosynthesis,[1026] although its substrate and product are still unknown. Considering the chemical structure, more enzymes are likely to be required for the biosynthesis of strigolactones from a carotenoid cleavage product, besides CCD7, CCD8, and CYP711A1.

In pea, petunia, and rice, genes encoding CCD8 are highly upregulated in the strigolactone-deficient and strigolactone-insensitive mutant backgrounds, suggesting a type of feedback regulation.[1019,1029] Consistent with this, *CCD8/D10* gene expression is downregulated by strigolactone treatment in rice.[3] Furthermore, strigolactone levels are substantially increased in the strigolactone-insensitive *d3* mutant in comparison with wild type.[3] These results indicate that there is a link between strigolactone action and biosynthesis in these plant species, as previously recognized for other plant hormones. However, there is no clear increase in the level of *CCD8/MAX4* gene expression in the *max* mutant background in *Arabidopsis*, suggesting a species-dependent difference in the regulation of strigolactone levels in plants.[1037]

The amount of strigolactones released from roots is drastically increased under deficiency of phosphate or nitrate in hosts of AM fungi.[1015,1033,1038,1039] This is thought to be an adaptive response of plants under nutrient deficiency in order to maximize the symbiotic interaction with AM fungi, which assist the uptake of mineral nutrients by the host plant. In fact, *Lupinus albus* (white lupin, a Fabaceae plant), a nonhost of AM fungi, does not

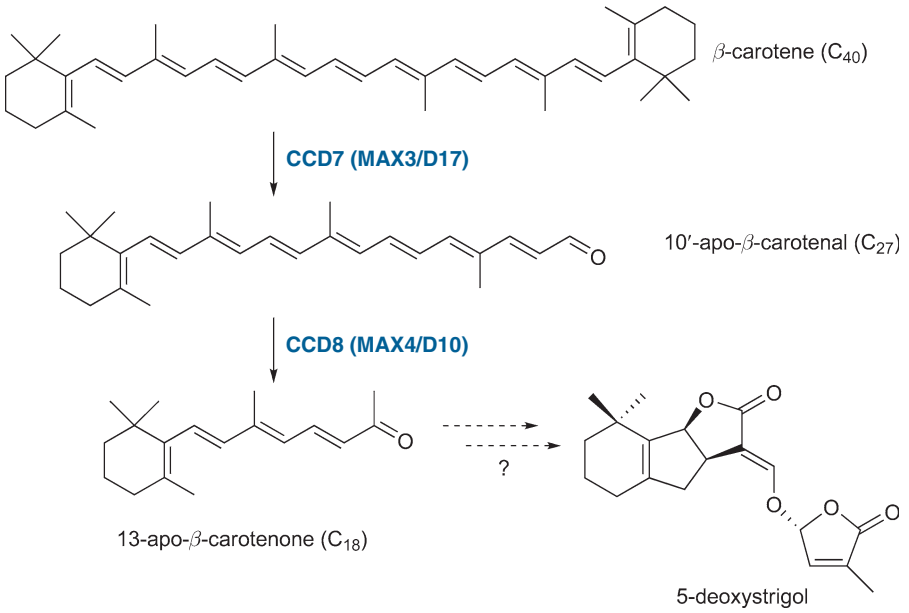

Figure 44 Oxidative cleavage of β-carotene by recombinant CCD7 and CCD8 proteins *in vitro* or in bacteria. Note that CCD7 can use other carotenoid substrates and the exact reactions catalyzed by these CCDs *in planta* have not been conclusive.

increase strigolactone production in response to phosphate deficiency, unlike the mycotrophic Fabaceae plant *Trifolium pratense*.[1015] These results indicate that strigolactone production is closely related to the strategy of plants for mineral nutrient acquisition.

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Biographical Sketches

Isomaro Yamaguchi is Professor of Department of Biotechnology, Maebashi Institute of Technology. He holds a Ph.D. degree in Agriculture from The University of Tokyo. He has served as Associate Professor of Department of Agricultural Chemistry, The University of Tokyo, Visiting Scientist of Institute for Biomedical Research, The University of Texas at Austin, and Associate Professor and Professor of Department of Agricultural and Biological Chemistry, The University of Tokyo. He has received young scientist award and senior scientist award from the Japan Society for Bioscience, Biotechnology, and Agrochemistry.

Jerry David Cohen is Gordon and Margaret Bailey Professor in the Department of Horticultural Science, University of Minnesota. He obtained his MS degree in Plant Physiology from San Diego State University and Ph.D. degree in Plant Biochemistry from Michigan State University. Later, he worked as a postdoctoral research associate in the Department of Botany and Plant Pathology, Michigan State University, research associate in Plant Hormone and Growth Regulators Laboratory, University of Maryland and Beltsville Agricultural Research Center, and plant physiologist/biochemist in the US Department of Agriculture. He is the President of International Plant Growth Substances Association and Director for Graduate Studies, Plant Biological Sciences Program, University of Minnesota. He has served as Editor-in-Chief, Plant Growth Regulation. He has won many awards and honors.

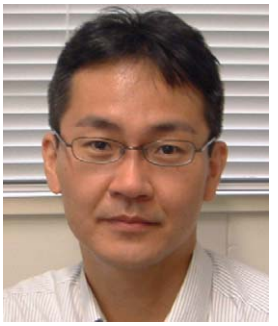
Angela Hendrickson Culler is a plant biochemist at Monsanto Company. She received her Ph.D. degree in Plant Biological Sciences, University of Minnesota. She has worked as a research assistant (Population Genetics, Hamline University), graduate research assistant (Phytohormone Biochemistry Lab and Plant Cytoskeleton (tubulin) Lab, University of Minnesota), visiting scientist (Plant Physiology Lab, Institut für Botanik, Technische Universität), and postdoctoral associate (Phytohormone Biochemistry Lab, University of Minnesota). She is a recipient of USDA National Needs Fellowship from University of Minnesota and Alderman Award for Outstanding Graduate Student Research.

Marcel Quint is currently working at the Leibniz Institute of Plant Biochemistry as a principal investigator. He received his Ph.D. degree in Agricultural Sciences (Plant Genetics) from Universität Hohenheim. He has worked as a visiting scientist in the Danish Institute of Agricultural Sciences and as DFG postdoctoral fellow and postdoc in the University of Minnesota-Twin Cities. He has been awarded Postdoctoral Fellowship from the Deutsche Forschungsgemeinschaft (DFG).

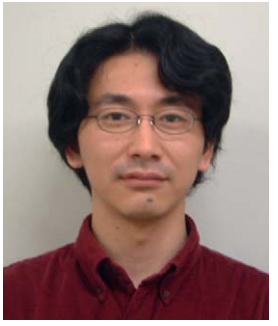
Janet P. Slovin is a plant molecular biologist in Genetic Improvement of Fruit and Vegetables Laboratory, USDA/ARS. She received her Ph.D. degree in Plant Sciences from Indiana University. She has worked as a research associate (University of California), NIH post-doctoral fellow (University of California), postdoctoral research associate (US Department of Agriculture), faculty research associate (University of Maryland), and plant biochemist/molecular biologist (USDA/ARS). She has received many honors and awards (Doctoral Student Grant-in-Aid for Research, Floyd Fellowship, NIH Postdoctoral Fellowship, Distinguished Service Award). She is a member of the Sigma Xi and a member in the Editorial Board of Plant Growth Regulation.

Masatoshi Nakajima is an Assistant Professor in the Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan. He received his Ph.D. degree in the field of applied biological chemistry in 1996 from the University of Tokyo. He is a chemical biologist who has studied (1) the localizations of some phytohormones in plants by using haptenic antibodies specific for them, (2) the function of gibberellin, a phytohormone, during seed development of morning glory and *Arabidopsis*, and (3) biological characterizations of the gibberellin receptors and some proteins related with the gibberellin signaling. He and some Japanese collaborators discovered GID1, a gibberellin receptor of rice, in 2005.

Shinjiro Yamaguchi is a team leader at RIKEN Plant Science Center, Japan. He graduated from the Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo in 1991. He obtained his Ph.D. degree in 1996 from the University of Tokyo. He then joined Frontier Research Program at RIKEN and contributed to the identification of gibberellin biosynthesis genes from plants. He worked at Duke University in the United States as a postdoctoral fellow from 1997 till 2000, and studied the regulation of gibberellin biosynthesis in *Arabidopsis* seeds. His research group has been working on the biosynthesis and metabolism pathways of terpenoid plant hormones at RIKEN Plant Science Center since July 2000.



Hitoshi Sakakibara was born in 1965. He graduated from the School of Agricultural Sciences, Nagoya University in 1988. He received his Ph.D. degree in agriculture from Nagoya University in 1995. He has worked as a research associate (1992) and Assistant Professor (1995) in the School of Agricultural Sciences, Nagoya University. He has served as the head of the Laboratory for Communication Mechanisms in RIKEN Plant Science Center (PSC) in 2000. He was the team leader of Biodynamics Research Team (2005) and group director of Plant Productivity Systems Research Group (2006). His major research interests concern biochemical and molecular physiological studies on the regulation mechanism of expression of nitrogen assimilatory genes, cytokinin-mediated nitrogen signaling, and cytokinin biosynthesis.



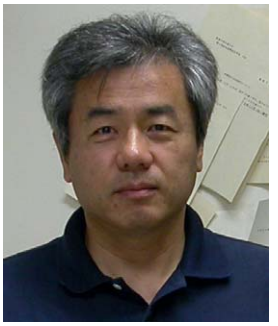
Takeshi Kuroha was born in 1977. He graduated from the School of Biological Sciences, University of Tsukuba in 1999. He obtained his Ph.D. degree from the University of Tsukuba in 2005. He studied the mechanisms of adventitious and lateral root formation in graduate school. In 2005, he joined the Biodynamics Research Team in RIKEN Plant Science Center as research associate and is involved in the study of enzymes related to cytokinin activation. His research interests concern genetic, biochemical, and molecular physiological studies on development, local and long-distance communication, and cytokinin biosynthesis in plants.

Nobuhiro Hirai is a Professor at the Graduate School of Agriculture, Kyoto University, Kyoto, Japan. He received his Ph.D. in Food Science and Technology from Kyoto University in 1982. He was a research fellow at the Department of Chemistry, Virginia Polytechnic Institute and State University, USA, from 1981 to 1983. He was an Instructor in 1983, and then an Associate Professor in 2002 at Kyoto University before being promoted to the present position in 2004. His research interest concerns chemistry and biology of plant hormones, allelochemicals, and phytoalexin. He has found novel metabolites of abscisic acid and was the first to develop long-lasting analogs of abscisic acid that resist metabolic inactivation. For this achievement, he received 'The Japan Society for Chemical Regulation of Plants Award' in 1995.

Takao Yokota is Professor of the Department of Biosciences, Teikyo University. He received his Ph.D. degree from the University of Tokyo in 1971. He became an Assistant Professor in the University of Tokyo in 1970, then a Lecturer in 1988. In 1990, he was assigned to the present position. He has been involved in brassinosteroid research for nearly 30 years. His major research interests concern the biosynthesis and physiological roles of brassinosteroids.

Hiroyuki Ohta was born in 1957. He obtained his Ph.D. degree in Agriculture from the Graduate School of Agriculture, Kyoto University in 1987. His doctoral study was on the formation and degradation of lipid hydroperoxide in rice grains. In 1987, he joined Kyoto University as Teaching Assistant. Later, he served in a postdoctoral position in Mitsui Plant Biotechnology Research Institute (1988-89) and National Institute of Basic Biology (1989-91). He worked in the Department of Bioscience and Biotechnology, Tokyo Institute of Technology as an Assistant Professor from 1991 to 1996 and as an Associate Professor from 1997 to 2003. In 2004, he joined Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology as an Associate Professor. Currently, he is a Professor in the Center for Biological Resources and Informatics, Tokyo Institute of Technology.

Yuichi Kobayashi was born in 1953. He received his Ph.D. degree in Engineering from Tokyo Institute of Technology in 1981. He joined Columbia University as a postdoctoral fellow in 1981. In 1982, he joined the Tokyo Institute of Technology as an Assistant Professor (1982–88) and was later promoted to Associate Professor (1988–present). He has won the CSJ Award for Young Chemists.



Hitoshi Mori received his Ph.D. degree in Agriculture from Nagoya University in 1990. He was a researcher in the Meiji Institute of Health Science from 1987 to 1989. He worked in the National Institute of Basic Biology, Okazaki as Assistant Professor from 1989 to 1993. In 1993, he was appointed as Associate Professor in the Graduate School of Agricultural Sciences, Nagoya. From 2005, he has been working as Professor in the Graduate School of Bioagricultural Sciences, Nagoya University.

Yoji Sakagami was born in 1948. He graduated from the Department of Agricultural Sciences, University of Tokyo in 1972. In 1979, he obtained Ph.D. in Agriculture from the University of Tokyo. In 1976, he was an Assistant Professor in the Department of Agricultural Chemistry, University of Tokyo. From 1983 to 1985, he was a visiting scientist at W. Alton Jones Cell Science Center, USA. He joined the School of Agricultural Sciences, Nagoya University in 1987 as Associate Professor. From 1994, he has been working as a Professor in the Graduate School of Bio-Agricultural Sciences, Nagoya University. He has been awarded Nougeikagaku Shoureishou (1979) and Nougeikagakukaishou (2003).

4.03 Insect Hormones

E. David Morgan, Keele University, Staffordshire, UK

4.03.1 Introduction

The extraordinary changes that occur in holometabolous insects as they pass from larva through pupa to adult have long excited the curiosity of scientists, to learn what prompts and regulates these changes. The discovery that they were hormone-regulated was the initiation of a fascinating pursuit of insect hormone chemistry. Vertebrate (and human) hormones display a wide variety of chemical structures that have been a great challenge to chemists in structure elucidation and synthesis. Insects, in contrast, at this time, have only revealed two classes of hormone compounds. The first group are the true hormones, produced by epithelial glands, the moulting hormone and juvenile hormone (JH), and the second are the neuropeptides produced by neurosecretory cells. The second half of the twentieth century was an active period for the discovery of insect hormones, their structures and action. That only two hormones were discovered gave a feeling of uniformity that was unwarranted. Recent work has shown how it is not helpful to regard such a large and ancient phylum, the arthropoda, to which insects belong, as a uniform group, either in terms of which compound or how it functions, when investigating hormones.

The two hormones regulate each step of development from embryo through larva or nymph to adult, and via pupa, where this is formed. At each stage the moulting hormone is produced when the old cuticle is cast off and a new one is formed. The presence of JH ensures that the new stage is also juvenile. A more complex action of JH allows the larva to develop into a pupa, and the absence of JH permits the formation of the adult form. The function of neither hormone ends there, for both are produced again in the adult insect, with other effects as described in a later discussion.

4.03.2 Neuropeptides

In addition to the true hormones, there are a vast number of internal messengers, produced by groups of cells in the brain and peripheral ganglia of the insect nervous system.[1,2] In a recent review by Gäde and Marco,[3] insect and crustacean neuropeptides are classified into 17 groups. The first discovered neuropeptide was the prothoracicotropic hormone (PTTH), produced by the brain, and acting on the prothoracic glands, to stimulate them to produce moulting hormone.[4] It now appears that another substance, an autocrine factor (roughly, an internal messenger within the cell) in the prothoracic gland, is also required.[5] PTTH is a homodimer of two chains of 108 or 109 amino acids with a chain of five sugar units attached. The structure varies with species. Another example is the adipokinetic hormone, which regulates the release of diglycerides for the energy requirements of the flight muscles.[6] It has been isolated from many species. It can be an octapeptide to decapeptide, always with a pyroglutamic acid at the N-terminus, and an amide function at the C-terminus. It provides an example of neuropeptides with similar structure in different insects or crustaceans, often with different functions (**Figure 1**). Of great interest in connection with pheromone production is the pheromone biosynthesis-activating neuropeptide (PBAN) of Lepidoptera, containing 18–36 amino acids.[7] Progress in neuropeptides has been most rapid, and several hundred peptide hormones are now known. The possibility of an aging hormone in insect has been considered. Kinetin, a plant hormone, which delays senescence in plants, prolongs the life of some insects, but at the cost of decreased reproductive activity. Insulin-like peptides are found in insects, and among their functions in *Drosophila*, they seem to control aging.[8] There seems to be a great deal of similarity in these neuropeptides between phyla from nematodes and arthropods to chordates.[9] One interesting observation is emerging: that many peptide hormones have been conserved during evolution, but that their structures are better conserved than their functions.

Advancing methods in mass spectrometry (MS) have made it easier to determine the amino acid sequence in these peptides, and on ever smaller amounts of the compound. Direct tissue and single neuron analyses by matrix-assisted laser desorption/ionization-MS are particularly successful (Chapter 9.10). By these methods about 440 neuropeptides have been identified, and some 450 by electrospray ionization (ESI) techniques. Further techniques hold promise for more peptides.[10] The neuropeptides have great importance for the insect physiologist,[11] but hold less interest for the structural chemist. Their three-dimensional folding is largely undetermined.

The possibility of using neuropeptides in pest control is a constant theme in research.[12,13] Their cost, stability, and the need to deliver them intact to the site of action are major problems with using neuropeptides. They must be able to cross the cuticle barrier or the gut epithelium in an active form. However, some peptides and proteins are able to pass the gut of insects intact, while others can be absorbed through the epithelium (e.g., PBAN[14]). Still others can be modified, as with allatostatins (which inhibit JH production), to which sterically bulky groups can be added to give analogues that resist breakdown by gut enzymes.[15] Such approaches are actively studied in the search for new methods of pest management.

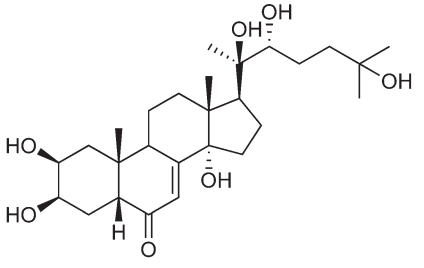
4.03.3 Moulting Hormone

Steroidal compounds, derived from cholesterol, known as ecdysteroids, control moulting or ecdysis in insects, arachnids, and crustaceans. The same hormone regulates metamorphosis, reproduction, and diapause. The structural characteristics of ecdysteroids are several hydroxy groups that render them water-soluble, a *cis*-fused AB ring junction, 7-ene-6-one unsaturated ketone group, and usually the full carbon skeleton of cholesterol. The most common example is 20-hydroxyecdysone (**1**), or a homologue with an additional side chain C-24 α-methyl or ethyl group, as in makisterone A (**2**) and makisterone C (**3**), may be used. For half a century, part of their biological function, and some of the chemical structures were known, but new aspects of their function and

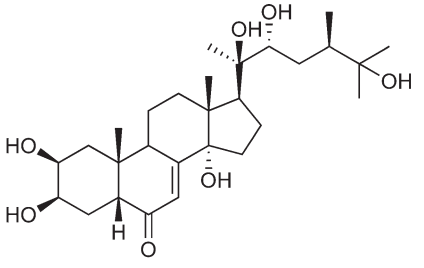
Adipokinetic hormone	*Locusta migratoria*	pGlu–Leu–Asn–Phe–Ser–Ala–Gly–TrpNH_2
Pigment-concentrating hormone	*Pandanus borealis*	pGlu–Leu–Asn–Phe–Ser–Pro–Gly–TrpNH_2
Adipokinetic hormone	*Manduca sexta*	pGlu–Leu–Thr–Phe–Thr–Ser–Trp–GlyNH_2
Hypertrehalosaemic hormone	*Tenebrio molitor*	pGlu–Leu–Asn–Phe–Ser–Pro–Asp–TrpNH_2

Figure 1 Examples of insect and crustacean neuropeptides of similar structure with the same or different functions. *Pandanus borealis* is a shrimp. pGlu, pyroglutamic acid.

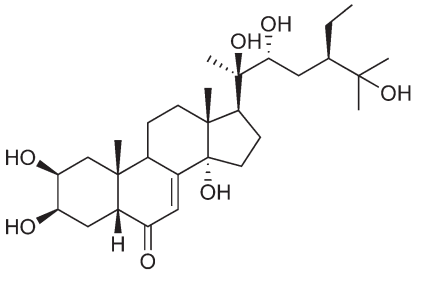
new, related compounds are as yet being discovered. Their identification in insects (*Bombyx mori*)[16] was almost synchronous with their identification in crustaceans (the crab *Jasus lalandii*)[17] and in a plant (the tree *Podocarpus elatus*).[18] Today there are about 300 compounds known, most of them derived from plants (see Section 4.03.3.5). Because of the ever-growing number of compounds identified in the group, the *Ecdysone Handbook*, which formerly tried to list all ecdysteroids, with available spectroscopic information and other data, had to be replaced by a website.[19] Lafont *et al.*[20] list 70 ecdysteroids found in insects and the species from which they were first isolated. The list includes inactive biosynthetic precursors, metabolites, ester conjugates with organic and inorganic acids, three adenosine monophosphates; 2-desoxyecdysone 22-adenosine monophosphate (**4**), ecdysone 22-adenosine monophosphate, and ecdysone 22-N^6-(isopentenyl) adenosine monophosphate (**5**), a cytokinin, found in *Locusta migratoria* eggs, plus two compounds of structure related to ecdysteroids, bombycosterol, and bombycosterol 3-phosphate (**6**), both found in *B. mori*, but of no known hormonal effect.[21,22]



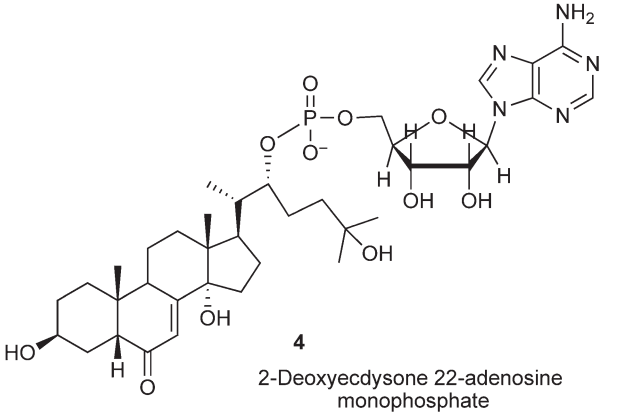
1
20-Hydroxyecdysone



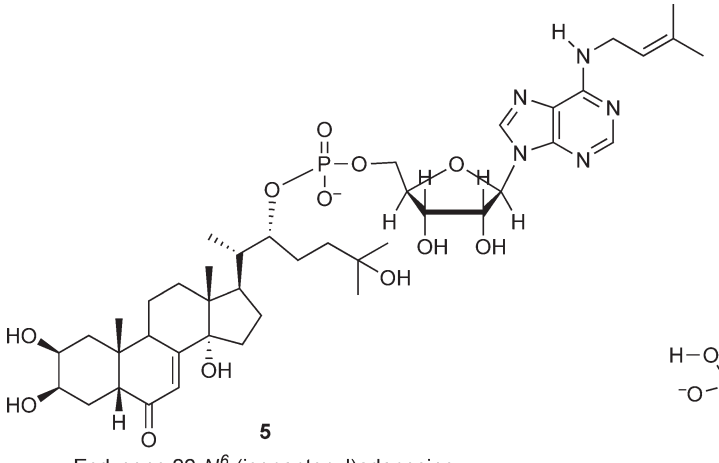
2
Makisterone A



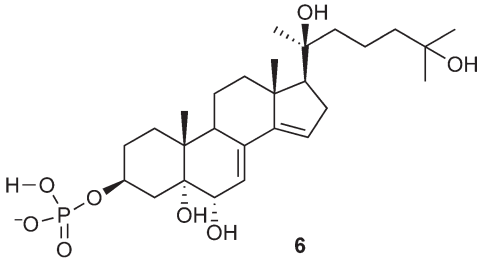
3
Makisterone C



4
2-Deoxyecdysone 22-adenosine monophosphate



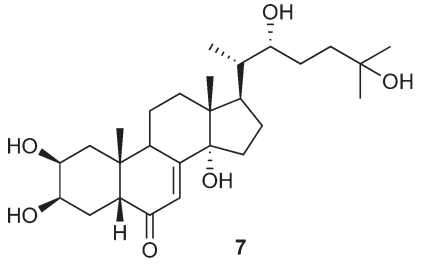
5
Ecdysone 22-N^6-(isopentenyl)adenosine monophosphate



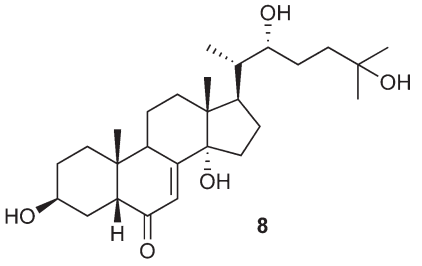
6
Bombycosterol 3-phosphate

4.03.3.1 Biosynthesis

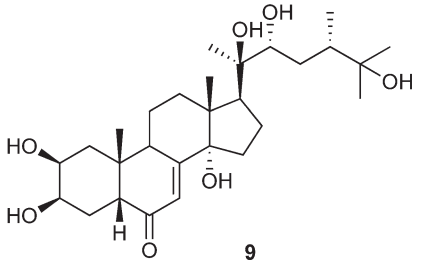
Ecdysteroids are produced in immature insects in the prothoracic glands, and in adults, in the gonads. The corresponding organ in crustaceans is the Y-organ. In some lower arthropods, ecdysteroids are produced by epidermal cells. In adult insects, the ecdysteroids control several reproductive processes. It is difficult to make general statements that apply to all insects, but the prothoracic gland usually produces ecdysone (**7**) (or 2-desoxyecdysone (**8**), oxidized to ecdysone in the hemolymph), a prohormone that is oxidized to active 20-hydroxyecdysone (**1**) in the fat body and Malpighian tubules. As arthropods are unable to synthesize sterols,[23] the starting material, cholesterol, or a close relative, must come from the diet. Since plant- or fungus-eating insects acquire phytosterols such as campesterol, β-sitosterol, or ergosterol, many insects from at least four orders (Coleoptera, Diptera, Hemiptera, and Hymenoptera) retain the extra methyl group and use makisterone A (**2**) for their moulting hormone. The fruit fly *Drosophila melanogaster*, for example, has a mixed diet of cholesterol and C_{29} phytosterols. It is unable to dealkylate the C_{29} sterols, so its moulting hormone is a mixture of 20-hydroxyecdysone (**1**) and makisterone A (**2**), depending upon its food. This mixture functions because the ecdysone receptors of *Drosophila* have very similar affinity for the two compounds.[24] The situation is similar in honeybees. Leaf-cutting ants raise fungus for food, and hence have an intake of 24β-methyl fungal sterols. *Acromyrmex octospinosus* therefore used 24-*epi*-makisterone A (**9**) as hormone.[25] Some insects are dependent on yeast-like symbionts to produce sterols. The symbionts of the beetles *Lasioderma serricorne* and *Stegobium paniceum* (Coleoptera: Anobiidae) contain ergosterol, the beetle sterols are predominantly cholesterol and 7-dehydrocholesterol.[26]



7
Ecdysone



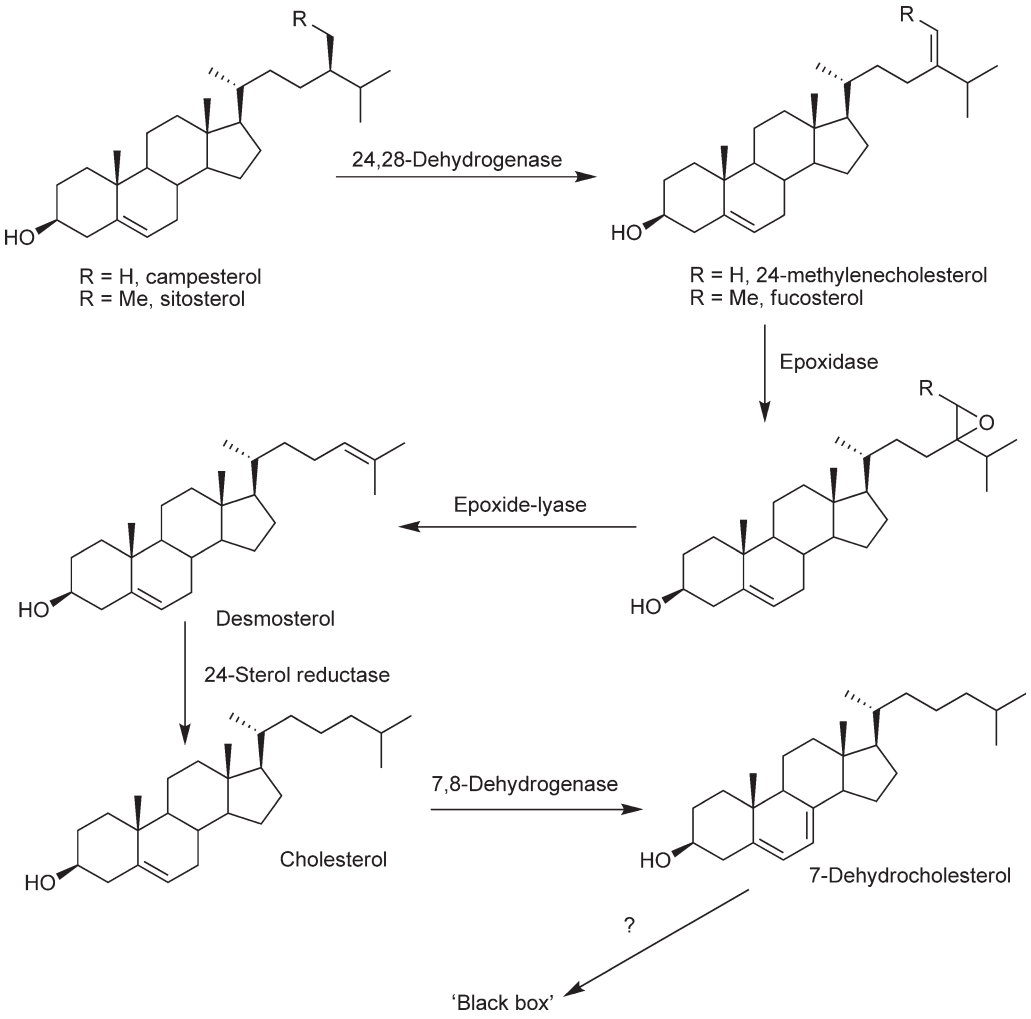
8
2-Desoxyecdysone



9
24-*epi*-Makisterone A

In spite of prodigious efforts in several laboratories, the full biosynthetic scheme from cholesterol to 20-hydroxyecdysone is as yet unknown, but the isolation of the enzymes catalyzing the various stages through molecular genetics and enzyme recognition is progressing, and the final answer cannot be far off. The scheme given earlier[27] has little modification after 10 years. The pathway can vary over several steps with different insect species (**Scheme 1**).[22,28] The intermediate steps between 7-dehydrocholesterol and 5β-ketodiol remain unknown and are often referred to as 'the black box'. The belief is held that either the intermediates are too unstable for easy isolation or the products of each step converts rapidly on to the next intermediate. The probable immediate precursor of the first recognizable compound in the pathway is the compound called Δ^4-diketol, which by the action of 5β-reductase gives the 5β-diketol. This is exchangeable with 2,22,25-tridesoxyecdysone, commonly known as 5β-ketodiol.

The choice of which compound is used for the next stages of hydroxylation depends upon the evidence. An enzyme, 3β-hydroxysteroid dehydrogenase, has been found in the crab *Carcinus maenas*, which can reversibly

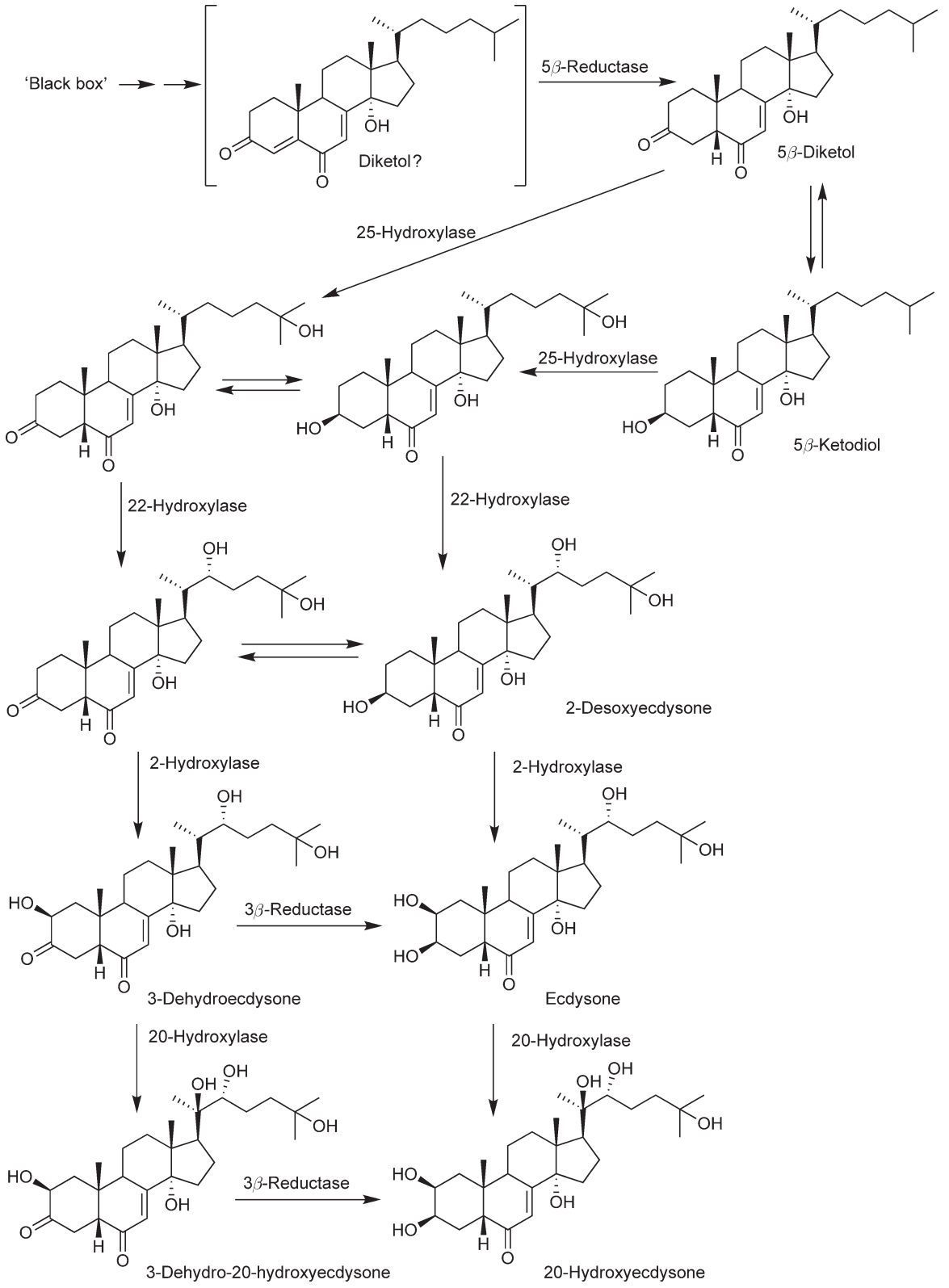


Scheme 1

catalyze the reduction of the next three suggested 3-keto-intermediates, but in one insect, *L. migratoria*, the 5β-ketodiol has definitely been shown to be present in the ovaries.[29] There seems to be an easy oxidation–reduction taking place that makes it difficult to know whether the 3-oxo- or 3β-ol is being hydroxylated (**Scheme 2**). The major step in hormone activation is the introduction of the 20-hydroxyl group, catalyzed by ecdysone 20-mono-oxygenase, the best-investigated biosynthetic enzymes in this series. It, like all the other hydroxylases in this sequence, is a cytochrome P-450 enzyme.

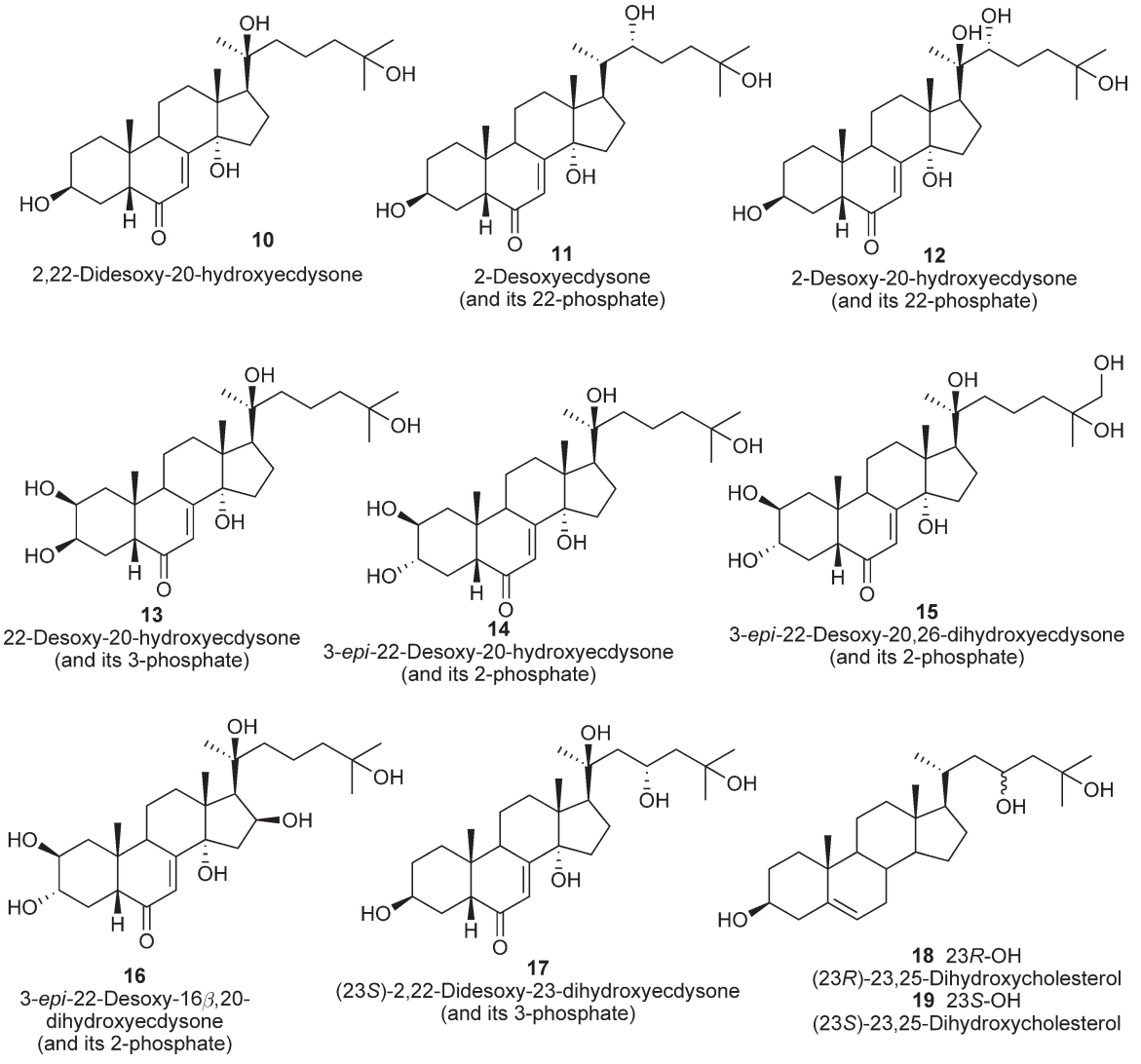
4.03.3.2 Storage Products

Ecdysteroids originate in the ovaries of adults, are converted into inactive conjugates, passed to the eggs and embryos, and later released for the early developmental stages of the embryo and the larva. These inactive conjugates usually have the 22-hydroxyl group converted into a phosphate, but no statement can cover all the possibilities of storage compounds. The greatest diversity of compounds is found in the eggs and embryos of Orthoptera and Lepidoptera. They contain unusually large amounts of ecdysteroids, up to 40 $\mu g\,g^{-1}$ fresh weight of eggs. That is about 10–100 times more than the concentration in corresponding larvae or pupae. Fatty acid esters of ecdysteroids are found in some orthopterans and in ticks. An extended study of the ovaries and eggs of the silkworm *B. mori* has led to a long list of compounds (structures **10**–**19**). In addition to the 2,22-didesoxy-20-hydroxyecdysone (**10**), 2-desoxyecdysone (**11**), 2-desoxy-20-hydroxyecdysone (**12**),


'Black box'
Diketol?
5β-Reductase
5β-Diketol
25-Hydroxylase
25-Hydroxylase
5β-Ketodiol
22-Hydroxylase
22-Hydroxylase
2-Desoxyecdysone
2-Hydroxylase
2-Hydroxylase
3β-Reductase
3-Dehydroecdysone
Ecdysone
20-Hydroxylase
20-Hydroxylase
3β-Reductase
3-Dehydro-20-hydroxyecdysone
20-Hydroxyecdysone

Scheme 2

ecdysone (7), and 20-hydroxyecdysone (1) and their 22-phosphates, 22-desoxy-20-hydroxyecdysone (13) and its 3-phosphate, 3-*epi*-22-desoxy-20-hydroxyecdysone (14) and its 2-phosphate, all found earlier, and the two nonhormonal compounds bombycosterol and its 3-phosphate[21] 6 (Section 4.03.3), we must add 3-*epi*-22-desoxy-20,26-dihydroxyecdysone (15), 3-*epi*-22-desoxy-16β,20-dihydroxyecdysone (16), their 2-phosphates (from diapausing eggs)[30] and (23*S*)-2,22-didesoxy-23-dihydroxyecdysone (17) and its 3-phosphate, (23*R*)- and (23*S*)-23,25-dihydroxycholesterol (18), and (19) (from mature ovaries).[31] On the other hand, there are many other insects in which no detectable maternal ecdysteroids are present in newly laid eggs.



4.03.3.3 Inactivation

There is little coherence or pattern in the way ecdysteroids are inactivated after they have served their purpose, they may be additionally hydroxylated, oxidized to carboxylic acids, conjugated with a water-solubilizing group, converted into water-insoluble fatty acid esters, or they may be excreted unchanged. Metabolic reactions are shown in **Figure 2**. When looking at available evidence across the arthropods, it is seen that the metabolic pathways differ strongly with the species investigated.[22] A frequently used strategy is the oxidation of 20-hydroxyecdysone to give 20,26-dihydroxyecdysone and further oxidation to ecdysonoic acid

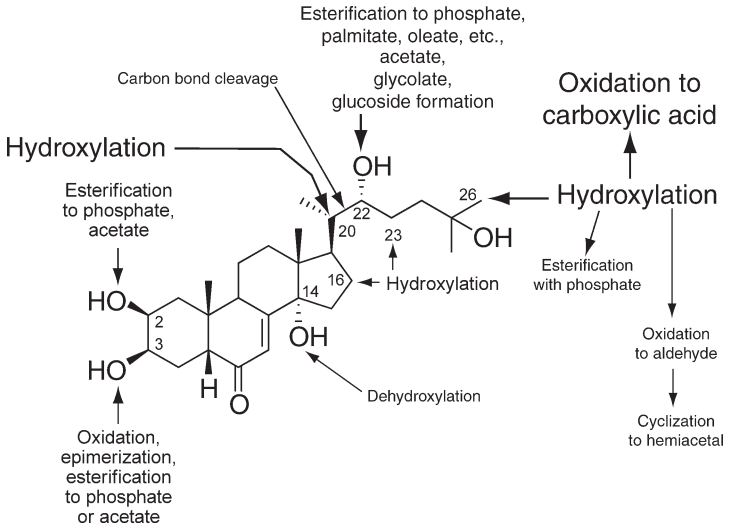
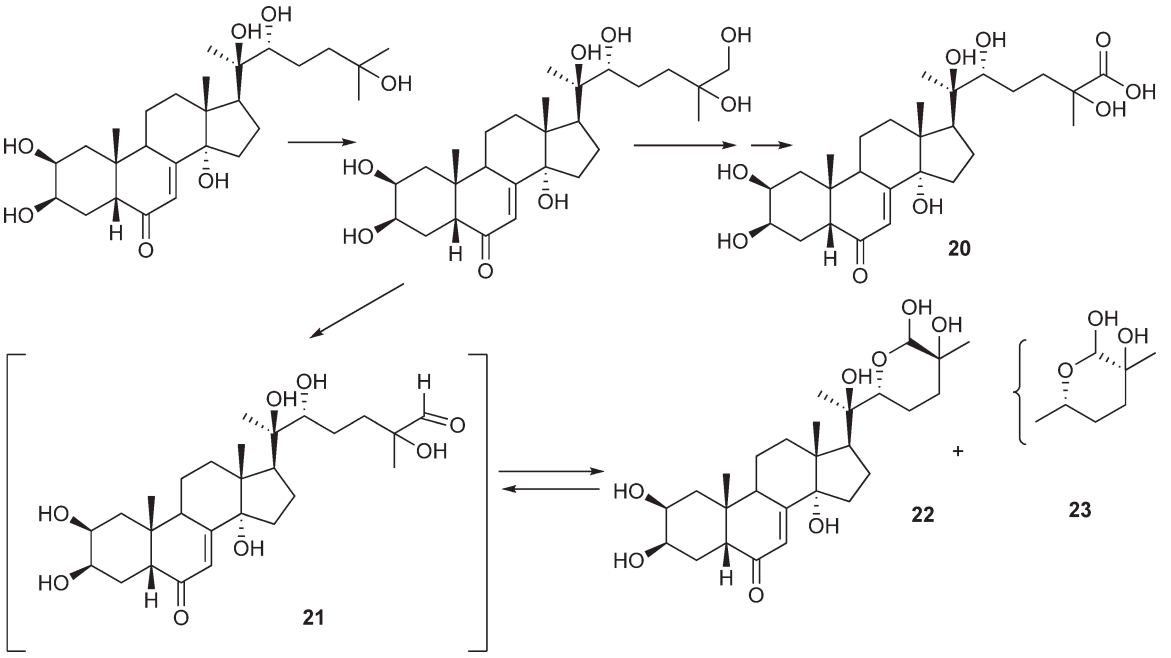


Figure 2 Principal reaction paths of metabolism of ecdysone. The larger the print, the more common the reaction in known examples. Modified from R. Lafont; C. Dauphin-Villemant; J. T. Warren; H. H. Rees, Insect Hormones. In *Comprehensive Molecular Insect Science*; Elsevier: Oxford, 2005; Vol. 3, pp 125–195, Fig. 15, Copyright Elsevier, 2004, in turn Modified from R. Lafont; J.-L. Connat, Pathways to Ecdysone Metabolism. In *Ecdysone: From Chemistry to Mode of Action*; J. Koolman, Ed.; Georg Thieme: Stuttgart, 1989; Chapter 14, Fig. 14.1.

(**20**) (**Scheme 3**). A clone of cells from the midge *Chironomus tentans* was found to be resistant to the effects of ecdysteroids because they metabolized 20-hydroxyecdysone rapidly. The initial oxidation product was 20,26-dihydroxyecdysone, but this was oxidized further to 20-hydroxy-26-oxo-ecdysone (**21**). This aldehyde (**21**) then formed a tautomeric equilibrium mixture of two cyclic hemiacetals (**22**) and (**23**), which were separable, isolated, and their structures determined (**Scheme 3**) with the use of acetonides (Section 4.03.3.6).[32] These are the first examples of ecdysteroids with side-chain hemiacetals. Although 20,26-dihydroxyecdysterone still



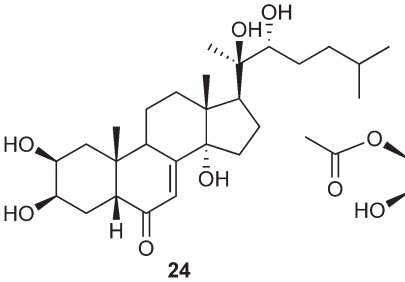
Scheme 3

retained some ecdysteroidal activity in the tests of Kayser *et al.*[32] (about one-tenth to one-hundredth of that of 20-hydroxyecdysone), the hemiacetals were inactive.

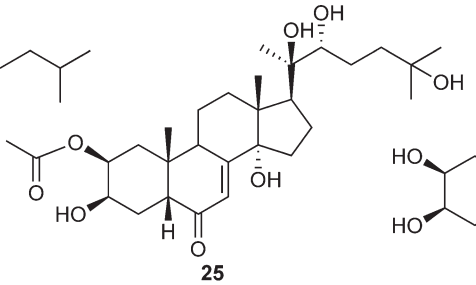
The existence of polar and nonpolar conjugates or metabolites of ecdysteroids makes it very difficult to search for them with chromatographic methods, even with linked MS. Many investigators resort to a mixture of radioimmunoassay and chromatographic methods, usually with antibodies that recognize different parts of the ecdysteroid molecule, so that conjugates with either the 3-position or the side-chain hydroxyls is blocked, can be recognized.[33]

4.03.3.4 Crustaceans and Other Phyla

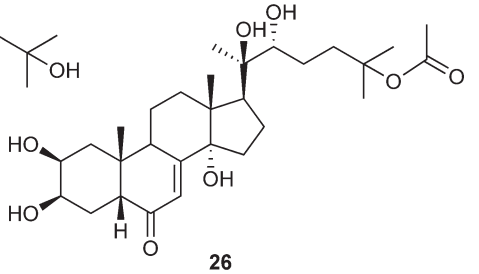
In evolutionary terms, endocrine glands are found in annelids onward. Ecdysteroids are known in other invertebrate groups such as coelenterates (polyps, corals, sea anemones, and jellyfish), platyhelminthes (flatworms), annelids (leeches), mollusks (snails, slugs, mussels, oysters, squid, octopus, and others), and echinoderms (starfish, sea urchins, sea cucumbers), although their functions are not yet fully understood. Ten known ecdysteroids (ponasterone A (**24**), 20-hydroxyecdysone 2-acetate (**25**), viticosterone E (**26**), integristerone A 22-acetate (**27**), 2-desoxy-20-hydroxyecdysone (**12**), ecdysone (**7**), ajugasterone C (**28**), dacryhainansterone (**29**), inokosterone (**30**), and 20-hydroxyecdysone (**1**)) and the new compound zoanthusterone (**31**) have all been isolated from the marine zoanthid *Zoanthus* sp. (Coelenterata: Anthozoa).[34]



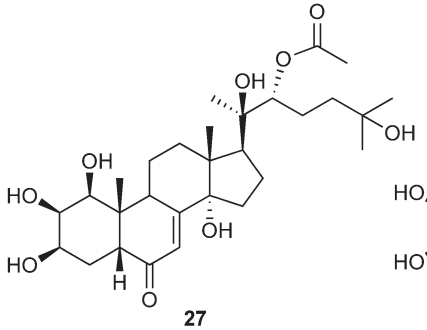
24
Ponasterone A



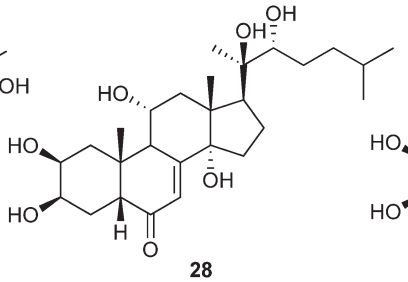
25
20-Hydroxyecdysone 2-acetate



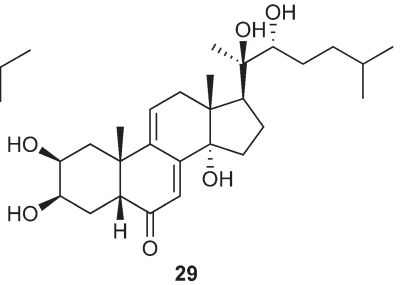
26
Viticosterone E



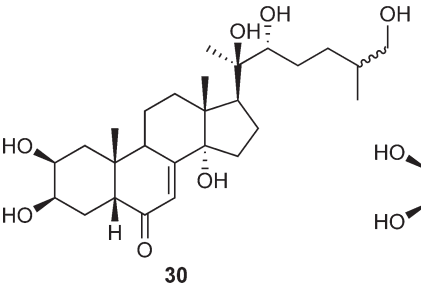
27
Integristerone A 22-acetate



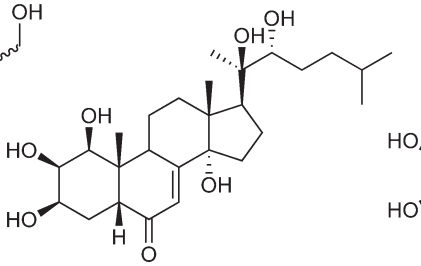
28
Ajugasterone C



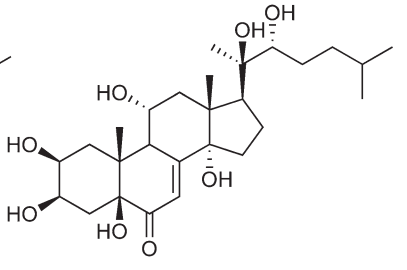
29
Dacryhainansterone



30
Inokosterone



31
Zoanthusterone



32
Muristerone A

In Crustacea, ecdysteroids are the moulting hormones, as in other arthropods, and are produced in the Y-organs. In most of the known cases, the Y-organs produce ecdysone, which is converted into 20-hydroxyecdysone in peripheral tissues, as in insects. However, in some cases (the crabs *Cancer antennarius*, *C. maenas*, and *Menippe mercenaria*) the Y-organs produce 3-desoxyecdysone and 25-desoxyecdysone, the latter gives active ponasterone A (**24**). In addition to ecdysone and 3-dehydroecdysone,[35] inokosterone (**30**) has been identified in the eggs and embryos of the giant freshwater prawn *Macrobrachium rosenbergii*.[36] Subramoniam[37] lists 15 species of crustaceans in which ecdysteroids and their conjugates have been identified. Both highly polar and nonpolar conjugates are found as inactivation and metabolized products, as in insects.[36] Although JH has not been found in ticks (Section 4.03.4.4), ecdysteroids in ticks function much as in insects, controlling production of sex pheromones, oogenesis, oviposition, and embryogenesis in females.[38]

In adult insects the prothoracic gland ceases to function, and the gonads take over ecdysteroid production. In adult crustacea moulting continues and the Y-organs continue to function, so that there is limited evidence about alternative sites of production. Lafont and Mathieu[39] point out that there is no clear evidence that crustacean ovaries produce ecdysteroids, and there is still less evidence that males produce them.

There are several reports of ecdysteroids isolated from sponges (phylum Porifera). For example, from the Caribbean sponge *Agelas dispar* 20-hydroxyecdysone and ajugasterone C (**28**) was isolated,[40] and from another Caribbean sponge *Iotrochota birotulate* ponasterone A (**24**), its 22-glycolate, 20-hydroxyecdysone (**1**), its 22-acetate and its 22-glycolate (Section 4.03.3.7).[41] In these sponges, there is no indication that they possess the hormone function or that the ecdysteroids serve as protection from predators. Sponges are known as rich sources of an array of sterols.[42,43]

4.03.3.5 Phytoecdysteroids

As plant-eating insects obtain their necessary sterols from plants to make moulting hormones, it is fitting that some plants, in return, make ecdysteroids to upset the development of insects feeding upon them. Ecdysteroids have been found in 5–6% of all the plants examined until now,[44] but there is evidence that all plants may be able to produce at least low levels of ecdysteroids.[45] The concentration of ecdysteroids in plants ranges from 50 ng g^{-1} to 30 mg g^{-1} dry mass, that is, 3%.[46] Plants usually contain one or a few major ecdysteroids, with a larger number of closely related minor products of similar structure,[46] which can be looked upon as metabolic by-products. The phytoecdysteroids are concentrated in leaves and flowers.[45,47] It is generally thought that they protect the plant from nonadapted insects,[23] and indeed this has been demonstrated in some examples. Many insects, particularly polyphagous ones (or their gut bacteria) appear to have developed detoxifying mechanisms that protect them from ingested ecdysteroids, even at high doses, by removing the C-14-OH group. Ecdybase attempts to keep up with new discoveries of phytoecdysteroids, with their spectral data and source.[19] There are many reviews available on methods for the isolation and identification of phytoecdysteroids.[46]

4.03.3.6 Structure Determination

There are many well-established methods for separation and structure determination of ecdysteroids.[20,27] A newly described method is two-dimensional thin-layer chromatography. It has been used to separate complex mixtures of phytoecdysteroids. Silica plates were developed first with toluene–acetone–ethanol–25% aqueous ammonia (100:140:32:9 v/v) and then developed in the other direction with chloroform–methanol–benzene (25:5:3 v/v).[48]

The advance of the so-called hyphenated methods have compressed the time required for the simultaneous separation and identification of ecdysteroids still further. Reversed phase high-performance liquid chromatography (HPLC) has been coupled to a diode array ultraviolet (UV) detector, with the effluent then passed to a proton nuclear magnetic resonance (NMR) spectrometer, Fourier transform infrared (IR) spectrometer, and a time-of-flight mass spectrometer. In this way, almost complete structural identification is possible without isolating any material.[49] There was some difficulty in getting good separation of ecdysteroids from other components of the crude extract. The process was improved by moving to normal phase HPLC and using

superheated deuterium oxide as the mobile phase.[50] The method still requires what are considered large amounts of ecdysteroids (100–400 μg injected).

Perhaps the lowest level of detection using MS has been with atmospheric pressure chemical ionization with selected ion monitoring. This technique gave a limit of detection (but not quantification) down to 10–100 pg per analysis.[51] This beats the mass spectral sensitivity achieved for ecdysteroids back in 1975,[52] and avoids the very difficult procedure of converting them into poly-(trimethylsilyl)ethers. Another development has been in electrospray injection (HPLC/ESI–MS). In this way, ecdysteroids can be divided into three groups. Those compounds with lower hydroxylation, including ecdysone (**7**), 2-desoxyecdysone (**11**), and 2,22-didesoxyecdysone, fit into the first category, with the $[M+H–H_2O]^+$ ion dominant. The second group, including 20-hydroxyecdysterone (**1**) and makisterone A (**2**), have the $[M+H]^+$ ion dominant, but with some contribution from $[M+H–H_2O]^+$, and the third group, including muristerone A (**32**) and ponasterone A (**24**), have $[M+H]^+$ dominant with no water loss evident.[53]

Large sample requirements have always been a drawback for ^{13}C-NMR spectra, where about 10 mg were required. These quantities have always been difficult to obtain from insects, but the introduction of ^{1}H–^{13}C correlation spectra has reduced sample size. Correlated spectroscopy (COSY), also called heteronuclear multiple-quantum correlation (HMQC), and long-range COSY, also called heteronuclear multiple-bond correlation (HMBC), have reduced sample size to less than 500 μg for ecdysteroids.[54] COSY spectra make use of direct ^{1}H–^{13}C coupling, and long-range COSY detects long-range ^{1}H–C–^{13}C (two bond) and ^{1}H–C–C–^{13}C (three bond) coupling.

A rapid method for making acetonides, coupled with liquid chromatography–MS (LC–MS) has been used to study the structure of ecdysteroid metabolites. Information about the structure can be deduced from chromatographic retention times. Mono-, di-, and tri-acetonides are well separated in HPLC.[55]

4.03.3.7 Ecdysteroids as Defensive Secretion

Beetles are always full of surprises for the investigator. We now know of a beetle (*Chrysolina carnifex*; Coleoptera: Chrysomelidae) that contains vast amounts of ecdysteroids in its defensive (elytral and prenotal) glands.[56] It has a concentration of 150 mmol l^{-1} of 20-hydroxyecdysone 22-acetate in its glands, but there is no evidence of its use in defense yet. Surprisingly, the Pycnogonida, an ancient branch of the Arthropoda that have existed for at least 500 million years, have their candidate, the sea spider *Pycnogonum litorale* (Chelicerata: Pycnonidae), which produces a range of eight ecdysteroids, including ecdysone 22-glycolate and 20-hydroxyecdysone 22-glycolate, with 20-hydroxyecdysone 22-acetate in greatest proportion (68–88%).[57] The total mass of ecdysteroids reach 0.1% of the body dry weight. This is about 1000 times greater than in most arthropods. The ecdysteroids accumulate in epidermal glands and are released on disturbance. Crabs (*C. maenas*) are the main predators in the environment of the sea spider, but the crabs will not eat them. Crabs also reject artificial food containing the *P. litorale* ecdysteroids.[58] The crabs are quickly able to detect the ecdysteroids if they bite the leg of a sea spider.

4.03.3.8 Ecdysteroids in Mammals

Although there is no suggestion that ecdysteroids occur in mammals, curiosity about possible effects of these sterols have led to experiments, not all of which have been conducted under well-controlled conditions. It is found that ecdysteroids have effects in mammals, and from available evidence, they influence or improve many physiological functions. They have a significant anabolic effect, and some 300 ecdysteroid-containing commercial products are available as a result.[59] However, there is too little good evidence to justify many of the claims. Injected or ingested ecdysteroids are quickly cleared from the blood, and our normal food plants are not the ones that contain significant amounts of ecdysteroids, with the exception of spinach, *Spinacia oleracea*.[60] Owing to the body-building craze, 20-hydroxyecdysone, the one ecdysteroid available in quantity, can be purchased in bulk at low prices.[61]

4.03.3.9 Mammalian Hormones in Arthropods

Many mammalian sterols have been identified in insects, particularly as defensive secretions in water beetles, but no hormonal function for mammalian sterols has yet been found. Estradiol was detected in the ovaries of the silkworm, *B. mori*, but had no significant effect on development.[62] A number of mammalian steroids have been found as defensive secretions of some water beetles.[63] Androgenic hormones have been found in crustaceans. Both estradiol and progesterone have been found in the hemolymph and ovaries of the giant tiger shrimp *Penaeus monodon*.[64] They appear to stimulate vitellogenin (egg protein) synthesis in the mole crab *Emerita asiatica* and the freshwater prawn *Macrobrachium rosenbergii*.[65] **Figure 3** shows the structures of mammalian steroids found in crustaceans.[37,66,67] There is no evidence of these being sex hormones. Toxic effects of mammalian steroids in crustaceans are discussed by LeBlanc.[68]

It has been proposed that insects do have sex hormones (ecdysone has been suggested[69]), but at present, it is generally accepted that sex differentiation in insects is a strictly genetic process, not requiring a hormone. However, an androgenic gland is present in some crustaceans and an isopod. When the androgenic gland of young males of the common wood louse *Armadillidium vulgare* (Isopoda: Armadillidiidae) is destroyed, it leads to complete feminization. The hormone responsible is a glycosylated protein.[70,71]

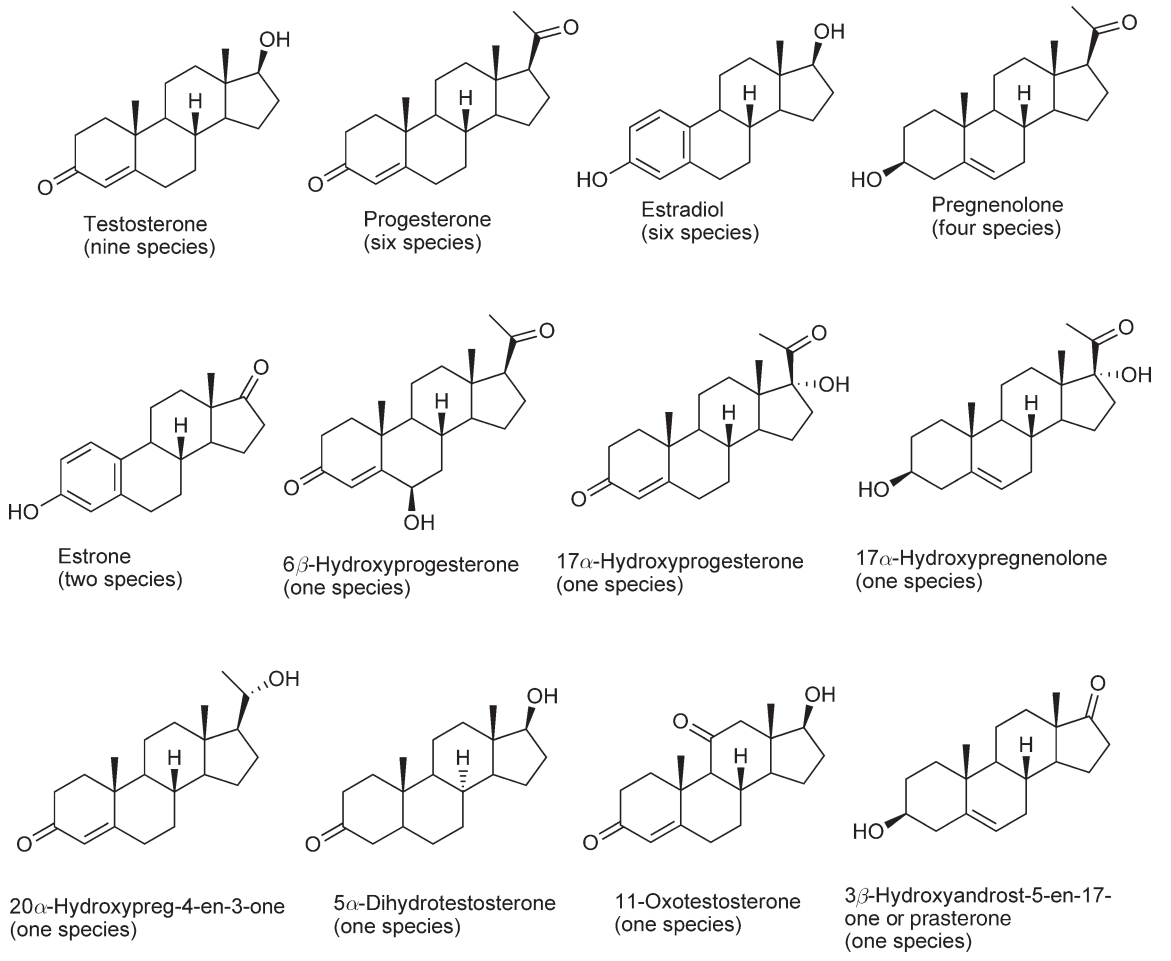
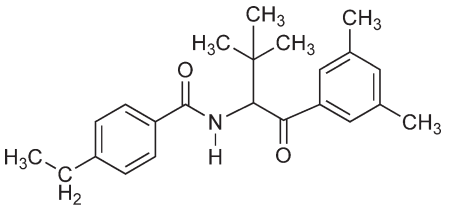


Figure 3 Some mammalian hormonal sterols found in arthropods.

4.03.3.10 Ecdysteroids and Pest Control

The possibility of using our knowledge of insect moulting and ecdysteroids to control pest species is a recurrent subject of consideration.[72,73] As yet no promising leads have followed from research. There are some purely synthetic compounds that are ecdysone antagonists or agonists, such as tebufenozide (RH 5992) (**33**), which causes premature moulting in lepidopteran species.[74] The cost of steroids and their possible effects on humans make them an unattractive starting point. It is possible that as our knowledge of hormone receptors and biosynthesizing enzymes grows (particularly through the use of mutant strains of *Drosophila*, which lack one or more of the enzymes necessary), we may learn the structure of the receptor sites and be able to design small synthetic molecules that will block these sites.



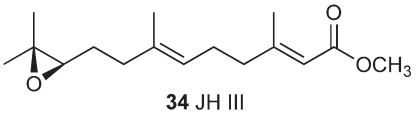
33

4.03.4 Juvenile Hormone

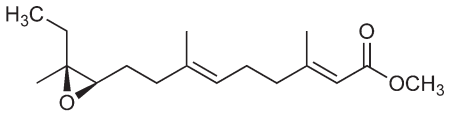
The term juvenile hormone (JH) results from an early stage of its study, when it was found to be important in each instar of larval or nymphal development, but it seemed to disappear at the pupal stage, hence the name. Later it was discovered in adult forms, but the name juvenile hormone had been established. Perhaps it is more accurate to say that it is the presence or absence of the hormone at critical periods that determines whether the subsequent molt is to a larval or adult form. In holometabolous insects, absence of JH in the final larval stage leads to pupal formation, and its absence in a pupa leads to adult development. There are two critical periods in the last larval instar, first with no JH formation, leading to pupal development, followed by a brief late peak of JH, causing the retention of imaginal discs, and preventing their development into adult structures. A number of other functions for this group of compounds are known, it covers embryogenesis, metamorphosis, and reproduction, including control of diapause, synthesis of egg proteins (vitellin), ovarian development, color, polymorphism, determination of phase in locusts and aphids, and of caste in honeybees and ants.[75] It regulates the production of sexual pheromones (in Lepidoptera) and response to them. It therefore has a dual function, in juvenile forms as a *status quo* hormone, and in adults, a number of regulatory tactics.

4.03.4.1 Structures

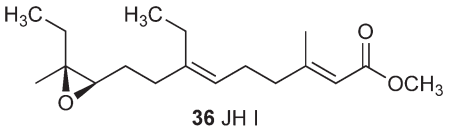
Formerly, six compounds in the series were known. JH III (**34**) is the most widely distributed JH homologue. It has been identified in most insect orders, particularly in Coleoptera, Hymenoptera, and Orthoptera, whereas JH 0 (**35**), JH I (**36**), JH II (**37**), and 4-Me-JH I (**38**) are found only in Lepidoptera, and JHB-3 (**39**) only in higher Diptera. Natural JHB-3 (**39**) has the configuration 2*E*,6*S*,7*S*,10*R*. JH III is derived from farnesoic acid (**41**) (**Scheme 4**) via three molecules of mevalonic acid (**41**). For JH 0, I, II, and 4-Me-JH I, homomevalonate (**42**) is required, derived from propionyl CoA (in turn from isoleucine or valine) and acetyl CoA. Since some insect embryos produce methyl farnesoate, and Diptera are considered the most highly evolved insects, there seems to be an evolution in JH, from methyl farnesoate through JH III to JHB-3 (**39**). JHs 0, I, II, and 4-methyl-JH I form a side branch that is peculiar to Lepidoptera. Other insect orders are capable of making and using homomevalonate. Hydrocarbons and oxygenated derivatives with the same carbon skeletons as JH 0, I, II, and 4-methyl-JH I are well known in ants,[76] and homomevalonate products are known in other Hymenoptera.[77]



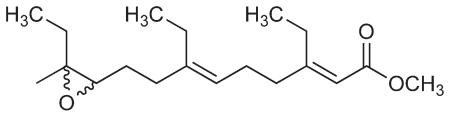
34 JH III



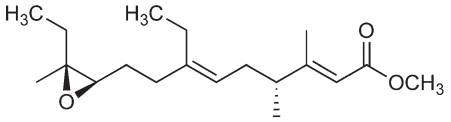
37 JH II



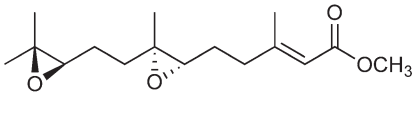
36 JH I



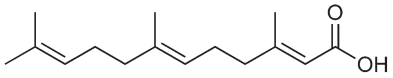
35 JH 0



38 4-Me-JH I



39 JHB-3

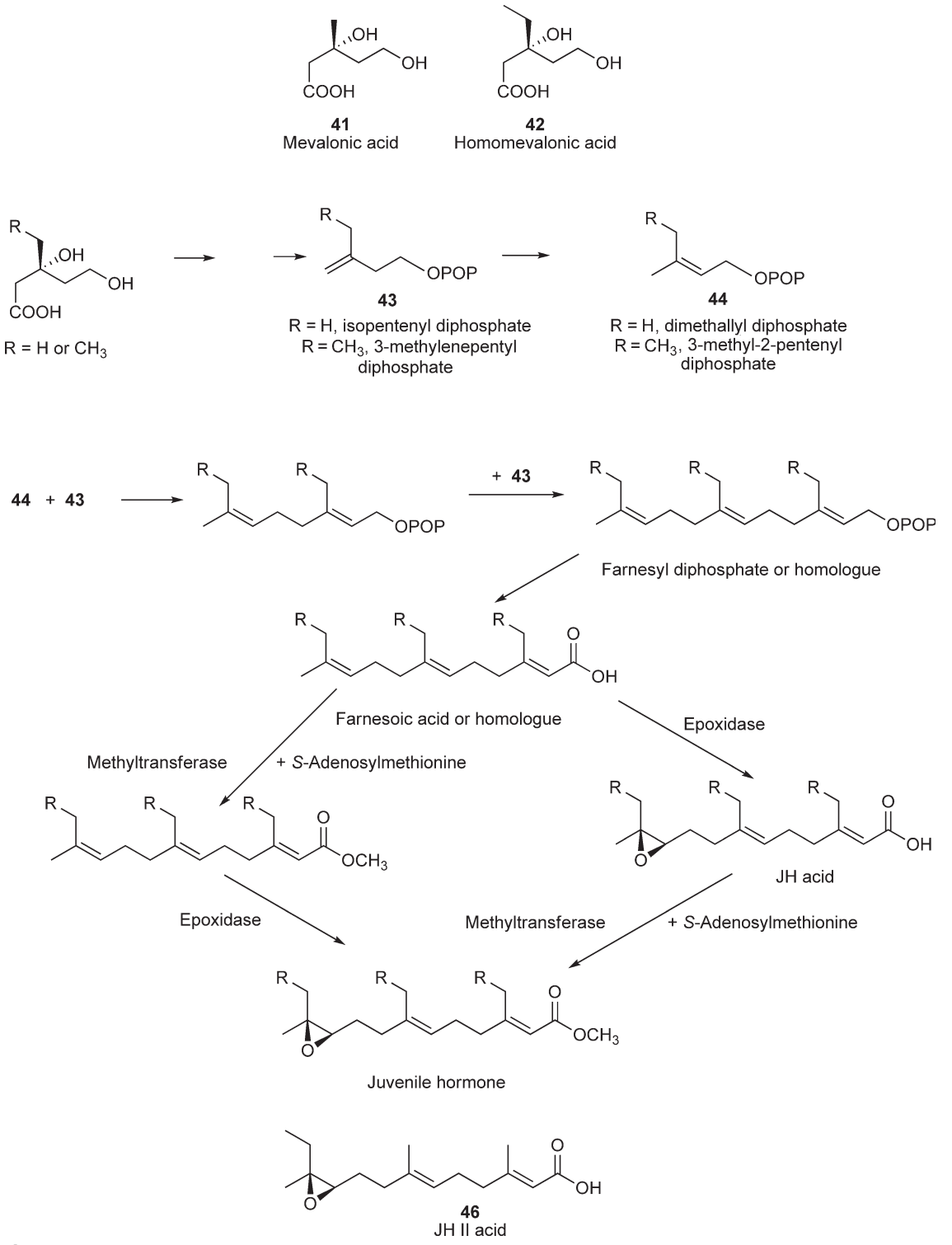


40 Farnesoic acid

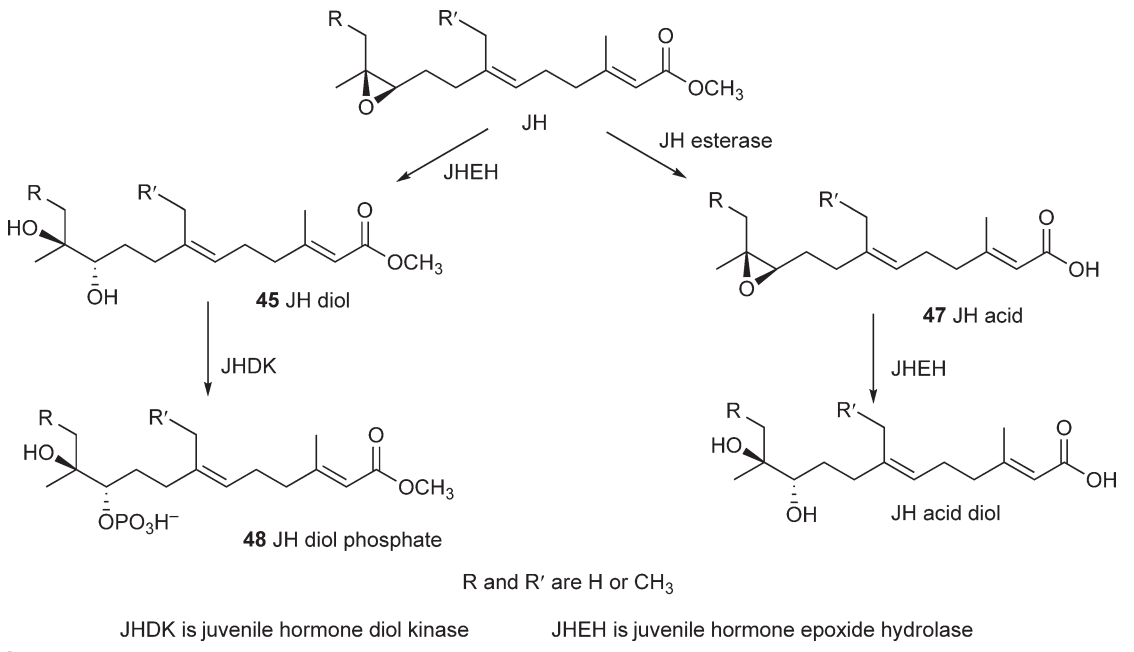
Of the best-known insect orders, only the Hemiptera, the true bugs, stand out as a group without a clearly recognized JH. An early report identified JH I as the principal hormone in the hemolymph of the adult female bean beetle, *Riptortus clavatus* (Hemiptera: Alydidae) by gas chromatography–MS (GC–MS).[78] It was reported that, of the three compounds JH I, II, and III, JH I was the most effective for inducing yolk protein synthesis in diapausing adults of this species.[78] More recently, it has been reported that a compound close to JH III, but not JH III, is the hormone in the two spotted stink bug *Perillus bioculatus* (Hemiptera: Pentatomidae). Production of this hormone is stimulated by farnesol, a known precursor of JH III.[79]

4.03.4.2 Biosynthesis and Degradation

JH is a product of the mevalonate biosynthetic pathway.[80] Its formation has been thoroughly investigated.[81] Farnesoic acid (**40**), a normal mevalonic acid product is converted by methylation and epoxidation in the pair of glands called the corpora allata, by *S*-adenosyl methionine, to yield JH (**Scheme 4**). In some insects it appears that JH acid is released by the corpora allata and converted by a methyltransferase in target tissues. In Lepidoptera, there may be a reversal in the final stages of biosynthesis, compared with other orders, that is, farnesoic acid to epoxyfarnesoic acid, then esterification to JH III. JHB-3 (**39**), likewise, is formed from farnesoic acid, epoxidation, and then methylation in *D. melanogaster*.[82] Higher homologues (**35**–**38**), with one or more extra methylene groups are produced in the same way, replacing mevalonic acid (**41**) by one or more molecules of homomevalonic acid (**42**), as building blocks. The enzymic aspects of the biosynthesis[83] and the physiology[84] have been reviewed. It is interesting to note that, the oxidative enzymes of both the ecdysteroid and JH series belong to the cytochrome P-450 group.[85] Steadily, through cloning and identification of the genes, more knowledge is gained about the enzymes catalyzing the various steps in the hormone synthesis.[86,87] The enzyme catalyzing epoxidation of methyl farnesoate in the corpora allata of the cockroach *Diploptera punctata* has been cloned and sequenced.[87] It contains 493 amino acids, and has a specificity that produces 98% of the natural (10R)-epoxide enantiomer of JH III (**34**). The final product of mevalonate biosynthesis in vertebrates is cholesterol, which for them is a key regulator. In arthropods, that route is blocked,[23,88] and the pathway ends with linear terpenes, such as JH.[88] The JH hormone does not accumulate in the gland, the rate of release depends upon the rate of production.[89] In Lepidoptera, the JH II acid (**46**) also seems to have some hormonal function.[90] Unlike the moulting hormone, which is produced by different tissues in immature and adult forms, the JH is biosynthesized in the corpora allata, throughout the life of the insect (**Scheme 4**).


OH
OH
COOH
41
Mevalonic acid
42
Homomevalonic acid
R = H or CH3
OPOP
43
R = H, isopentenyl diphosphate
R = CH3, 3-methylenepentyl diphosphate
44
R = H, dimethallyl diphosphate
R = CH3, 3-methyl-2-pentenyl diphosphate
44 + 43
+ 43
Farnesyl diphosphate or homologue
Farnesoic acid or homologue
Epoxidase
Methyltransferase
+ S-Adenosylmethionine
JH acid
Juvenile hormone
46
JH II acid

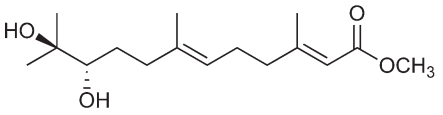
Scheme 4



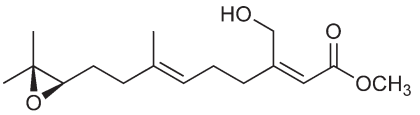
Scheme 5

JH can be inactivated by opening of the epoxide ring to a diol **45**, or by hydrolysis to the free acid **47**, and sometimes further by phosphorylation of the diol (**Scheme 5**). As the isolation and identification of enzymes involved in hormone synthesis and catabolism advances, a JH diol kinase has been isolated from the tobacco hornworm *Manduca sexta* that converts JH I diol into the phosphate **48**.[91] This enzyme is probably the first example of a phosphotransferase directly involved in the catabolism and inactivation of a lipid-soluble hormone. It was much less active in catalysing the phosphorylation of JH II or JH III diols and was inactive with the free JH acids.[91]

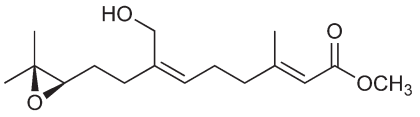
Juvenile hormone JH III 10,11-diol (**49**) is synthesized and released by the corpora allata of the migratory locust *L. migratoria*.[92] Identification was based on, first, similar ratio of labeled carbon and tritium incorporated in the new compound as in JH III, similar chromatographic properties of it and its acetate to the synthetic diol, and its acetate, and the mass spectrum of the new compound. It was possible to show that it was not a degradation product of JH III in the corpora allata by incubating JH III with corpora allata and no diol was produced. Then more recently, in a lepidopteran, the fall armyworm *Spodoptera frugiperda*, it has been shown that adult females produce and release JH III diol and JH II diol in the ratio 4:1. Only traces of JH III and JH II could be detected.[93] Also, three new compounds in the series, 4′-OH-JH III (**50**), 8′-OH-JH III (**51**), and 12′-OH-JH III (**52**) have been isolated from the corpora allata of *L. migratoria*.[94,95] The (10*R*,11*R*) enantiomer of 12′-OH-JH III had already been synthesized in a labeled form *en route* to JH III,[96] and was available for comparison, and shown to be identical. The Mauchamp group suggested that different patterns, or 'bouquets', of JH compounds may change with insect species or within the same species according to sex and growth stage.[95] There is no further evidence reported. In some insects, the corpora allata secrete JH acid **45**, which converts into the methyl ester in some target tissue. There is a similar situation with the moulting hormone, where ecdysone is secreted by the postpharyngeal glands, to be further hydroxylated in the hemolymph.



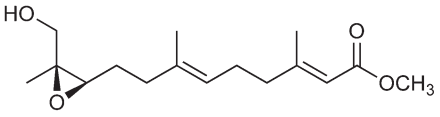
49 JH III 10,11-Diol



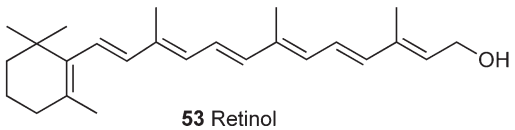
50 4′-OH-JH III



51 8′-OH-JH III



52 12′-OH-JH III



53 Retinol

A further complication arose when it was discovered that in the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Trypetidae) the corpora allata of adult virgin females produce, in addition to JHB-3, smaller amounts of methyl palmitate and less of JH III.[97] Mated females produced much less methyl palmitate. It is suggested that methyl palmitate may be a default product of methylation, in the absence of JH, but it does not rule out the possibility that it also participates in some way in reproductive maturation and control, because its presence is correlated with the period of nonreceptivity toward mating in adult females of *C. capitata*.[98]

Retinol (**53**), retinal, and retinoic acid appear to be present in insects, and these substances, particularly retinoic acid, have been shown to have some JH effect.

4.03.4.3 Transfer of Juvenile Hormone

Insect chemists may be familiar with the work of Eisner and Meinwald[99] on the accumulation of alkaloids by certain male Lepidoptera, and that of Dettner[100] on cantharidin-carrying beetles, and the transfer of these at copulation to the female, followed by incorporation of the alkaloid into the eggs. It has been known for some time that male abdomens contain more JH than those of females in about 13 species of Lepidoptera. However, it now emerges that in some species, this JH is synthesized in the male accessory sex glands, and transferred by the male to the female at copulation.[101] In *Heliothis virescens* (Lepidoptera: Noctuidae) the male-produced JH stimulates the production of more JH in the female and egg development.[102] JH I was the compound transferred in large quantities, and which had the greatest effect in the females. JH in the males decreased to very low level after copulation. In contrast, with *Choristoneura fumiferana* (Lepidoptera: Tortricidae) and *Choristoneura rosaceana*, it was found that males had little or no capacity to produce JH in their male accessory sex glands and did not transfer JH on copulation.[103] Once again we seem to have an example of retention of structure but not of function. These two species produced mostly JH II (**37**) in both males and females.[103]

4.03.4.4 JH in Arthropods

Earlier work on arthropod hormones began with an observation of function and that led to identification of a source and isolation of a substance. More recently, investigation has proceeded by observing a function of an exogenous compound and then seeking the hormone. For example, JH was first found only in insects, but insect JH and some analogues have been shown to produce precocious moulting in barnacle larvae.[104] Later, methyl farnesoate, which could fill the requirement of JH for crustaceans, was found to be present in several crustacean species.[105–107] It has been shown that methyl farnesoate is synthesized in the mandibular organs of a number of

crustaceans and has been found in the hemolymph of females and males. It is also found to be actively synthesized by females during vitellogenesis.[105] In males, higher titers of methyl farnesoate are associated with reproduction and aggressive mating behavior.[105] It stimulates general protein synthesis and promotes the moult cycle.[106] Methyl farnesoate has been identified in the cyprid (laval) forms of the barnacle *Balanus amphitrite* by selective ion monitoring with GC–MS and shown to have a JH effect.[108] As in insects, part of the function of JH is to maintain juvenile morphology;[109] however, the situation is more complicated in that there are many more early stages in crustacea than in insects.

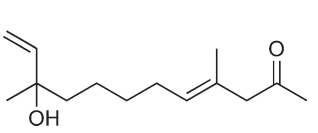
Methyl farnesoate is also found in some insect embryos. On the other hand, a thorough investigation of JH in two species of tick, representing hard and soft ticks, the American dog tick *Dermacentor variabilis* (Acari: Ixodidae) and the argasid tick *Ornithodoros parkeri* (Acari: Argasidae), using L-[methyl-^{3}H]methionine, farnesoic acid, and [1-^{14}C]acetate, found no evidence of JH I, II, or II or methyl farnesoate, whereas JH III and farnesol were found in three insect controls.[110] It was concluded that these ticks do not have the ability to make the common insect JHs, and these hormones do not regulate tick metamorphosis or reproduction as had been suggested earlier.

4.03.4.5 Structure Determination and Analysis

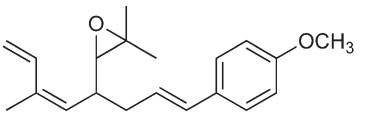
Chromatographic and mass spectrometric methods (both GC–MS and LC–MS, frequently with selected ion monitoring) are important in the study of JH; the cool, on-column injection method of Lefevere *et al.*[111] remains probably the most sensitive method for quantification of the range of JHs, but radiolabeling, radioimmunoassay, and bioassay methods still continue to be important tools for their study. The accuracy of, and problems in, the radiochemical assay for JH synthesis have been discussed.[112] At some concentrations, there is a preferential incorporation of methyl-^{14}C over methyl-^{3}H from labeled methionine.[112] A comparison of HPLC and radioimmunoassay methods showed no significant difference in quantifying JH III and minimal cross-reaction with JH precursors.[113] Since some insects seem to produce JH diols and not the epoxides (see above), a specific method, using reverse-phase HPLC and ESI–MS, has been described for the quantification of the JHs, their diols, and their acids. The limit of detection was 6 pg for JHs and 8 pg for diols.[114]

4.03.4.6 Phytojuvenoids

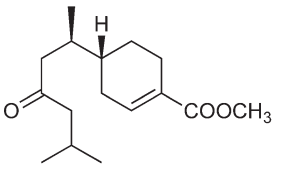
It is a well-established observation that one of the defensive strategies of plants is to produce secondary metabolites that interfere with the normal development of insects. Ecdysteroids in plants are a common phenomenon (Section 4.03.3.5). Plants also make JH mimics, such as echinolone (**54**) from the American coneflower *Echinacea angustifolia*, juvocimene-2 (**55**) from oil of sweet basil *Ocimum basilicum*, or juvabione (**56**), from the balsam fir *Abies balsamea*,[115] and antagonists to JH, such as the precocenes (**57**, **58**) of *Ageratum houstonianum*.[115] JH has itself been discovered in a plant, along with farnesol and methyl farnesoate.[116] JH III (**34**) was discovered in the grasshopper's sedge *Cyperis iria* and also *C. aromaticus*.[116] The JH has the same stereochemistry as insect JH III.[109] Levels of 151 $\mu g\,g^{-1}$ fresh weight of tissue of JH III and 14 $\mu g\,g^{-1}$ of methyl farnesoate were found,[116] and still higher levels of 193 $\mu g\,g^{-1}$ fresh weight of JH III in 1-month-old plants.[117] Later it was discovered that most of the JH was in the roots (43.5 $\mu g\,g^{-1}$ fresh weight).[118] For comparison, insects typically have 0.1–100 $ng\,g^{-1}$ fresh weight. Five other species of *Cyperus* did not contain detectable amounts of JH,[117] and although this information has been available for nearly 20 years, there has not been a rush of discoveries of JH in other plants. When a particular species of grasshoppers *Melanoplus sanguinipes* were fed on the plant, 90% of them displayed abnormal effects on moulting to adults, and ovaries of adult females were markedly underdeveloped.[116] A series of studies by Bede *et al.*[113,119–121] including tissue culture of *C. iria*[119] demonstrate that the biosynthetic route was the same in plant as insect, via mevalonate, and not by the alternative route of methylerythritol 4-phosphate, a route which is also available to the plant.[120]



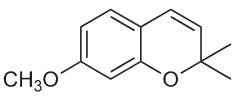
54 Echinolone



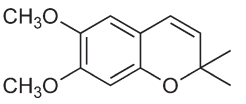
55 Juvocimene-2



56 Juvabione



57 Precocene I



58 Precocene II

4.03.4.7 JH and Pest Control

There is continuing interest in the use of knowledge gained from study of JH, its genes and biosynthesis, similar to the studies conducted on ecdysteroids, to discover new ways to control insect pests.[121] It might be thought difficult to see how such knowledge of a universal hormone could be applied selectively to pest species, but insects vary widely in their sensitivity to the phytojuvenoids. Interest in the precocenes **57** and **58** as tools in studying JH effects in insects continues, but there is little activity in finding new natural compounds with juvenilizing effects. There are numerous reports on the precocene content of the plant *Ageratum conyzoides* (Asteraceae). The leaf oil of *A. conyzoides* from northern Brazil consists exclusively of precocene I (**58**; 95.4%) and precocene II (**57**; 4.5%).[122]

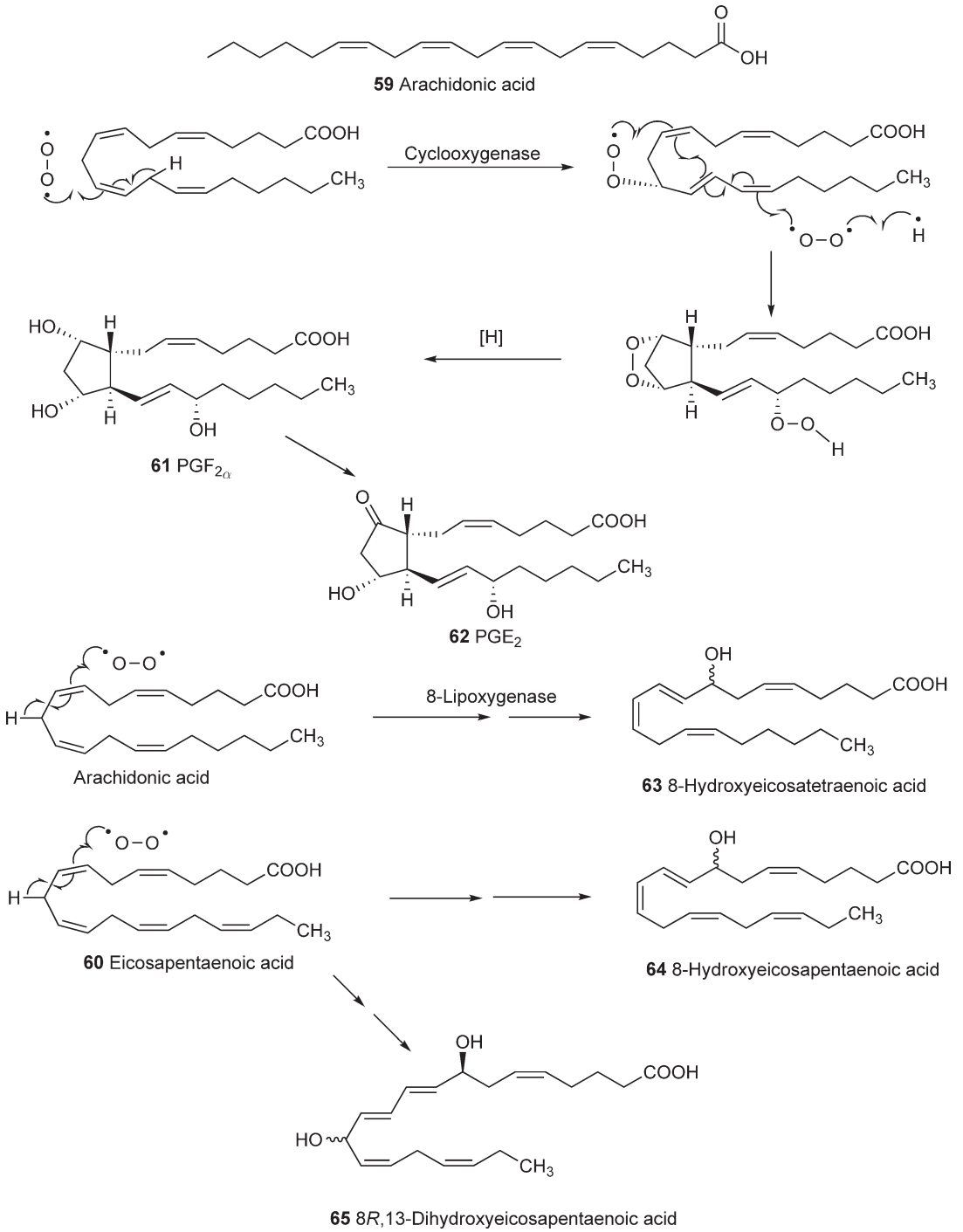
4.03.5 Eicosanoids

The eicosanoids are a group of lipid compounds based on arachidonic (5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,11,13-tetraenoic acid (**59**) and (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosa-5,8,11,14,17-pentaenoic acid (**60**; Chapter 1.03). They can be subdivided into three groups: prostaglandins, epoxyeicosatrienoic acids, and products of lipoxygenase enzymes. The physiological effects of prostaglandins from humans were first recognized in the 1930s, and the first chemical structure was identified in 1962. In subsequent years, owing to rapid advances, prostaglandins have been recognized in vertebrates, invertebrates, and even in microbes. Research on insects has revealed that they have several actions there, most notably concerned with the immune reaction and response to infection, but they also have a hormonal role in some places, and therefore deserve consideration here.[123,124]

In the cricket *Achaeta domestica*, it was found that an enzyme was transferred from males to females at copulation, and this enzyme was responsible for converting arachidonic acid into prostaglandin E_2 (PGE_2) (**62**), which induced egg-laying, and could therefore be considered a primer pheromone.[125] It was concluded that egg-laying in the Australian field cricket *Teleogryllus commodus*, was released by PGE_2 in a mode more like a broadly circulating hormone.[126] According to D. Stanley, this point can be extended to research on the mode of action, at the whole-organism level, of eicosanoids in invertebrates.[127] Prostaglandins release egg-laying behavior in some, but not all, insects. More recently, prostaglandins have been found to control uptake of a specific yolk protein, *Rhodnius* heme-binding protein.[128] Prostaglandins are also detected in salivary glands, endocrine glands, Malpighian tubules, testes, and ventral nerve cords.[129]

The early stages in the biosynthesis of prostaglandins and other eicosanoids in mammals are outlined in **Scheme 6**. From what is known at this stage, the pathway is very similar in invertebrates.[130]

The substance that induces hatching of eggs in the barnacle *Semibalanus* (*Balanus*) *balanoides* is identified as a prostaglandin.[131] In the intertidal barnacle *Balanus perforatus* the compounds identified were



Scheme 6

8-hydroxyeicosatetraenoic acid (**63**) and 8-hydroxyeicosapentaenoic acid (**64**), formed through the effect of an 8-lipoxygenase. Their production was linked to seawater temperature.[132] It seems that arachidonic acid and eicosa-8,11,14-trienoic acid act as juvenoids in annelid worms, and may also function in crustaceans and insects, a parallel with methyl farnesoate and the JH compounds.[109] 8,13-Dihydroxyeicosapentaenoic acid (**65**) has

been shown to control part of the post-copulation behavior of the subtidal barnacle *Balanus balanus* and causes muscular contractions.[133] It is suggested that new and different juvenoids await discovery.

Many of the studies on prostaglandins have been made using either a bioassay, immunoassay, sometimes with the enzyme cyclooxygenase, or using injected or added arachidonic acid or an eicosanoid, so the evidence for a prostaglandin is sometimes indirect. This has sometimes led to a mis-identification.[130] Both GC–MS and LC–MS have been used for direct identification. Typically identifications are based upon mass spectra, tandem MS (MS/MS), UV spectra, and chromatographic behavior.[134] Recent ESI–MS/MS linked to HPLC has provided detection over the range 0.5–50 pg and quantification from 2 to 100 pg.[135] Eicosanoids in invertebrates, other than insects, have been reviewed recently.[133]

Some interesting ideas have been presented by Schultz and Appel[136] in an article on hormones shared by plants and their insect herbivores. Proposing that signaling compounds have an early origin in evolution, such compounds can therefore be shared by microbes, plants, and animals, and that both plants and animals can manipulate these compounds to their advantages. This is particularly true of eicosanoids, which seem to be present in primitive and advanced organisms. The authors point to diverse evolutionary and ecological implications of shared signals and signal-stealing. It should now not be surprising that one eicosanoid ($PGF_{2\alpha}$ **61**) has been found in at least one flowering plant, the popular pot plant *Kalanchoe blossfeldiana* (Crassulaceae).[137] Several classes of prostaglandins have been identified in fresh vegetable oils by GC–MS[138] and may act as growth regulators.[139]

4.03.6 Conclusion

Developments in the chemistry of hormones of insects and other arthropods have shown that the simple picture of a few compounds and a few actions has become less simple. Advances in analytical methods and the increasing number of species studied have thrown up more variations on the simple rules. We are learning more about the interactions between plants and insects, and the conservation of biosynthetic routes between them, and across the phyla. The subject of eicosanoids in insects and lower animals is going to demand further investigation.

Glossary

androgenic development of male character
Annelid the phylum of ringed worms
corpus allatum (pl. corpora allata) endocrine gland behind the brain of insects that secretes juvenile hormone
diapause a period of delayed growth or development in unfavorable conditions such as drought or winter
elytra in beetles, the hardened, chitinous forewings, which act as wing covers
hemimetabolous insects that develop adult characteristics gradually, and do not go through a pupal stage (see *nymph*)
hemolymph the blood-like fluid in insects
holometabolous insects that undergo complete metamorphosis, passing through a larval, pupal, and an adult stage
imaginal disc a clump of tissue in an insect pupa that develops into an adult organ
midge tiny biting fly of the order Diptera
nymph the immature form of a hemimetabolous insect, corresponding to the larva of holometabolous insects
oogenesis the development of ova or eggs
Orthopteran insect order that includes locusts, grasshoppers, and crickets
phase locusts can exist either in solitary or gregarious phase, depending upon their behavior
polyphagous feeding on different kinds of food
prothoracic gland an endocrine gland in the prothorax of insects that secretes moulting hormone
vitellin protein of egg

vitellogenesis the production of egg protein in the mature female
Y-organ one of the pair of organs in crustaceans that secrete moulting hormone
zoanthid kind of marine coral

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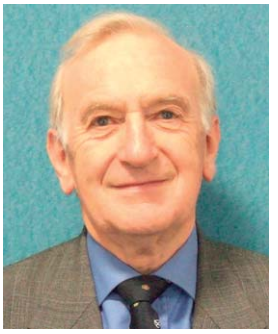
Biographical Sketch



David Morgan is a member of the Chemical Ecology Group at Keele University. He was born in Newfoundland and had his university education there, at Dalhousie University and University of King's College in Halifax, Nova Scotia, and at Oxford. His doctorate thesis was on the lipids of *Mycobacterium tuberculosis*. He then worked at the National Institute for Medical Research in London, and for Shell Chemical Company and Shell Research under the direction of Sir Robert Robinson, O.M., Nobel Laureate. From 1966 he has been at Keele in Staffordshire as lecturer, senior lecturer, reader, and professor. He discovered the natural pesticide azadirachtin and collaborated with S.V. Ley in its final structural elucidation. He isolated and identified the moulting hormone in the first hemimetabolous insect and charted the hormone titer throughout the embryonic, nymphal, and adult life cycle of the desert locust, *Schistocerca gregaria*. He has collaborated with entomologists in the identification of

numerous social insect secretions, including several ant trail pheromones. He has been an invited lecturer and examiner at a number of institutions and in 1997 was president of a Jacques Monod Conference on *Chemical Communication in Vertebrates and Invertebrates*. He is the author of over 300 papers and reviews, mostly on insect chemistry, editor and contributor to several volumes, and author of the book *Biosynthesis in Insects*. Recently, he developed an interest in secretions of stingless bees, and has throughout maintained an interest in organic archaeological chemistry.

4.04 Pheromones of Terrestrial Invertebrates

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4.04.1 Introduction

This chapter is a continuation and an updated version of our earlier discussion of pheromones.[1] Covering the literature of the past decade until the end of 2008, it predominantly deals with structures of new compounds that have been identified to play a role as (components of) pheromones in systems of chemical communication among arthropods. Structures are arranged and grouped in the same way as in the previous review, that is, according to structures of carbon skeletons and (presumed) biosyntheses. Short essays provide a survey of recent developments in the field of chemical ecology and some fringe areas as well as progress in analytical techniques. Subsequently, semiochemicals showing 'unbranched carbon chains' followed by 'terpenes' (formally made up of isoprene units), and 'propanogenins and related compounds' (made up of propanoate units) as well as pheromones produced by 'mixed biosyntheses' are summarized, whereas some structures that do not fit this scheme are summarized as 'other structures'. To complete the survey that we are aiming at and to provide a better overview over the state of the art, each of these sections starts with a discussion of selected structures and corresponding references that have been discussed in our earlier review.[1]

Since the synthesis of pheromones has been extensively reviewed,[2–8] corresponding aspects will not be the subject of this chapter.

During the past 10 years, several monographs and reviews dealing with pheromones have been published. They cover a broad spectrum[9–16] or focus on pheromones and their functions in special orders, or families of arthropods such as Hymenoptera,[17–19] acridids,[20] bark beetles,[21] and crustaceans[22] as well as pheromone-mediated aggregation in nonsocial arthropods in general,[23] or on the importance of chemical signals at different hierarchic levels.[24]

4.04.2 Pheromone Biology

4.04.2.1 Endocrinology

The endocrinological background of the production of sex pheromones has been extensively studied in a number of moth species.[25] Neurosecretory cells located in the subesophageal ganglion release a pheromone biosynthesis-activating neuropeptide (PBAN). After mating, the production of PBAN can be drastically reduced, due to male-produced accessory gland factors that are transferred during copulation.[26] A most frequently found structural element in the PBAN family is the C-terminal pentapeptide sequence FXPR/KL amide, which is the active core required for the stimulation of pheromone biosynthesis in female moths.[27] In the silkworm, as in other moth species, diel changes in the firing activity of PBAN-producing cells suggest that the neurosecretory system is under the control of a circadian pacemaker.[28] A radio-receptor assay has been developed to partially characterize the PBAN receptor in the pheromone gland of *Heliothis* species.[29] The identification of an age- and female-specific putative PBAN membrane-receptor protein in the pheromone glands of the American bollworm *Helicoverpa armigera* and its possible upregulation by juvenile hormone have been described,[30] and similarities between *H. armigera* and the corn earworm *Heliothis zea* have been shown.[31] A full-length cDNA encoding PBAN in the giant silkworm moth *Samia cynthia ricini* based on reverse transcriptase-PCR and rapid amplification of cDNA end strategies was obtained.[32] A gene encoding a G-protein-coupled receptor was identified from the pheromone glands of females of *H. zea*. Subsequently, the full-length PBAN receptor was cloned, expressed in Sf9 insect, and shown to mobilize calcium in response to PBAN.[33] Similar investigations suggested the PBAN receptor in *Bombyx mori* to be both structurally and functionally distinct from that of *H. zea*.[34] To help reveal the regulatory mechanisms of cell-specific expression of PBAN and related hormones, a recombinant AcNPV-mediated gene transfer system and a gel-mobility

shift assay have been used. Results may give rise to speculations that functional conservation of Pitx family members on neuropeptide gene expression occurs through a 'combinatorial code mechanism' in neuroendocrine systems of both vertebrates and invertebrates.[35] Recently, the PBAN receptor from the tobacco budworm *Heliothis virescens* has been identified, functionally expressed, and investigated with respect to structure–activity relationships of ligand analogues.[36] Using [2-^{14}C]-acetate, the control of pheromone biosynthesis by PBAN was followed up in *H. virescens*. Results indicate that PBAN controls an enzyme involved in the synthesis of fatty acid precursors, probably acyl-CoA synthase, and another one, perhaps the reduction of acyl-CoA moieties.[37]

The biosynthesis of pheromones has been reviewed several times focusing on different aspects.[38–42] A more general overview over biosyntheses of natural products with special emphasis on semiochemicals has been provided by Morgan.[43]

4.04.2.2 Neurophysiology

Perception of semiochemicals by insects is remarkably selective. Detection of target compounds involves highly specialized, extremely sensitive sensilla that are situated along sensory hairs on the insect's antenna. The sensilla carry odorant receptor neurons (ORNs) whose odorant receptors (ORs) are isolated by an aqueous sensillar fluid (lymph). Odorant- and pheromone-binding proteins (OBPs and PBPs) serve as mediators between the external environment and ORs carrying semiochemicals through the antennal lymph to the receptors. Earlier understanding on the insect olfactory system, its sensory function, morphology, and development as well as the pheromone-specific/host-related detection and processing of odor information, has been carefully discussed.[44] Recent reviews deal with odor detection and central processing of natural odor mixtures in insects.[45–48] Several excellent reviews compiling current knowledge on OBPs, PBPs, and chemosensory-specific proteins (CSPs) have been published.[49–53] Specialized ORNs or OBPs mediating intra- and interspecific chemical communication have been found in many insect species: ants,[54] honeybees,[55] termites,[56] beetles[57] (according to database search, the termite OBPs are homologues of the PBPs from scarab beetles and antennal binding proteins from moths), fruit flies,[58] and mosquitoes.[59–61] A crystal structure of a PBP could be obtained from cockroaches.[62]

Moths represent by far the most extensively investigated insects. Studied species include the sphinx moth *Manduca sexta*,[63] the gypsy moth *Lymantria dispar*,[64] the wild silk moth *Antheraea polyphemus*,[65,66] and above all, the silk moth *B. mori*. In *B. mori* species, which served as a model in many investigations, female-biased ORNs, which may detect odors that encode oviposition cues or male-produced courtship pheromones,[67] could be identified as well as male-specific PBPs, which may account for sex recognition.[68] Two male-specific ORs have been described, one being specifically tuned to bombykol, the sex pheromone, and the other to bombykal[69] whereas *in vitro* competitive binding assays showed that both bombykol and bombykal bind to the *B. mori* PBP with similar affinity.[70] Similar to the *B. mori* PBP,[68] the *A. polyphemus* PBP consists of six α-helices, which are arranged in a globular fold that encapsulates a central helix formed by the C-terminus. The three-dimensional arrangement of these helices is anchored by three disulfide bonds.[66] This suggests that the two PBPs use the same mechanism of ligand binding and ejection: the polypeptide fold caused by the disulfide bridges encloses a large hydrophobic cavity that accommodates the natural ligand, the pheromone. Disulfide connectivities were also found in the two PBPs of the gypsy moth.[64] Generally, PBPs can undergo striking pH-dependent conformational changes that play a decisive role in the transport and release of the pheromone to the membrane-standing pheromone receptor.[71] Assignments for the *B. mori* PBP fragment BmPBP(1–128) at pH 6.5 as well as the *A. polyphemus* PBP1 at pH 4.5 have been reported.[72,73]

In *B. mori* an aldehyde oxidase has been identified that may be involved in the degradation of bombykal.[74] Esterases have been described from *A. polyphemus*,[75] the cabbage moth *Mamestra brassicae*,[76] as well as from the Egyptian armyworm *Spodoptera littoralis*, and the Mediterranean corn borer *Sesamia nonagrioides*.[77] All these moth species use acetates of long-chain unsaturated alcohols as pheromones. In the Japanese beetle *Popillia japonica*, antennae-specific esterases distinguish between the enantiomers of its pheromone. Inactivation of the (*R*)-enantiomer is preferred over its antagonistically active enantiomer, which suggests that kinetics of pheromone degradation may play a significant role in chiral discrimination.[52]

4.04.2.3 Pest Management

As an alternative or supplement to the exclusive application of broad-spectrum toxicants, interference with insect semiochemicals may be favorably used in integrated pest management (IPM). At a physiological level, targets may be at the early stages in the biosynthesis of semiochemicals such as switching off pheromone production by the inhibition of PBAN-mediated physiological functions[78] or by blocking pheromone perception.[79] At present, apart from biological control measurements, IPM frequently relies on the use of pheromones for the manipulation of insect populations. In this context, the fact that pheromone communication is highly species-specific is particularly advantageous. Basically, there are three ways:

- Trapping: Pheromones are released to trap target insects or to attract them into devices where pathogens (trap and affect) or insecticides (trap and kill) can be deployed.
- Monitoring: Trap catches are used to detect or monitor populations, to check for an optimal time to apply pesticides or to control the efficacy of earlier treatments.
- Disruption: High dosages of pheromones in a selected area may reduce the population of the target species because of mating disruption, probably due to disorientation.

Much has been learnt in this field during the past decade. It starts with the development of dispensers and other release systems. Earlier controlled-release devices for pheromones have been summarized in several reviews.[80] More recent approaches in the formulation techniques use zeolites,[81] microparticle dispensers,[82] sol–gel formulations,[83] or microencapsulation[84] of pheromones. Trap devices and dispensers have to be carefully optimized according to the target insect species. Spreading of the Swede midge *Contarinia nasturtii*, a pest of cruciferous plants that has been recently introduced to the United States, could be followed by monitoring with pheromone traps.[85] Population dynamics in stored-product pests in food processing plants has been successfully monitored.[86] Similarly, flight periods and population densities of the pine sawfly *Diprion jingyuanensis* could be monitored.[87] In the case of sawflies – as in other cases – large-scale applications are hampered by problems concerning bulk synthesis. This is in contrast to the pheromones of several weevil species, which, consequently, have been very successfully used in mass trapping.[88–91] Refined approaches include host compounds to manipulate the behavior of phytophagous insects as they often synergize or otherwise enhance insect responses to sex pheromones.[92] On the contrary, nonhost compounds may be used as repellents and applied in push–pull systems, where certain stimuli act to make the protected resource unattractive or unsuitable to the pests whereas others lure them to an attractive source.[93] An interesting variant is opened by the fact that semiochemicals may exhibit interspecific activities – it may be between closely related species (e.g., disruption of response to the natural pheromone because of application of the 'wrong' enantiomeric composition of a chiral semiochemical, which, in turn, is used by a competing species) or between very different taxa.[94] Disruption is most widespread in the control of lepidopteran pests for stored products[95] and for pests in cotton fields, orchards, vineyards, and so on. As important tools, analytical instrumentation has been developed to measure pheromone concentrations in the field after treatment for mating disruption,[96] whereas spatial and temporal structures of pheromone plumes could be analytically followed up in fields and forests.[97] Cheap large-scale syntheses of the compounds that will be applied and cheap devices for large-scale deployment are prerequisite.[98] During recent years, the oriental fruit moth *Grapholita molesta*, and the codling moth *Cydia pomonella* have been successfully targeted.[99,100] An exemplary review on codling moth management has been recently published by Witzgall.[101]

4.04.3 Isolation and Structure Elucidation

Reliable bioassays are indispensible for a successful isolation and identification of semiochemicals, especially pheromones. Recent improvements range from the construction of new olfactometers[102] and the development of a delivery system for laboratory bioassays[103] to measurements of odor plume structures in the wind tunnel[104] and to a new method to improve olfactory responses to gas

chromatography (GC) effluents.[105] Coupled GC/electroantennography (EAD) has become a standard method in many laboratories. It detects those volatile compounds eluting from the GC that are perceived by the antenna of the target insect – without saying anything about their biological significance (i.e., the activity of the compound as a chemical signal). The method works well when a preparation of the biological detector is feasible – and when the target compounds survive GC. Although an insect antenna is most frequently used, it is also possible to prepare a whole head or even an intact insect.[106]

Rapid progress has been made in the collection and isolation of insect pheromones and other volatiles.[107] After the pioneering work of Pawliszyn[108] and first steps in insect pheromone analyses,[109,110] the application of solid-phase microextraction (SPME) is now widespread. Advantages are simple handling and the fact that the method is noninvasive, that is, it does not harm the target organism, which, therefore, can be analyzed several times, for example, when the production of certain compounds (pheromones) is followed up over a certain period of time with the same individual. The tremendous sensitivity of modern analytical instruments renders this solvent-free technique superior to many conventional headspace techniques and over solvent extraction. Based on the type of fiber used, highly volatile compounds may be trapped from headspace[111] or high-boiling cuticular lipids may be obtained after just touching the surface of an insect.[112] SPME has been successfully applied to monitor allelochemicals in plants.[113,114] Loaded fibers can be easily transported from the field to the laboratory or mailed from one laboratory to the other. Because of the selectivity of some commercially available fibers toward certain chemical classes of compounds, quantitative analyses using SPME can be problematic. Some improvements have been described.[115] A further development is represented by membrane extraction with sorbent interface (MESI), which can be used in the field to monitor very small amounts of volatiles.[116] Condensation of volatiles and subsequent thermal desorption GC[117] or trapping of volatiles with silicone rubber tubes[118] are modern alternatives, whereas solid sample injection can also be highly successful.[119] A limitation of SPME and other solvent-free techniques is caused by the fact that it is (almost) impossible to carry out derivatization of the target compounds. However, even simple derivatization of double bonds (hydrogenation, addition of dimethyl disulfide), carbonyl groups (reduction, hydrazone formation), or hydroxyl functions (esterification, silylation) providing the most important information about the chemical nature of unknown compounds can be most helpful in structure elucidation.

Isolation and structure determination of minute amounts of volatile organic compounds, including pheromones, have been reviewed.[120,121] Systematic compilations of retention indices of selected target compounds[122–124] can be particularly helpful during GC investigations. General numerical methods for the estimation of retention indices of (methyl)alkanes and their derivatives have been presented by Schulz[125] and Junkes *et al.*[126] Derivatization techniques used in structure elucidation or quantitation of volatiles have been summarized.[127] Procedures range from single standard methylation or silylation of polar compounds (which facilitates GC) to the transformation of naturally occurring methyl ethers and alcohols to nitriles[128] (which facilitates mass spectrometric (MS) investigations). Transformation of secondary alcohols into the corresponding trifluoroacetates provides a reliable method for quantitative GC analyses.[129] As the receptors of chemical messages at the receiver's side are made up of proteins, it is quite understandable that the enantiomeric composition of chiral semiochemicals plays a decisive role. Several comprehensive reviews, especially those written by Mori, emphasize the importance of chirality in pheromone science.[130–133] This is underlined by the fact that the two enantiomers of linalool induce activity in different parts of the brain of the hawk moth *M. sexta*[134] and that the esterase that accounts for the degradation of the enantiomers of japonilure, the pheromone of the Japanese beetle *P. japonica* acts enantioselectively.[52] Techniques for the separation of enantiomers and the determination of the absolute configuration of optically active compounds have been carefully compiled.

Investigation on the enantiomeric composition of chiral secondary alcohols will, however, require either derivatization with an optically active reagent and separation on a conventional column or enantioselective GC using an optically active stationary phase. Today, the latter approach most frequently involves modified cyclodextrins.[135] Enantioselective HPLC has also been successfully applied to separate enantiomers.[136,137] Several reagents have been used in the transformation of chiral alcohols into diastereomers. Among these, acetyllactic acid[138] or chlorofluoroacetic acid[139] furnish volatile derivatives of pheromone

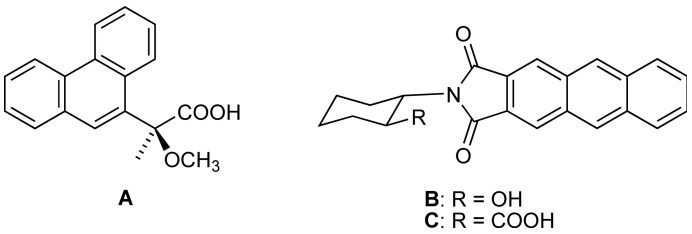


Figure 1 Optically active reagents for the derivatization and separation of chiral compounds.

alcohols. Resolution of 1,3-diols could be achieved after reaction with chiral bidentate silyl reagents.[140] Chiral resolving agents yielding nonvolatile derivatives that can be separated upon conventional HPLC at low temperature have also been developed. The aromatic compounds (*S*)-2-methoxy-2-(9-phenanthryl) propionic acid **A**[141] as well as the enantiomers of 2-(2,3-anthracenedicarboximido)cyclohexanol **B**[142] and 2-(2,3-anthracenedicarboximido)cyclohexanecarboxylic acid **C** proved to be highly efficient.[143] (see **Figure 1**). The use of compounds **B** and **C** as chiral reagents has been particularly suitable for the separation of chiral compounds with a stereogenic center remote from the functional group.[144] Because of their fluorescent activity, detection of the derivatives is possible with extremely small amounts. Compound **B** has been used to determine the stereochemical composition of the copulation-releasing pheromones of *Callosobruchus* weevils.[145,146]

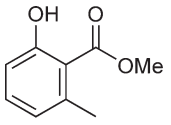
During recent years, NMR techniques have been tremendously improved, and modern high-field instruments, equipped with microcoil probes are extremely sensitive.[147] A few insects (or glands), sometimes only a single individual, may be enough for structure elucidation of new compounds even in crude mixtures obtained from natural sources.[148–150] New chiral silylation reagents have been developed to determine the absolute configuration of chiral compounds through NMR spectroscopy.[151–153] Online assignments of the absolute configuration of natural products through HPLC–circular dichroism (CD) has been achieved with very small amounts of material,[154] and vibrational CD spectroscopy (VCD) has been successfully applied in pheromone chemistry.[155,156]

Despite the enormous progress in the abovementioned techniques, the sensitivity of MS remains unequalled. Apart from more conventional derivatization procedures,[127] determination of double bond positions in complex mixtures of alkenes has been achieved upon chemical ionization ion-trap MS using acetonitrile as a reagent gas.[157] Two-dimensional GC in combination with a time-of-flight (TOF)-MS detector greatly facilitates the analyses of complex mixtures and may become a powerful tool in the analysis of semiochemicals.[158] Matrix-assisted laser desorption/ionization (MALDI)-TOF-MS has been successfully used to analyze cuticular lipids of insects and revealed the presence of high-boiling compounds with more than 70 carbon atoms.[159] This makes an area of natural products accessible that had previously been almost ignored or overlooked. Atmospheric pressure chemical ionization (APCI)–MS allows real-time monitoring of volatiles released by single insects. Rapid changes in the emitted signal can be followed with a minimal detection delay and high sensitivity.[160,161] A similar approach is feasible through techniques summarized as direct analysis in real time (DART)–MS.[162] Unfortunately, compared to NMR spectrometry, information about structural features of a given target compound, provided by the obtained data, is less definite. As a consequence, thorough studies concerning the mass spectrometric fragmentation pattern of pheromones and analogue compounds may largely facilitate structure elucidation of hitherto unknown compounds.[163–166]

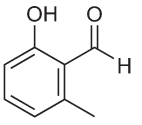
Remark: At the beginning of each of the following sections, a selected compilation of chemical compounds is presented that have been treated in our earlier review.[1] In this context, chemical structures are shown, and the Latin names of the species and, where possible, common names are given. If only genus, family, or order is provided, the occurrence of the questionable compound is not restricted to one species. Corresponding references are cited for further information.

4.04.4 Aromatic Compounds

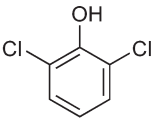
In our earlier review,[1] the compounds presented in the following figures were discussed in more detail.



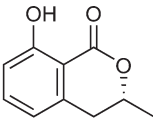
Ar1: ants;[167]



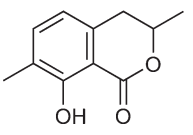
Ar2: mites;[168]



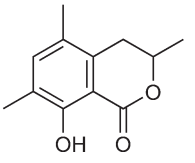
Ar3: ticks;[169]



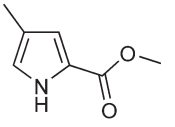
Ar4: *Lasius fuliginosus* ant;[170]



Ar5: *Camponotus* ants;[171]



Ar6: *Camponotus* ants;[171]



Ar7: leaf-cutting ants[172]

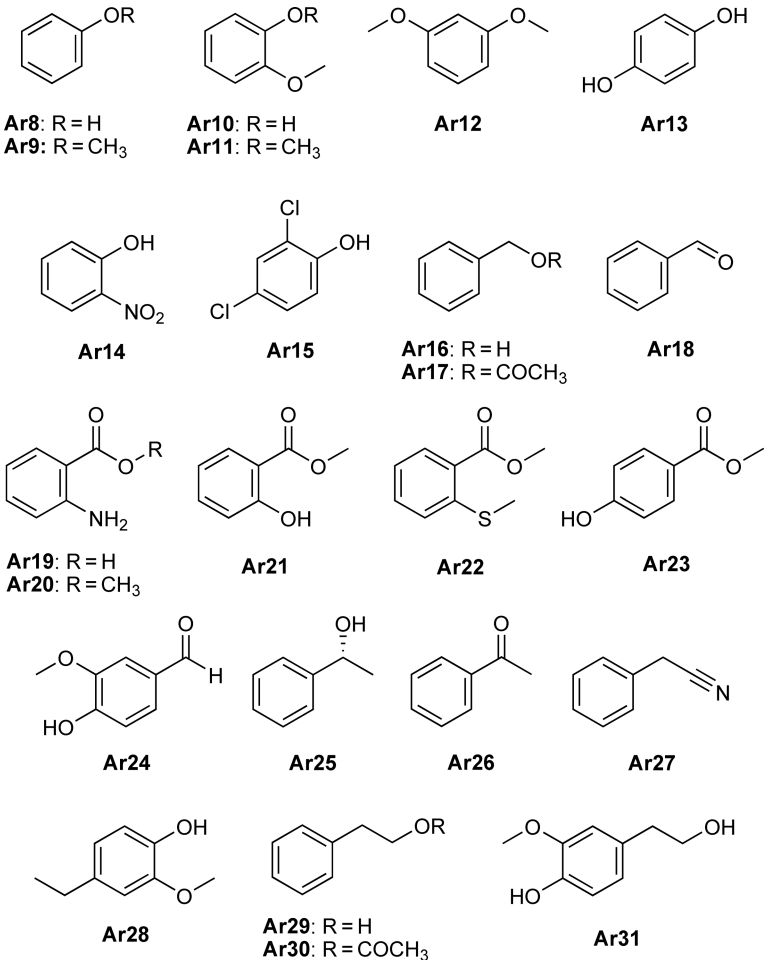
Phenol (**Ar8**) is the pheromone of the grass grub beetle *Costelytra zealandica*,[173] while anisol (**Ar9**) is the pheromone of two scarab beetles *Holotrichia reynaudi*[174] and *Holotrichia consanguinea*.[175] In the desert locust *Schistocerca gregaria*, the aggregation pheromone of mature males consists of **Ar8**, benzyl cyanide (**Ar27**), guaiacol (**Ar10**), and **Ar18**.[176] Compounds **Ar8** and **Ar10** are synergists to aldehydes and acids acting as aggregation pheromones of the fifth instar larvae of the species.[177] Acetophenone (**Ar26**) and veratrole (**Ar11**) have been identified as the two major behaviorally active components of the oviposition aggregation pheromone of *S. gregaria*. Both compounds elicited aggregation of gravid females but did not act synergistically.[178] The function of **Ar27** in *S. gregaria* is controversial. Recently, it has been found to be a courtship inhibition pheromone of mature locusts.[179,180] The same function is found for this compound in the butterfly *Pieris brassicae*.[181] 1,3-Dimethoxybenzene (**Ar12**) is an alarm pheromone component of the springtail *Neanura muscorum*.[182]

Hydroquinone (**Ar13**) is a food-marking pheromone used by *Mastotermes darwiniensis* termites. Interestingly, it is active in many termite species from all over the world, obviously used as a general signal.[183] *o*-Nitrophenol (**Ar14**) is part of the aggregation-attachment pheromone of *Amblyomma* ticks.[184,185] 2,6-Dichlorophenol (**Ar3**) is a female sex pheromone of the ticks *Anocentor nitens*[186] and *Amblyomma cajennense*,[187] as well as of other ticks.[188,189] The American dog tick *Dermacentor variabilis* also contains this phenol, and 2,4-dichlorophenol (**Ar15**) is another component of the pheromone.[190]

Benzaldehyde (**Ar18**), emitted by males of the bug *Triatoma infestans* during courtship and copula, attracted female conspecifics. Highest response was observed in a mixture with hexanal (**A68**) (ratio 1:20).[191] It also recruits workers for defense in the stingless bee *Trigona angustula*.[192] Together with benzyl alcohol (**Ar16**) it is part of the aggregation pheromone of the common bedbug *Cimex lectularius*.[193] Benzyl acetate (**Ar17**) occurs in the stinging alarm pheromone of the honeybee *Apis mellifera* and induces flying.[194] Anthranilic acid (**Ar19**) is the pheromone of the black chafer *Holotrichia loochooana*,[195] while a 100:1 mixture of methyl anthranilate (**Ar20**) and **Ar41** comprises the trail pheromone of an *Aenictus* sp.[196] Methyl salicylate (**Ar21**) was identified as an antiaphrodisiac of male *Pieris napi*[197] and *Pieris rapae* butterflies.[181] The binary blend of **Ar21** and iridodial (**T128**), an iridoid that may play a role in the context of chemical communication among aphids, seems to attract the aphid predator *Metasyrphus americanus*, a hoverfly.[198] Methyl 2-(methylthio)benzoate (**Ar22**) is a rare sulfur-containing sex pheromone of the scarab beetle *Phyllophaga crinita*.[199] Methyl 4-hydroxybenzoate (**Ar23**) and 2-(4-hydroxy-3-methoxyphenyl)ethanol (**Ar31**) are constituents of the retinue response pheromone of the honeybee *A. mellifera*.[200] Vanillin (**Ar24**) is a pheromone component of the oil palm bunch moth *Tirathaba mundella*.[201]

(*R*)-1-Phenylethanol (**Ar25**) is the trail pheromone of *Aphaenogaster cockerelli*.[202] 2-Ethylguaiacol (**Ar28**) is a male sex pheromone component of the cockroach *Nauphoeta cinerea*.[203] 2-Phenylethanol (**Ar29**) is a component of the aggregation pheromone of male *Megacyllene caryae*.[204] The acetate (**Ar30**) was identified as part of a male-produced attracting pheromone for both sexes in the bug *Neacoryphus bicrucis*.[205] 2-Hydroxy-6-

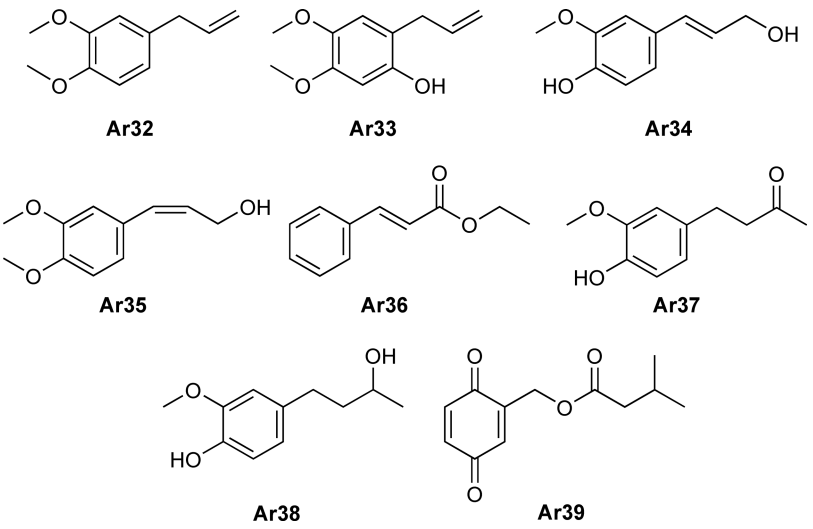
methylbenzaldehyde (**Ar2**) was proposed as the female sex pheromone of the house dust mite *Dermatophagoides farinae*[206] and has been identified as an alarm or sex pheromone in several other mite species.[207–212] Methyl 6-methylsalicylate (**Ar1**) (the methyl ester of the acid corresponding to **Ar2**), a component of the poison gland secretion, elicited trail following in the ants *Mayriella overbecki*[213] and *Tetramorium* cf. *impurum*.[214] In the slave-making ant *Polyergus rufescens*, it is a component of the male-attracting pheromone of the queen,[215] while it serves the same function in *Polyergus breviceps*, acting only in combination with 3-ethyl-4-methylpentan-1-ol.[216]



Males of the oriental fruit fly *Bactrocera dorsalis* (*papayae*) take up methyl eugenol (**Ar32**) pharmacophagously and use it together with its hydroxylated analogue, 2-allyl-4,5-dimethoxyphenol (**Ar33**) as sex and aggregation pheromones.[217] Other pheromonally active components are (*E*)-3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol (**Ar34**) (coniferyl alcohol),[218] which is also a synergistic component of the honeybee retinue response signal of workers,[219] and (*Z*)-3-(3,4-dimethoxyphenyl)-2-propen-1-ol (**Ar35**).[220] Males of the oriental fruit moth *G. molesta* use ethyl cinnamate (**Ar36**) as courtship pheromone, together with **Ar4**, **A45**, or **A46**.[221] Zingerone (**Ar37**) and zingerol (**Ar38**) are attractant pheromone components of males of the melon fly *Bactrocera cucurbitae*, and potentially other *Bactrocera* species.[222,223] The sex pheromone of the German cockroach *Blattella germanica* has been characterized as gentisyl quinone isovalerate (blatellaquinone, **Ar39**). Because of its surprising instability, this unique compound was particularly difficult to isolate and to characterize.[224]

3,4-Dihydro-8-hydroxy-3-methylisocoumarin (**Ar4**) (mellein) is the trail pheromone of the ants *Formica rufa*[225] and *Lasius (Dendrolasius) fuliginosus*.[226] It is also the male pheromone of the moth *Aphomia sociella*[227] and a

male pheromone component of the moth *G. molesta*, enhancing the activity of **Ar36**.[221] Several ants of the genus *Camponotus* use 3,4-dihydro-8-hydroxy-3,7-dimethylisocoumarin (**Ar5**)[228] or 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (**Ar6**).[228,229] The (*R*)-enantiomer is most active in the ant *Lasius niger*.[230]



4.04.4.1 Nitrogen-Containing Aromatic Compounds

Methyl 4-methylpyrrole-2-carboxylate (**Ar7**) was the first trail pheromone identified in ants. It is used by *Atta texana*,[231] *Atta cephalotes*,[232] and *Acromyrmex octospinosus*.[233] Two *Metapone* ant species use the unsubstituted derivative **Ar40** as the trail pheromone.[234] Methyl nicotinate (**Ar41**) is the minor component of the trail pheromone of an *Aenictus* sp.[196]

Various pyrazines act as trail pheromones of ants. 3-Ethyl-2,5-dimethylpyrazine (**Ar47**) has the same function in *Atta sexdens*,[235,236] *Manica rubida*,[237] *Myrmica incompleta*,[238] and *Messor bouvieri*,[239] as well as in several *Myrmica* spp.[236] It is also the recruitment pheromone of *Pogonomyrmex* harvester ants.[240] A 7:3 mixture of **Ar47** and 2,5-dimethylpyrazine (**Ar45**) comprises the trail pheromone of *Tetramorium caespitum*.[241] 2,3-Dimethyl-5-(2-methylpropyl)pyrazine (**Ar48**) is the trail pheromone of the ant *Eutetramorium mocquerysi*.[242] 2-Isopropyl-3-methoxypyrazine (**Ar42**), a widespread warning odor of insects, acts as a pheromone in the seven-spotted ladybird beetle *Coccinella septempunctata*.[243]

Indole (**Ar53**) is part of the antiaphrodisiac pheromone of male *P. rapae* butterflies.[181] Together with methylpyrazine (**Ar44**), **Ar45**, trimethylpyrazine (**Ar46**), and **Ar47**, it represents the trail pheromone of the ant *Tetramorium meridionale*.[244]

The mixture of *N*-isopentyl-2-phenylethylamine (**Ar49**), anabasine (neonicotine, **Ar58**), anabaseine (nor-nicotine, **Ar59**), and 2,3′bipyridyl (**Ar60**) constitutes the trail pheromone of the ant *Aphaenogaster rudis*.[245]

Danaidone (**Ar50**), the male courtship pheromone of the queen butterfly *Danaus gilippus*, was one of the first pheromones identified.[246,247] Despite its presence in scent glands of many danaine butterflies, the pheromonal function of **Ar50** was shown in only one additional species *Idea leuconoe*.[248] A more widespread dihydropyrrolizine pheromone is hydroxydanaidal (**Ar51**). It is used by many male danaine, ithomiine, and arctiine butterflies, preferentially as part of a courtship pheromone.[249–251] The absolute configuration of **Ar51** was determined in a few species only, but proved to be (*R*) in all cases.[252–254] Sometimes, the related compound danaidal (**Ar52**), lacking the hydroxy group, is used by arctiine moths too.[253] The butterflies sequester precursors of the dihydropyrrolizines **Ar50–Ar52** from plants and convert them into the pheromones.[254] It is interesting to note that butterflies producing such compounds may differentiate between the precursor alkaloids: *Parantica sita* is able to process the alkaloids intermedine or lycopsamine (monoesters that carry a (7*R*)-OH group) in contrast to heliotrine (monoester with (7*S*)-OH group) and retrosine or monocrotaline

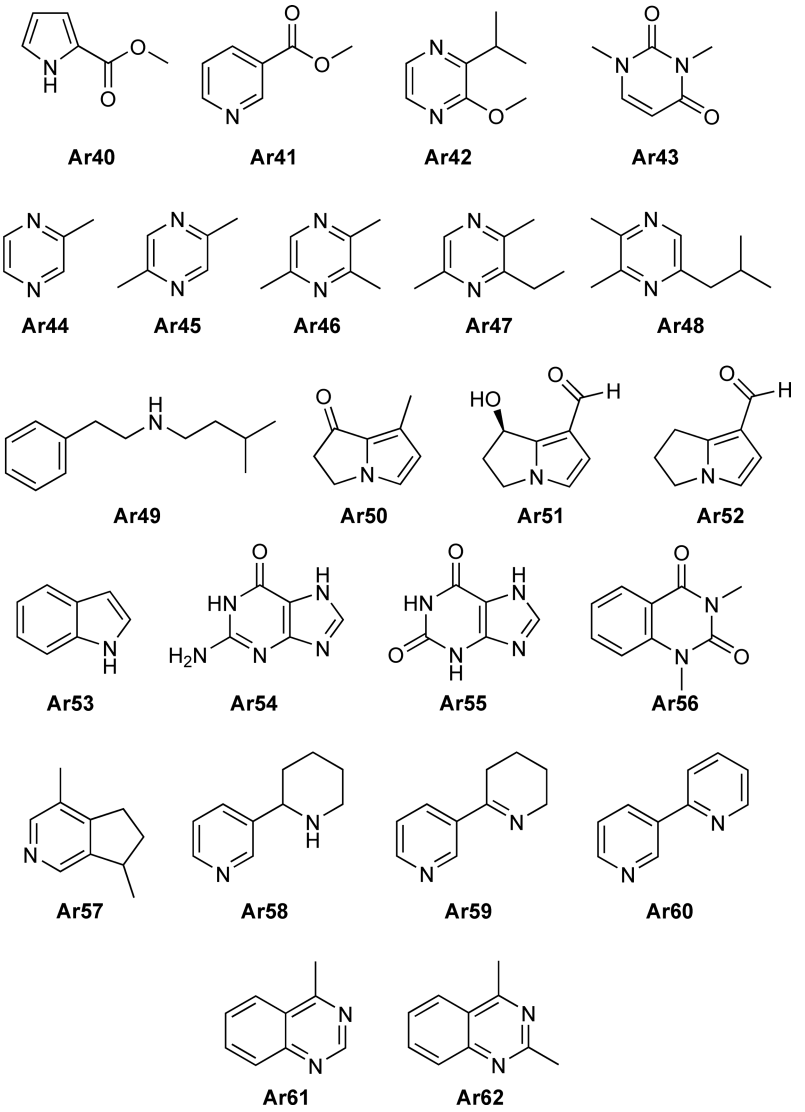
(diesters that carry a (7*R*)-OH group).[255] Some other species do not distinguish. It is remarkable that caterpillars of the arctiid moth *Utetheisa ornatrix*, monophagous on *Crotolaria* species, can taste the alkaloids contained in its host plant.[256] Whether consumed pyrrolizidine alkaloids are degraded upon saponification and further processing or via a transesterification step[257,258] may need further investigations.

N,*N*-Dimethyluracil **Ar43** is the trail pheromone of the ant *Pachycondyla analis*, whereas actinidine (**Ar57**) serves as foraging stimulation signal.[259]

A unique pheromone structure is represented by the alkaloid 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione (**Ar56**), which is the sex pheromone of the pale-brown chafer *Phyllopertha diversa*.[260] Its degradation with special enzymes on the antennae leading to signal inactivation has been described.[261]

4-Methylquinazoline (**Ar61**) is a minor component of the male sex pheromone of the parasitoid *Nasonia vitripennis*.[262] It is also found in the feces of *T. infestans*, together with 2,4-dimethylquinazoline (**Ar62**), attracting different stages of this hematophagous bug.[263]

Guanine (**Ar54**) and xanthine (**Ar55**) are components of the arrestment pheromone of the tick *Ixodes scapularis*.[264]



4.04.5 Unbranched Aliphatic Compounds

4.04.5.1 Mixtures of Hydrocarbons Acting as Pheromones

Chemical communication in social insects is often more complex as compared to solitary species, using different signals in a varying context. Signals in ants, bees, wasps, or termites may not only be represented by single compounds or mixtures of a few components, but also comprise complex multicomponent bouquets. The obvious place to present such bouquets is the epicuticle, allowing easy detection upon direct contact or short-range olfaction.[265] The insect cuticle is commonly covered by hydrocarbons, consisting most often of *n*-alkanes, methyl-branched alkanes, and *n*-alkenes with various numbers of double bonds.[266,267] Qualitatively and quantitatively defined patterns of hydrocarbons make up specific chemical signals.[268,269] Unusual compounds occur, as for example, allenic structures like (*R*)-9,10-tricosadiene and similar components on the cuticle in Australian scarab beetles,[270] or oligoprenylsesquiterpenes on the cuticle of the collembola *Podura aquatica*.[271] In addition, oxygenated compounds derived from hydrocarbons have been frequently identified on insect surfaces,[272–274] but their attribution to the signature of complex bouquets used in chemical communication remains largely unclear.

Social interactions of insects are particularly well investigated in ants. Several aspects have been found to be mediated through hydrocarbon profiles unique to certain colonies, castes within a colony, tasks of ants, or sex. A sensillum that discriminates between nestmate and nonnestmate cuticular hydrocarbon patterns has been described in the carpenter ant *Camponotus japonicus*.[275] Some examples dealing with the importance of hydrocarbons in social insects are given below. Harvester ants, *Pogonomyrmex barbatus*, use hydrocarbon profiles to discriminate between nestmates and aliens,[276] as does *Cataglyphis niger*.[277] In the ant *Formica japonica*, nestmates are detected by variations in *n*-alkanes and (*Z*)-9-alkenes. Artificial alteration of the colony bouquet by changing relative proportions of selected components induces increased aggression in the ants.[278] In contrast, only the (*Z*)-9-alkenes were found to be responsible for nestmate recognition in *Formica exsecta*.[279] In other ants, various classes of hydrocarbons are involved,[269] as seems to be also the case for honeybees.[280] When added to the extracts of host hydrocarbons, (*Z*)-9-tricosene (**A190**) induces aggression in the ant *Camponotus floridanus*.[265] Furthermore, task decisions can be mediated by hydrocarbon profiles in the ant *P. barbatus*.[281] In the termite *Macrotermes subhyalinus*, complex regression analysis showed that primarily alkenes, present only in minor amounts, seem to be crucial for colony detection.[268]

Aggression between members of different colonies was also observed in the eusocial wasp *Ropalidia opifex*. They use different hydrocarbon patterns for recognition, consisting of straight-chain and methyl-branched alkanes.[282] In the European hornet *Vespa crabro*, alteration of the hydrocarbon profile by the application of additional components changes the response from tolerance to aggression.[283] Reproductive and nonreproductive individuals of the ant *Myrmecia gulosa* are differentiated by conspecifics using hydrocarbon profiles.[284] Young females of the alfalfa leaf-cutter bee *Megachile rotundata* attract males by cuticular alkenes, while fatty acids or alkanes that are also present on the cuticle proved to be inactive.[285]

In certain cases, compositions of hydrocarbon patterns are changed during interactions. The paper wasp *Polistes atrimandibularis*, a social parasite of *Polistes biglumis bimaculatus*, controls the host nest by sequentially changing the composition of its cuticular hydrocarbons during the colonial cycle. The parasite can switch on and off alkene biosynthesis, thus becoming undetected by the host.[286] Intercolonial aggression in the stingless bee *Scaptotrigona bipunctata* was assigned to statistically significant quantitative differences in the composition of hydrocarbon patterns isolated from the wings of guard bee workers. Unfortunately, no bioassays were carried out to test the biological activity of the investigated extracts, and, similar to many other investigations, no experiments using synthetic compounds were performed.[287] Hydrocarbon profiles also play a role in nonsocial species. Males of two hybridizing *Chrysochus* leaf beetles are influenced in their mate choice and sexual isolation by hydrocarbon profiles of females.[288] Several other pheromonal functions of cuticular hydrocarbon mixtures have been described, for example, trail following by wasps.[289]

More detailed aspects of hydrocarbon bouquets acting as pheromones and various problematic aspects in revealing their true biological function have been discussed.[266,290] In many cases, however, only extracts of the insect cuticle are used for bioassays, sometimes roughly separated by chromatography into different chemical classes. Tests with synthetic blends have been less frequently performed; only such approaches would allow unequivocal assignment of activity to the compounds tested. Actually, in whole extracts or fractions thereof

only a single or a few compounds might be active, and often it is still unclear whether certain compounds included in a mixture or the whole bouquet are active and whether relative proportions really matter. Nevertheless, the study on the hydrocarbon receptor in *C. japonicus* showed that patterns can indeed be perceived and that the receptor is well suited to detect mixtures.[275] Finally, many studies show qualitative differences in hydrocarbon patterns depending on status, sex, age, physiological state, social group, or colony, but experimental evidence for corresponding biological activities using defined (synthetic) mixtures in biotests is often lacking. In the ant *F. exsecta*, it could be shown that profiles of cuticular hydrocarbons may not only be colony-specific but also depend on the task of the individual (e.g., foraging vs. nonforaging) within the same colony.[291] Consequently, (sufficient numbers of collections of) homogenous samples and a careful evaluation of analytical results as well as scrutinized application of statistical methods such as discriminant analyses or principal component analyses are indispensible.[292]

Recently, very long hydrocarbons consisting of more than 70 carbon atoms have been described as constituents of the insect cuticle. These compounds cannot be analyzed by conventional GC/MS methods; instead MALDI-TOF-MS is used.[160] Whether these compounds are involved in pattern recognition of hydrocarbons is unknown. Nevertheless, their sheer size may inhibit them from entry into the recognition size of proteins. Small molecules like the secondary metabolites discussed in this chapter are detected usually at the recognition site inside the protein, because otherwise their affinity to the protein would not be sufficient for complexation.

4.04.5.2 Female Lepidopteran Sex Pheromones

Sex pheromones of female moth constitute the best-investigated group of insect pheromones, primarily because of their economic importance. Chemically they are very uniform, and their occurrence and biosynthesis have been discussed in detail in our previous review.[1] Recently, an excellent review has been published by Ando.[40] A complete searchable database on the pheromones of about 1800 species is found in the Pherobase database, which is freely available via the Internet.[293]

About 75% of the known pheromones are represented by even-numbered straight-chain alcohols, aldehydes, or acetates. The chain length varying between C_{10} and C_{18}, and one to three double bonds in specific geometries are found along the chain. Apart from some few exceptions, these pheromones, which have been termed Type I compounds,[40] will not be covered in the following discussion. The reader should instead consult the Pherobase[293] for more information on Type I compounds.

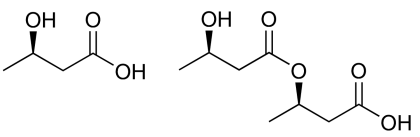
The biosynthesis of Type I components has been recently reviewed.[41] The high structural variability is achieved by combinations of chain shortening steps and desaturation reactions of fatty acid coenzyme A esters. These precursors are then reduced to alcohols, which can be converted into either aldehydes or acetates. Athough Δ11-desaturases are most prominent among Lepidoptera, enzymes that generate double bonds at positions 5, 9, 10, 12, 13, or 14 have been described too.[41] Evolutionary aspects of the desaturases have been discussed,[294,295] as have been some mechanistic aspects. In all cases investigated so far, double bonds are formed upon abstraction of the pro-*R* hydrogens from the methylene groups that are involved.[296–298] The hydrogen closer to the carboxyl-end of the chain is removed first, exhibiting a large isotope effect.[299] Especially interesting is the finding of multifunctional desaturases in the Lepidoptera. Bombykol ((10*E*,12*Z*)-10,12-hexadecadien-1-ol), the first insect pheromone identified, is biosynthesized from palmitate. After transformation into (*Z*)-11-hexadecenoate, the same enzyme induces 1,4-elimination of hydrogen, yielding (10*E*,12*Z*)-hexadecadienoate, the bombykol precursor.[300] A similar enzyme was described from *S. littoralis*.[301] Another bifunctional desaturase is involved in the biosynthesis of (*Z*)-13-hexadec-13-en-11-ynyl acetate, the unique pheromone of the processionary moth *Thaumetopoea pityocampa*. A single desaturase accounts for three desaturation reactions: Palmitate is first transformed into (*Z*)-11-hexadecenoate, followed by additional dehydrogenation into 11-hexadecynoate. The third dehydrogenation step at C13 yields (*Z*)-13-hexadecen-11-ynoate. Activities of other enzymes complete the biosynthesis by reduction to the corresponding alcohol and final acetylation.[302] Very recently, it was shown that in the European corn borer *Ostrinia nubilalis*, male-produced pheromones that account for the acceptance by females are structurally very similar to the female-produced sex pheromone of the species – which may give rise to further speculations on evolutionary aspects of the *Ostrinia* phenomenon.[303]

The second largest group, called Type II compounds, is represented by odd-numbered unbranched hydrocarbons with two to five double bonds, most of which being homoconjugated (skipped conjugated, two

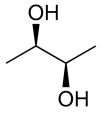
double bonds being separated by one methylene group).[42] The chain length varies between C_{17} and C_{25}. One or two of the double bonds can be stereospecifically epoxidized. In addition, a formal rearrangement of the epoxide to a carbonyl group generates unsaturated ketones, which may also act as sex pheromones. Compounds of this type identified during the past decade are listed in the following sections. Results of investigations concerning their biosynthesis have been discussed.[41] Type II compounds are derivatives of linolenic or linoleic acid.[304] During biosynthesis, carbon C1, bearing the carboxylic acid function, is lost, so that the even-numbered fatty acid is converted into an odd-numbered hydrocarbon.[305] Subsequently, one of the double bonds may be regiospecifically and enantiospecifically attacked by a monooxygenase.[306] In a few cases, an additional α-oxidation step converts an even-numbered fatty acid into an odd-numbered one, and, consequently, even-numbered hydrocarbons, as for example, **A155**, are produced during the subsequent steps.[307]

Finally, a small part of the sex pheromones of female moth do not fit into this scheme. They show additional methyl branches along the chain or functional groups at unusual positions. Examples can be found in the following sections.

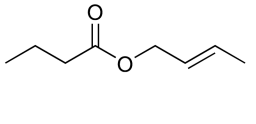
In our earlier review,[1] the compounds presented in the following figures were discussed in more detail.



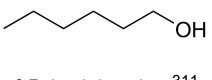
A1, A2: *Linyphia*, spiders;[308]



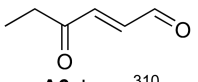
A3: *Eurycotis floridana*, cockroach;[309]



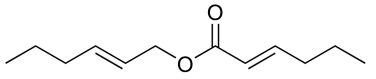
A4: *Campyloma verbasci*, stink bug;[310]



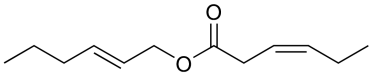
A5: bark beetles,[311] stink bugs;[312]



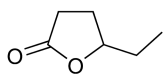
A6: bugs;[310]



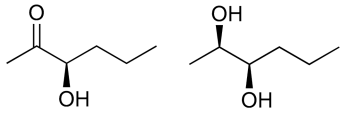
A7: *Rhiptortus*, bugs;[313]



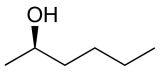
A8: *Rhiptortus*, bugs;[313]



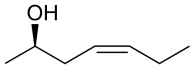
A9: *Trogoderma*, beetles;[314]



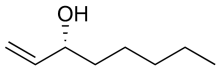
A10, A11: long-horned beetles;[315,316]



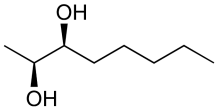
A12: *Rhyacophila*, caddis flies;[317]



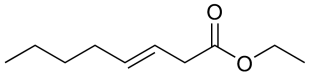
A13: *Eriocrania*, moths;[318]



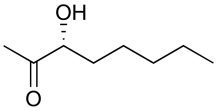
A14: *Oryzaephilus*, cucujiid beetles;[319]



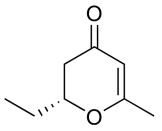
A15, A16: *Xylotrechus*, beetles;[320]



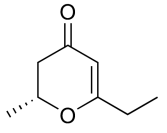
A17: *Ceratitis capitata*, Mediterranean fruit fly;[321]



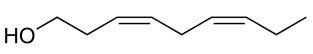
A18: *Anaglyptus subfasciatus*, scarab beetle;[322]



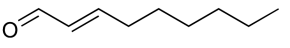
A19: *Hepialus californicus*, moth;[323]



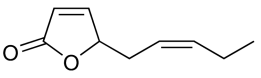
A20: *Hepialus hecta*, hepialid moth;[324]



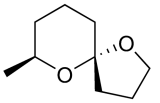
A21: *Anastrepha suspensa*, fruit fly;[325]



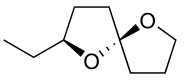
A22: *Anomala*, scarab beetles;[326]



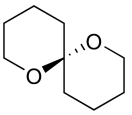
A23: *Aphomia gularis*, moth;[327]



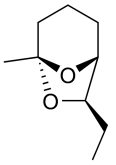
A24: bark beetles;[328,329]



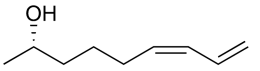
A25: bark beetles;[330]



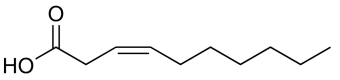
A26: *Bactrocera* (*Dacus*) *oleae*, olive fruit fly;[331]



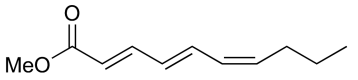
A27: bark beetles;[332]



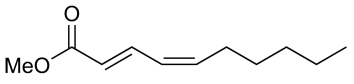
A28: *Stigmella malella*, moth;[333]



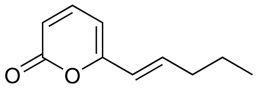
A29: *Anthrenus flavipes*, beetle;[334]



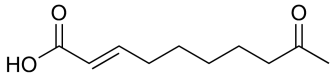
A30: *Plautia stali*, stink bug;[335]



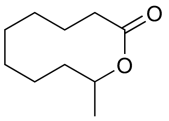
A31: *Pityogenes chalcographus*, bark beetle,[336] *Euschistus*, bugs;[310]



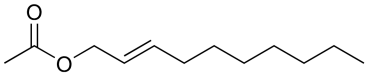
A32: *Solenopsis*, fire ants;[337]



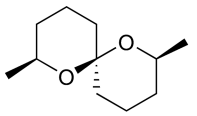
A33: *Apis mellifica*, honey bee;[338,339]



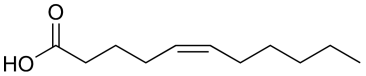
A34: *Phoracantha synonyma*, long-horned beetle;[340]



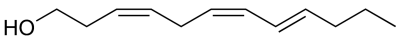
A35: *Apis mellifica*, honey bee;[341]



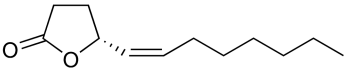
A36: *Andrena*, bees;[342]



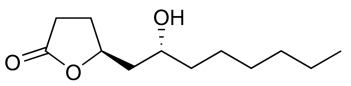
A37: *Anthrenus verbasci*, beetle;[343]



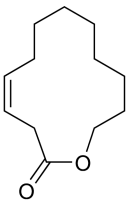
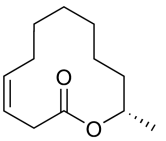
A38: *termites*;[344,345]



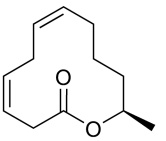
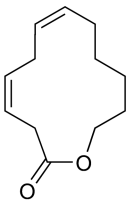
A39: *Anomala*, scarab beetles,[326] *Idea leuconoe*, butterfly;[346]



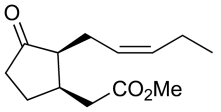
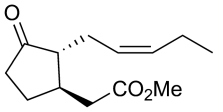
A40: *Cryptolestes*, cucujiid beetles;[347]

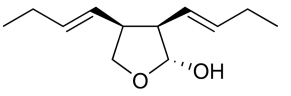
A41, A42: *Cryptolestes*, cucujiid beetles;[347]

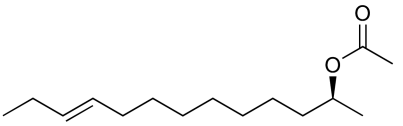
A43, A44: *Orycaephilus*, cucujiid beetles;[348]

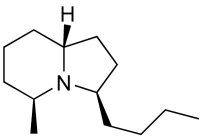
A45, A46: *Grapholita molesta*, moth;[349]



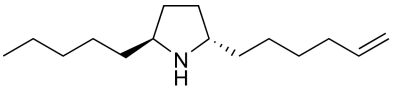
A47: *Biprorulus bibax*, bug;[310,350]



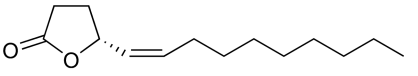
A48: *Mayetiola destructor*, gall midge;[351]



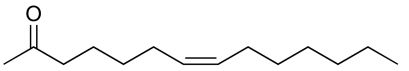
A49: *Monomorium*, ants;[352]



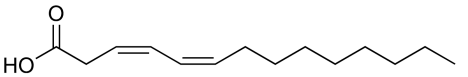
A50: *Monomorium*, ants;[352]



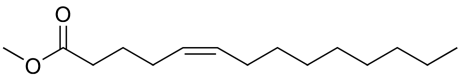
A51: *Popillia japonica*,[353]
Anomala octiescostata, scarab beetle;[354]



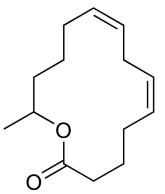
A52: *Anomala orientalis*, scarab beetle;[355]



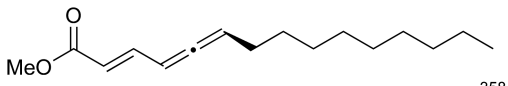
A53: *Attagenus elongatulus*, dermestid beetle;[356]



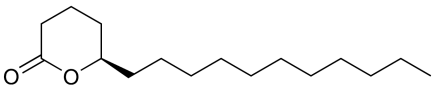
A54: *Anomala rufocuprea*, scarab beetle;[357]



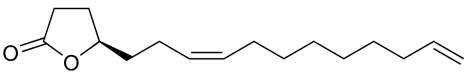
A56: cucujiid beetles;[347]



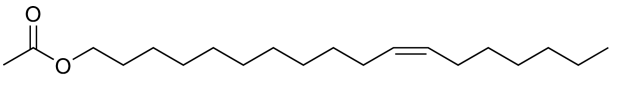
A55: *Acanthoscelides obtectus*, dried bean beetle;[358]



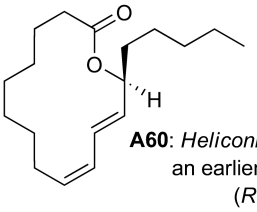
A57: *Vespa orientalis*, oriental hornet;[359]



A58: *Heptophylla picea*, scarab beetle;[360]



A59: *Drosophila*, fruit flies;[361,362]



A60: *Heliconius pachinus*, butterfly;[363] an earlier report had assigned (*R*)-configuration to **A60** (Coriolide).[364]

4.04.5.3 Pheromones According to Carbon Chains

4.04.5.3.1 C_1-units

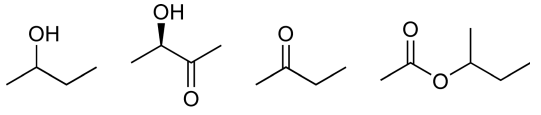
Formic acid seems to be a recruitment signal of several *Camponotus* ants.[229,365] In contrast, it has been described as a signal to repel conspecifics in *Camponotus obscuripes*.[366]

4.04.5.3.2 C_2-units

Acetic acid occurs in Brindley's gland of male and female *Rhodnius prolixus* bugs, a vector of the Chagas disease. Males and females are attracted to this compound in a dose-dependent manner.[367]

4.04.5.3.3 C_4-units

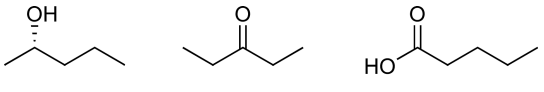
Males of the scarab beetle *Amphimallon solstitialis* are attracted to the pheromone (*R*)-3-hydroxy-2-butanone (**A62**), released by both males and females.[368] Although the (*S*)-enantiomer and 2,3-butanediol (**A4**) are also produced, they were not active in the field. 3-Hydroxy-2-butanone of unknown enantiomeric composition is the major sex pheromone component of male cockroach *Leucophaea maderae*.[203] The rhinoceros beetle *Scapanes australis* uses a 84:12:4 mixture of 2-butanol (**A61**) (*R*/*S* 67:33), 3-hydroxy-2-butanone (**A62**), and 2,3-butanediol (**A4**, (*R,R*/*S,S*/*meso* 43:17:40), whereas the pheromone of another rhinoceros beetle *Strategus aloeus* is a 95.5:4.0:0.5 mixture of 2-butanone (**A63**), 3-pentanone (**A66**), and 1-methylpropyl acetate (**A64**). As mixtures of all components of the natural secretion were used in this study, it is not clear whether all compounds really possess pheromonal activity.[369]



A61 **A62** **A63** **A64**

4.04.5.3.4 C_5-units

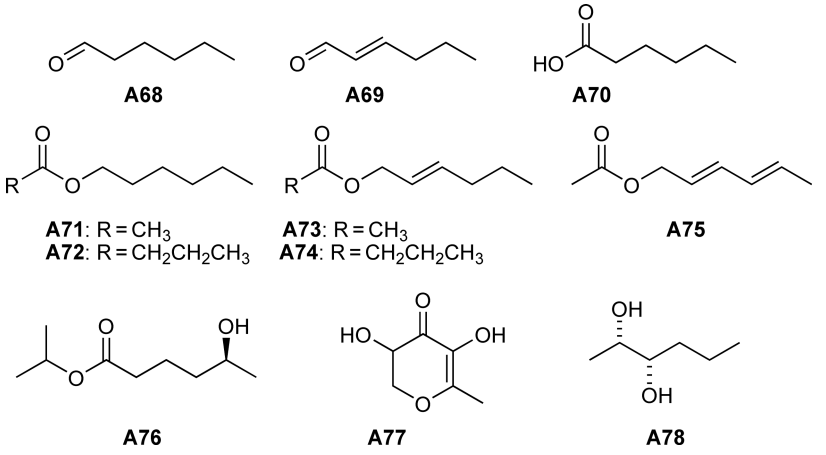
(*S*)-2-Pentanol (**A65**) is the major component of the alarm pheromone of the giant hornet *Vespa mandarinia*. The (*R*)-enantiomer occurs in minor amounts and is equally effective.[370] 3-Pentanone (**A66**) is part of the pheromone of *S. aloeus* (see Section 4.04.5.3.3). Pentanoic acid (**A67**) was discussed as a compound stimulating premating behavior in the desert locust *S. gregaria*.[371]



A65 **A66** **A67**

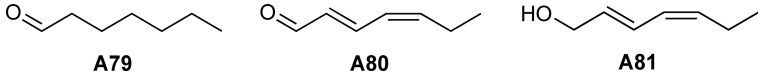
4.04.5.3.5 C_6-units

Hexanal (**A68**), emitted during courtship and copula of the bug *T. infestans*, attracted female conspecifics.[191] The common bedbug *C. lectularius*, uses (*E*)-2-hexenal (**A69**) as part of its aggregation pheromone.[193] Hexanoic acid (**A70**) occurs in males and females of the bug *R. prolixus*. Males were attracted to this compound in a dose-dependent manner.[367] Hexyl acetate (**A71**) is a female pheromone component of several bugs of the genus *Phytocoris*.[372–374] (*E*)-2-Hexenyl acetate (**A73**) is a pheromone component of *Phytocoris difficilis*.[374] (2*E*,4*E*)-2,4-Hexadienyl acetate (**A75**) was identified as part of a male-produced attracting pheromone for both sexes in the bug *N. bicrucis* (Lygaeidae).[205] 1-Methylethyl (*R*)-5-hydroxyhexanoate (**A76**), produced by adult male asparagus flies *Plioreocepta poeciloptera* elicits arrestment in females and attracted conspecific males, but not females.[375] Hexyl butyrate (**A72**), (*E*)-2-hexenyl butyrate (**A74**), and (*E*)-4-oxo-2-hexenal (**A6**) constitute the female sex pheromone of the sorghum plant bug *Stenotus rubrovittatus*.[376] Males of the long-horned beetle *Neoclytus acuminatus* and *M. caryae* produce (2*S*,3*S*)-2,3-hexanediol (**A78**) as an aggregation pheromone,[377] while the (2*S*,3*R*)- and (2*R*,3*S*)-enantiomers are part of the aggregation pheromone of male *M. caryae*.[204] The related (*R*)-3-hydroxy-2-hexanone (**A10**) is the aggregation pheromone of the long-horned beetle *Neoclytus mucronatus mucronatus*.[378] 2,3-Dihydro-3,5-dihydroxy-6-methylpyran-4-one (**A77**) shows trail pheromone activity in the ant *Camponotus socius*.[365]



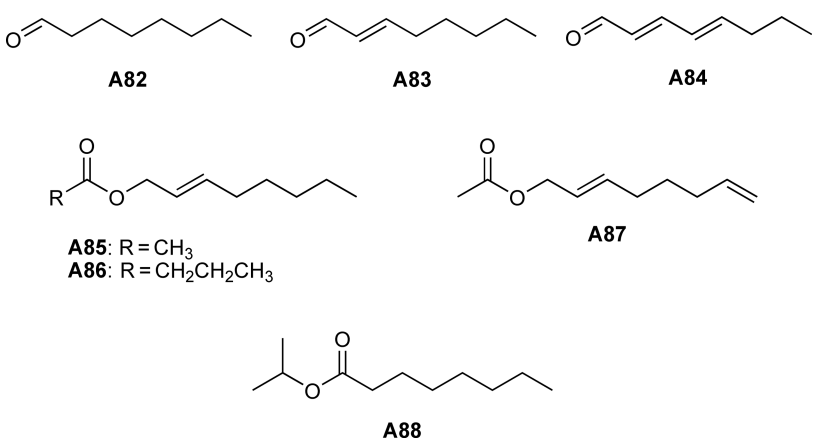
4.04.5.3.6 C_7-units

Heptanal (**A79**), emitted by the bug *T. infestans* during courtship and copula, attracted female conspecifics.[191] (2*E*,4*Z*)-2,4-Heptadienal (**A80**) and the respective alcohol **A81** are produced by males of the leaf beetle *Diorhabda elongata* and are attractive to both sexes.[379] (*R*)-2-Heptanol (**A12**) is a female sex pheromone component of the caddis fly *Molanna angustata*.[380]



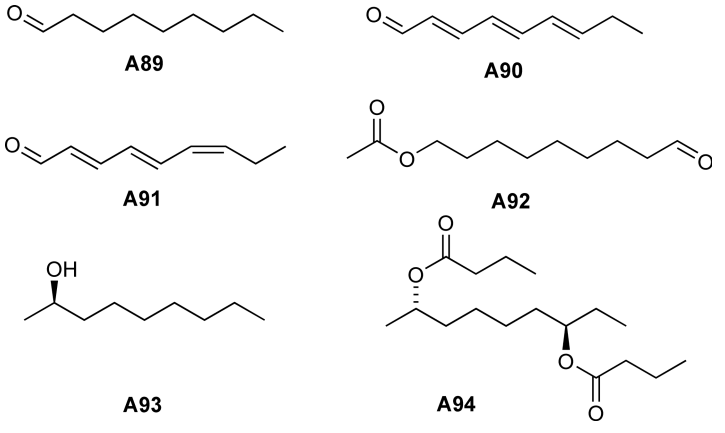
4.04.5.3.7 C_8-units

Octanal (**A82**) and (*E*)-2-octenal (**A83**), a pheromone component of the insidious flower bug *Orius insidiosus*,[381] are aggregation pheromone components of the fifth instar larvae of the codling moth *C. pomonella*, along with additional aldehydes and terpenes.[382] Compound **A83** and (2*E*,4*E*)-2,4-octadienal (**A84**) are components of the complex aggregation pheromone of the common bedbug *C. lectularius*.[193] A 1:10 mixture of (*E*)-2-octenyl acetate (**A85**) and (*E*)-2,7-octadienyl acetate (**A87**) was identified as a pheromone attractive to both sexes of the lygaeid bug *Tropidothorax cruciger*.[383] The latter compound is also part of the pheromones of the bugs *Oncopeltus fasciatus*,[384] *Geocoris punctipes*,[385] *Phytocoris* spp.,[373,374] and *O. insidiosus*.[381] The respective butyrate, (*E*)-2-octenyl butyrate (**A86**), is a female sex pheromone component of another bug *Phytocoris relativus*.[372] 1-Octen-3-ol (**A14**) is an attraction pheromone of *Amblyomma* ticks.[386] The sex pheromone of the female bagworm moth *Megalophanes viciella* is 1-methylethyl octanoate (**A88**), an unusual lepidopteran sex pheromone because it represents an ester of a fatty acid with a short-chain alcohol.[387]



4.04.5.3.8 C_9-units

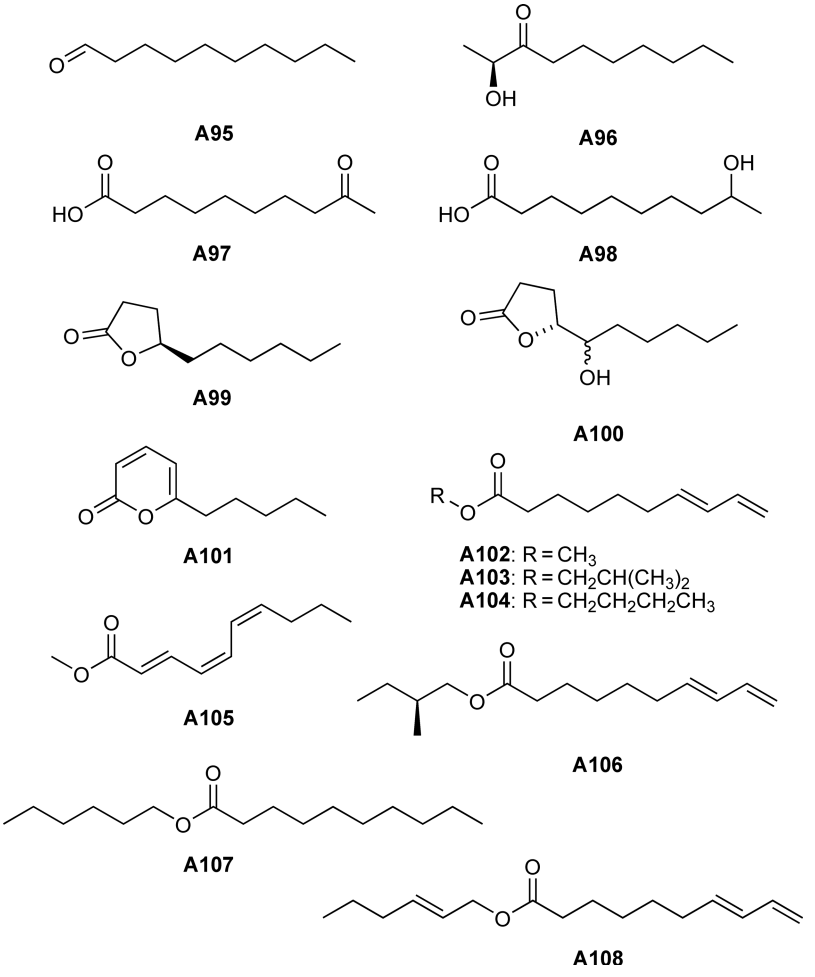
(*R*)-2-Nonanol (**A93**) is a female sex pheromone component of the caddis fly *M. angustata*.[380] Nonanal (**A89**), emitted during courtship and copula of *T. infestans*, attracted male conspecifics.[191] It is also used by another bug *C. lectularius*, as part of its aggregation pheromone.[193] Both (*E*)-2-nonenal (**A22**) and **A89** are constituents of the larval pheromone of *C. pomonella* (see Section 4.04.5.3.7).[382] The eggplant flea beetle *Epitrix fuscula* uses (2*E*,4*E*,6*E*)- and (2*E*,4*E*,6*Z*)-2,4,6-nonatrienal (**A90** and **A91**) as male sex pheromone.[388] (+)-*exo*-Brevicomin (**A27**) produced by males is part of the aggregation pheromone complex of the bark beetle *Dendroctonus jeffreyi*.[389] The unusual diester (2*S*,7*S*)-2,7-nonanediyl dibutyrate (**A94**), a pheromone structure typical for midges, is the sex pheromone of the female orange wheat blossom midge *Sitodiplosis mosellana*.[390] 9-Acetyloxynonanal (**A92**) is an attraction pheromone for the wheat stem sawfly *Cephus cinctus*.[391,392] Interestingly, it seems to be formed upon oxidative cleavage of unsaturated cuticular lipids.



4.04.5.3.9 C_{10}-units

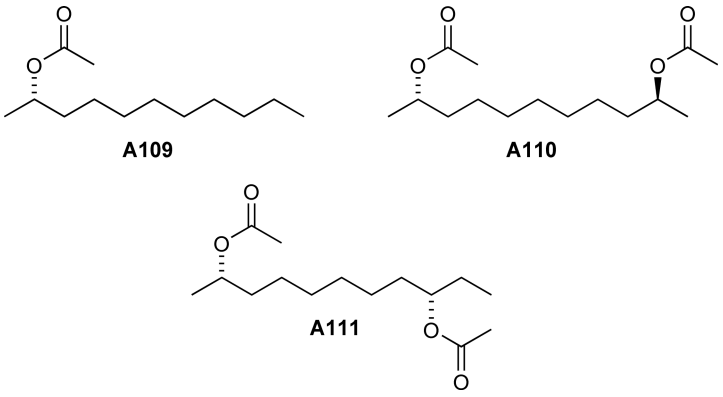
Decane is an alarm pheromone of the ant *C. obscuripes*.[366] Decanal (**A95**) is a pheromone component of larvae of *C. pomonella* (see Section 4.04.5.3.7),[382] and is used by the red bug *C. lectularius* as an aggregation pheromone component.[193] Males of a long-horned beetle, the coffee white stem borer *Xylotrechus quadripes*, a serious coffee pest in India, produce (*S*)-2-hydroxy-3-decanone (**A96**) as sex pheromone to attract female beetles.[393] 9-Oxo- (**A97**) and 9-hydroxydecanoic acid (**A98**) are the major sex pheromone components of females of the scoliid wasp *Campsoscolia ciliata*. Interestingly, the orchid *Ophrys speculum* also produces these compounds to attract males of *Campsoscolia* for pollination.[394] (*R*)-4-Decanolide (**A99**)

is the male-produced pheromone of the scarab beetle *Osmoderma eremita*.[395] Male jewel wasps, *N. vitripennis*, release a sex pheromone consisting of a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (**A100**) to attract juvenile females. These compounds become repellent to females after copulation.[396] The myrmicine ant *Pristomyrmex pungens* marks recruitment trails with the poison gland constituent 2,4-decadien-5-olide (6-pentyl-2-pyrone) (**A101**) as the trail pheromone.[397] Hexyl decanoate (**A107**) is a trail pheromone of the stingless bee *Trigona recursa*.[398] A structural deviation from typical female lepidopteran pheromones is represented by the pheromone of female nettle caterpillars. *Darna pallivitta* uses butyl (*E*)-7,9-decadienoate (**A104**) as female sex pheromone.[399] Similarly, (*S*)-2-methylbutyl (*E*)-7,9-decadienoate (**A106**) in combination with (*E*)-2-hexenyl (*E*)-7,9-decadienoate (**A108**) proved to be attractive to males of *Darna trima*, while 2-methylpropyl (*E*)-7,9-decadienoate (**A103**) was attractive to *Darna bradleyi*, enhanced in activity by methyl (*E*)-7,9-decadienoate (**A102**).[400] Together with sesquiterpenes, the related trienoate methyl (2*E*,4*Z*,6*Z*)-2,4,6-decatrienoate (**A105**) forms the male-produced sex pheromone of the red-shouldered stink bug *Thyanta pallidovirens*,[401] while it is per se active as a male sex pheromone of the red-shouldered stink bug *Thyanta perditor*.[402]



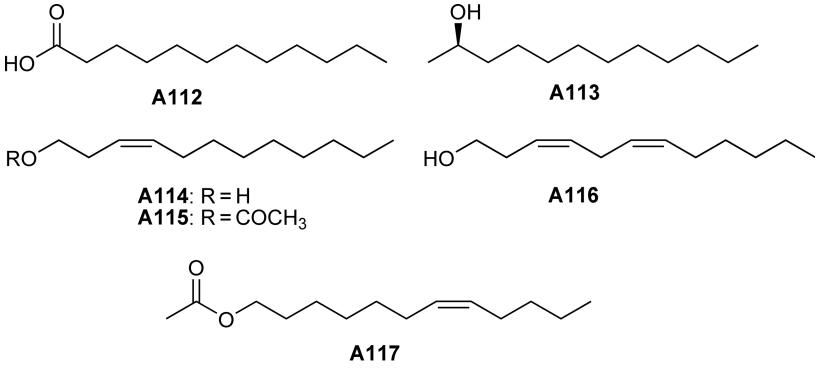
4.04.5.3.10 C_{11}-units

Undecane is an alarm pheromone of the ant *C. obscuripes*.[366] The sex pheromone of the Swede midge *C. nasturtii* consists of a mixture of three components (2*S*,9*S*)-2,9-diacetoxyundecane (**A111**), (2*S*,10*S*)-2,10-diacetoxyundecane (**A110**), and (*S*)-1-methyldecyl acetate (**A109**). Only formulations that contain around 1% of **A109** are attractive in the field.[403] A carbonyl derivative of **A109**, (*S*)-2-acetoxyundecan-5-one proved to be the sex pheromone of the raspberry cane midge *Resseliella theobaldi*.[404]



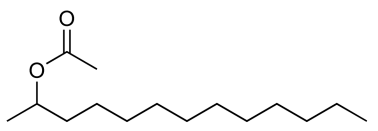
4.04.5.3.11 C_{12}-units

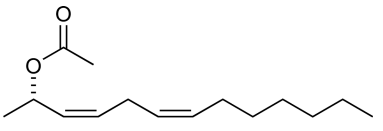
The trail pheromone of the myrmicine ant *Crematogaster castanea* has been identified as (*R*)-2-dodecanol (**A113**).[405] Dodecanoic acid (**A112**) was identified as the oviposition pheromone of the sand fly *Lutzomyia longipalpis*.[406] Although (*Z*)-7-dodecenyl acetate (**A117**) is used by many Lepidoptera as a pheromone component, it is interesting to know that it has also been identified as the sex pheromone of female Asian elephant *Elephas maximus*.[407] (*Z*)-3-Dodecen-1-ol (**A114**) is a trail pheromone of the termite *Macrotermes annandalei*;[408] whereas the related (3*Z*,6*Z*)-3,6-dodecadien-1-ol (**A116**) serves this function in the termite *Ancistrotermes pakistanicus*.[409] (3*Z*,6*Z*,8*E*)-3,6,8-Dodecatrien-1-ol (**A38**) has the same function in *Reticulitermes lucifugus grassei* and *R. santonensis*. It also seems to function as sex pheromone in both sympatric species[410] and is a trail pheromone of other termites as well.[411,412] (*Z*)-3-Dodecenyl acetate (**A115**), representing the structure of a typical moth pheromone, is a female-attracting pheromone of males of the flour beetle *Tenebrio molitor*.[413] (*R*)-1-Methylpropyl (*Z*)-7-dodecenoate is a component of the female-produced sex pheromone of the zygaenid moth *Illiberis rotundata*.[414]

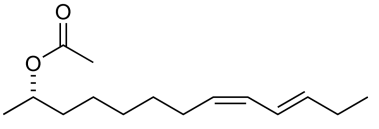


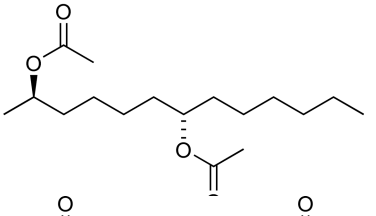
4.04.5.3.12 C_{13}-units

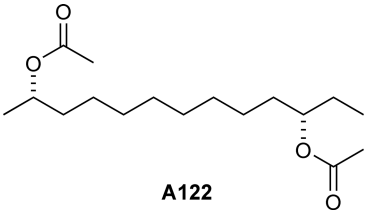
1-Tridecene is the male sex pheromone of the tenebrionid beetle *Parstizopus transgariepinus*.[415] A blend of the three compounds 2-acetoxytridecane (**A118**), (2*S*,11*S*)-2,11-diacetoxytridecane (**A122**), and (2*S*,12*S*)-2,12-diacetoxytridecane (**A123**) comprise the pheromone of the female pea midge *Contarinia pisi*.[416] Interestingly, only a 0.1:7:10 mixture proved to be fully attractive in the field, while omission of the minor component reduces catches to almost zero.[417] The sex pheromone of the Hessian fly *Mayetiola destructor* proved to be a mixture of **A48**, small amounts of its *Z*-isomer and (2*S*,8*Z*,10*E*)-2-acetoxy-8,10-tridecadiene (**A120**) as well as its (2*S*,8*E*,10*E*)-stereoisomer and the saturated compound.[418] Females of the aphidophagous midge *Aphidoletes aphidimyza* produce (2*R*,7*S*)-diacetoxytridecane (**A121**) as sex pheromone. The presence of the (2*R*,7*R*)- and (2*S*,7*R*)-stereoisomers inhibit trap catches.[419] (1*S*,3*Z*,6*Z*)-1-Methyl-3,6-dodecadienyl acetate (**A119**) is the sex pheromone of the Douglas-fir cone gall midge *Contarinia oregonensis*.[420]

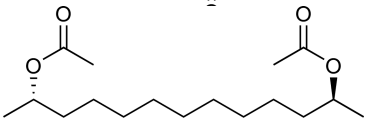

A118


A119


A120

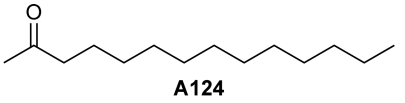


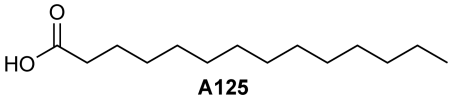

A122

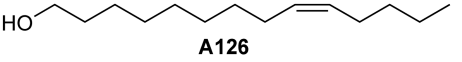

A123

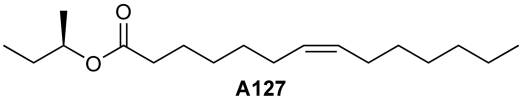
4.04.5.3.13 C_{14}-units

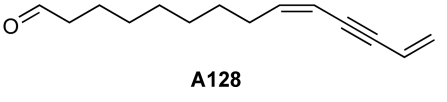
2-Tetradecanone (**A124**) is the female sex pheromone of the scarab beetle *Hoplia equina*.[421] The female sex pheromone of the vine bud moth *Theresimima ampellophaga* (Zygaenidae), (*R*)-1-methylpropyl (*Z*)-7-tetradecenoate (**A127**), initially erroneously designated to be (*S*)-configured, is unusual for a lepidopteran sex pheromone because it comprises an ester of a long-chain fatty acid and a short unbranched alcohol,[422,423] a feature that is also found in **A88** and **A106**. Tetradecanoic acid (**A125**) is part of the male hairpencil bouquet of the moth *H. virescens*. This compound can induce behavioral changes and together with C_{16} and C_{18} (see Sections 4.04.5.3.15 and 4.04.5.3.17) components, determines either accepting behavior or dispersal of the females.[424] (*Z*)-9-Tetradecenol (**A126**) is a male courtship pheromone component of the butterfly *Bicyclus anynana*. (*Z*)-9,13-Tetradecadien-11-ynal (**A128**) is the unusual female-produced sex pheromone of the avocado seed moth *Stenoma catenifer*. The corresponding alcohol serves as an antagonist.[425] (*R*)-1-Methylpropyl (*Z*)-9-tetradecenoate is a second component of the female-produced sex pheromone of the zygaenid moth *I. rotundata*.[413]


A124


A125

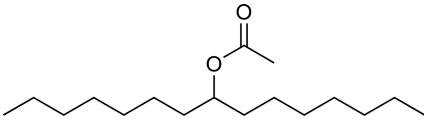

A126


A127


A128

4.04.5.3.14 C_{15}-units

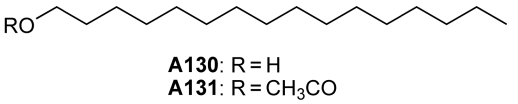
Pentadecane is a pheromone of the ant *C. obscuripes* that seems to calm down ants after the more volatile alarm signals decane and undecane have been evaporated.[366] 1-Heptyloctyl acetate (**A129**) is part of the trail pheromone of the ant *Leptogenys peuqueti*.[426]



A129

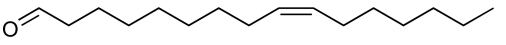
4.04.5.3.15 C_{16}-units

1-Hexadecanol (**A130**) is a synergistic component of the honeybee retinue response signal of workers.[219] Along with its acetate (**A131**) and palmitic acid (**A137**), it is part of the male pheromone of *H. virescens* (see Section 4.04.5.3.13).[424] The corresponding aldehyde, hexadecanal (**A132**) is part of the courtship pheromone of *Bicyclus anynana*.[427] The acid **A137** was claimed to be an oviposition-deterring pheromone of the female cotton bollworm moth *H. armigera*.[428] (*Z*)-7,15-Hexadecadien-4-olide (**A58**) is the female sex pheromone of the yellowish elongate chafer *Heptophylla picea* (Scarabaeidae). Its absolute configuration is unknown.[429] Isopropyl (*Z*)-9-hexadecenoate (**A141**) is the male attractant pheromone of the rove beetle *Aleochara curtula*.[430] The grape leaffolder moth *Desmia funeralis* uses the unusual triple bond-containing pheromone component 11-hexadecynal (**A135**) together with more common compounds such as (*Z*)-11-hexadecenal (**A134**) and (11*Z*,13*Z*)-11,13-hexadecadienal (**A136**).[431] The corresponding acetate **A139**, ethyl palmitate (**A138**), and ethyl (11*Z*,13*Z*)-11,13-hexadecadienoate (**A140**) are part of the sex pheromone of *Amyelois transitella* (see Section 4.04.5.3.22).[432] (*Z*)-9-Hexadecenal (**A133**) is part of the trail pheromone of the ant *Dolichoderus thoracicus*.[433]

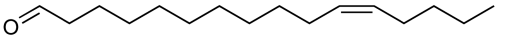


A130: R = H
A131: R = CH_3CO

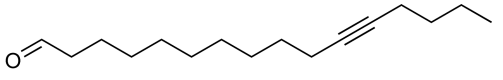
A132



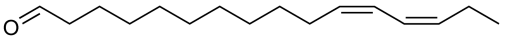
A133



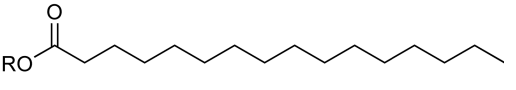
A134



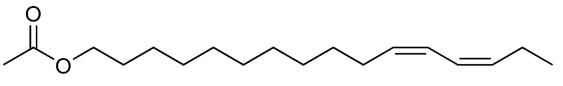
A135



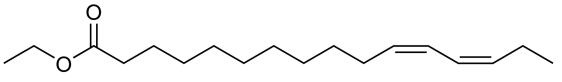
A136



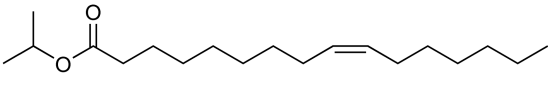
A137: R = H
A138: R = C_2H_5



A139



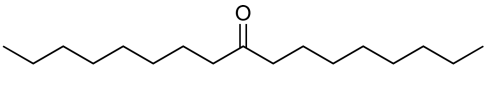
A140



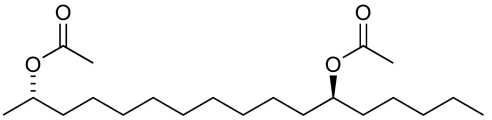
A141

4.04.5.3.16 C_{17}-units

9-Heptadecanone (**A142**) is the trail pheromone of the ant *Pachycondyla (Paltothyreus) tarsata fabricius*.[434] The sex pheromone of females of the red cedar cone midge *Mayetiola thujae* consists of a mixture of 2,12-, 2,13-, and 2,14-diacetoxyheptadecane (**A143–A145**). Only the (*S*,*S*)-enantiomers are active.[435] Following a related biogenesis, the sex pheromone of the pistachio twig borer *Kermania pistaciella* is (2*S*,12*Z*)-2-acetoxy-12-heptadecene (**A146**). The enantiomer inhibits trap catches.[436] A similar structure is represented by (2*S*,8*Z*)-2-butyroxy-8-heptadecene, the female sex pheromone of a *Rhopalomyia* gall midge.[437]



A142



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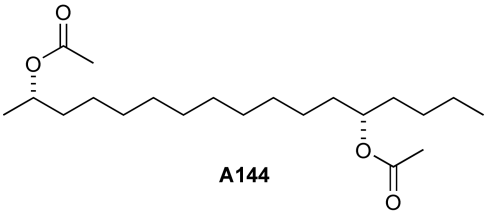
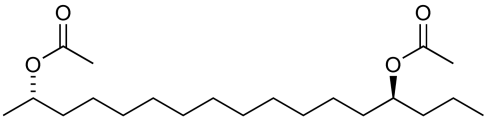
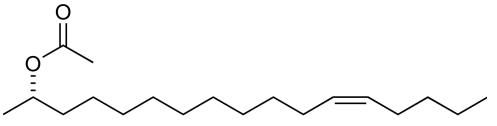


A144



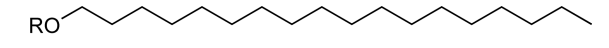
A145



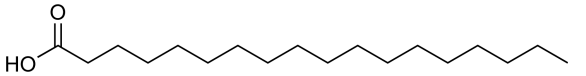
A146

4.04.5.3.17 C_{18}-units

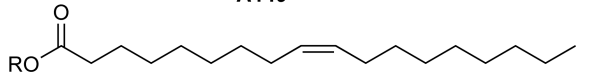
1-Octadecanol (**A147**), its acetate (**A148**), and stearic acid (**A149**) are part of the male pheromone of *H. virescens* (see Section 4.04.5.3.13).[424] Methyl oleate (**A151**) and linolenic acid (**A152**) are synergistic components in the honeybee retinue response signal of workers.[438] (*Z*)-9-Octadecenal (**A153**) is part of the trail pheromone of the ant *D. thoracicus*.[433] (9*Z*,12*Z*)-9,12-Octadecadienal (**A154**) is a pheromone component of the noctuid moth *Achaea janata*.[439] (3*Z*,6*Z*,9*Z*)-3,6,9-Octadecatriene (**A155**) constitutes the female sex pheromone of the winter moth *Erannis bajaria* together with **A159**.[307] This compound is quite unusual because it shows an even number of carbon atoms in the chain, just in contrast to common Type II sex pheromones of moth. (+)-Monachalure ((7*R*,8*S*)-7,8-epoxyoctadecane) (**A156**) is part of the pheromone system of the nun moth *Lymantria monacha*.[440] Oleic acid (**A150**) was claimed to act as an oviposition-deterring pheromone of the female cotton bollworm moth *H. armigera*.[99] The sex pheromone of the Australian guava moth *Coscinoptycha improbana* comprises (*Z*)-11-octadecen-8-one (**A157**) and the homologue ketones **A167** and **A194**.[441]



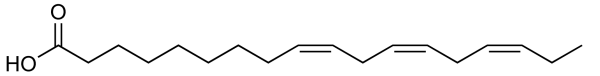
A147: R = H
A148: R = CH_3CO



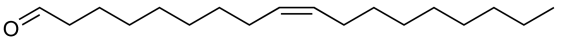
A149



A150: R = H
A151: R = CH_3



A152



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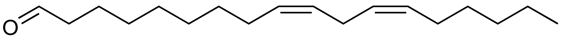
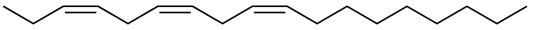
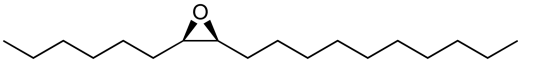


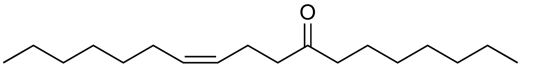
A154



A155



A156

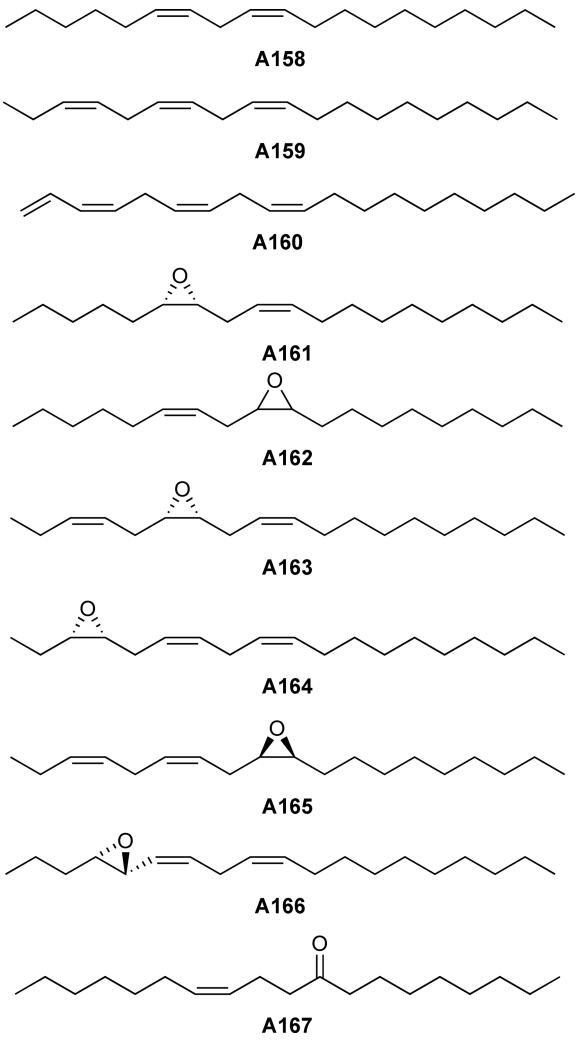


A157

4.04.5.3.18 C_{19}-units

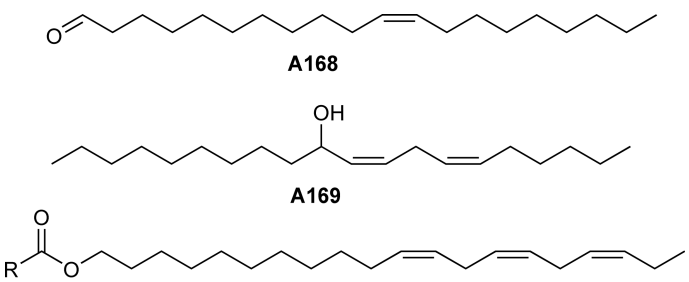
The pheromone of the geometrid moth *Biston robustum* consists of (6*Z*,9*Z*)-6,9-nonadecadiene (**A158**), (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (**A159**), (6*S*,7*R*,9*Z*)-6,7-epoxy-9-nonadecene (**A161**), and (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxy-3,9-

nonadecadiene (**A163**).[442] (*Z*)-*cis*-9,10-Epoxy-6-nonadecene (**A162**) is a female sex pheromone component of the common forest looper *Pseudocoremia suavis*.[443] The northern winter moth *Operophtera fagata* uses a 10:1 mixture of **A158** and (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**A160**) as sex pheromone.[444] (6*Z*,9*Z*)-*cis*-3,4-Epoxy-6,9-nonadecadiene (**A164**) is the female sex pheromone of the Japanese giant looper *Ascotis selenaria cretacea*. It is produced in a (3*S*,4*R*):(3*R*,4*S*) ratio of 53:47, although the pure latter enantiomer was most attractive in the field. In Israel, only the (3*S*,4*R*)-epoxide was attractive. The epoxide is obviously formed by PBAN-regulated oxidation of (**A159**).[445] The geometrid moth *Milionia basalis pryeri* uses the (3*S*,4*R*)-enantiomer of **A164** as female sex pheromone.[446] Another regioisomer, (3*Z*,6*Z*,9*R*,10*S*)-9,10-epoxy-3,6-nonadecadiene (**A165**), (+)-mathuralure, and its (−)-enantiomer in a ratio of 4:1 comprise the pheromone of *Lymantria mathura*.[447] Only this enantiomeric ratio is active, whereas pure enantiomers proved to be inactive. In another study, only the (−)-enantiomer was active in single-cell recordings, while both the pure enantiomer and the racemate captured males.[448] An unusual *trans*-configured vinyl epoxide is represented by the pheromone of *Bupalus piniarius*, (4*S*,5*S*,6*Z*,9*Z*)-4,5-epoxy-6,9-nonadecadiene (**A166**).[449] (*Z*)-12-Nonadecen-9-one (**A167**) was identified as the female sex pheromone of the raspberry budmoth *Heterocrossa rubophaga*[450] and as part of the sex pheromone of the moth *C. improbana*.[441]



4.04.5.3.19 C_{20}-units

(*Z*)-11-Icosenal (**A168**) is a component of the trail pheromone of *D. thoracicus*.[433] (6*Z*,9*Z*)-6,9-Icosadien-11-ol (**A169**) is a synergistic component of the female-produced sex pheromone component of the fir tussock moth *Orgyia detrita*.[451] (11*Z*,14*Z*,17*Z*)-11,14,17-Icosatrienyl 2-methylpropanoate (**A170**) and 3-methylbutyrate (**A171**) compose the pheromone of the tussock moth *Euproctis pulverea* in a 1:1 ratio.[452]



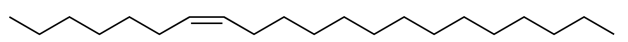
A168

A169

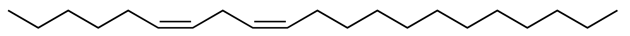
A170: R = $CH(CH_3)_2$
A171: R = $CH_2CH(CH_3)_2$

4.04.5.3.20 C_{21}-units

Henicosane is part of the female sex pheromone of the bee *Andrena nigroaenea*.[453] (*Z*)-7-Henicosene (**A172**) is the major alkene in the sex pheromone of the bee *Colletes cunicularius* inducing courtship in males. Along with the bishomologues **A189** and **A195**, it showed the highest activity among several blends tested. Related alkanes exhibit synergistic effects, whereas alkenes with double bond positions other than C7 seem to reduce activity.[454] (6*Z*,9*Z*)-6,9-Henicosadiene (**A173**), (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene (**A174**), and henicosane are sex pheromone components of the noctuid moth *A. janata*.[439] Compound **A174** is also the pheromone of the geometrid *Mnesampela privata*,[455] whereas **A173** is a major sex pheromone component of the painted apple moth *Teia anartoides*.[456] (3*Z*,6*R*,7*S*,9*R*,10*S*)-6,7-9,10-Diepoxy-3-henicosene (**A179**) (leucomalure) is the major female-produced sex pheromone component of the Satin moth *Leucoma salicis* (Lepidoptera), showing an unusual diepoxide structure.[457,458] The related monoepoxide, (3*Z*,6*Z*)-*cis*-9,10-epoxy-3,6-henicosadiene, is also present.[457] (3*Z*,6*Z*,9*S*,10*R*)-9,10-Epoxy-3,6-henicosadiene (**A175**) and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-1,3,6-henicosatriene (**A176**) are sex pheromone components of the fall webworm *Hyphantria cunea* in China.[459] In the clear-winged tussock moth *Perina nuda*, (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxy-3,9-henicosadiene (**A177**) is the main pheromone component, whereas its activity can be enhanced by the addition of the minor components, (3*R*,4*S*,6*S*,7*R*,9*Z*)-3,4-6,7-diepoxy-9-henicosene (**A180**), and/or its (3*S*,4*R*,6*S*,7*R*,9*Z*) diastereomer (**A181**).[460] The rare vinyl epoxide structural motif is found in (6*Z*,9*Z*,11*S*,12*S*)-11,12-epoxy-6,9-henicosadiene (posticlure, **A178**), the sex pheromone of the tussock moth *Orgyia postica*[461] (compared with the structures of the abovementioned *Bupalus* pheromone[449]). Interestingly, ketone **A183**, produced by the Ishigaki strain, reduces attractivity in the Okinawan strain when added to posticlure.[462] The highly unstable (6*Z*,8*E*)-6,8-henicosadien-11-one (**A182**) is a synergistic sex pheromone component of the Douglas-fir tussock moth *Orgyia pseudotsugata*. It seems to add specificity to the major pheromone component, (*Z*)-6-henicosen-11-one (**A183**).[463] A 100:5 mixture of the latter compound with (*Z*)-6-henicosen-9-one (**A184**) (thyellinone) is the pheromone of *Orgyia thyellina*.[447] The white-marked tussock moth *Orgyia leucostigma* uses (6*Z*,9*Z*)-6,9-henicosadien-11-one (**A185**) as a single component female sex pheromone.[464] The corresponding alcohol, (6*Z*,9*Z*)-6,9-henicosadien-11-ol (**A186**), is the pheromone of another tussock moth *O. detrita*. Its racemate is more attractive than the natural 1:3.5 *R*/*S*-mixture.[451] Ketone **A185** is a major component of the sex pheromone of *Teia anartoides*.[456,465]



A172



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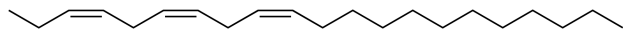
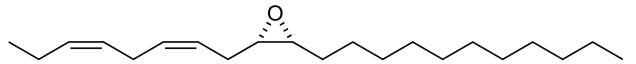


A174



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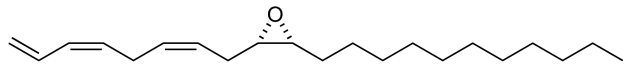
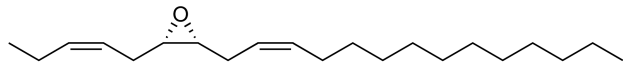


A176



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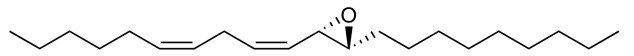

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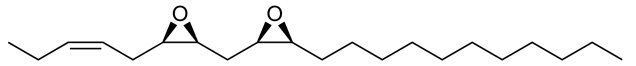

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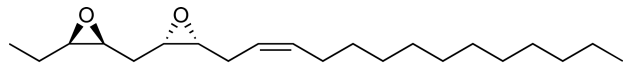

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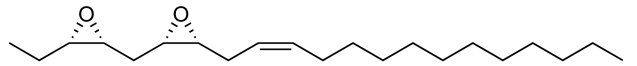

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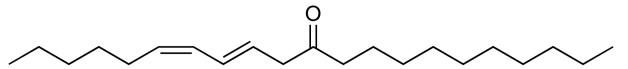

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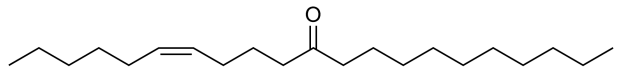

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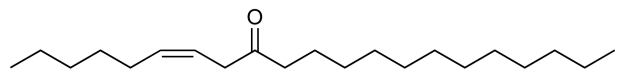

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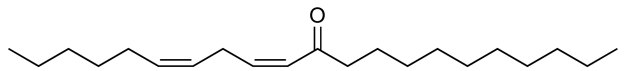
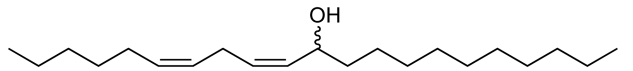


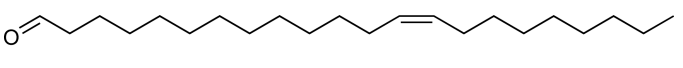
A185



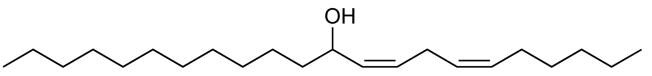
A186

4.04.5.3.21 C_{22}-units

Docosane is a component of the female sex pheromone of the bee *Andrena nigroaenea*.[453] (*Z*)-13-Docosenal (**A187**) is part of the trail pheromone of the ant *D. thoracicus*.[433] (6*Z*,9*Z*)-6,9-Docosadien-11-ol (**A188**) is a synergistic sex pheromone component of female *O. detrita*.[451]



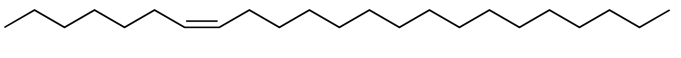
A187



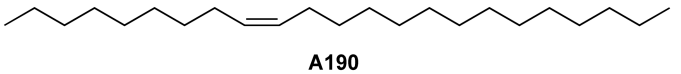
A188

4.04.5.3.22 C_{23}-units

Tricosane and (*Z*)-9-tricosene (**A190**) are components of the female sex pheromone of the bee *A. nigroaenea*.[453] The latter compound also occurs in the contact sex recognition pheromone of the Asian long-horned beetle *Anoplophora glabripennis*.[466] (*Z*)-7-Tricosene (**A189**) is a component of the sex pheromone of the bee *C. cunicularius* (see Section 4.04.5.3.20)[454] and the Australian guava moth *C. improbana*.[441] (6*Z*,9*Z*)-6,9-Tricosadiene (**A191**) is a component of the female-produced pheromone of the wasp *Eurytoma amygdali*,[467] whereas (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene (**A192**) is a synergistic pheromone component of the tomato fruit borer *Neoleucinodes elegantalis*.[468] It enhances the activity of the main pheromone component, (*E*)-11-hexadecen-1-ol. This activity enhancement of a typical lepidopteran pheromone by a hydrocarbon is quite unusual. The highly unsaturated polyene (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-tricosatriene (**A193**) together with **A199** and the three C_{16} compounds **A138**, **A140**, and (11*Z*,13*Z*)-11,13-hexadecadienyl acetate comprise the female sex pheromone of the navel orangeworm *A. transitella*.[432] (*Z*)-7-Tricosen-11-one (**A194**) is a component of the sex pheromone of the Australian guava moth *C. improbana*.[441]



A189



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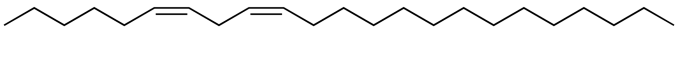

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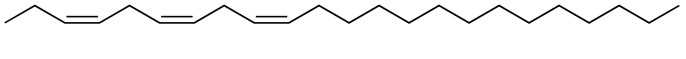

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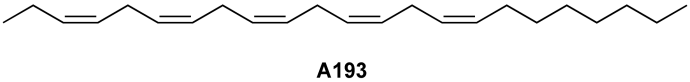
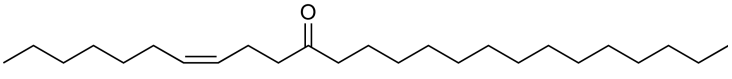


A193



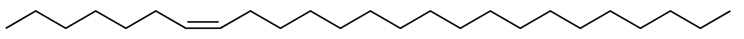
A194

4.04.5.3.23 C_{24}-units

Tetracosane is another component of the female sex pheromone bouquet of the solitary bee *A. nigroaenea*.[453]

4.04.5.3.24 C_{25}-units

Pentacosane is part of the female sex pheromone of the bee *A. nigroaenea*,[453] and of females of the long-horned beetle *Xylotrechus colonus*.[469] (*Z*)-7- (**A195**) and (*Z*)-9-pentacosene (**A196**) are components of the contact sex recognition pheromone of the Asian long-horned beetle *Anoplophora glabripennis*,[466] while male locust borer *Megacyllene robiniae* exclusively uses (**A196**).[470] The alkene **A195** is a component of the sex pheromone of *C. cunicularius* (see Section 4.04.5.3.20).[454] The isomer (*Z*)-12-pentacosene (**A197**) is an oviposition-deterrent pheromone of the coccinellid beetle *Cheilomenes sexmaculata*.[471] (6*Z*,9*Z*)-6,9-Pentacosadiene (**A198**) is a female pheromone component of the wasp *E. amygdali*.[467] (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-Pentacosapentaene (**A199**) is a component of the sex pheromone of the navel orangeworm *A. transitella* (see Section 4.04.5.3.22),[432] and a sex pheromone component of the pyralid moth *Dioryctria abietivorella*.[472]



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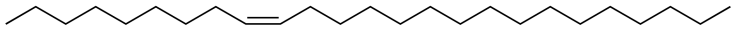

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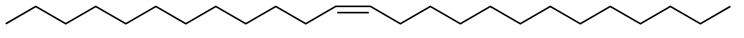
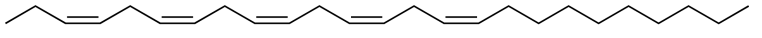


A197

A198



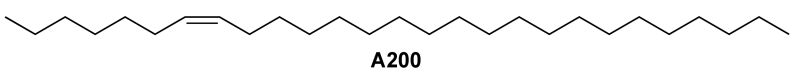
A199

4.04.5.3.25 C_{26}-units

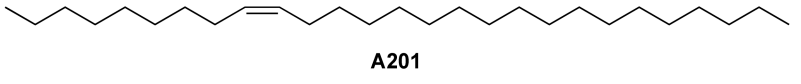
Hexacosane is part of the female sex pheromone of the bee *A. nigroaenea*.[453]

4.04.5.3.26 C_{27}-units

Heptacosane and (*Z*)-9- (**A201**), (*Z*)-11- (**A203**), and (*Z*)-12-heptacosene (**A202**) are important components of the female sex pheromone of the bee *A. nigroaenea*.[453] (*Z*)-7-Heptacosene (**A200**) and **A201** are part of the contact sex recognition pheromone of the Asian long-horned beetle *Anoplophora glabripennis*.[466] 10-Heptacosanone (**A204**), (*Z*)-18-heptacosen-10-one (**A205**), (18*Z*,21*Z*)-18,21-heptacosadien-10-one (**A206**), and (18*Z*,21*Z*,24*Z*)-18,21,24-heptacosatrien-10-one (**A207**) occur on the cuticle of females of the white-spotted longicorn beetle *Anoplophora malasiaca*.[473] They act as contact pheromone components and can evoke precopulatory behavior in males.



A200



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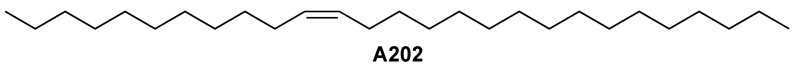

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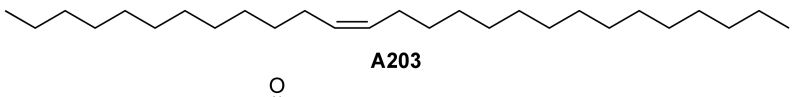
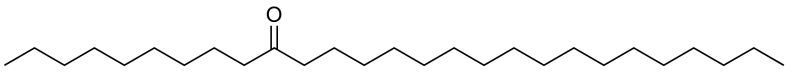


A203



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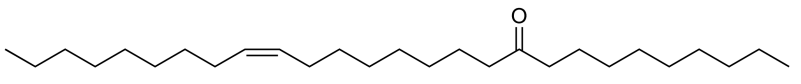

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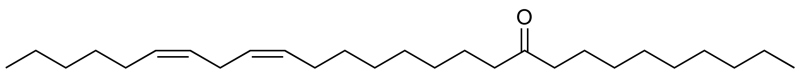

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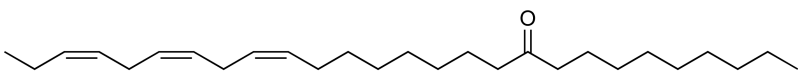
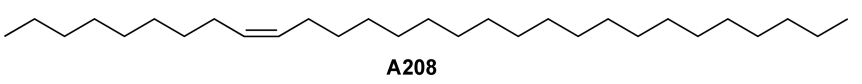


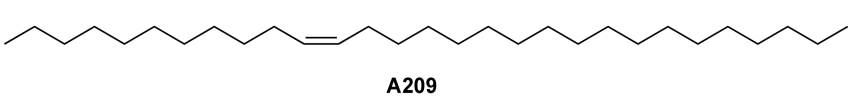
A207

4.04.5.3.27 C_{29}-units

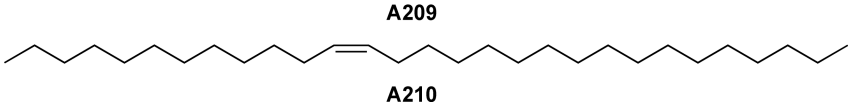
Nonacosane and (*Z*)-9- (**A209**), (*Z*)-11- (**A211**), and (*Z*)-12-nonacosene (**A210**) are parts of the female sex pheromone of the bee *A. nigroaenea*.[453] The alkene **A209** is a contact pheromone of male cerambycid beetle *M. caryae*.[474]



A208



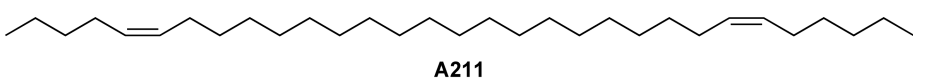
A209



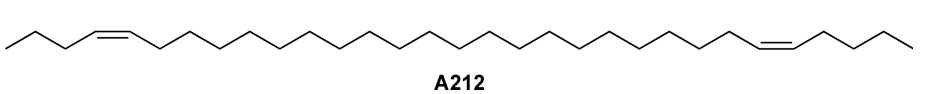
A210

4.04.5.3.28 C_{31}-units

(5*Z*,25*Z*)-Hentriaconta-5,25-diene (**A211**) as well as the less active (4*Z*,26*Z*)-4,26-hentriacontadiene (**A212**), components of the mixture of cuticular hydrocarbons present in females of the fruit fly of *Drosophila ananassae*, release courtship of males.[475]



A211



A212

4.04.6 Terpenes

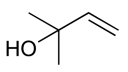
Volatile isoprenoids that control insect behavior and development have been reviewed.[476] Information on the biosynthesis of terpenoids with special emphasis on beetle pheromones has been compiled by Seybold and Vanderwel.[477–479]

The majority of newly assigned structures represent monoterpenes and sesquiterpenes.

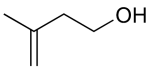
A new component of the alarm pheromone of Africanized honeybee *Apis mellifica* contained in the sting apparatus, was identified to be **T117**, 3-methyl-2-butenyl acetate.[480] In workers of giant hornets, *Vespa mandariana*, components of the alarm pheromone were shown to be 3-methyl-1-butanol, 2-pentanol, and its 3-methylbutanoate **T118** (stereochemistry unknown).[481] Whether the branched compounds represent isoprenoids at all or they are produced from the amino acid leucine remains an open question. The same holds true for structures **T4–T6** and **T8**. Despite the fact that **T7** and **T9** are clearly not terpenoids, we earlier listed them among the homoterpenes to point to certain structural relations between biologically active C5-units.[1] New results concerning the role of amino acid derivatives in systems of chemical communication are given in Section 4.04.9. Actually, there are direct biogenetic links between isovaleryl-CoA, derived from leucine, and the mevalonate pathway.[482,483]

A few diterpenes have been described from bumblebees[484,485] and stingless social bees;[486] however, the biological significance of these compounds remained a speculation. Neocembrene **T73** was found to be a major component of the trail-following pheromone in the genus *Prorhinotermes*.[487] A new diterpene, (4*R*,7*S*,8*R*,11*E*,15*S*,16*S*)-trinervita-1(14)2,11-triene, has been found in the termite *Nasutitermes ephratae*; however, conclusive bioassays assigning its biological function have not been reported.[488]

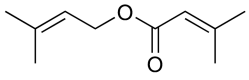
In our earlier review,[1] the compounds presented in the following figures were discussed in more detail.



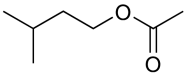
T1: bark beetles,[489] *Vespa crabro*, European hornet;[490]



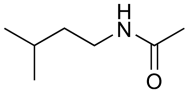
T2: *Ips*, bark beetles;[491]



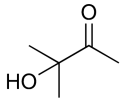
T3: *Vespa crabro*, European hornet;[492]



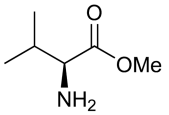
T4: *Apis mellifica*, honey bee;[493]



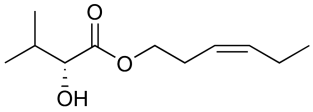
T5: *Paravespula*, yellowjackets,[494]



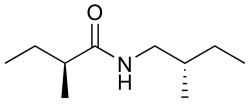
T6: *Trypodendron domesticum*, bark beetle,[495] *Rhyssa persuasoria*, parasitic wasp;[496]



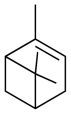
T7: *Phyllophaga anxia*, scarab beetle;[497]



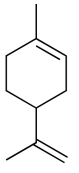
T8: *Pristhesancus plagipennis*, assassin bug;[498]



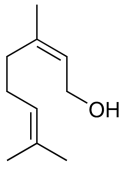
T9: *Migdolus fryans*, long-horned beetle;[499]

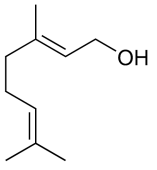


T10: bark beetles,[500] termites,[501] ants,[501] tenebrionid beetles;[502]

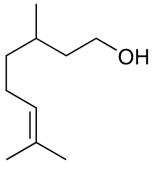


T11: *Myrmicaria eumenoides*;[503]

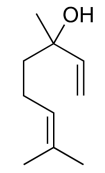




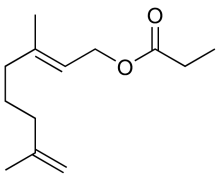
T12, T13: many ants and bees;[501]



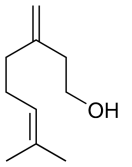
T14: bees, ants,[501] *Tetranychus urticae*, spider mite;[504]



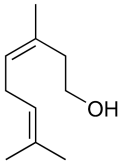
T15: bees,[501] *Colletes* bees,[505] parasitic wasps;[506]



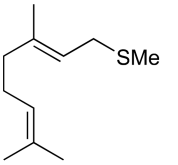
T16: *Quadraspidiotus perniciosus*, San José scale;[507]



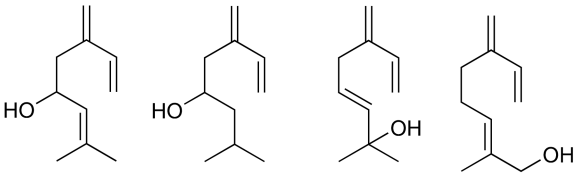
T17: *Pityogenes quadridens*, bark beetle;[508]



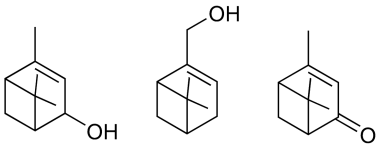
T18: *Leptogenys diminuta*, ant;[509]



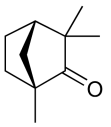
T19: *Idea leuconoe*, butterfly;[510]



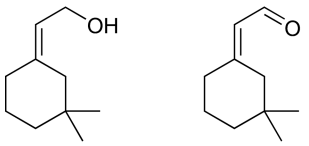
T20–T23: *Ips*, bark beetles;[329,511–514]



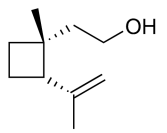
T24–T26: *Scolytidae*, bark beetles;[515,516]

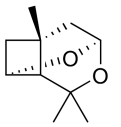


T27: *Dendroctonus micans*, bark beetle;[517]

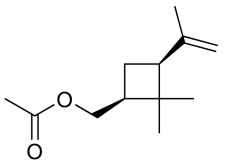


T28–30: weevils,[518–520] *Coleomegilla maculata*, lady beetle;[521]

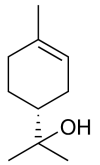




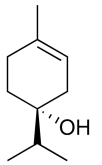
T31: *Trypodendron lineatum*, bark beetle;[522,523]



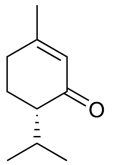
T32: *Planococcus citri*, mealy bug;[524]



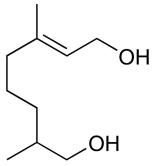
T33: *Podisus maculiventris*, soldier bug;[525]



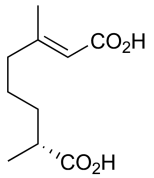
T34: *Polygraphus poligraphus*, bark beetle;[526]



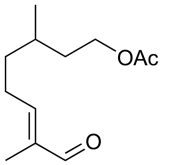
T35: *Tyrophagus similis*, acarid mite;[527]



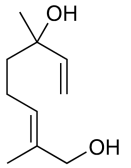
T36: monarch, butterflies,[528] diacetate: *Oechalia schellenbergii*, predaceous bug;[529]



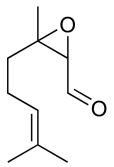
T37: *Callosobruchs chinensis*, azuki bean weevil;[530]



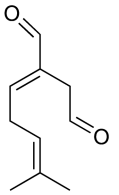
T38: rove beetles;[531]



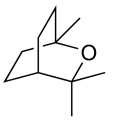
T39: *Chrysomela*, leaf beetles;[532]



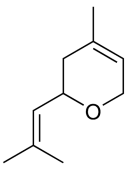
T40: *Caloglyphus*, acarid mites;[533]



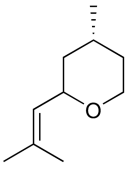
T41: *Caloglyphus polyphyllae*, mite;[534]



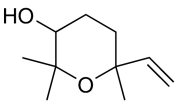
T42: bugs;[535]

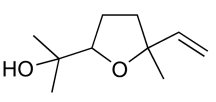


T43: *Euroleon*, ant lions;[536]

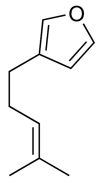


T44: *Aromia moschata*, long-horned beetle,[537] *Bembix rostrata*, digging wasp;[538]

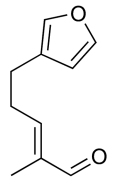




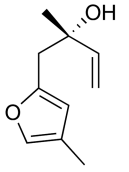
T45, T46: *Amauris*, butterflies;[539]



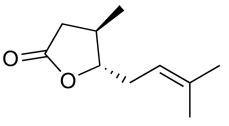
T47: ants;[540]



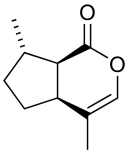
T48: sawflies;[541]



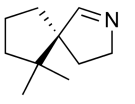
T49: *Elymnias thryallis*, butterfly;[542]



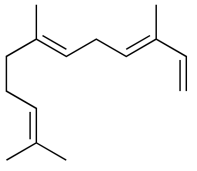
T50: *Eldana saccharina*, moth;[543]



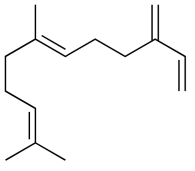
T51: aphids;[544,545]



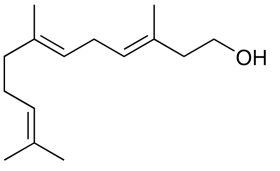
T52: *Polyzonium rosalbum*, millipede. [546]Natural **T52** was found to keep (*S*)-(+)-configuration;[547,548]



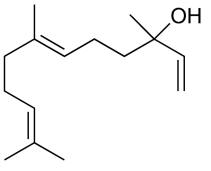
T53: *Ceratitis capitata*, Mediterranean fruit fly,[549] *Hepialus humuli*, hepialid moth;[324]



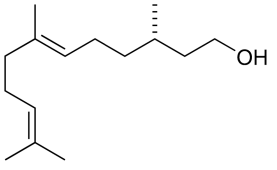
T54: aphids;[550]



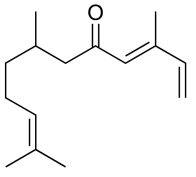
T55: bees,[551–553] ants;[554]



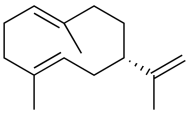
T56: *Kheper*, dung beetles,[555] danaid butterflies,[539] *Tetranychus urticae*, spider mite;[556]



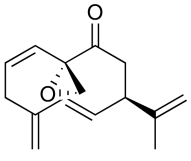
T57: *Bombus*, bumblebees;[557]

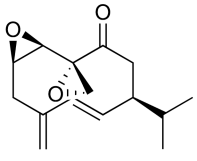


T58: *Nomada*, cleptoparasitic bees;[558]

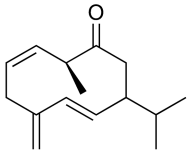


T59: *Therioaphis maculata*, alfalfa aphid,[559] *Syntermes*, termites,[560] *Papilio*, swallowtail butterflies;[561]

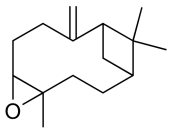




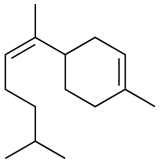
T60, T61: *Periplaneta americana*, American cockroach;[562]



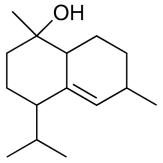
T62: *Periplaneta fuliginosa*, smoky brown cockroach;[563]



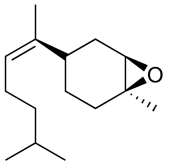
T63: *Papilio memnon*, swallowtail butterfly;[561]



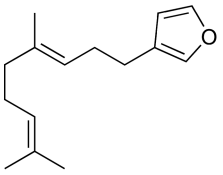
T64, T65: termites;[564]



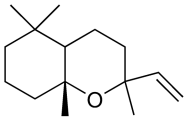
T66: *Subulitermes*, termites,[565] Lycaenid butterflies;[566]



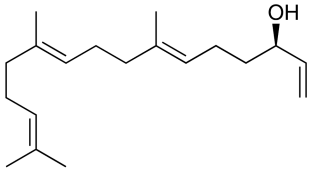
T67: *Nezara*, stink bugs;[567–569]



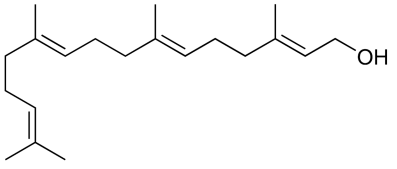
T68: *Lasius*, ants;[570]



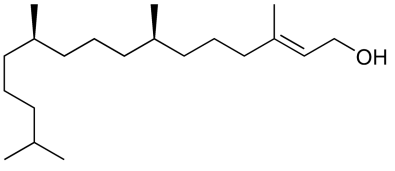
T69: *Amitermes*, termites;[571]



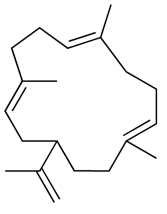
T70: *Reticulitermes lucifugus*, termite;[572]



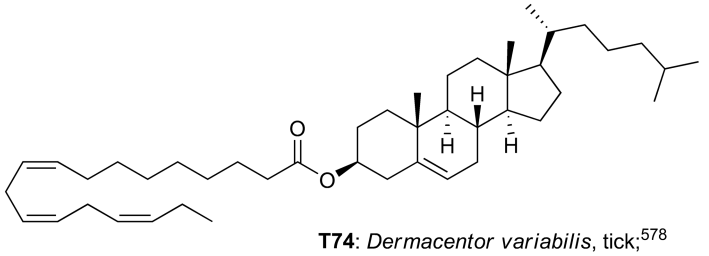
T71: *Formica*, ants,[573] bumblebees;[557]



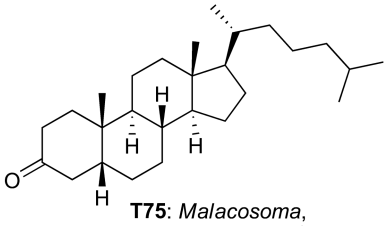
T72: *Ephestia elutella*, pyralid moth;[574]



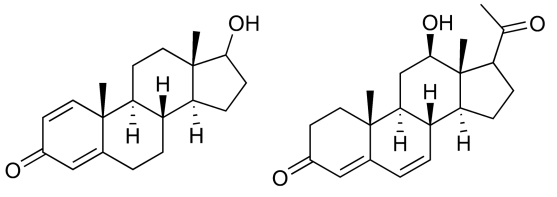
T73: *Trinervitermes bettonianus*,[575] *Cubitermes umbratus*,[576] termites, *Monomorium*, ants;[577]



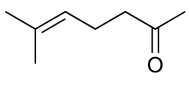
T74: *Dermacentor variabilis*, tick;[578]



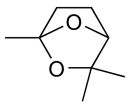
T75: *Malacosoma*, tent caterpillars;[579]



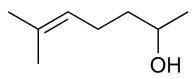
T76, T77: Dytiscidae, water beetles;[580–582]



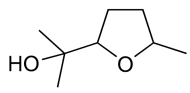
T78: very widespread;[583–592]



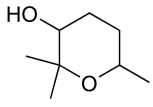
T79: *Aenictus rotundatus*, ant;[593]



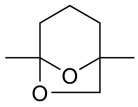
T80: *Gnathotrichus*, bark beetles;[594,595]



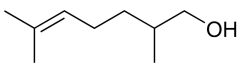
T81: *Pityophthorus pityographus*,[596] *Conophthorus*, bark beetles;[597,598]



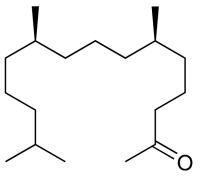
T82: *Pteleobius vittatus*, bark beetle;[584]



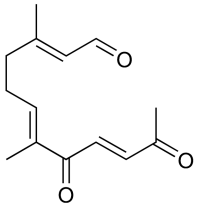
T83: *Dendroctous*, bark beetles;[329,599]

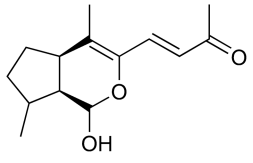


T84: bees, ants;[583]

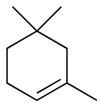


T85: *Corcyra cephalonica*, rice moth;[600]

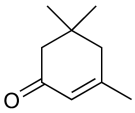




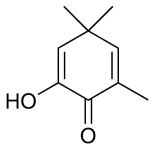
T86, T87: *Gyrinus, Dineutes,* water beetles;[601–603]



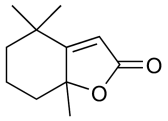
T88: *Ancistrotermes cavithorax*, termite;[604]



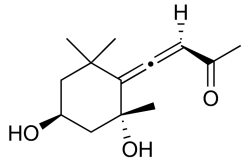
T89: *Ips typographus*, bark beetle,[605] Danaid and Ithomiid butterflies;[539]



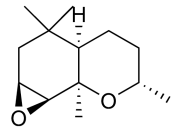
T90: *Ips pini*, bark beetle;[606]



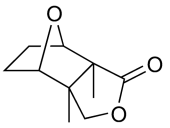
T91: *Solenopsis invicta*, fire ant,[607] *Idea iasionia*, butterfly;[508]



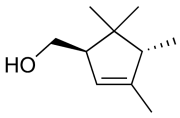
T92: *Romalea* sp., grasshopper;[608]

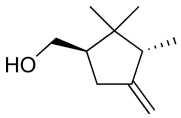


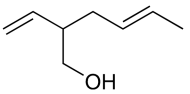
T93: *Euploea klugii*, butterfly;[609]



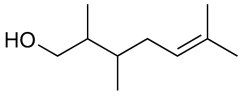
T94: *Lytta vesicatoria*, 'Spanish fly' beetle;[610,611]



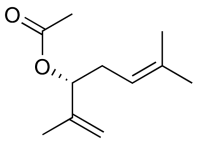




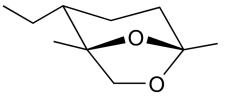
T95, T96, T97: *Necrodes surinamensis*, carrion beetle;[612,613]



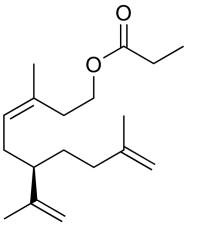
T98: *Lasius meridionalis*, ant.[614,615]



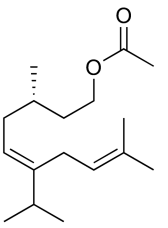
T99: *Pseudococcus comstocki*, Comstock mealybug;[616,617]



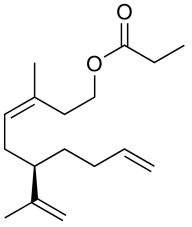
T100: *Taphrorychus bicolor*, bark beetle;[618]



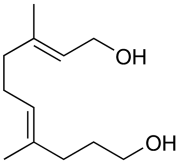
T101: *Pseudaulascaspis pentagona*, white peach scale;[619]



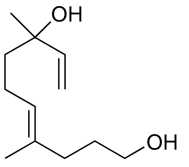
T102: *Aonidiella citrina*, yellow scale;[620]



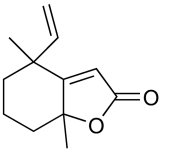
T103: *Aonidiella aurantii*, California red scale;[621,622]

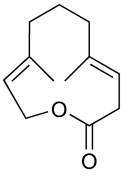


T104: Danaid butterflies;[623]

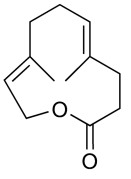


T105: *Euploea sylvester*, Danaid butterfly;[539]

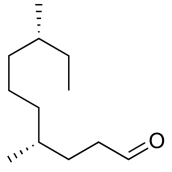




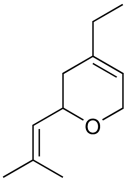
T106, T107: *Anastrepha suspensa*, Caribbean fruit fly;[624,625]



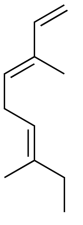
T108: *Cryptolestes ferrugineus*, rusty grain beetle;[626]



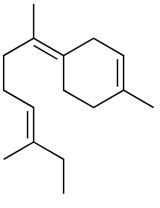
T109: *Tribolium*, flour beetles;[627,628]



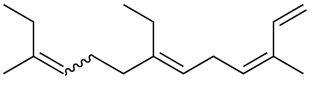
T110: *Grocus bore*, ant lion;[629]



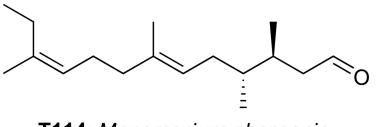
T111: *Labidus praedator*, ant;[630]



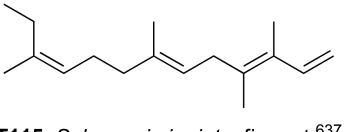
T112: *Eryogaster integriceps*, sun bug;[631]



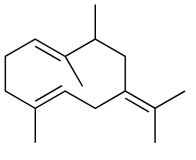
T113: *Myrmicaria*, ants;[632–635]



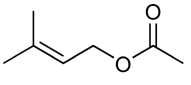
T114: *Monomorium pharaonis*, Pharaoh's ant;[636]



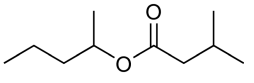
T115: *Solenopsis invicta*, fire ant;[637]



T116: *Lutzomyia longipalpis*, sand fly;[638]



T117

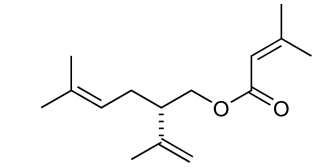


T118

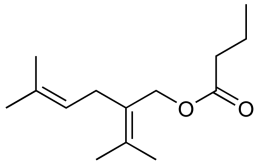
4.04.6.1 Monoterpenes

A compound consisting of a hemiterpene and a monoterpene is the female-produced sex pheromone of the vine mealybug *Planococcus ficus*.[639,640] Its structure, (*S*)-lavandulyl senecioate (**T119**), is somehow related to that of the female-produced pheromone of the comstock mealybug *Pseudococcus comstocki*, **T99**, which is the acetate of an (*R*)-configured nor-lavandulol. The structure of **T119** is even closer to that of the pheromone of the passion vine mealybug *Planococcus kraunliae*, **T120**,[641] and that of *Planococcus minor*,[642] **T121**, which are all esters of lavandulyl derivatives and short-chain carboxylic acids.

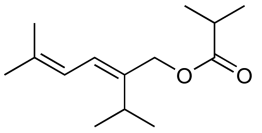
Another unusual group of pheromones of mealybugs (Homoptera: Pseudococcidae) shows cyclopentanoid structures: the sex pheromone of the obscure mealybug *Pseudococcus viburni* is (2,3,4,4-tetramethylcyclopentyl)methyl acetate[643] (**T122**) showing (1*S*,2*S*,3*R*)-configuration.[156] A similar structure is found in (R^*,R^*)-*trans*-(3,4,5,5-tetramethylcyclopent-2-enyl)-2-methylpropanoate (*trans*-α-necrodyl acetate) (**T123**) the female-produced sex pheromone of the grape mealybug *Pseudococcus maritimus*.[644] A cyclobutane structure is represented by the sex pheromone of *Planococcus cryptus*, **T124**,[645] the alcohol part of which being identical to that of the pheromone of *Planococcus citri* **T32**. The sex pheromone of the pink hibiscus mealybug *Maconellicoccus hirsutus* is made up of esters of (*S*)-2-methylbutyric acid and [(*R*)-2,2-dimethyl-3-(1-methylethylidene)cyclobutyl]-methanol (maconelliol) (**T125**) or (*R*)-lavandulol (**T126**), respectively.[646,647] Reactions of the insects to different stereoisomers of the pheromone have been investigated.[648] The biosyntheses of such unusual terpenes have been described.[649] (1*R*,2*S*)-Grandisol (**T30**) and grandisal are components of the male-produced pheromone of the Brazilian papaya weevil *Pseudopiazurus obesus*,[650] which also contains the interesting new bicyclic ether **T127**. This compound, keeping (1*R*,2*R*,6*R*)-configuration, may be produced from grandisol upon epoxidation of the double bond, followed by intramolecular ring closure.[651] Grandisol, **T30**, along with other typical boll weevil pheromone components and ochtodenol/ochtodenal (**T28**/**T29**) were found to be attractive to both sexes of sugar beet weevils.[652,653] A similar bouquet, additionally containing lavandulol, the alcohol component of **T119**, was found to be male-specific in the strawberry blossom weevil *Anthonomus rubi*.[654] Already, (1*R*,2*S*)-grandisoic acid has been identified as the sex pheromone of the plum weevil *Conotrachelus nenuphar*.[655] Investigations on the attractivity of lineatin (**T31**), the female-produced aggregation pheromone of the ambrosia beetle *Trypodendron lineatum*, showed only the naturally occurring (+)-enantiomers to be active.[656]



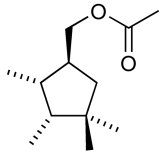
T119



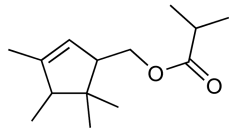
T120



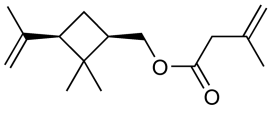
T121



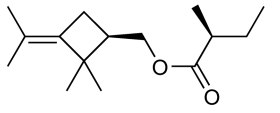
T122



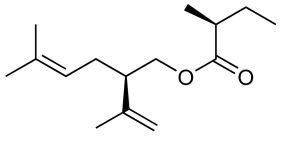
T123



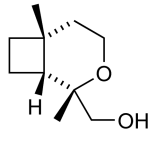
T124



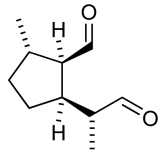
T125



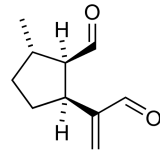
T126



T127



T128



T129

Much has been learnt about the chemical ecology and pheromone biology of aphids during the past years. Earlier investigations on sex pheromones of aphids have been briefly summarized.[657] Many species use the (4a*S*,7*S*,7a*R*)-stereoisomer of nepetalactone (**T51**) and the corresponding lactol, which frequently keeps (1*R*)-configuration.[658–661] A very careful and most detailed study on the pheromones of *Dysaphis plantaginea* has been carried out by Stewart-Jones *et al.*[662] The influence of stereochemistry and purity of the compounds on field catches may be explained by the fact that some species, for example, *Phorodon humuli* uses other stereoisomers such as (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol.[663] Interestingly, (1*R*,4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactol (**T130**) was found to attract three different predatory lacewing species.[664] It turned out that predatory lacewings are attracted to volatiles produced by aphid prey and their host plants.[665] In this context, (1*R*,2*S*,5*R*,8*R*)-iridodial (**T128**) has been reported to attract males of several *Chrysopa* species.[666,667] Methyl salicylate (**Ar21**) may act synergistically, and the binary blend of **T128** and **Ar21** seemed to attract the predatory hoverfly *M. americanus*.[198] Recently, (1*S*,2*R*,3*S*)-dolichodial **T129**, released by *D. plantaginea* ovipare, has been identified to elicit behavioral response from males and naive-mated female parasitoids, *Aphidius ervi*.[668] While **T51** and **T128**/**129**, and other iridoid pheromones that play a role in the context of aphids, are *cis*-configured with respect to the two oxygen-carrying substituents, cephalic secretions of the hyperparasitoid wasp *Alloxysta victrix* (a parasitoid on *Aphidius* spp. that in turn are parasitoids on *Aphid* spp.) contain small amounts of (4*R*,4a*S*,7*R*,7a*S*)-dihydronepetalactone (**T131**) while (4*S*,4a*R*,7*S*,7a*R*)-iridomyrmecin (**T132**) forms a major constituent[669] and its (4*S*,4a*S*,7*R*,7a*S*)-diastereomer (**T133**) a minor one.[670] Because of the structural relations to behaviorally active iridoids known so far, these *trans*-fused iridoids may well play a role in the tetratrophic relations made up of plant – aphid – *Aphidius* – *Alloxysta*; however, the actual biological significance of the compounds is yet unknown.

During the past decade, the literature dealing with the behavior-mediating capacity of monoterpenes has largely been dominated by investigations on bark beetles. This includes response to host- and nonhost volatiles as well as interspecific attraction and repellency. Since the subject has been comprehensively reviewed,[671] only more recent papers are cited here.[672–675] It could be shown that the enantiomeric composition of chiral host monoterpenes, for example, α-pinene may influence feeding preference.[676] The biosynthesis and enantiomeric composition of α-pinene (**T30**) and β-pinene in loblolly pine *Pinus taeda* has been carefully studied.[677] A mixture of ethanol and (−)-α-pinene, a widespread constituent of the resin of coniferous trees, was shown to be attractive to a large number of coleopterans including Buprestidae, Cerambycidae, Curculionidae, and Elateridae.[678] Once again, the effect of verbenone (**T26**) on the attraction/repellency of wood-boring beetles and their predators has been investigated.[679,680] Similarly, nonhost leaf and bark volatiles as well as verbenone have been described as disruptants/repellents.[681–683] The role of bark beetle pheromones and host volatiles as kairomonal attractants of long-horned beetles, especially *Monochamus*, has been discussed.[684,685] Investigations on the attraction of pine engravers and associated bark beetles to ipsdienol (**T20**) and ipsenol (**T21**) are still hampered by high costs of pure enantiomers.[686] Comprehensive investigations using pure enantiomers or defined mixtures thereof are still rare.[687]

The biosynthesis of ipsdienol (**T20**) has been intensively investigated during recent years. While in earlier times, it was thought that bark beetles use the host monoterpene myrcene as the precursor, which is just oxidized to the pheromone,[688] following a breakthrough in 1995,[689,690] the work of Blomquist, Seybold, and Tittiger proved a *de novo* biosynthesis by the beetles.[691] Their investigations revealed that the compound is definitely produced in the midgut of the beetles,[692,693] while juvenile hormone III stimulates its biosynthesis.[694] A sex-specific and inducible monoterpene synthase activity associated with the pine engraver *Ips pini* could be demonstrated[695] as well as a full-length cDNA encoding 3-hydroxy-3-methyl-glutaryl CoA synthase could be isolated, and its genomic structure was examined.[696] It is now evident that the beetles do produce myrcene *de novo*, and the activitiy of a myrcene hydroxylase could be detected; however, the mechanism determining the enantiomeric composition of ipsdienol in *Ips* spp. will still need further investigations. Male *Ips* bark beetles, exposed to myrcene vapors, did not contain the same enantiomeric composition of ipsdienol as during their activities in the host trees and myrcene hydroxylases do not seem to control the final enantiomeric composition of ipsdienol.[697] Following way A (see **Figure 2**) myrcene could be nonenantioselectively hydroxylated at position 5, followed by oxidation to ipsdienone and a subsequent stereoselective reduction to furnish the 'correct' enantiomeric composition. An alternative (way B, **Figure 2**) would be a regio- and stereospecific hydroxylation of an activated geranyl precursor (e.g., geranyl diphosphate). Subsequent 1,4-elimination of diphosphate could directly yield the ipsdienols in the naturally occurring proportions. Maybe both ways exist – oxygenation of myrcene being the more archaic one because 'ancient' species could certainly have used myrcene directly from the host tree. It should be noted that the bark beetle pheromone lineatin

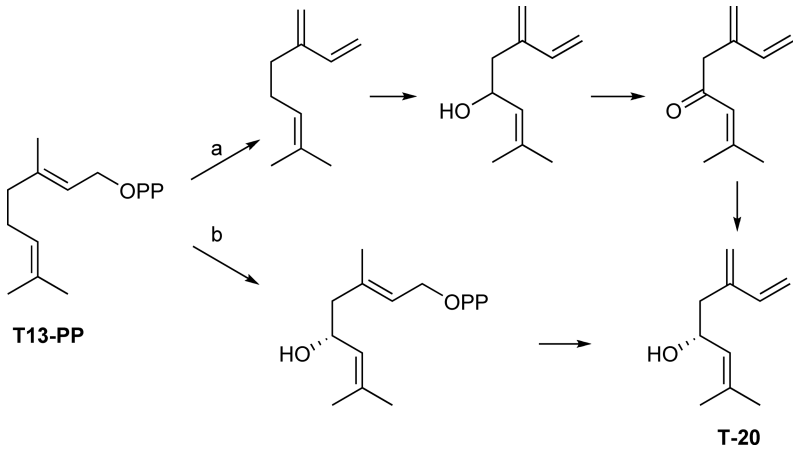


Figure 2 Possible biosynthetic pathways leading to ipsdienol (**T20**).

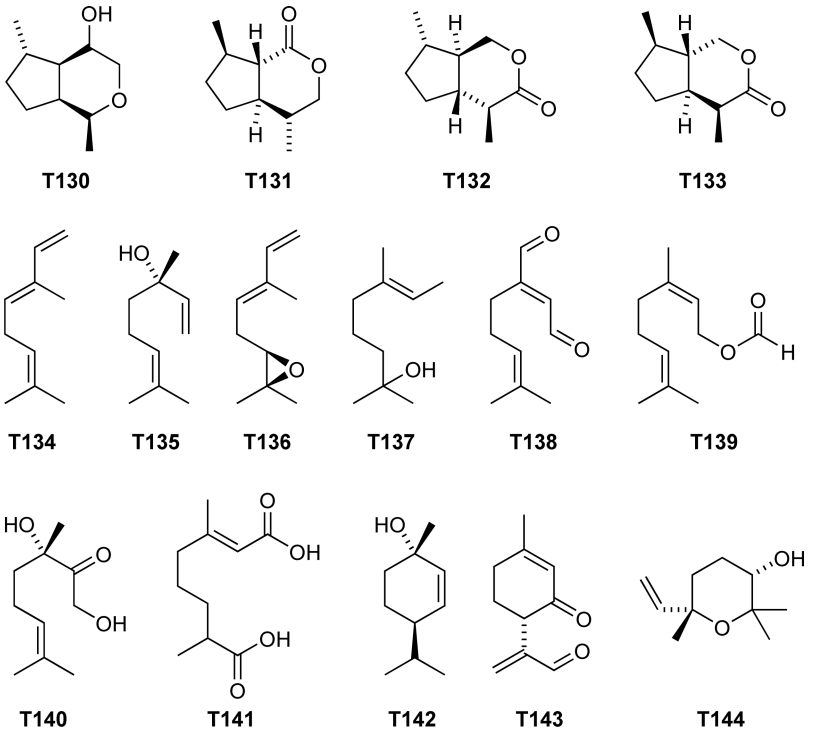
(**T31**) shows a 5-hydroxygeranial structure (carrying an additional oxygen function at position 7). An antiaphrodisiac, *trans*-β-ocimene (**T134**), that male *Heliconius* butterflies transfer to the females during copula is also biosynthesized *de novo*.[698]

A formal hydroxylation product of myrcene/ocimene is (*S*)-linalool (**T135**) a volatile signal in the communication system of the solitary bee *C. cunicularius*.[699] The ocimene epoxide (**T136**) showing (3*S*,5*E*)-configuration was identified among the headspace volatiles released by males of the coreid bug *Amblypelta nitida*.[700] The biological function of the compound remained unknown. The closely related terpenol, **T137**, (*E*)-subaenol, is produced by males of the dung beetle *Kheper subaeneus*. It is active on the antennae of both sexes, however, its behavior-mediating capacity is unknown.[701] The female sex pheromone of the mite *Rhizoglyphus robini* was identified to be α-acaridial (**T138**), which stimulated males.[702] This was the first time that two pheromones, the alarm pheromone neryl formate (**T139**) and the sex pheromone **T137**, have been demonstrated to be compounds of the same gland secretion in a mite.[702]

A highly oxygenated monoterpene is (*S*)-3,7-dimethyl-2-oxooct-6-ene-1,3-diol (**T140**) the aggregation pheromone of the Colorado potato beetle *Leptinotarsa decemlineata*.[703] The identification of this male-produced compound – the first male pheromone identified for a chrysomelid beetle – marked a true breakthrough, as for a long time it was believed that the pheromone is produced by the females. Open chain monoterpenes are also represented by close-range pheromones of *Callosobruchus* weevils. In the azuki bean weevil *Callosobruchus chinensis*, the dicarboxylic acid **T141**, callosobruchusic acid, shows an enantiomeric composition of *R*:*S* ~3.5:1.[704] The dihydro product of **T141**, forming a mixture of stereoisomers, acts as a pheromone of the cowpea weevil *Callosobruchus maculatus*.[145,146] The natural product is represented by a blend of (2*R*,6*S*)-:(2*S*,6*R*)-:(2*S*,6*S*)-:(2*R*,3*R*)- = 43:38:18:trace.[146]

Only one new structure of a bark beetle pheromone could be assigned during the past decade: the aggregation pheromone of the ambrosia beetle *Platypus quercivorus* was shown to be (1*S*,4*R*)-1-methyl-4-(1-methylethyl)cyclohex-2-en-1-ol (**T142**), quercivorol.[705] Another oxygenated *p*-menthene is vesperal (**T143**) (*R*)-3-methyl-6-(1-formylethenyl)-2-cyclohexen-1-one, 10-oxoisopiperitenone) and the corresponding alcohol (vesperol). The two compounds constitute the female-produced sex pheromone of the long-horned beetle *Vesperus xatarti*.[706]

Males of the palm bunch moth, *T. mundella*, contain (3*S*,6*S*)-6-ethenyl-2,2,6-trimethyl-tetrahydropyran-3-ol (**T144**), a pyranoid form of linalool oxide.[707] Apart from vanillin (**A31**), the bis-nor-diterpenoid 6,10,14-pentadecan-2-one and the corresponding secondary alcohol were found to be present. The stereochemistry of the latter two compounds remained undetermined, and bioassays with mixtures of stereoisomers failed.

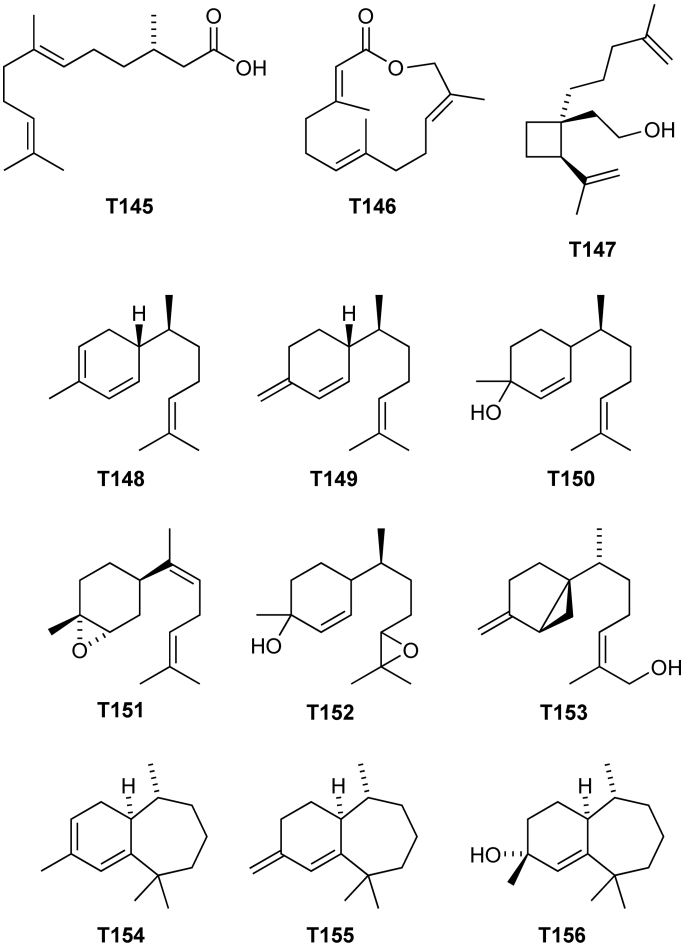


4.04.6.2 Sesquiterpenes

Because of the development of highly sensitive instruments and advanced techniques, several new sesquiterpenes could be identified that play a role in systems of chemical communication among insects. For some known compounds, enantiomeric compositions could be determined.

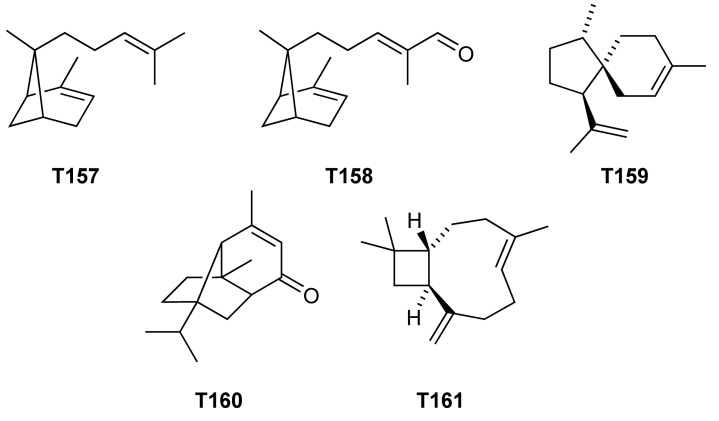
(*E*,*E*)-α-Farnesene (**T53**) was identified as an alarm pheromone of the termite *Prorhinotermes canalifrons*.[708] Once again, (*E*)-β-farnesene (**T54**) has been reported to be a widespread alarm pheromone of aphid species.[709–711] However, in some species other terpenes were found to be released in addition.[710] Moreover, (*E*)-β-farnesene was described as an insect behavior-mediating volatile released by stressed plants (e.g., upon insect attack).[711] The compound may also act as a kairomone for predators of (*E*)-β-farnesene-producing insects.[712,713] Interestingly, **T53** and **T54** were also detected in the urine of female African elephants. However, no behavior releasing effects of the compounds on elephants have been reported so far.[714] Along with geranyl butyrate, (*E*,*E*)-α-farnesyl butyrate was identified as the female-produced pheromone of click beetle species (Elateridae).[715,716] Similar terpene esters were used to attract several click beetle species in the field.[717] (*E*,*E*)-α-Farnesyl acetate proved to be a component of a bouquet of volatiles predominantly made up of oxygenated straight-chain compounds that are released by eggs of the apple moth *C. pomonella* and that appear to serve as a kairomone in host location by the egg-larval parasitoid *Ascogaster quadridentata*.[718] Apart from several straight-chain aliphatics, secretions of the European beewolf *Philanthus triangulum* contain (*S*)-2,3-dihydrofarnsoic acid (**T145**),[719] the oxidation product of (*S*)-2,3-dihydrofarnesol (terrestrol), the typical marking substance of male bumblebees. The biological significance of the acid is not quite clear. The methyl ester of racemic **T145** is the male-produced pheromone of the stink bug *Chlorochroa sayi*,[720] whereas the (*R*)-enantiomer, along with methyl farnesoate and the nor-sesquiterpene **T165** (see below), constitutes the pheromone of *Chlorochroa ligata*.[721] Hydroxylation of (*E*,*E*)-farnesoic acid at the ω-position followed by ring closure would yield the interesting 13-membered ring lactone **T146**. The compound was identified in males of the African butterfly *Amauris niavius* and was called niaviolide.[722] Along with its 10,11-epoxide, keeping (2*E*,6*E*,10*S*,11*S*)-configuration, it may play a role in courtship. The sex pheromone of the yellow scale *Aonidiella citrina*, **T102**, was successfully used to monitor population dynamics of the insect pest in the field.[723] Pheromones of scale insects have been reviewed.[724]

An unusual functionalized cyclobutane is **T147**, the female-produced pheromone of the oleander scale *Aspidiotus nerii*. The structure represents the prenyl homologue of grandisol (**T30**), the sex pheromone of the boll weevil.[725] Several sesquiterpenes have been identified as components of bug sex pheromones.[726] The male-produced sex pheromone of the red-shouldered stink bug *T. pallidovirens* was shown to consist of a blend of methyl (2*E*,4*Z*,6*Z*)-decatrienoate (**A105**), (−)-zingiberene (**T148**), as well as β-sesquiphellandrene (**T149**) and its aromatic derivative, α-curcumene. The pure compounds were not attractive to females, but mixtures of **A105** and any of the sesquiterpenes proved to be attractive.[727] Recently, (7*R*)-(+)-β-sesquiphellandrene, the enantiomer of **T149** has been identified as a male-produced sex pheromone of the red-banded stink bug *Piezodorus guildinii*.[728] A sesquiterpene showing the carbon skeleton of **T148–T149** is zingiberenol (**T150**), a male-produced pheromone component of the Brazilian rice stalk stink bug *Tibaraca limbativentris*. The attractive bouquet seems to be a mixture of stereoisomers, among which at least one shows (1′*S*)-configuration as indicated in structure **T150**.[729] Similarly, bisabolene as well as *cis*-*Z*-bisabolene epoxide (**T151**) and its *trans*-*Z*-isomer, showing the opposite configuration at the epoxide moiety, are major pheromone components in several bug species of the genera *Nezara* and *Acrosternum*.[726] An even higher oxygenated derivative is represented by murganitol (**T152**), the male-specific aggregation pheromone of the harlequin bug *Murganita histrionica*.[730] As indicated by bioassays, the natural product seems to show (1′*S*)-configuration. A bicyclic relative of this group is the sesquiterpene **T153** ((2*Z*,6*R*,1′*S*,5′*S*)-2-methyl-6-(4′-methylenebicyclo[3.1.0]hexyl)hept-2-en-1-ol, sesquisabinen-1-ol), the male aggregation pheromone of the stink bug *Eysarcoris lewisi*.[731,732] Male-produced pheromone candidates of flea beetles of the genera *Phyllotreta* and *Apthona* are himachalene derivatives such as **T154–T156**. The stereochemical composition of the natural products does not seem to be clear in all cases;[733–735] in *Phyllotreta crucifera*, **T154** keeps (5*R*,5a*S*)-configuration.[736] The aromatic compound corresponding to **T154–T156** has also been identified in some species.



The prenyl homologues of α- and β-pinene, α- and β-*trans*-bergamotene (**T157**), and its isomer with an exocyclic double bond of unknown configuration have been described as a male-produced attractant of the parasitic wasp *Melittolia digitata*.[737] The aldehyde, (*Z*)-*exo*-α-bergamotenal (**T158**) was isolated from male *Eysarcoris* stink bugs.[738] The correct structure of the male-produced pheromone of the broad-horned flour beetle *Gnathocerus cornutus* was shown to be (1*S*,4*R*,5*R*)-α-acoradiene (**T159**).[739] The interesting novel sesquiterpene **T160** has been reported to be a putative sex pheromone of the stink bug *Thynacantha marginata*,[740] and the structure of the compound has been confirmed upon synthesis. Despite both enantiomers being prepared,[741] no bioassays have been reported so far.

The (−)-enantiomer of β-caryophyllene (**T161**) is a female-specific volatile in the multicolored Asian lady beetle *Harmonia axyridis*;[742] however, no biological activity has been reported.



4.04.6.3 Norterpenes

The most widespread norterpene in insects and plants is 6-methyl-5-hepten-2-one, sulcatone (**T78**); however, not much is known about its biological significance. For some references see Francke and Schulz;[1] recently, sulcatone has been found in myrmecophilous rove beetles to avert attacks of their host ant *L. fuliginosus* by mimicking the alarm pheromone of the ants.[743] Recently, the same compound has been identified as an essential component of the pheromone bouquet of the bedbug *C. lectularius*.[744] It is generally accepted that sulcatone represents a degradation product of an (activated) monoterpene precursor, which lost two carbon atoms upon either degradative oxidation or retro-aldol reaction. However, a mixed biosynthesis from a prenyl unit and an acetate unit may also be conceivable: acetoacetate could well be alkylated with 3,3-dimethylallyl diphosphate, and the intermediate β-ketoacid could form sulcatone upon decarboxylation (see **Figure 3**). In this context, it should be noted that sulcatone may be found in relatively high concentrations in a certain organism while the same individual does not seem to contain any (mono)terpenoid that could serve as a realistic precursor. On the contrary, sulcatone can be rather rapidly formed (from an unknown stock?) by plants upon damage, which indicates a short biosynthetic access.

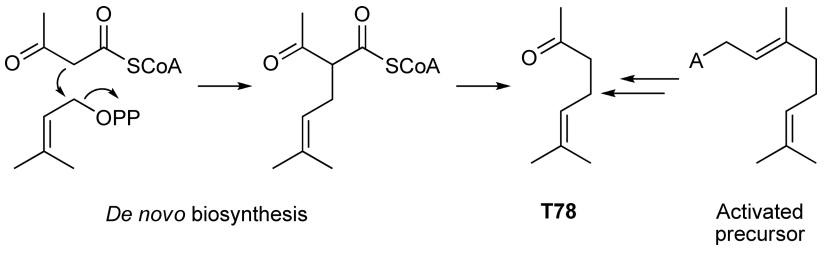


Figure 3 Possible biosynthetic pathways leading to sulcatone (**T78**).

The bark beetle pheromone frontalin (**T83**), which is widespread among *Dendroctonus* species, is easily formed from the epoxide of 6-methyl-6-hepten-2-one, an isomer of sulcatone.[745] Using radiolabeled precursors, it could be shown that the beetles produce frontalin *de novo*.[746] Male Asian elephants, *E. maximus*, release frontalin from the temporal gland on the face. The enantiomeric composition of the compound varies with the physiological state of the emitter, and conspecific males and females are able to smell whether he is in musth. Specific enantiomeric compositions are particularly attractive to ovulating females.[747] The reduction product of sulcatone, the chiral secondary alcohol, sulcatol is also frequently found in ambrosia bark beetles of the genus *Gnathotrichus* where it serves as an aggregation pheromone.[748] A biotransformation of sulcatol to the epoxide followed by ring closure would yield pityol (**T81**), a pheromone of scolytid beetles of the genera *Pityographus* and *Conophthorus*,[749] that has been successfully used in pest management.[750] Another C8 compound, possibly a bis-nor-terpenoid, is (*R*)-3-ethyl-4-methylpentanol (**T162**), which along with methyl-6-methyl salicylate (**Ar1**) plays a decisive role as a sex pheromone in slave-making ants of the genus *Polyergus*.[215,216] The compounds per se were found completely unattractive.

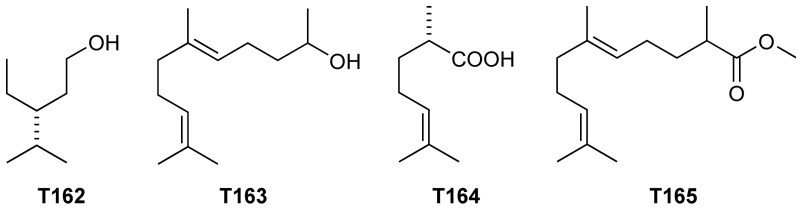
The prenyl homologue of sulcatol, (5*E*)-tangerinol (**T163**), and its (*Z*)-isomer were identified in *Polistes* paper wasps;[751,752] however, the biological significance of the compound is not clear. The corresponding ketone, geranylacetone, is rather widespread in nature, and in some cases biological activity could be assigned.[753]

A nor-terpenoid corresponding to the alcohol **T84** is the acid **T164**, identified as an electrophysiologically active volatile in the dung beetle *K. subaeneus*.[701] A prenyl homologue of this compound is the methyl ester **T165** of unknown configuration, which was identified as a minor component among the volatiles released by stink bugs *Chlorantha* spp.[721] The aldehyde and the alcohol corresponding to **T165** (stereochemistry unknown) have been found to be involved in sex pairing or trail-laying in several termite species.[754,755]

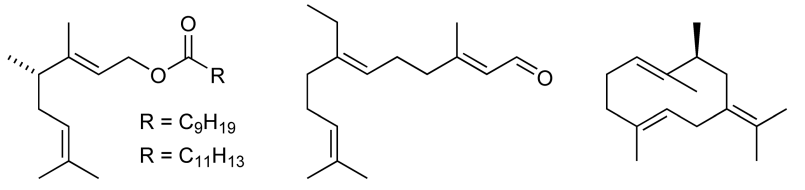
4.04.6.4 Homoterpenes

The homoterpenes **T166** represent a mixture of the decanoate and dodecanoate of (*S*)-4-methylgeraniol. In addition, esters of (*E*,*E*)-3,4,7-trimethyl, a *bis*-homogeraniol, showing a terminal ethyl group, were found to be present.[756] The compounds are trail pheromones of the ponerine ant *Gnamptogenys striatula*.[757] With respect to the biosynthesis of the compounds, it is not clear whether homomevalonate is involved or whether the additional methyl group in **T166** results from a methylation reaction. Methylation of monoterpenes is known: in the myxobacterium *Nannocystis exedens*, the additional methyl group in 2-methylisoborneol is derived from (*S*)-adenosylmethionine.[758] In *Gnamptogenys*, the structure of the *bis*-homoterpene, however, indicates homomevalonate. This may also be true for the homofarnesal **T167** and its (5*Z*)-isomer, which are female-produced sex attractant pheromones of the southern cowpea weevil *C. chinensis*.[759] Earlier, the terpenedioic acid **T37** had been identified as a close-range contact pheromone of this beetle.[760] The *L. longipalpis* complex comprises sand fly species that are the main vector of Chagas disease caused by *Leishmania* spp. in South America. In these sand flies, male sex pheromones belonging to different chemotypes may not only differ in quantitative composition of the attractive bouquet but also show qualitative differences.[761,762] Major homosesquiterpenes are (*S*)-9-methylgermacrene B, **T168**[763,764] and (1*R*,3*R*,7*S*)-3-methyl-α-himachalene (**T169**).[765,766] Ferrulactone (**T107**), a species-specific component of the aggregation pheromone of the rusty grain beetle *Cryptolestes ferrugineus*,[767] has been identified in males of the butterfly *P. rapae*, where it contributes to success in courtship. Whether this is a true norterpene (produced upon degradation of a higher terpene) or whether it originates from a mixed biogenesis involving propionate and acetate remains an open question. However, it is interesting to note that **T170** (brassicalactone), a 'prenyl homologue' of **T107**, was found as a behaviorally active volatile in *P. brassicae* males enhancing male courtship success.[768,769]

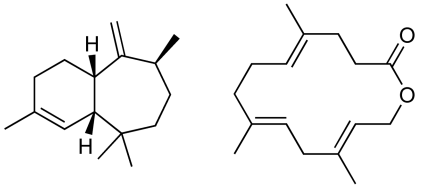
Tribolure (**T109**), 4,8-dimethyldecanal, an important pheromone component in *Tribolium* spp. shows the same carbon skeleton as ferrulactone.[770]



T162 **T163** **T164** **T165**



T166 **T167** **T168**



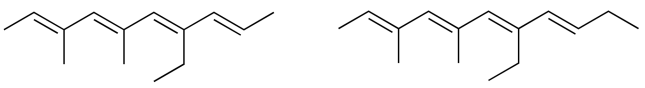
T169 **T170**

4.04.7 Propanogenins and Related Compounds

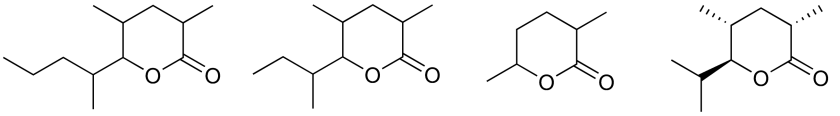
The following section reviews structures of pheromones that are biosynthesized from propanoate or clearly result from a mixed propanoate/acetate (methylmalonate/malonate) sequence. Several compounds showing corresponding carbon skeletons are known from various insect taxa, especially from ants and beetles.[1] As some important stored-product pests use compounds like **P7**, **P8**, or **P11** (see below), efforts have been made to use such compounds in IPM. Consequently, investigations covered (flight)behavior of the insects in the presence of pheromones and host odors[771–773] as well as longevity, pheromone production, and signaling.[774,775]

For compounds such as **P1**–**P32**, showing a distinct branching pattern, polyketide biosyntheses have been postulated earlier;[1] however, unequivocal proof has been shown only in a few cases (see below). The ecological role of polyketides in insects and evolutionary aspects of their biogenesis have been reviewed.[776] It should be pointed out that structural features verified in this group of volatile signals strongly resemble those of secondary metabolites that are typically found in microorganisms. This may indicate biosyntheses involving hitherto unknown endosymbionts, while the stereotypic straight-chain pheromones that are derived from fatty acids may be produced from any organism that uses an acetate pool. A corresponding remark has also been made by Pankewitz and Hilker[776] in their comprehensive review. In this context, it should be noted that the lactone **A39** known as a sex pheromone of scarab beetles[777,778] and 2-ethyl-3,6-dimethylpyrazine (**Ar47**), a component of the trail pheromone of myrmicine ants[779] – and other pyrazines known as insect volatiles – have also been identified in the headspace of marine bacteria. The authors carefully discuss the coincidence of volatile compounds produced by insects and microorganisms and point to possible consequences.[780]

In our earlier review,[1] the compounds presented in the following figures were discussed in more detail.



P1, P2: *Carpophilus*, nitidulid beetles;[781–783]



P3: *Camponotus*, ants,[784,785] *Solenopsis invicta*, fire ant;[786–788]

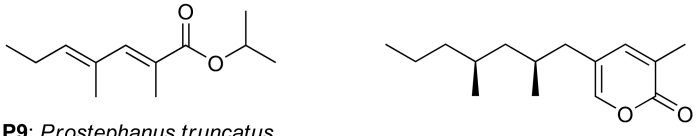
P4: *Camponotus*, ants;[784]

P5: *Xylocopa hirsutissima*, carpenter bee;[789]

P6: *Macrocentrus grandis*, parasitic wasp;[790]

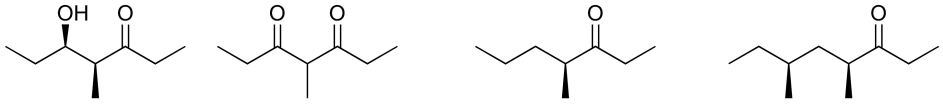
P7: *Sitophilus granarius*, granary weevil;[791,792]

P8: *Rhizoperta dominica*, lesser grain borer;[793]



P9: *Prostephanus truncatus*, Bostrychid beetle;[794]

P10: *Supella longipalpa*, brown-banded cockroach;[795–797]

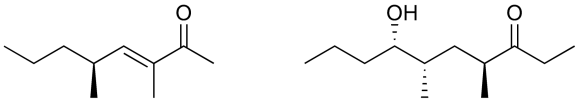


P11: *Sitophilus oryzae*, rice weevil;[798,799]

P12: *Sitona lineatus*, pea weevil;[800]

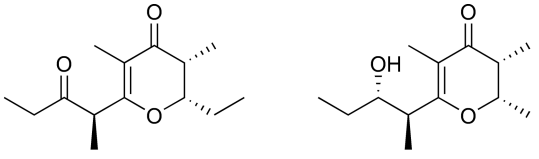
P13: leaf-cutting ants,[801–804] 'daddy long-legs,' opilionid spiders,[805] wasps;[806]

P14: *Leiobunum*, daddy long-legs;[805]



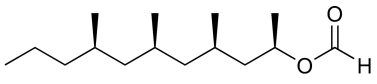
P15: *Manica*, ants;[807]

P16: *Lasioderma serricorne*, cigarette beetle;[808]



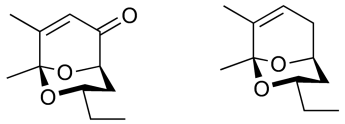
P17: *Lasioderma serricorne*, cigarette beetle;[809,810]

P18: *Stegobium paniceum*, drug store beetle,[811,812] *Anobium punctatum*, anobiid beetle;[813]

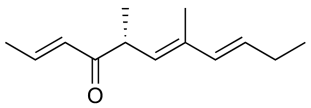


P19: *Lardoglyphus konoi*, acarid mite;[814,815]

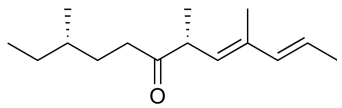
P20: *Scolytus*, elm bark beetles;[816,817]



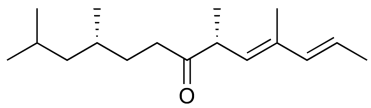
P21, P22: *Hepialus hecta*, hepialid moth;[818]



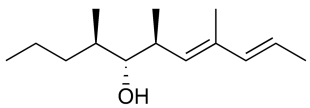
P23: *Matsucoccus josephi*, Israeli pine scale;[819]



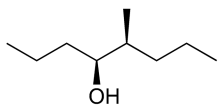
P24: *Matsucoccus feytaudi*, maritime pine scale;[820,821]



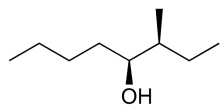
P25: *Matsucoccus*, pine bast scales;[822–824]



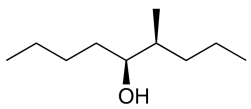
P26: *Cryptocercus punctulatus*, wood roach;[825,826]



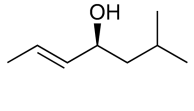
P27: *Rhynchophorus cruentatus*, palmetto weevil;[827,828]



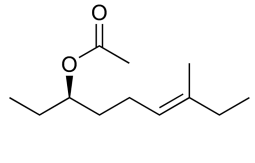
P28: *Rhynchophorus phoenicis*, African palm weevil;[829,830]



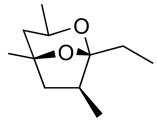
P29: *Rhynchophorus*, palm weevils;[831,832] *Metamasius hemipterus*;[833–835]



P30: *Rhynchophorus palmarum*, American palm weevil;[836–838]



P31: *Cathartus quadricollis*, square-necked grain beetle;[839]



P32: *Cosmopolites sordidus*, banana weevil;[840,841]

Components of the aggregation pheromone of the ambrosia beetle *Megaplatypus mutatus* were shown to be sulcatone (**T78**) and sulcatol (**T80**) as well as pentan-3-ol.[842] The latter, which was already known as the alcohol component of the polyketide ester **P7**, may well be formed from two propanoate units (after decarboxylation and reduction of the corresponding intermediates). Using stable isotope-labeled probes, (*S*)-4-methyl-3-heptanone (**P13**), a widespread pheromone of leaf-cutting ants,[1] was shown to be biosynthesized from three propanoate units following a polyketide/fatty acid-type route.[843] Similarly, a start from acetate followed by the incorporation of four propanoate units proved to form (4*R*,6*R*,8*R*)-4,6,8-trimethyldecan-2-one

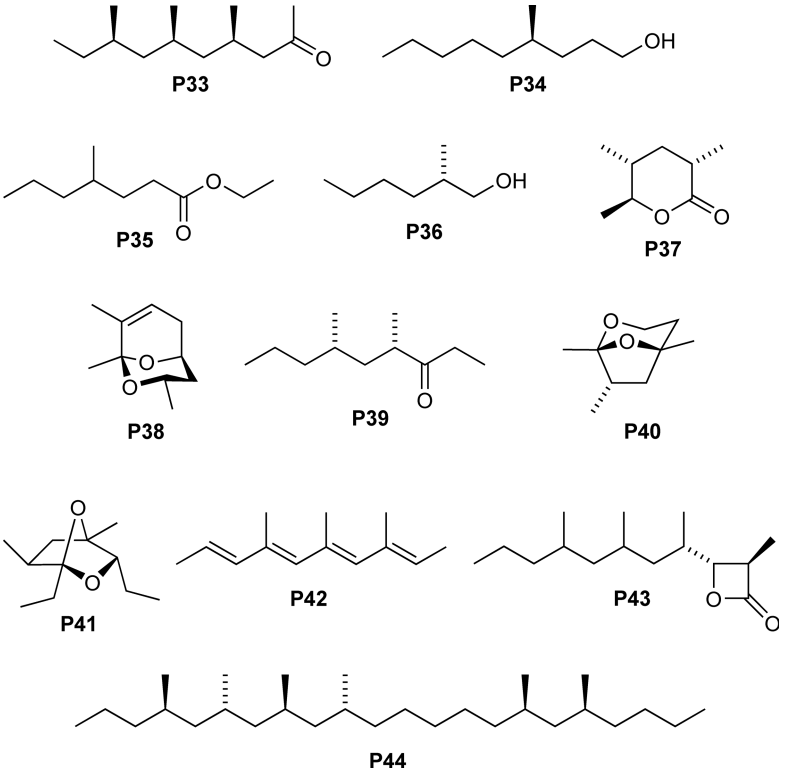
(chortolure) (**P33**), a component of the aggregation pheromone of the storage mite *Chortoglyphus arcuatus*.[844] The ketone **P33** is the carbonyl analogue of the formate **P19**, the pheromone of another mite species. It is accompanied by traces of the corresponding alcohol (2*S*,4*R*,6*R*,8*R*)-4,6,8-trimethyldecane-2-ol and by (4*R*,6*R*,8*R*)-4,6,8-trimethylundecan-2-one as well as higher homologues. In another case, using labeling techniques, a sequence of propanoate–acetate–propanoate–acetate could be shown to yield (*R*)-4-methylnonanol, the female-produced sex pheromone of the yellow mealworm beetle *T. molitor*.[845]

The pheromone of the palm fruit stalk borer *Oryctes elegans* consists of a mixture of closely related compounds, namely the methyl- and ethyl ester of 4-methyloctanoic acid, the corresponding alcohol, and its acetate.[846] The biosyntheses of the carbon skeleton of these compounds may start with butanoate (or two acetate equivalents) followed by propanoate and another acetate equivalent. A male-produced pheromone of the beetle *Nicrophorus vespilloides* (Silphidae), ethyl 4-methylheptanoate (**P35**),[847] may be formed from two propanoate units and acetate. Butanoate (or two acetate units) coupled to propanoate, followed by the reduction of the acid intermediate would yield (*S*)-2-methyl-1-hexanol (**P36**), the almost-only volatile substance present in the mandibular gland of several *Cataglyphus* ants.[848] Trace amounts of some esters of this alcohol have been found in Dufour glands.[849] An acetate–propanoate–propanoate sequence is obvious in (2*S*,4*R*,5*S*)-trimethyltetrahydropyran-2-one (**P37**),[850] whereas in **P4** an acetate starter appears to be linked to three propanoate units (stereochemistry of the natural compound still unknown).[851] Both of these tetrahydropyran-2-ones are reported to be associated with trail following in *Camponotus* ants. Propanoate, accounting for the branched carbon skeleton, may well be involved in the biosyntheses of (1*R*,3*S*,5*S*)-1,3,8-trimethyl-2,9-dioxabicyclo[3.3.1]nonan-7-ene **P38**. This bicyclic acetal acts as a male-produced pheromone in the hepialid moth *Endoclita excrescens*.[852,853] It is structurally close to **P22**, another hepialid pheromone. Both **P22** and **P38** occur in *Hepialus hecta*.[818] Apart from (*S*)-4-methyl-3-heptanone (**P13**) and a higher homologue (4*S*,6*S*)-4,6-dimethyl-3-nonanone (**P39**) as well as (4*S*,6*S*)-4,6-dimethyl-3-octanone (present in minor amounts), are released by caddis flies *Potamophylax* spp. and *Glyphotaelius pellucidus*.[854] In *Potamophylax*, **P39** (possibly derived from four propanoate units) is accompanied by the new bicyclic acetal (1*R*,3*S*,5*S*,7*S*)-1-ethyl-3,5,7-trimethyl-2,8-dioxabicyclo[3.2.1]octane (**P40**), whereas *Glyphotaelius pellucidus* contains the new (1*S*,3*S*,4*R*,6*S*)-1,3-diethyl-4,6-dimethyl-2,8-dioxabicyclo[2.2.1]heptane (**P41**) or its (1*R*,3*R*,4*S*,6*S*)-stereoisomer.[855] The ketones as well as the bicyclic acetals proved to be electrophysiologically active; however, their biological significance remained unknown. The bicyclic acetal (**P41**) may be produced from (*E*)-2,4-dimethyl-6-nonen-3-one, which was found to be present in the caddis flies in small amounts.[856] Epoxidation followed by intramolecular ring closure would produce the target compound following a mechanism similar to that yielding the bark beetle pheromones brevicomin (**A27**) and frontalin (**T83**) from unsaturated ketones (see Francke and Schulz[1]). However, the formation of **P40** from a 4,6-dimethyl-3-nonanone precursor is not immediately obvious. The compound is a stereoisomer of sordidin (**P32**), the male-produced aggregation pheromone of the banana weevil *Cosmopolites sordidus*.[840,841] Both compounds may be produced from different stereoisomers of the same principal precursor.

According to the careful investigations of Bartelt and his group, using isotope labeling, the branched polyenic pheromones of sap beetles (Nitidulidae) such as **P1** and **P2** were found to be made up of propanoate/methylmalonate units, and (in some cases) acetate and/or butyrate.[856] The structures of these chemical signals are rather stereotypic,[857] and consequently cross attraction between species is not a rare case.[858,859] A certain specificity at the receptor site is, however, evident. Males of *Colopterus truncatus* produce a mixture of four compounds, among which (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**P42**) is the main component. Two additional compounds, (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-octatriene and its homologue (2*E*,4*E*,6*E*)-2,4,6-nonatriene, add to the aggregation pheromone, while the fourth compound, (2*E*,4*E*,6*E*,8*E*)-4,6,8-trimethyl-2,4,6,8-undecatetraene, the homologue of the main component, was found to be not registered by the beetles' antennae.[860] It may, however, have an interspecific function.

The final examples of polyketide structures in pheromone chemistry are represented by two β-lactones that are male-specific, electrophysiologically active volatiles released by feeding striped cucumber beetles, *Acalymma vittatum*.[861] The minor component, **P43**, appears to be made up of five propanoate units. The substitutents at the oxetan-2-one ring were found to keep (3*R*,4*R*)-configuration while the stereochemistry along the side chain remained undetermined. The major component carries an additional methyl group giving rise to a dimethyl formation at the end of the side chain. The structure of the latter compound was verified by independent syntheses of (3*R*,4*R*,1′*R**,3′*R**,5′*R*)-3-methyl-4-(1,3,5,7-tetramethyloctyl)oxetan-2-one, which

perfectly matched the analytical data of the natural product.[862] Because of the terminal branching, the starter of the biosyntheses of the main compound cannot be propanoate; however, an amino acid precursor like valine seems to be conceivable. The *Acalymma* compounds show the highest number of stereogenic centers found in insect pheromone chemistry, so far. At this point it should be noted that (4*R*,6*S*,8*S*,10*R*,16*R*,18*S*)-4,6,8,10,16,18-hexamethyldocosane, **P44** (or its enantiomer), a cuticular hydrocarbon of the cane beetle *Antirogus parvulus* shows even six stereogenic centers. This hydrocarbon is accompanied by smaller amounts of the 18-nor-compound; however, both are not sex specific, and no behavior-mediating capacity has been reported.[863]

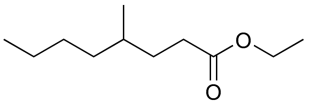


4.04.8 Mixed Structures

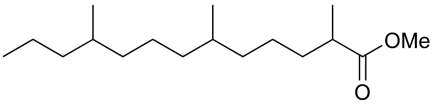
This section summarizes structures of pheromones showing methyl-branched carbon skeletons, whereas the extra-methyl groups cannot be immediately associated with the incorporation of propanoate during biosynthesis. A terminal branching (*iso*-branching) may be caused when the biosynthesis starts with an 'isoprene unit' or with a valine or leucine precursor. After transamination and decarboxylation, valine may yield a branched C4 starter in which leucine may be the precursor of a corresponding C5 unit. Finally, starting with a derivative of isoleucine, the biosynthesis of the chain would result in *ante-iso*-branching. Methyl groups along the chain, for example, in (mono)methylalkanes, may be introduced upon incorporation of propanoate (methylmalonate/succinate – see Section 4.04.7) or upon 'chain methylation' with C1 units such as methionine or fragmented acetate[864] or other alkyl-transferring agents. Blomquist and Howard[865] indicate that the biosynthesis of branched hydrocarbons, showing several methylene groups between two methyl branchings, incorporate propanoate. It is obvious that internally branched carbon skeletons of pheromones constantly show an uneven number of methylene groups between the carbons that carry the methyl groups – which would perfectly fit a biogenesis involving propanoate.

Esters of fatty alcohols and 4-methyloctanoic acid (the acid part of **M1** – possibly derived from a sequence involving acetate–acetate–propanoate–acetate) are components of the Dufour gland secretion of the ant *Gnamptogenys moelleri*.[866]

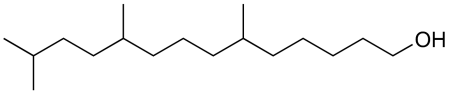
In our earlier review,[1] the compounds presented in the following figures were discussed in more detail.



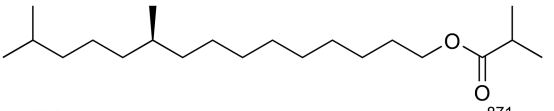
M1: *Oryctes*, rhinozeros beetles;[867,868]



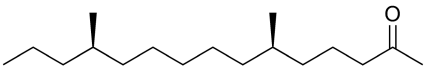
M2: *Euschistus*, stink bugs;[869]



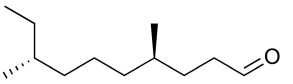
M3: *Stiretrus anchorago*, stink bug;[870]



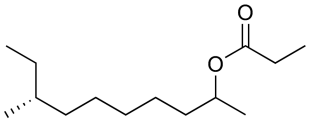
M4: *Euproctis pseudoconspersa*, tussock moth;[871]



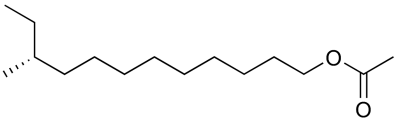
M5: *Diabrotica balteata*, corn rootworm;[872,873]



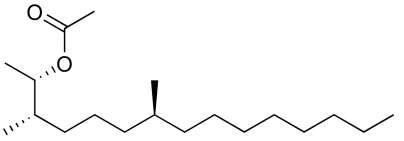
M6: *Tribolium castaneum*, red flour beetle;[874]



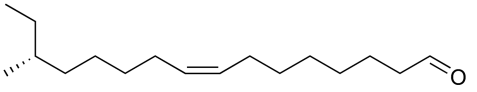
M7: *Diabrotica*, corn rootworms;[875]



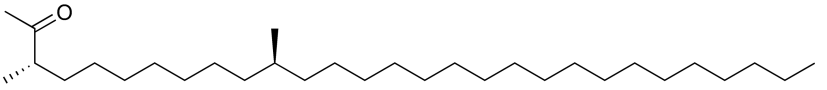
M8: *Adoxophyes* sp. Smaller tea, tortrix moth;[876]



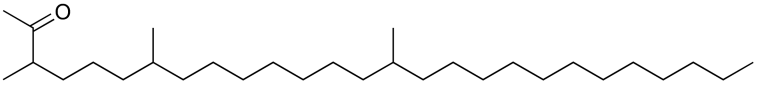
M9: Diprionidae, pine sawflies.[877,878]



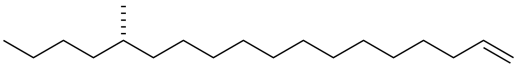
M10: *Trogoderma*, khapra beetles;[879,880]



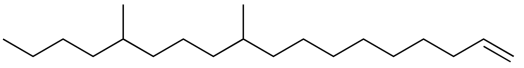
M11: *Blattella germanica*, German cockroach;[881–883]



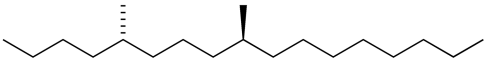
M12: *Schistocerca gregaria*, desert locust;[884]



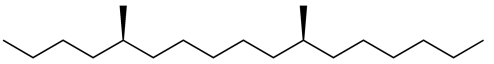
M13: *Lyonetia clerkella*, leaf miner moth;[885]



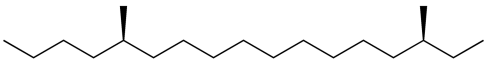
M14: *Lyonetia prunifoliella*, leaf miner moth;[886]



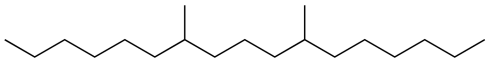
M15: *Leucoptera malifoliella*, mountain-ash bentwing;[887,888]



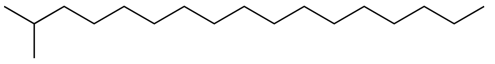
M16: *Lambdina fiscellaria*, western hemlock looper.[889]



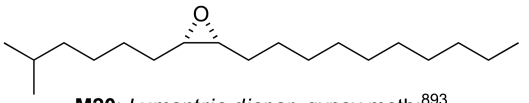
M17: *Nepytia freemani*, false hemlock looper;[890]



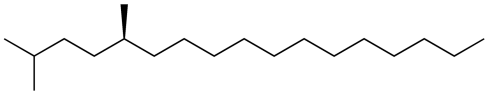
M18: *Lambdina athasaria*, spring hemlock looper;[891]



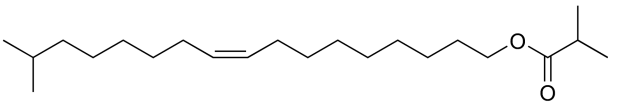
M19: *Holomelina* sp., arctiid moth;[892]



M20: *Lymantria dispar*, gypsy moth;[893]



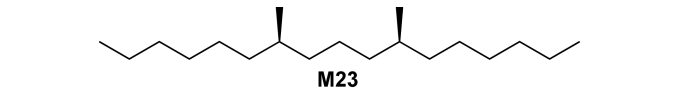
M21: *Lambdina fiscellaria*, western hemlock looper;[894]



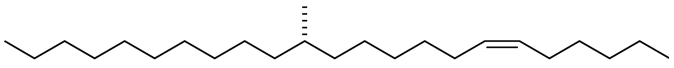
M22: *Euproctis taiwana*, tussock moth.[895]

A bunch of monomethylalkanes acts as a contact sex pheromone in the chrysomelid beetle *Gastrophysa atrocyanea*. The compounds, methylheptacosanes and methylnonacosanes, are associated with the females' cuticular surface lipids and release mating behavior in the males. Synthetic 9- and 11-methylheptacosane as well as 9- and 11-methylnonacosane showed biological activities.[896] Similarly, 9-methylpentacosane is the first component of a contact pheromone identified in buprestid beetles. It is produced by females of the emerald ash borer *Agrilus planipennis*.[897] Hydrocarbons of slightly high volatility, 7-methylheptadecane and 7,11-dimethylheptadecane, comprise the female-produced sex pheromones of both, the spring hemlock looper *Lambdina atarsia*, and the pitch pine looper *Lambdina pellucidaria*.[898] Bioassays using pure stereoisomers showed that only (*S*)-7-methylheptadecane and *meso*-7,11-dimethylheptadecane (**M23**) are registered by the males' antennae, and only the binary mixture of the two compounds proved to be attractive in the field.[899] The 7,11-substitution pattern may suggest a propanoate–acetate–propanoate sequence to be involved in the formation of the C7–C12 part along the chain. A methyl-branched alkene (*Z*)-13-methyl-6-icosene (**M24**) is the female-produced sex pheromone of the herald moth *Scoliopteryx libatrix*.[900] Results of bioassays with synthetic stereoisomers suggest the natural product to keep (*S*)-configuration.[901]

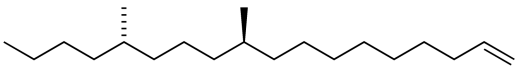
More chemical specificity is represented by the structure of the sex pheromone released by females of the Korean population of the apple leaf miner *Lyonetia prunifoliella*. The main compound is 10,14-dimethyl-1-octadecene (**M25**), which is accompanied by minor amounts of the saturated hydrocarbons, 5,9-dimethyloctadecane and 5,9-dimethylheptadecane.[902] Earlier, the three compounds were reported to be components of the sex pheromone for the North American population of the moth.[903] During bioassays in Korea, all (*S*,*S*)-configured isomers proved to be electrophysiologically active, whereas (10*S*,14*S*)-dimethyloctadec-1-ene elicited the strongest response. In contrast to the North American insects, (10*S*,14*S*)-**M25** was found to attract the moths as a single compound. In the case of the *Lyonetia* compounds, the structure **M25** suggests the incorporation of two propanoate units interrupted by an acetate unit.



M23



M24



M25

Arrangements involving the formation of two (or more) methyl groups and formal interruptions by acetate will typically result in an uneven number of methylene groups between the methyl branchings – just as it is shown by most of the branched-chain insect pheromones.

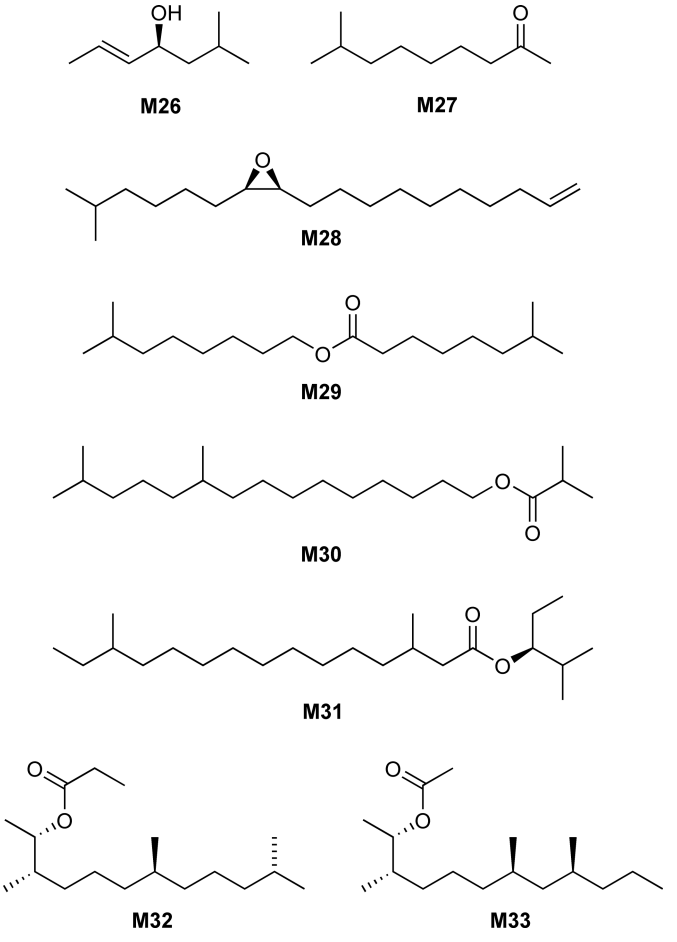
In the abovementioned hydrocarbons, acting as pheromones of certain moth species, the biologically active stereoisomers could be identified because of the bioassays with pure compounds. Nevertheless, the stereoisomeric composition of the natural products remains unknown. Very unfortunately, today there is no way to unambiguously determine the enantiomeric composition of very small amounts of mono- or dimethylalkanes containing more than 10 carbon atoms. Enantioselective GC, usually the method of choice, will not work in these cases, as chiral discrimination of the known stationary phases is too small. As a result, enantiomers will not be separated.

Males of the palm weevil *Rhabdoscelus obscurus* release 2-methyl-4-octanol as a pheromone. Although the Hawaiian strain of the species has only this compound, the Australian strain additionally contains 2-methyl-4-heptanol and (*E*)-6-methyl-2-hepten-4-ol (**M26**, rhynchophorol).[904] In the American palm weevil, rhynchophorol keeps (*S*)-configuration.[905] A similar bouquet, including 4-methyl-5-nonanol (possibly (4*S*,5*S*)-configuration), was found in the West Indian sugarcane weevil *Metamasius hemipterus*.[906] Attraction of palm weevils to pheromone sources is quite often strongly enhanced by host odors that act as synergists.[907] The sugarcane weevil *Sphenophorus levis*, uses (*S*)-2-methyloctan-4-ol,[908] that is, the configuration at the hydroxyl group is opposite to that in rhynchophorol. Although it appears that palm weevils are not very sensitive to the stereochemistry of their pheromones, the fact that different stereoisomers (enantiomers) may be found in different species indicates that these insects may very well distinguish.

Involvement of an amino acid starter is even more obvious in 8-methylnonan-2-one (**M27**), the female sex pheromone of the desert spider *Agelenopsis aperta*.[909] A minor component, 6-methylheptan-3-one does not seem to play a role in the spider's communication system. The same starter may also account for the biosynthesis of (*Z*)-2-methyl-7-octadecene, the sex pheromone of two allopatric moth species *Lymantria lucescens* and *Lymantria serva*.[910] The closely related (11*S*,12*R*)-11,12-epoxy-17-methyl-1-octadecene, **M28**, 'terminally' unsaturated disparlure, is a trace component in the secretion of the sex pheromone gland of the gypsy moth *L. dispar*.[911] The female-produced sex pheromone of the European click beetle *Elater ferrugineus* is a mixture of esters of 7-methyloctanol, among which 7-methyloctyl 7-methyloctanoate (**M29**) and 7-methyloctyl (Z)-4-decenoate are the main components, accompanied by minor amounts of 7-methyloctyl 5-methylhexanoate and 7-methyloctyl octanoate.[912] The terminal branching in those compounds may well be due to a biogenetic start from leucine. The isobutyrate of 10,14-dimethylpentadecan-1-ol (**M30**) has been identified in the pheromone gland of females of the tea tussock moth *Euproctis pseudoconspersa*. During field tests, the (*R*)-enantiomer was found to be more attractive than the

(*S*)-enantiomer or the racemate.[913] In the biosynthesis of **M30**, valine may act as a starter of the chain and at the same time account for the formation of the isobutyric acid part. Compared with the common structures of conventional moth pheromones, the structure of the *Euproctis* compound looks rather strange. An even more unusual case is represented by **M31**, the pheromone of the Paulowina bagworm *Clania variegata*: an ester made up of (*S*)-2-methyl-3-pentanol and 2,13-dimethylpentadecanoic acid (stereochemistry not assigned).[914]

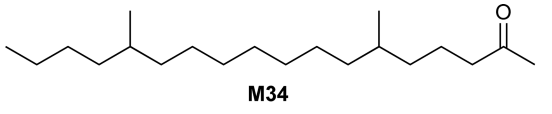
The *ante-iso* branching found in **M9** is a stereotypic feature of female sawfly pheromones. The compounds are acetates or propanoates of methylcarbinols showing (*S*)-configuration at the oxygen moiety (the presence of compounds with (*R*)-configuration could be shown in one case[915]). Apart from a methyl group at C3, there is at least one additional methyl group along the saturated chain, which contains 11–15 carbon atoms. Analyses of sawfly pheromones are extremely difficult due to small amounts available and problems in the separation of stereoisomers of the target compounds. Bioassays are hampered by the sensitivity of the insects against nonnatural stereoisomers of their pheromone.[916] However, examining the pheromone composition released by *Dendrolimus pini*, it could be shown that this sawfly species is not very specific with regard to the structure of the functional group – as long as it is an ester.[917] Pronounced geographic variation has been found between strains: with respect to both the composition of the pheromones and the response at the receptor site.[918] Nevertheless, several new structures could be identified during recent years: (1*S*,2*R*,7*R*)-1,2,7-trimethyldecyl propanoate is the pheromone of *Diprion nipponica*;[919] (1*S*,2*R*,6*R*)-1,2,6-trimethyldodecyl propanoate and the identically substituted tridecyl ester are most important in *Gilpinia pallida*;[920] (1*S*,2*S*,6*S*,10*R*)-1,2,6,10-tetramethyldodecyl propanoate (**M32**) is the pheromone of *Microdiprion pallipes*;[921] and that of *Macrodiprion nemoralis* is (1*S*,2*R*,6*R*,8*S*)-1,2,6,8-tetramethyldecyl acetate (**M33**).[920] Interestingly, egg parasitoids may use sawfly pheromones as kairomones to locate their host.[922]



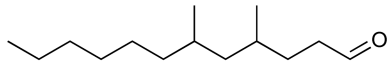
Another unusual pheromone blend comprising a female-produced moth pheromone is the mixture of 6-methyloctadecan-2-one, 14-methyloctadecan-2-one, and 6,14-dimethyloctadecan-2-one (**M34**) (stereochemical composition unknown) identified in the arctiid moth *Lyclene dharma dharma*.[923] The compounds are reported to be electrophysiologically active; however, no results of behavior experiments have been described. It should be noted that **M34** is structurally close to the pheromone of the corn rootworm, **M5**. A mixed biosynthesis is also shown by 4,6-dimethylnonanal (**M35**), sex pheromone of several termite species.[754] The biogenetic scheme of this compound may follow an acetate–acetate–acetate–propanoate–acetate–propanoate way; however, chain methylation cannot be excluded. Major components of the male-produced pheromone of the stink bug *Euschistus obscurus* (Pentatomidae) are reported to be a mixture of predominantly methyl 2,6,10-tridecanoate (**M2**) and methyl (2*E*,4*Z*)-dodecadienoate.[924] The same blend has also been described from closely related stink bugs including *P. guildinii*.[925] The identification of β-sesquiphellandrene as a male-produced sex pheromone of the latter[728] sheds, however, a new light on problems concerning the communication system of this bug. Recently, **M2** has been erroneously termed 'methyl-2,4,6-trimethyl tridecanoate,'[926] which may cause misunderstandings by the nonexperts.

The New World screwworm fly *Cochliomyia hominivorax* is a serious pest to livestock in Central and South America. An attractive fraction containing seven acetates of methylnonacosanols carrying a secondary hydroxyl group were isolated from mature females.[927] A series of papers focusing on the syntheses of various positional isomers and stereoisomers revealed the most active compound to be 6-acetoxy-19-methylnonacosane.[928–931] Using the method of Ohrui and Akasaka,[145,146] the natural product exhibited (6*R*,19*R*)-configuration[133] as shown in **M36**.

In addition to 3,11-dimethylnonacosan-2-one (**M12**) and 3,11-dimethylheptacosan-2-one, products of ω-oxidation, that is, 29-hydroxy-3,11-dimethylnonacosan-2-one and 19,27-dimethyl-28-oxononacosanal (**M37**) as well as the two corresponding C27 compounds are components of a multicomponent contact pheromone contained in the cuticula of sexually mature females of the German cockroach *B. germanica*.[932]

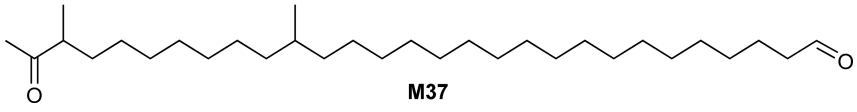


M34



M35

M36



M37

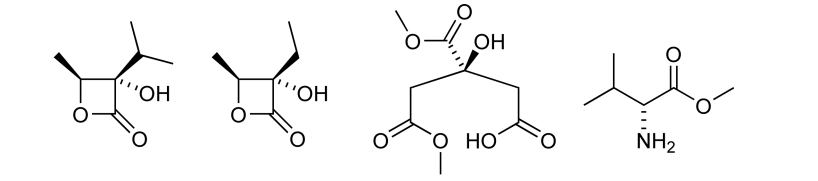
4.04.9 Other Structures

One of the few nonvolatile pheromones discussed in this chapter is a 14.5 kDa lysozyme protein identified as an egg recognition pheromone of the termite *Reticulitermes speratus*.[933]

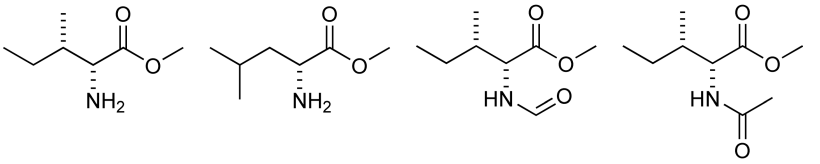
A complex mixture of oligosaccharides and phospholipids acts as a pheromonal phagostimulant, produced by males of the German cockroach *B. germanica*. This secretion functions in the precopulatory behavior, strongly eliciting feeding response in the females.[934,935]

During larval stage or as adults, males of some butterfly species sequester pyrrolizidine alkaloids from their host trees. Subsequently, the nitrogen-containing part of the molecule, the so-called necine bases, are transformed into the volatiles **Ar50–Ar52** that play a role in courtship of the species (see Section 4.04.4.1). Although the biological significance of these compounds has been well established, the metabolic fate and possible biological role of the aliphatic acid part of the alkaloids remained largely unclear. In the giant danaid butterfly *I. leuconoe*, it could be shown that the degradation products 2-hydroxy-2(1-methylethyl)-3-butanolide (viridifloric acid β-lactone) (**O1**) and the corresponding nor-compound 2-hydroxy-2-ethyl-3-butanolide (**O2**) are components of a complex mixture present in the hairpencil glands of the males.[248] Viridifloric acid β-lactone elicited a strong electrophysiological reaction in the antennae of both males and females. A mixture containing mellein (**Ar8**), phenol (**Ar4**), danaidone (**Ar50**), farnesol (**T55**), geranyl methyl sulfide, and viridifloric acid β-lactone proved to be highly attractive to females. The natural β-lactones keep (*S,S*)-configuration as shown in the depicted structures.[936]

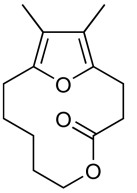
Another small and unusual compound is the chiral citric acid dimethylester **O3**, named cupilure. The natural product showing (*S*)-configuration is the female-produced sex pheromone of the wandering spider *Cupiennius salei*.[937,938]



O1 **O2** **O3** **O4**



O5 **O6** **O7** **O8**



O9

Female-produced pheromones of several scarab beetles (*Phyllophaga* spp.) have been shown to be derivatives of amino acids. The sex pheromone of the cranberry white grub *Phyllophaga anxia* is a mixture of the methyl esters of L-valine (**O4**) and L-isoleucine (**O5**).[497] Recently, it became evident that at least three pheromone races exist that respond differently to the two compounds or blends thereof.[939] While valine methyl ester, possibly its L-enantiomer **O**4, was identified to be the sex pheromone of *Phyllophaga georgiana*, *P. anxia*, *Phyllophaga gracilis*, and *Phyllophaga postrema* were attracted to **O5**.[940] The methyl ester of L-leucine (**O6**) proved to be the sex pheromone of *Phyllophaga lanceolata*.[941] Addition of **O4** or **O5** to **O6** in 1:1 mixtures completely inhibited attraction of males. The pheromone bouquet of *Phyllophaga elenans* adds a special feature: Apart from the ester **O5**, the *N*-formyl- and *N*-acetyl-products **O7** and **O8** could be identified.[942] Although **O7** was shown to attract males, **O8** proved to be behaviorally inactive. In field tests, addition of either **O7** or **O8** to **O5** in 1:1 mixtures did not increase the attractivity of **O5**.

Another unusual structure is represented by the male-produced pheromone of the chrysomelid beetles, *Galerucella calmariensis* and *Galerucella pusilla*. The compound, 12,13-dimethyl-5,14-dioxabicyclo[9.2.1]tetradeca-1(13),11-dien-4-one, (**O9**) is a lactone including a 3,4-dimethylfuran substructure.[943] Similar

compounds have been described as the so-called furan fatty acids (F-acids) occurring in plants[944] and fish[945] as well as in marine and freshwater invertebrates.[946] In the urine of cattle, dicarboxylic acids were identified representing products of an ω-oxidation at the alkyl chain of F-acids.[947] Similarly, **O9** may be formed from an F-acid precursor upon oxidation to a corresponding ω-hydroxy acid that would yield **O9** after lactonization. Plants and microorganisms produce F-acids from polyunsaturated fatty acids, while the two methyl groups at positions 3 and 4 of the furan ring are introduced through methylation.[948] It may well be that 2,5-dialkylfuran components of the cuticular lipids of Lepidoptera[272] are biogenetically related to **O9** and, therefore, similar unusual pheromone structures may be found in the future.

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Biographical Sketches

Professor Wittko Francke, retired professor of organic chemistry at the Universität Hamburg started his chemistry-oriented investigations in chemical ecology as early as 1969. His main research activities cover structure elucidation and synthesis of semiochemicals from insects and (their host) plants. Investigations are carried out in close cooperation with biologists all over the world. Honorary doctoral degrees of Gothenburg University (1997) and Lund University (2005), the honorary medal of the International Society of Chemical Ecology (1995) as well as the Otto-Wallach-Medal of the German Society of Chemistry (GDCh 1996), and the Karl-Escherich-Medal of the German Society of General and Applied Entomology (GDaaE 2005) reflect his scientific standing.

Professor Stefan Schulz studied chemistry at the Universität Hamburg where he got his Ph.D. degree in 1987 under the guidance of Professor W. Francke. After a postdoctoral work with Professor J. Meinwald at the Cornell University, he returned to Hamburg, where he founded his independent research group. In 1994, he received a habilitation degree and *venia legendi*, and in 1997, he was appointed as a full professor of organic chemistry at the Technische Universität Braunschweig. His main research areas are the identification, synthesis, and biosynthesis of extracellular signal compounds in arthropods, bacteria, and vertebrates.

4.05 Pheromones in Vertebrates

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4.05.1 Introduction

4.05.1.1 Definition of a Pheromone

About 50 years ago, Karlson and Lüscher coined the term 'pheromone' to describe chemicals that are 'excreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction.'[1] They created this term in conjunction with the then recent structure elucidation of 10*E*, 12*Z*-hexadecadien-1-ol (bombykol), a long-chain 16-carbon alcohol that female silkmoths (*Bombyx mori*) produce to attract mates. Notably, the concept is a biological one. Since that discovery many hundreds of structures with similar types of activity on conspecifics (members of the same species) have been identified, and it is now commonly accepted that most animals use chemicals to mediate a wide variety of intraspecific social interactions. Many hundreds of invertebrate pheromones have been identified. However, in spite of compelling evidence that with the possible exception of birds, vertebrates commonly use chemical cues to communicate (exchange information) with conspecifics, only a handful of vertebrate pheromones have been identified. The complication seems to be related to the biological complexity of vertebrates whose large brains and complex sensory systems allow them to process more complex cues and use them in complex and subtle manners that are difficult for biologists to measure. Indeed, many mammalian pheromones might best be considered as subtle modulators, rather than drivers, of behaviors. Thus, the original definition of a pheromone has been frequently revisited and many revisions suggested;[2–4] however, no new definition has gained acceptance and the term, pheromone, is now commonly used to describe almost any chemical cue that mediates information transfer between members of the same species and to which organisms are in some manner predisposed to respond. We use this broad definition here.

4.05.1.2 Biological Activity of Pheromones: Defining Principles

Pheromones are typically defined by the biological actions they induce, which may be either behavioral and/or physiological and need not always be immediate. To understand pheromones, one must understand their biological function. These functions are as diverse as the life histories of the species that use them. Generally, the actions of pheromones are species specific, although there are exceptions because the ecology of animals

does not always mandate that they be so. This point is sometimes misunderstood because most insect pheromones are species specific (see Chapter 4.04). Generally, pheromones are quite potent, although there are exceptions in cases where animals do not have to communicate over large distances. Another complex and at times confusing issue is that roles of pheromones can be subtle; cognitive function, including memory and perception of ecological context, play important roles in the behavior of 'higher' vertebrates and thus may modulate their responsiveness to pheromones. The latter attribute complicates our ability to determine whether and how species use pheromonal compounds. Additionally, most vertebrates have evolved to use mixtures of compounds as pheromonal cues, the meaning of which may even change with composition, further complicating isolation, identification, and understanding. Although vertebrates use mixtures in many ways, we focus on two proposed by Johnston:[2,5] 'blends' in which ratios of two or more pheromonal compounds determine overall activity, and 'mosaics' in which a variety of components need to be present in order to convey the message, such as tiles in mosaic painting. These terms are not mutually exclusive; there is a continuum of ways in which multicomponent pheromones are used and full understanding is often difficult to reach because odor perception is complex and, consequently, poorly understood. Here, we will not restrict the requirement that the components of blends need to be present in highly specific ratios to be pheromonally active or that all components of a mosaic need be present to convey a message. Rather, we subscribe to the simple notion that pheromones are odors, and, as such, represent perceptual entities that originate from numerous stimuli but that the brain integrates as one. Notably, it is clear that the vertebrate nervous system is elegantly suited for discriminating complex odor mixtures. Moreover, more complex vertebrates tend to perceive and use more complex mixtures.

Pheromones that elicit behavioral responses are often termed 'releasers,' whereas those with largely physiological effects are often called 'primers.'[6] However, pheromones often induce both short-term behavioral and long-term physiological effects,[3,4] and it may not always be appropriate to separate the two, so here we do not emphasize these differences. Further, behavioral activity driven by pheromones is often relatively subtle and seems to require cognitive recognition and processing, leading many to employ the term 'signaler' instead of releaser,[7] a term we favor and use here. Behaviorally active pheromones are best known and include sex pheromones, cues that facilitate or drive reproductive behaviors. Aggregation pheromones, cues that stimulate aggregation independent of mating activity, are common, especially in fishes. Many mammals use complex scent marks that serve as identifying and territorial cues. Components of these scent marks can also be pheromonal.[3] Many vertebrate species appear to use alarm pheromones, chemical cues that injured or alarmed animals release and, once perceived by conspecifics, alert them to danger. Here, pheromonal function can be intertwined with self-defense. Priming pheromones are employed by a wide variety of vertebrate species, and their actions may be dramatic. For example, urinary odors of male mice advance puberty in juvenile females (the 'Vandenbergh effect'),[8,9] whereas female urinary odors have the opposite effect. Similarly, male goldfish synchronize their endocrine/reproductive cycles with those of ovulatory females by detecting hormonal sex pheromones released by the latter.[10,11] Not surprisingly, evolution apparently has favored social organisms that can achieve behavioral and physiological coordination through the use of pheromones.

Studies of mammalian pheromones increasingly highlight the roles that conspecific odors, including pheromones, can play in mediating social awareness and that often seem to go beyond the classical definition of a pheromone.[2,4] For example, among rodents it is now clear that individuals are readily discerned by their odors and that kin-related odors are part of this process. Evidence suggests that peptides associated with the major histocompatibility complex (MHC) have a role(s) in these complex cues, but small volatile compounds have also been identified. Modulator pheromones have also been suggested to affect mood and thought processes in humans, although these have not yet been identified or gained wide acceptance.[4]

Because olfactory receptors can detect a wide array of structural types (in principle, any), the evolution of pheromones has been influenced primarily by ecological factors. First, certain types of chemical compounds can travel great distances and convey information in complex environments, including those that lack light. These compounds can be adapted, through evolution, to the medium within which the animal functions (e.g., water or air). Second, other types of chemicals are sufficiently stable that they can convey information for extended periods; terrestrial species benefit most from these. Third, complex structures and/or mixtures of smaller structures can be information-rich and highly specific (i.e., immune to eavesdropping by other species because of the selective nature of olfactory receptors). Finally, products that are readily and naturally produced by

organisms (perhaps to serve metabolic functions) are favored for use in pheromones because the biosynthetic pathways that produce them would already be in place.[11] Indeed, evolution of pheromone systems is complex because they ultimately require simultaneous production and detection of cues; structurally complex compounds are thus not favored and are rarely seen.

4.05.1.3 Biological Activity of Pheromones: Neural Basis of Detection

To have function, pheromones must be produced, detected, and discerned and they must drive a biological response. This requires a specialized nervous system that is highly sensitive to sensory cues. Vertebrates possess multiple, sophisticated chemosensory systems that have become increasingly complex with evolution, giving them the ability to discern complex chemical compounds and mixtures with considerable precision. Because multiple components are often involved and olfactory receptors are not perfectly specific, a type of neural pattern recognition is required.[5,12]

Vertebrates possess three primary chemosensory systems: gustation ('taste'), trigeminal, and olfaction ('smell'); but only one of these, the olfactory system, mediates responses to pheromones. Chemicals that stimulate the olfactory system are known as odorants and comprise one type of biological cue (any entity that stimulates a sensory system). Bouquets of odorants that can be discriminated as specific entities are termed odors. The olfactory system contains olfactory receptor neurons (ORNs) that comprise cranial nerve I and project directly to the forebrain. ORNs are now known to express only one to a few olfactory receptor proteins ('receptors'), which means that the chemoreceptive range of each neuron can be very narrow. The olfactory system also has several subcomponents including the vomeronasal organ, which is described below.

Neurons expressing the same receptors project to brain areas where they both amplify each other's activity and discern complex mixtures of odorants. Nevertheless, the primary unit of discrimination are the olfactory receptors. Fish have only about 100 olfactory receptors whereas mammals have hundreds. Although the specificities of these several classes of receptors are not well-studied, it is clear that they range from being highly specific to rather promiscuous. Small chemical structures can be discriminated by several ORNs/receptors, although single-neuron studies suggest that pheromone receptors are narrowly tuned. ORNs then project to the olfactory bulb where odor processing occurs. In fish, pheromone mapping appears to occur in medial regions of the olfactory bulb. However, in tetrapods (vertebrates with legs), the situation is more complex, because they have a multicomponent olfactory system comprised of the main olfactory epithelium, several small ancillary systems, and the vomeronasal system (VNO or Jacobsen's organ). The last is located in the roof of their mouths.[4,13] Both the main olfactory system and the VNO can mediate responses to social odors (pheromones),[4] especially in experienced animals (e.g., pigs and rabbits). In some species, a single pheromone odor may be perceived in two different brain regions, perhaps as two different but related entities. The VNO appears to be an important system for discriminating pheromones and is required for naive rodents to respond to priming cues. The VNO possesses only microvillar receptor neurons that project to a specific set of mitral (output) cells of the accessory olfactory bulb (AOB). It also has a specialized duct system with a pumping mechanism that links directly with the oral cavity, allowing VNO-equipped animals to sample nonvolatile compounds by physical contact (odorants are pumped through the duct in mucus). The behavior animals exhibit when activating their VNO pump is known as a 'flehmen' and is often associated with a distinctive curling of the lips as they strive to bring nonvolatiles into this organ. Flehmening behavior is commonly measured to quantify the possible presence of a pheromone. Human beings have a VNO *in utero*, but it typically fails to develop after birth and appears nonfunctional.[13]

4.05.1.4 Primer on Chemistry Issues

Most chemists 'grow up' with the notion that a pheromone is a chemical compound. As we have discussed above, the more broadly held view is that a pheromone is a meaningful chemical stimulus, which means that it can comprise more than one compound. Thus, we have attempted to draw clear distinction here between 'pheromone' and 'pheromonal component(s)' (or constituents), the latter being the individual compound(s) that are the causative agent(s) of the behavioral response associated with the pheromone's activity. To be clear, a

pheromone may be single- or multicomponent. Because vertebrates are complex organisms, it is common for their pheromones to be multicomponent mixtures of chemical compounds.

Several issues related to the isolation and structure determination of pheromonal components are the same as those for isolation of any natural product. There are some notable exceptions. Because pheromones often operate with exquisite sensitivity, the pheromonal components are often present at very low concentrations. As with other natural products whose concentrations are very low in the producing organism, it is usually essential that a biological assay be used to guide the fractionation and purification at nearly all stages (see the following Section 4.05.1.5). In the case of pheromones, these assays are often quite time consuming because they require measurement of behavioral changes in whole animals. Thus, the feedback loop for guiding isolation often has a long timeline. The importance of objectivity and sound statistical analysis here cannot be overstated – 'how meaningful was the observation' is a question to which the answer must be clear and must be supported by 'error-barred' measurements. It is common for the pheromonal components to be observable only after they have been emitted from the animal into its environment; when so, the accompanying dilution adds to the challenge of isolation and stands in stark contrast to nearly all other natural product isolation work. A corollary is that the task of isolation usually is made considerably easier in cases where the organ, tissue, or specific body fluid of origin can be identified (at least in the cases of organisms that can be sacrificed). In many instances, mass spectrometry plays a more important role in guiding the intermediate phases of the isolation work. Additionally, while invertebrate pheromones nearly operate in the vapor phase and, therefore, comprise volatile chemicals, because of the frequent use of the VNO by vertebrates, there is, in principle, no limit on the vapor pressure of the compounds vertebrates can use. Of course, when higher molecular weight pheromonal components are used, they must be emitted to the aqueous or terrestrial environment through various excretions.

Pheromonal components tend to belong to well-established structural classes of compounds. This is reasonable from an evolutionary viewpoint. Organisms presumably have evolved to utilize ways to use compounds already available to them through their basic biosynthetic and metabolic machinery (e.g., hormones or bile acids) to gain further advantage through pheromone development and use. These compounds can be used in either unmodified (e.g., see discussion of hormonal pheromones in goldfish) or modified/derivatized (e.g., see discussion of sex and migratory pheromones in sea lamprey) form. Since pheromonal components tend to belong to known structural families, the task of determining the structure once appropriate spectroscopic data for sufficiently pure compounds are at hand is often simpler than for other classes of natural products, where the metabolic structural space that needs to be considered is much greater. Thus, the structural complexity of pheromonal compounds parallels that dictated by the organism's basic biochemical pathways.

Finally, pheromonal compounds tend to have relatively high chemical stability. This is because they must survive, sometimes for long period, in an exposed state in the environment; evolution would disfavor the use of labile compounds. In particular, it is interesting to note that few have a significant chromophore. This is reasonable at a fundamental level because light absorption in an aerobic environment inherently renders a molecule vulnerable to decomposition. A speculative corollary is that there are likely vertebrate pheromones that make use of compounds that have undergone spontaneous chemical modification (e.g., photochemically, oxidatively, and/or hydrolytically) once they have been released to their surroundings by the originating animal as a 'propheromone.'

4.05.1.5 Identifying a Pheromone

Pheromones are defined by the biological actions they induce. To identify a pheromone one must be able to identify and quantify odor-driven behavioral activity. As such, efforts to establish pheromone existence require an interdisciplinary approach that relies heavily on both biology and chemistry. To understand the relevance of the chemistry described in this chapter, the reader must appreciate some biology. The first step in identifying a pheromone is to recognize, often through ethological (or natural behavioral) description of species, that one might exist. A protocol for systematically measuring biological responses that are pheromonally driven then needs to be developed. Bioassays need to be tightly controlled and reproducible, especially for higher vertebrates whose behavior is complicated by cognitive function. Generally, behavioral responses serve as the focus of bioassays, but sometimes physiological (neural and endocrinological) measures are deployed as

well; these are often advantageous because they can be more easily interpreted in an objective fashion. All types of bioassays exist (many are represented in the case studies below). In general, the more meaningful bioassays will examine both laboratory and wild animals to confirm relevance and will use several different but complementary measurements. Unfortunately, instances of falsely identified pheromones attributable to artifacts of domestic animals and/or unnatural laboratory scenarios are not rare. Additionally, because of the vagaries of behavior, a powerful and useful approach commonly used to complement behavioral assay using an endocrinological measure and/or to measure the chemosensitivity of the subject's nervous system. The latter approach can provide considerable insight because olfactory receptor systems are extremely specific and sensitive. This permits screens of putative pheromonal compounds and confirmation of their likely function by testing to see if sensitivity is restricted to neural pathways known to convey social information (e.g., medial olfactory tracts of fish or VNO of mammals).

Often electrophysiological recording from various levels of the subject's nervous system is made to help assess pheromonal activity/presence. The use of electro-olfactogram recording (EOG), which measures voltage changes thought to reflect olfactory receptor activity, is an especially popular screen for aquatic organisms because it works well under water. Neural recording is often employed in combination with various behavioral (or endocrinological) assays to identify biologically relevant fractions resulting from various rounds of purification of the chemicals emitted by the species being studied. This approach is known as bioassay-guided fractionation. It is usually necessary to isolate each individual pheromonal component with a sufficient level of purity to simplify its identification. This is nearly always considerably easier for cases where the active compound is already known and structurally characterized. Mass spectrometric and comparative chromatographic analyses are often sufficient in these instances. For the case of new compounds, it is nearly always necessary to isolate a sufficient quantity (of sufficiently pure) material to permit characterization by nuclear magnetic resonance (NMR) spectroscopy (e.g., 0.1–1 mg, depending on molecular complexity and size). This can be a daunting task since the high sensitivity of the receiving animal often means that pheromone chemicals are present in very low concentration, especially so once they have been emitted to the donor animal's environment. In the most comprehensive studies, new compound identification and structure proof is complemented with chemical synthesis of isolated pheromonal components. Isolation of multicomponent pheromones in complex organisms is challenging, especially so when they are present in their natural environment in low concentrations and operate synergistically so that more than one component needs to be present to elicit full response.

4.05.1.6 The Aims and Organization of This Chapter

In this chapter we review the handful of vertebrate pheromonal cues for which both biological activity and chemical structures are relatively well-established, emphasizing those identified cues for which there is clear behavioral evidence that animals are genetically predisposed to respond to them. For nearly all of the cases discussed, the chemicals that comprise the pheromone were identified either in fluids being released by the research subject or from the environment itself (studies of biological incubates are not addressed). The organization of the discussion for each pheromone consists of a brief introduction to the organism, an overview of important biological aspects of the pheromone, and a summary of the (largely structural) chemical facts associated with each. Only well-understood systems are reviewed for which both behavior and chemistry are established; this is not a comprehensive review of the chemistry of the plethora of compounds proposed to have pheromonal function but for which association with specific behavioral evidence is scant (e.g., many of the constituents of scent glands).

4.05.2 Pheromones in Ancient Vertebrates

Vertebrates (animals with a vertebral spinal column) evolved from a group of ancestral cartilaginous craniates (animals with a cranium or head) approximately 500 million years ago. Only one true group of cartilaginous craniates survived to the present day – the hagfish. This unusual group of jawless, boneless craniates persists in the deep oceans where they probably rely on pheromones because of the lack of light and their possession of a

well-developed olfactory organ. The identity of the putative cues used by hagfish remains totally unknown. The first 'true' vertebrates were the Ostracoderms, a group of jawless cartilaginous fishes from which modern jawed vertebrates evolved. Lampreys are the only surviving members of this group and approximately 40 species exist today. One of these, the sea lamprey, has been studied for pheromonal function. This species relies heavily upon unique, sulfated steroids, as reviewed next.

4.05.2.1 Sea Lamprey

The sea lamprey (*Petromyzon marinus*) has a fascinating migratory life history.[14] It breeds in coastal freshwater streams where its males build simple nests, which females locate using pheromones. Both die a few days after mating. Surviving eggs hatch into filter-feeding, blind larvae that burrow in stream bottoms and grow at varying rates for 3–20 years before eventually metamorphosing into a parasitic form having eyes, a very developed nose, and a sucker-like rasping mouth. Parasitic phase lampreys leave their streams and enter the ocean (or large lakes in the case of landlocked populations) where they locate and prey on other fishes. Sea lampreys grow rapidly and mature within 1–2 years, before returning in the spring to streams to spawn (and die). The tasks of finding suitable spawning streams and then mates are essential and demanding processes. Both of these activities are mediated by potent, now well-understood, pheromones. Both the gender-specific sex pheromone and gender-neutral migratory pheromone systems have been the subject of considerable study because the sea lamprey is an invasive species in the North American Great Lakes. The sea lamprey invaded the Great Lakes about a century ago and the fisheries there have become severely threatened by this species.[15]

4.05.2.1.1 Male sex pheromone: Biology

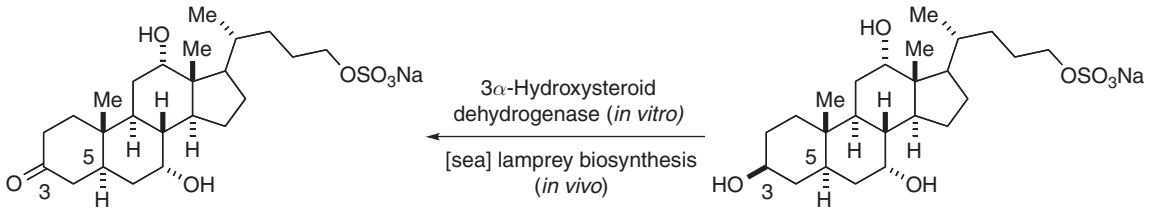
During upstream migration, male and female sea lamprey undergo final maturation and develop behavioral responsiveness to the odor of mature conspecifics of the opposite sex during which time these cease responding to larvae and their odor.[16–19] Males build simple nests and females follow with spawning lasting only a few days. French lamprey fishermen, originally caught females by placing males into traps, a response that has recently been duplicated and attributed to pheromonal odors.[18] While male sea lamprey release attractants in their urine,[18] more recent work has focused on components released through the gill.[16] Using a fractionation scheme based upon EOG recording and Y-mazes, Li and coworkers discovered that male sea lamprey release a substance that is attractive to females. That new compound was shown to be a ketone and was named 3-ketopetromyzonol sulfate (3K-PS, **1**). EOG recording has shown that this steroid is detected by adult lamprey at approximately 10^{-12} mol l^{-1} and that it is attractive to ovulated females in laboratory mazes and in streams.[20–22]

Behavioral studies suggest that 3K-PS is synergized by the presence of other steroidal odorants, perhaps including another ketone, 3-keto-allocholic acid. Immunoassay shows that nonspermiated males (whose odor does not attract ovulated females in the maze) do not release appreciable quantities of 3K-PS, whereas spermiated males release large quantities of immunoreactive 3K-PS. It is estimated that the quantity produced is sufficient to create a large active space (the volume of water that contains detectable concentrations of pheromone) of greater than 10^6 l h^{-1}.[16,23] Not surprisingly, this cue is being explored for use in lamprey control in the Great Lakes.[24] Presently, the lamprey is the only vertebrate for which identified pheromones are being actively considered for managing wild animals.

4.05.2.1.2 Male sex pheromone: Chemistry

To isolate the putative male sex pheromone, the organic content of water conditioned by spermiating male sea lampreys was concentrated by passage through a C18 resin. Chromatographic purification, guided by EOG recording and mass spectrometry, provided a sufficient quantity of material for NMR analysis. Thereby the structure of 3K-PS, **1** was deduced. This was confirmed by preparation of an authentic sample of **1** through the action of 3α-hydroxysteroid dehydrogenase on petromyzonol sulfate (PS, **2**) (Equation 1). The latter was identified over 40 years ago as a major constituent of the larval sea lamprey bile.[25] It is interesting that, like many higher organisms, the sea lamprey uses a bile acid family consisting of allocholic rather than cholic acid (i.e., having a 5α- rather than 5β-hydrogen substituent) derivatives. PS (**2**) is the probable biosynthetic

precursor to the male sex pheromone component 3K-PS (1), and it is also noteworthy that PS has been found nowhere else in nature other than in this group of ancient vertebrates.



1 3-Ketopetromyzonol sulfate (3K-PS) **2** Petromyzonol sulfate (PS)

(1)

4.05.2.1.3 Migratory pheromone: Biology

Migratory adult lampreys are now known to locate suitable riverine spawning habitat using a pheromone released by stream-dwelling larval lampreys. So important is this cue that lampreys cannot find suitable streams if their olfactory systems are ablated (blocked). Indeed, the vast majority of adult lacustrine lampreys select and then spawn in only a few streams, all of which have very high densities of larvae.[26] This ecological strategy makes good evolutionary sense for a species whose juveniles may become widely distributed and whose adults need to quickly locate suitable spawning/nursery habitat because the presence of such habitat correlates strongly with the presence (odor) of larval conspecifics living in it.[15] Although the presence of a migratory pheromone system in sea lamprey was first suggested by historical fisheries records in the Great Lakes, which showed that eradication of larvae from streams using poisons resulted in reduced adult migration, direct evidence has come from behavioral laboratory studies. These key studies have used mazes and natural waters into which larval odors have been added (**Figure 1**).[19] First, low but realistic concentrations of larval lamprey

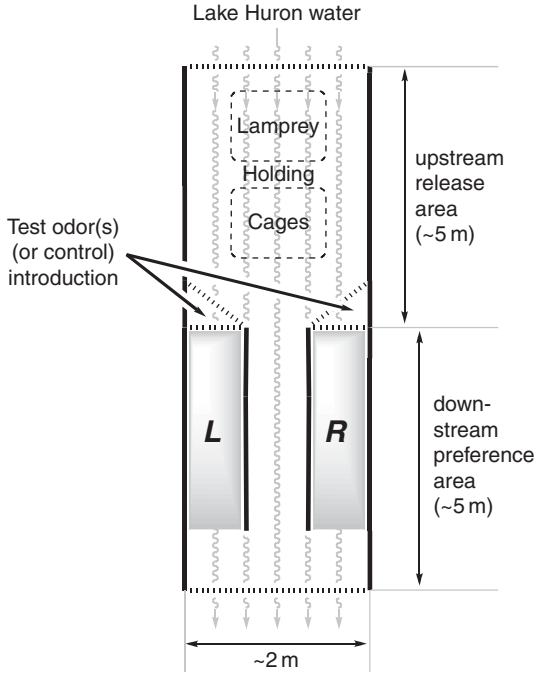


Figure 1 Schematic of the behavior maze used to guide isolation and identification of the sea lamprey pheromone.[19,27,28] Lake water mixed with a small amount of nonlamprey river water flows slowly from top to bottom at a depth of *c*.10 cm in this two-choice maze, where preference of adult lamprey is assessed by measuring the ratio of time in the right channel to time in the left (t_R/t_L), while odor is into one side or the other.

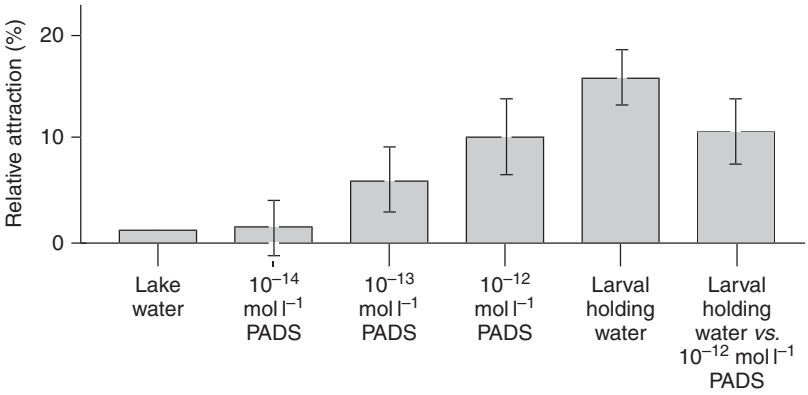


Figure 2 Behavioral responses of migratory sea lamprey tested in the maze to various concentrations of PADS, a concentration of larval holding water known to contain approximately picomolar PADS (the concentration thought to be present in many spawning streams), and an extract of larval holding water versus PADS alone (showing that PADS does not account for all activity).[19,29]

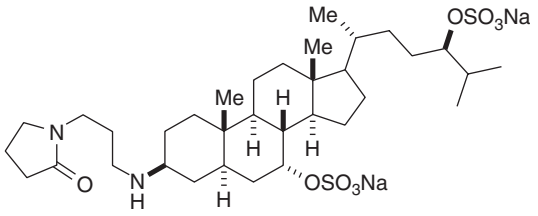
holding water (1 g of larva in 1000 l of water per hour) is attractive to migratory adult lamprey (**Figure 2**). Second, water from streams containing larvae is more attractive to adults than water from streams without larvae, and the addition of larval odor from larval holding water to the stream water increases its attractiveness. This migratory pheromone appears to function within a matrix of natural stream odor(s) and does not appear to be species specific, which perhaps is not surprising given that all North American lampreys use similar spawning and nursery habitats.[15,27]

A bioassay-guided fractionation scheme, based on results from the behavioral maze and electrophysiological recording from the surface of the lamprey olfactory organ (EOG), was used to isolate and then identify three key components of the sea lamprey larval pheromone.[28] These components account for the majority of the activity of the larval pheromone when tested as a mixture at a concentration of 10^{-13} mol l^{-1} in behavioral mazes (i.e., adult lamprey do not distinguish between the synthetic and natural cue) (**Figure 2**). All three components are sulfated steroid derivatives (see below) and each is detected by an independent olfactory receptor.[29] Together, the components function as a synergistic mixture; that is, individual components are not as behaviorally active as the natural mixture even at higher than normal concentrations.[28,30] Because pheromone component ratios do not appear to be critical to activity but the presence of most, if not all, components is, this pheromone mixture might be considered a mosaic. While all three of the migratory pheromone components are biosynthesized by larvae, there is no evidence that any is produced in the parasitic or adult phases[30,31] various components of the pheromone have also been measured in natural waters.[30,32] Finally, behavioral studies have shown that mixtures of the migratory pheromone introduced into the wild attract adults, suggesting that these pheromonal constituents have considerable potential for use in trapping and controlling adults.[24]

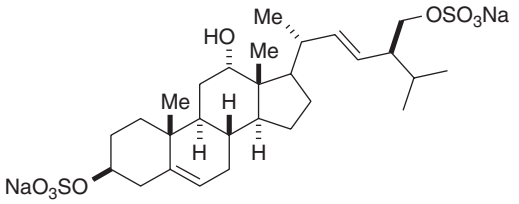
4.05.2.1.4 Migratory pheromone: Chemistry

Lamprey larvae were captured and placed in holding tanks, where they can be maintained indefinitely. Larval holding water was passed through an XAD7HP resin to sequester the organic contents. Subsequent methanol elution brought down material that accounted for essentially all pheromonal activity. Repeated chromatographic fractionations, finally with a shallow gradient on a C18 high-pressure liquid chromatography (HPLC) support allowed identification of three principal components, each of which was both behaviorally active and able to elicit an EOG response. Early mass spectrometry studies suggested that the compounds were probably sulfated steroids, and the first was shown to be petromyzonol sulfate (PS, **2**). The isolation was then scaled up in order to obtain sufficient quantities for NMR analysis. Given the very low quantities present in the larval holding water and the fact that it was not possible to identify the presence of either of the two remaining components in any part of larval tissue, it was necessary to process

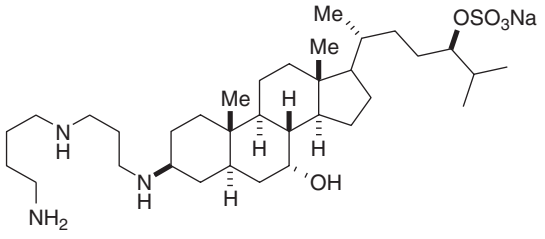
8000 l of the holding water. This yielded approximately 600–800 μg of each of the two unknowns. Extensive spectroscopic studies[33] led to the identification of two unique structures: petromyzonamine disulfate (PADS, **3**) and petromyzonol disulfate (PSDS, **4**).



3 Petromyzonamine disulfate (PADS)



4 Petromyzonol disulfate (PSDS)



5 Squalamine

PADS (**3**) is an aminosterol that is detected by the lamprey olfactory system at concentrations ranging down to 10^{-13} mol l^{-1} (1 g in approximately 70 billion liters of water) and appears to be the single-most important component; the second-most important one is PSDS (**4**). Although PSDS has a similar level of olfactory activity as that of PADS, it only attracts lampreys down to a concentration of 10^{-11} mol l^{-1}, while PADS is attractive to 10^{-13} mol l^{-1}.[28] The third component, the lamprey-specific bile acid petromyzonol sulfate (PS, **2**), is detected with less sensitivity and is the least important of the three. Intriguingly, PADS (**3**) is closely related in structure to squalamine (**5**), a unique aminosterol originally isolated from dogfish sharks.[34] The *N*-(3-aminopropyl)pyrrolidinone sidechain in PADS (**3**) is a unique substructural unit in natural products. It is probable that PADS is derived from squalamine biosynthetically; oxidation of the spermidine side chain in **5** (perhaps, by a primary amine oxidase) and sulfation of the C7-hydroxyl group is all that is necessary. In this light, it is particularly interesting that the shark family is also ancient, with origins dating back over 300 million years. Squalamine has antimicrobial (and antiangiogenic) properties and may well have provided chemical protection against microbes, a feature essential for evolution of higher organisms. Indeed, squalamine has recently been identified in sea lamprey, lending support to this hypothesis of biosynthetic linkage.[35] Thus, it is tempting to speculate that PADS is a metabolite that evolved in lamprey larvae from a self-defense agent and that adults have evolved to discern because it correlates with abundance of larval and spawning habitat.

4.05.3 Pheromones in Advanced (Teleost) Fishes

Fish, here defined as vertebrates with fins and gills that live in water, dominate the waters of the world. Fish are the ancestral group of all vertebrates, and they represent the majority of vertebrate biodiversity. Half a dozen major groups of fishes survive, including the Chondrichthyes (sharks and rays), Sacropterygii (coelacanths, lungfishes), Chondrostei (sturgeons and bichirs), and Holostei (gars, bowfins). Although the species found in these groups possess extremely well-developed olfactory systems and probably use pheromonal signaling systems, we know nothing of their chemistry. Understanding of fish pheromones is largely restricted to a few species of boney fishes (the teleosts), which dominate modern waters and comprise over 23 000 species.[11,36] It is

difficult to provide generalities about this enormous, highly diverse group, which comprises over 400 families. Nevertheless, most fish probably use pheromones to influence many aspects of their behavior, and these can be broadly categorized according to three biological functions:

1. *Signaling pheromones*. Boney fishes commonly use pheromones to mediate many behaviors, including species and mate selection or recognition. Often the pheromonal aromas are complex and may simultaneously convey multiple pieces of information. However, with the exception of cues associated with reproduction, none has been clearly identified. Reproduction is, arguably, the most important moment in an animal's life, and there is strong evidence that many species of fish (perhaps all) use sex pheromones to mediate it. These cues are used to locate mates (releasers), identify their reproductive status (signalers), and recognize competitors. Half a dozen relatively well-described structures have been identified, most of which are hormonal metabolites. Indeed, it is probable that hormonal products are commonly used as sex pheromones by fish.[37] Several hormone-based pheromones are reviewed below along with one nonhormonal example (the sex pheromone of the masu salmon). Although it is clear that many of these cues are discerned as a matrix, only for the goldfish has the chemical identity been partially defined (see below).
2. *Reproductive priming pheromones*. Many species of fish are external fertilizers, and they spawn only a few times, which means that it is essential that male and female gamete outputs coincide. There is strong evidence that synchronization is mediated by sensory cues, including pheromones, most of which appear to be hormonally derived.
3. *Alarm cues*. Recognition of danger is fundamental to survival. Frisch[38] noted that, upon injury, minnows release chemical stimuli that cause others located downstream to startle. Presumably, fish evolved to recognize compounds in each other's skin as an indicator of danger. This phenomenon has been noted in dozens of descriptive behavioral studies, and, while it has been suggested that nitric oxides are involved, definitive establishment of the pheromonal component(s) has yet to be established using bioassay-guided fractionation so this story is not reviewed.

4.05.3.1 Masu Salmon, a Pacific Salmon

Fishes from the genus *Oncorhynchus*, often termed Pacific Salmon, are distributed across the northern Pacific. While some of the dozen or so species are now landlocked (e.g., rainbow trout), all are/were once (or still are) anadromous; that is, they reproduce in freshwater but spend the majority of adult lives feeding in the oceans, feeding only to return to freshwater to spawn and die. Unlike sea lamprey, Pacific salmon return to their streams of birth to reproduce, a feat that is attributed to odor recognition. While conspecific pheromones have been suggested to play a role in this process, the evidence is not compelling. Nevertheless, after returning to home streams salmon, like lamprey (see above), must find mates and reproduce. One of the most-studied examples is the masu salmon, *O. masou*, of Japan.

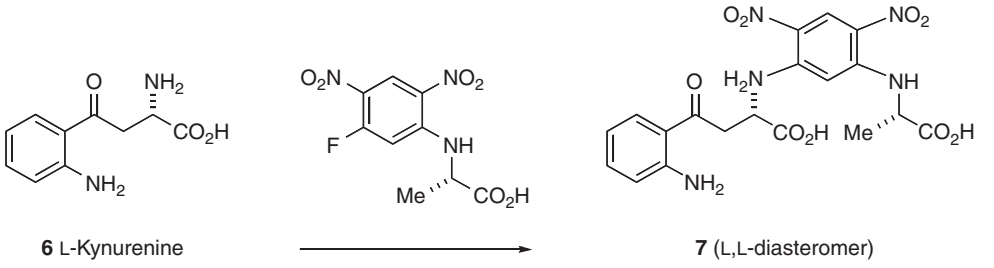
4.05.3.1.1 Female sex pheromone: Biology

During their reproductive season, female masu salmon build simple nests (redds) and males fight to gain access to them. Using a series of behavioral tests (Y-mazes), Yambe *et al.*[39] have demonstrated that ovulated female masu salmon, release an attractant in their urine. Urine was fractionated and the fractions tested both by EOG recording, followed by and behavioral assays, leading to the identification of a novel amino acid, L-kynurenine (**6**). Although this amino acid is active at a concentration adequate to explain activity in the laboratory, it seems probable that this cue is supplemented by other factors which might give it greater specificity.

4.05.3.1.2 Female sex pheromone: Chemistry

The urine (20 ml) of anadromous, ovulated females was collected by catheterization. Repeated fractionation, guided by male behavioral response, over complementary chromatographic supports led to the isolation of a sufficient quantity of pure substance for full spectroscopic characterization. The active pheromonal compound was shown to be L-kynurenine (**6**). It was estimated to be present at a concentration of 1.1 mg 100 ml^{-1} of urine. Interestingly, that is a hundred or more times higher than the concentration in the ovulated female urine of rainbow or brown trout. The absolute configuration of **6** was determined by Marfey's analysis,[40] which involves

characterization of the chromatographic behavior of the L,L- versus the D,L-diastereomers of **7** (Equation 2) *vis-a-vis* authentic samples.



6 L-Kynurenine → **7** (L,L-diasteromer) (2)

L-Kynurenine (**6**) is a known oxidative metabolite of L-tryptophan. This study for the first time appears to represent this amino acid metabolite that has been identified as a pheromone in a vertebrate. Although additional minor constituents present in the active fraction were identified (*N*-formylkynurenine, tyrosine, cystathionine, and prostaglandin $F_{2\alpha}$), none was observed to synergize the sex pheromonal activity of L-kynurenine (**6**).

4.05.3.2 Atlantic Salmon and a Relative, the Brown Trout

Salmonid fishes from genus *Salmo*, originated in the Atlantic Oceans approximately 60 million years ago and although they share many ecological and morphological features with Pacific salmon, their pheromones appear to be quite different. Five salmonine species are found in this genus (*Salmo*) with the brown trout (*Salmo trutta*) and Atlantic salmon (*S. salar*) being the best understood.

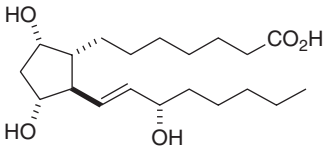
4.05.3.2.1 Female sex pheromone: Biology

Both Atlantic salmon and brown trout probably use pheromones to mediate many aspects of their biology, including migration (the Atlantic salmon is anadromous), but none has been clearly identified. Interestingly, brown trout and Atlantic salmon will hybridize, suggesting they employ very similar sex pheromones, a fact also supported by electrophysiological recordings[41] and behavior (below). Females of both species construct nests when spawning (like Pacific salmon) and males fight for access to females, which they seem to recognize largely by odor. EOG recording has established that the mature male Atlantic salmon is sensitive (10^{-11} mol l^{-1} threshold) to both prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and $PGF_{2\alpha}$.[42] Further, when exposed to female urine or either of these compounds, mature male salmon experience increased hormone levels and sperm production, leading to the suggestion that F prostaglandins serve as priming pheromones, an hypothesis that immunological measurement of female holding water seems to confirm (see below). It makes sense that fishes should use a PGF-based sex pheromone system because, as originally shown in the goldfish (see below),[43] these fatty acids have prominent endocrine roles mediating ovulation and sexual receptivity. Evidence using EOG recording and a hormone/sperm production bioassay also suggests that the closely related brown trout employs a similar F prostaglandin-derived sex pheromone system.[41,44–46] In one report using precociously mature Atlantic salmon, it was reported that 17α,20β-dihydroxy-4-pregnen-3-one 20 sulfate (**12**) was detected by the olfactory system.[47]

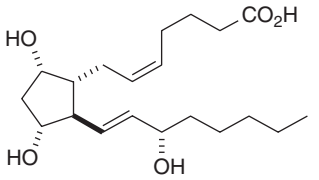
4.05.3.2.2 Female sex pheromone: Chemistry

To test the possibility that $PGF_{1\alpha}$ (**8**) and $PGF_{2\alpha}$ (**9**) might have pheromonal relevance in Atlantic salmon and brown trout, as they do in goldfish (below), ovulatory fluid and urine from both species were collected and the possible presence of these products tested using radio-immunoassays directed against $PGF_{2\alpha}$. These assays had notable affinity for both $PGF_{2\alpha}$ and $PGF_{1\alpha}$ (the latter being slightly lower) but very little affinity for a half dozen other prostaglandin metabolites. Several tens of nanograms of immunoreactive prostaglandin F (IR-PGF) was measured per milliliter of urine in the Atlantic salmon,[48] and IR-PGF was detected in both the urine and ovulatory fluid of brown trout.[44] Olsén *et al.*[45] also measured IR-PGFs in brown trout using different

immunoassays, which included a chemical reduction step to evaluate the presence of E series prostaglandins. In conclusion, it seems clear that F-series prostaglandins function as pheromones in this species, but their precise identities and functions await further characterization and confirmation.



8 Prostaglandin $F_{1\alpha}$



9 Prostaglandin $F_{2\alpha}$

4.05.3.3 African Catfish

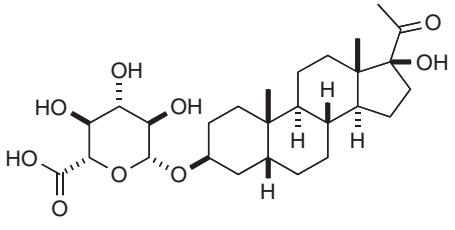
The African catfish (*Clarias gariepinus*) is native to subtropical Africa but is now cultured across the planet. Males and females of this species spawn as pairs in flooded vegetation in turbid waters and appear to use pheromones to locate and identify each other.

4.05.3.3.1 Male sex pheromone: Biology

Various laboratory behavioral studies suggest that both males and females release sex pheromones, but most of the attention has focused on the males, which have enormously developed seminal vesicles and whose fluid is attractive to ovulated females in mazes. *In vitro* incubation demonstrates that these glands possess the ability to produce numerous steroids, many of which are conjugated with glucuronic acid, and, thus, highly water soluble.[49] EOG recording shows that many of these conjugates are detected at subpicomolar concentrations,[50] and a mixture of them attracted females in a laboratory maze.[51] One of these compounds, 3α,17α-dihydroxy-5β-pregnan-20-one-3-glucuronide (**10**), is of special interest because it has high olfactory activity and also was found in African catfish holding water. Studies also suggest that this catfish possesses a maturation inducing pheromone that accelerates the onset of puberty.[52]

4.05.3.3.2 Male sex pheromone: Chemistry

Holding water from the adult conspecifics was collected, freeze-dried, and further concentrated through a C18-resin. The concentrate was then partitioned between methylene chloride and water to provide separate free steroid and glucuronidated steroid fractions, respectively. The latter was treated with β-glucuronidase to release the steroidal aglycones. Both fractions of parent steroids were derivatized (through oxime formation and exhaustive trimethylsilyl etherification) and subjected to GC–MS analysis, which relied substantially on comparison with authentic standards. Numerous hormonal steroids and steroidal glucuronides, previously known to be present in seminal vesicle incubates,[49] were identified. The most potent of these from an olfactory perspective (EOG measurement) was 3α,17α-dihydroxy-5β-pregnan-20-one-3-glucuronide (**10**).



10 3α,17α-Dihydroxy-5β-pregnan-20-one-3-glucuronide

4.05.3.4 Goldfish

Goldfish (*Carassius auratus*) and its immediate relatives, the crucian carp (*C. carassius*), and the common carp (*Cyprinus carpio*) are three closely related species that will hybridize and appear to use very similar hormonally derived pheromones. The goldfish, a temperate East Asian species, is the best understood of these species (and perhaps all bony fishes) and thus serves as a model for understanding hormonal pheromones more generally. The carps live in turbid, temperature waters. Because they spawn one to several times a year and because their gametes are short-lived, reproductive synchrony between and within genders is of paramount importance to their reproductive success. For example, pheromones are so important that male goldfish do not spawn if their olfactory system is blocked.[53] Female goldfish live in unstructured groups and spawn infrequently. Males actively seek and then compete for access to ovulated females, a process that has favored a heightened awareness of body odors and hormonal products in particular. Male goldfish discriminate between priming cues, which allows their hormone systems to cycle with that of the females so that they produce maximum amounts of fertile sperm, and signaling cues, which they use to locate females. Several of the sex pheromones used by goldfish are unmodified hormones, which are naturally released at times that correlate directly with the female's reproductive state and, therefore, have intrinsic biological relevance. The same chemicals are thus used twice, first hormonally as internal synchronizers and then pheromonally as external synchronizers. This elegant parsimony is an example of how two signaling systems can co-evolve. Other species also appear to use hormonal metabolites as sex pheromones, presumably for similar reasons, but the most comprehensive evidence for this has been obtained in goldfish. This discovery serves as an important model of 'hormonal sex pheromones,' a topic of other reviews.[10,11]

4.05.3.4.1 Male and female sex pheromones: Biology

Both male- and female-derived cues have been extensively characterized in the goldfish. These cues represent a now well-understood hormone-based system that synchronizes female and male behaviors and physiologies. We discuss both sets of cues together because they comprise closely related and overlapping hormonal systems that function as priming cues (i.e., they stimulate hormone production). Interestingly, one of the female priming pheromone components (17,20β-dihydoxy-4-pregnen-3-one) is an unmodified hormone, which is excreted through the gill during final maturation. In effect, this hormonal pheromone stimulates its own production in conspecifics, resulting in massive synchronization of entire populations. In other words, some goldfish hormones and pheromones are comprised of one and the same set of components. Understanding of the goldfish system is of added importance because the goldfish and its relative, the carp, are commercially important. More in depth understanding of this pheromone was made possible, in part, from the fact that the endocrinology of goldfish was well understood. This allowed adoption of techniques developed in endocrinological studies for use in the subsequent investigations of hormonal pheromones. Indeed, internal (hormonal) and external (pheromone) chemical signaling systems are tightly linked in this species resulting in positive feedback loops.[11,37] As its internal hormonal composition changes so does the externally released pheromonal blend, and different messages are produced and perceived over the cycle. Specifically, 17,20β-dihydroxy-4-pregnen-3-one; (17,20βP, **11**) mediates final gamete maturation and is found in high levels in the blood for just a few hours,[54] and PGF_{2a} (**9**) mediates follicular rupture and drives female sexual behavior.[43] Both structures and their metabolites are then cleared to the outside to function as pheromones. Immunoassays have shown that ovulatory female goldfish rapidly clear dozens of steroid hormones and metabolites to the water (below),[55,56] where three of them [17,20βP (**11**), 17,20β-dihydroxy-4-pregen-3-one-20-sulfate (17,20βP-S, **12**) and androstenedione (AD, **13**)] are discerned with sensitivities ranging between 10^{-11} and 10^{-12} mol l^{-1} (**Figure 3**[57]).[55,58] Depending on context and timing these steroids can function as pheromones in their own right or as components of more complex multicomponent cues with various meanings.[59] Species identity seems to be portrayed by the nonhormonal components of this mixture.[60]

Bioassays using either ovulatory female holding water or the steroids they contain have demonstrated that 17,20βP (**11**) functions as a priming pheromone that triggers hormonal surges in exposed males and

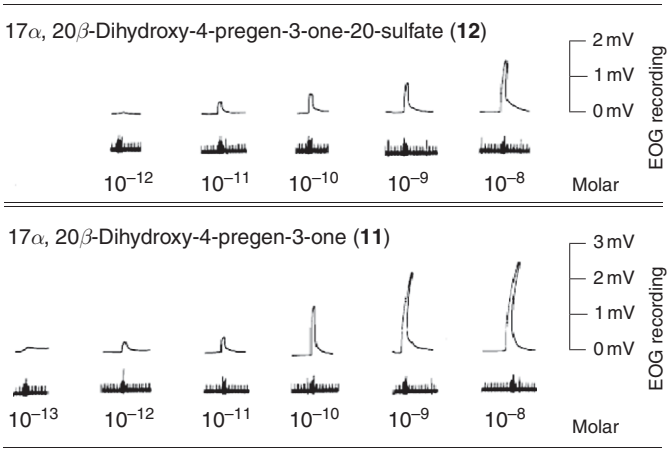


Figure 3 An example of electro-olfactogram (EOG) data; chart recordings of responses of male common carp whose sensitivity to hormonal compounds **12** and **11** is very similar to that of goldfish.[57]

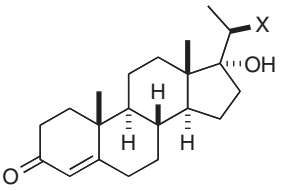
females, thereby driving synchronized gamete maturation and spawning.[54,61] The other two gonadal steroids serve as modulatory components, with 17,20βP-S (**12**) enhancing behavioral responses, and AD (**13**) suppressing responses at different times in the female cycle.[10] Ratios determine function and change during the reproductive cycle. Later at the time of ovulation, which coincides with spawning, females stop releasing steroids and instead produce and release prostaglandin F2α (**9**) and its metabolites. The male goldfish nose is extremely and specifically sensitive to one of these, 15keto-prostaglandin $F_{2\alpha}$ (15K-$PGF_{2\alpha}$, **14**).[62] The latter, postovulatory cue elicits strong behavioral responses and allows males to locate females, which release it, exclusively, in their urine. Behavioral studies confirm that 15K-$PGF_{2\alpha}$ (**14**) elicits similar responses as those of ovulated female holding water, at least when presented in a matrix of body odor.[11,43]

Finally, immunoassay shows that male goldfish release AD (**13**) and other androgens in the absence of pregnane (21 carbon) gonadal steroids. Together this specific mixture (i.e., blend) signals gender identity.[55] There is no evidence that hormonal metabolism is highly specialized in the goldfish,[56] but mixture recognition is very important.[59] Since the discovery of this sophisticated chemical signaling system in the goldfish, EOG recording has shown that many (perhaps most) fishes detect hormonal cues (cf. Atlantic salmon and brown trout, above) and probably use them as hormonal pheromones, but only for goldfish has production, release, detection, and biological responsiveness been established fully.[37] Carps are of economic importance and their pheromones are being considered for various applications.[57]

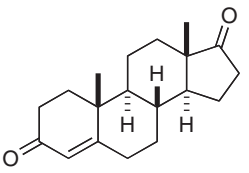
4.05.3.4.2 Male and female sex pheromones: Chemistry

Sex pheromone identification in the goldfish is based on the coordinated use of over a dozen highly specific antisera developed against a variety of fish gonadal steroids and prostaglandins. Characterization of all components of the cue using mass spectrometry has not been achieved (except for $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$, (P.W. Sorensen, unpublished)), but characterization using immunoassays has been completed and correlates with olfactory sensitivity and specificity. For steroid analysis, water samples were collected at appropriate times during the goldfish reproductive cycle, extracted using reversed-phase C18 resins, and then deconjugated (or not) using either acid hydrolysis (sulfates) or treatment with appropriate enzymes (glucuronidase).[55,61] With few exceptions, the antisera used in these studies were very specific (e.g., they were sensitive to single changes in side chain structure or orientation).[63,64] The prostaglandin assays were also specific; separate antisera were used to assay for $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$.[65,62] Further, goldfish injected with radiolabeled $PGF_{2\alpha}$ (**9**), excreted labeled 15K-$PGF_{2\alpha}$ (**14**) in their urine thereby establishing that the former is oxidatively metabolized into the latter and that it

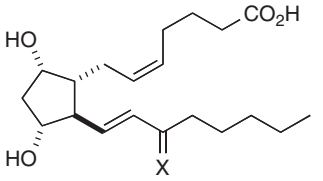
has pheromonal function.[66] Finally, unlike *Salmo*, the goldfish olfactory system has been extensively characterized and shown to discriminate among 17,20βP (**11**), 17,20βP-S (**12**), AD (**13**), $PGF_{2\alpha}$ (**9**), and $PGF_{1\alpha}$ (**8**). Thus, data from electrophysiological, behavioral, endocrinological, and immunological assays all are in agreement that these five hormonal compounds serve as a pheromonal mixture in the goldfish.



11 17, 20β-Dihydroxy-4-pregnen-3-one, X = OH
12 17,20β-Dihydroxy-4-pregnen-3-one-20-sulfate, X = OSO_3Na



13 Androstenedione



9 Prostaglandin F2α, = αH, βOH
14 15-Ketoprostaglandin F2α, X = O

4.05.4 Pheromones in Amphibians

The amphibians (Amphibia) evolved from the ancient fishes approximately 400 million years ago. There are about 6000 species of amphibians in five groups comprising the frogs, toads, salamanders, newts, and gymnophiona. Amphibians possess four legs, and most have a life history that includes a juvenile water breathing form that eventually metamorphoses, mandating an aquatic pheromone system. Several aquatic sex pheromones have been fully identified in the newts and frogs and a few protein cues have been characterized in terrestrial salamanders.

4.05.4.1 Asian Red-Bellied Newt and Its Relative the Sword-Tailed Newt

There are seven species of fire belly newts (*Cynops*), all of which are found in semi-tropical regions of Japan and China and spend most of their lives in water.

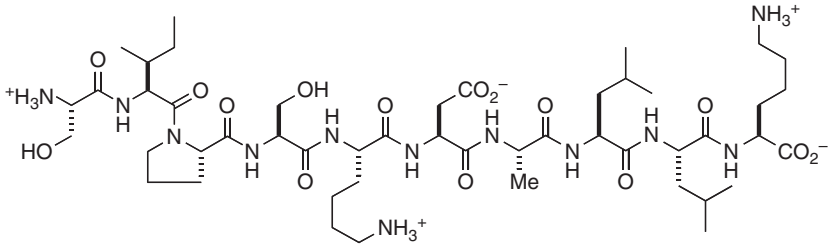
4.05.4.1.1 Male sex pheromones: Biology

Mating in *Cynops* newts occurs in the water during which time males actively court females, blocking their passage and using their tails to then fan water toward the females' snouts. Interested females respond by following a male and touch their snouts to his cloaca. The male eventually releases a sticky spermatophore in the females' path, which can stick to the lips of their cloaca and be absorbed. A few weeks later the female will deposit eggs. Although salamanders have long been suspected to employ pheromones, Kikuyama *et al.*[67] were the first to test this possibility by extracting newt holding waters and their cloacal glands. Activity was monitored by observing the snout position of sexually receptive females in 3-l aquaria containing sponges soaked with glandular extracts, pheromonal candidates, or various controls. A decapeptide, termed sodefrin (**15**), was eventually isolated from red-bellied newt water and found to have behavioral activity in the picomolar range. This represents the first example of a peptide used by any organism as a component of a pheromone. Later, EOG recording from the vomeronasal system of mature female newts confirmed their sensitivity to this novel peptide.[68] Tests using the congeneric species, the sword-tailed newt, subsequently led to the discovery that this species is behaviorally insensitive to sodefrin and that it uses a different but related peptide, silefrin (**16**).[69] Most recently, a population-specific variant of sodefrin [(Val8)-sodefrin] has

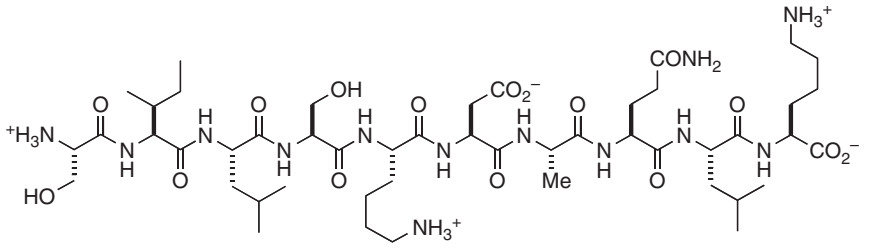
been isolated from two specific red-bellied newt populations,[70] demonstrating how flexible yet specific a peptide-based pheromone system can be.

4.05.4.1.2 Male sex pheromone: Chemistry

Sodefrin (15) is the first peptide pheromone to be discovered in a vertebrate.[67] An aqueous extract of male abdominal cloacal glands was shown to be behaviorally attractive to female red-bellied newts when presented on a sponge block in a 3-l holding tank. Approximately 0.1% of the extracts of one male gland was sufficient to elicit response. Partitioning the extract with organic solvent left the active fraction in the water phase and pronase digestion destroyed activity. Thus it was hypothesized that the active component(s) was peptidic. Fractionation of the water-soluble portion by gel permeation chromatography showed that activity remained with material of size <5 kDa. Regimens of reversed-phase chromatographic separation led to isolation of a behaviorally active peptide of nominal mass 1071 (FAB-MS) that could be sequenced directly. The resulting structure was new, so, to pay homage to its activity, the peptide was named sodefrin (15), a derivative of the ancient Japanese word 'sodefuri,' which means 'soliciting.' An authentic sample of sodefrin was prepared by synthesis. In an elegantly simple and effective experiment, a sponge block doped with 10 ng of sodefrin (15) was placed in a tank holding 3 l of 0.1 pmol l^{-1} background level of sodefrin (15) and females were attracted to the source; however, at background concentrations above 1 pmol l^{-1}, females could no longer distinguish the concentration gradient emitted from the sponge block.



15 Sodefrin (Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys)



16 Silefrin (Ser-Ile-Leu-Ser-Lys-Asp-Ala-Gln-Leu-Lys)

A related species, the sword-tailed newt, was studied leading to the isolation and sequencing of the homologous peptide silefrin (16).[69] Peptides 15 and 16, each produced in the abdominal gland of male red-bellied or sword-tailed newts, respectively, differ in only two of their ten amino acid residues (positions 3 and 8), yet neither attracts females of the other species. Furthermore, none of the 'foreign' peptide could be observed by cross radioimmunoassay experiments with the abdominal gland from each species of male. The specificity of these otherwise closely related systems is elegant.

4.05.4.2 Jordan's Salamander

Woodland and slimy (Plethodon) salamanders are lung-less and lack an aquatic phase, reproducing in moist regions of the forest. They have evolved to employ large peptide contact pheromones, which are best understood in the Jordan's salamander and partially described in the Ocoee salamander.[71]

4.05.4.2.1 Courtship pheromone: Biology

During the courtship season males of the terrestrial Jordan's salamander, which is native to the Pacific Northwest, develop a gland under their chins that they rub on a female's nares (nostrils) while performing a courtship behavior known as the straddling walk. This chin gland contains two peptides, a 22-kDa protein called plethodontid receptivity factor (PRF)[72] and a smaller 7-kDa protein called plethodon modulatory factor (PMF).[73] Assays using purified peptides placed on the female nares have shown that the former shortens female receptivity while the latter lengthens female courtship behavior, one counterbalancing the effect of the other. Application of a neural label (agmatine) to the vomeronasal organ has confirmed the presence of ORNs that are specifically sensitive to either PRF or PMF in this species.[74]

4.05.4.2.2 Courtship pheromone: Chemistry

Both PRF and PMF were isolated by a similar protocol.[72] The proteinaceous material from excised mental (submandibular) glands from male *P. jordani* was extracted with acetylcholine chloride and purified by NaCl gradient HPLC on a MonoQ HR 5/5 anion exchange column. Further purification of the PRF-rich fractions was performed on a Sephadex G-75 gel filtration column. SDS–PAGE and HPLC analyses confirmed the presence of several isoforms of 22 kDa PRF. The DNA sequences corresponding to four of these were determined, and sequence homology with members of the interleukin-6 (IL-6) cytokine family was noted. More recently, the behavioral properties of the purified 7 kDa protein component (PMF) were reported.[73] Together, PRF and PMF comprise approximately 85% of the total protein composition of the chin (mental hedonic) gland extract. The ratio of PRF:PMF (~1:2) was constant in collections from different years.

4.05.4.3 Australian Tree Frog

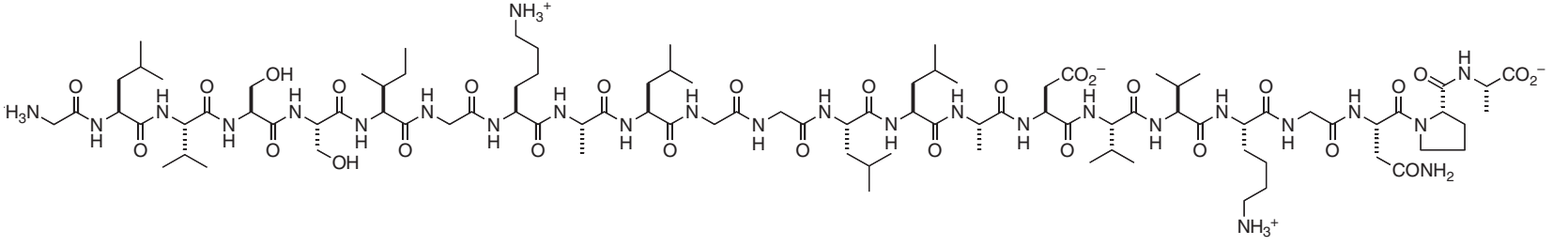
The Australian tree frog (*Litoria splendida*) is native to Western Australia and spends much of its day in caves. The first anuran (frog or toad) pheromone was identified in this species.

4.05.4.3.1 Male sex pheromone: Biology

The Australian magnificent tree frog breeds in the water, where males court females. A peptide, now called splendipherin, has been extracted from the male parotid and rostral glands and found to attract females in glass tanks at concentrations of approximately 10^{-13} mol l^{-1}.[75] This pheromone attracts females from distances of up to a meter within minutes.

4.05.4.3.2 Male sex pheromone: Chemistry

Because anurans, including *L. splendida*, often breed in water, the peptide components of the secretions of the paratoid and rostral glands were purified directly by HPLC. The active component was sequenced with the aid of electrospray ionization mass spectrometry (ESI-MS) and given the name splendipherin (**17**).[76] The structure of this 25-amino acid peptide subsequently was confirmed by comparison with a synthetically prepared sample, which further established that all amino acids were of the L-configuration.



17 Splendiferin (GLVSSIGKALGGLLADVVKSKGQPA)

4.05.5 Pheromones in Reptiles

The reptiles (Reptilia) evolved from the early vertebrates about 300 million years ago. Reptiles have four legs and are air-breathing, cold-blooded vertebrates that have skin covered with scales. Most are exclusively terrestrial. Modern reptiles inhabit every continent except Antarctica and are represented by four living groups: the crocodiles and alligators, lizards and snakes, turtles and tortoises, and the Sephondontia (New Zealand only). Except for the last group there is compelling behavioral data that suggest that all groups use pheromones to mediate sexual interactions. However, few have been studied at the chemical level. All members of this group have a vomeronasal organ and appear to use nonvolative cues. Only in one species of snake, the Canadian red-sided garter snake, is the chemical identity of the pheromones well-established. Reptilian pheromones have been reviewed.[77]

4.05.5.1 Red-Sided Garter Snake

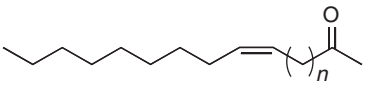
Although it is quite possible that most, if not all, reptiles employ pheromones to mediate sexual interactions, only for the red-sided garter snake (*Thamnophis sirtalis parietalis*) have both the identities of the cues and their functions been established.[78] This species is one of the most northern living reptiles in the Northern Hemisphere and has evolved an unusual life history; it hibernates in large underground hibernacula for most of the year, emerging in the spring to mate and then feed. Males emerge first from hibernacula and aggregate in thousands waiting for females, which they recognize using mixtures of ketones found in the skin of the female. Males sample other snakes by traversing the lengths of their bodies while rubbing them with their chins and flicking their tongues, thereby bringing samples to the vomeronasal organ (VNO). Thus, this is a nonvolatile contact pheromone.

4.05.5.1.1 Female sex pheromone: Biology

Hundreds of males may aggregate around females during the mating process, forming 'mating balls,' but ultimately only one male will mate with any given female. Analyses of the snake skin lipid components showed the presence of a series of nonvolatile, nonpolar long-chained methyl ketones (see **18–30** below).[79,80] Several were synthesized. Males specifically respond to individual ketones but more strongly to artificial mixtures of these ketones, suggesting that mixture composition is important.[79] Pheromone blends are probably discerned with considerable sophistication because males are known to be able to distinguish the trails of larger females and even females from different locations, all of which have slightly different skin lipid compositions.[81,82] Interestingly, while most males are ignored by other males because their skin lipids are distinguished by the presence of squalene,[83] certain males known as 'she-males' function as female mimics, attracting other males giving them certain advantages.[84] She-males lack squalene, and it is speculated that they possess a unique blend of the lipid ketones, although this has yet to be documented. Neurophysiological recording has also not been reported so it is unclear how sensitive the nervous systems of snakes are. This interesting system, variations of which are probably operative in other snakes, has considerable potential for further development.

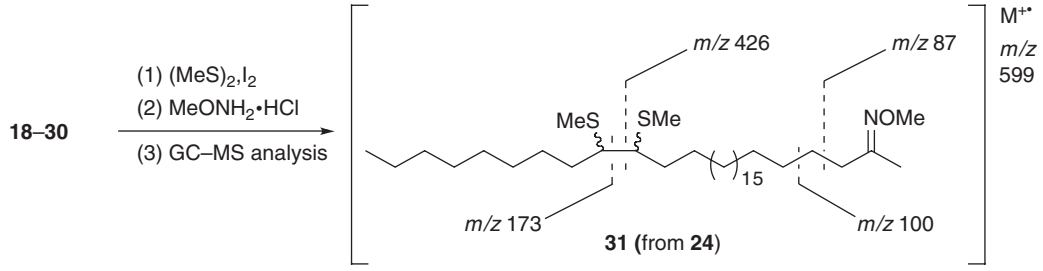
4.05.5.1.2 Female sex pheromone: Chemistry

The red-sided garter snake pheromone components were isolated by hexane extraction of the skin lipids of sacrificed snakes. Female snakes yielded more total lipid than males (38.4 versus 8.4 mg snake^{-1}).[80] Initial fractionation on an activity III alumina column gave a fraction (eluted with 98:2, hexanes:ethyl ether) that was attractive to courting males. NMR and infrared (IR) spectra of this fraction were suggestive of the presence of methyl ketones, straight chain alkyl lipid subunits, and *Z*-alkenes. GC–MS analysis, including extensive consideration of the fragmentation of the electron impact MS data, led to the identification of a family of relatively nonvolatile (C_{29}–C_{37}) lipid methyl ketones. Specifically, the individual components of a mixture of saturated and monounsaturated methyl ketones **18–30** were identified.



Saturated methyl ketones			Mono unsaturated methyl ketones		
Str	*m*	Tot C#	Str	*n*	Tot C#
18	24	27	**21**	19	33
19	26	29	**24**	21	33
20	27	30	**26**	22	34
22	28	31	**27**	23	35
23	29	32	**29**	24	36
25	30	33	**30**	25	37
28	32	35			

To deduce the location of the double bond within the lipid backbone, the mixture (500 ng) was subjected to consecutive bisthiomethylation of the alkene[85] and *O*-methyloxime formation (Equation 3). GC–MS study of the fragmentation of these derivatives (e.g., see **31**, derived from **24**) allowed simultaneous determination of the cleavage site (between C_{24} and C_{25}) and of which portion contained the original ketone (i.e., the odd versus even mass fragments of 173 and 426 for **31**). All of the monounsaturated lipid ketones had the alkene in the same 'downstream' location; in other words, they varied in the number of methylene units between the ketone and alkene functional groups but were constant in their *n*-octyl terminal alkyl moiety. The four most major components (**24**, **25**, **27**, and **28**) were prepared by chemical synthesis and used to confirm their identity in the natural pheromone and their pheromonal activity both alone and in admixtures.



(3)

4.05.6 Pheromones in Birds

Birds (class Aves) evolved from the therapsid dinosaurs (lizards) approximately 200 million years ago. Birds are warm-blooded and bipedal (two legs) and lay eggs. Most modern birds have feathers and fly, sometimes migrating enormous distances. There are about 10 000 species of birds, making them the second largest group of vertebrates after the fishes. They are extremely mobile and have excellent visual and auditory systems suited to aerial communication. Not surprisingly, with only a few exceptions (water/sea birds and flightless birds), birds have poorly developed olfactory systems and chemical signaling systems. Although there is good evidence for the existence of pheromones in several species of ducks, only in the communal nesting auklet is the associated chemistry well-developed. The use of body odors by birds may nevertheless be more common than has been shown to date.[86] Birds produce many chemicals for the purposes of defense from insects, and it is possible that some of these have become secondarily specialized for chemical signaling, although evidence to support that hypothesis is, as yet, correlational. This hypothesis and the bird pheromone field have been reviewed recently.[86]

4.05.6.1 Crested Auklet

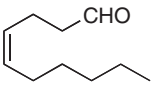
The crested auklet (*Aethia cristatella*) is a small seabird from the Sea of Okhotsk and the Bering Sea in the western and northern Pacific. It nests in huge (>1 million) colonies that are often co-occupied with their congeners, the least auklets. During its mating season, the highly social crested auklet develops a characteristic group of bristled feathers on its head and it emits an odor that has been described as tangerine-like.

4.05.6.1.1 Ornamental pheromone: Biology

Crested auklets produce large quantities of various volatile odorants in their plumage, which gives them a tangerine-like odor. This mixture is comprised mainly of octanal and *cis*-4-decanal and is not gender specific. It is associated with seasonal display and courtship behavior when birds place their bills under the nape of their partner's plumage.[87] Bioassays using models of birds show that birds of both sexes approach models of males for longer periods of times and more closely when the models are scented with natural or synthetic plumage odors; female models, similarly scented, elicit little response.[87,88] Scents did not increase courtship behaviors, which suggests that this odor serves as a social 'ornament' facilitating social aggregation, perhaps akin to scent marks in mammals. Questions about the true function of this odor remain as does the neural basis of its detection, and it can be debated whether it has been shown to be a 'true' pheromone. Nevertheless, we include it here because it is the best example of possible pheromone usage amongst the birds.

4.05.6.1.2 Ornamental pheromone: Chemistry

Plumage from test (breeding season) and control (off-breeding season) auklets were collected and placed in a closed vessel. Headspace was collected, concentrated with solid-phase microextraction, and subjected to GC–MS analysis. Correlation with library MS data and, ultimately, authentic samples led to compound identification. Twenty volatile compounds (generally, fatty acids and aldehydes) were present in sufficient concentration for reliable quantification (>0.1 $\mu g\,g^{-1}$ feathers). Meaningful seasonal variation was observed for ten of them. Of the compounds present only in the feathers collected during breeding season, *cis*-4-decenal (**32**) was the most prevalent (1.1 $\mu g\,g^{-1}$ feathers), and octanal (**33**), while present in samples from both seasons, was present in the highest concentration overall (3.0 $\mu g\,g^{-1}$ feathers).



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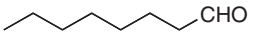

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4.05.7 Pheromones in Mammals

The mammals (Mammalia) evolved from the amniotes, a sister group of the sauropsids (from which the reptiles and birds evolved), approximately 300 million years ago. This group contains >5000 species in a few dozen orders. Mammals are homeothermic (warm blooded) and are characterized by the presence of hair, sweat glands (some of which are modified for milk production), three middle ear bones, and a well-developed brain with a neocortex. The latter is responsible for their complex cognitive function and the ability to process and remember a wide variety of sensory stimuli to effect a broad array of sophisticated behaviors. With the exception of the monotremes (platypus), all mammals give birth to live young and have well-developed family structures, a feature that also requires behavioral sophistication and the ability to recognize kin. Mammals have keenly developed chemosensory systems that often include a vomeronasal system. Accordingly, it should be no surprise that odors, including pheromones, play important and complex roles in the everyday lives of most mammals. These roles generally defy simple definition. The odors often function as subtle signaling cues and often are context dependent. Mixtures play important and varying roles. Pheromones are especially important in small burrowing nocturnal creatures (e.g., rodents). Many have evolved various skin glands that either produce various compounds themselves (sometimes by providing a haven for microbes) and/or shuttle

products of various excretory pathways to their surrounds, where they then adopt the role of scent marker. Although some of these products can be considered pheromones because there appears to be specific disposition to respond to them (e.g., frontalin in the elephants), others have less-defined functions related to individual identification. Other mammalian pheromones are found in urinary or fecal excretions. The combinations are complex, and context (odor and other cues) is often important to pheromone function in mammals.[2,5]

Recent reviews of mammalian semiochemicals[89] and of mammalian pheromones[8,90,91] have appeared. Vandenbergh groups mammalian pheromones into various categories,[8] which we have used here as a basis for organization into three main and four subcategories:

1. **Signaling pheromones**. Communication of individual identity and status is of enormous importance to most mammals, and they have evolved a variety of chemical cues and chemosensory systems to accomplish this task. Although in many instances the message conveyed is multifaceted, complicating its study, several subtypes can be identified, some of which can have overlapping pheromone function.
   (1) *Individuality signals*. Information of precise genetic identity and relationship is especially important to mammals that live in tight family groups (often in dark burrows) and need to outbreed and recognize young and mates. In many mammals, including mice and humans, unknown components of body odor identify genetic identity. These distinctive odors (which some also term 'signature cues'), may, depending on context, mediate maternal relationships between mothers and their young, mate selection by females seeking unrelated fathers, or recognition of the fathers of young by pregnant females (the 'Bruce Effect').[92] Although the identity of this odor has not yet been identified, its production is now known to be strongly correlated with the MHC, a set of genes that encodes for self-recognition and is associated with immunity. This conclusion is rooted in the observation that mice, which select mates based on odor, can distinguish between urine of mice that differ at one gene position in their MHC.[93] Recent discoveries have shown that the vomeronasal system is sensitive to peptide fragments of proteins encoded by the MHC.[94] It is possible that other components, including microbial breakdown products, also contribute to MHC-associated individuality signals, which may, in fact, be learned.[4]
   (2) *Territoriality pheromones*. Many mammals maintain feeding and/or reproductive territories, which they mark with excretions derived from specialized scent glands. These scent gland excretions contain a rich array of phenolics, fatty acids, lipids, and small alcohols; territoriality odors are used by beavers, dogs, rats, hamsters, deer, and perhaps half a dozen other species.[3] Often these chemicals are bound to and transported by special carrier proteins and can be long-lived, permitting rodents to engage in competitive 'over-marking.'[2,95] Some systems simultaneously convey information on social dominance, but no compound with clear direct relevance has been identified.[91]
   (3) *Sex attractants*. As in most animals, mating in mammals is typically highly competitive and its duration short-lived. Males of many (most) species of mammals have evolved acute abilities to detect and identify ovulating or recently ovulated (fertile) females using special types of signaling pheromones. A good example of such a female cue is found in the Asian elephants (below). Additionally, in a few instances, female mammals have evolved the ability to discern males so that they perform appropriate submissive or receptive behaviors. One of these cues has been identified in the pig (below).
   (4) *Alarm pheromones*. Many mammals live in family groups that are prone to attack. Some of these species have evolved chemical signaling systems that are triggered when danger is perceived. This is best described perhaps in deer, which release a series of odorants from their tarsal glands when startled, although the active component(s) have not yet been definitively isolated and identified.[96]
2. **Priming pheromones**. Many species of mammals live in small social groups that stand to benefit if they can synchronize their reproductive cycles so that optimal numbers of females come into reproductive condition at the same times. Examples of such benefit is so that they give birth when food is available or that they might care for each other's young. Accordingly, many mammalian pheromonal systems serve to either accelerate or inhibit the onset of puberty, mediate synchronous ovulation in groups of females, or block pregnancy.[91] These cues appear to use complex suites of inter-related compounds (somewhat akin to goldfish priming pheromones). Although such systems have been described in at least half a dozen mammals, chemical components have only been identified for the house mouse (below).

3. **'Nipple search' pheromones**. Females of many mammals give birth to poorly developed young, which often are blind and have difficulty finding the mother's teats. In at least one of these instances, the rabbit is an example (below), where the mother releases chemicals to guide this process.

4.05.7.1 European Rabbit

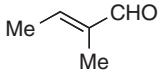
Rabbits are small, rather unusual, herbivorous mammals found in the order Lagomorpha. They live in burrows and lead nocturnal existences. Rabbits breed young numerous times, giving birth to litters of half a dozen, poorly developed young called 'kits.' In addition to having an excellent sense of hearing, rabbits have a superb sense of smell. They appear to mark territories with odors that they use (in conjunction with sound) to recognize each other, although none of the chemicals in these cues has been clearly identified. However, a pheromone released by nursing mothers that attracts their newborn young has been identified.

4.05.7.1.1 Nipple search pheromone: Biology

Female European rabbits (*Oryctolagus cuniculus*) give birth in burrows but quickly leave to continue foraging. They only visit their young for 4–5 min each day, when they return to the nest to allow the young to feed on their rich milk. During these brief intervals, young blind rabbits need to quickly find the mother's teats in the face of competition from their littermates in the darkness of the den. A pheromone released in the female's milk guides this process. It was identified by passing milk through a gas chromatograph and directing fractions onto the noses of young rabbits whose head movements and tendencies to grab the odor port with their mouths were quantified.[97]

4.05.7.1.2 Nipple search pheromone: Chemistry

A stream of nitrogen gas was passed through freshly collected mother's milk and the effluent collected by adsorption on a Tenax® TA resin (2,6-diphenylene oxide based). Subsequent desorption into a GC–MS instrument led to the identification of 21 volatile components (low-molecular-weight aldehydes, ketones, alcohols, and lactone) in the milk headspace. When each was screened for behavioral activity, one, 2-methylbut-2-enal (**34**), was clearly the main compound responsible for eliciting searching–grasping responses in the rabbit pup test subjects.



34
2-methylbut-2-enal (2MB2)

4.05.7.2 Golden Hamster

Rodents comprise a large order of mammals (over 2000 species or 40% of all mammals). The golden hamster (*Mesocricetus auratus*), which comes from the steppe region of central Asia, is a rather typical rodent; it is not to be confused with the golden gopher, which resides in the homeland of the authors. It relies heavily on pheromones.[98] Hamsters are solitary animals, which spend the day in their burrows and most of the night gathering food across large feeding territories (up to 12 km across). It marks and maintains these areas using scent marks. Although scent marking behavior is extremely sophisticated and well-documented,[98] the identities of the key compounds are not understood. Conversely, information is available on some female sexual attractants. The hamster is now globally distributed and is used as a laboratory model for studying chemical signaling.

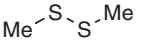
4.05.7.2.1 Female sex pheromone: Biology

Female hamsters, like the males, are solitary, and they only allow males to approach when they are ovulating. This species has evolved to use vaginal odors to signal receptivity. These odors appear to be very complex and context dependent, although a single component, dimethyldisulfide (DMDS, **35**), has been isolated and identified during the course of three decades of study. Briefly, experienced male hamsters prefer the odor of estrus females over that of pregnant or lactating females, and, in the presence of the bedding of such females,

males exhibit increased levels of scent-marking activity.[98] Further, when nerves of the vomeronasal system of males are severed, they show reduced courtship behavior.[99] The chemical underlying this behavior appears to be largely associated with female hamster vaginal discharge. Application of this discharge to males (but not to inanimate objects) causes them to be inspected by females, without necessarily promoting attraction.[100] DMDS is a volatile component of the discharge, and it has been shown to promote male investigation behavior at femtomolar concentrations.[101,102] However, it appears to be only one component of a mosaic because, when tested on its own, it is relatively inactive, causing some to question its true function.[103] Interestingly, male rats also find DMDS attractive, although here it is clear that it is only one component of many released by females. Following the discovery of this role of DMDS, many other complex odors that convey individual identity were described in various rodent species, further suggesting that these pheromone signaling systems are very complex.[91,104]

4.05.7.2.2 Female sex pheromone: Chemistry

In one of the earliest studies focused on identifying a specific chemical perhaps responsible for a pheromonal activity in a vertebrate,[105] vaginal secretions from estrus hamsters was collected on a filter paper. To isolate volatile components, the paper was immersed in water, nitrogen gas was passed through the water, and the effluent was adsorbed on a Tenax resin. Behavioral response of male hamsters to the volatiles fractionated by gas chromatographic separation and analysis of the active fraction by mass spectrometry led to the identification of DMDS **35**. An authentic sample was then used to assess that this agent elicited behavioral response at least similar in nature to that of the intact vaginal odor set.[105] Later studies have led to the suggestion that DMDS may largely serve simply to stimulate investigation.[2,103]



35
Dimethyl disulfide (DMDS)

4.05.7.3 House Mouse

The most well-known species of mouse is the common house mouse (*Mus musculus*). Its ecology and behavior have been carefully documented. Because it is easily cultured and its genome has been sequenced, the mouse serves as an important biomedical model. It also serves as a good model to understand mammalian pheromone systems. Unlike the golden hamster, the house mouse is a highly social rodent that lives in large colonies where individuals live in close contact with each other. The social and reproductive structure of these colonies is controlled by complex mixtures of signaling and priming pheromones that mediate individual recognition, the timing of puberty, reproductive cycling, mate selection, and sexual attraction. Two complex male-derived and female-derived priming cues with complex functions are now relatively well understood and are described below. One incompletely identified pheromone, which will not be reviewed further, is responsible for pregnancy block (the 'Bruce effect'). In pregnancy block pregnant females spontaneously abort after they are exposed to the odor of alien males that are not the father of their young.[4,92] It is possible that small peptides produced by the MHC complex have a role in this system, and electrophysiological recording has shown both the main olfactory system and the vomeronasal system to be acutely sensitive to MHC (nona)peptides.[94] Most of the other pheromone used by mice are released in the urine, even though they may be produced in any of several specialized glands. They also are transported by carrier proteins, which may have pheromonal properties of their own. Scent glands are also important to mice, although their role appears associated with recognition of individuals, and they are not further discussed here. Mice also appear to be using male and female signaling pheromones, although understanding of these is not well-developed.[106] Female mice prefer the odor of males, and both the male urine and preputial glands have been implicated.[107] It is probable that the types of pheromonal cues employed by mice are typical of those used by many rodent species.

4.05.7.3.1 Male-derived priming pheromone: Biology

Social mammals that live in colonies, such as mice, must orchestrate complex series of physiological and behavioral events to ensure reproductive success of the unit. Of special importance is the timing of puberty in

females and the subsequent cycling of their estrus cycles. This can both divert the attention of males and determine the need for maternal care. In many mice, these phenomena are linked and driven by a common set of pheromonal cues released by mature males and females. These have opposing stimulatory and inhibitory functions and act as a coupled oscillator.[9] Only in mice have the identities of these cues been partially deciphered. Four decades ago, Vandenbergh discovered that the onset of puberty in colonies of female mice is accelerated by as much as 10 days if they are exposed to trace amounts of mature male urine.[108] Odorous cues coming from dominant males are the most effective. Even earlier, Whitten had demonstrated that urine of male mice can stimulate the onset of estrus in mature females (the 'Whitten effect'), thereby inducing cycling within the colony.[109] From a systematic set of studies conducted by Novotny and colleagues, we now know that both phenomena are driven by a common set of chemical cues.

As detailed below, early studies of volatiles found in male mouse urine led to the isolation of two unique compounds: 2-(*sec*-butyl)-4,5-dihydrothiazole (**36**) and 3,4-dehydro-*exo*-brevicomin (**37**). These compounds were found to attract females and stimulate aggression in males when added to a matrix of castrated male urine; thus they have mixed priming and signaling functions.[110] Next, it was discovered that when these components were introduced into cages of grouped females (no effect was seen in single females), they shortened the duration and increased the number of estrus cycles, resulting in an effect similar to the Whitten effect.[111] Examining male odor even more closely, Novotny and colleagues later identified two terpene compounds from the male preputial gland (an exocrine gland associated with the genitalia).[112] These were *E,E*-α-farnesene (**38**) and *E*-β-farnesene (**39**), which also stimulate estrus synchronization on their own. More recently, examining uterine weights of groups of prepubescent female mice exposed to these stimuli, the same group established that all four compounds advance the time puberty (uterine growth) and cycling in females (**Figure 4**).[113] A fifth compound, as well as the fifth male urinary bladder component, 6-hydroxy-6-methyl-3-heptanone (**40**) was later identified in male urine. It was also found to have priming effects on uterine development.[114] All five of these components are now known to be detected with extreme sensitivity by the mouse vomeronasal system.[115] Exactly why and how the house mouse uses these complex matrices of cues, and whether more components exist, remains an open question.

The function of many of the five identified male-derived pheromonal components requires the presence of male urinary protein (MUP), a conglomerate of highly variable polypeptides found in male urine that bind **36** and **37**.[116] Although the role of MUP has been not fully elucidated (e.g., there is evidence that MUPs have pheromonal activity in their own right[95] but this interpretation is not universally held[113]), Novotny *et al.*[113] established that recombinant MUPs selectively complex with the five male pheromones, subsequently releasing them to elicit priming activity on females. It makes sense for a terrestrial vertebrate that engages in scent marking to possess a sophisticated system for tailoring the release of the individual chemicals, the suite of which comprise a fundamental priming cue. Presumably, subtly different messages can be sent by variation of the composition of the suite.

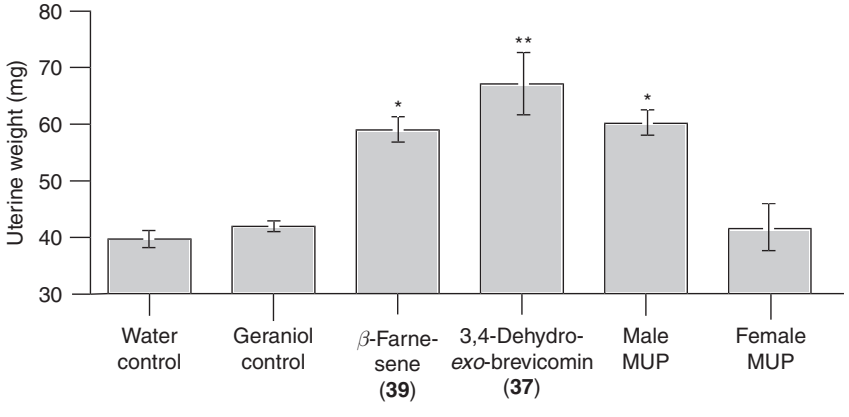
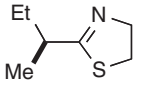


Figure 4 Uterine growth rates of female mice exposed to various pheromones and MUPs ($^{*}p < 0.05$, $^{**}p < 0.01$).[113]

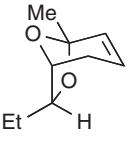
4.05.7.3.2 Male-derived priming pheromone: Chemistry

Thiazoline **36** was first isolated from mouse urine in 1977 by a protocol that was similar to that used (and discussed above) for volatile compounds **34** and **35**.[117,118] Namely, the contents of headspace from 15 ml of mouse urine was purged with nitrogen through a Tenax column, concentrated onto a cold trap precolumn, and injected for GC and mass spectrometric analysis. Bioassay-guided fractionation was not used; instead attention was focused on two components that were absent or present in only trace amounts in female but predominant in male urine samples. Since differential detection by flame ionization versus 'sulfur-specific' detection was used, it was certain that each of the two compounds contained sulfur. Detailed analyses of the accurate masses (10 millimass units) of the nine and seven most major (parent and fragment) ions led to the conclusion that the compounds were 2-(*sec*-butyl)-4,5-dihydrothiazole (**36**) and its lower homologue, 2-isopropyl-4,5-dihydrothiazole. Neither had previously been reported as a constituent of any animal's urine. Behavioral responses directly associated with **36** were first described in 1985.[119] While a natural sample of nonracemic **36** was characterized by retention on a chiral gas chromatographic column, a number of early attempts to synthesize an enantioenriched sample failed because of the configurational lability of the stereogenic center in synthetic precursors and/or **36** itself under conditions used to close the heterocyclic ring. It was not until 1999 that an enantioselective synthesis was achieved.[120] During that study, it was observed that a neat sample of **36** racemized within days at ambient temperature, but that storage in the cold or in dilute solution slowed that process, which presumably occurred through the exocyclic enamine tautomer of **36**. Finally, in 2003 the absolute configuration of the **36** produced in male mice was reported,[121] having been determined through study of the retention times of the synthetic sample of known configuration[120] versus the natural sample on an enantioselective capillary GC column.

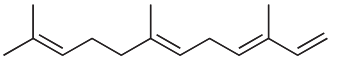
In 1984, Novotny and coworkers reported the first of their series of studies describing mouse pheromones.[117,122] Specifically, they described the isolation and structure determination of 3,4-dehydro-*exo*-brevicomin (**37**) and the synthesis of a racemic sample for structure confirmation. Volatiles were isolated in much the same manner as described for **36**. As with thiazoline, a direct bioassay was not used to focus attention on the component of greatest interest; rather the presence of what eventually proved to be **37** in male but not female urine, established by comparative chromatographic analysis, guided that decision. Precise mass measurement of the active component pointed to a molecular formula of $C_9H_{14}O_2$. Interestingly, GC-FTIR (Fourier transform infrared spectroscopy) was then used to show the absence of carbonyl or hydroxyl functionality, implicating a ketal. This further informed analysis of the electron-impact mass spectrometry (EIMS) fragmentation pattern, leading to the tentative assignment of the constitution of **37**, which was then confirmed by synthesis of the (±)-**37**.[117,122] The levels of **37** in urine from castrated male mice was substantially lower, but could be restored by subsequent administration of testosterone to those castrates. Thus, the testes are not the location of biosynthesis of **37**. Subsequently, numerous research groups reported syntheses of **37**, both in racemic and nonracemic[120,123–125] forms. Comparison of synthetic samples of known absolute configuration with the natural material established the latter as the (1*R*, 5*S*,7*R*)-isomer shown in **37**.[126] Curiously, females showed a preference for urine samples from castrated male mice that had been spiked with racemic **37** but not when spiked with either individual enantiomer when compared with the unspiked urine alone.[127]



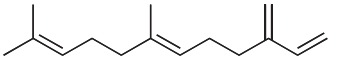
36 (*S*)-2-(*sec*-Butyl)-4,5-dihydrothiazole



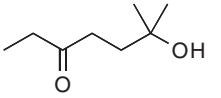
37 3,4-Dehydro-*exo*-brevicomin



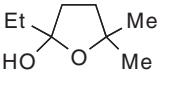
38 *E,E*-α-Farnesene



39 *E*-β-Farnesene



40a 6-Hydroxy-6-methyl-3-heptanone



40b 2-Ethyl-2-hydroxy-5,5-dimethyltetrahydrofuran

In subsequent studies the simple terpenes *E,E*-α- and *E*-β-farnesene (**38** and **39**, respectively) were identified in dominant male urine.[128] These odoriferous terpenes had long been recognized as components of cues released by a variety of other organisms (red fire ants, aphids, wild potato plants, fruit flies, and springbok). Because neither was detected in male bladder urine, attention was focused on the preputial glands as the source. Volatile components from dissected, fat-free preputial glands of dominant male mice were, again, preconcentrated on Tenax. Subsequent GC analysis readily allowed identification of known **37** and **38**. None of the earlier two components **36** or **37** was observed in the preputial volatiles, but both were present in the bladder urine of the same animals.

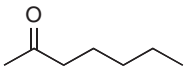
More recently, a fifth principle component, the spontaneously equilibrating mixture of ketoalcohol **40a** and hemiketal **40b**, also was isolated from male mouse urine.[114] In this instance, frozen urine sample was lyophilized and the residue reconstituted in aqueous buffer and subjected to size-exclusion chromatography on Sephadex G-10. Each resulting fraction was then subjected headspace analysis (Tenax/GC/MS protocol). A uterine weight response assay was used to focus attention on a fraction that contained **40a/b**. The tentative structural assignment was confirmed by synthesis of an authentic sample.[114] Ketone/hemiketal **40a/40b** was found to accelerate puberty in female mice. It was proposed that the ability of **40a/40b** to bind to urinary protein and peptide constituents that have very low volatility would explain the long-standing confusion over whether all constituents of the male-derived priming pheromone are volatile.

4.05.7.3.3 Female-derived puberty inhibiting pheromone: Biology

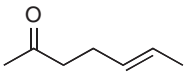
Although the presence of male mice in the colony accelerates puberty onset in juvenile females, the presence of mature females has the opposite effect – it inhibits the onset of puberty in juvenile females in the colony. Urinary cues detected by the vomeronasal organ of females were found to be the cause.[129,130] Presumably, this system has evolved to prevent excessive numbers of females from breeding at the same time, thereby increasing the chances of other females in the colony to raise young. Once again, the chemical basis of this effect is most clearly understood in the mouse. After earlier surprise discoveries that adrenalectomy but not ovariectomy had affected the production of the female cue, Novotny and colleagues focused on identifying adrenal metabolites in female mouse urine, some of which were shown to inhibit puberty in female mice.[131] It was established that one of these, 2,5-dimethylpyarzine (**46**), was especially active and suppressed both ovarian and testicular growth in young grouped mice.[132] Vomeronasal recording was used to confirm that **46** is detected at a concentration of 10^{-8} mol l^{-1}.[115]

4.05.7.3.4 Female-derived puberty inhibiting pheromone: Chemistry

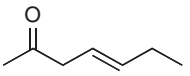
The volatile components from the urine of normal and adrenalectomized female mice were compared and chemical differences identified through gas chromatographic analysis.[131] Six simple compounds accounted for the majority of the difference, at least from the perspective of concentration. These were 2-heptanone (**41**), (*E*)-hept-5-en-2-one (**42**), (*E*)-hept-4-en-2-one (**43**), *n*-pentyl acetate (**44**), (*Z*)-pent-2-enyl acetate (**45**), and 2,5-dimethylpyrazine (**46**). When authentic samples of **41–46** were added to urine from adrenalectomized animals and their concentrations restored to the levels of normal urine, full recovery of biological activity (delay of first estrus) was observed.[131] Subsequent studies have confirmed the biological responses to pyrazine **46**.[132]



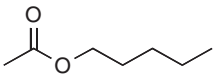
41
2-Heptanone



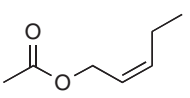
42
(*E*)-Hept-5-en-2-one



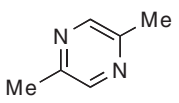
43
(*E*)-Hept-4-en-2-one



44
n-Pentyl acetate



45
(*Z*)-Pent-2-enyl acetate



46
2,5-Dimethylpyrazine

4.05.7.4 Domestic Pig

Pigs (genus *Sus*) are among a group of approximately a dozen ungulates (mammals that walk on two digits); they are from subtropical Eurasia and have tusks. One pig species (*Sus scrofa*) has a long history of domestication, although the wild form is common and often invasive. The domestic pig/wild boar species is known for its large head, high fecundity, large snout, and intelligence. Adult males (boars) are large, solitary, and aggressive. Receptive females ('sows') have developed an acute ability to identify appropriate mates, allowing them to approach and mate. Through early ethological studies it was shown that sows recognize mature males using a variety of visual, tactile, auditory, and odor cues.[133] They then assume a stationary vertical position ('stand') and allow the male to mount. Given the importance of artificial breeding and insemination in the domesticated species and the brevity of the sow's reproductive cycle, it has become important for farmers to be able to predict sow receptivity and then induce standing behavior. A series of studies in the early 1970s led to the discovery of a steroidal pheromone released by boars, which is now used for this purpose.[3] This cue may be the first example of a bona fide vertebrate pheromone to have been identified; it is also the first to be used in a commercial setting.

4.05.7.4.1 Male pig (boar) pheromone: Biology

Dominant boars are large and loud and drool continuously. They have a preputial diverticulum, an invagination of the skin where the penis emerges, where urine, seminal fluid, and sweat accumulate. They look and smell obnoxious. Nevertheless, receptive females of their species show a great deal of interest in preputial fluid, as well as the boar's breath, which has a strong musky odor to the human nose.[134] Interestingly, even the muscle (meat) of these animals has a strong musky aroma ('boar taint'), making it of little commercial value. In the 1960s it was established that boar odor alone could effectively stimulate standing behavior in females and that stimulated males often 'clamp' their jaws causing saliva to be released.[133,134] Subsequently, it was discovered that the distinctive, permeating odor of boars is largely attributable to a variety of odorous 16-androstene steroids related to testosterone.[135,136] These accumulate in the muscle, fat, and sweat glands after biosynthesis in the testis.[137]

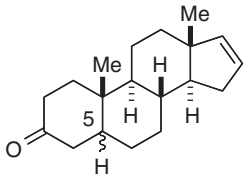
Assuming these odorous steroids might be the causative agents in the boar pheromone, Melrose *et al.*[138] tested the ability of 5α-androst-16-en-3-one (**47**, a prominent fat steroid absent in castrates and females) and 3α-hydroxy-5α–androst-16-ene (**48**, a prominent salivary steroid) to elicit a standing response in sows that were otherwise unresponsive to sexual cues. These steroids were administered in aerosol. Artificial insemination was attempted in those sows that responded positively. Both steroids were effective at identifying fertile sows. These results were later confirmed and extended by Reed *et al.*,[139] who tested another five steroids, either alone or as mixtures. They found that while most were active, androsta-4,16-dien-3-one (**49**) and 5β-androst-16-en-3-one (**50**) were particularly so. Interestingly, **49** and **50** were tested not because they were known to be present in boars but, rather, because of their similar structure and, especially, their similar odor to the human nose as that of **48** and **47**. Although detailed studies linking biological production and behavioral activity of these steroids have not been reported, 5α-androst-16-en-3-one (**47**) is now in commercial use (Boar-Mate™). It is notable that single steroids have activity in this species. Further, Dorries *et al.*[140,141] have used operant conditioning to demonstrate that sows are more sensitive than boars to androstenone **47** and that both the vomeronasal and olfactory systems discriminate this steroid. Interestingly, sows are commonly used to hunt truffles in Europe, the odor of which they probably are able to learn easily because this delectable fungus also produces large quantities of 3α-hydroxy-5α-androst-16-ene (**48**).[142]

4.05.7.4.2 Male pig (boar) pheromone: Chemistry

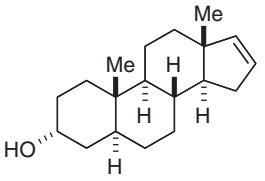
The isolation of 5α-androst-16-en-3-one (**47**) from boar fat was first reported in 1968, following a search for the agent responsible for the tainted odor of that source.[135] It had long been recognized that a foul odor of perspiration (boar taint) was associated with large male pigs, but not with hogs or gilts (immature males or females). Boar fat from several tissues was frozen, minced, thawed, filtered ('through layers of butter muslin supported on a warmed Büchner funnel'[135]), dried over sodium sulfate, and refiltered. The colorless oily residue was passed through a fat stripper, which allowed removal and cold trapping of volatiles from a thin film of fat (*c.*150 ml) that was slowly dripped through a vertical tube at 90 °C and *c.*10^{-4} torr. GC analysis of those volatiles was guided by the nose of the investigator:

> "The odor of each compound (or coincident compounds) producing a chromatographic peak was evaluated as it was eluted from the column by extinguishing the flame of the detector after the apex of the peak had been recorded and smelling the effluent. Continuous olfactory examination of the effluent of the chromatograph during duplicate analyses provided an important supplementary means of detecting odorous compounds which were not present in sufficient quantity to produce a response in the flame detector and hence a peak on the chromatogram. The odor recognized in the fat as boar taint was first located in the effluent of the gas chromatograph in this way although no peak was observed on the recorder chart."[135]

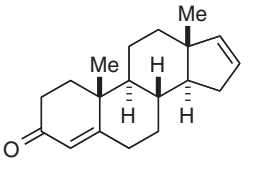
Collection of the GC effluent and subsequent MS analysis allowed assignment of a possible molecular formula as $C_{19}H_{28}O$. Since boar taint can be eliminated by castration of male pigs, attention was focused on the testosterone and androsterone family of compounds as possible candidates. When the crude volatiles were treated with 2,4-dinitrophenylhydrazine, the boar taint odor was completely removed, implicating a ketone functionality for the lone oxygen atom. Anecdotal information implicated several androstene derivatives, including **47**, which was described as having an 'intense, urine-like odor.'[143] An authentic sample of **47** was prepared, and comparison of the GC and MS properties allowed the definitive structural identification of the boar taint compound.



47 5α-Androst-16-en-3-one (H5-down)
50 5β-Androst-16-en-3-one (H5-up)



48 3α-Hydroxy-5α and rost-16-ene



49 Androsta-4,16-dien-3-one

Patterson[136] also examined the odorous salivary glands of boars, a source of an unpleasant smell referred to as 'sex odor' in reports from the 1930s.[144–147] Soxhlet extraction of minced submaxillary glands with diethyl ether provided a solution that contained an unpleasant odor. The contents were tested for relative volatility by a simple experiment.

> "A glass rod, dipped in the solution, acquired and retained the musk odor for an hour or more after the solvent and other volatile compounds had evaporated."[136]

Again, GC and MS analyses led to the identification of the compound responsible for the odor, alcohol **48**, the likely precursor to the 3-keto compound **47**, which accumulates in the fat and should be less easily excreted compared to **48**.

4.05.7.5 Asian Elephant

The elephants are very large land mammals that have trunks and well-developed chemosensory systems. Today only two species are commonly recognized, the Asian elephant (*Elephas maximus*) and the African elephant (*Loxodonta africana*) although the latter is often further subdivided. The two species have very similar behaviors and ecologies; the Asian elephant better studied, probably because it is both smaller and more tranquil. Female elephants live in tightly knit family groups made up of mothers and their young, sisters, and aunts and these groups are led by the eldest female, or matriarch. In contrast, adult male elephants live solitary lives, occasionally living in loosely knit bachelor herds and fighting amongst each other for dominance and access to females. They can be very dangerous, especially during reproductive season when their hormone levels are extremely high. Female elephants have a several-month long estrus cycle that is punctuated by a brief period of fertility. Gestation lasts nearly 2 years and typically produces a single calf. Mate selection and conception is thus of great importance and is known to be strongly influenced by a variety of male and female pheromonal cues released in the urine and well-developed glands in both species.[148,149] However, only for the Asian elephant is there strong biological data to support the chemistry. Here both a male and female signaling

pheromone have been identified. Studies of elephant biology have been complicated by the fact that elephants are highly intelligent and acutely aware of their own identities as well as those of other elephants, who they remember for many years.

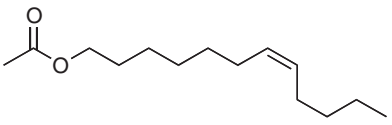
4.05.7.5.1 Female sex pheromone: Biology

As a consequence of their solitary lifestyle, male Asian elephants are under substantial evolutionary pressure to develop the means to locate fertile females whose period of fertility is quite brief. Conversely, female elephants are under pressure to be able to identify the most fertile male amongst many (violent and dangerous) suitors. The process by which females signal their reproductive condition to males is mediated by both sound and chemical cues. Specifically, Rasmussen and Krishnamurthy[148] have discovered that female elephants release distinctive blends of volatile odorous compounds in their urine (45–65 l day^{-1}) and that these suites change during the estrus cycle. Initial tests involved exposing lone male elephants in zoos to various urine samples and controls. Reproductive males in musth (see Section 4.05.7.5.3) responded by exhibiting olfactory sampling and then flehmening at which time they placed the tips of their trunks, first, on or above the odor source and, then, into their mouth over their vomeronasal duct.[150] In later work using fractioned female urine, the key chemical components were identified and then used in sophisticated flehmen assays that carefully controlled simple olfactory inspection behavior. Through these studies the active component was found to be (*Z*)-7-dodecen-1-yl acetate (**51**).[151,152] It is interesting that this is the same pheromonal compound used by many lepidopteran moths, although, of course, context plays a role; there is little likelihood that an elephant would confuse a moth for another of its own kind.[3,151] It is likely that **51** is complemented by other urinary cue components because, when tested alone, it is not as active as whole female urine. Interestingly, the mucous of the male trunk contains an 18.5 kDa olfactory binding protein that appears to further regulate the availability of **51**.[153]

4.05.7.5.2 Female sex pheromone: Chemistry

Fresh urine was collected from Asian elephant females that were at the height of estrus.[150] Urine was extracted with an immiscible organic solvent and concentrated. Bioassay involved reconstitution of the extract concentrate in acetone, addition of the acetone solution to (in active) urine samples from females that were not in estrus, and application to concrete slabs or logs. Observers, who were unaware of the sites where samples had been placed, recorded the flehmening responses of test bull elephants. These studies convincingly demonstrated that 'in Asian elephants the estrous state, and probably the receptivity of the female, is revealed by a substance or substances that can be extracted from the urine'[150] of females in estrus.

This bioassay was used to monitor fractionation, which mostly involving normal-phase separations on silica gel. Eventually both ^{1}H NMR and Gas chromatography–mass spectrometry (GC–MS) analyses, the latter supported by bisthiomethylation methodology of Buser (cf., Equation 3),[85] were used to secure the structure of *Z*-7-dodecen-1-yl acetate (**51**).[152] The ability of this acetate ester to induce overt flehmening was established in a large number of test animals. Varying levels of **51** in urine collected from females at different times during the estrus cycle were measured using a solid-phase microextraction and gas chromatographic protocol. These varied from being nondetectable in the luteal phase through 0.48 and 13 μg ml^{-1} in the early and mid-follicular stages to a high of 33 μg ml^{-1} just before ovulation.[152]



51 *Z*-7-Dodecen-1-yl acetate

4.05.7.5.3 Male sex pheromone: Biology

Mature male elephants (bulls) have active endocrine systems and experience huge increases in circulating testosterone as they mature. Levels peak dramatically during the reproductive season, a condition known as 'musth.' Bulls in musth are very aggressive and they develop a number of glandular secretions, amongst which that of the facial temporal gland (scent glands located near their eyes) is well studied. Rasmussen *et al.*[154] have

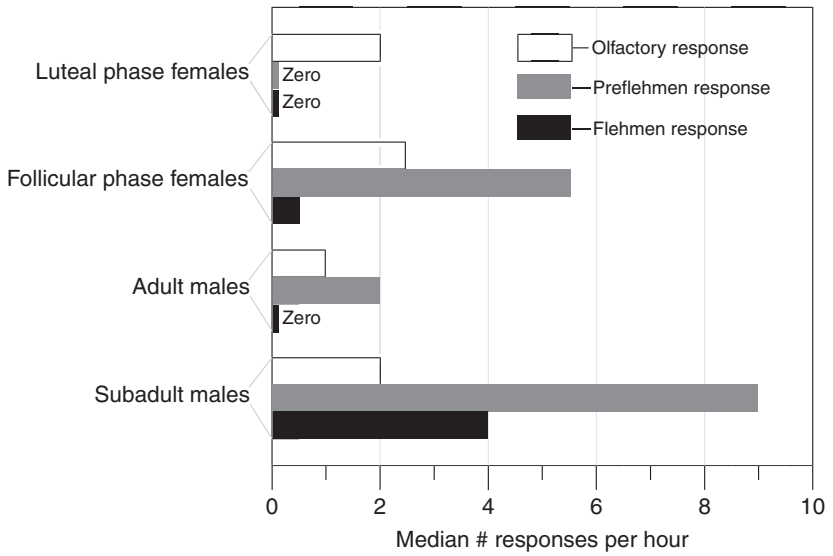
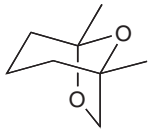


Figure 5 Chemosensory inspection behaviors by male and female elephants to frontalin (**52**), demonstrating specific and varying responses to this male pheromone during different life history stages.[155]

discovered that subordinate females and young males will strongly avoid these excretions. Bioassay-driven fractionation studies by this group led to the eventual isolation and structure determination of frontalin, 1,5-dimethyl-6,8-dioxabicyclol[3.2.1]octane (**52**). More specifically, **52** will elicit strong, prolonged flehmening responses in both subadult males and females that are either in the follicular (preovulatory) phase of estrus or pregnant; dominant males and ovulating females do not respond in notable ways (**Figure 5**).[155] The activity of this structure, which also serves as an aggregation pheromone in the bark beetle, is probably complemented by other compounds and could have other effects that have not yet been studied.

4.05.7.5.4 Male sex pheromone: Chemistry

First identified in Asian elephants during a headspace analysis of volatiles collected from secretions of the musth temporal gland of adult males,[156] frontalin (**52**) is a bicyclic ketal, which is structurally reminiscent of the male mouse priming pheromone component 3,4-dehydro-*exo*-brevicomin (**37**). Frontalin (**52**), *Z*-7-dodecen-1-yl acetate (**51**), was already known because of its chemosensory role in the insect world; it is an aggregation pheromone in bark beetles.[157] Interestingly, the ratio of the two enantiomers of frontalin (**52**) changes with age and stage of musth and elicits different behavioral responses.[158]



52 1,8-Dimethyl-6,8-dioxabicyclo[3.2.1]octane (frontalin)

4.05.7.6 Humans

Human beings, *Homo sapiens*, are highly intelligent primates whose behavior is complex and strongly influenced by experience (learning) as opposed to instinctual mechanisms. Additionally, humans do not appear to possess a functional vomeronasal system and express few, if any, of the olfactory receptor genes (the V1Rs) that have been associated with pheromone detection in other mammals.[4] Nevertheless, there is good evidence that humans subtly use a variety of pheromones whose identities have not yet been established in the peer-reviewed

literature. We mention here cues for which biological responses have been repeatedly observed. A recent review of human pheromonal cues has appeared.[159]

Family relationships are important to humans, and numerous studies have now shown that we can discern familial odors.[159] Perhaps of special interest is the observation that women tend to chose mates whose MHC complex is dissimilar to their own;[160,161] whether MHC-associated peptides are involved remains to be tested. Similarly, the odor of breast milk has been shown to attract babies, much like the nipple search pheromone used by rabbits as discussed above.[162] Although the chemistry of human underarm (axillary gland) odor and its signaling pheromone-like effects is the subject of a multibillion dollar industry, and sex-specific differences in the production of dozens of volatiles have been noted, individual cues with specific activity have yet to be identified.[159] Nevertheless, it is interesting that similar to many rodent pheromones, some human axillary gland odorants bind to apolipoproteins.[163] Also intriguing, is the recent observation that high concentrations of the aromatic axillary gland steroid 4,16-androstadioen-3-one (androstenone, **44**) causes subtle, seemingly subconscious, changes in human mood and brain glucose levels.[164,165] Human sensitivity to certain odors is also known to vary with endocrine state.[166] Mood-altering effects of underarm odor also have been noted,[167] and have tentatively been referred to as 'modulator pheromones;'[164] however, this term is not commonly used.

In contrast to the incomplete state of the evidence for putative human signaling pheromones just discussed, evidence for human priming pheromone that influences estrus cycling is strong. The similarities to cues with similar function used by mice and goldfish are noteworthy. Numerous studies have shown that women who share the same environment (e.g., roommates, mothers, and daughters), experience a shortening and synchronization of their estrus cycles.[168,169] Further, women simply exposed to axillary odor from donor females will, over the course of just a few cycles, develop estrus cycles that match that of the donor female.[169,170] There is evidence for both cycle accelerators and inhibitors (blends). Other studies appear to demonstrate that, as in mice, male odors may accelerate female cycles as well.[171] Finally, this effect has been confirmed by studies in which hospital-bound women have been exposed to axillary gland odor and their circulating luteinizing hormone (LH) then monitored.[172] None of these priming cues has been identified at the chemical level. Some speculate that they may have evolved in early humans whose tightly knit family groups would function better if women shared common reproductive cycles.[173]

4.05.8 Overview

Understanding of vertebrate pheromones has increased dramatically since the first cue was identified in the pig over 40 years ago. Nevertheless, pheromonal cues have only been definitively identified in just over a dozen of the approximately 58 000 species of vertebrates – much fundamental work remains. Perhaps the most significant advance in our understanding of vertebrate pheromones is the realization that most comprise multiple components and that these are found in complex mixtures that vertebrate chemosensory systems discern with precision. Further, it is now clear that the neural basis for pheromone function is mediated by multiple nervous systems (e.g., different ORNs and even organs, as in the vomeronasal system); we should expect the relatively sophisticated vertebrate brain to be employing pheromones in sophisticated and diverse manners. Indeed, the examples of both goldfish and mice show that mixture composition and context both contribute to the determination of function.

Although with a few exceptions (e.g., PADS (**3**) in sea lamprey or splendipherin (**17**) in frogs) most of the individual compounds that comprise vertebrate pheromones are relatively simple, the variety is impressive. Alcohols, ketones, lipids, peptides, and steroids are of special importance. Interestingly, the majority of these structures are found in various excretions and secretions whose production often appears to be specialized (e.g., prostaglandin pheromones in goldfish, frontalin (**52**) in the Asian elephant). How this specialization occurred from an evolutionary perspective and its biochemical basis are largely unknown. However, as these facts become better understood, future researchers will be better informed of where/what to look for and how to assess new vertebrate pheromones and be able to isolate and identify their components with greater ease. Here, the importance of peptide cues (e.g., sodefrin (**15**) in the salamander or MHC-derived peptides (MUPs) in the mouse) may prove to play special roles and are promising lines of investigation because of the relative ease with which the tools of molecular biology can be used once the peptide has been identified. There also appears to be

a need for additional work on steroid biochemistry (e.g., PADS in the lamprey), because novel unexpected products are being isolated. Protein binding transport systems now have been described in the mouse and elephant. How these systems work is not yet well understood, but is clearly a fascinating aspect of pheromone function.

Our knowledge of vertebrate pheromonal communication systems is in its infancy. Certainly, many significant and fascinating questions about the basic nature of the evolutionary and biochemical mechanisms that underlie pheromone evolution and function await answers.

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Biographical Sketches

Peter W. Sorensen was born in Billings, Montana, USA but spent his childhood outside of New York City before moving to Europe for high school. He received a B.A. in biology from Bates College in 1976 where he was introduced to fish sensory biology. In 1984, Peter received a Ph.D. in biological oceanography from the University of Rhode Island, where he studied the use of olfactory cues (including pheromones) by the American eel under the guidance of Howard Winn, Yuzuru Shimizu, and Saul Saila. In 1984, Peter was awarded a postdoctoral fellowship by the Alberta Heritage Foundation for Medical Research to study links between hormones and pheromones at the University of Alberta with Norm Stacey. Together, they discovered that hormones serve as substrates for sex pheromones in goldfish. Peter assumed a faculty position at the University of Minnesota in 1988 where he since has been working with its fisheries, ecology, and neuroscience programs. His interests are broad but revolve around chemical mechanisms that drive fish behavior. Tom Hoye and he collaborated to identify lamprey migratory pheromones. Presently, Peter's group focuses on the chemical ecology and physiology of common carp while addressing whether pheromones might be used to control invasive fishes.

Thomas R. Hoye was born in New Wilmington, PA, USA. He earned B.S. and M.S. degrees in 1972 at Bucknell University and the Ph.D. in 1976 from Harvard University, studying in the laboratories of Professors Harold W. Heine and Robert B. Woodward, respectively. That fall he joined the faculty at the University of Minnesota. His teaching interests span synthetic and mechanistic organic chemistry as well as the continuing evolution of advanced and honors undergraduate organic chemistry laboratory courses. He was recognized as a University of Minnesota Distinguished Graduate Teaching Professor in 1999, the inaugural year of that award. In 2007, he received the Horace T. Morse-University of Minnesota Alumni Association Award for Outstanding Contributions to Undergraduate Education. His research interests are broad. They include the total synthesis and structure determination of natural products, the discovery of new synthetic methods, the advancement of new strategies in organic synthesis, the development of new methods in NMR spectroscopy, organometallic chemistry, peptides and peptidomimetics as antiangiogenic and antibacterial agents, polymer synthesis, polymeric materials from renewable resources, the design of functional block copolymers and nanoparticles useful in drug and vaccine delivery, and the study of fish pheromones and odorants.

4.06 Pheromones of Marine Invertebrates and Algae

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4.06.1 Introduction

This chapter focuses on the study of pheromones of marine invertebrates and algae. The term 'pheromone' refers to an intraspecific chemical signal, and includes alarm pheromones, food trail pheromones, sex pheromones, and many others that affect behavior or physiology. Up to the present moment, various pheromones have been identified in marine invertebrate and algae communication systems, particularly sex pheromones.[1] Researchers in marine chemical ecology have been attracted to reproductive systems as well as predator–prey and competitive interactions, settlement cues, and defense substances against infection by microorganisms.[2–7] To sustain precious marine natural resources, it is important to have a broad understanding of systematic biology and marine ecology. To achieve this, clarification of the function and role of chemical cues that significantly influence biological and physiological phenomena in marine creatures are becoming increasingly essential.

Several books and reviews dealing with general or specialized subjects in pheromones of marine invertebrates and algae have been published.[8–13] Here we describe the recent progress regarding such pheromones, with a special focus on their structural and functional diversity.

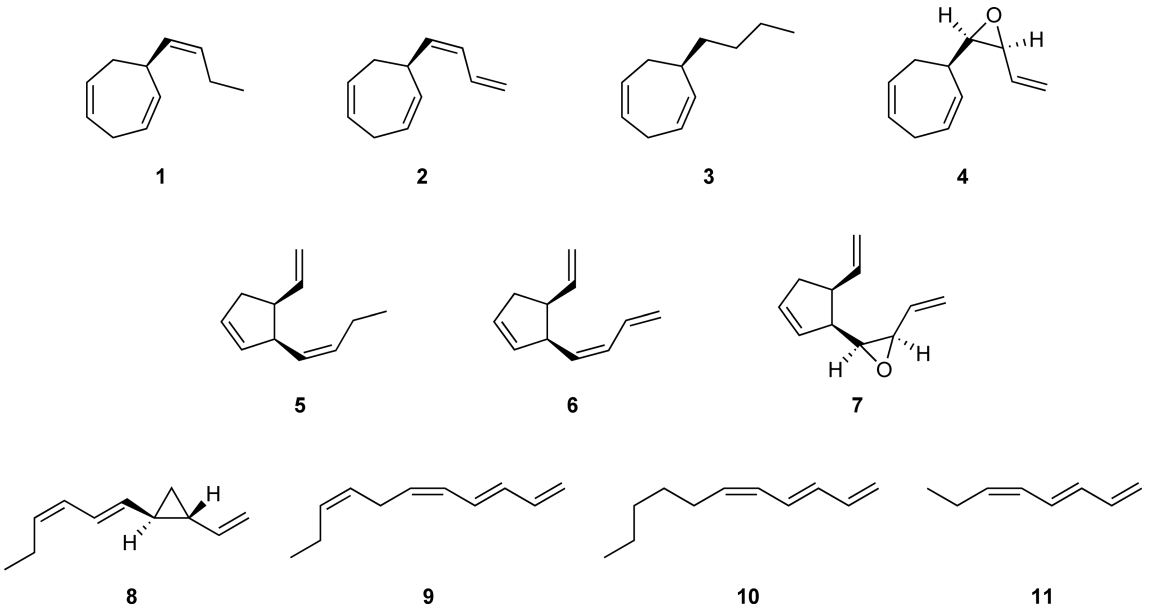
4.06.2 Algal Sex Pheromones

4.06.2.1 Brown Algae

The presence of spermatozoid-releasing and spermatozoid-attracting substances released from the eggs of marine brown algae was first suggested for the *Fucus* species in the first half of the twentieth century.[14] In 1971, Müller *et al.*[15] reported the isolation of ectocarpene (**1**) from fertile female gametophytes of the cosmopolitan

brown alga *Ectocarpus siliculosus*. They observed that culture dishes of mature gynogametes of *E. siliculosus* released a faint aromatic fragrance, which was not perceived by the male culture. This isolation technique exploits the volatility of the compounds by collecting them in a closed system with a stream of air continuously being circulated, and the collection done on a filter of activated carbon. After desorption with dichloromethane, the volatile substances are analyzed by glass capillary gas chromatography. Müller also devised a quantitative assay for testing chemotaxis. Volatile substances to be tested are dissolved in an inert fluorocarbon which is insoluble in, and more dense than, water. When samples were injected with a syringe, male gametes immediately congregated around the outlet of the syringe. When this phenomenon was used as a biological test in *Ectocarpus*, *c.*1 kg of cultured mature gynogametes gave 92 mg of the active attractive substance, which was named as ectocarpene (**1**). The chemical structure of **1** was established to be (+)-(6*S*,1′*Z*)-6-(1′-butenyl)-1,4-cycloheptadiene, thereby making it the first sex pheromone from algae whose chemical structure was introduced.

The structure of the sex pheromone for the *Fucus* species, fucoserratene (**11**), was elucidated in 1973.[16] The positions and geometries of alkenes were revealed by comparison of the gas chromatographic behavior with those of the isomeric conjugated 1,3,5- and 2,4,6-octatrienes. To date, a series of hydrocarbons and epoxides **1**–**11** and their stereoisomers have been identified within the pheromone bouquets of more than 100 different species of brown algae.[17–23] Identification of these compounds was based on a combination of gas chromatography–mass spectrometry (GC–MS) analysis and by comparison with authentic synthetic compounds. These sex pheromones were all lipophilic, volatile compounds that consisted of C_8 or C_{11} linear or monocyclic hydrocarbons or their epoxides. The monocyclic compounds have a cyclopropane, cyclopentene, or cycloheptadiene structure. Interestingly, the relationships between the chemical structures of pheromones and the taxonomical classifications of algae are unclear (**Table 1**).



Mature female gametophytes of brown algae of the order Laminariales, which includes the large kelps used as food, such as tangle, secrete a highly volatile material that induces an explosive discharge of antheridia and spermatozoids. The active substance was investigated using mass cultures of female gametotypes of *Laminaria digitata* and was identified as lamoxirene (**4**) in 1978.[24] Lamoxirene (**4**) induced the mass release of male gametes of *L. digitata* within 8–12 s at a threshold of *c.*50 pmol. Lamoxirene (**4**) is also active against five species of brown algae of the order Laminariales. The culture suspensions of mature female gametophytes of eight species of Japanese brown algae, belonging to the order Laminariales, induced the release of spermatozoids from

Table 1 C_{11} and C_8 pheromones from marine brown algae

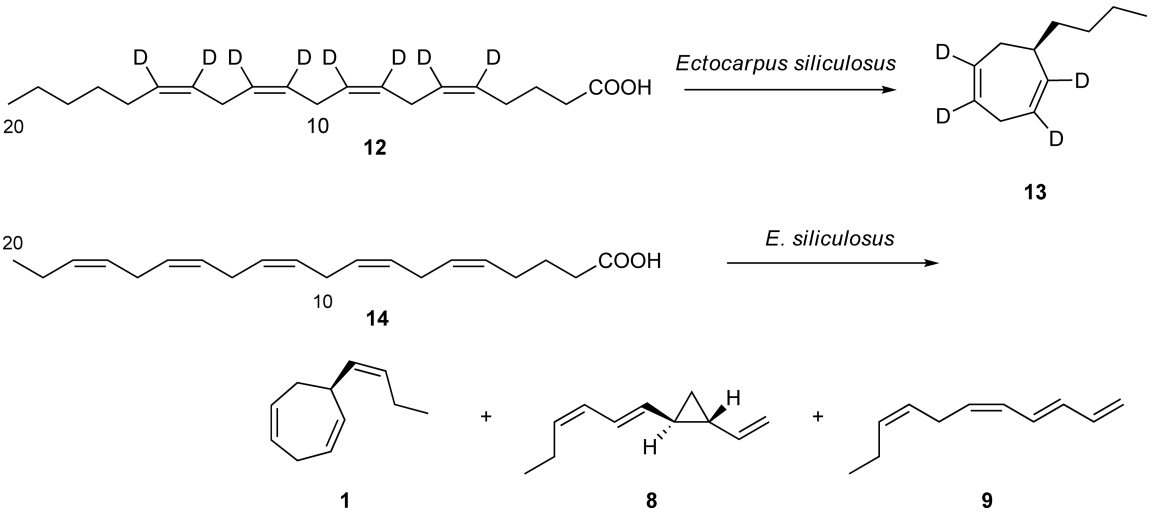
Pheromone	*Release (R)/ attraction (A)*	*Algal species*	*Pheromone*	*Release (R)/ attraction (A)*	*Algal species*
Ectocarpene (**1**)	A	*Ectocarpus* spp.	Viridiene (**6**)	A	*Syringoderma phinneyi*
	A	*Adenocystis utricularis*		R/A	*Desmarestia virdis*
	A	*Sphacelaria rigidula*	Caudoxirene (**7**)	R	*Perithalia caudata*
Desmarestene (**2**)	R/A	*Desmarestia* spp.			*Sporochnus radciformis*
	A	*Cladostephus spongiosus*	Hormosirene (**8**)	A	*Hormosira banksiii*
Dictyotene (**3**)	A	*Dictyota dichotoma*		A	*Durvillea* spp.
	A	*Dictyota diemensis*		A	*Xiphophora* spp.
Lamoxirene (**4**)	R/A	*Laminaria* spp.		A	*Scytosiphon lomentaria*
	R/A	*Alaria* spp.		A	*Colpomenia perergrina*
	R/A	*Undaria pinnatifida*	Finavarrene (**9**)	A	*Ascophyllum nodosum*
	R/A	*Macrocystis pyrifera*		A	*Sphaerottichia divaricata*
	R/A	*Nereocystfs luetkeana*	Cystophorene (**10**)	A	*Cystophora siliquosa*
Multifidene (**5**)	A	*Cutleria mulffida*	Fucoserratene (**11**)	A	*Fucus serratus*
	R/A	*Chorda tomentosa*			
	A	*Zonana angustata*			

antheridia of all eight species.[13] Thus, this sex pheromone proved to be a common spermatozoid-releasing and spermatozoid-attracting substance in brown algae of the order Laminariales.

Recent advances in the gas chromatographic separation of enantiomers have allowed precise determination of the enantiomeric purity of algal pheromones.[17] The *cis*-disubstituted cyclopentenes, such as multifidene (**5**), viridiene (**6**), and caudoxirene (**7**), are of high optical purity (>95% enantiomeric excess (e.e.)) whenever they have been identified.[18,19] However, the situation is different with the cyclopropanes and cycloheptadienes. For example, the enantiomeric composition of hormosirene [(−)-(1*R*,2*R*)-**8** and (+)-(1*S*,2*S*)-**8**] from female gametes or thalli of brown algae varied with the species (among the genera *Dictyopteris*, *Analipus*, *Durvillaea*, *Haplospora*, *Hormosira*, and *Xiphophora*) and even depended on the locality. It is assumed that for brown algae the production of characteristic enantiomeric mixtures represents a simple means for individualization of the signal blends, although this is not supported by any experimental results.

To date, it has been shown that these algae pheromones have at least three well-defined biological functions: (1) synchronization of the mating of male and female cells by the controlled release of male spermatozoids, (2) enhancement of mating efficiency by attraction, and (3) chemical defense of the plant due to the presence of high amounts of pheromones within and released into the environment. Interestingly, the occurrence of C_{11} hydrocarbons is not limited to marine brown algae. The same compounds are also found in cultures of diatoms and among the volatile compounds released during blooms of microalgae in freshwater lakes. C_{11} hydrocarbons have also been identified in roots, leaves, blossoms, and fruit of higher plants, although their specific biological functions have not been characterized.

The biosynthesis of these marine algal pheromones has been well investigated. In marine algae, it is shown that C_{11} hydrocarbons are derived from polyunsaturated C_{20} fatty acids. Exogenously supplied [$^2H_8$]-arachidonic acid (**12**) was efficiently converted into labeled [$^2H_4$]-dictyotene (**13**), which indicated that the $C_{11}H_{18}$ hydrocarbons are formed from the C-10 to C-20 positions of arachidonic acid (**Scheme 1**).[25] Thus,



Scheme 1

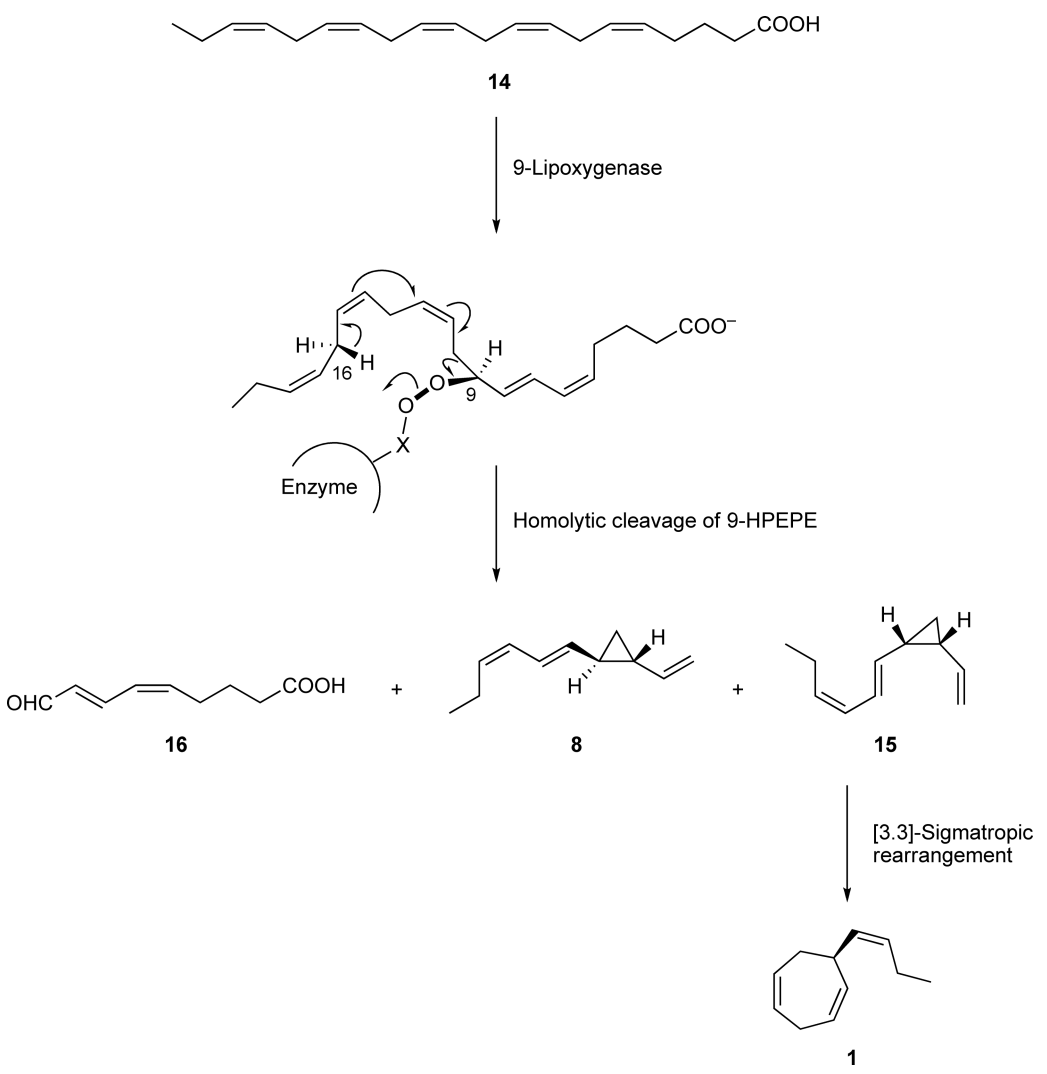
$C_{11}H_{16}$ hydrocarbons with one additional degree of unsaturation, such as (*S*)-ectocarpene (**1**), hormsirene (**8**), and finavarrene (**9**), are expected to be formed from eicosapentaenoic acid (**14**).

The first functionalization of the eicosanoid (**14**) has been assumed to involve 9-lipoxygenase to yield 9-hydroperoxyicosa-(5*Z*,7*E*,11*Z*,14*Z*,17*Z*)-pentaenoic acid (9-HPEPE) (**Scheme 2**). If we consider the 6*S* configuration of ectocarpene (**1**), a homolytic cleavage of 9-HPEPE with hydroperoxide lyase gives an allyl radical intermediate, which cyclizes to the 1*R*,2*S*-*cis*-disubstituted cyclopropane (**15**) with some amount of hormsirene (**8**).[26] The sequence is terminated by transfer of a hydrogen atom from C16 to the enzyme -X-O^- function. In addition to the C_{10}–C_{20} cyclopropane fragments, the C_1–C_9 dicarbonyl fragment **16** would be released.

Furthermore, the *cis*-cyclopropane **15** is thermolabile, and thus a subsequent spontaneous [3.3]-sigmatropic rearrangement (Cope rearrangement) is assumed to proceed via a *cis–endo* transition state to give (*S*)-ectocarpene (**1**). This hypothesis was verified by the synthesis and rearrangement reactions of thermally labile *cis*-divinylcyclopropane **15** and its analogues. For instance, the Cope rearrangement of **15** occurred spontaneously at ambient temperatures to afford **1**. The half-life of **15** for these transformations was 56 min at 8 °C and 21 min at 18 °C.[27] These results suggested that other 6-substituted cyclohepta-1,4-dienes such as desmarestene (**2**), dictyotene (**3**), and lamoxirene (**4**) are also biosynthesized via similar rearrangements of *cis*-cyclopropane derivatives. Surprisingly, comparative biological assays using male gametes of *E. siliculosus* revealed that the unstable *cis*-cyclopropane **15** was much more active than the stable cycloheptadiene **1**. The threshold concentration of **1** was estimated to be 10 nmol l^{-1} in seawater, whereas that of **15** was significantly lower, ~5 pmol l^{-1}. The release of pheromones by female gametes, chemotactic orientation of male gametes, and fertilization all occur within a few minutes. Consequently, it is possible that thermally labile *cis*-divinylcyclopropyl precursors may be the actual pheromones even when the cycloheptadienes were identified as active pheromones.

4.06.2.2 Green Algae

The volvocine algae range in complexity from unicellular *Chlamydomonas* through colonial genera (such as *Gonium*, *Pandorina*, and *Eudorina*) to multicellular organisms and are capable of both asexual and sexual reproduction.[28] *Volvox carteri* reproduces asexually most of the time in nature, but would die in minutes once their pond dried up in the heat of late summer. However, *V. carteri* can survive by switching to a sexual life cycle shortly before the water disappears, to produce dormant zygotes that survive the drought. When rain fills the pond again in spring, the zygotes hatch out to establish a new generation of asexually reproducing individuals. In the early 1970s, sexual development in *V. carteri* was found to be initiated by a sex-inducing pheromone, a



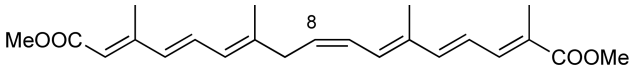
Scheme 2

32-kDa glycoprotein.[29,30] On the basis of the results of cDNA cloning and sequence analysis, its primary structure was deduced to contain 208 amino acids including a signal sequence and six putative N-glycosylation sites.[31,32] It has been shown that this sex-inducing pheromone leads to gamete production at a concentration of about 10^{-16} mol l^{-1}. Thus, this pheromone is one of the most potent biological effector molecules known.

Although it is still unknown why this sex-inducing pheromone is active at such a low concentration in *V. carteri*, it has been demonstrated that the sexual cycle is initiated by heat shock that causes the somatic cells of the asexual *Volvox* spheroid to produce the sex-inducing pheromone.[33] The first biochemical response to this pheromone was shown to be the synthesis of specific extracellular matrix glycoproteins, which have been designated pherophorins.[34,35] Since pherophorins contain a domain that is homologous to the sex-inducing glycoprotein pheromone, the level of pheromone may be further amplified by the ability of sperm cells to produce more sex-inducing pheromone.

The sexuality and genetics of the freshwater green flagellate, *Chlamydomonas eugametos*, *C. moewusii*, and *C. reinhardtii*, have also been extensively studied since the 1930s. Early studies mentioned that a 3:1 mixture of (8*Z*)-crocetin dimethyl ester (**17**) and its (8*E*)-isomer activated and attracted the female gametes of *C. eugametos*, whereas a 1:3 mixture was effective for its male gametes.[36,37] However, these observations have not been shown in a reproducible manner, and their sex pheromones were still unclear. In 1995, the attractant of male gametes

produced by female gametes of the green flagellate, *Chlamydomonas allensworthii*, was successfully isolated. Its structure was elucidated as (4*E*,8*E*,12*E*)-14-[2′-hydroxy-3′,4′-dimethyl-5′-(1″-*β*-D-xylo-pyranosyloxy)phenyl]-4,8,12-tri-methyltetradeca-4,8,12-trienoic acid (lurlenic acid) (**18**).[38,39] Additionally, the primary alcohol **19**, lurlenol, was identified to be a sex pheromone from another strain of *Chlamydomonas*.[40] Both lurlenic acid (**18**) and lurlenol (**19**) showed significant attractant activity at concentrations as low as 1 pmol l^{-1}. The threshold concentrations of synthesized **18** and **19**, which show the attractant activity were similar to natural compounds.[41] Furthermore, structure–activity relationship studies on lurlenic acid (**18**) revealed that the important structural features for attractant activity were the sugar moiety, the phenolic hydroxy group, the appropriate length, and the unsaturation of the side-chain group, and the presence of a polar group at the terminal position of the side chain.[42,43] Notably, analogues of **18** with a different sugar moiety, including L-xylose, D-glucose, D-galactose, and D-arabinose, were almost inactive, which strongly suggested that the male gametes of *C. allensworthii* are highly specific to the D-xyloside.



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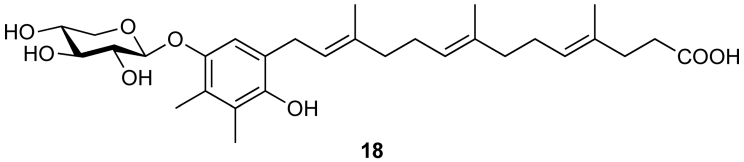

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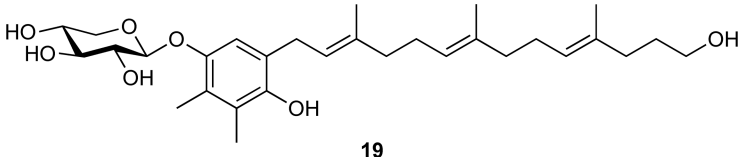

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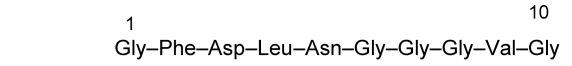
4.06.3 Sperm Chemotaxis in Marine Invertebrates

Sperm are attracted by chemical substances, which are released by eggs. This process is called chemotaxis and is a well-known phenomenon in most animals and lower plants.[44] The attraction of sperm to eggs should counteract the effects of dilution and increase the probability of fertilization in free-swimming invertebrates. Since the fertilization rate may be low under natural conditions, the presence of such chemoattractants is believed to be important. Sperm-attractant substances have been identified in several marine invertebrates, including hydrozoans, mollusks, ascidians, starfish, sea urchins, and urochordates.

4.06.3.1 Sea Urchin

It has been known for nearly a century that soluble factors associated with the eggs of certain species of sea urchins enhance the respiration and motility of their spermatozoa.[45] These factors have been shown to be diffusible in dialysis, heat-stable, alcohol-stable, and nonvolatile.[46] The sea urchin egg is surrounded by an extracellular investment known as the jelly layer, the major components of which are high-molecular-weight fucose sulfate-rich glycoconjugates. Kopf *et al.*[47] found that the egg jelly of the sea urchin *Strongylocentrotus* has a factor that elevates the respiratory rate and cAMP and cGMP levels of homologous spermatozoa. Thus, constituents of their egg jelly, especially relatively low-molecular-weight peptides, have been well investigated.

To date, about 80 peptides that affect sperm motility have been identified from two different phyla, Echinodermata and Cnidaria, and these have been referred to as sperm-activating peptides.[48,49] For example, speract (**20**), or sperm-activating factor H2, is a decapeptide that was isolated from the jelly layer of *Strongylocentrotus purpuratus* and *Lytechinus pictus*.[47,50–52] Treatment of *S. purpuratus* with **17** significantly increases sperm respiration and motility. Meanwhile, resact (**21**), which was isolated from the jelly layer of *Arbacia punctulata* eggs, is one of the rare sperm-activating peptides whose chemotactic function has been unequivocally demonstrated.[53,54] Resact (**21**) is a 14-amino acid peptide with an intramolecular disulfide bond between Cys^1 and Cys^8, and the carbonyl-terminal leucine is amidated.[55] Resact (**21**) stimulates sperm respiration rates by 5–10-fold, and increases the cGMP levels of *A. punctulata* spermatozoa at 0.5 nmol l^{-1}.[56] It has also been shown that resact (**21**) induces a change in the phosphorylation state and enzymatic activity of sperm guanylate cyclase, which is highly enriched in the sperm flagella.[57,58]



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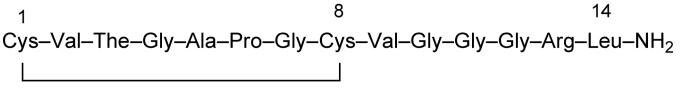

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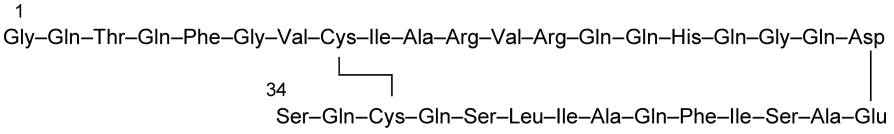
4.06.3.2 Starfish

Various sperm-activating peptides of starfish have also been identified. Among them, however, asterosap (P15) (**22**), which was isolated from the egg jelly coat of the starfish *Asterias amurensis*, is the only example for which the chemotactic function has been unequivocally demonstrated.[59,60] Asterosap is a glutamine-rich tetratriacontapeptide with an intramolecular disulfide linkage between Cys^8 and Cys^{32}. Sperm respond with high sensitivity to picomolar concentrations of asterosap (**22**).[61]



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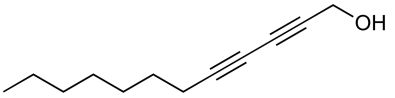
On the basis of the cloning of cDNA and chemical cross-linking analysis, the receptors for both asterosap (**22**) and resact (**21**) in the sperm flagellum were elucidated to be guanylyl cyclases that synthesize cGMP from GTP after binding to the receptor peptide.[62,63] Meanwhile, stimulation of the sea urchin *A. punctulata* and the starfish *A. amurensis* by the chemoattractant or by an intracellular cGMP evokes Ca^{2+} spikes in the flagellum, which elicits a turn in the trajectory followed by a period of straight swimming.[64] Notably, when sperm swim in a concentration gradient of the attractant, the Ca^{2+} spikes and the stimulus function are synchronized, which suggests that Ca^{2+} spikes in the flagellum control navigation.

4.06.3.3 Coral

Sexual reproduction with eventual larval dispersion in corals plays an important role in the recruitment of new genets to other reefs and ensures genetic vitality.[65,66] As for coral, the chemical structure of sperm chemoattractants in the scleractinian coral *Montipora digitata* has been identified. *M. digitata* is a hermaphroditic coral, which reproduces bi-annually, releasing egg–sperm bundles during mass spawning in late spring–early summer and autumn each year. The buoyant egg–sperm bundles float to the surface where they break apart, releasing eggs and sperm into the ocean. From the dichloromethane extracts of lyophilized unfertilized eggs, an

unsaturated fatty alcohol, dodeca-2,4-diyn-1-ol (**23**), was identified as the chemoattractant of *M. digitata* sperm.[67] Notably, a natural mixture of **23**, tetradec-13-ene-2,4-diyn-1-ol (**24**), and (*Z*)-heptadeca-14,16-diene-2,4-diyn-1-ol (**25**) in a ratio of 1:4:9 was more effective at attracting sperm from *M. digitata* than those from other *Montipora* species. Consequently, it is believed that sperm attractants act to reduce the incidence of hybridization between different species of *Montipora.*



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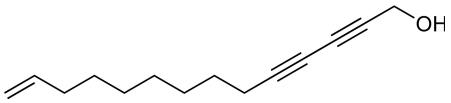

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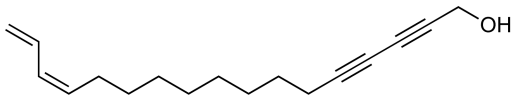

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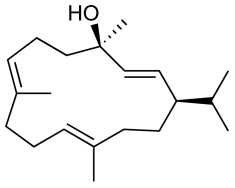
As structurally related compounds for the sperm chemoattractants **23**–**25**, several cytotoxic and antibacterial unsaturated fatty acids, such as montiporic acids, have been isolated from the eggs of *Montipora* sp., in which they have been suggested to serve as defense materials. However, it was recently found that sodium salts of montiporic acids A (**26**) and C (**27**) isolated from the breeding seawater of *Montipora* sp., showed potent feeding-attractant activity toward *Drupella cornus* at doses of 1 and 0.1 mg 20 ml^{-1} agar, respectively.[68] Among several coral-feeding marine creatures, the crown-of-thorns starfish *Acanthaster planci* and the muricid gastropods genus *Drupella* are well known to be voracious coral predators. Interestingly, the structures of the coral sperm chemoattractants are similar to those of the feeding attractants for their predators.

26

27

Soft corals (Cnidaria, Octocorallia, and Alcyonacea) are important contributors to the attached benthic communities on shallow Indo-Pacific reefs. The eggs of *Lobophytum crassum* contain significant amounts (6% dry weight) of (−)-*epi*-thunbergol (**28**), which has been shown to attract the sperm of *L. crassum* at concentrations as low as 3.25 μg ml^{-1}.[69] This is the first evidence for sperm chemotaxis in the Alcyonacea. Interestingly, (−)-thunbergol (**29**) from the eggs of *Lobophytum compactum* and (+)-thunbergol from a Douglas fir tree both showed levels of attraction similar to (−)-*epi*-thunbergol (**28**) against *L. crassum* sperm. These results suggest that *L. crassum* sperm are neither stereo- nor enantio-specific in their sensitivity toward the attractants. While the sperm attractant in the scleractinian coral *M. digitata* was a series of unsaturated fatty alcohols, the sperm-attracting agent for *L. crassum* was cembranoid diterpenes.



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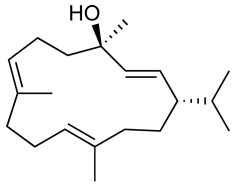
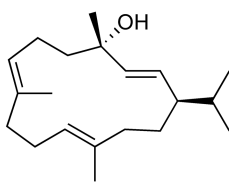


(−)-**29**

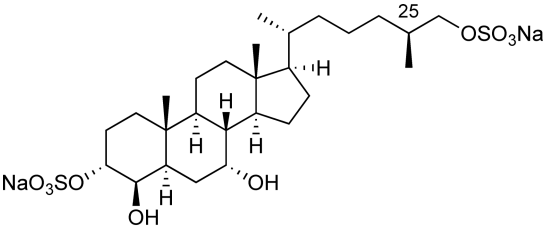


(+)-**29**

4.06.3.4 Ascidian

The chemotactic behavior of ascidians is also interesting, and extensive investigations have been carried out. It has been shown that spermatozoa of the ascidians *Ciona intestinalis* and *Ciona savignyi* near an unfertilized egg are intensely activated and begin to show chemotactic behavior toward the egg.[70–72] The eggs release a sperm-activating and sperm-attracting factor (SAAF) from their vegetal pole. SAAF induces Ca^{2+}-influx and membrane hyperpolarization, which causes a transient increase in intracellular cAMP in the sperm, and induces the activation of sperm motility.

In 2002, through the use of nuclear magnetic resonance (NMR) and mass spectrometry/mass spectrometry (MS/MS) analysis, the planar and partial stereostructure of a SAAF from the egg-conditioning medium of *C. intestinalis* was elucidated to be a previously uncharacterized sulfated steroid: 3,4,7,26-tetrahydroxycholestane-3,26-disulfate (**30**).[73] Its structure was deduced from only ~4 μg (6 nmol) of sample. Thus, SAAF may represent the smallest amount of sample used in the structure elucidation of novel nonpeptidic or nonoligosaccharide natural products.[74]



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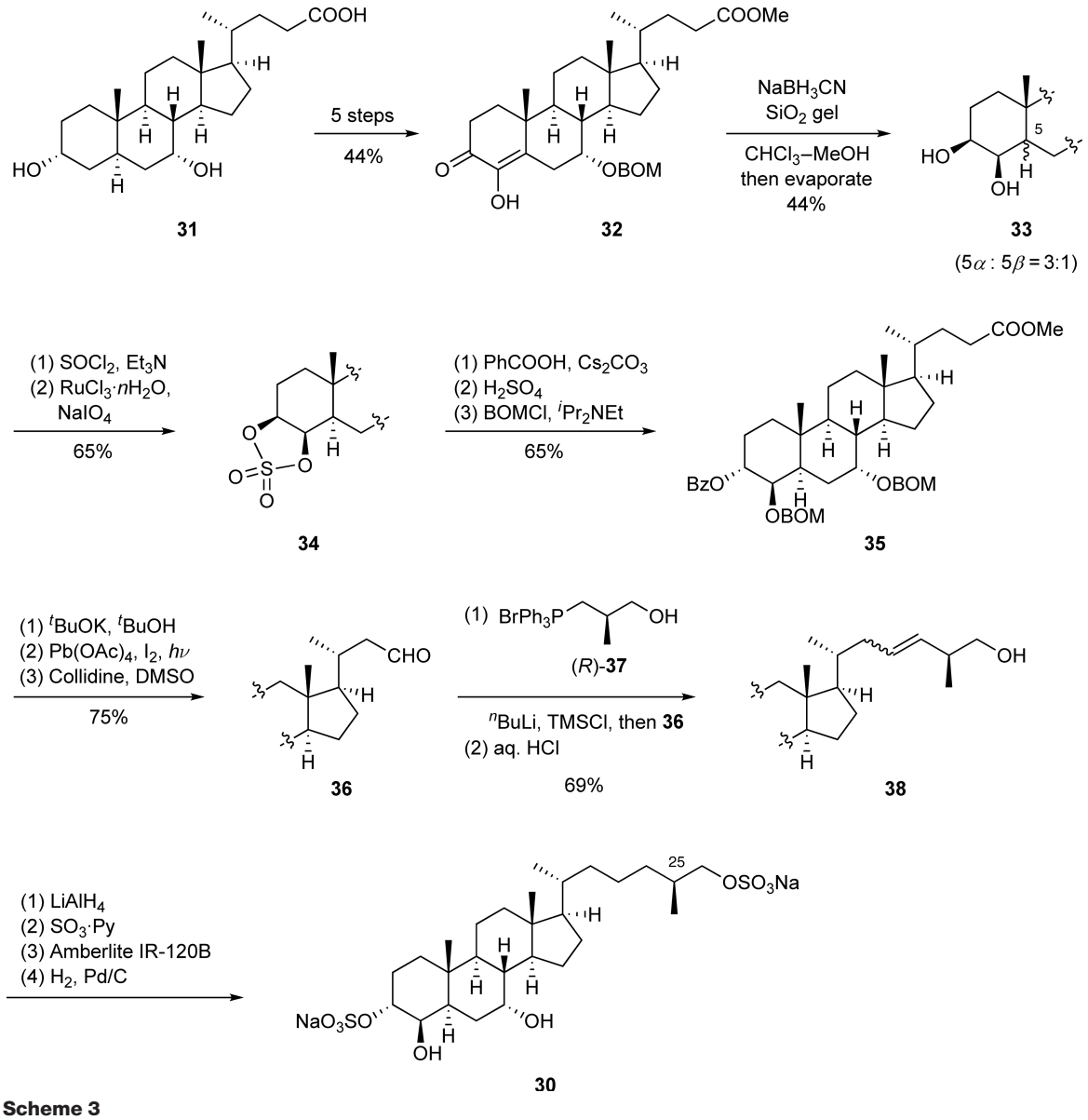
To confirm the stereochemistry of SAAF, both 25*S*- and 25*R*-stereoisomers were synthesized (**Scheme 3**).[75,76] Chenodeoxycholic acid (**31**) was converted into a 3-keto-4-enol derivative **32** in five steps via selective oxidation at C-4 carbon as a key step. A solid-phase reduction of **32** with $NaBH_3CN$ on silica gel afforded the desired diol **33** in a regio- and stereo-selective manner. Reaction with thionyl chloride followed by ruthenium oxidation gave cyclic sulfate **34**, whose C-5b epimer was separated by silica gel column chromatography. Regioselective opening of the cyclic sulfate in **34** and a benzyloxymethyl (BOM) protection afforded benzoate **35**. Selective hydrolysis using potassium *tert*-butoxide followed by decarboxylation and dimethyl sulfoxide oxidation (DMSO) yielded **36**. Wittig reaction of the aldehyde **36** with an ylide generated from (*R*)-phosphonium salt **37** afforded olefin **38** in moderate yield. Finally, removal of the benzoyl group, conversion into the corresponding sodium bis-sulfate, and successive olefin hydrogenation and concomitant removal of the BOM groups afforded 25*S*-isomer **30**. Similarly, the 25*R*-isomer was synthesized by using (*S*)-phosphonium salt.

A comparison of the structure of synthetic and natural compounds confirmed that the stereochemistry of SAAF was (3*R*, 4*R*, 7*R*, 25*S*)-3,4,7,26-tetrahydroxycholestane-3,26-disulfate. The synthetic pure sample was also used to confirm that a single compound possesses both sperm-activation and sperm-attraction properties. Synthetic SAAF (**30**) activated *C. intestinalis* sperm at 3.7 nmol l^{-1} and concurrently exhibited attracting activity at a concentration less than 10 nmol l^{-1}.

From the viewpoint of the steroid structure, SAAF (**30**) has several unique features. The hydroxylation pattern at the 3, 4, 7, and 26 positions of a cholestane skeleton has not been reported in any other natural products.[73] The positions of the C-3 and C-26 sulfate esters are also unique among sulfated polyhydroxysterols of marine origin. In general, steroid hormones act on nuclear receptors and activate gene expression. However, the chemotactic behavior of sperm occurs within a few seconds, and the sperm nucleus is condensed, indicating that genes could not be expressed in the sperm. Furthermore, since SAAF has a hydrophilic nature due to the presence of two hydroxyl and two sulfated esters, it may bind to receptors located on the sperm plasma membrane.

4.06.3.5 Abalone

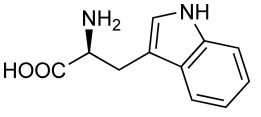
More recently, an abalone sperm attractant has been identified. Sperm of the red abalone *Haliotis rufescens* responds to soluble factors released into the seawater by conspecific eggs. Bioassay-guided purification revealed



31 **32** **33** (5α : 5β = 3:1) **34** **35** **36** **38** **30**

Scheme 3

that the free amino acid L-tryptophan (**39**) was a natural sperm attractant in *H. rufescens*.[77] L-Tryptophan released from eggs triggered both significant activation and chemotaxis in sperm at a concentration of 10 nmol l^{-1}. The D-isomer of tryptophan was inactive, which shows that the sperm response was stereospecific. Furthermore, with the addition of tryptophanase, an enzyme that selectively digests tryptophan, the sperm failed to navigate toward live eggs. Thus, a natural gradient of L-tryptophan is considered to be essential.



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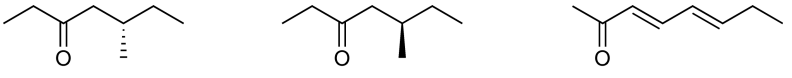
4.06.4 Sex Pheromones of Marine Invertebrates

Sex pheromones attract a sexual partner and ensure the coordinated release of gametes by both sexual partners. The function of sex pheromones in a variety of marine invertebrates and their role in the timing of reproduction has fascinated many researchers. However, studies on courtship and mating pheromones between individual adult organisms have been limited by the unreliability of the bioassays used and the difficulty of securing materials.[8] Remarkably, few attempts have been made to characterize the structures of sex pheromones other than sperm attractants. Although, outstanding progress has been made toward elucidating the structures of sex pheromones in the Polychaeta, Gastropoda, and Crustacea.

4.06.4.1 Polychaete Worm

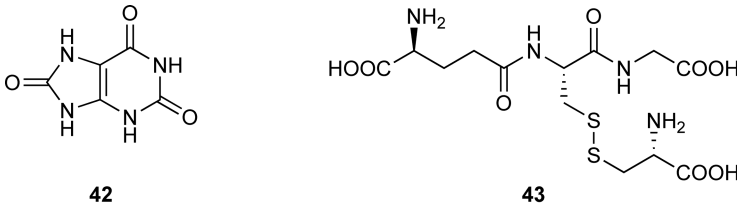
Most nereidid polychaetes undergo a metamorphosis to a sexually mature heteronereis stage. This specialized heteronereis usually reproduces by performing a typical behavior, the 'nuptial dance', at the water surface.[78,79] This spawning behavior is characterized by the sexual partners swimming around each other in circles of decreasing size prior to the release of gametes. It was found that this 'nuptial dance' swimming behavior in *Platynereis dumerilii* was triggered by the release of an attractant pheromone which is secreted from the coelomic fluid of gravid specimens.[80–82] Gas chromatographic analysis revealed that the sex pheromone of *P. dumerilii* is the volatile 5-methyl-3-heptanone (**40**).[83,84] This is the first water-borne sex pheromone to be identified in a marine invertebrate. Interestingly, the *S*-isomer (+)-**40** was produced only by males and was attractive only for females, while the *R*-isomer (−)-**40** was produced by females and acted only on males.[85] The pheromone is released in nanogram quantities, and the biological activity threshold was 3.5 pmol l^{-1}. The 'nuptial dance' was induced in animals separated by up to 50 cm upon the addition of 5-methyl-3-heptanone (**40**). Interestingly, the structure of **40** is very similar to those of alarm pheromones found in ants and many insects.[86]



(+)-**40** (−)-**40** **41**

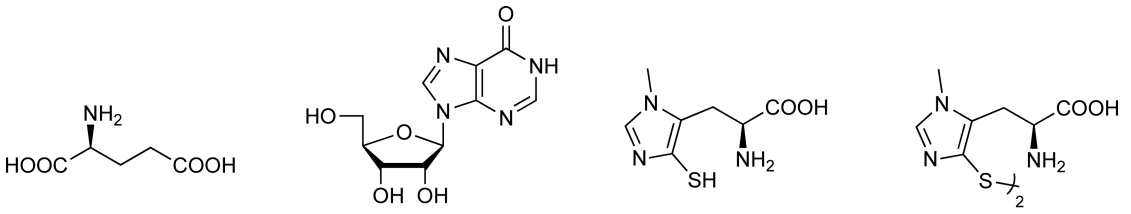
Meanwhile, Pacific species *Nereis japonica* did not show the spawning behavior when exposed to **41**. In this species, the induction of swarming behavior was controlled by 3,5-octadiene-2-one (**41**), which was previously detected as a major volatile constituent of the eggs in *P. dumerilii*.[87]

It has been shown that the reproduction process is controlled by the coelomic fluid derived from the opposite sex, even after mate recognition. At the moment of recognition, the male discharges the egg-release pheromone. The female is then stimulated to swim quickly in narrow circles surrounded by swarming males followed by spawns. The discharged egg contains the sperm-release pheromone. Finally, males achieve fertilization by circling the eggs and emitting the sperm clouds. It has been demonstrated that these gamete-release pheromones of both males and females are nonvolatile and water soluble.[88,89] In the case of *P. dumerilii*, uric acid (**42**) was identified as the sperm-release pheromone.[90,91] This pheromone is effective at a threshold concentration of 0.6 µmol l^{-1}. It is surprising to detect microgram levels of uric acid (**42**) in mature marine invertebrates since most of them, including the Nereididae, are strictly ammoniotelic, and release nitrogenous waste from purine metabolism in the form of ammonia.[88]



42 **43**

Sperm-release pheromones



44 **45** **46** **47**

Egg-release pheromones

In the case of *Nersis succinea*, the earliest studies showed that pig liver or any sample containing glutathione could induce the release of gametes in spawning *N. succinea* males, consequently the natural pheromone was hypothesized to be 'glutathione-like'.[80] Indeed, the structure of the sperm-release pheromone derived from the female *N. succinea* has been elucidated to be cysteine–glutathione disulfide (**43**).[92]

Purification of the egg-release pheromone from the male heteronereid has been attempted. In previous work, L-glutamic acid (**44**) and inosine (**45**) were detected as the main components of the coelomic fluid of sexually mature *N. succinea* males.[90] Recently, however, the egg-release pheromone was identified to be L-ovothiol A (L-1-methyl-4-mercaptohistidine) (**46**).[93] This compound was first isolated in an inactive form, L-ovothiol A disulfide (**47**). Notably, however, after **47** was treated with sodium borohydride, the highly active reduced form of the amino acid **46** was recovered, which suggests that the active form was a thiol. Ovothiols are thiohistidine compounds that were previously isolated from sea urchin eggs, and which confer NAD(P)H-O_2 oxidoreductase activity on ovoperoxidase by rapidly reacting with H_2O_2.[94] Notably, pheromones in nereidids include a series of diverse molecules, from volatile lipophilic compounds to water-soluble acids and peptides, although their roles and mode of action remain unclear.

4.06.4.2 Sea Hare

The marine mollusk *Aplysia*, found in ocean waters in many areas of the world, is a simultaneous hermaphrodite that does not normally fertilize its own eggs. It is a solitary animal during most of the year, but moves into breeding aggregations during the reproductive season.[95] Most of the egg-laying animals mate simultaneously as females, which suggests the presence of some chemical mediators that establish and maintain the aggregation. Recently, attractin was identified as a potent sex pheromone from the eluates of *Aplysia californica* egg cordons that stimulates sexually mature animals to approach egg cordons.[96,97] Attractin is the first water-borne peptide pheromone to be characterized in mollusks. Attractin from *A. californica* is a 58-residue N-glycosylated protein with three intramolecular disulfide bonds (**Figure 1**).[98] The NMR solution structure of *A. californica* attractin demonstrates that it has two helices, and the latter contains the IEECKTS motif.[99] To date, a family of attractins has been characterized in five species of the genus *Aplysia*. The six cysteines, three charged residues (Asp^5, Asp/Glu^{22}, and Glu^{39}), and the sequence of $I^{30}EECKTS^{36}$ are conserved in all five *Aplysia* attractins. Interestingly, a synthetic constrained cyclic peptide that contains the conserved heptapeptide sequence also showed significant attractive activity, which suggests that the IEECKTS sequence is important for this property.

```
                    1         10        20        30        40        50        58
                    |         |         |         |         |         |         |
Aplysia californica DQNCDIGNITSQCQMQHKNCEDANGCDTIIEECKTSMVERCQNQEFESAAGSTTLGPQ
A. brasiliana       DQNCDIGNITSQCQMQHQNCDDANGCDTIIEECKTSMVERCQNQEFESASGSTTLGPQ
A. fasciata         DQNCDIGNITSQCEMQHQNCDDANGCNTIIEECKTSMVERCQNQEFESASGSTTLGPQ
A. vaccaria         NNKCDIEFATSECEMRYQDCGEASSCTALIEECKTSLQEECN--QASSDESSTTVRPE
A. depilans         NNKCDLEFASSECQMRYQDCGEASNCTALIEECKTSLQEECD--QASSESSSTTIRPE
                       **     * * *    *  *  *   *******  * *      *   ***  *
```

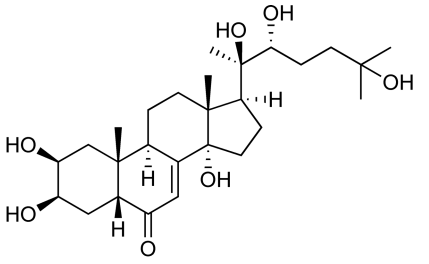
Figure 1 Sequence alignment of attractins.

Individual *Aplysia* have been shown to be attracted to the pheromone attractin in the presence of a nonlaying conspecific, but not to attractin alone.[100] In contrast, *Aplysia brasiliana* are attracted to egg cordons alone, suggesting that additional pheromonal factors may work synergistically with attractin to attract *Aplysia* to reproductive aggregates. Consistent with the prediction, three albumen gland proteins (enticin, temptin, and seductin) have recently been identified.[101] They are mature proteins that consist of 69, 103, and 192 amino acid residues, respectively. Through the use of their recombinant proteins or glutathione *S*-transferase fusion proteins, it has been shown that binary blends of attractin (1 nmol) and either enticin, temptin, or seductin (1 nmol each) stimulated mate attraction, as in the case of egg cordons. The N-terminal region of enticin aligns well with the conserved epidermal growth factor (EGF)-like domain of mammalian reproductive proteins known as fertilins, which may mediate intercellular adhesion interactions between eggs and sperm. Although the biological functions or roles of these additional proteins have not been well characterized, they may act in concert with attractin.

4.06.4.3 Crab

Some crustacean females have been shown to emit sex pheromones that elicit precopulatory behavior in conspecific males, including mate searching, courtship display, and guarding. This phenomenon has been observed in detail, especially in many marine brachyuran crabs.[102] Once the male detects the premolt female, it grasps and guards her under his abdomen until molting. Mating takes place shortly after ecdysis. Moreover, it has been shown that sex pheromones are released into the female urine. To date, various crustaceans have been demonstrated to show sexual behaviors, such as the lobster *Homarus americanus*,[103] *Pachygrapsus crassipes*,[104] *Macropipus holsatus*,[105] *Portunus sanguinolentus*,[106] the snow crab *Chionoecetes opilio*,[107] the shore crab *Carcinus maenas*,[108,109] and the helmet crab *Telmessus cheiragonus*.[110–112]

Early studies reported that the crustacean molting hormone, 20-hydroxyecdysone (**48**), was a sex pheromone in the shore crab *C. maenas* and was also found in several other crab species.[104] However, many authors have provided convincing evidence that this is not the case for most crab species. Despite their intriguing reproductive behavior, crustacean sex pheromones have rarely been elucidated.



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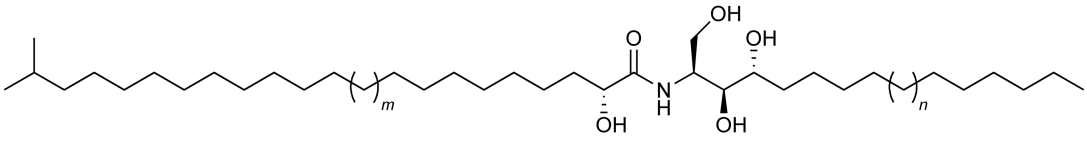


49 ($m=n=1$)

Recently, a sexual pheromone of the hair crab *Erimacrus isenbeckii* has been identified from the feeding seawater of pre- and postmolt females.[113] The purified sample elicited guard behavior in male hair crabs at a dose of 2.0 mg per sponge and was composed of 13 ceramides. On the basis of detailed NMR and fast atom bombardment tandem mass spectrometry (FAB-MS/MS) analysis, the structure of one of the major ceramides, ceramide A (**49**), was determined to be (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-21-methyldocosanoylamino]-1,3,4-pentadecane-triol.[114] Similarly,

Table 2 Structures of ceramide components in *Erimacrus isenbeckii* sexual pheromone

Ceramides[a]	*Sphingosines/fatty acids combination*	*Relative abundance (%)*
A (**49**)	*n*-C_{15}/*i*-C_{23}	18
B	*n*-C_{15}/*n*-C_{23}	2
	n-C_{16}/*n*-C_{22}	2
C	*n*-C_{15}/*i*-C_{23}	12
	n-C_{16}/*i*-C_{23}	16
D	*n*-C_{15}/*n*-C_{25}	1
	n-C_{16}/*n*-C_{25}	2
E	*n*-C_{15}/*i*-C_{25}	6
	n-C_{16}/*i*-C_{24}	15
F	*n*-C_{16}/*n*-C_{25}	2
G	*n*-C_{16}/*i*-C_{25}	19
H	*n*-C_{16}/*i*-C_{26}	2
	n-C_{17}/*i*-C_{25}	4

[a] Ceramides B, C, D, E, and H were inseparable mixtures of two components.

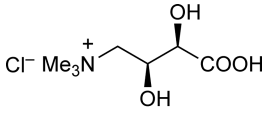
the structures of ceramides B–H were also elucidated to be a combination of C_{15}–C_{17} sphingosine and C_{22}–C_{26} branched fatty acid (**Table 2**). These structures are supported by comparison with those of synthetic compounds.

4.06.5 Alarm Pheromones

In aquatic environments, chemical cues serve as an important source of information for the detection of predation risk. Chemical signals released by disturbed or injured conspecifics may provide prey animals with an early warning of danger.[2,115] We now discuss two kinds of well-established alarm pheromones from sea anemone and sea slug.

4.06.5.1 Sea Anemone

The colonial sea anemone *Anthopleura elegantissima* responds with characteristic contraction to a pheromone released by wounded conspecifics. This alarm response is highly characteristic, includes rapid bending and shortening of the tentacles and depression of the oral disk. In 1975, by extensive ion exchange column chromatography, (3-carboxy-2,3-dihydroxy-*N*,*N*,*N*-trimethyl)-1-propanaminium chloride (**50**) was isolated as a pure crystalline substance.[116] It showed alarm pheromone activity with a median concentration of 0.35 nmol l^{-1} and was named anthopleurine. Comparison of spectral data between natural and synthetic compounds revealed that anthopleurine had a structure of 4-amino-4-deoxy-L-threonic acid betaine hydrochloride.[117]

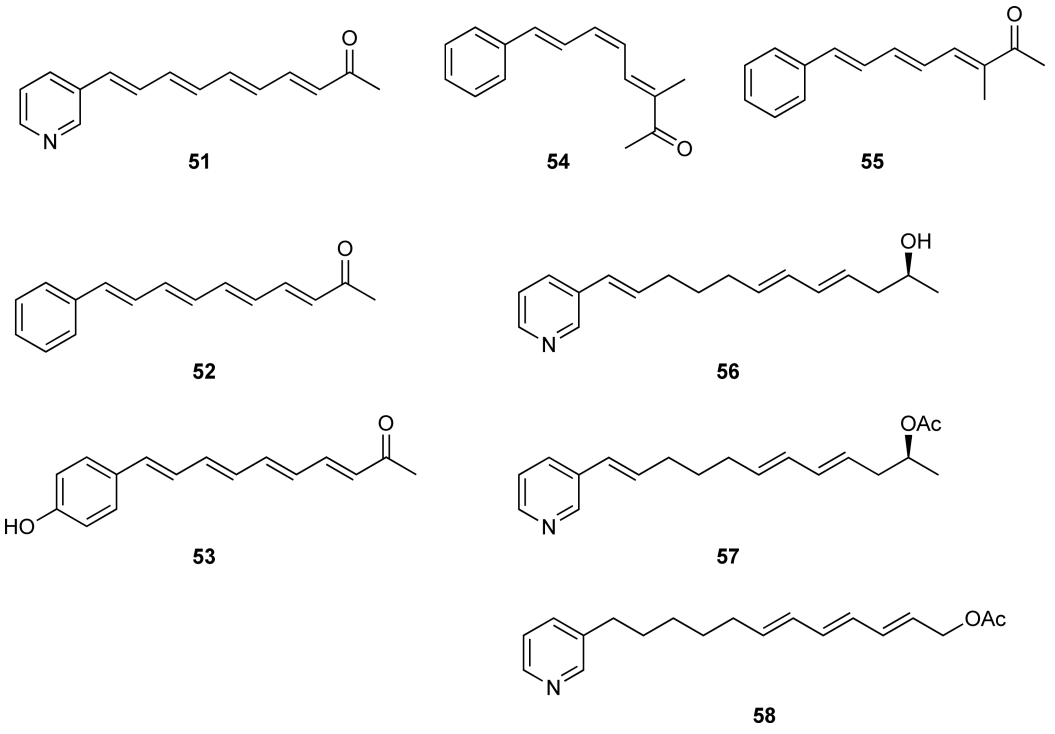


50

A. elegantissima is a preferred prey of the aeolid nudibranch *Aeolidia papillosa*. Interestingly, anthopleurine (**50**) remains in the tissue of nudibranch, and leakage of the pheromone causes the alarm response in other anemone individuals for several days.[118,119] Consequently, the predator may help in transmission of the alarm pheromone, which can reduce the severity of predation on *Anthopleura*.

4.06.5.2 Sea Slug

Opisthobranch are marine mollusks that are scarcely protected by a shell, which suggests that they possess defensive substances.[120,121] For instance, when the Pacific aglajid *Navanax inermis* is damaged by enemies, it secretes a bright-yellow water-insoluble mixture into its slime trail. From the extracts, three major compounds have been isolated and identified as polyenic compounds **51**–**53**, navenones A–C.[122,123] It has been demonstrated that these secreted constituents induce an avoidance-alarm response in a trail-following *Navanax* species at a concentration of 10 μmol l^{-1}.[124]



More recently, structurally related metabolites have been found in Mediterranean cephalaspideans, that is, phenyl conjugated trienones, lignarenones A (**54**) and B (**55**) in the Cylichnidae *Scaphander lignarius*,[125] and polyenic pyridines in the Haminoeidae: haminols A (**56**) and B (**57**) in *Haminoea navicula*, haminol C (**58**) in *H. orteai*.[126,127] Haminols A (**56**) and B (**57**) also induce alarm response at concentrations of 0.3 and 0.1 mg, respectively. Thus, navenones and their structural congeners may serve as a communication tool to indicate the presence of predators.

4.06.6 Summary and Future Prospects

Recent technological advancements including spectroscopic analyses and genetic approaches have provided outstanding opportunities for new discoveries in marine natural products chemistry, thus allowing the quantification of interactions between hydrodynamic, chemical, and biological factors at numerous spatial and temporal scales. However, a prominent question in chemical signaling that remains unresolved is the species-specificity of chemical cues including pheromones and the scale of their distribution in the vast marine environment. Further studies on the mode of action of such physiologically active substances as well as the interaction with their target molecules are becoming essential.

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Biographical Sketches

Masaki Kita was born in Nagoya in 1976, and received his Ph.D. in 2005 from Nagoya University under the supervision of Professor Daisuke Uemura. He joined the Graduate School of Science, Nagoya University as an Assistant Professor in 2001. He moved to the Department of Chemistry, University of Tsukuba in 2007, where he is currently an Assistant Professor of Chemistry. He received Inoue Research Award for Young Scientists in 2005 and Young Scientist's Research Award in Natural Products Chemistry in 2006. His research interest is in natural products, which regulate biologically and physiologically intriguing phenomena.

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Daisuke Uemura was born in Gifu in 1945 and received his Ph.D. in 1975 from Nagoya University under the supervision of Professor Yoshimasa Hirata. He was an assistant professor at Nagoya University (1973–79), associate professor at Shizuoka University (1979–91), professor of chemistry at Shizuoka University (1991–97), and professor of chemistry at Nagoya University (1997–2008). Since 2008, he has been a professor of Biosciences and Informatics, Keio University. He is a Professor Emeritus of Nagoya University. His research interest is in the diverse chemical structures and bioactivities of marine natural products. He received The Chemical Society of Japan Award for Young Chemist in 1977, and The Chemical Society of Japan Award in 2006.

4.07 Cell-to-Cell Communications among Microorganisms

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4.07.1 Introduction

Sociomicrobiology of bacteria is a recent field of research and it involves the study of communication and cooperative behavior of bacteria. Cell-to-cell communication between bacteria, usually referred to as quorum sensing, was initially described as a means by which bacteria achieve signaling in microbial communities to coordinate gene expression within a population.[1] Quorum sensing involves small molecules produced by bacteria. The first experimental observation of quorum sensing, or autoinduction, was for the bioluminescent system of a luminous marine bacterium, *Vibrio harveyi*, in which an extracellularly produced autoinducer acted as the specific inducer. The definitive involvement of the autoinducer at a very low concentration was proved by using a weakly luminescent variant of *Vibrio fischeri* as an indicator for the assay of the autoinducer. The progress in molecular biological techniques for microorganisms led to the isolation and expression of the *lux* genes of *V. fischeri*, which was eventually used for establishment of the bioluminescent system as an autoinduction or a quorum sensing system that involves a small molecule having an acylhomoserine lactone structure. This class of molecules is now known to function in a wide range of microbial processes carried out by Gram-negative bacteria (see Section 4.07.5).

In the 1960s and 1970s, in addition to the luminescent marine bacteria, the Gram-positive cocci and the Myxobacteria were studied for extracellular signaling systems, by which these unicellular microorganisms coordinate the activities of individuals within a population. The enterococcal sex pheromones were the first peptide signals identified, and peptides are now known to function as signaling molecules in a variety of Gram-positive bacteria (see Section 4.07.4). Subsequently, the identification of extracellular signaling mechanisms in a wide variety of bacteria has made the signaling systems one of the most important areas of current microbiology. There has been an explosion of new information on phenomena controlled by quorum sensing, the expression of target genes, signal receptors, and mechanisms of signal transduction. Recent information on bacterial genomes and DNA microarray analysis of the whole open reading frames of a bacterium has accelerated the study of quorum sensing. We can expect that there is a remarkable diversity among different microorganisms with regard to molecular structures of the signals, the networks connecting a quorum sensing system with other regulatory systems, and the target phenotypes controlled.[2]

Recent evidence shows that quorum sensing signaling is not restricted to bacterial cell-to-cell communication, but also allows communication between microorganisms and their hosts.[3] In addition, the topic of multicellular cooperative behaviors among bacteria has been increasingly considered in the context of evolutionary biology.[4] Further comprehensive study of these new topics will establish 'interkingdom' signaling.[4] In this chapter, signal molecules involved in quorum sensing in microorganisms are listed, with emphasis on the chemistry of the signal molecules.

4.07.2 γ-Butyrolactones in *Streptomyces*

4.07.2.1 Introduction

The Gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* shows characteristic morphological differentiation resembling that of filamentous fungi (**Figure 1**). Early in the life cycle on solid medium, *Streptomyces* grows as a branching, multinucleoid substrate mycelium mainly by cell wall extension at the hyphal tips. As older parts of the substrate mycelium produce aerial mycelium, most cells of the substrate mycelium die because of apoptotic lysis of proteins, lipids, and carbohydrates to supply materials for the generation of aerial hyphae. Septa are subsequently formed at regular intervals along the hyphae to produce uninucleoid compartments, each of which develops into a spore, thus resulting in the formation of spore chains. The complex morphogenesis of *Streptomyces* has made this genus one of the model organisms to study the molecular mechanisms of multicellular differentiation in prokaryotes. In addition to this characteristic morphogenesis, *Streptomyces* is also characterized by its ability to produce a wide variety of secondary metabolites, such as antibiotics, immunosuppressants, and other biologically active substances. For example, a single species of *Streptomyces griseus* is known to produce not only streptomycin but also about 180 other secondary metabolites. *Streptomyces* is thus one of the most important resources for compounds useful in medicinal, agricultural, and industrial applications.

The two characteristics, morphological differentiation and secondary metabolism, of *Streptomyces* are controlled by environmental conditions including nutritional conditions, such as carbon energy, nitrogen and phosphorus nutrients, and trace elements. The two biological aspects are also controlled by diffusible low-molecular-weight γ-butyrolactones called autoregulators, in addition to the environmental conditions. These

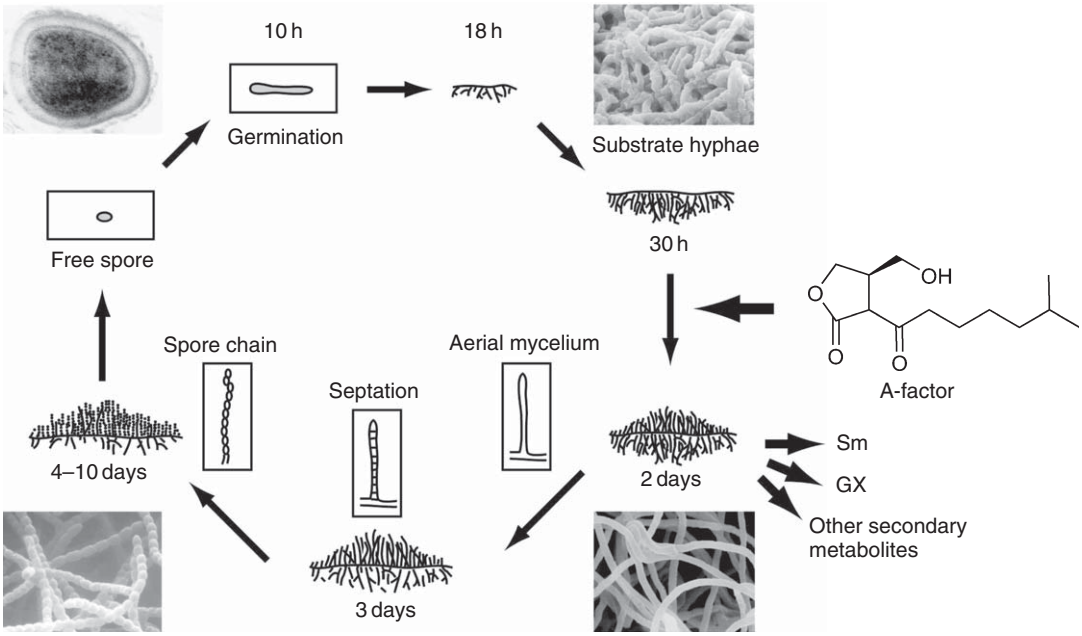


Figure 1 Life cycle of *Streptomyces*. The life cycle of *S. griseus*, a representative of *Streptomyces*, is shown. A-factor is essentially required for the progression of the step from substrate mycelium to aerial mycelium. Sm, streptomycin; GX, grixazone. Reproduced from Y. Ohnishi; S. Horinouchi, *Biofilms* **2004**, *1*, 319–328.

autoregulators are effective at extremely low concentrations and are essentially required as intrinsic factors for triggering morphogenesis and/or secondary metabolism. Such properties of autoregulators are akin to those of hormones in eukaryotic organisms, rather than *N*-acylhomoserine-type quorum sensors seen in a variety of Gram-negative bacteria (see below).

A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone; for the structure, see **Figure 1**) is representative of substances that trigger secondary metabolism or aerial mycelium formation, or both, in *Streptomyces*. A-factor was originally discovered by Khokhlov in the 1960s as a diffusible self-regulatory substance in *S. griseus*, which simultaneously induces sporulation and streptomycin production in the same organism.[5] Although the pioneer work of Khokhlov had long been neglected, Hara and Beppu[6,7] confirmed his study through genetic study of an industrial streptomycin-producing strain of *S. griseus*. Many of the streptomycin-nonproducing and nonsporulating mutants they isolated actually responded to exogenously supplemented A-factor; chemically synthesized (3*R*)-A-factor, provided by Mori,[8] at a concentration of 1 nmol l^{-1} simultaneously restored the defects in streptomycin production and sporulation. A subsequent study established the molecular mechanisms of the A-factor regulatory cascade for secondary metabolism and morphological differentiation in *S. griseus* (**Figure 2**).[9,10]

A-factor homologues with a γ-butyrolactone ring have been found to act as similar autoregulatory factors, and this has opened a new window to a characteristic regulatory system for physiological and morphological differentiation in *Streptomyces*.[9,10] On the other hand, similar diffusible factors, *N*-acylhomoserine lactones also having a γ-butyrolactone ring, have been found to act as signaling molecules in many Gram-negative bacteria and are involved in the so-called quorum sensing to induce diverged cellular functions, such as bioluminescence and toxin production, depending on the cell density.[11,12] Because the biosynthesis of *N*-acylhomoserine lactones as quorum sensors in Gram-negative bacteria and γ-butyrolactones as self-regulatory factors in *Streptomyces* (see below) and the gene regulation by the respective receptors are totally different, these two regulatory systems have evolved from different ancestors. It is evident that various bacterial species have characteristic chemical signaling systems, which enable their cells to communicate and cross talk with one another to control their functions in response to the environment.

In this section, an overall picture of the γ-butyrolactone regulatory systems in *Streptomyces*, focusing on A-factor in *S. griseus*, is described, because the A-factor regulatory system has been most intensively studied and because the regulatory mechanism by A-factor can be applied to all the γ-butyrolactone regulatory systems in various *Streptomyces* spp. Major regulatory steps in the A-factor regulatory cascade involve the following: AfsA, a key enzyme for A-factor biosynthesis; ArpA, the A-factor-specific receptor protein; and AdpA, a key transcriptional activator in the cascade. The regulatory cascade clearly shows how A-factor determines the timing of the onset of secondary metabolite formation and morphological differentiation and how A-factor triggers simultaneous expression of a number of genes of various functions. As an example of the phenotypes controlled by A-factor, a signal relay from A-factor to the streptomycin biosynthesis genes is described.

4.07.2.2 General Properties of γ-Butyrolactones in *Streptomyces*

The genetic study of A-factor biosynthesis in *S. griseus* by Hara and Beppu[6,7] showed that the (3*R*)-form of A-factor at a concentration as low as 10^{-9} mol l^{-1} restores all the phenotypic defects in streptomycin production and sporulation of an A-factor-deficient mutant strain HH1. A-factor-deficient mutants, like mutant HH1, were readily obtained by treatment of the wild-type strain with UV irradiation or on incubation at 32 °C.[6] The extreme instability of the A-factor productivity was later explained in terms of the location of *afsA*, a gene encoding a key A-factor biosynthesis enzyme, AfsA,[13,14] which is located in the vicinity of one end of the linear chromosome.[15] The ends of the *Streptomyces* chromosomes are deleted at high frequency due to homologous recombination between long repeat sequences on both ends of the linear chromosome. The genome sequence of *S. griseus* with a total of 8 545 929 bp[16] shows that *afsA* is located at a distance of 272 kb from one end of the chromosome. The location of *afsA* near one end of the linear chromosome of *S. griseus* is the molecular basis of the simultaneous loss of streptomycin production and sporulation that has long been empirically observed by those who are engaged in streptomycin fermentation. The extreme instability of A-factor production appears to be specific to *S. griseus*, because *afsA* orthologues in other *Streptomyces* species are present in the core region of the chromosome and because some *Streptomyces* strains contain multiple *afsA* homologues.

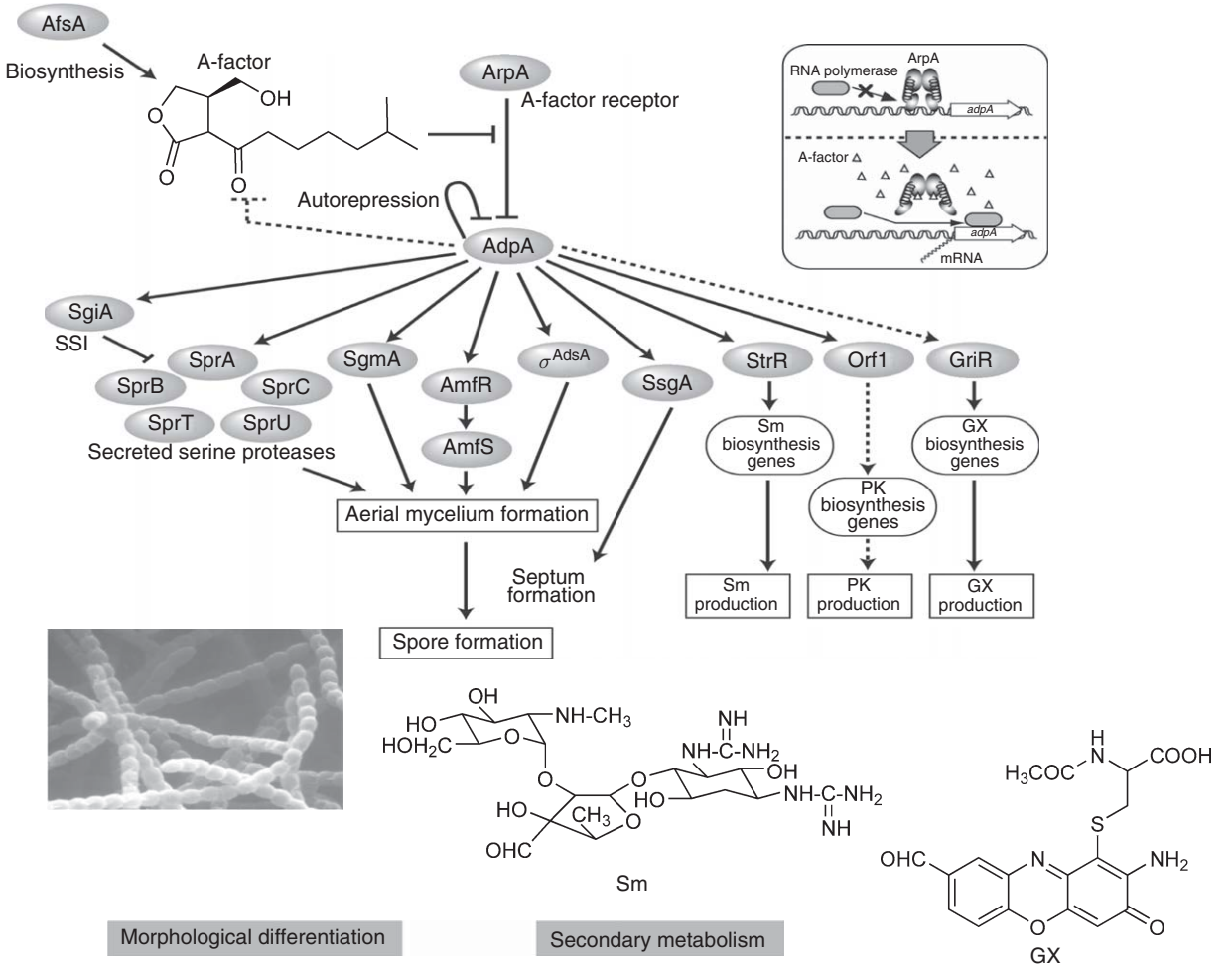


Figure 2 The A-factor regulatory cascade. The A-factor signal, starting with the A-factor biosynthesis gene *afsA*, is transferred to the receptor ArpA, to the transcriptional activator AdpA, and finally to a variety of genes required for morphological development and secondary metabolite formation. See the text for details of the target genes of AdpA. Through this cascade, morphological and physiological differentiation occurs at a specific time of growth, when the intracellular concentration of A-factor reaches a critical level at or near the middle of the exponential growth. Reproduced from S. Horinouchi, *Biosci. Biotechnol. Biochem*. **2007**, *71*, 283–299.

A-factor and its homologues produced at a portion of a hypha can move freely within the individual hypha and spread into neighboring hyphae. Owing to filamentous growth, *Streptomyces* might have developed diffusible γ-butyrolactone regulatory systems that facilitate communication between the cells at a distance within an individual hypha and between different hyphae, as a consequence of convergent evolution. The filamentous mycelia of *Streptomyces* are close enough to communicate with one another. The signaling system between physically separate individual cells in the same mycelium can be termed hormonal regulation, rather than quorum sensing regulation found in Gram-negative single-cell bacteria growing in liquid culture.[11,12] On the other hand, A-factor is also important in cell–cell communication between neighboring mycelia, similar to the quorum sensing system. This system also facilitates discrimination of signals originating from neighboring living things, thus allowing the cell to recognize the neighbor as a member of the same species or not, since a given *Streptomyces* strain contains its own γ-butyrolactone and its receptor with strict ligand specificity. In addition, the regulatory system employing chemicals is also advantageous for survival in the ecosystem; A-factor produced at an extremely low concentration by a cell is accepted by several hyphae and causes rapid sporulation of the whole population, which is advantageous compared with piecemeal sporulation of individual hyphae induced by environmental stimuli such as nutritional limitation.

4.07.2.2.1 A-factor homologues in Actinomycetes

A-factor homologues having a γ-butyrolactone structure have been found in various *Streptomyces* species, such as *S. bikiniensis*, *S. coelicolor* A3(2), *S. cyaneofuscatus*, *S. lavendulae*, *S. virginiae*, and *S. viridochromogenes* (reviewed in the works of Horinouchi[17,18] and Yamada and Nihira[19]). Examples include virginiae butanolides (VBs), controlling virginiamycin production in *S. virginiae*;[20] inducing material (IM)-2, controlling pigment production in *S. lavendulae*;[21] and *S. coelicolor* A3(2) butyrolactone (SCB1), controlling actinorhodin and undecylprodigiosin production in *S. coelicolor* A3(2).[22] Their chemical structures are shown in **Figure 3** and the chemistry will be discussed below in relation to the key enzyme AfsA for biosynthesis of γ-butyrolactones. These strains contain homologues of *afsA* and *arpA*, encoding the A-factor receptor, which implies that the mechanism of regulation by these γ-butyrolactones is the same as that for the A-factor regulatory system including *afsA* and *arpA* in *S. griseus* (see below). As described above, *afsA* is located near one end of the linear chromosome in *S. griseus*, and *arpA* is located in the middle of the chromosome. Some *afsA* homologues are located near *arpA* homologues, but others are not. The former includes, for example, the *afsA*/*arpA* homologous system for the production of virginiamycin by *S. virginiae*,[23,24] a pigment by *S. venezuelae*,[25] jadomycin B by *S. venezuelae*,[26] and methylenomycin by *S. coelicolor* A3(2) (accession number

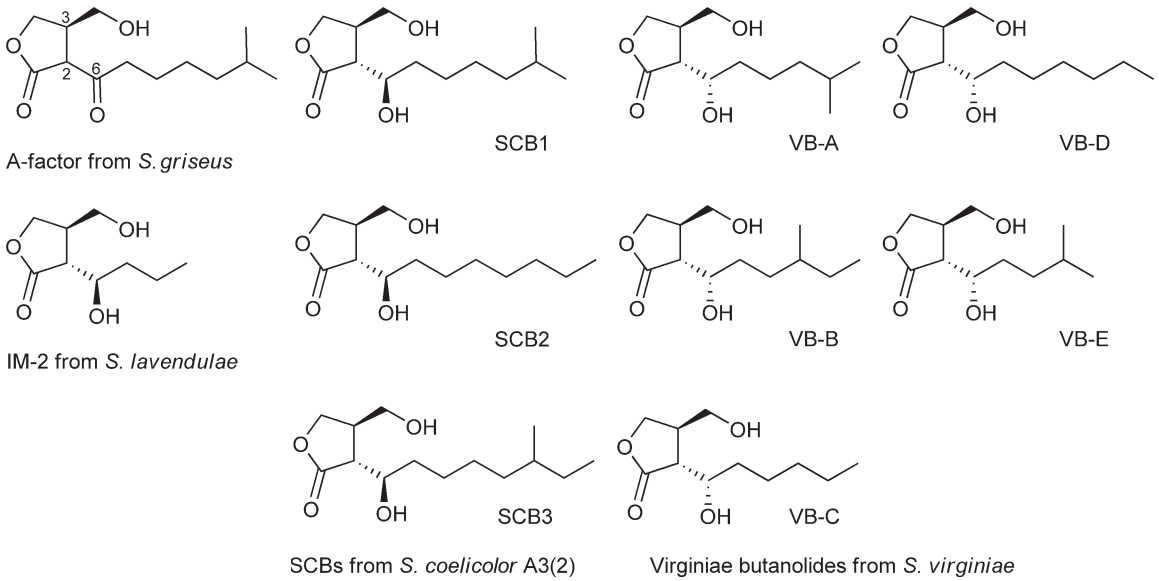


Figure 3 γ-Butyrolactones in *Streptomyces*. The differences in chemical structure among the γ-butyrolactones are the length and branching of the acyl chain and the reduction state, either a keto or a hydroxyl group, at position 6.

AJ276673), and the latter includes the system for the production of pristinamycin by *S. pristinaespiralis*[27] and tylosin by *S. fradiae*.[28] Apparently, the *afsA*/*arpA* system in the former case is specific for the adjacent gene cluster for production of a certain secondary metabolite, and the system in the latter case exerts pleiotropic effects on both secondary metabolism and morphological differentiation.

The A-factor and receptor system in *S. griseus* acts as an all-or-nothing switch (i.e., a crucial switch) for both morphological and physiological differentiation. On the other hand, CprA and CprB, both of which are A-factor receptor homologues, act as tuners for these processes in *S. coelicolor* A3(2); a *cprA* or *cprB* mutant still produces a small amount of antibiotics and forms less abundant spores.[29] As described above, some γ-butyrolactone regulatory systems control the timing of antibiotic production, but not morphological development, and others control both antibiotic production and morphological differentiation. These observations imply that *Streptomyces* has evolved the γ-butyrolactone regulatory system to control different steps in the regulatory hierarchy for healthy growth, as an all-or-nothing switch for some phenotypes and as just a tuner for other phenotypes. This may be the reason why a *Streptomyces* strain contains redundant γ-butyrolactone regulatory systems.

afsA encoding an A-factor biosynthesis enzyme has been found only in *Streptomyces* and its closely related genera,[30] which is consistent with the idea that the γ-butyrolactone regulatory cascades are confined to *Streptomyces*. In contrast, the receptor protein ArpA and its homologues, especially proteins having high similarity in DNA-binding domains, are distributed rather widely among various bacteria. In addition, some *Streptomyces* strains contain multiple *afsA–arpA* pairs. Phylogenetic analysis of the γ-butyrolactone synthases and receptors suggests that the ancestral ArpA protein had existed as a DNA-binding protein, not as a γ-butyrolactone receptor, before the appearance of a γ-butyrolactone receptor in the course of the bacterial evolution.[30] Once a *Streptomyces* strain acquired a γ-butyrolactone as a chemical signaling molecule during the evolution, the preexisting ArpA ancestor employed it as a ligand to modulate its own DNA-binding activity. Because the combination of *afsA* and *arpA* in a given *Streptomyces* strain is greatly different in the topology of the phylogenetic tree,[30] *Streptomyces* has changed the combination of *afsA* and *arpA* when it acquired a new pair of *afsA–arpA*, during which it has selected the best-fit pair by trial and error. This is in clear contrast to the *luxI–luxR* systems that mediate quorum sensing via *N*-acylhomoserine lactones.[11,12] The inducer–receptor elements in a quorum sensing system in various Gram-negative bacteria have evolved concomitantly, as revealed by phylogenetic analysis.[26]

4.07.2.3 Biosynthesis of γ-Butyrolactones

4.07.2.3.1 AfsA as the key enzyme for A-factor biosynthesis

afsA was cloned as a gene that restored streptomycin production by an A-factor-deficient mutant strain HH1.[32] This gene appeared to encode an A-factor biosynthesis enzyme because (1) *afsA* mutants lost A-factor productivity, (2) introduction of *afsA* into A-factor-nonproducing *Streptomyces* species caused overproduction of A-factor with a gene dosage effect, and (3) introduction of *afsA* into *Escherichia coli* caused the host to produce a substance having A-factor activity.[13] Structure modeling of AfsA by S. Nakamura (unpublished data) showed that, like β-hydroxyacyl acyl carrier protein (ACP) dehydratase, AfsA has a tunnel that can accept an acyl chain of acyl-ACP. The presence of such a tunnel led to the prediction that AfsA might be involved in the condensation of a three-carbon (C_3) compound and a C_{10} fatty acid derivative containing a β-ketoacyl chain, as suggested by Sakuda *et al.*[33]

Consistent with the prediction, *in vitro* A-factor synthesis with a purified AfsA protein showed that it catalyzes acyl transfer between 8-methyl-3-oxononanoyl-ACP (**Figure 4**; **3**) and the hydroxyl group of dihydroxyacetone phosphate (**2**), forming a fatty acid ester (**4**).[14] The ester (**4**) is nonenzymatically converted to a butenolide phosphate (**5**) by intramolecular aldol condensation. The butenolide phosphate (**5**) is then reduced by the *bprA* product, which is encoded just downstream of *afsA*. The *bprA* product was tested as a candidate of the reductase of **5**, based on the assumption that functionally related genes are, in many cases, encoded as neighbors on the bacterial chromosome. The stereoselectivity, the *R*-form, at position 3 is determined by this reduction step. The phosphate group on the resulting butanolide (**6**) is finally removed by a phosphatase, resulting in the formation of A-factor (**1**). The fatty acid ester (**4**) is also converted into A-factor in an alternative way. The phosphate group on the ester is first removed by a phosphatase and the

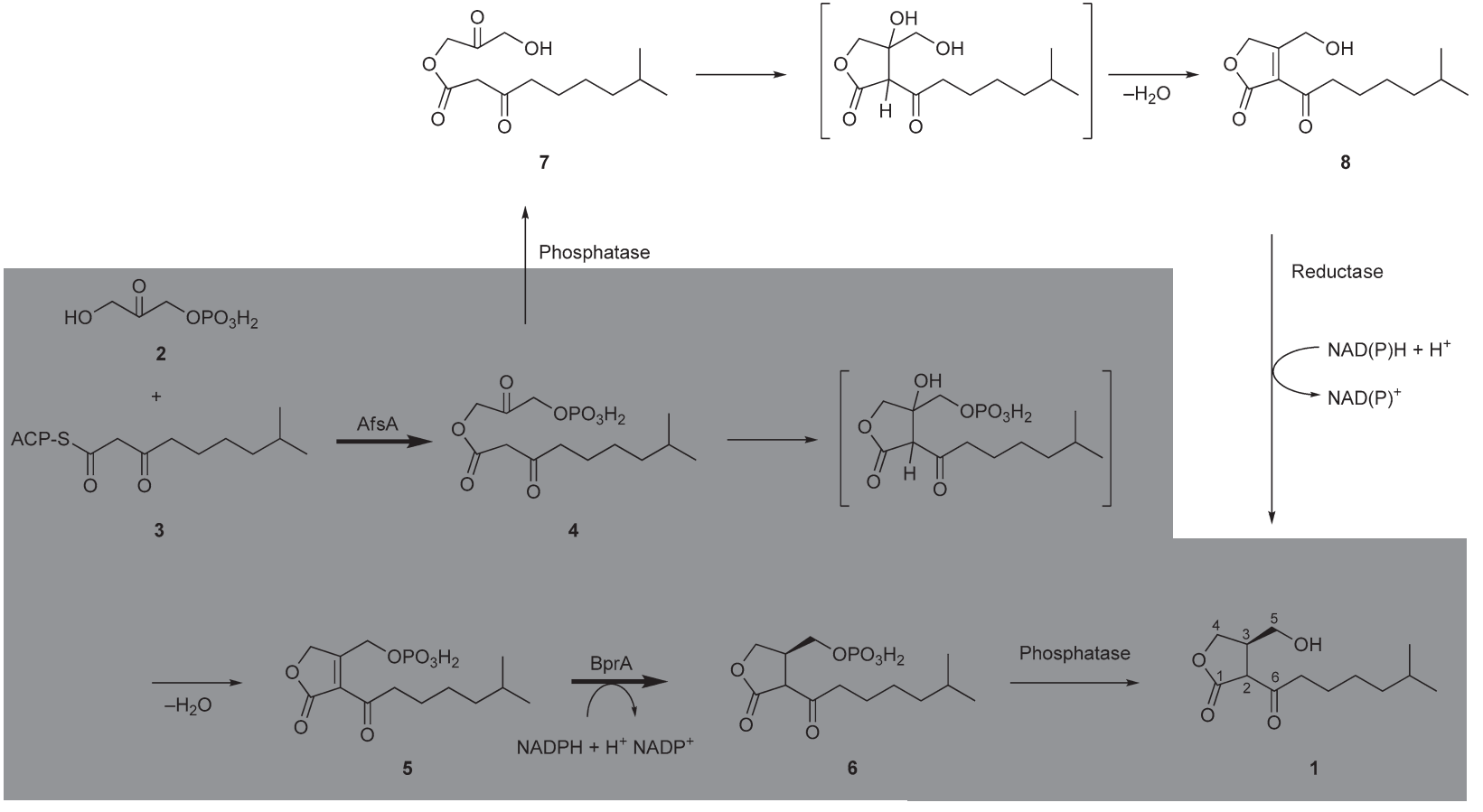


Figure 4 The whole A-factor biosynthesis pathway. The major pathway, highlighted by hatching, and an alternative pathway are shown.

dephosphorylated ester (**7**) is converted nonenzymatically to a butenolide (**8**), which is then reduced by a reductase different from BprA, resulting in A-factor (**1**). The phosphatase and reductase in the latter route are not specific to A-factor biosynthesis but are generally present in bacteria. This may be the reason why *afsA* alone causes *E. coli* to produce substances having A-factor activity (see below). Because of the operon structure of *afsA–bprA*, we assume that the former route is important.

Escherichia coli carrying *afsA* alone produces substances having A-factor activity.[13] Mass spectrometry suggested that the active substances are A-factor homologues with a C_{10} straight side chain (m/z 241) and a C_8 straight side chain (m/z 213).[14] The straight side chains of the A-factor homologues reflect the fact that *E. coli* produces no branched fatty acids.

4.07.2.3.2 Structural variety of γ-butyrolactones

Figure 3 lists several A-factor homologues in *Streptomyces*.[16,17] There are two structural differences in the γ-butyrolactone signal molecules: one is the length and branching of the fatty acid side chain and the other is the reduction state of the 6-oxo group. On the basis of the A-factor biosynthesis pathway, we can ascribe the difference in side chain to the variety of the β-ketoacyl-ACPs, one of the substrates used by AfsA and its homologues. The branching of the side chain reflects the fact that the fatty acids of *Streptomyces* consist primarily of branched-chain fatty acids that are synthesized from isobutyryl- and methylbutyryl-CoA. The difference at position 6, either a keto or a hydroxyl group, can be ascribed to the existence and stereoselectivity of the 6-keto reductase that reduces this position. VBs in *S. virginiae* and SCBs in *S. coelicolor* A3(2) are reduced by BarS1-type reductases.[34] Owing to the absence of such reductases in *S. griseus*, position 6 of A-factor remains as a keto group.

4.07.2.3.3 Regulation of A-factor biosynthesis

A-factor is accumulated in a growth-dependent manner and reaches its maximum, 25–30 ng ml^{-1} (about 100 nmol l^{-1}), at or near the middle of exponential growth.[14,35] An interesting question is why A-factor is produced in such an extremely small amount? Because the transcription of *afsA* is almost constant throughout growth,[14] it is most likely that the extremely small amount of A-factor is due to the availability of the substrates. 8-Methyl-3-oxononanoyl-ACP (**3**), which is synthesized by condensation of three acetate units with the starter substrate isobutyryl-CoA, is an intermediate in the fatty acid biosynthesis, and is leaked from the pathway. Therefore, the intracellular pool of the acyl-ACP (**3**) must be extremely small, therefore serving as a bottleneck. Dihydroxyacetone phosphate (**2**), another substrate of AfsA, is derived from glycolysis. Thus, the A-factor biosynthesis by AfsA reflects the growth period and determines the timing of secondary metabolism and morphological differentiation. The biosynthesis of γ-butyrolactones reflective of the amounts of the intermediates of primary metabolism is analogous to that of *N*-acylhomoserine lactone quorum sensors in Gram-negative bacteria; *N*-acylhomoserine lactones are synthesized from *S*-adenosylmethionine derived from amino acid biosynthesis and the diverse intermediates in fatty acid biosynthesis.[36]

4.07.2.4 Receptors of γ-Butyrolactones

4.07.2.4.1 Properties of ArpA

Miyake *et al.*[37] detected A-factor-binding activity in the cytoplasmic fraction of *S. griseus* by using [^{3}H]A-factor. The A-factor-binding protein was found to serve as a repressor of streptomycin production and aerial mycelium formation, since mutants deficient in the binding protein produced streptomycin in a larger amount and formed spores more abundantly than the wild-type strain, even in the absence of A-factor.[38] The gene for the A-factor-binding protein, named *arpA*, was then cloned,[39] and the DNA-binding activity of ArpA and a consensus ArpA-binding sequence were determined.[38] The consensus ArpA-binding sequence was a 22 bp palindromic site with the sequence 5′-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3′ as one half of the palindrome.[40] ArpA binds this site in the absence of A-factor, and the exogenous addition of A-factor to the ArpA–DNA complex induces immediate release of ArpA from the DNA. These observations were in agreement with the idea that ArpA acts as a repressor-type regulator of secondary metabolism and morphological differentiation by preventing the expression of a certain key gene(s) during the early growth phase. A-factor, produced in a growth-dependent manner, releases ArpA from the DNA, thus switching on the

expression of the key genes, leading to the simultaneous onset of secondary metabolism and morphogenesis at a certain timing during growth. *adpA* was later identified as the sole target of ArpA.[41]

The domain structure of ArpA was predicted by site-directed mutagenesis of the helix–turn–helix DNA-binding motif of ArpA and by analysis of *arpA* mutations. A mutant ArpA protein (Val41Ala) lacked DNA-binding ability but still retained A-factor-binding ability.[42] Conversely, mutant Pro115Ser lacked A-factor-binding ability but retained DNA-binding ability.[43] Mutant Trp119Ala also lacked A-factor-binding ability but retained DNA-binding ability, indicating that Trp-119 is essential for A-factor binding.[42] These observations predict that ArpA contains two independently functional domains, a DNA-binding domain and an A-factor-binding domain.

4.07.2.4.2 Crystallography of CprB, an ArpA homologue

CprB, an ArpA homologue in *S. coelicolor* A3(2),[29] was crystallized and its structure was solved.[44] CprB, consisting of 215 amino acids, shows about 30% identity in amino acid sequence to ArpA. It recognizes and binds the same nucleotide sequence as ArpA,[42] although its ligand still remains unknown. In addition, CprB serves as a negative regulator of both morphological differentiation and secondary metabolism in *S. coelicolor* A3(2), as ArpA does in *S. griseus*. The crystal structures of three different forms, Ia, Ib, and II, were determined at 2.4 Å resolution,[44] and they turned out to be a dimer with an 'Ω' shape (**Figure 5**). The two subunits are bound via six hydrogen bonds and three water-mediated hydrogen bonds. In addition, a disulfide bond via Cys-159 links the subunits. This disulfide bridge is specific to CprB because typical γ-butyrolactone receptors contain no Cys residue at this position. The DNA-binding domain is composed of three N-terminal helices, α1, α2, and α3. Of the three, α2 and α3 form a typical helix–turn–helix motif. The amino acid residues on helix α3 are

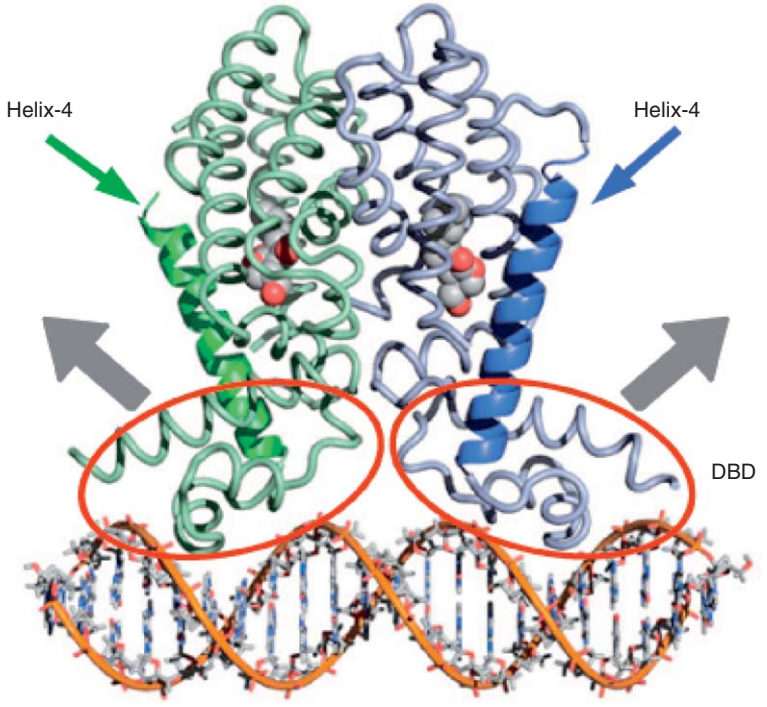


Figure 5 Overall structure of CprB, an ArpA homologue. CprB constitutes a dimer, each subunit of which contains a ligand-binding pocket in the C-terminal portion and a helix–turn–helix DNA-binding domain in the N-terminal portion. The receptor dimer binds the same face of the DNA by inserting the DNA-binding helices in the major groove. The A-factor molecule in the pocket is illustrated with a ball model. The binding of A-factor so that it is embedded completely in the pocket relocates the DNA-binding domains (DBD) outside the molecule via the long helix-4, thus dissociating ArpA from the DNA. This computer-modeled structure was provided by R. Natsume.[44] Reproduced from S. Horinouchi, *Biosci. Biotechnol. Biochem*. **2007**, *71*, 283–299.

completely conserved among the receptors, in agreement with the observation that γ-butyrolactone receptors recognize the same nucleotide sequence.

A large cavity is present in the regulatory domain, which we assume is a ligand-binding pocket 5 Å in diameter and 20 Å long. Trp-127, corresponding to Trp-119 of ArpA, which has been found to be essential for A-factor binding by site-directed mutagenesis,[42] participates in forming the pocket. This pocket is completely embedded in the molecule, and a flexible loop covers the entrance to it, serving as a lid for the pocket. The docking study suggests that a γ-butyrolactone molecule binds to the pocket in an extended manner and Trp-127 causes a hydrophobic interaction with the alkyl chain of the γ-butyrolactone molecule. The hydrophobic interaction between Trp-127 and the alkyl chain of the ligand stabilizes the ligand binding.

4.07.2.4.3 How A-factor dissociates ArpA from DNA

A database search for structural comparison revealed that the overall structure of CprB is similar to that of the TetR family proteins, TetR and QacR. One of the crystal structures of CprB, form Ib, is closely related to that of QacR in complex with its target DNA.[45] We can hence predict how γ-butyrolactones dissociate their cognate receptors from DNA upon binding the ligands, on the basis of the mechanism of the conformational changes of TetR upon tetracycline binding.[45,46] Ligand binding induces relocation of a long helix α4 that links the ligand-binding pocket with the DNA-binding domain. As a result of the relocation of the DNA-binding domain, ArpA dissociates from the DNA (**Figure 5**).

4.07.2.5 AdpA Regulon

4.07.2.5.1 Properties of AdpA

The transcription of streptomycin biosynthetic genes is presumably controlled by A-factor because A-factor switches on streptomycin production. The data from our laboratory[47] and a detailed study by Distler *et al.*[48] on the transcriptional organization of part of the streptomycin biosynthetic gene cluster showed that one mRNA species covering a regulatory gene (*strR*) and the streptomycin-6-phosphotransferase (*aphD*) gene was dependent on A-factor, as determined by S1 nuclease mapping. We then tried to detect and purify a protein that might bind the promoter region of *strR–aphD*, based on the assumption that the A-factor signal is transmitted via ArpA and some other regulatory proteins to the A-factor-dependent promoter. As a result, an A-factor-responsive protein (AdpA) able to bind the upstream activation sequence, about 270 bp upstream of the transcriptional start point of *strR*, was detected by gel mobility shift assay.[49] StrR was later found to be a pathway-specific transcriptional activator for all streptomycin biosynthetic genes.[50,51] AdpA was detected only in the presence of A-factor. After purification of AdpA and partial amino acid sequence determination, the *adpA* gene was cloned by PCR.[52] AdpA encoding a 405-amino acid protein with a helix–turn–helix DNA-binding motif at the central portion showed sequence similarity to transcriptional regulators belonging to AraC/XylS family.

The −35 and −10 regions of *adpA* contained a 22 bp palindrome, caggcAGGAACGGACC*GCGCGG TACGCt (the underlines indicate the −35 and −10 promoter elements; * indicates a dyad axis), which showed similarity to the consensus sequence of the ArpA-binding site, (A/C)C(A/G)(T/A)ACCC(A/G)CC*GG(T/C)CGGT(A/T)(T/C)G(T/G).[52] As expected, ArpA bound the promoter region of *adpA* in the absence of A-factor but did not bind in the presence of A-factor. In addition, the exogenous addition of A-factor to the ArpA–DNA complex induced immediate release of ArpA from the DNA. Thus, the promoter of *adpA* turned out to be a target of ArpA. Consistent with this, S1 nuclease mapping showed that *adpA* was transcribed only in the presence of A-factor and *strR* was transcribed only in the presence of intact *adpA*. Furthermore, *adpA* disruptants produced no streptomycin and overexpression of *adpA* caused the wild-type *S. griseus* strain to produce streptomycin at an earlier growth stage in a larger amount.

Our next question was whether ArpA targets only *adpA*. Because ArpA acts as a repressor of aerial mycelium formation and secondary metabolism, an *arpA* disruptant forms aerial hyphae and spores earlier than the wild-type strain and overproduces streptomycin and other secondary metabolites. On the other hand, mutant KM2, expressing a mutant ArpA (Trp119Ala), neither produces secondary metabolites nor forms aerial hyphae, since this A-factor-insensitive mutant ArpA always binds to and represses the *adpA* promoter. Trp-119 of ArpA is essential for A-factor binding, and replacement of this Trp residue with Ala abolishes its A-factor-binding

ability, resulting in the formation of a mutant ArpA that binds the target DNA irrespective of the presence of A-factor.[42] When *adpA* under the control of a foreign, constitutively expressed promoter is introduced into mutant KM2, all the phenotypes that we can observe are restored.[41] These results show that the only significant target of ArpA is *adpA*.

4.07.2.5.2 *Autoregulation of* adpA

Because *adpA* targets many genes, the intracellular concentration of AdpA must be important for ordered gene expression at a specific timing. We found that *adpA* acts as a repressor of its own transcription by binding to three sites within its promoter and forming a DNA loop via two molecules of AdpA dimer.[53] The cooperative binding of AdpA to two sites allows effective regulation to result from small alterations in the AdpA concentration and serves as a fine sensor of the AdpA concentration.

4.07.2.5.3 *AdpA regulon*

adpA disruptants failed to produce streptomycin and a yellow pigment, suggesting that the biosynthetic genes for the pigment were under the control of AdpA.[52] Furthermore, disruption of *adpA* caused the host to show a bald phenotype. These observations implied that AdpA controls multiple, unlinked genes necessary for physiological and morphological differentiation. We started isolating multiple genes as DNA fragments bound by AdpA by a combination of gel mobility shift assay, immunoprecipitation with the anti-AdpA antibody, and PCR. Repeated experiments yielded more than 60 DNA fragments that were specifically bound by AdpA.[54] The presence of many genes, all of which are simultaneously activated by AdpA at a specific point in the growth phase, means that the signal from A-factor is greatly amplified at this regulatory step via AdpA as an amplifier. We have so far analyzed several of these isolated fragments, which are essential or important for secondary metabolite formation and morphological differentiation (**Figure 2**).

The targets of AdpA required for morphological differentiation are the following: *adsA*, encoding an extracytoplasmic function (ECF) σ factor;[54] *amfR*, encoding a transcriptional activator that activates the *amf* operon;[55,56] extracellular proteases, including a metalloendopeptidase,[57] two trypsin-type proteases,[58] and three chymotrypsin-type proteases;[59] a *Streptomyces* subtilisin inhibitor (SSI) gene;[60] and *ssgA*, which is essential for spore septum formation.[61] These AdpA-dependent proteases, which are produced at the time of aerial mycelium formation, may be involved in hydrolysis or apoptosis of proteins in substrate mycelium for reuse during aerial hyphae formation. The SSI is supposed to modulate the activities of the proteases. The *amf* operon is for the production of a hydrophobin, AmfS, that is essential for the erection of aerial hyphae into the air.[62] The σ^{AdsA} presumably transcribes specific genes, which suggests the presence of an additional regulatory step downstream of this σ factor.

The targets of AdpA for secondary metabolism are the following: *strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis (see below);[51] and a gene encoding a transcriptional factor probably for biosynthesis of a polyketide compound.[63] *griR*, the pathway-specific transcriptional activator, is also indirectly activated by AdpA.[64] In addition to these genes, almost all gene clusters for biosynthesis of a certain secondary metabolite in *S. griseus* are activated by AdpA, as revealed by our DNA microarray analysis on the basis of the whole genome sequence of *S. griseus*.[16,65]

The AdpA target genes contain one or more AdpA-binding sites upstream of their promoters, and some genes require simultaneous binding of a dimer of AdpA to multiple sites. All the target sites contain a consensus AdpA-binding sequence, 5′-TGGCSNGWWY-3′ (S: G or C; W: A or T; Y: T or C; N: any nucleotide). The binding sites are, for example, 200 bp upstream and 25 bp downstream from the transcriptional start points. Despite the differences in binding position with respect to the promoter and in the number of binding sites, AdpA recruits RNA polymerase to the promoter of the target genes and facilitates isomerization of the RNA polymerase–DNA complex into an open complex competent for transcriptional initiation.[44,57]

The genome of *S. griseus* consists of 8 545 929 bp containing about 8000 open reading frames.[16] Preliminary DNA microarray analysis has shown that more than 600 genes are activated by AdpA and about 200 genes are relatively downregulated in the Δ*adpA* background. The number of genes whose expression is switched-on or affected by *adpA* (actually, A-factor itself) is amazing, indicating that A-factor is a true microbial hormone.

4.07.2.6 The A-Factor Signal Relay Leading to Streptomycin Biosynthesis

The streptomycin biosynthesis gene cluster, consisting of 27 genes, is transcribed by 9 polycistronic mRNA species (**Figure 6**).[51] As described above, the A-factor signal, starting with *afsA* encoding an A-factor biosynthesis enzyme, is transferred first to ArpA, the A-factor receptor protein, and then to AdpA, a key transcriptional activator. The pathway-specific transcriptional activator, *strR*, for streptomycin biosynthesis is a member of the AdpA regulon and is induced by AdpA. Two AdpA dimers bind the upstream activation sequences of *strR*, approximately at nucleotide positions −270 and −50 with respect to the transcriptional start point of *strR*, and activate its transcription. For transcriptional activation, the two AdpA-binding sites should be occupied by an AdpA dimer, speculatively because the two AdpA dimers form a complex, as a result of which a DNA loop via the AdpA complex is formed. The DNA-bound AdpA molecules assist RNA polymerase in forming an open complex competent for transcriptional initiation.[44] StrR thus induced binds and activates the nine promoters that cover all the streptomycin biosynthesis genes, triggering biosynthesis of streptomycin from glucose. The signal relay for streptomycin biosynthesis has thus been elucidated: from AfsA to A-factor to ArpA to AdpA to StrR, and finally to the streptomycin biosynthesis genes.

The major streptomycin resistance determinant, *aphD*, encoding streptomycin-6-phosphotransferase is encoded just downstream of *strR* and cotranscribed with *strR* by read-through from the AdpA-dependent *strR* promoter.[47] The cotranscription of *strR* and *aphD* accounts for the prompt induction of streptomycin resistance by A-factor and achieves a rapid increase in self-resistance just before induction of streptomycin biosynthesis.

4.07.3 Signaling Molecules in High-GC Gram-Positive Bacteria

4.07.3.1 Introduction

The high-GC Gram-positive bacterial taxon Actinomycetes includes a number of industrially important organisms. The best characterized genus is *Streptomyces*, which consists of the largest number of species including several model organisms whose genome has been completely sequenced. *Streptomyces* and related bacteria are renowned for their ability to produce a wide variety of secondary metabolites, which have many industrial applications.[66] This group of bacteria are also characterized by their ability to perform complex cellular differentiation resembling that of filamentous fungi. In the initial stages of life cycle, the organism grows as a branching multinucleoid substrate mycelium on solid media.[67] The substrate hypha then produces

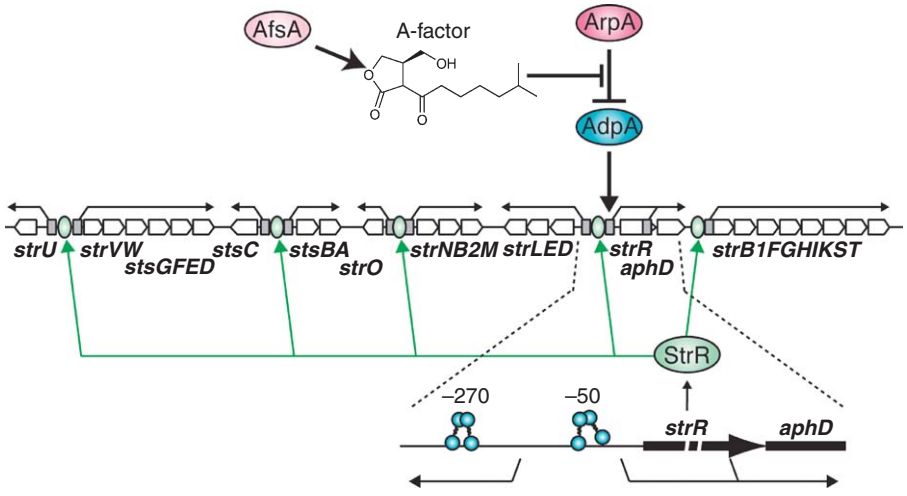


Figure 6 The A-factor signal relay to the streptomycin biosynthesis genes. The streptomycin biosynthesis genes, in a total of 27 genes, including the pathway-specific transcriptional activator *strR*, are shown. The A-factor signal relay starts with A-factor and is then transmitted to ArpA to AdpA to StrR, and finally to the nine transcriptional units covering the whole gene cluster.

aerial mycelium, which culminates in a long spore chain by forming septa at regular intervals. The complex metamorphosis activity programmed in prokaryotic genetic background is an attractive research target in terms of cellular development.

Both morphological differentiation and secondary metabolite formation in this group of bacteria take place in response to environmental stimuli such as nutritional limitation and desiccation. To date, many genes that regulate morphogenesis and secondary metabolism have been identified and their physiological roles have been characterized in several model organisms. The lines of evidence have demonstrated that the genetic control of morphological development and that of secondary metabolism are linked to each other and comprise a complex regulatory network. It is probable that various environmental stimuli are sensed by signaling systems that have connections to the complex regulatory network, but the details of such signaling mechanisms have not yet been well characterized.

In contrast to the relatively poor understanding of environmental signal sensing, the molecular mechanism of hormonal sensing has been well studied in some model organisms.[68] As described in the previous section, the general autoinducer functioning in this group of bacteria retains a γ-butyrolactone structure. The best studied autoinducer, A-factor (2-isocapryloyl-3*R*-hydroxymethy-γ-butyrolactone), controls the onset of both morphological differentiation and secondary metabolite formation in *S. griseus*. The studies by Horinouchi and coworkers[18,69] have identified the A-factor-dependent transcriptional regulators and characterized their precise roles and functions. Accumulating evidence has shown that similar hormonal control of secondary metabolite formation occurs widely in *Streptomyces*.[70]

This section reviews the current understanding of interspecific signal transfer mediated by small molecules that stimulate the initiation of secondary metabolism and cell differentiation in *Streptomyces* and related bacteria. In contrast to the deep understanding of intraspecific hormonal sensing, this research field is yet under development; however, several new approaches have started to unveil the unseen nature of cross-talking in this group of bacteria.

4.07.3.2 Gräfe's Factors

It is generally understood that the above-mentioned γ-butyrolactone sensing occurs in a species-specific manner. This is supported by the fact that A-factor of *S. griseus* and VBs of *S. virginiae* do not exhibit cross-reaction: A-factor does not induce virginiamycin production in *S. virginiae*, and none of the VBs stimulates aerial mycelium formation or streptomycin production in *S. griseus*.[69] This is explained by the diversity of side chain structure of these factors and ligand selectivity of the corresponding receptor proteins (see Sections 4.07.2.2 and 4.07.2.4).

On the other hand, the extensive study by Gräfe *et al.*[71] on the structural and functional diversity of γ-butyrolactones in the early 1980s showed the presence of not only intraspecific but also interspecific factors. For example, they showed that anthracycline production by a mutant strain of *S. griseus* was induced by γ-butyrolactone factors produced by several other *Stretomyces* spp.[71] Hara and Beppu[6] also suggested that A-factor or an alternative factor is produced by several *Streptomyces* species other than *S. griseus*. Recently, Choi *et al.*[72,73] reported that known types of γ-butyrolactones function in several non-*Streptomyces* Actinomycetes, including *Amycolatopsis*, *Actinoplanes*, and *Kitasatospora*. Lines of evidence strongly suggest that γ-butyrolactones serve as a communication factor that functions widely in Actinomycetes.

4.07.3.3 Antibiotics as Signal Molecules

A long-lasting open question in the study of Actinomycetes is why this group of bacteria produce such highly diverged compounds as secondary metabolites. It is well known that some of the compounds exhibit antimicrobial activities, which leads to a plausible argument that the defensive function of these compounds is essential for the life of this kind of bacteria in the natural environment, although this simple idea does not fully explain the marked diversity of structure and activity of the secondary metabolites.

On the other hand, another attractive idea for the role of secondary metabolites has been propounded by Davies and coworkers.[74] They demonstrated that some antibiotics at low concentrations alter global bacterial transcription patterns.[74] The precedent discovery was done by Murakami *et al.*[75] on the binary activity of

thiostrepton, a cyclic peptide produced by *Streptomyces azureus*. Thiostrepton not only exhibits an antibiotic activity by inhibiting the function of prokaryotic ribosomes but also induces the activity of a specific promoter in *Streptomyces lividans*.[76] Similarly, antibiotics including erythromycin, rifampicin, and the so-called macrolide–lincosamide–streptogramin (MLS) antibiotics have been shown to act as global regulators that modulate transcription of bacterial cells.[77] Furthermore, it has been shown recently that β-lactam antibiotics stimulate transcription in eukaryotic cells.[78] Lines of evidence strongly suggest that the unseen functional diversity of secondary metabolites constitutes an unexpectedly wide and complex signaling network in the natural environment, although the details still largely remain unknown.

4.07.3.4 Wide Occurrence of Interspecific Cross-Talking in the *Streptomyces* Community

To assess the occurrence of interspecific cross talk in the *Streptomyces* community, Kawai at Nihon University performed comprehensive cross-feeding experiments using a number of culture collection strains and fresh environmental isolates.[79] The result showed unexpectedly wide occurrence of interspecific stimulation of antibiotic production and/or morphological differentiation. The especially high frequencies were observed with environmental isolates; more than 90% of the strains examined exhibited a stimulatory activity(ies) against other strain(s), and more than 30 and 50% of the strains were promoted for antibiotic production and cell differentiation, respectively, when they were grown in close proximity of other strains.[73] The evidence strongly supports the idea that cross-talking generally takes place in the community of this group of soil bacteria.

4.07.3.5 Role of Ferrioxamines

As had been observed with some γ-butyrolactones and antibiotic substances, it appeared likely that the above interspecific stimulation events are mediated by some diffusible metabolites produced by stimulator strains. To study the details of the interspecific stimulation events, Yamanaka *et al.*[80] isolated the activity principle that stimulated growth and development of *Streptomyces tanashiensis* from culture supernatant of *S. griseus*. As a result of structural analysis of the purified preparation, the substance was determined to be desferrioxamine E (synonym, nocardamine; **Figure 7**).

Desferrioxamine E and its analogues are siderophores widely produced by *Streptomyces* and related bacteria.[81] Their major role is ferric transportation; cells secrete these cage compounds to their environment and then uptake their ferric-bound form (ferrioxamines) by the activity of a specific transporter to utilize ferric. Probably, the organisms had developed this complex retrieval system due to the insolubility and low availability of ferric in the natural environment.

Notably, the effect of exogenous supply of ferrioxamine in *Streptomyces* is diverse: it promotes growth in some strains, while it does not affect growth but stimulates cellular differentiation and/or secondary metabolism in

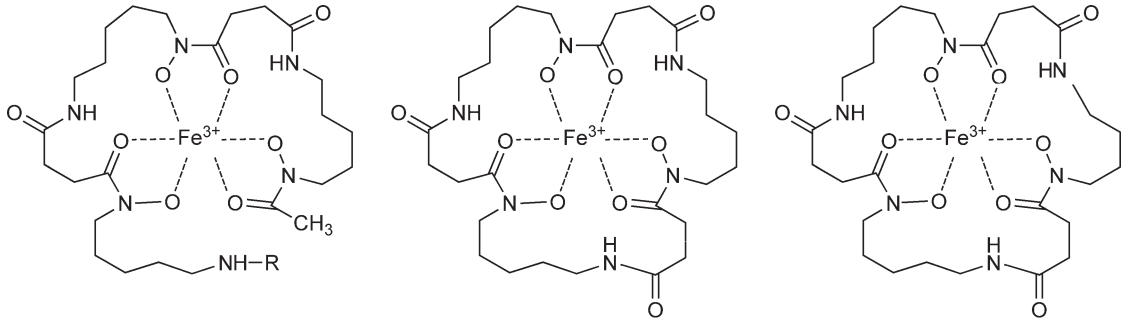


Figure 7 Structures of representative ferrioxamines (ferric-containing desferrioxamines) produced by *Streptomyces*.

other strains.[80] This fact suggests that the ferric uptake system plays different roles in this group of bacteria. Desferrioxamines are known to promote growth of some fungi[82–84] and bacteria[85,86] that do not have the ability to produce the siderophore. Hence, it is likely that desferrioxamines produced by *Streptomyces* and several other bacteria are utilized by a wide variety of microorganisms, constituting a basis of commensalism in microbial community. The diverse effect also implies that the substance has additional function other than ferric transportation.

4.07.3.6 Other Factors

In addition to the above factors, several substances have potential to be signal molecules that mediate cell–cell communication in Actinomycetes. Onaka *et al.*[87] isolated an activity principle that stimulated secondary metabolite formation and/or cell differentiation in various *Streptomyces* strains from the cell extract of a *Streptomyces* sp. The substance, named goadsporin, was a peptidic molecule consisting of 19 amino acids.[88] Although evidence is not yet available for its secretion, the multifunctional or pleiotropic effect of the exogenous supply of goadsporin suggests its unique and general function as a signal substance.

It has been known that a secreted hydrophobic peptide called SapB induces the cellular development of the model organism *S. coelicolor* A3(2). SapB probably reduces surface tension of substrate mycelium and induces the erection of aerial mycelium. Recently, Kodani *et al.*[89] revealed the chemical structure of SapB to be a lantibiotic-like cyclic peptide that contains two unusual amino acids, dehydroalanine and lanthionine (for a detailed description of lantibiotics, see the following section). Since an activity alternative to SapB has been observed with other surface-active agents including hydrophobins produced by some kind of fungi,[90] it is possible that some kind of secreted hydrophibic molecules are also involved in cell–cell interaction in microbial community.

Like other organisms, it is likely that cyclic AMP (cAMP) functions as a second messenger in *Streptomyces*. Evidence has suggested that cAMP acts as an extracellular signal for the onset of morphological development and antibiotic production in *S. coelicolor* A3(2)[91] and *S. griseus*.[92] Although little genetic and biochemical information is available on the secretion of cAMP, it is possible that this general signal substance plays some role in microbial community structuring in *Streptomyces*.

Actinomycetes include unicellular organisms that comprise industrially and clinically important genera such as *Corynebacterium*, *Rhodococcus*, and *Mycobacterium*. To date, little information is available with regard to the social behavior of these kinds of bacteria. The genome sequencing studies on the representative species did not discover any known autoinducer systems. Hence, it is possible that a different signaling system controls the community structuring of these organisms. An unknown diversity of communication mechanism may exist within this group of bacteria.

4.07.4 Signaling Molecules in Low-GC Gram-Positive Bacteria

4.07.4.1 Introduction

The first example of chemical communication in low-GC Gram-positive bacteria was discovered in 1978 by Dunny, Clewell and their colleagues.[93] It was found that conjugative plasmid transfer of *Enterococcus faecalis* was induced by a chemical substance, termed sex pheromone, which is secreted from plasmid-free recipient cells. Several years later, the structure of the sex pheromone was elucidated to be an oligopeptide by Suzuki *et al.*[94] Since this discovery, a variety of cell-to-cell communication events in Gram-positive bacteria have been found to be mediated by peptidic substances.[95–97]

While Gram-negative bacteria use nonpeptidic small compounds represented by *N*-acylhomoserine lactone for cell-to-cell communication, Gram-positive bacteria mainly use peptidic compounds for this purpose with the exception of butyrolactones in *Streptomyces* (see Section 4.07.2) and furanosyl borate diester, autoinducer-2 (AI-2), which is the universal signal molecule common in both Gram-positive and Gram-negative bacteria.[98] Although peptides are ubiquitous compounds in the living organism, their structures can be diverse not only at the primary structure level but also at the higher order structure level. This means that it is relatively easy for peptides to gain diversity in their structure during the course of molecular evolution. Probably, this has allowed to accomplish specific interaction between signal and receptor and may provide considerable advantage to

achieve specific and selective cell-to-cell communication in natural biosphere comprising complex biota. The diversity in peptide structure can also be created by posttranslational modification. The peptides involved in cell–cell communication of Gram-positive bacteria are often posttranslationally modified to form lanthionine, thiolactone, isoprenylated tryptophan, and so on. Those posttranslational modifications indeed seem to allow stable interaction of peptide signal molecules with their receptors.

Peptide signal molecules are mainly classified into two categories based on their mode of action. The first category includes nonmodified small oligopeptides that are reimported into the cells and directly trigger the signal transduction pathway by binding to intracellular target molecules. The other category includes a variety of peptides but the signals of all peptides are commonly transduced through a two-component regulatory system consisting of membrane histidine kinase and intracellular response regulator. The latter category is classified into four different types in terms of chemical structure as follows: (1) bacteriocin-inducer pheromone and competence-stimulating peptides (CSPs); (2) lantionine-containing peptides; (3) cyclic thiolactone and lactone peptides; and (4) prenylated peptides. In addition to these peptide signal molecules, a recent topical compound, AI-2, will be reviewed in Sections 4.07.4.7 and 4.07.5.3.1.

4.07.4.2 Nonmodified Small Oligopeptides

Nonmodified small oligopeptides are reimported into responder cells after the secretion. The imported peptides directly bind to intracellular receptors that regulate transcription of target genes directly or indirectly.

4.07.4.2.1 Sex pheromones of E. faecalis

Recent outbreak of drug-resistant enterococci is outstanding. It is well known that enterococci are like a reservoir of drug-resistant genes and sometimes also play a pivotal role as a spreader of resistant genes not only in the clinical area but also in the natural environment including animal gastrointestinal tract.[99,100] In enterococci, drug-resistant genes are often encoded by mobile genetic elements.[101] Pheromone-responsive plasmids are one of the mobile genetic elements carrying drug-resistant genes and some other pathogenicity-related genes such as hemolysin.[102] Host cell harboring a pheromone-responsive plasmid expresses a series of genes required for conjugative plasmid transfer in response to subnanomolar concentrations of the sex pheromone, that is, the concentrations surrounding the pheromone-producing recipient cells in nature. As a result of the pheromone response, cell clumps are formed between plasmid donor and recipient cells, which enable high-frequency plasmid transfer in a liquid culture.

Thus far, five peptide sex pheromones have been identified (**Figure 8(a)**).[94,103–106] It is interesting to note that each plasmid specifically responds to only the corresponding pheromone. Each peptide sex pheromone is named 'cX', which induces plasmid 'pX'. For example, cAD1 induces conjugative transfer of pAD1. The initial letter of cX is named after the initial letter of 'clumping' because these peptide sex pheromones drastically induce cell clumping between donor and recipient cells.

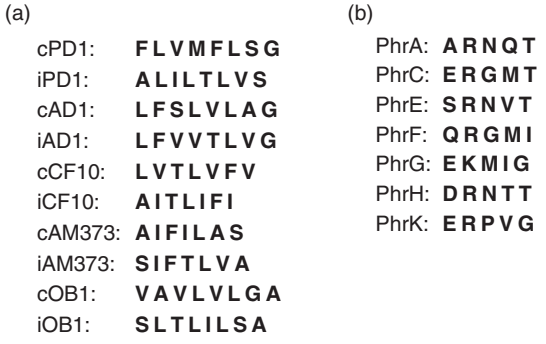


Figure 8 Structures of nonmodified oligopeptide signal molecules mediating intraspecies cell-to-cell communication in Gram-positive bacteria. (a) Peptide sex pheromones and their inhibitors involved in the regulation of conjugative plasmid transfer in *Enterococcus faecalis*. (b) Competence- and sporulation-stimulating factors of *Bacillus subtilis.*

Once the host cell acquires the pheromone-responsive plasmid, it apparently shuts off the production of the corresponding pheromone. It has been revealed that two systems are involved in this phenotypic change. One is the production of pheromone inhibitor; each pheromone-responsive plasmid encodes a pheromone inhibitor, termed 'iX', which is an antagonist peptide of sex pheromone. The other one is shutdown of pheromone production, which is accomplished by a plasmid-encoded protein (TraB for pAD1 and pPD1 and PrgY for pCF10).[107–110]

As shown in **Figure 8(a)**, sex pheromones and their inhibitors are hepta- or octapeptides. All are rich in hydrophobic amino acid residues and no charged amino acid residues are contained in these peptides. It has been found that these peptide sex pheromones are encoded in the N-terminal leader moiety of lipoproteins. The leader peptides (about 20 amino acids) are cleaved off after the translocation of lipoproteins, further processed to generate the pheromones, and eventually they are excreted.[110,111] The secreted pheromones are reimported into the plasmid-donor cells and bound to their receptors, as shown in **Figure 9**.

The pheromones and their inhibitors are suggested to be imported through an oligopeptide permease (Opp). Leonard *et al.*[112] demonstrated that an insertional inactivation of the chromosomal *opp* operon reduced the sensitivity of the host cell to cCF10 by about one order of magnitude, suggesting that the Opp contributes to sex pheromone uptake. Pheromone-responsive plasmids encode accessory proteins, pheromone-binding proteins (TraC for cAD1 and cPD1, and PrgZ for pCF10), involved in pheromone uptake. These pheromone-binding proteins showed high similarity to oligopeptide-binding proteins encoded by chromosomal *opp* gene cluster. This similarity suggested the model that sex pheromone is initially bound to the pheromone-binding protein, transferred to Opp complex located in the membrane, and eventually internalized into the cell. However, it should be noted that the *opp*-knockout mutant still has partial ability to respond to sex pheromone. Considering that a series of peptide sex pheromones commonly show highly hydrophobic profile, it is likely that they are somehow permeable through the cytoplasmic membrane and can be internalized without any transporter. Indeed, Nakayama *et al.*[113] have demonstrated that *opp*-knockout mutant of *E. coli* is still able to import a tritium-labeled cPD1 as does wild-type *E. coli*.

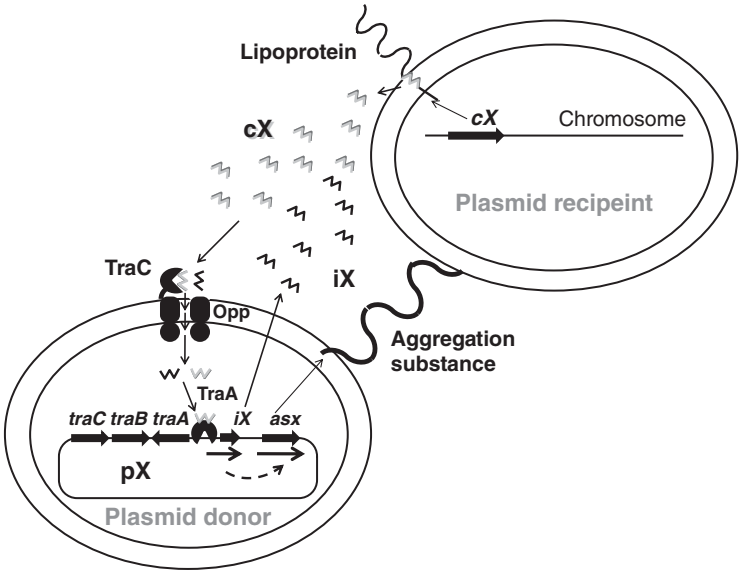


Figure 9 Sex pheromone signaling leading to conjugative plasmid transfer in *Enterococcus faecalis*. Sex pheromone (cX) is encoded in the N-terminal leader moiety of lipoprotein. The leader peptide is cleaved off after the translocation of lipoprotein and further processed to generate the pheromone, which is eventually excreted. The secreted pheromone is reimported into the plasmid-donor cell and bound to TraA, which is a transcriptional regulator. Eventually, aggregation substance is induced on the donor cell surface, which leads to plasmid transfer to recipient cell.

After the pheromone is imported into the plasmid-donor cells, it directly binds to an intracellular receptor. The receptor is designated as PrgX for cCF10[114] and TraA for cAD1[115] and cPD1.[116,117] Nakayama *et al.*[116] have proved, in an *in vivo* experiment using tritium-labeled cPD1, that cPD1 binds to TraA. It was also demonstrated that TraA of pPD1 shows affinity only to cPD1 and iPD1 but not to other pheromones and inhibitors, by *in vitro* competitive binding assay with recombinant TraA and the tritium-labeled cPD1.[116] This indicated that TraA recognizes the corresponding pheromone cPD1 and that iPD1 is a receptor antagonist of TraA. It is known that each sex pheromone induces specifically the conjugative transfer of the corresponding plasmid. These experiments showed that the pheromone receptor, that is one of the plasmid determinants, is responsible for the specific signal transduction of sex pheromone. Recently, crystal structures of PrgX and PrgX–cCF10 complex have been determined.[118] Comparison of their structures suggests that pheromone binding destabilizes PrgX tetramers, opening a 70-bp pCF10 DNA loop required for conjugation repression. As for the cases of cAD1 and cPD1, it was also demonstrated that pheromone binding modulates the interactive property of TraA to the operator site.[115,119] As a consequence of signal transduction of those pheromones, transcription of the aggregation substance gene, designated *asa1* for pAD1, *asp1* for pPD1, and *asc10* for pCF10, is induced, resulting in cell aggregation between donor and recipient cells.

4.07.4.2.2 *Competence- and sporulation-stimulating factors of* Bacillus subtilis

Bacillus subtilis also uses oligopeptide signal molecules for intraspecies cell–cell communication, which is occasionally called quorum sensing. It is well known that *B. subtilis* forms spore and also develops genetic competence in the stationary phase.[120] The finding that mutation of an oligopeptide permease gene, *spo0K*, abolished those phenotypic changes clearly indicated that those changes are mediated by oligopeptides imported into the cells.[121,122] Later, a series of Phr pentapeptides were identified as inducers of sporulation or competence development.[123–129] Thus far, it has been found that PhrA and PhrE are involved in sporulation;[130,131] PhrC, PhrF, PhrG, and PhrK are involved in competence development;[123,129,132] and PhrH is involved in both phenotypes (**Figure 8(b)**).[133]

Figure 10 shows a schematic of signal transduction of Phr peptides together with a competence pheromone, ComX, which is described in Section 4.07.4.6. Each Phr peptide is encoded in an operon with a Rap protein and inhibits the activity of its cotranscribed Rap. PhrA, PhrC, PhrF, and PhrG comprise the five C-terminal residues of the precursors, whereas PhrE and PhrH are derived from internal five-residue fragment of their precursors. The secreted Phr pentapeptides are reimported via the oligopeptide permease (Opp) and bind to the corresponding Rap. PhrA and PhrE peptides bind to RapA and RapE, respectively, and interfere with the function of these Rap proteins to promote dephosphorylation of phosphorylated Spo0F, which is an intermediate response regulator of the phosphorelay signal transduction system involved in sporulation initiation.[125,126,134] Consequently, the phosphorelay cascade among a subset of Spo0 proteins is triggered and, eventually, the resulting phosphorylated Spo0A induces the expression of a series of genes involved in sporulation.[126]

The other series of Rap–Phr system is associated with *com* signal transduction pathway. Thus far, it has been demonstrated that PhrC, PhrF, PhrG, and PhrK pentapeptides interfere with the function of the corresponding Rap proteins, each inhibiting the DNA-binding activity of ComA.[121,122] Furthermore, it was recently found that RapH/PhrH system is involved in both signaling pathways by dephosphorylating the Spo0F-P and inhibiting the DNA-binding activity of ComA.[127] ComA is a response regulator constituting a two-component regulatory system with a histidine kinase ComP, which is the main signaling pathway from ComX pheromone to competence development. ComA free from Rap proteins induces transcription of *srfA* operon, which leads to the development of genetic competence.[125] This signal transduction pathway is described in detail in Section 4.07.4.6.

It is noticeable that some bacteria of the *Bacillus cereus* group also use the pentapeptide as a quorum sensing signal.[135] The pentapeptide is a processed product corresponding to the C-terminal five-residue peptide of a 48-residue propeptide, PapR. Similar to Phr peptide family, the PapR pentapeptide is imported into the cell and interacts with a transcriptional regulator, PlcR, and regulates its activity. This type of small oligopeptide-mediated quorum sensing may be commonly used among bacilli.

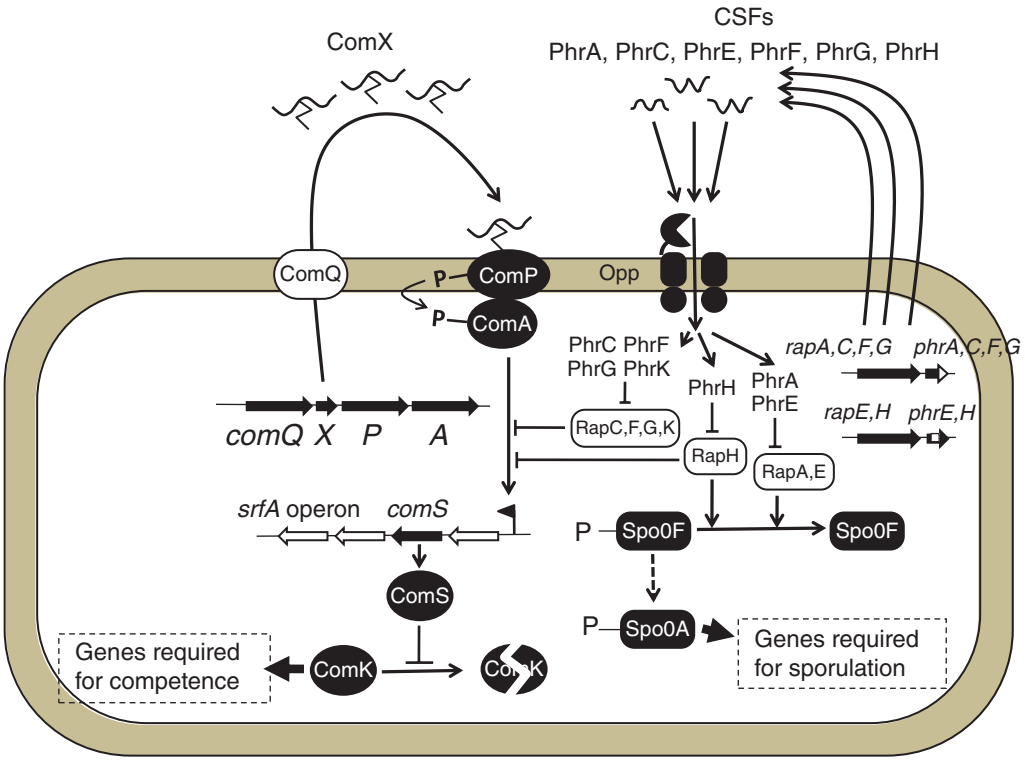


Figure 10 Signal transduction pathways of Phr peptides and competence pheromone (ComX) in *Bacillus subtilis*. After secretion, Phr pentapeptides are reimported by the oligopeptide permease (Opp). The imported PhrA and PhrE peptides bind to RapA and RapE, respectively, which promote dephosphorylation of phosphorylated Spo0F. As a result of Phr binding, the dephosphorylation of Spo0F-P is inhibited and the phosphorelay cascade among a subset of Spo0 proteins is triggered. Phosphorylated Spo0A is resulted at the end of this cascade and eventually induces the expression of a series of genes involved in the sporulation. The imported PhrC, PhrF, PhrG, and PhrK pentapeptides bind to the corresponding Rap proteins and inhibit their interaction with ComA. ComA free from these Rap proteins induces transcription of *srfA* operon containing *comS*. ComS prevents the degradation of the competence transcription factor ComK, which regulates the expression of gene set encoding the transformation machinery. PapH–PhrH system is involved in both signaling pathway by dephosphorylating the Spo0F-P and inhibiting the DNA-binding activity of ComA.

4.07.4.3 Inducer Peptide Pheromones of Class II Bacteriocins and Competence-Stimulating Peptides

Although these two types of peptide signals are involved in the regulation of different phenotypes, that is bacteriocin biosynthesis and development of genetic competence, they can be categorized in the same group in terms of structure and mode of action. These peptides are about 2–3 kDa and presumably form amphiphilic α-helical structure in the middle region. These peptides are commonly translated as propeptides containing double glycine leader at their N-terminal side, which is processed concomitantly with export.

4.07.4.3.1 Inducer peptide pheromones of class II bacteriocins

Bacteriocins are antimicrobial peptides ribosomally synthesized in bacteria. These are classified into four or five classes.[136] Class II bacteriocins are heat-stable nonlantibiotic peptides. Unlike lantibiotics described in the next section, class II bacteriocins are less modified; disulfide bridge and some N-terminal modifications are known in some class II bacteriocins. It is well known that the biosynthesis of some of the class II bacteriocins is regulated by quorum sensing mediated by inducer peptide pheromones.[95,137–140]

In 1996, Eijsink *et al.*[140] found that a certain level of inoculum size (more than 0.05% (v/v) of final volume) was necessary for the production of bacteriocin in a liquid culture of *Lactobacillus sake* LTH673, which is a producer of sakacin P. When the conditioned medium was added to the culture medium to reach a final

concentration of 0.1–1.0%, the bacteriocin biosynthesis was recovered. This suggested that the producer strain secreted an inducer triggering bacteriocin biosynthesis. The inducer, the so-called 'inducer pheromone', was purified and identified to be a 19-amino acid residue peptide. Its synthetic peptide induced sakacin P production at subnanomolar concentrations.

Thus far, a number of inducer pheromones of class II bacteriocin have been identified as shown in **Figure 11(a)**. These peptides are simulated to form amphiphilic α-helical structure.[140,141] In some peptides, there are two conserved cysteine residues, which probably form a disulfide bridge. These structural features resemble the N-terminal parts of class II bacteriocins, although the sizes of inducer pheromones (19- to 30-amino acid residues) are considerably smaller than those of class II bacteriocin peptides (mostly ranging from 35- to 70-amino acid residues). The biosynthetic feature of this type of inducer pheromones also resembles that of class II bacteriocins; in both cases, peptides are translated as propeptides with an N-terminal leader and maturated by cleaving off the leader moiety.[95,137–139] It is known that some of this type of inducer peptides show antibacterial activity.[95,142] It is likely that the inducer peptides achieve antibacterial activity with the amphiphilic feature allowing pore formation in the bacterial cytoplasmic membrane.[141,143] It is interesting to note that the D-enantiomeric form of an inducer pheromone, plantaricin A, consisting of D-amino acids for all residues showed the same level of antibacterial activity as the native form and also antagonistic activity to native plantaricin A.[141] This suggests that an initial nonchiral interaction with membrane lipids induces α-helical structure, allowing it to be properly positioned in the membrane interface, thus enabling it to engage in a chiral interaction with its receptor in or near the membrane–water interface. This membrane-interacting mode of action explains why some peptide pheromones sometimes display antimicrobial activity in addition to their pheromone activity.

Figure 12 shows a schematic of signal transduction pathway of the inducer pheromone (IP-673) in *L. sake* LTH673, as a representative of quorum sensing involved in class II bacteriocin biosynthesis. The receptor of this type of inducer pheromone belongs to the protein family of histidine protein kinase. The histidine kinase is located in the cytoplasmic membrane and transduces the inducer signal from outside to inside the cell by transferring phosphate group from histidine residue of the histidine kinase to aspartate residue of the response regulator. The set of receptor kinase and response regulator is the so-called two-component regulatory system. By using the inducer pheromone and the two-component regulatory system, the bacteriocin-producing cell can sense the density of cells carrying the same bacteriocin gene, and when the cell density reaches a certain threshold level, the producer cells living in the same niche can start to produce bacteriocin concomitantly.[137] In most cases, genes for inducer pheromone and the two-component regulatory system are encoded adjacently and the quorum sensing gene cluster is located adjacent to the bacteriocin biosynthesis gene cluster encoding bacteriocin structural gene, its transporter, and immunity proteins, as in the case of sakacin P in **Figure 12**.[95] Also in most cases, some sets of bacteriocin biosynthetic genes are encoded as operons and are transcriptionally regulated by the same promoter and operator controlled by the two-component regulatory system. This system allows the concerted control of expression of genes involved in bacteriocin biosynthesis and autoimmunity.[95,137]

4.07.4.3.2 Competence-stimulating peptides of streptococci

In 1928, Griffith[144] discovered genetic transformation in *Streptococcus pneumoniae*. In the 1960s, it was suggested that this phenomenon depends upon cell density and the inducer peptide named CSP mediates this quorum sensing.[145,146] Thirty years later, CSP was found to be a linear peptide consisting of 17-amino acid residues.[147] Gene analysis revealed that CSP is translated as a 40-amino acid propeptide containing the double glycine leader in the N-terminal part as found in the inducer peptide pheromones of class II bacteriocins (**Figure 11(b)**).[147]

Figure 13 shows a schematic of the signal transduction system, the so-called '*com* system', which is involved in competence development in *S. pneumoniae*. CSP is encoded by *comC* and the translated precursor is secreted and processed by an ATP-binding cassette (ABC) transporter composed of ComA and ComB.[147,148] The secreted CSP triggers the ComD–ComE two-component regulatory system.[149] The phosphorylated ComE induces transcription of early genes, including *comAB* and *comCDE*; this positive feedback causes increased signal production and sensing, which enables rapid response to a certain cell density.[149] The early gene subset also includes *comX* and *comW*. ComX plays a central role in the *com* signal transduction pathway as a competent-specific alternative sigma factor that controls the transcription of a series of genes involved in DNA uptake

Figure 11 Sequences of inducer pheromones of class II bacteriocin (a) and competence-stimulating peptides of streptococci (b) and their leader peptides. Cysteine residues putatively formed disulfide bridge are boxed. The name in parentheses represents the bacteriocin induced by the pheromone.

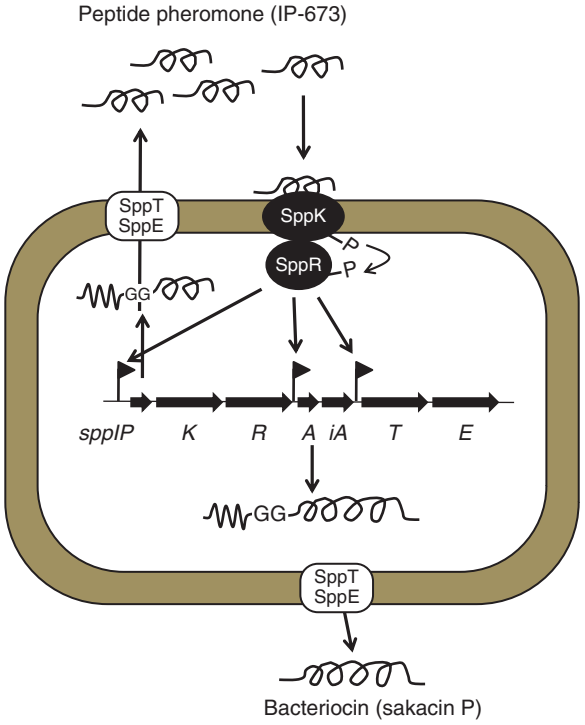


Figure 12 Signal transduction pathway of the inducer pheromone (IP-673) for the control of sakacin P production in *Lactobacillus sake* LTH673. *sppIP* encodes the precursor of IP-673. After translation, the precursor is processed and secreted via the ABC-transporter complex of SppT and SppE. The secreted IP-673 binds to SppK and triggers the phosphorelay to SppR. The phosphorylated SppR induces the transcription of three operons, *sppIP-K-R* encoding the pheromone and two-component regulatory proteins, *sppA-iA* encoding sakacin P and the immunity protein, and *sppT-E* encoding the ABC transporter for the pheromone and bacteriocin. As a consequence, production of sakacin P is started concomitantly.

together with the core enzyme of RNA polymerase.[150] ComW also functions as a key protein that may act in the stabilization as well as activation of ComX, allowing a high level of competence.[151,152] The subset of CSP-responsive genes includes a few encoding proteins involved in killing noncompetent cells. This mechanism is called fratricide and is considered to be used by competent cells to acquire DNA from noncompetent cells.[153]

The cognate *com* gene cluster was also found in other streptococci, including *S. mutans*, *S. gordonii*, *S. mitis*, and *S. oralis*.[154–158] **Figure 11(b)** shows the sequences of some CSPs found in these *com*-like gene clusters. The sequences of these CSPs are varied at the strain level. Each strain specifically responds to the corresponding CSP, suggesting coevolution of *comC* and *comD*. Structure–activity analysis of *S. mutans* CSP identified two functionally important structural motifs in this peptide: one is the core amphiphilic α-helical structure extending from residue 5 to the end of the peptide, which is important for binding to the receptor, and the other is the C-terminal structural motif consisting of a sequence of polar–hydrophobic–charged residues, which is crucial for the activation of the signal transduction pathway.[159] It is interesting to note that these important motifs are well conserved among the streptococcal CSPs.

In the past decade, new aspects of *com* quorum sensing system have been explored in *S. mutans*. Besides development of genetic competence, *com* signaling system has been found to play a regulatory role in biofilm formation,[160,161] stress responses,[162] and bacteriocin production,[163] which are key virulence factors in *S. mutans* pathogenesis. Biofilm formation is a marked phenotype of *S. mutans*. Knockout mutants defective in *com* genes were remarkably attenuated in biofilm formation, and addition of CSP restored the wild-type biofilm formation of *comC* mutants, indicating that biofilm formation is regulated by the *com* quorum sensing system.[160,161] It is known that most clinical isolates of *S. mutans* produce one or few kinds of bacteriocins called mutacins. Currently, two classes of mutacins have been characterized, the lantibiotics and the nonlantibiotics. It was demonstrated that expression of two sets of bacteriocin gene clusters, *nlmAB* encoding mutacin IV and

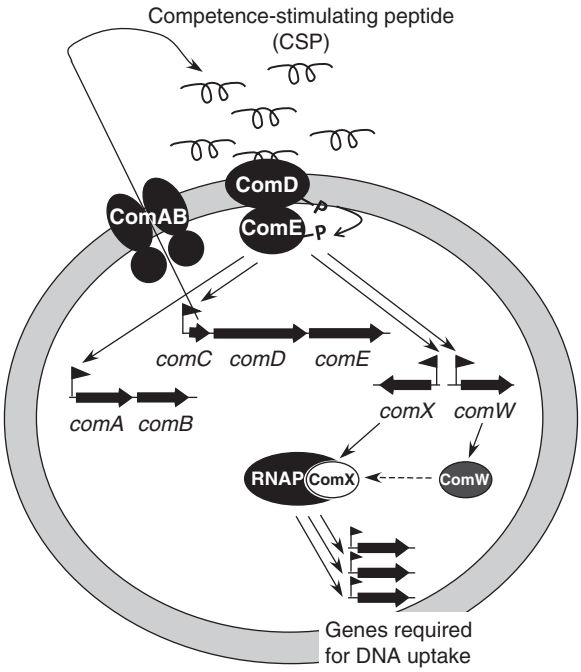


Figure 13 Signal transduction pathway of *com* system in *Streptococcus pneumoniae*. Competence-stimulating peptide (CSP) is encoded by *comC* and the translated precursor is secreted and processed by an ABC transporter composed of ComA and ComB. The secreted CSP triggers the ComD–ComE two-component regulatory system. The phosphorylated ComE induces the transcription of early genes, such as *comAB* and *comCDE*; this positive feedback causes increased signal production and sensing. The early gene subset also includes *comX* and *comW*. ComX plays a central role in the *com* signal transduction pathway as a competent-specific alternative sigma factor that controls the transcription of a series of genes involved in DNA uptake together with the core enzyme of RNA polymerase. ComW also functions as a key protein that may act in the stabilization as well as activation of ComX, allowing a high level of competence.

bsmA–immA encoding other class IIb bacteriocins and its immunity protein, is induced upon addition of CSP and that inactivation of the *com* two-component regulatory system abolishes the production of these bacteriocins.[163] It is quite interesting that development of genetic competence, bacteriocin production, and biofilm formation are under the same control. As described in Section 4.07.4.7, it is also known that AI-2 is related to the regulation of biofilm formation and bacteriocin production in *S. mutans*.

4.07.4.3.3 Bacteriocin-like peptides encoded by streptococcal genomes

The genome sequence of *S. pneumoniae* revealed the existence of 13 sets of two-component regulatory systems.[164] An analysis of these two-component regulatory gene mutants in a mouse model suggested that eight of these 2CSs were involved in respiratory tract infection.[165] Among them, TCS13 is best studied. TCS13 is encoded in *blp* locus including a number of bacteriocin-like genes. Downstream of TCS13 genes, *blpR* (response regulator gene) and *blpH* (histidine kinase gene), there are *blpC* (encoding ComC-like peptide) (**Figure 11(a)**) and *blpAB* (encoding a putative protein complex of ABC transporter). This organization is similar to the streptococcal *com* gene cluster, suggesting that this *blp* gene cluster is involved in quorum sensing other than *com* system. DNA microarray experiment demonstrated that 16 genes in 8 operons including *blpABC* and *blpRH* themselves were induced by this *blp* regulatory system.[166] Three of these operons code seven open reading frames encoding bacteriocin-like peptides carrying putative double glycine leader. Recently, it was demonstrated that *blpM* and *blpN*, both encoding class II bacteriocin-like peptides, are responsible for bacteriocin activity against a heterologous strain.[167] A mutant in the *blpMN* operon was unable to compete with its parent as well as a different serotype strain during cocolonization in a mouse model experiment. These results suggest that the *blp* peptides mediate intraspecies competition in nature.

A *blp* gene cluster orthologue, designated blp_{St}, was also identified in the genome sequence of *Streptococcus thermophilus*. The blp_{St} cluster of strain LMD-9 contains all the genetic information required for the production of bacteriocin, and is regulated at the transcriptional level by a quorum sensing mechanism in which the mature form(s) of the induction factor $BlpC^{*}{}_{St}$ (**Figure 11(a)**) triggers the expression of bacteriocin and immunity genes through the $BlpH_{St}/BlpR_{St}$ TCS.[168]

4.07.4.4 Lanthionine-Containing Peptides

Lanthionine is 3,3′-thiodialanine, which is composed of two alanine residues that are crosslinked on their β-carbon atoms by a thioether linkage as the monosulfide analogue of cystine. This structure occasionally gives rise to not only highly stable properties but also unique biological activity to the peptides. Lantibiotics are lanthionine-containing peptide antibiotics. They are classified as class I bacteriocin. Production of some lantibiotics is known to be regulated by quorum sensing in which the lantibiotics function as autoinducers as well as antimicrobial peptides.

4.07.4.4.1 Nisin and subtilin

Nisin is the most famous and best studied lantibiotic and it has high antibactericidal activity against a wide range of Gram-positive bacteria. Thus far, three nisin variants, nisin A,[169] nisin Z,[170] and nisin Q,[171] have been found from *Lactococcus lactis* (**Figure 14**). It is known that the production of these nisins is controlled by quorum

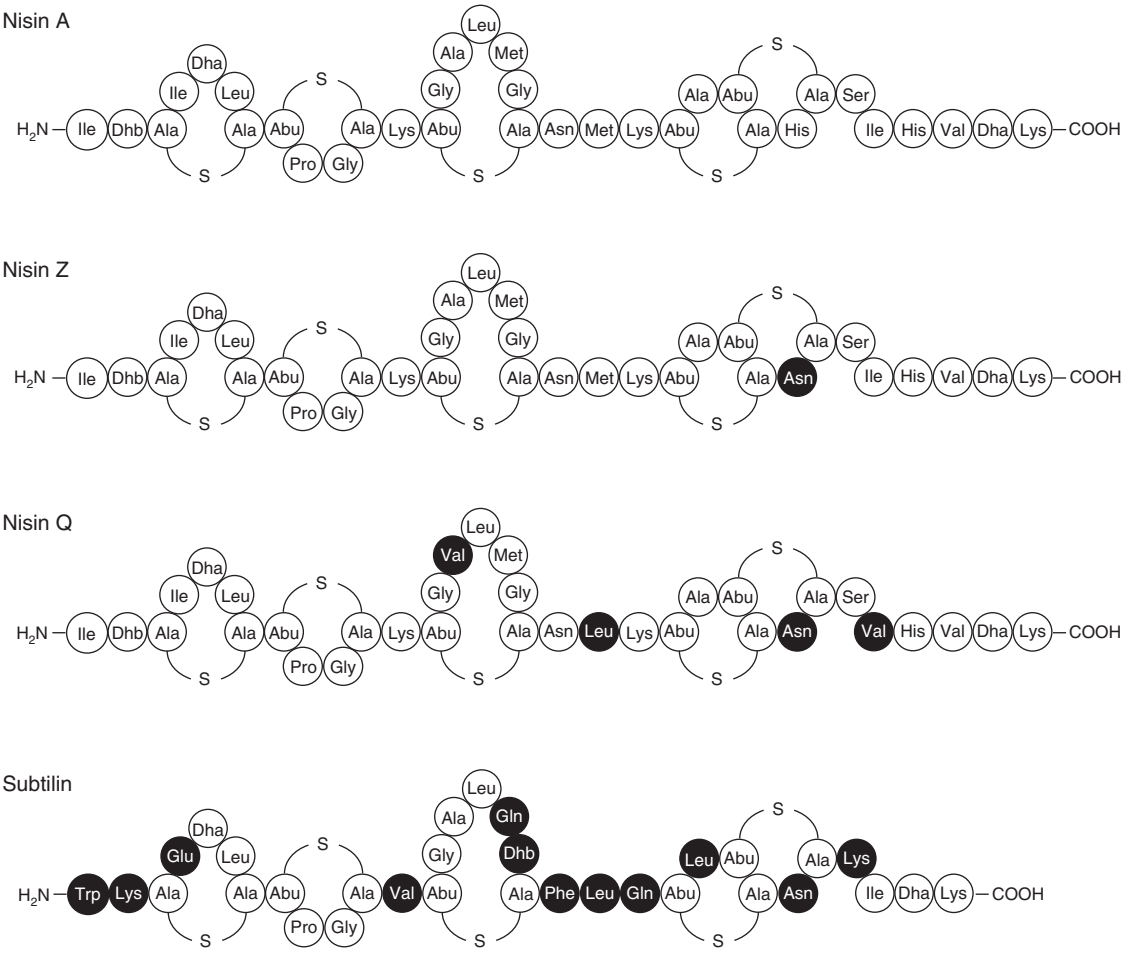


Figure 14 Structures of nisins and subtilin. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine. Filled circles are substituted amino acids from the corresponding residues of nisin A.

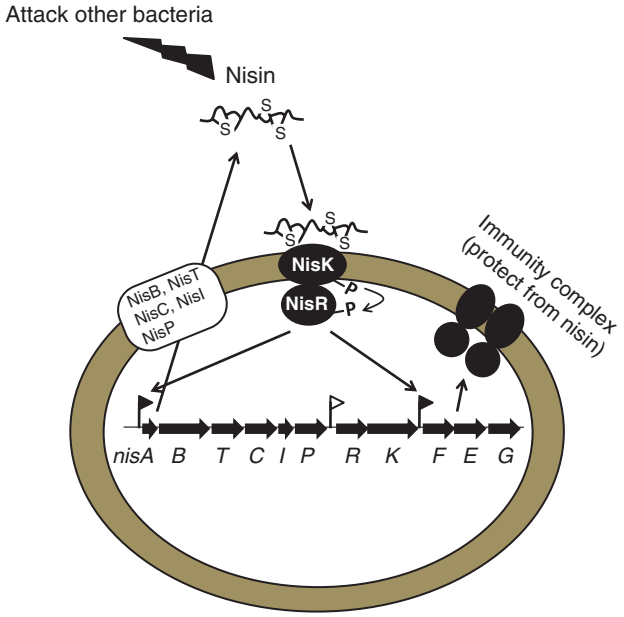


Figure 15 Quorum sensing system involved in the regulation of nisin A. After translated from *nisA*, prepropeptide of nisin is modified, processed, and secreted via NisB, NisC, NisT, and NisP. The mature nisin A binds to NisK and triggers the phosphorelay to NisR. The phosphorylated NisR induces transcriptions of two operons: *nisABTCIP* and *nisFEG* encoding immunity protein complex.

sensing.[172–174] Unlike the case of quorum sensing for class II bacteriocin biosynthesis, nisins themselves function as inducers (**Figure 15**). But like the inducer pheromones of class II bacteriocins, the signal is transduced through the two-component regulatory system encoded by *nisR* and *nisK* genes. The phosphorylated NisR induces transcriptions of two operons: *nisABTCIP* operon, encoding nisin propeptide (NisA), biosynthetic enzymes (NisB, NisC), transporter (NisT), immunity protein (NisI), and processing enzyme (NisP), and *nisFEG* operon, encoding immunity protein complex. As a consequence of this concerted control, the positive feedback loop was established to allow efficient biosynthesis concomitantly with the quorum sensing. The genetic organization was common among the three nisin variants. The three variants were cross-active with each other, suggesting that NisK sensor kinases do not discriminate but commonly recognize these variants.

The production of subtilin (**Figure 14**) in *B. subtilis* is also controlled by the same system. The gene organization of subtilin gene cluster is highly similar to that of nisins.[95,175] Also at the amino acid sequence level, NisR and NisK exhibit marked similarities to SpaR and SpaK, respectively.[176] NisK and SpaK belong to EnvZ-type histidine kinase subfamily having two transmembrane segments in the N-terminal sensor domain, whereas the histidine kinases involved in the regulation of class II bacteriocins belong to HPK_{10} having multiple transmembrane segments in the sensor domain.[177]

4.07.4.4.2 Cytolysin

Cytolysin of *E. faecalis* is also a lantibiotic and it shows cytotoxic activity against both bacterial and mammalian cells and is considered as a virulence factor. Cytolysin consists of two different peptide chains, $CylL_L$, which is the large subunit, and $CylL_S$, which is the small subunit, and both contain lanthionine.[178] $CylL_S$ functions as an autoinducer triggering the two-component regulatory system of CylR1 and CylR2, but the complex of $CylL_L$ and $CylL_S$ does not show the inducer activity (**Figure 16**).[179] The CylR1–CylR2 two-component regulatory system is unusual in that it does not seem to use a recognizable, highly conserved two-component signal transduction system consisting of a histidine kinase and a response regulator. Instead, an apparently small helix–turn–helix DNA-binding protein, CylR2, and an apparent transmembrane protein of unknown function, CylR1, work together to repress the cytolysin operon. This system has another interesting property.[180] CyL_L

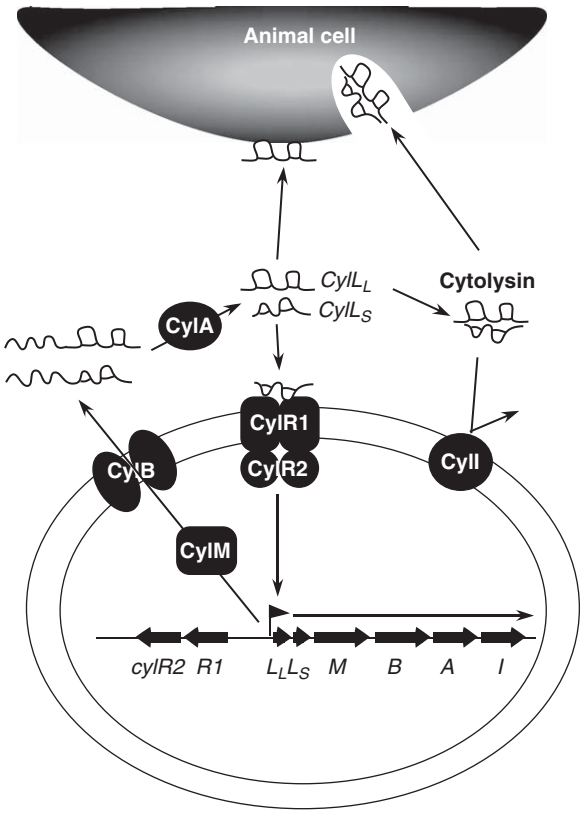


Figure 16 Regulatory mechanism of cytolysin biosynthesis. Cytolysin consists of two different peptide chains, CylL$_L$ and CylL$_S$. When animal cells approach an *Enterococcus faecalis* cell, CylL$_L$ specifically binds to the animal cells and CylL$_S$ becomes a free form, which is able to trigger the two-component regulatory system of CylR1 and CylR2. Consequently, CylR2 activates the expression of operon encoding cytolysin biosynthesis.

and CylL$_S$ usually form complex but in the presence of animal cells, CylL$_L$ is specifically bound to the animal cells and CylL$_S$ becomes a free form and consequently activates the expression of operon encoding cytolycin biosynthesis. It seems that *E. faecalis* cells use this mechanism to control the biosynthesis of cytolysin in response to the approach of target animal cells.

4.07.4.5 Cyclic Thiolactone and Lactone Peptides

It is well known that the expression of virulence factors in staphylococci is regulated by quorum sensing mediated by cyclic thiolactone peptide as an autoinducing peptide (AIP). This type of AIP had been known only in staphylococci before a cyclic lactone AIP, gelatinase biosynthesis-activating pheromone (GBAP), was found from *E. faecalis*. The components necessary for this quorum sensing are encoded in a gene cluster encoding propeptide of AIP, its biosynthetic enzyme, and two-component regulatory proteins. Recently determined genome sequences have revealed the four-component gene cluster orthologues in some low-GC Gram-positive bacteria, suggesting widespread presence of this type of cyclic peptide-mediated quorum sensing. Figure 17 shows the structures of known cyclic thiolactone and lactone AIPs.

4.07.4.5.1 Autoinducer peptides in staphylococci

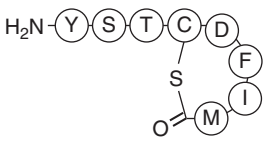
In *in vitro* culture of *Staphylococcus aureus*, the production of different exoproteins follows a quorum sensing program in which adhesins are made before hemolysins or proteases and other degradative enzymes. A thiolactone peptide functions as an AIP in this system.[97,181] The quorum sensing system is encoded by *agr* (accessory gene regulator) gene cluster, which is found not only in *S. aureus* but also in other species of this

genus.[97,181,182] The AIPs are seven- to nine-amino acid residue cyclic peptides as shown in **Figure 17**.[97,181] In all AIPs except for a few cases carrying lactone instead of thiolactone,[183] C-terminal five-amino acid residues form thiolactone ring in which the α-carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of cysteine residue, which is always located at the fifth position from C-terminus.[97,181]

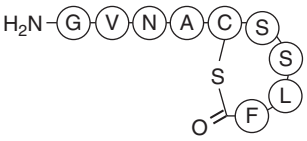
In *S. aureus*, the sequences of AIPs are diverse depending on strains and these are classified into four groups in terms of their cross-activity.[97,181,182,184] Antagonism among different AIP groups is also known: For example, AIP-I inhibits the *agr* system of strains belonging to AIP-II and AIP-III, while AIP-III inhibits the *agr* system of strains belonging to AIP-I and AIP-II.[184,185] It is also known that AIPs of *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* (**Figure 17**) show antagonistic activity against AIPs of *S. aureus*.[181] These cross-inhibition among different AIP groups may correlate with a competition at infection or colonization sites, or both, called bacterial interference.

The structure–function relationship studies with synthetic AIP analogues suggested that the ring moiety of AIPs is important for binding to the receptor and tail moieties are important for its activity.[186] Based on this knowledge, a truncate peptide that had only the ring moiety of AIP-II was designed and synthesized as a global inhibitor and was proved to show inhibitory activity against all four classes of AIPs.[186]

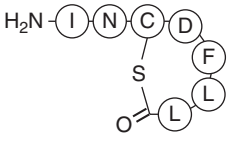
Figure 18 shows the molecular mechanism of *agr* regulatory circuit in *S. aureus*. AIP propeptide is translated from *agrD* and processed and cyclized to the mature form. Thus far, it has been demonstrated that at least processing at C-terminal side is performed by AgrB and processing at N-terminal side is performed by Type I signal peptidase.[187,188] It is in question what molecules are involved in the secretion and cyclization of AIP. The secreted AIP triggers a two-component regulatory system consisting of AgrC and AgrA. The phosphorylated AgrA induces the expression of two transcripts, RNA II encoding the operon of *agrBDCA* and RNA III encoding δ-hemolysin. In addition to the mRNA function, RNA III acts as a regulatory RNA molecule that controls the translation of a series of target genes called virulon.[182] It is known that RNA III acts reciprocally upregulating transcription of most of the extracellular protein genes and downregulating transcription of many surface protein genes. As the translational control, RNA III is known to anneal to the transcriptional start region of *hla* encoding β-hemolysin, causing a conformational change that unmasks the ribosomal binding site and



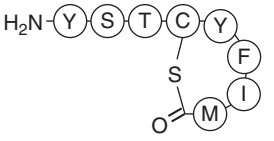
Staphylococcus aureus AIP-I



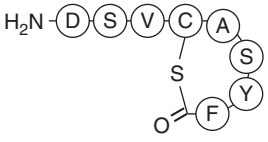
Staphylococcus aureus AIP-II



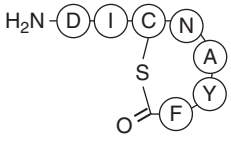
Staphylococcus aureus AIP-III



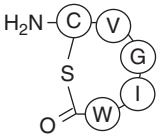
Staphylococcus aureus AIP-IV



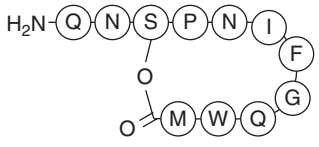
Staphylococcus epidermidis AIP



Staphylococcus lugdunensis AIP



Lactobacillus plantarum LamD558



Enterococcus faecalis GBAP

Figure 17 Structures of cyclic AIPs.

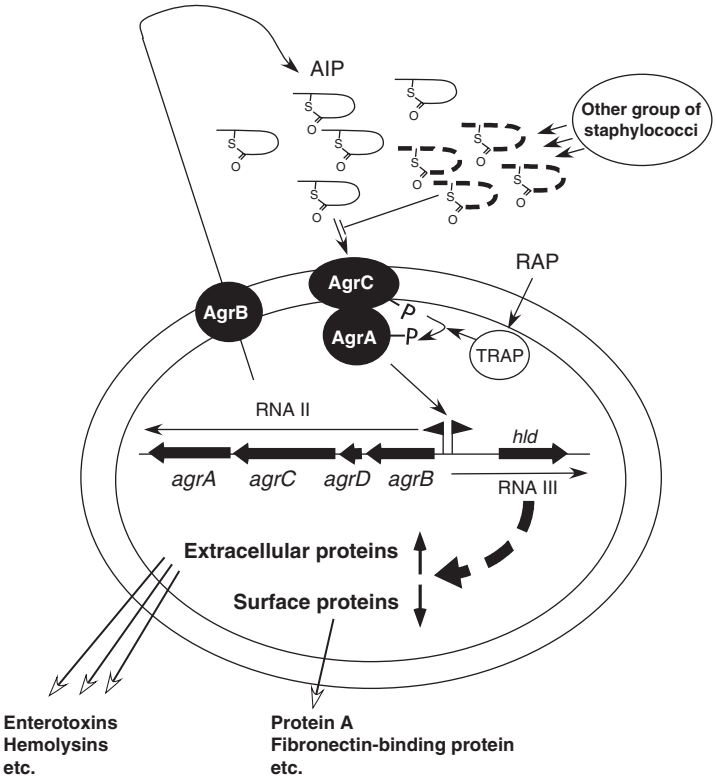


Figure 18 *agr* quorum sensing system in *Staphylococcus aureus*. AIP propeptide is translated from *agrD* and processed and cyclized to the mature form via AgrB and some other processing enzyme(s). The secreted AIP binds to AgrC and triggers the phosphorelay to AgrA. The phosphorylated AgrA induces the transcription of RNA II (*agrBDCA*) and RNA III encoding δ-hemolysin. In addition to the mRNA function, RNA III reciprocally upregulates transcription of most of the extracellular protein genes including α-hemolysin and downregulates transcription of many surface protein genes including protein A. RAP also activates the AgrC–AgrA two-component regulatory system via TRAP. Cross-inhibition by other group AIPs is also known and is recognized as bacterial interference.

upregulates its translation. It is also known that RNA III downregulates *spa* encoding protein A and a pleiotropic transcription factor *rot*, which is a repressor of toxins, by binding to their mRNAs.

RNA III-activating protein (RAP) activates the AgrC–AgrA two-component regulatory system via TRAP, which is a target of RAP.[189] It has been demonstrated that RNA III-inhibiting peptide (RIP), which is a heptapeptide originally isolated from culture supernatant of *Staphylococcus xylosus*, efficiently inhibits RNA III transcription by targeting TRAP.[189,190] It was also demonstrated that RIP prevents biofilm formation both *in vitro* and *in vivo*.[191] Furthermore, a recent paper has demonstrated that RIP injected systemically into rats has strong activity in preventing methicillin-resistant *S. aureus* graft infections, suggesting that RIP is useful as a therapeutic agent.[192]

4.07.4.5.2 Gelatinase biosynthesis-activating pheromone in E. faecalis

Gelatinase is one of the virulence factors in *E. faecalis*. The production of gelatinase is active only in late-log to early-stationary phase. Addition of conditioned medium shifts the onset of the production to mid-log phase, suggesting the presence of an induction factor. Nakayama *et al.*[193] purified this induction factor and identified it as an 11-residue cyclic lactone peptide named GBAP. This structure was confirmed by chemical synthesis.[194] As shown in **Figure 17**, GBAP consists of an N-terminal two-amino-acid tail moiety and a nine-amino acid ring moiety. The ring moiety is constructed by ester linkage instead of thioester linkage in staphylococcal AIPs. The ester linkage is constructed between C-terminal α-carboxyl group and hydroxyl group of the third serine

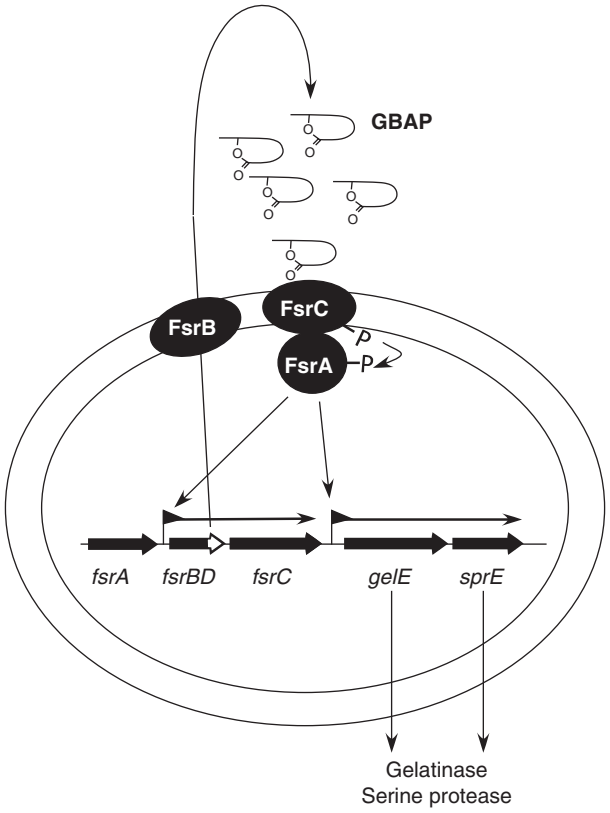


Figure 19 *fsr* quorum sensing system in *Enterococcus faecalis*. After translation, FsrD is processed, cyclized, and secreted via FsrB and some other enzymes and transporters. Eventually, mature form of GBAP is secreted and accumulated outside the cells. GBAP binds to FsrC and triggers the phosphorelay to FsrA. The phosphorylated FsrA induces the transcription of *fsrBDC* and *gelE–sprE* encoding two pathogenicity-related extracellular proteases.

residue. A study indicated that two aromatic residues in the ring region, Phe-7 and Trp-10, are important for the induction activity (K. Nishiguchi and J. Nakayama *et al.*, unpublished data).

As shown in **Figure 19**, the GBAP-mediated quorum sensing system is encoded by *fsr* gene cluster.[193,195,196] The nucleotide sequence of *fsr* gene cluster identified three open reading frames designated *fsrA*, *fsrB*, and *fsrC*; *fsrA* and *fsrC* encode the two-component regulatory proteins and *fsrB* encodes a protein showing high similarity to staphylococcal AgrB.[195] However, small open reading frame encoding AIP propeptide, corresponding to AgrD of staphylococci, was missing in the predicted open reading frame organization of *fsr* gene cluster. Amino acid sequence alignment between AgrB and FsrB indicates about 50-residue C-terminal extension of FsrB, which is not present in AgrB but shows some similarities to AgrD. The 11-amino acid sequence of GBAP was found in the C-terminal region of FsrB. Recently, it was demonstrated that the C-terminal region is translated independent of FsrB even though it was encoded in-frame of FsrB.[197] Thus, the newly found small open reading frame, designated *fsrD*, encodes GBAP propeptide as staphylococcal *agrD* encodes the AIP propeptide.

The presence of two-component regulatory genes in the *fsr* gene cluster suggested that GBAP signal is transduced through the FsrC–FsrA two-component regulatory system. The two-component regulatory system controls the transcription of an operon encoding a serine protease in addition to gelatinase, which is located immediately downstream of *fsr* gene cluster.[193,195,196] These two extracellular proteases appear to function synergistically to efficiently digest a wide range of protein substrates.

Gelatinase-negative *E. faecalis* is frequently isolated from nature including patients with enterococcal infection and also healthy volunteers. Nakayama *et al.*[198] investigated the presence of *fsr* gene cluster and *gelE–sprE* gene cluster in gelatinase-negative urine isolates. Most of the gelatinase-negative isolates possess intact *gelE–sprE* gene cluster but carry a 23.9-kb deletion covering the whole region of *fsrA*, *fsrB*, and *fsrD* and a

5′-end part of *fsrC*. Seventy-nine percent of gelatinase-negative isolates tested had the 23.9-kb deletion. In addition to urine isolates, the 23.9-kb-like deletion was also a main determinant of gelatinase-negative phenotype in endocarditis, blood, and healthy stool isolates.[199] These results imply that gelatinase-negative phenotype is mainly determined not by lack of gelatinase gene but by lack of quorum sensing gene. A known fungal secondary metabolite, ambuic acid, has been also found to be an inhibitor of GABA biosynthesis (Nakayama *et al.*)[200]

Some recent studies have indicated the involvement of the *fsr* system in virulence, which includes not only the effect of the induced extracellular proteases but also the effect of expression of other genes controlled by *fsr* system.[195,201–203] It was also demonstrated that *fsr* system is involved in biofilm formation of *E. faecalis* cells.[204,205]

Inhibitors targeting bacterial quorum sensing offer a novel means of treating virulent and/or antibiotic-resistant infections. This would be the case of enterococci. Nakayama *et al.*[206] performed screening of targeting natural compounds for the inhibitor of *fsr* quorum sensing and found that a known peptide antibiotic, siamycin, produced by *Streptomyces* sp., efficiently inhibits *fsr* quorum sensing at sublethal concentrations of around 0.1–1 $\mu mol\,l^{-1}$. Siamycin appeared to inhibit signal transduction of the FsrC–FsrA two-component regulatory system; however, the mode of action was not in a competitive manner, suggesting that siamycin is not an antagonist of FsrC but modulates the FsrC–FsrA two-component regulatory system.

4.07.4.5.3 Cyclic AIPs in other Gram-positive bacteria

Recent bacterial genome sequencing studies have revealed a number of *agr*-like loci in the genomes of low-GC Gram-positive bacteria other than staphylococci and *E. faecalis*, for example, *Clostridium acetobutylicum*, *Clostridium thermocellum*, *Listeria innocua*, *Listeria monocytogenes*, *Lactobacillus plantarum*, *Bacillus cereus*, and *Roseburia inulinivorans*. The presence of gene cassette of *agrB* and *agrD* homologues suggests the potential to produce cyclic peptide, and the presence of two-component regulatory gene orthologue suggests the potential to sense the produced cyclic peptide autoinducer. In the case of *B. cereus*, the *agr*-like gene cluster is found only in a highly pathogenic strain, G9241. It is interesting that the gene cluster is located adjacent to a putative lantibiotic gene cluster, suggesting that the encoded *agr*-like quorum sensing system might regulate the production of lantibiotics in this highly pathogenic *B. cereus* strain.

The *agr*-like gene cluster of *L. plantarum* was termed *lam* (*L. plantarum agr*-like module). *lam* consists of four genes: *lamB* encoding biosynthetic enzyme involved in the biosynthesis of cyclic AIP like *agrB*, *lamD* encoding propeptide of cyclic AIP like *agrD*, *lamC* encoding histidine kinase like *agrC*, and *lamA* encoding response regulator like *agrA*. Sturme *et al.*[207] constructed *lamBD*-overexpressing *L. plantarum* and analyzed its culture supernatant by liquid chromatography–mass spectrometry. As a result, three peptides, named LamD558, LamD558B, and LamD677, were found to be produced from *lamBD*. The structure of LamD558, which was the main product, was determined to be a five-amino acid residue thiolactone peptide (**Figure 17**).[207] Similar to staphylococcal AIPs, LamD558 has a ring structure consisting of five amino acids in which the thiolactone linkage is formed between C-terminal α-carboxyl group and sulfhydryl group of N-terminal cysteine residue; however, it lacks the two- or three-amino acid tail moiety found in staphylococcal AIPs and *E. faecalis* GBAP.[181,193] Microarray analysis of *lamA*-knockout strain suggested a number of genes regulated by *lam* system, including a subset of putative capsular polysaccharide biosynthetic genes.[207] It should be noted that the *lamA*-knockout mutant displayed less adherent phenotype compared to wild type, suggesting correlation between the quorum sensing-regulated capsular polysaccharide biosynthesis and biofilm formation.

In *L. monocytogenes*, a knockout mutant of *agrA*-like gene was created.[208] The production of several secreted proteins was modified in the *agrA* mutant, indicating that the *agr*-like locus influenced protein secretion. The inactivation of *agrA* did not affect the ability of the pathogen to invade and multiply in mammalian cells *in vitro*. However, the virulence of the *agrA* mutant was attenuated in the mouse (a 10-fold increase in the 50% lethal dose by the intravenous route), demonstrating a role for the *agr*-like locus in the virulence of *L. monocytogenes*. Also, a recent paper reported that in-frame deletion of *agrA* and *agrD* resulted in an altered adherence and biofilm formation on abiotic surfaces, suggesting the involvement of the *agr* system of *L. monocytogenes* during the early stages of biofilm formation.[209] The production of thiolactone peptide was also confirmed in the culture supernatant of *L. innocua* and *L. monocytogenes* (N. Sujaku, J. Nakayama *et al.*, unpublished data).

4.07.4.6 Isoprenylated Peptide as a Competence Pheromone of *B. subtilis*

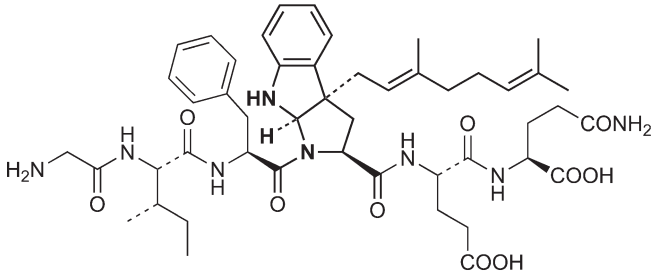
Thus far in bacteria, prenylation to peptide signal molecule is known only in the competent pheromone (ComX) of *B. subtilis*, although prenylated peptides are well known in fungal mating hormones. The structure of prenylated moiety is quite different from that of eukaryotes (**Figure 20**).[210]

As described in Section 4.07.4.2.2 (**Figure 10**), development of genetic competence is controlled by two quorum sensing pathways: one is the Phr–Rap signaling pathway and the other is the *com* signaling pathway. The former is triggered by the small oligopeptide imported via oligopeptide permease. The latter consists of the ComP–ComA two-component regulatory system that is triggered by the ComX pheromone. The phosphorylated ComA appears to directly activate transcriptions of more than 20 genes.[131] *srfA* operon including *comS* is a well-studied target regulated by ComA.[212,213] Once produced, ComS prevents the degradation of the competence transcription factor ComK, thereby affecting expression of more than 100 genes, including those encoding the transformation machinery.[131,214,215]

The competence pheromone ComX was purified from a culture medium of *B. subtilis* growing to high cell density.[216] As a result, two peptides having pheromone activity were isolated. The amino acid sequence of these two peptides suggested that they are 9- and 10-amino acid residue peptides that differed only in their N-terminal residue. These N-terminal amino acid sequences were found in the C-terminus of a 55-codon open reading frame, designated *comX*. Masses of these two peptide pheromones were 206 Da greater than those expected based on the sequence of *comX*, suggesting some posttranslational modification on these two peptides. Besides *comX*, *comQ*, the gene immediately upstream of *comX*, is required for the production of the pheromone. ComQ shows an amino acid sequence similarity to isoprenyl diphosphate synthases found in *Methanobacterium thermoautotrophicum* and some other bacteria, suggesting that ComX is isoprenylated.

The comQXP loci of a set of natural *Bacillus* isolates have been sequenced and shown to possess a striking polymorphism that determines specific patterns of both activation and inhibition of the quorum sensing response.[217] Genetic and biochemical evidence demonstrate that all the ComX variants are isoprenylated by the posttranslational modification of a conserved tryptophan residue and that the modifications on the ComX peptide backbones vary in mass among the various phenotypes.

The exact structure of modification had been unclear for a long time. Okada *et al.*[210] recently reported the precise structure of competence pheromone of *B. subtilis* RO-E-2, which contains a geranylated tryptophan with an unusual tricyclic architecture. The structure reported was somewhat surprising, involving an apparent cyclization reaction followed by transfer of the geranyl moiety to the modified tryptophan. This finding has an intriguing parallel in the discovery of the prenylated peptide mating pheromones of yeast variants, which were shown to contain an isoprenoid modification of a cysteine residue over two decades ago.[211] Okada *et al.*[218] also synthesized various ComX(RO-E-2) analogues and examined their biological activities to investigate structure–activity relationships. Surprisingly, the minimal active unit was the tripeptide [3-5]ComX(RO-E-2) and all residues except the modified tryptophan residue were replaceable by alanine without total loss of activity.



Gly-Ile-Phe-**Trp (modified)**-Glu-Gln

Figure 20 Structure of ComX pheromone. Bold part shows modified tryptophan residue with a geranyl group in the $ComX_{RO\text{-}E\text{-}2}$.

4.07.4.7 Autoinducer-2

Autoinducer-2 is suggested to be a global communication signal because *luxS* presumably encoding an enzyme involved in the biosynthesis of AI-2 is widespread among Gram-positive and Gram-negative bacteria.[219] The chemical structure of AI-2 of *V. harveyi* was elucidated to be furanosyl borate diester,[220] as described in Section 4.07.5.3.1. Although the existence of *luxS* is found in the genomes of a variety of bacteria including a number of Gram-positive species, the production and chemical structure of AI-2 have not been confirmed in many Gram-positive species. In most cases, the production of AI-2 was examined by using a reporter strain of *V. harveyi* BB170.[221] Indeed, AI-2 activity has been confirmed in some species of streptococci including *S. pneumoniae*[222] and oral streptococci,[223] lactobacilli,[224] clostridia,[225] and listeria.[226]

In *S. mutans*, it has been reported that AI-2 is involved in the regulation of bacteriocin production and biofilm formation.[227] In *Streptococcus anginosus*[228] and *L. monocytogenes*,[229] it was also reported that AI-2 is involved in the regulation of biofilm formation, while virulence of *S. pneumoniae*[222] and *Streptococcus pyogenes*[229,230] and toxin production in *Clostridium perfringens*[225] were reported to be regulated by AI-2-mediated quorum sensing. It should also be noted that the biofilm defect of the *S. mutans luxS* mutant was complemented by other oral strains of *S. gordonii*, *Streptococcus sobrinus*, *S. anginosus*, *Porphyromonas gingivalis*, and *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*.[223] Furthermore, while *luxS* is dispensable for monospecies biofilm formation in *P. gingivalis* and *S. gordonii*, its expression is required in one of the two species for mixed biofilm formation.[231] These results suggested AI-2-mediated interspecies communication in oral biofilm.

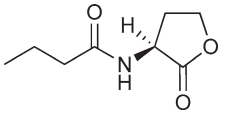
4.07.5 Signaling Molecules in Gram-Negative Bacteria

4.07.5.1 Introduction

For cell–cell communication in Gram-negative bacteria, many host-associated bacteria use chemical signals to monitor the population density of their own species and to control expression of specific genes in response to their population density. This type of bacterial cell–cell communication and gene regulation was initially found in the luminescence bacterium *V. fischeri* in the early 1970s and was termed quorum sensing in the 1990s.[232] In *V. fischeri* and also *V. harveyi*, another luminescence bacterium, the signal compound, named autoinducer, is synthesized inside the cell and diffuses out of the cell. Bacteria accumulate the autoinducer and can recognize their population density as the concentration of the autoinducer. The autoinducers of quorum sensing in Gram-negative bacteria were identified as a series of *N*-acyl-L-homoserine lactones (AHLs) and regarded as species-specific molecules in the beginning. But, until now, it has been found that the same kind of AHL is synthesized by several kinds of bacteria to regulate their own different quorum sensing systems and some bacteria produce several kinds of AHLs to regulate their own same or different gene expressions. Quorum sensing system is a cell population density-dependent system. But every study about mutants of quorum sensing-related genes showed that quorum sensing system did not affect the bacterial growth itself. AHLs have an effect not only on the gene expression of bacteria but also on the modulation of immune system of the host cell.[233,234] Moreover, other autoinducers, AI-2 and other compounds, and the related systems were found.[235,236] In this section, the structures and functions of autoinducers in Gram-negative bacteria are described.

4.07.5.2 *N*-Acylhomoserine Lactones as Autoinducers of Quorum Sensing

It has been reported that several dozen species of bacteria produce AHLs.[237] Until now, AHLs containing 4–14 carbon acyl chains with or without modification have been identified. Some of the reported AHLs and related regulatory proteins are shown in **Figure 21**.[238–245] The general concept of quorum sensing system is illustrated in **Figure 22**. AHL is synthesized in the cell by the AHL synthase called the I-protein family, such as LuxI of *V. fischeri*. AHL diffuses out of the cell through the membrane by passive diffusion or is exported by efflux pumps and also accumulated by the cell. When the concentration of AHL in the cell exceeds the threshold level, AHL binds to receptor protein such as LuxR protein (R-protein) family. Then, AHL–R-protein complex induces the target gene expression. R-protein mainly acts as a positive regulator for the gene expression, but in some cases, acts as a negative regulator. In general, the diffused AHL is at nanomolar or lower concentrations. As the concentration

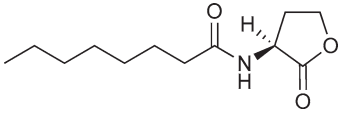


***N*-butanoyl-L-HSL**
(C4-HSL)

Pseudomonas aeruginosa
RhlI/RhlR

Aeromonas salmonicida
AsaI/AsaR

Serratia liquifaciens
SwrI/SwrR

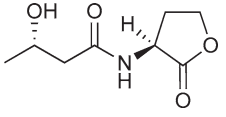


***N*-octanoyl-L-HSL**
(C8-HSL)

Vibrio fischeri
AinS/AinR

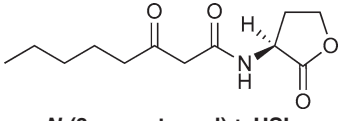
Burkholderia cepacia
CepI/CepR

Ralstonia solanacearum
SolI/SolR



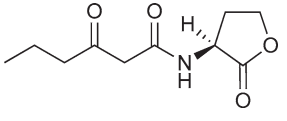
***N*-(3-β-hydroxy-butanoyl)-L-HSL**
(3-β-OH-C4-HSL)

Vibrio harveyi
LuxM/LuxN–LuxO–LuxR



***N*-(3-oxooctanoyl)-L-HSL**
(3-oxo-C8-HSL)

Agrobacterium tumefaciens
TraI/TraR

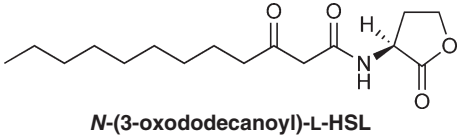


***N*-(3-oxohexanoyl)-L-HSL**
(3-oxo-C6-HSL)

Vibrio fischeri
LuxI/LuxR

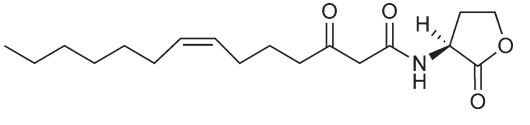
Erwinia carotovora
ExpI/ExpoR
CarI/CarR

Enterobacter agglomerans
EagI/EagR



***N*-(3-oxododecanoyl)-L-HSL**
(3-oxo-C12-HSL)

Pseudomonas aeruginosa
LasI/LasR



***N*-(3*R*-hydroxy-7-*cis*-tetradecanoyl)-L-HSL**
(3-*R*-hydroxy-7-*cis*-C14-HSL)

Rhizobium leguminosarum
RhiI/RhiR

Figure 21 The structures of AHLs isolated from Gram-negative bacteria and their regulatory proteins.

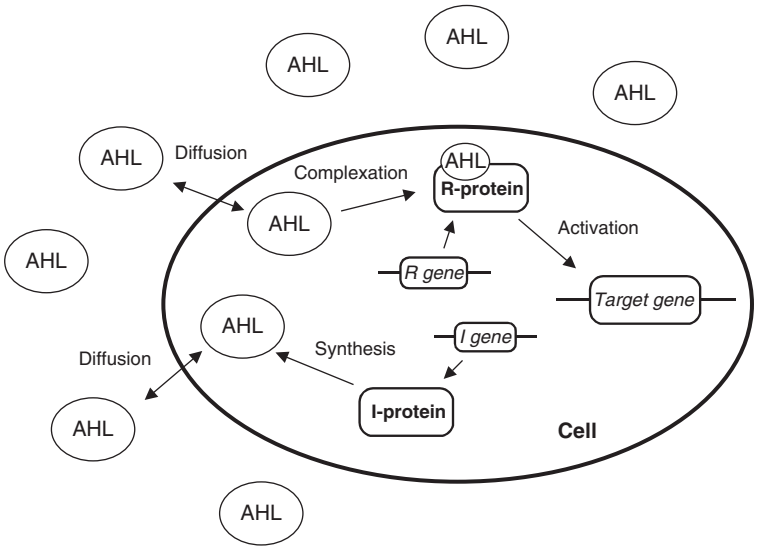


Figure 22 AHL-mediated quorum sensing in Gram-negative bacteria. I-protein encoded by the *I gene* is an AHL synthase and R-protein encoded by the *R gene* is a receptor of AHL. The AHL–R-protein complex activates the expression of target genes.

of AHL in the cell reaches the threshold level at the late logarithmic and early stationary phases of growth, the target gene expression regulated by quorum sensing usually occurs during or after the late logarithmic and early stationary phase of growth.

Natural degradation of AHLs was first reported as hydrolysis of the lactone ring by the lactonase AiiA identified in *Bacillus* species (**Figure 23(a)**).[246,247] Another AHL-degrading enzyme was also isolated from *Ralstonia* species as AiiD, which is an acylase and hydrolyzed the amide bond of AHL to remove the acyl chain from the lactone ring (**Figure 23(b)**).[248] These AHL-degrading enzymes have been isolated from a wide range of bacterial species including AHL-producing Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*.[249] These AHL-degrading enzymes may act as one of the control factors of quorum sensing intra- and interspecies in the several environments.

4.07.5.2.1 Vibrio fischeri *and* Vibrio harveyi

Vibrio fischeri is a symbiotic bacterium isolated from a light organ of a squid (*Euprymna scolopes*).[250] In *V. fischeri*, several kinds of quorum sensing system were identified.[251–258] At first, *lux* system was found. The *lux* system regulates the luciferase operon and light production. LuxI was isolated as an AHL synthase and directs the synthesis of *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL). A biosynthetic scheme of 3-oxo-C6-HSL in *V. fischeri* is shown in **Figure 24**.[259,260] LuxI binds an acylated ACP and *S*-adenosylmethionine. The acyl chain is transferred from ACP to LuxI and forms an amide bond with *S*-adenosylmethionine. Then, AHL is

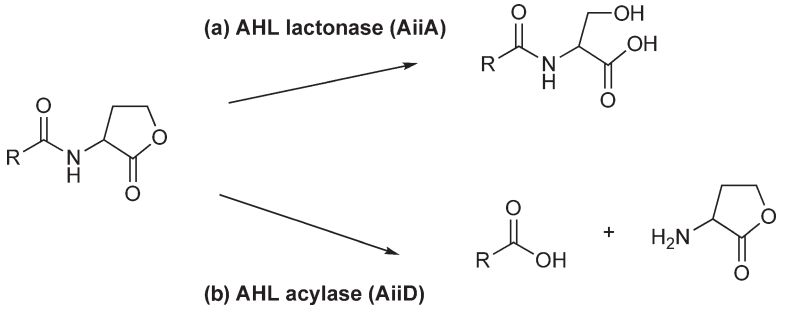


Figure 23 Degradation of AHL by (a) AHL lactonase (AiiA) and (b) AHL acylase (AiiD).

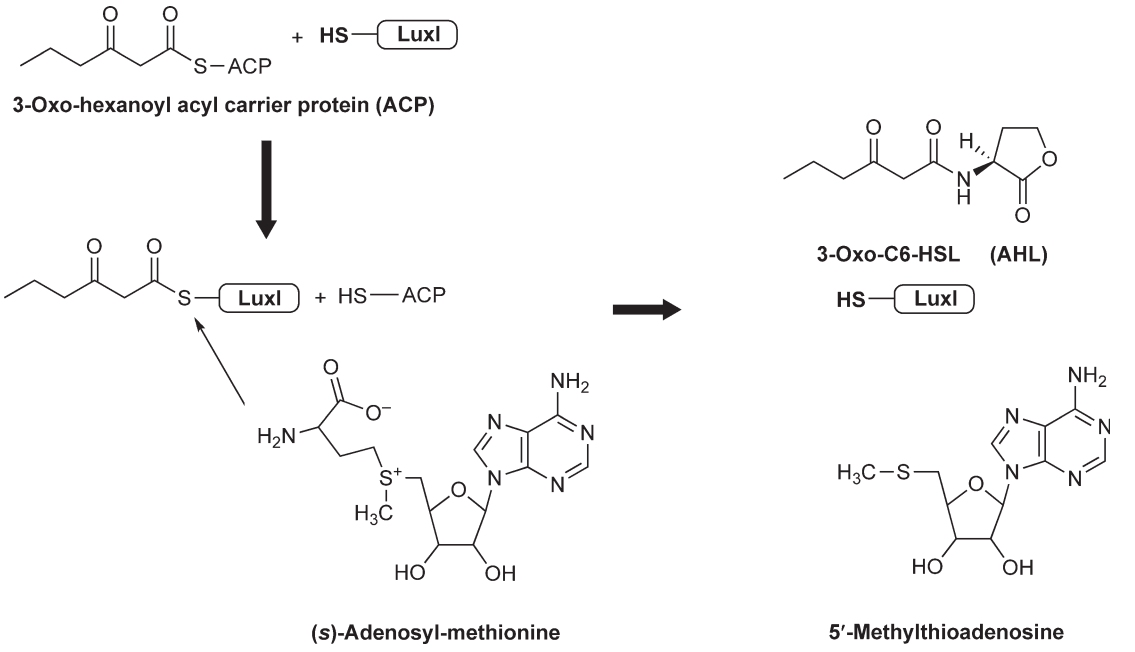


Figure 24 The synthetic pathway of AHL (3-oxo-C6-HSL in this case).

formed with the formation of lactone ring and release of 5′-methylthioadenosine. LuxI consists of 193 amino acids including an active site for amide bond formation and a binding site of acylated-ACP. LuxR was isolated as a transcriptional activator of the luciferase operon. 3-Oxo-C6-HSL synthesized by LuxI binds to LuxR and the 3-oxo-C6-HSL–LuxR complex binds to DNA at the region named *lux* box and activates the transcription of the luminescence operon *luxICDABEG*. LuxR consists of 250 amino acids and has two domains. The N-terminal region is an AHL-binding site and the C-terminal region is a DNA-binding site with a helix–turn–helix motif. The Lux system also has effects on colonization factors.

AinS was also isolated as another AHL synthase in *V. fischeri*. AinS directs the synthesis of C8-HSL. While the Lux system regulates the bioluminescence at high cell density, the Ain system effects at lower cell density. These two quorum sensing systems are connected and regulate sequential induction (**Figure 25**). At low cell density, LuxO represses LitR, which is a positive regulator of the expression of LuxR. During the cell growth, even at the lower cell density, C8-HSL produced by AinS binds to AinR, which is a homologue of LuxN, and C8-HSL–AinR complex inactivates LuxO. Increased expression of LitR results in increased expression of LuxR. C8-HSL also binds to LuxR directly and slightly activates bioluminescence. At high cell density, Lux system mainly regulates bioluminescence as described previously.

The third system of quorum sensing in *V. fischeri* using AI-2 was also reported. AI-2 system in *V. fischeri* is described in Section 4.07.4.3.1.

Vibrio harveyi is a marine bioluminescent bacterium and regulates bioluminescence, production of siderophore, polysaccharide and metalloprotease, and type III secretion through quorum sensing.[261–265] 3-β-OH-C4-HSL is synthesized by LuxM. LuxN is identified as a receptor of 3-β-OH-C4-HSL. Without 3-β-OH-C4-HSL, that is, at low cell density, through LuxU and LuxO, LuxN leads to inactivation of LuxR, which is required for transcription of genes for bioluminescence, production of siderophore, polysaccharide and metalloprotease, and type III secretion. At high cell density, 3-β-OH-C4-HSL binds to LuxN and such 3-β-OH-C4-HSL–LuxN complex leads to inactivation of LuxO through LuxU and then LuxR is activated to promote transcription of genes (**Figure 26**). AI-2-mediated quorum sensing system was first found in *V. harveyi*. AI-2 quorum sensing system in *V. harveyi* is described in Section 4.07.5.3.1.

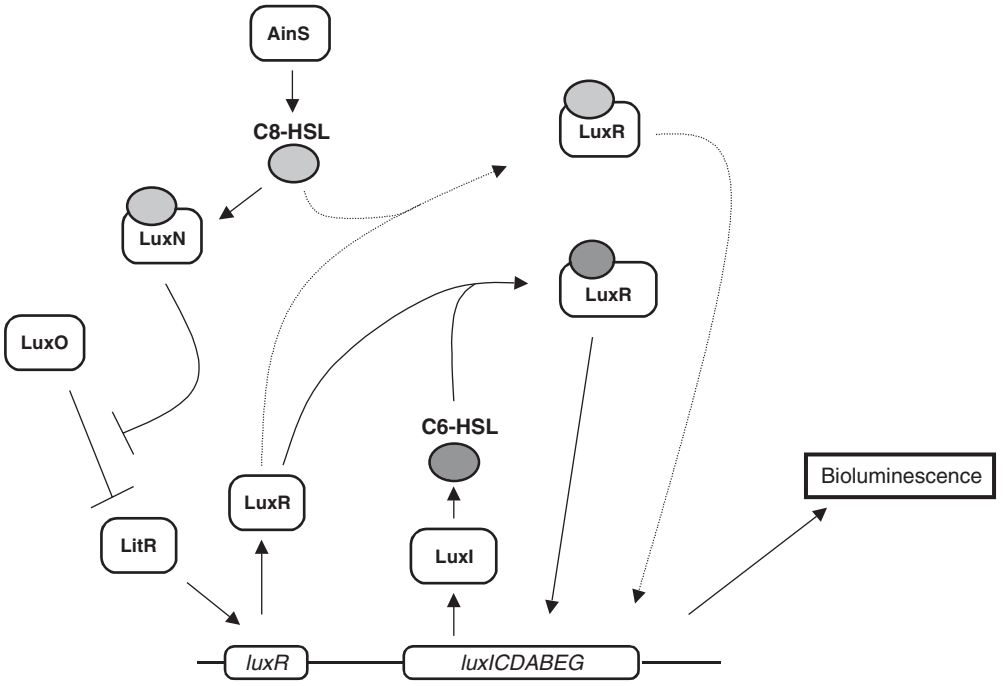


Figure 25 The relation of Lux and Ain quorum sensing systems in *Vibrio fischeri* for bioluminescence.

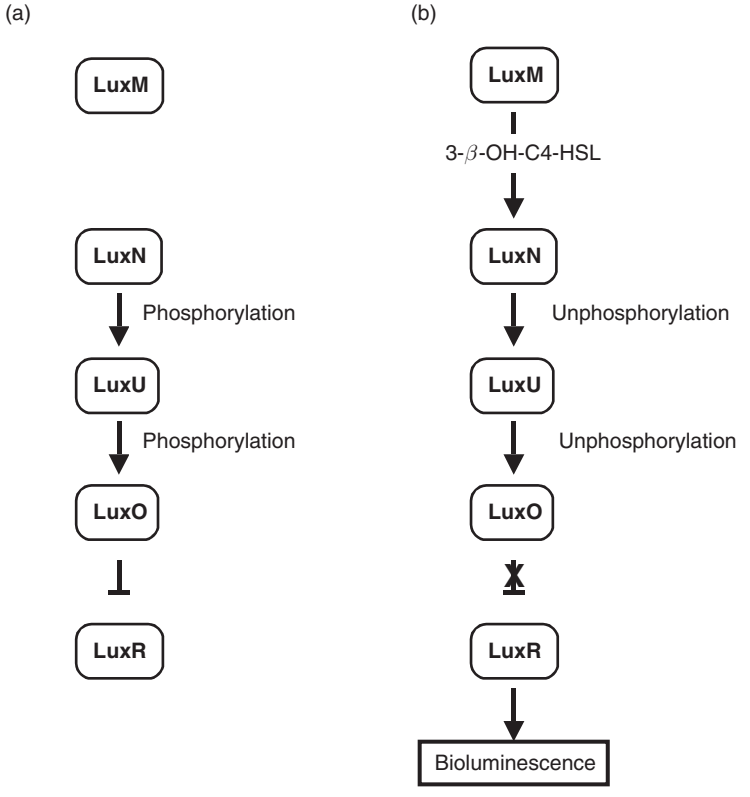


Figure 26 Regulation for bioluminescence in *Vibrio harveyi* (a) at low cell density and (b) at high cell density.

4.07.5.2.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen and infects especially immunocompromised patients.[266–270] Many clinical isolates of *P. aeruginosa* are identified not only from the inside of the body but also from the biofilm formed on indwelling catheter or medical devices. Quorum sensing system of *P. aeruginosa* has been extensively studied and several kinds of systems have been reported.[271–292]

The first found system is the Las quorum sensing system, which consists of LasI and LasR, the homologues of LuxI and LuxR of *V. fischeri*. LasI is a synthase of 3-oxo-C12, which binds to LasR. 3-Oxo-C12–LasR complex regulates the expression of many virulence genes such as *lasB*, *lasA*, *aprA*, and *toxA*, and also *lasI* (**Figure 27**). On the contrary, 3-oxo-C12–LasR complex also regulates the expression of *rsaL*, which inhibits the expression of *lasI*.

The second system consists of RhlI and RhlR, which are also the homologues of LuxI and LuxR. The synthase RhlI directs the synthesis of C4-HSL. C4-HSL–RhlR complex regulates expression of *rhlAB*, *aprA*, the stationary-phase sigma factor RpoS, and the production of pyocyanin and cyanide (**Figure 27**).

In general, it is thought that AHLs freely diffuse out of the cell. In *P. aeruginosa*, C4-HSL diffuses rapidly across the cell membranes. But it was discovered that 3-oxo-C12-HSL was actively pumped out by the MexAB-oprM pump, which is one of the multidrug efflux pumps in *P. aeruginosa* to export a wide range of antimicrobial agents.[293–297] According to the difference in the length of the acyl chain, 3-oxo-C12-HSL is more hydrophobic and hard to pass through the cytoplasmic membrane than C-4-HSL. Therefore, the active export of 3-oxo-C12-HSL by the pump is needed.

The relationship between these two quorum sensing systems, Las and Rhl, is very interesting. C4-HSL does not activate LasR, but 3-oxo-C12–LasR complex showed a low level activation of RhlR. The Las system

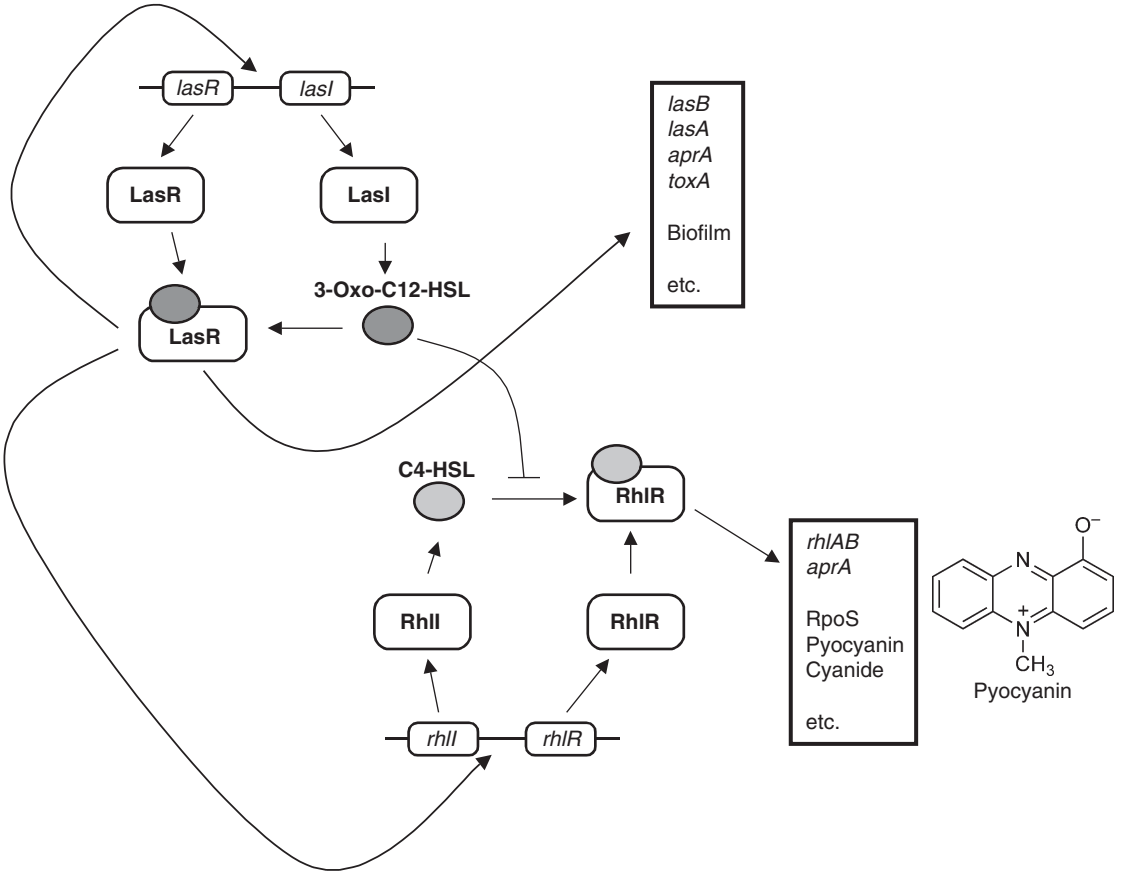


Figure 27 Quorum sensing in *Pseudomonas aeruginosa*.

positively regulates the expression of both RhlI and RhlR. Moreover, 3-oxo-C12 acts as an antagonist of C4-HSL, because 3-oxo-C12 competes with C4-HSL for binding to RhlR. A *lasR* mutant of *P. aeruginosa* showed decreased virulence compared with the parent strain. A *lasI* mutant, an *rhlI* mutant, and a *lasI rhlI* double mutant also showed decreased virulence, and a double mutation was the most effective in the reduction of the virulence factor.

P. aeruginosa produces not only 3-oxo-C12-HSL and C4-HSL but also 3-oxo-C10-HSL, 3-oxo-C8-HSL, and 3-oxo-C6-HSL as minor products. As two genes encoding LuxR type proteins with high homology have been identified in *P. aeruginosa*, these minor AHLs may activate such LuxR-type proteins. However, the function of such minor AHLs in *P. aeruginosa* is unclear.

The third system is also related to both Las and Rhl systems, but not to the AHL-mediated one. The signal molecule is 2-heptyl-3-hydroxy-4-quinolone (PQS). PQS quorum sensing system is described in Section 4.07.5.3.2.

4.07.5.2.3 Erwinia *species*

Erwinia species, *Erwinia carotovora* subspecies *carotovora* (*Ecc*), *Erwinia carotovora* subspecies *atroseptica* (*Eca*), *Erwinia chrysanthemi* (*Echr*), and *Erwinia amylovora* (*Ea*), are phytopathogens and regulate the production of carbapenem antibiotic and exoenzymes, such as pectate lyases, cellulase, and proteases, through quorum sensing.[298–304] Each strain normally synthesizes one or two major AHLs and also minor AHLs. *Ea* strain produces 3-OH-C6-HSL or 3-oxo-C6-HSL by the synthase LuxI homologue EamI. In the case of *Echr* strain, ExpI directs the synthesis of 3-oxo-C6-HSL and C6-HSL. C10-HSL is also synthesized by an uncharacterized LuxI homologue in *Echr* strain. *Ecc* and *Eca* strains are classified into two groups depending on the kind of AHLs produced. One group of strains synthesize mainly 3-oxo-C8-HSL and also smaller amounts of 3-oxo-C6-HSL. On the other hand, another group of strains produce 3-oxo-C6-HSL with little or no 3-oxo-C8-HSL. One AHL synthase, which is a LuxI homologue, produces all kinds of AHLs in these *Ecc* and *Eca* strains. The LuxI homologues identified from each strain are different from strain to strain, such as CarI, AhlI, and HslI.

Ecc strains produce the β-lactam antibiotic, carbapenem, through quorum sensing. 3-Oxo-C6 produced by CarI binds to CarR, which is a LuxR homologue, and such 3-oxo-C6–CarR complex activates the transcription of the *carA-H* operon to produce carbapenem. The second LuxR homologue in *Ecc* strain was identified and named VirR or ExpR. 3-Oxo-C6–VirR or 3-oxo-C6–ExpR complex activates the production of virulence factors, such as pectate lyases, cellulases, and proteases. There is a big difference between the action mechanism of CarR and that of VirR or ExpR. The deletion mutants of the AHL synthase in *Ecc* strains reduce the production of carbapenem and virulence factors. CarR mutants also show the reduction of carbapenem production. But VirR or ExpR mutants show no effect on the production of virulence factors. These phenomena reveal that CarR acts as a transcriptional activator of the production of carbapenem like other luxR homologues, but VirR or ExpR acts as a repressor of the production of virulence factors. This repression by VirR or ExpR is reduced by complexation with 3-oxo-C6. Moreover, it was revealed that VirR or ExpR did not directly repress the expression of the virulence factors, but activated the transcription of *rsmA*. RsmA is a direct repressor of the production of virulence factors. At low cell population density, that is, at low concentrations of 3-oxo-C6, free VirR or ExpR activates the transcription of *rsmA*, resulting in the reduction of virulence factors production. At high cell population density, that is, at high concentrations of 3-oxo-C6, VirR or ExpR forms a complex with 3-oxo-C6 and this complex does not activate the transcription of *rsmA*, and hence the production of the virulence factors is activated (**Figure 28**). These R-proteins as repressors were also found in *Serratia* species as described in Section 4.07.5.2.4.

4.07.5.2.4 Serratia *species*

Serratia species, *Serratia marcescens*, *Serratia liquefaciens*, *Serratia plymuthica*, *Serratia rubidaea*, *Serratia fonticola*, *Serratia marnorubra*, *Serratia proteamaculans*, and *Serratia odorifera*, are opportunistic pathogens and can be isolated from water, soil, plants, and air. *Serratia* species secrete several virulence factors, such as DNase, lipase, gelatinase, hemolysin, proteases, chitinase, chloroperoxidase, and multiple isozymes of alkaline phosphatase, and also produce carbapenem antibiotics, a red pigment named prodigiosin (**Figure 29**), and biosurfactants. Besides these phenotypes, biofilm formation and swarming motility are also regulated by quorum sensing. LuxR and LuxI homologues have been identified from each species, such as SmaI and SmaR in *Serratia* sp.

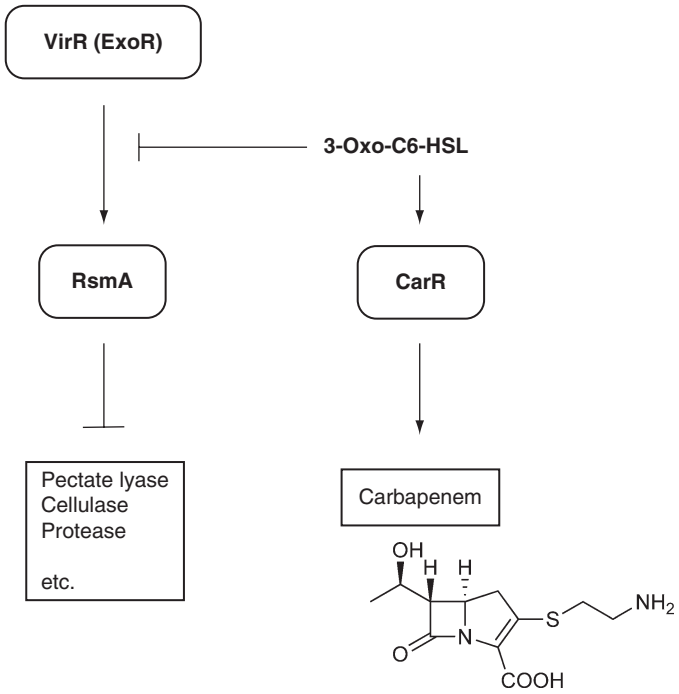
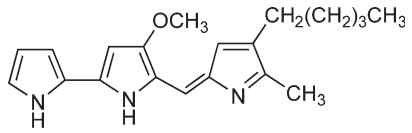


Figure 28 3-Oxo-C6-HSL-mediated quorum sensing in *Erwinia* species. The 3-oxo-C6-HSL–CarR complex activates the production of carbapenem. On the contrary, complexation of 3-oxo-C6-HSL and VirR (or ExoR) reduces the activity of RsmA and then the production of the virulence factors is activated.



Prodigiosin

Figure 29 The structure of the red pigment prodigiosin.

ATCC39006, SpnI and SpnR in *S. marcescens*, SprI and SprR in *S. proteamaculans*, and SwrI and SwrR in *S. liquefaciens*. SmaI and SwrI produce C4-HSL and C6-HSL. SpnI produces C6-HSL, C7-HSL, C8-HSL, and 3-oxo-C6-HSL. C7-HSL, borne with odd number of acyl chain, is very rare among natural products. SprI produces only 3-oxo-C6-HSL. SwrR in *S. liquefaciens* acts as an activator like LuxR, but SamR, SpnR, and SprR act as a repressor like VirR or ExpR in *Erwinia* species.[305]

AI-2-mediated quorum sensing system in *Serratia* species was also found and is described in Section 4.07.5.3.1.

4.07.5.2.5 Burkholderia *species*

The genus *Burkholderia* contains more than 30 species isolated from many places such as water, soil, plants, insects, animals, and also human beings. *Burkholderia mallei* and *Burkholderia pseudomallei* are revealed as pathogens of glanders and meliodosis. Several other *Burkholderia* species have emerged also as a pathogen of cystic fibrosis. These *Burkholderia* strains called *Burkholderia cepacia* complex (BCC) consist of at least nine species and show high sequence similarity of 16S rDNA. *Burkholderia cepacia* was isolated as one of the BCC strains. As cystic fibrosis patients are infected with *B. cepacia* and *P. aeruginosa* together in many cases and the supernatant of

P. aeruginosa culture medium has an effect on the protease activity and siderophore production of *B. cepacia*, it was expected that there was interspecies cell–cell communication between *B. cepacia* and *P. aeruginosa*. Actually, CepI and CepR have been identified from BCC strains as LuxI and LuxR homologues. CepI directs the synthesis of C8-HSL as a major product and also C6-HSL as a minor product. The amounts of AHLs produced by BCC strains vary largely from micromolar level to below nanomolar level depending on the strain. For instance, the amount of C8-HSL produced by *B. cepacia* is 1000-fold less than that of 3-oxo-C12-HSL and C4-HSL produced by *P. aeruginosa*.[306,307]

The quorum sensing of BCC strains shows both positive and negative regulatory role, increasing protease and chitinase production, activating swarming motility, and biofilm formation, while simultaneously decreasing siderophore production. The quorum sensing in BCC strains is illustrated in **Figure 30**. C8-HSL binds to the receptor protein CepR and C8-HSL–CepR complex induces or represses the target genes including *cepI*. *Burkholderia vietnamiensis*, one of the BCC strains, also produces C10-HSL for another quorum sensing system consisting of BviI and BviR. Studies on chronic coinfection showed that BCC strain could use exogenous AHLs produced by other bacteria, such as *P. aeruginosa*. But *P. aeruginosa* did not respond to AHLs produced by BCC strain. These results revealed that this interspecies cell–cell communication was unidirectional.[308,309]

4.07.5.2.6 Agrobacterium tumefaciens *and* Chromobacterium violaceum

Agrobacterium tumefaciens is a pathogen of plants using tumor-inducing Ti plasmid to transfer oncogenic DNA. The copy number and conjugal transfer of Ti plasmid are regulated by quorum sensing in *A. tumefaciens*. TraI, which is a LuxI homologue, is a synthase of 3-oxo-C8-HSL. TraR, which is a LuxR homologue, is an AHL receptor and a transcriptional regulator. Interestingly, the TraR genes are expressed only in the presence of specific opines called conjugal opines produced by plants. The 3-oxo-C8-HSL–TraR complex regulates expression of the *tra* regulon as well as the *traI* gene. The *tra* genes are required for conjugal transfer of Ti plasmid.[310–314]

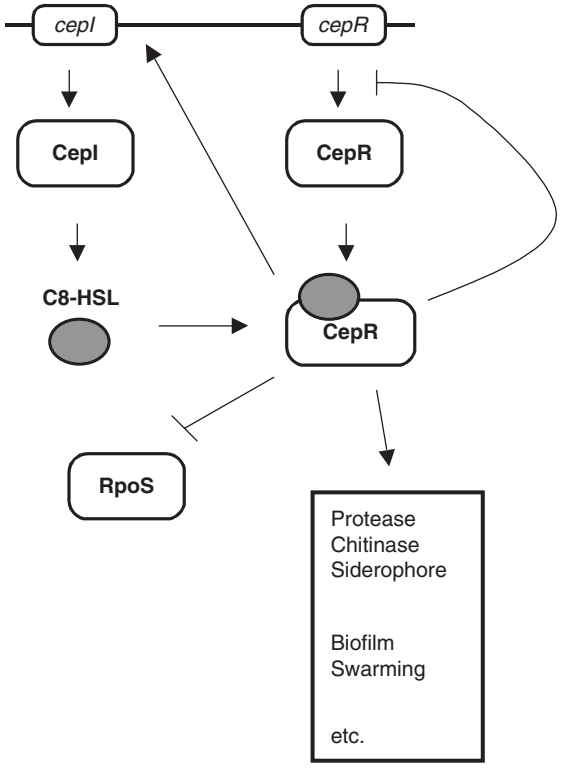


Figure 30 Quorum sensing in *Burkholderia cepacia* complex.

The crystal structure of TraR was the first reported structure among AHL receptor protein. 3-Oxo-C8-HSL–TraR complex forms a homodimer, which binds to DNA (*tra* box). TraR consists of two domains as LuxR. The N-terminal domain is an AHL-binding site and the C-terminal domain is a DNA-binding site with a helix–turn–helix motif (**Figure 31**).[315]

Chromobacterium violaceum is commonly found in soil and water and produces a purple pigment named violacein (**Figure 32**), which is water insoluble and has an antibacterial activity. Violacein production is regulated by CviI–CviR quorum sensing system with C6-HSL in *C. violaceum*.[316]

The mutants of *A. tumefaciens* and *C. violaceum* are well known as AHL reporter strains for screening AHLs.[317,318] *A. tumefaciens* NTL4 (*pZLR4*) cannot produce its own AHL (3-oxo-C8-HSL), but can induce *traG::lacZ* by exogenous AHLs. It is very interesting that *A. tumefaciens* NTL4 (*pZLR4*) can respond to not only 3-oxo-C8-HSL but also to other kinds of AHLs. *C. violaceum* CV026 is a mini-Tn5 mutant and cannot produce its own AHL (C6-HSL). *C. violaceum* CV026 can produce violacein when exogenous AHLs are supplied. Different from *A. tumefaciens* NTL4 (*pZLR4*), *C. violaceum* CV026 can respond to short acyl chain AHLs only (shorter than C8) and cannot respond to longer acyl chain AHLs (longer than C10). Moreover, such longer acyl chain AHLs inhibit violacein production of *C. violaceum* CV026. These results suggest that many kinds of AHLs can be detected by the combination of *A. tumefaciens* NTL4 (*pZLR4*) and *C. violaceum* CV026.

4.07.5.3 Other Types of Autoinducers

Until now, it has been reported that several dozens of Gram-negative bacteria produce AHLs. But it has also been reported that many Gram-negative bacteria including *E. coli* and *Salmonella* species did not produce AHLs. The analysis of genome showed the presence of *lux* homologues even in non-AHL-producing bacteria and several kinds of quorum sensing system with autoinducers except AHLs have been reported.

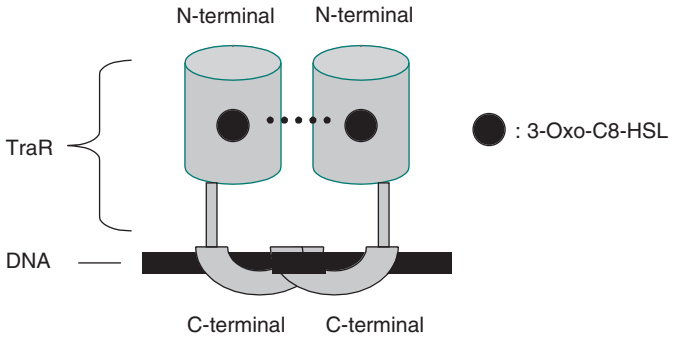
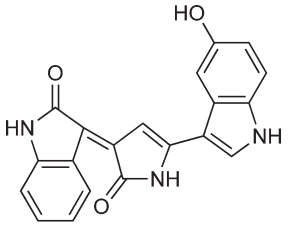


Figure 31 The image of TraR–DNA complex. A homodimer of 3-oxo-C8-HSL–TraR binds to DNA at its C-terminal domain.



Violacein

Figure 32 The structure of the purple pigment violacein.

4.07.5.3.1 Autoinducer-2

As already described in Section 4.07.4.7, AI-2 is thought to be a global communication signal among both Gram-positive and Gram-negative bacteria. AI-2-mediated quorum sensing system was first found in *V. harveyi* as the third system as mentioned in Section 4.07.5.2.1. AI-2 was identified from the X-ray analysis of the crystallographic structure of LuxP, which was a periplasmic protein of *V. harveyi*.[319] The crystallogram showed the complex of LuxP and AI-2. The structure of AI-2 was identified as (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxtetrahydrofuran-borate (furanosyl borate diester) (**Figure 33**). It was also a first report about a borated compound identified from bacteria. AI-2 is synthesized by LuxS from (4*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) derived from (*S*)-adenosyl methionine and the proposed synthetic pathway is shown in **Figure 33**. AI-2–LuxP complex interacts with the sensor kinase LuxQ. Then through a dephosphorylation cascade with LuxU and LuxO, bioluminescence is activated.[265]

In *V. fischeri*, LuxS, LuxP, and LuxQ were also identified and AI-2 was produced by LuxS. In contrast to *V. harveyi*, the effects of AI-2 on the bioluminescence in *V. fischeri* are smaller than that of AHL (**Figure 34**).[258,320]

LuxS homologue was also found in *Ecc* and *Eca* strains. *luxS* mutants in each of these strains showed reduced production of some exoenzymes and some virulence factors. But the complete function of AI-2-mediated quorum sensing in *Erwinia* species is not known in detail.[304]

In *S. marcescens*, AI-2-mediated quorum sensing also regulates the production of carbapenem antibiotic, prodigiosin, hemolysin, and other virulence factors. But such AI-2-mediated regulation is not for all *Serratia* strains, that is, it is a strain-dependent phenotype.[305,321]

Salmonella species and *E. coli* do not produce AHLs, but produce AI-2 by LuxS.[322,323] In *Salmonella typhimurium*, the periplasmic protein LsrB was identified as a receptor protein of AI-2. Although of low sequence homology with LuxP, the X-ray analysis of the crystallographic structure of LsrB showed that the overall protein structure of LsrB was similar to that of LuxP. But the X-ray analysis also showed a big difference between LuxP and LsrB. LuxP bound furanosyl borate diester, but LsrB bound the molecule without borate.

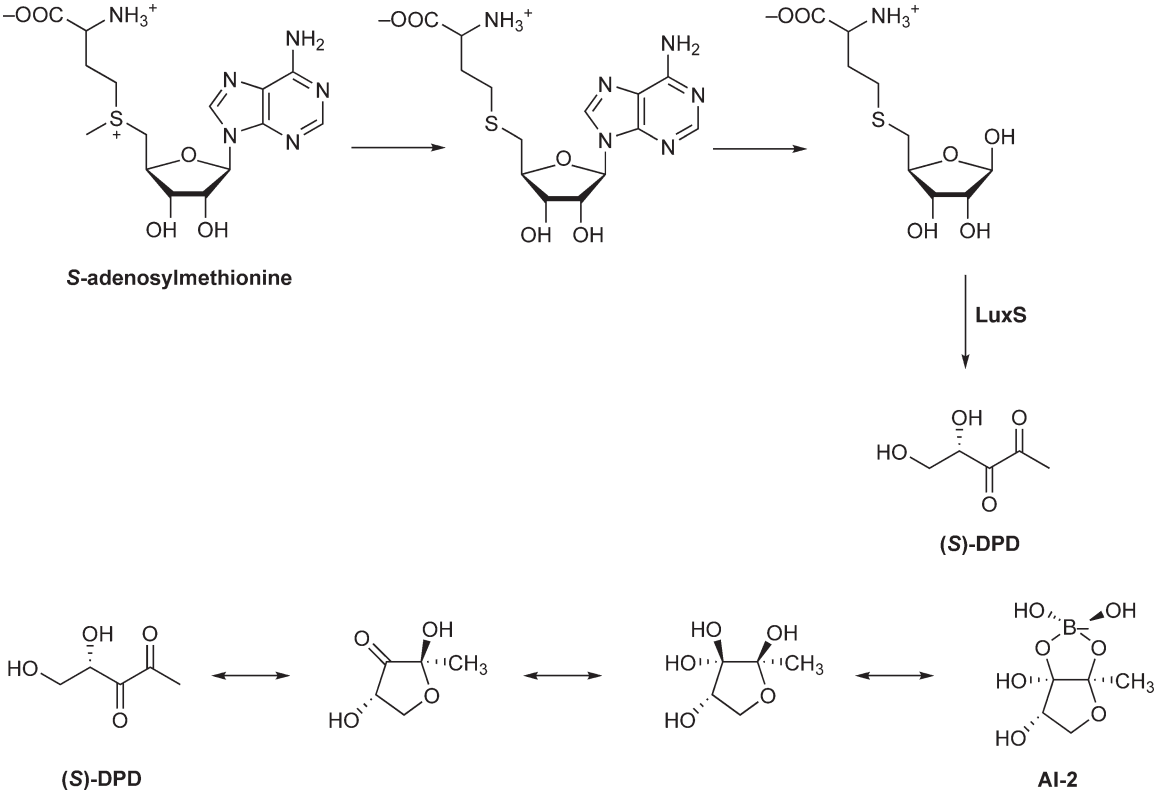


Figure 33 The pathway of AI-2 synthesis.

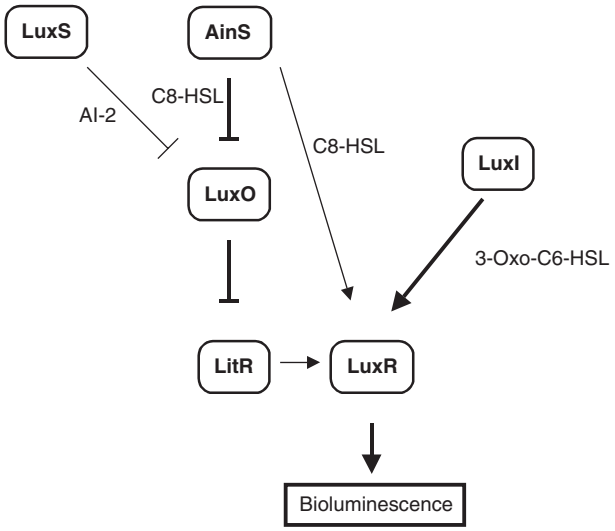


Figure 34 Quorum sensing in *Vibrio fischeri* for bioluminescence included AI-2. The effect of AI-2 is smaller than that of AHLs.

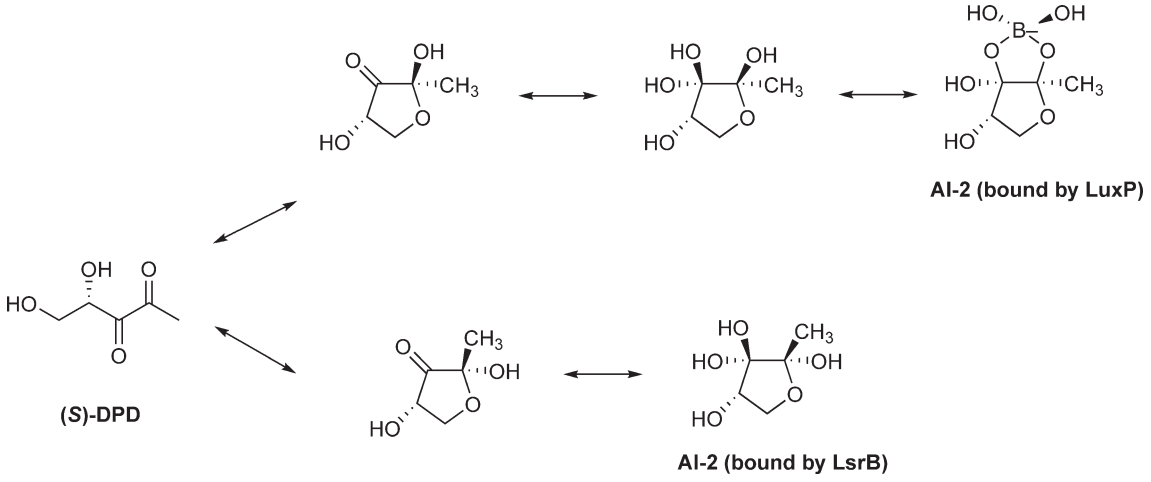


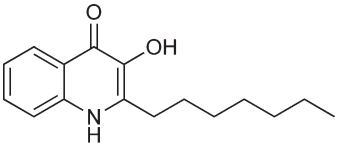
Figure 35 The synthetic pathway of the molecule bound by LsrB.

Moreover, the configuration of the molecule bound by LsrB was different from that of the precursor of furanosyl borate diester. The structure and proposed synthetic pathways of these ligands are shown in **Figure 35**. It is uncertain whether this unborated compound also acts as AI-2, but many phenotypes in *S. typhimurium* seemed to be regulated by AI-2 activities.[324]

To screen AI-2 production, a reporter strain based on *V. harveyi* is used not only for Gram-negative bacteria, but also for Gram-positive bacteria.[325] At present, AI-2 is thought to be a group of compounds synthesized by LuxS from DPD with or without boron. More detailed studies will show the world of AI-2 in more depth.

4.07.5.3.2 PQS

The autoinducer of the third quorum sensing system in *P. aeruginosa* is PQS. (**Figure 36**) PQS is involved in *lasB* expression, and RhlR is required for PQS activity. The structure of PQS is similar to that of antimicrobial quinolones, but PQS shows no antimicrobial activity. PQS is converted from 2-heptyl-4(1*H*)-quinolone (HHQ) by PqsH, which is activated by 3-oxo-C12-HSL–LasR. PQS regulates the expression of elastase, rhamnolipid, pyocyanin, and LecA. As PQS regulates LasB expression and the synthesis of PQS is regulated by LasR and



PQS

Figure 36 The structure of PQS.

RhlR, PQS-mediated regulation is not independent but linked to both *las* and *rhl* quorum sensing systems.[326–329] Until now, PQS quorum sensing has been reported only in *P. aeruginosa*, but PQS gene homologues were found in *Burkholderia* species. PQS-mediated quorum sensing may be extended to other bacterial species in future.

4.07.5.3.3 Indole

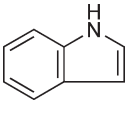
Escherichia coli produces indole (**Figure 37**) from tryptophan by tryptophanase and exports it by AcrEF pump. Indole acts as an autoinducer to regulate transcription of *tnaAB*, *astD*, and *gabT* in *E. coli*.[330] As *tnaA* encodes tryptophanase, indole signaling enhances the production of indole itself. *astD* and *gabT* involve the production of pyruvate and succinate from the amino acid. Indole signaling also has an effect on the multidrug exporters and biofilm formation in *E. coli*.[331–333] Moreover, it was also reported that indole enhanced biofilm formation of *P. aeruginosa*, in which indole is not produced.[333] Indole may act as an interspecies signaling compound.

4.07.5.3.4 3-OH PAME

Ralstonia solanacearum is a phytopathogen and regulates virulence factors, extracellular polysaccharide (EPS), and endoglucanase, by quorum sensing.[334] The autoinducer in *R. solanacearum* was identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME) (**Figure 38**). Proposed 3-OH PAME regulation is shown in **Figure 38**. At low concentrations of 3-OH PAME, PhcR is phosphorylated by PhcS and represses PhcA, which regulates virulence factors. At high concentrations of 3-OH PAME, phosphorylation of PhcR is reduced and PhcA is activated to produce EPS and endoglucanase. The degradation of 3-OH PAME by esterase identified from *Ideonella* species reduced EPS production of *R. solanacearum*.[335] It is very interesting that such a simple fatty acid derivative as 3-OH PAME acts as an autoinducer.

4.07.5.3.5 Cholerae autoinducer-1

Vibrio cholerae is a pathogen of the human disease cholera and regulates virulence factors and biofilm formation by quorum sensing.[336–338] *Vibrio cholerae* uses two quorum sensing systems. One system is AI-2-dependent system with LuxS, LuxP, and LuxQ and the other system uses CAI-1 (cholerae autoinducer-1) as a signaling molecule (**Figure 39**). CAI-1 is produced by CqsA and identified as 3-hydroxytridecan-4-one.[339] In *V. cholerae*, the CAI-1 system is more effective than the AI-2 system. At low cell density, that is, at low concentrations of CAI-1 and AI-2, *V. cholerae* enhances virulence factors and biofilm formation but no bioluminescence. At high cell density, that is, at high concentrations of CAI-1 and AI-2, LuxO is inactivated and then HapR is activated. HapR represses the gene expressions of virulence factors and biofilm formation and expresses bioluminescence (**Figure 39**). Using these quorum sensing systems, *V. cholerae* can leave from the host to infect other new hosts in large numbers and start a new infection cycle.



Indole

Figure 37 The structure of indole.

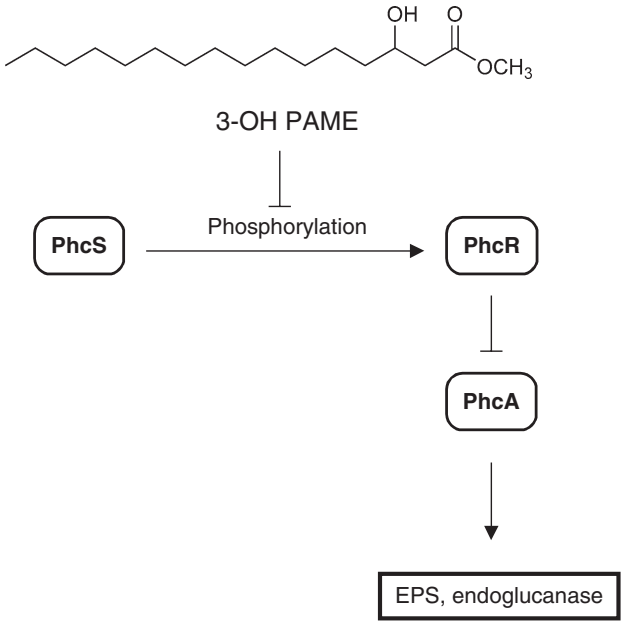


Figure 38 Quorum sensing in *Ralstonia solanacearum* mediated by 3-OH PAME.

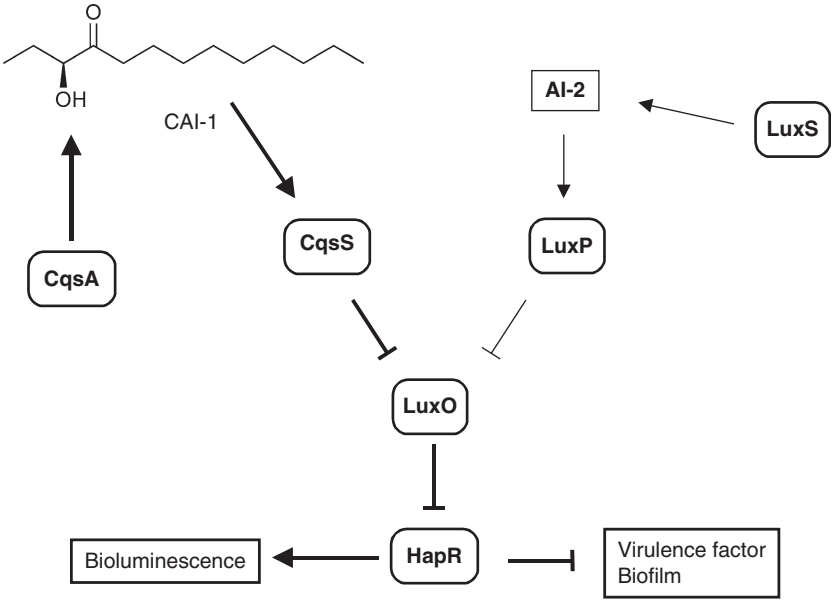


Figure 39 Quorum sensing in *Vibrio cholerae* mediated by CAI-1 and AI-2.

4.07.5.3.6 Stigmolone

Myxobacteria are Gram-negative slime molds that are on the borderline between unicellular and multicellular organisms.[340] Upon nutrient starvation, cells cooperate to build up multicellular fruiting bodies comprising several sporangioles that contain myxospores. When nutrients are available again, the myxospores germinate to build up a new swarm of vegetative cells. In the early 1980s, a pheromone activity of a myxobacterium,

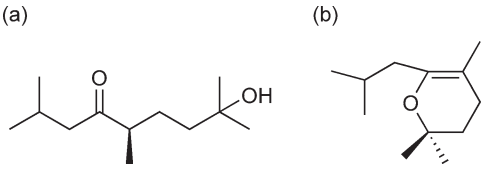


Figure 40 Stigmolone, an inducer of fruiting bodies of myxobacteria. Equilibrium between the hydroxy ketone stigmolone (a) and dihydropyran (b) is shown.

Stigmatella aurantiaca, was detected and was shown to be involved in the formation of fruiting bodies.[341] The existence of a secreted low-molecular-weight substance(s) was suggested by a dialysis experiment, which showed that *Stigmatella* cells cannot aggregate if an extracellular substance(s) is removed by dialysis.[342] Thus, the pheromone was found to be required for the aggregation of the cells at the beginning of fruiting body formation.

The pheromone was purified and its chemical structure was determined as a hydroxy ketone, 2,5,8-trimethyl-8-hydroxy-nonan-4-one, which was named stigmolone (**Figure 40**).[342,343] The equilibrium between stigmolone and the dihydropyran (**Figure 40**) has impeded the structural elucidation for a long time. However, the equilibrium seems to be relevant only in dry organic solvents; in an aqueous environment, that is, under the physiological conditions, the hydroxy ketone stigmolone is by far the predominant compound. Chemical synthesis of stigmolone and the dihydropyran showed that stigmolone is active at a concentration of 1 nmol l^{-1} and the dihydroxypyran is inactive even at a concentration of 10 nmol l^{-1}.[342,343]

4.07.5.3.7 *Extracellular death factor in* E. coli

Programmed cell death (PCD) is known as a system in eukaryotic multicellular organisms,[344,345] but PCD has also been observed in bacteria.[346,347] The toxin–antitoxin module (*mazEF*) in *E. coli* is one of the PCD systems. It was observed that *mazEF*-mediated cell death in *E. coli* was dependent on the cell population density and required a signal compound named extracellular death factor (EDF).[348,349] EDF was identified as a linear pentapeptide with a sequence Asn-Asn-Trp-Asn-Asn (**Figure 41**). EDF is one of the autoinducers of quorum sensing in *E. coli*. As already described in Section 4.07.4.2, peptides are known as autoinducers in Gram-positive bacteria, but EDF was the first peptide that was identified as an autoinducer in Gram-negative bacteria.

4.07.5.3.8 *Aryl-HSL in* Rhodopseudomonas palustris

Rhodopseudomonas palustris is an anoxigenic phototrophic soil bacterium. *Rhodopseudomonas palustris* has *luxI* and *luxR* homologues, *rpaI* and *rpaR*,[350] and the expression of *rpaI* is activated by adding *p*-coumarate into the growth medium.[351] It was found that *R. palustris* produced *p*-coumaroyl-HSL (**Figure 42**) by RpaI using

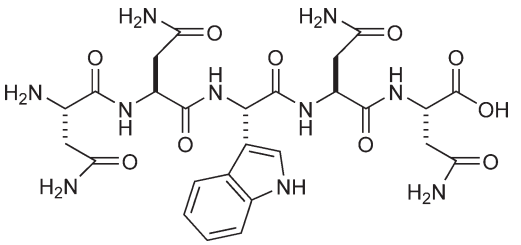


Figure 41 The structure of EDF required for *mazEF*-mediated cell death in *Escherichia coli*.

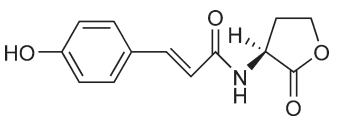


Figure 42 The structure of *p*-coumaroyl-HSL produced by *Rhodopseudomonas palustris*.

environmental *p*-coumaric acid.[352] Several kinds of aryl-HSLs have been chemically synthesized as the artificial analogues of AHLs, but this was the first report of a natural aryl-HSL. Even RpaI and RpaR are the homologues of the synthase and the receptor of AHL (fatty acyl-HSL), and RpaI and RpaR produce and respond to such a new type of quorum sensing signal (non-fatty acyl-HSL).[352] As not only *R. palustris* but also other bacterial species, such as *Bradyrhizobium* sp. and *Silicibacter pomeroyi*, seem to produce *p*-coumaroyl-HSL, *p*-coumaroyl-HSL does not seem to be unique to *R. palustris*.[352] Because the synthesis of *p*-coumaroyl-HSL needs *p*-coumarate and *p*-coumarate is usually produced by plants in the environment, *p*-coumaroyl-HSL-mediated quorum sensing may play a role not only in bacterial interspecies signaling but also in signaling between bacteria and plants.

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4.08 Chemical Defence and Toxins of Plants

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4.08.1 Chemical Defense against Herbivores

4.08.1.1 Introduction

An important component determining plant growth and reproduction is the defense against herbivores. Direct lines of defense against herbivores have been well documented. In addition, several methods of indirect defense have been noted in which the herbivores' natural enemies are exploited.[1–5] Initially, it was thought that both direct and indirect defenses were constitutive in nature. However, evidence is accumulating that both direct and indirect defenses can be either constitutive or inducible by herbivores. Furthermore, exposure to volatiles emitted by neighboring plants also induces plant defense against herbivores (priming). Here, direct chemical defense and indirect chemical defense of plants against herbivores are reviewed. A complete review is not provided, but rather the processes of some examples are outlined.

4.08.1.2 Direct Defense of Plants against Herbivores

Plants contain a large variety of secondary metabolites that are not involved in primary metabolisms necessary for maintaining life activities and survival. Over the last several decades, a number of studies, including the pioneering study by Feeny[6] on the ecological roles of plant tannins, have reported that many of the plant secondary metabolites play important roles in plant defense activities against herbivores, including insects, mammals, and snails. By 1980s, major groups of defense chemicals such as alkaloids, nonprotein amino acids, cyanogenic glycosides, glucosinolates, terpenoids, coumarins, cardenolides, iridoid glycosides, tannins, phenolics, flavonoids, and phytoecdysteroids became well known, and their defensive roles against insects have been well established.[4,5,7,8] Since then, the discovery of totally new groups of secondary metabolites is rare; however, there is considerable progress in our understanding of the modes of action of toxicity, target sites in herbivores, precursors of toxic molecular species and enzymatic activation, plant tissues specialized in defense and localization of secondary metabolites, inducible defense and induction of secondary metabolites in plants, evolution of the synthetic pathway of defense chemicals, synergism of defense chemicals, and molecular-level adaptive mechanisms in specialist herbivores. The progress made is mainly due to new experimental methods, especially molecular biology techniques. Furthermore, it has become more apparent that plant defense does not depend fully on secondary metabolites and that various defense proteins are also important. Such recent progresses as well as important basic knowledge on plant defense based on plant ingredients both chemicals and proteins are discussed in this section.

4.08.1.2.1 Secondary metabolites

4.08.1.2.1(i) Alkaloids Alkaloids are a diverse group of secondary metabolites and more than 12 000 alkaloids have been reported from 150 families of plants and more than 20% of all plant species contain alkaloids.[9–11] According to Pelletier an alkaloid is "a cyclic compound containing nitrogen in a negative oxidation state";[12] however, recently other nitrogen-containing compounds not classified into other groups of compounds, such as amino acids, amines, and cyanogenic glycoside, are sometimes classified as alkaloids.[10] Alkaloids are quite diverse in structure and are classified into several groups: tropane alkaloids (e.g., atropine), quinolizidine alkaloids (e.g., lupinine), pyridine alkaloids (PAs) (e.g., nicotine), indole alkaloids (e.g., harman), pyrrolizidine alkaloids (e.g., senecionine-*N*-oxide), isoquinoline alkaloids (e.g., morphine), purine alkaloids (e.g., caffeine), and polyhydroxy alkaloids (e.g., 1-deoxynojirimycin (DNJ), 1,4-dideoxy-1,4-imino-D-arabinitol, and castanospermine), and other groups (**Figure 1**). Most alkaloids are derived from amino acids (predominantly ornithine, lysine, tyrosine, phenylalanine, and tryptophan), apart from those that involve insertion of nitrogen into preformed skeletons, notably diterpene and steroidal alkaloids.[13] This is also the case for sugar-mimic alkaloids such as DNJ, at least in bacteria.[14]

The modes of action of different alkaloids are diverse. For example, nicotine binds to and affects nicotinic acetylcholine receptors and shows toxicity. A recent molecular 3D model suggests that both acetylcholine and nicotine bind to the same pocket formed in a nicotinic acetylcholine receptor.[15] Morphine binds to and activates opioid receptors, transmembrane-spanning G protein-coupled receptors, in the central nervous system of humans.[16] Caffeine, which is structurally similar to adenine, inhibits cyclic AMP phosphodiesterase activity and inhibits the degradation of cAMP, thus exerting a toxic effect on insects;[17] in human beings, binding of caffeine to the adenosine A2A receptor induces wakefulness.[18] Atropine binds to muscarinic acetylcholine receptors, competing with acetylcholine, and blocks neurotransmission.[19]

Although many alkaloids exert their toxic effects by affecting the nervous system, there are other alkaloids that show toxicity in completely different ways. Colchicine binds to tubulin and inhibits its polymerization, and thus inhibits the formation of microtubules in cells.

A new and interesting group of alkaloids is polyhydroxy alkaloids, such as DNJ and 1,4-dideoxy-1,4-imino-D-arabinitol (**Figure 1**), which are also called sugar-mimic alkaloids or iminosugars. Because polyhydroxy alkaloids resemble the structures of sugars, they are very potent inhibitors of sugar-metabolizing enzymes.[20] The IC_{50} values (the concentration necessary for inhibiting 50% of enzyme activities) of polyhydroxy alkaloids for disaccharidases such as amylase, sucrase, and trehalase from human beings and many insects are as low as 10^{-4}–10^{-6} mol l^{-1}.[20,21] However, some plants contain polyhydroxy alkaloids in very high concentrations. For example, the seeds of *Castanospermum australe* contain 0.06% of castanospermine,[20,21] and the latex of mulberry trees (*Morus* spp.) contains up to 2.5% (0.2 mol l^{-1}) of 1,4-dideoxy-1,4-imino-D-arabinitol and DNJ in

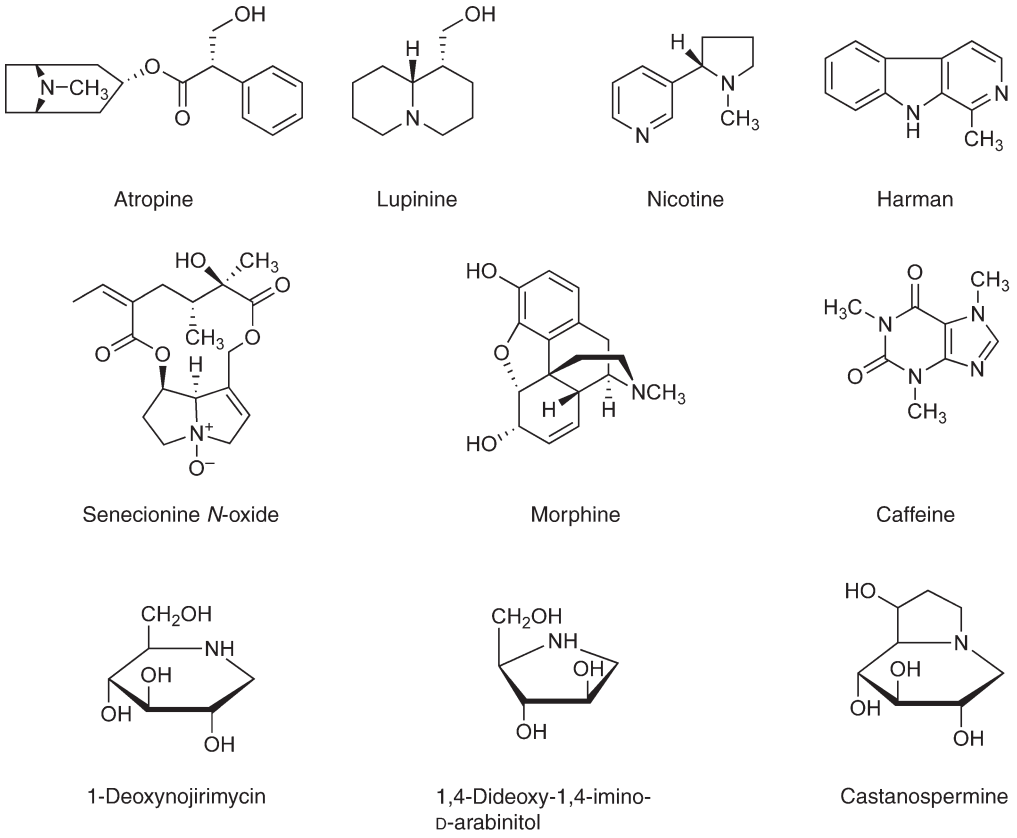


Figure 1 Alkaloids.

combination.[22] The sugar-mimic alkaloids in mulberry latex inhibit both sucrase in the midgut, thereby inhibiting sugar digestion, and trehalase in various tissues, thereby inhibiting the utilization of trehalose, the major blood sugar in many insects, and thus mulberry trees are well protected from herbivores.[23] However, the silkworm *Bombyx mori*, a specialist of mulberry leaves, is tolerant to sugar-mimic alkaloid because they have developed sucrase and trehalase that is insensitive to sugar-mimic alkaloids.[23]

Pyrrolizidine alkaloids are unique among alkaloids in that evolution of synthetic process, toxicology, ecological impacts on herbivorous insects, adaptation, detoxification, sequestration, and utilization as defense chemicals by insects are all well studied (**Figure 2**).[11,24–26] The synthesis of necine base, the characteristic structure of PA, starts from the synthesis of homospermidine, catalyzed by homospermidine synthase (HSS), using primary metabolites spermidine and putrescine.[24] Sequence data indicated that HSSs from Boraginaceae, Asteraceae, and Orchidaceae have high homologies with deoxyhypusine synthase (DHS), an enzyme involved in the post-transcriptional activation of eukaryotic initiation factor 5A (elF5A), and have evolved several times independently from elF5A.[24,25] Thus in this case, the key enzyme involved in the synthesis of a secondary metabolite has evolved from an enzyme necessary for a basic biological function by change of function. After several steps, necine base is synthesized. PA has two molecular forms: the protoxic free base and the nontoxic *N*-oxide.[26] In most plants, PA is stored as *N*-oxide produced by oxidation of free base. When herbivores feed on a PA-containing plant, *N*-oxide is reduced into free base automatically and is then converted by cytochrome P-450 monooxygenase of herbivores into an unstable toxic pyrrolic intermediate that alkylates cellular nucleophiles (i.e., –OH, $–NH_2$, –SH residues of proteins and nucleic acids); therefore, PAs show toxicity to herbivores.[26] Insects specialized in feeding on PA-containing plants have developed physiological mechanisms to avoid the toxicities of PAs.[26] For example, PAs absorbed in the form of the protoxic free base in the larvae of the arctiid moth *Tyria jacobaeae* feeding on the host plant *Senecio jacobaea* (Asteraceae) were converted in hemolymph by a specific NADPH-dependent monooxygenase into *N*-oxide form and then stored in the larval

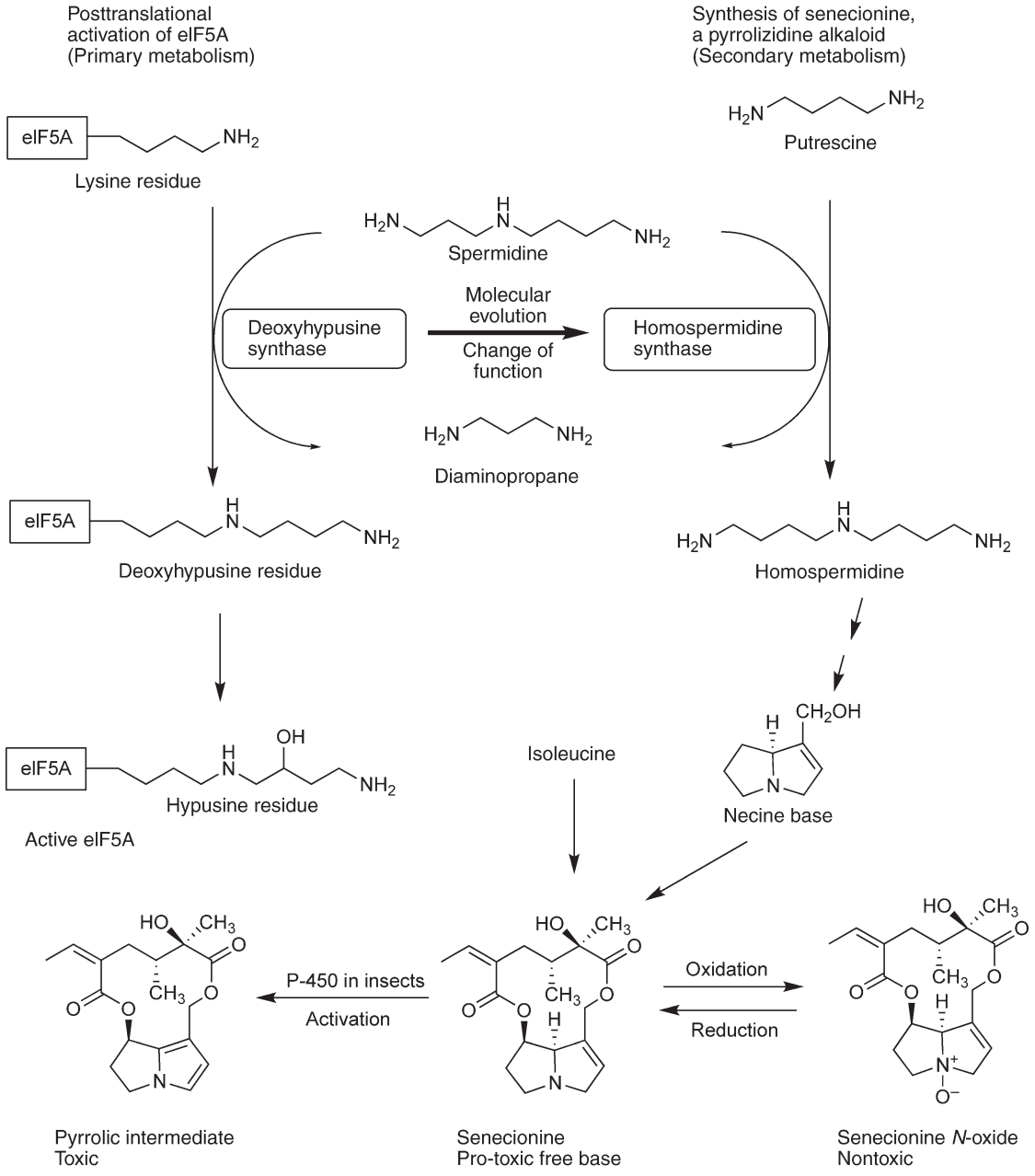


Figure 2 Synthetic pathway and activation process of pyrrolizidine alkaloid.[24–26]

body, with about 75% in the integument. A chrysomelid leaf beetle, *Oreina cacaliae*, feeding on the PA-containing plant *Adenostyles alliariae* (Asteraceae) is able to suppress the autoreduction of *N*-oxide in the gut lumen and then absorb and store it in the body. The stored *N*-oxide functions as a reservoir for exocrine defense glands. Any absorbed PAs in free base form are efficiently detoxified by glucosylation. A neotropical beetle, *Platyphora boucardi*, has developed a completely different strategy to cope with and sequester PAs. The beetle absorbs PAs in protoxic free base. Different from *T. jacobaeae*, the beetle does not have the ability to convert free base into *N*-oxide; however, in *P. boucardi*, the protoxic free base is efficiently pumped into the exocrine defense gland and the concentration of the free base in hemolymph is always low. Furthermore, females of *Utethesia ornatrix* transmit PAs that have been transmitted by males during copulation to eggs for protection of eggs.[27]

4.08.1.2.1(ii) Nonprotein amino acids Nonprotein amino acids are amino acids other than the 20 amino acids incorporated in protein (**Figure 3**). More than 900 nonprotein amino acids have been reported from a wide variety of plants including Leguminosae, Liliaceae, Sapindaceae, Cycadaceae, Compositae, Rubiaceae, and Lecythidaceae.[28–30] However, nonprotein amino acids are most often found in legumes and are mainly abundant in seeds.

The typical toxic mechanism of nonprotein amino acids is that they function as mimics of 20 protein amino acids and are mistakenly incorporated in protein in the place of the corresponding protein amino acids similar in structure, thereby leading to the production of unnatural proteins that cannot function properly. It is the case with azetidine-2-carboxylic acid found in *Convallaria majalis* (Liliaceae), which is a mimic of proline, and a legume toxin canavanine that occurs in the seeds of jackbean, *Canavalia ensiformis*, and *Dioclea megacarpa* in very high concentrations (up to 6 and 10%, respectively), which is a mimic of arginine.[8]

Other nonprotein amino acids exert toxicity in different ways. For example, 3,4-dihydroxyphenylalanine (L-DOPA), which is found in high concentrations (6–9%) in *Mucuna* seeds, shows strong toxicity to insects, but not to mammals, presumably by affecting tyrosinase activity, which is important in the hardening of insect cuticle.[8] Mimosine, which was first isolated from *Mimosa pudica* and which exists in the seeds and leaves of *Leucaena leucocephala* in high concentrations (8–10% dry weight in leaves), causes loss of hair in mammals when fed.[30] This phenomenon is explained by the fact that mimosine prevents initiation of DNA replication.[31] The fruit of *Blighia sapida* is consumed especially in Jamaica. The unripe fruits, however, are toxic, because hypoglycine, which exists in unripe fruit arils in relatively high concentrations (0.1% dry weight), induces severe hypoglycemia or very low blood sugar concentrations.[29,30] In mammals, hypoglycine is degraded into α-methylenecyclopropylacetic acid and this compound completely blocks fatty acid oxidation; thus, sugar is consumed intensively as the sole energy source and as a result all glycogen in the liver is exhausted, leading to hypoglycemia.[29,30,32]

The above toxic nonprotein amino acids serve as defense against mammals and insects in general; however, the herbivorous insects specialized in feeding on plants containing nonprotein amino acids have developed physiological adaptation to nonprotein amino acids. For example, the larvae of the bruchid beetle *Caryedes brasiliensis*, a specialist feeder of *D. megacarpa* seeds rich in canavanine, are able to avoid misincorporation of canavanine into proteins, because the arginyl-tRNA synthetase of the beetle can discriminate between arginine and canavanine.[30,33]

4.08.1.2.1(iii) Cyanogenic glycosides Cyanogenic glycosides are nitrogen-containing secondary metabolites that have an ability to produce highly toxic hydrogen cyanide when degraded by plant enzymes. More than 75 different cyanogenic glycosides have been reported from at least 2650 plants from 130 families, including Euphorbiaceae, Rosaceae, Asteraceae, Passifloraceae, Fabaceae, and Poaceae.[34–36] These compounds are typically *O*-β-glycoside of α-hydroxynitriles (cyanohydrins) and are kept in plant tissues. Linamarin and

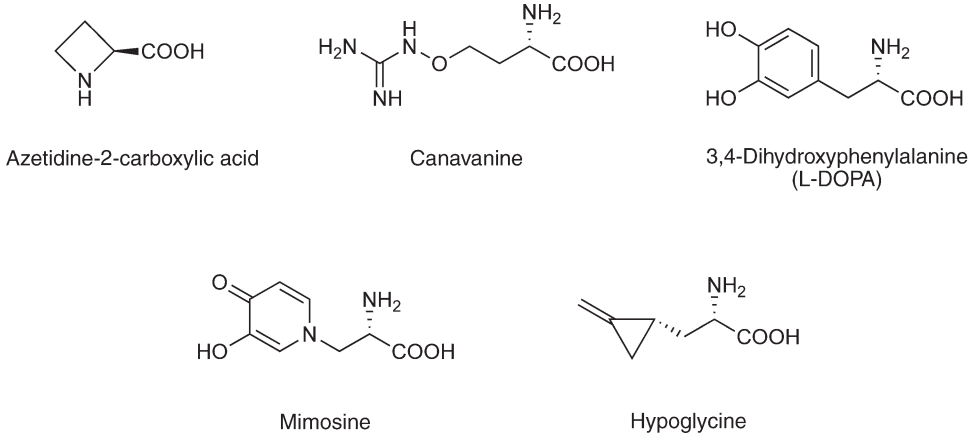


Figure 3 Nonprotein amino acids.

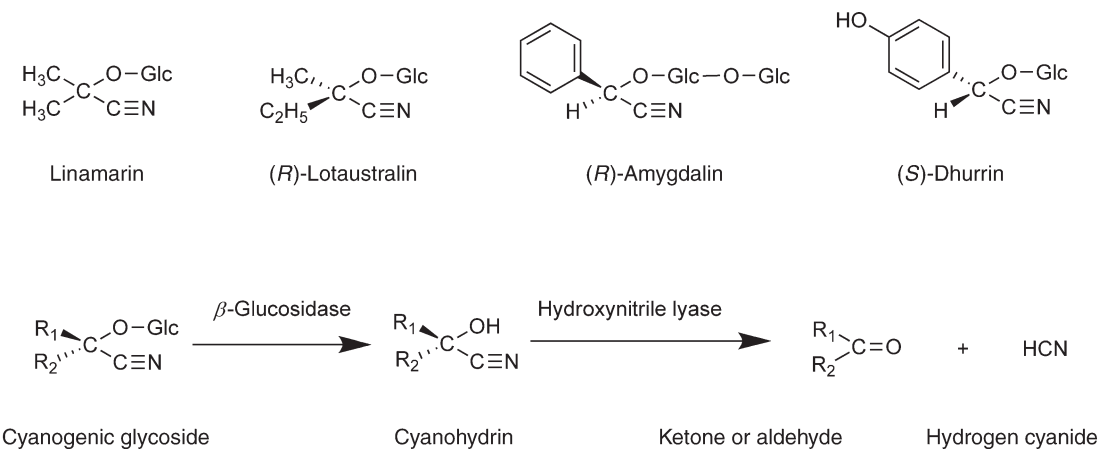


Figure 4 Cyanogenic glycosides and release of cyanide.

lotaustralin are the two most commonly found cyanide glycosides and are reported from many plant families, whereas other cyanide glycosides such as amygdalin common in Rosaceae plants and dhurrin often found in Poaceae plants are more confined in distribution (**Figure 4**). Cyanide glycosides are synthesized from amino acids.

Cyanide glycosides function as defense chemicals because they produce hydrogen cyanide, which is highly toxic to most living organisms because of its ability to inhibit the electron transport system by binding to cytochromes. When plant tissues are damaged by herbivory, β-glycosidases that are kept in other tissues separate from cyanogenic glycosides come in contact with and cleave sugars from cyanide glycoside and, as a result, cyanohydrins (hydroxynitriles) are formed (**Figure 4**).[34–36] Then, the cyanohydrins are degraded by a second enzyme, hydroxynitrile lyase, into hydrogen cyanide and ketones (or aldehydes) (**Figure 4**), although the last degradation occurs spontaneously but slowly.[34–36] The details of compartmentation differ among plant species. In the shoot of sorghum, *Sorghum bicolor*, dhurrin is contained in the vacuoles of epidermal cells, whereas β-glucosidase is kept in the chloroplasts of mesophyll cells and hydroxynitrile lyase is kept in the cytosol of mesophyll cells.[37] In cassava, *Manihot esculenta*, linamarin and lotaustralin are widely distributed among tissues,[38] whereas linamarase is concentrated in laticifer and latex[39] and hydroxynitrile lyase is concentrated in the cell wall of leaf tissues but absent in the root.[40] As hydrogen cyanide is highly toxic to most animals and insects, cyanide glycoside-borne defense systems are effective against most herbivores. Nonadapted insects and mammals have limited ability to detoxify hydrogen cyanide by means of the enzyme rhodanese or β-cyanoalanine synthase, which converts cyanide ion into less toxic thiocyanate. Moreover, insects specialized in feeding on plants with cyanide glycoside-borne defense systems can tolerate the toxicity, and some highly specialized insects such as *Heliconius* butterflies can even sequester cyanide glycosides for their own defense from predators. For example, the larvae of *Heliconius sara* sequester up to 1.5 mg CN dwg^{-1} of cyanide glycoside, mostly epivolkenin, from their host plant *Passiflora auriculata*.[41] The same larvae can further convert a substantial amount of epivolkenin into the corresponding thiol compound sarauriculatin by enzymatic replacement of the nitrile group by a thiol group.[42]

4.08.1.2.1(iv) Glucosinolates Glucosinolates are reported from a few plant families including Brassicaceae, Capparaceae, Tovariaceae, and Caricaceae, most of which belong to the order Brassicales in APG II system of plant taxonomy. In spite of their rather limited distribution, glucosinolates have been studied intensively because Brassicaceae includes many important crops such as cabbage and rapeseed and an important model plant species, *Arabidopsis thaliana*.

Glucosinolates such as sinigrin are molecules that consist of a β-thioglucose moiety, a sulfonated oxime, and a variable side chain derived from various amino acids (**Figure 5**).[43,44] Glucosinolates themselves are not toxic to herbivores and are widely distributed in plant tissues. On the contrary, the enzyme called myrosinase or thioglucosidase is distributed in myrosin cells that do not contain glucosinolates. In the flower stalk of

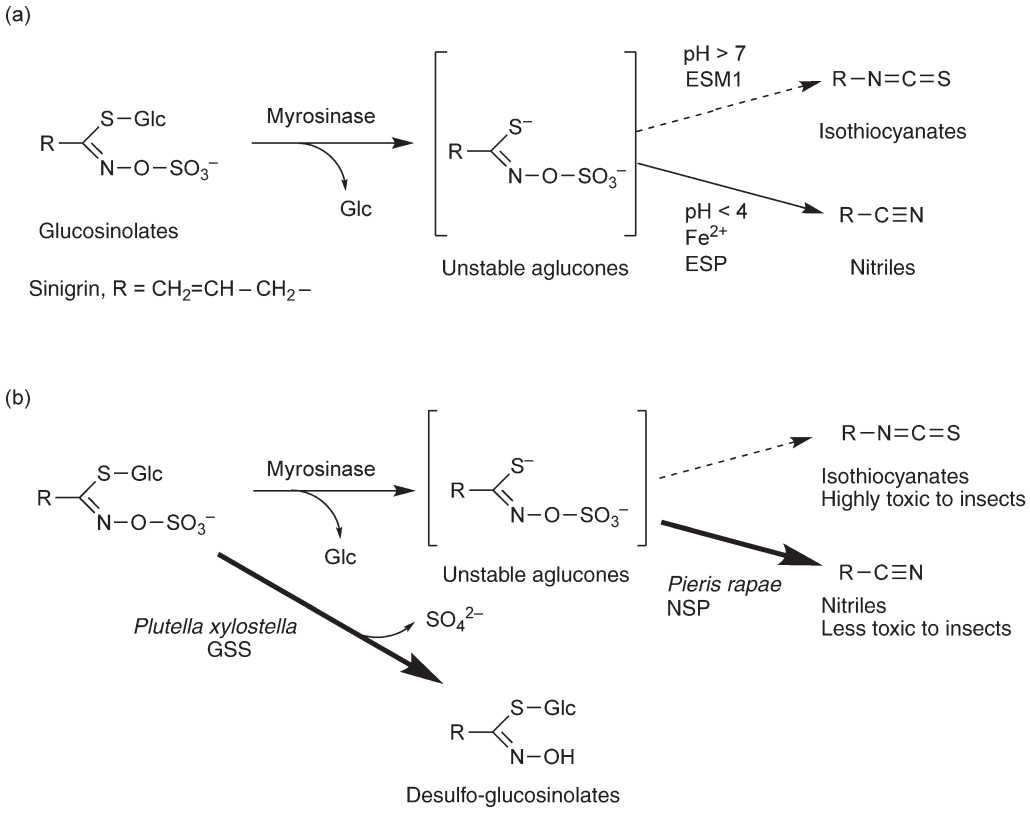


Figure 5 Enzymatic activation of glucosinolates (a) and adaptation of specialist herbivores (b).

A. thaliana, sulfur-rich cells (S-cells) that exist between the phloem and the endodermis contain high concentrations of glucosinolates, and myrosinase exists in adjacent phloem parenchyma cells.[45] When plant tissues are broken by herbivory, myrosinase cleaves the β-thioglucose moiety. The aglycones are unstable and further degrade in most cases into either isothiocyanates or nitriles depending on pH and other conditions (**Figure 5(a)**), although in rarer cases depending on R groups, thiocyanates and epithionitriles are formed.[43,44] Isothiocyanates, which are also called mustard oils, are highly toxic and even lethal to most unadapted generalist herbivores, especially to herbivorous insects,[46,47] whereas nitriles are less toxic.[46] Chemical conditions have been shown to be important in determining whether isothiocyanates or nitriles are formed: acidic conditions and existence of Fe^{2+} favor nitrile formation and alkaline conditions favor isothiocyanate formation (**Figure 5(a)**).[43,44] Recently, however, particular plant proteins associated to myrosinase are found to determine the final product, nitriles or isothiocyanates. The epithiospecifier protein (ESP) found in a certain line of *A. thaliana* promotes the production of nitriles, whereas another myrosinase-associated protein EPITHIOSPECIFIER MODIFIER1 (ESM1) recently found in certain *A. thaliana* lines represses nitrile formation and favors isothiocyanate production (**Figure 5(a)**).[48] The herbivory by the larvae of generalist lepidopteran species *Trichoplusia ni* or *Spodoptera littoralis* is greater on *A. thaliana* lines expressing ESP than on those not expressing ESP, and is greater on lines not expressing ESM1 than on those expressing ESM1.[48] It is interesting that the larvae of small white butterfly, *Pieris rapae*, a specialist on Brassicaceae plants, adapt themselves to glucosinolate-borne defense by modifying the final product to nitriles and inhibiting the production of isothiocyanates by the activity of nitrile specifier protein (NSP) in the larval gut, and as a result isothiocyanates are not formed and less toxic nitriles are excreted in feces (**Figure 5(b)**).[49] The larvae of diamondback moth, *Plutella xylostella*, another specialist species, adapt themselves with gut glucosinolate sulfatase (GSS), which cleaves the sulfate moiety (sulfonated oxime) of glucosinolates and forms desulfoglucosinolates (**Figure 5(b)**). The plant myrosinase cannot cleave the glucose moiety any more from desulfoglucosinolates, and thus mustard oils (isothiocyanates) are not formed (**Figure 5(b)**).[50]

4.08.1.2.1(v) Coumarins Coumarins are chemicals that have a 2*H*-1-benzopyran-2-one structure. Coumarins are grouped into simple coumarins, which have only two rings, such as coumarin, and those with an additional ring on benzopyran-2-one ring. Those with an additional furan ring attached at the 6,7 positions are called linear furanocoumarins, such as xanthotoxin, and those with an additional furan ring attached at the 7,8 positions are called angular furanocoumarins, such as angelicin (**Figure 6**).

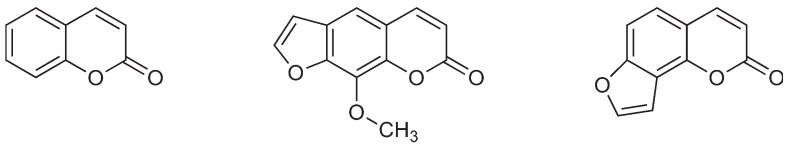
Simple coumarins are toxic or deterrent to insects and are widely distributed among plant families, being found in more than 79 families of angiosperms, whereas furanocoumarins are more restricted to a few plant families such as Rutaceae and Umbelliferae.[51] Furanocoumarins have a unique phototoxicity, which occur under UV radiation. Under UV radiation, furanocoumarins crosslink both strands of DNA by binding to pyrimidine bases. The crosslinking inhibits DNA replication and transcription, and thus furanocoumarins are toxic to generalist insects under UV radiation.[51] To avoid phototoxicity, some insects roll the leaves and feed on the inside of the leaves, thereby avoiding UV radiation.[52] Specialist insects feeding on furanocoumarin-containing plants (Umbelliferae and Rutaceae) such as swallowtail butterflies detoxify them by P-450 oxygenases. Angular furanocoumarins are more difficult to detoxify than linear ones,[53] and some specialists can feed only on linear furanocoumarin-containing plants. Further, in Umbelliferae plants, chemicals that inhibit the detoxification of furanocoumarins and enhance the toxicity of furanocoumarin by inhibiting P-450 activity coexist with furanocoumarin.[53]

4.08.1.2.1(vi) Phenolics and tannins Phenolics, including tannins, are found in plant tissues in very large amounts. The concentration of tannin in oak reaches 5% dry weight in late summer and autumn.[6,8] Tannins bind to proteins noncovalently, precipitate dietary proteins, and inhibit many digestive enzymes, and therefore inhibit the digestive processes and growth of herbivorous mammals and insects.[8] Herbivorous mammals adapted to tannin-rich diets secrete several kinds of tannin-binding proteins, such as proline-rich proteins that precipitate with tannin as an adaptation to tannin.[54]

Phenolics not only exert negative growth effects by noncovalent interactions, but also by covalent interactions. Oxidative enzymes such as polyphenol oxidases (PPO) and peroxidases (POD) oxidize phenolics, monophenols, and diphenols into reactive molecular species such as quinones and quinone methides, which covalently bind to nucleophilic residues in dietary proteins such as –SH, $-NH_2$ residues of nutritionally important amino acids such as cysteine and lysine, thereby decreasing the nutritive values of dietary proteins (**Figure 7**).[55]

4.08.1.2.1(vii) Terpenoids Terpenoids are chemicals that basically consist of isoprene (C5) units. Despite this similarity in basic units, terpenoids are extremely diverse in their structures and biological activities and include a large number of chemicals; at least 15 000 terpenoids are found in plants. Because of this diversity, terpenoids are divided into several groups according to their structures and biological functions. For example, monoterpenoids (C10) include volatile terpenoids rich in conifer resins, essential oil, and exudates of glandular trichome such as α-pinene, β-pinene, limonene, and menthol (**Figure 8**), and confer defense on plants.[56,57] Iridoids are also included in monoterpenoids. Sesquiterpenoids (C15) include sesquiterpene lactones, phytojuvenile hormones, and others; diterpenoids (C20) include clerodanes, tiglianes, daphnanes, and others; and triterpenoids (C30) include cardenolides, cucurbitacins, limonoids, phytoecdysteroids, saponins, and others. As it is not possible to describe all these diverse terpenoids, some characteristic groups of terpenoids are briefly described here.

4.08.1.2.1(vii)(a) Hormone analogues: Phytojuvenile hormones and phytoecdysteroids Insect development is controlled by several hormones, for example, juvenile hormone (JH) and two closely related steroid



Coumarin Xanthotoxin Angelicin

Figure 6 Coumarins.

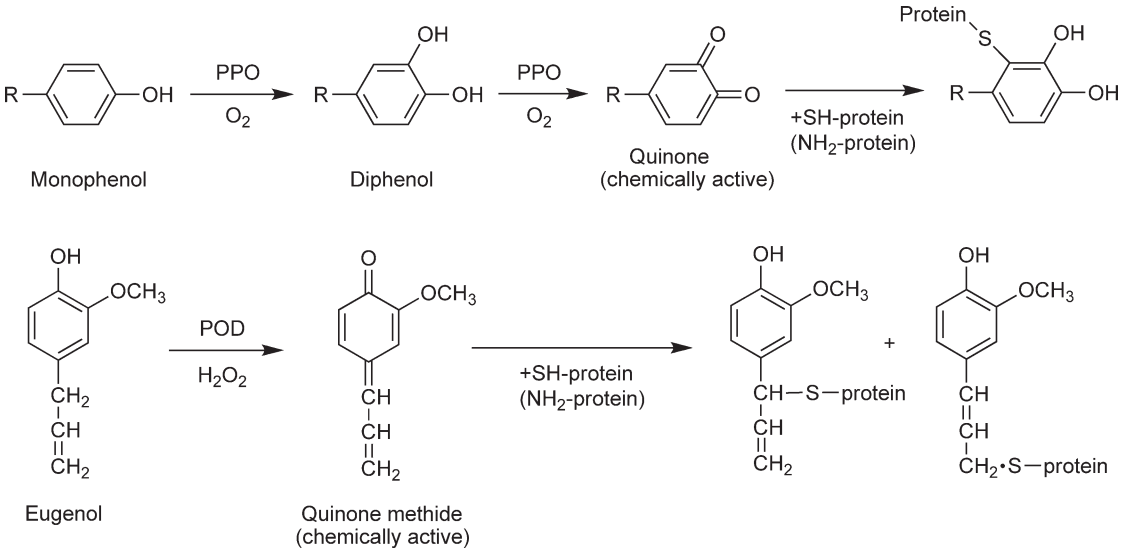


Figure 7 Activation of phenolics by oxidative enzymes such as polyphenol oxidases (PPO) and peroxidases (POD) and reaction of activated phenolics with protein.

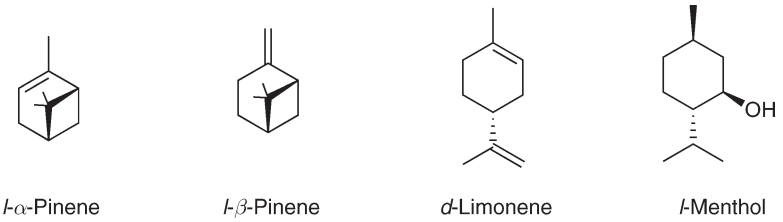


Figure 8 Volatile monoterpenoids often found in conifer resins, essential oils, and glandular trichomes that function as defense chemicals of plants against insect herbivores.

molting hormones, ecdysone and 20-hydroxyecdysone (commonest in insects). JH III is important for maintaining juvenile forms during molting and 20-hydroxyecdysone is important in molting and metamorphosis (**Figure 9**).

From the balsam fir tree *Abies balsamea*, a sesquiterpenoid, juvabione (**Figure 9**), was isolated as a compound that has a JH activity that causes the last instar larvae of European linden bug, *Pyrrhocoris apterus*, to molt into nymphal-adultoid forms.[58,59] Since then, a number of phytojuvenile hormones have been detected[56] such as juvocimene I and II from the sweet basil *Ocimum basilicum*.[60,61]

A compound that closely resembles the structure of molting hormone 20-hydroxyecdysone was first reported from the leaves of *Podocarpus nakaii* in large quantities and was called ponasterone A (**Figure 9**).[62] Starting from this discovery, more than 200 ecdysteroids have been found, most of which are from plants, most often in ferns and conifers.[59,63] These phytoecdysteroids have molting hormone activity and disturb the normal development of insects and in many cases the insects die. In the flowers of *Serratura inermis*, 20-hydroxyecdysone is found in very high concentrations of up to 2%.[64] In spinach, the amount of phytoecdysteroids increases in response to herbivory.[63,65] Both the induction of phytoecdysteroids in response to insect herbivory and the insect-specific toxicity of phytoecdysteroids support the idea that phytoecdysteroids are defense chemicals against insect herbivory.

4.08.1.2.1(vii)(b) Cardenolides Cardenolides are a group of cardiac-active steroids that have a five- or six-membered lactone ring and, in most cases, a sugar moiety, and are found mainly in plants belonging to Asclepidaceae and Apocynaceae, such as ouabain (**Figure 10**) from an African plant, *Acokanthera ouabaio*

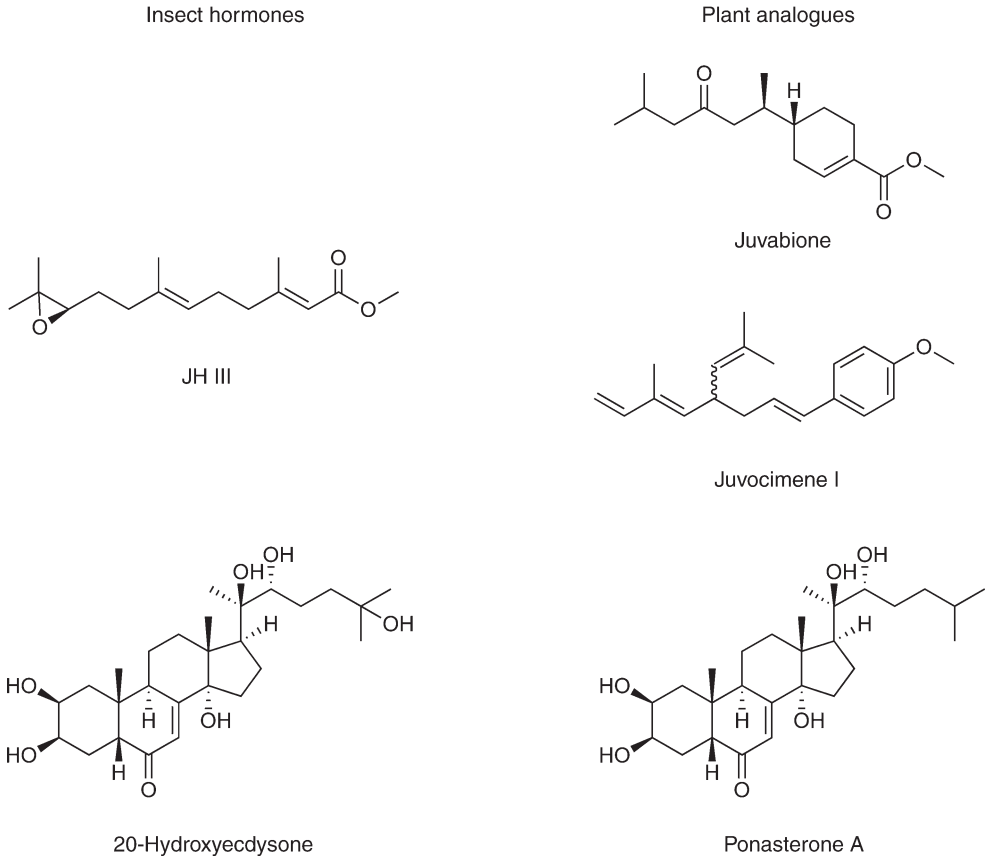


Figure 9 Insect hormones and plant analogues.

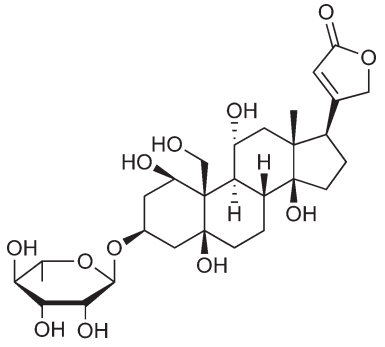


Figure 10 Ouabain, a cardenolide found in *Acokanthera ouabaio* (Apocynaceae).

(Apocynaceae), but also in plants belonging to Moraceae and other families.[66] The latex of several plants belonging to Asclepidaceae and Apocynaceae often contains very high concentrations of cardenolides.[66,67] The toxicity of cardenolides resides in their inhibitory activity against $Na^+ - K^+$ ATPase (or $Na^+ - K^+$ pump) involved in maintenance of ion concentrations in cells and neurotransmission, and thus cardenolides are generally toxic to animals, including insects.[66,68] Cardenolides bind specifically to membrane extruding part of $Na^+ - K^+$ ATPase; however, several insect species specialized in feeding on cardenolide-containing plants have developed cardenolide-insensitive $Na^+ - K^+$ ATPase, which commonly have single amino acid substitutions in target sites (cardenolide-binding sites).[69]

4.08.1.2.1(vii)(c) Iridoid glycosides Iridoid glycosides are a group of compounds that have a structure related to iridodial (**Figure 11**).[70] Almost 600 iridoid glycosides have been described from 57 families of plants.[70] Iridoids have been regarded as defense chemicals against herbivores and pathogens, since iridoid glycosides generally have bitter taste and have antifeedant and growth inhibitory activities against insects.[70] The observation of induction of iridoid glycosides in *Lonicera implexa* leaves bearing naturally laid eggs of the specialist herbivore *Euphydryas aurinia* and the observation that iridoid glycosides such as secologanic acid are present in these leaves in high concentrations (15-fold the total concentration, which is 15% of dry weight) compared to other leaves[71] support the idea that iridoid glycosides play a role in plant defense.

Iridoids are ecologically very interesting, because several insect herbivores from different families specialized in feeding on iridoid glycoside-containing plants sequester iridoid glycosides in higher concentrations. The adults of checkspot butterflies, *Euphydrias* spp., feeding on iridoid glycoside-containing plants contain as much as 9% dry weight of iridoid glycosides and are unpalatable to birds, and the larvae and pupae are warning colored and gregarious,[70] which are the typical characteristics of unpalatable insects. The molecular mechanism of sequestration has recently been studied in leaf beetles (Chrysomelidae) by feeding the larvae with structurally different artificial thioglucosides resembling natural iridoid glucosides (*O*-glucosides).[72]

In spite of the above biological phenomena, the reason why iridoid glycosides show such effects are not explained at the molecular level. Several studies have shown that at least some defense activities of iridoid glycosides could be attributed to their unstable aglycones that have glutaraldehyde-like structures and have alkylating activity against nucleophilic residues in biomolecules, in particular, amino residues in amino acids, proteins, and nucleic acids (**Figure 12**).[73,74] For example, two iridoid glycosides, aucubin and catalpol, in *Plantago lanceolata* showed growth inhibitory activity on specialist fungus *Diaporthe adunca* only in aglycone form and not in glycoside form.[74] Oleuropein, a secoiridoid glycoside with a diphenolic moiety, exists in high concentrations (3% of leaf dry weight) in *Ligustrum obtusifolium* and shows a strong growth inhibitory activity against herbivorous insects after being activated by an oleuropein-specific β-glucosidase and by a polyphenol oxidase (**Figure 12**).[73] Since the aglycone of oleuropein is a very strong electrophile due to its glutaraldehyde and α,β-unsaturated aldehyde structures, and covalently binds to amino residues of side chains of lysine, which is an essential amino acid, lysine becomes unavailable and privet leaves become nonnutritive to generalist insects (**Figure 12**).[73] The specialist caterpillars that feed on privet tree, such as *Brahmeae wallichii*, secrete high concentrations of free glycine, which also has amino residues, in the digestive juice as neutralizer to prevent the loss of lysine by competing with the amino residues in the side chain of lysine (**Figure 12**).[75,76]

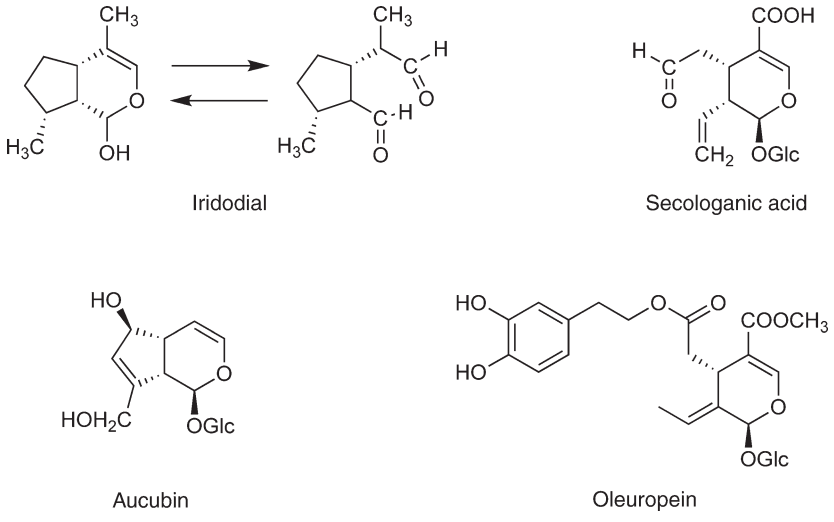


Figure 11 Iridoid glycosides.

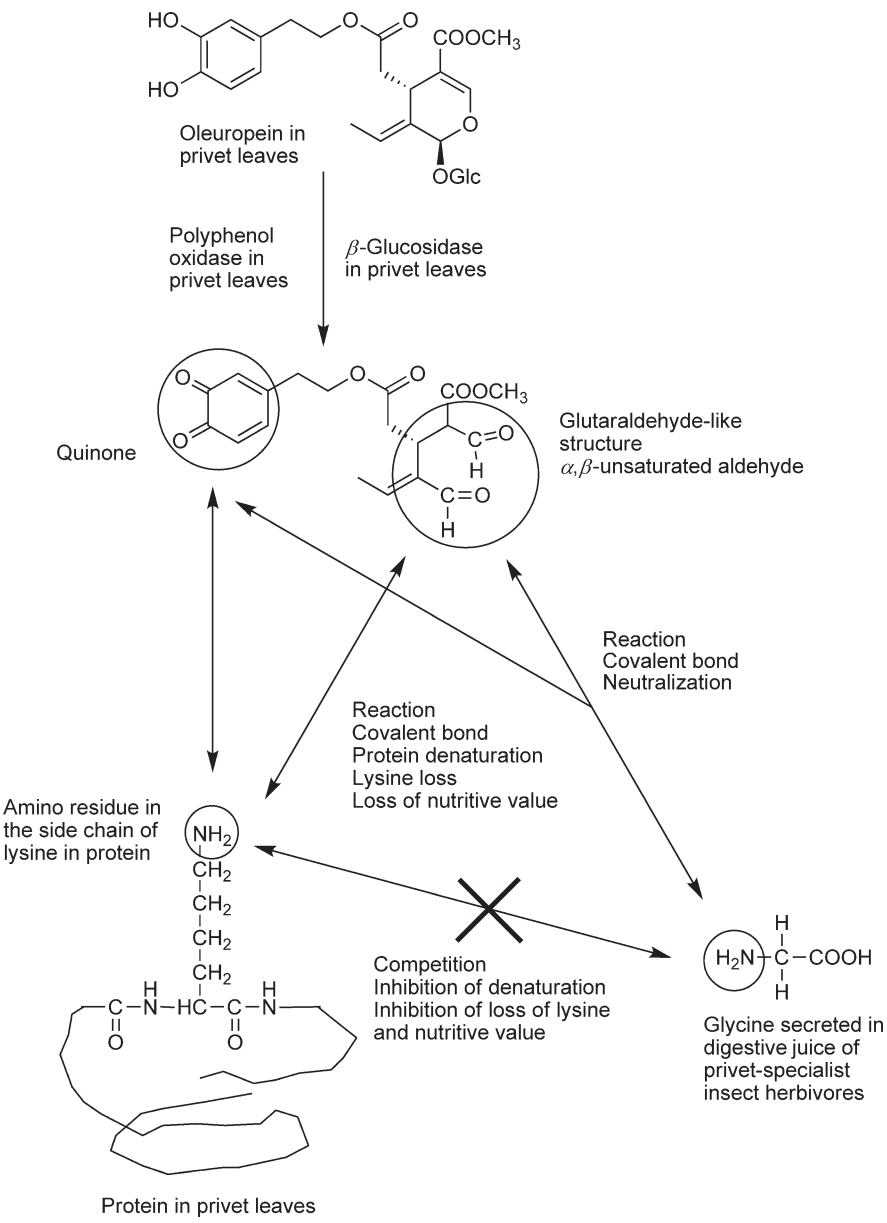


Figure 12 Enzymatic activation of oleuropein in the defense system of the privet tree *Ligustrum obtusifolium* and adaptation of privet specialist insects.

4.08.1.2.2 Defense proteins

Recently, the importance of defense proteins in plant defenses against herbivores has become more recognized. Although there have been some well-known defense proteins such as proteinase inhibitors (PIs), the progress in studies on defense proteins had been slow until recently compared with those on secondary metabolites involved in defense, probably because methodologies used in protein sciences and organic chemistry are very different. For example, before performing bioassays of defense substances, the chemist often performs leaf extraction with an organic solvent such as methanol; however, the same extraction may denature and inactivate defense proteins irreversibly. Recently, DNA technology and gene expression analyses showed that many genes are induced when plants are damaged by herbivores, and that quite a few of these genes code defense proteins.

4.08.1.2.2(i) Proteinase inhibitors Among defense proteins, the best known and widely distributed are PIs, which are also called protease inhibitors.[55,77,78] Serine proteinase inhibitors inhibit serine proteinases such as trypsin and chymotrypsin, the major digestive proteinases in lepidopteran and a part of coleopteran insects, and many of which are classified into Bowman–Birk inhibitors and Kunitz inhibitors according to the structures, but some, such as potato inhibitors, are not included in these classes.[77] Serine proteinase inhibitors are most abundant in storage tissues such as seeds and tubers, and their concentrations reach a few percent of the total protein. Legume seeds are a rich source of various serine proteinase inhibitors.

In foliage, the concentrations of serine proteinase inhibitors are lower than that in seeds; however, the induction of serine proteinase inhibitors has been reported in many plants including tobacco and tomato, and the inhibitors have been proven to function as a plant defense system.[79–81] Leaf damage by the tobacco hornworm *Manduca sexta* was much bigger in tomato plant line deficient in proteinase inhibitor induction than in those that can induce proteinase inhibitor on herbivory.[82]

Some lepidopteran larvae such as *Helicoverpa zea* and *P. xylostella* adapt themselves to plant PIs by changing digestive midgut proteinases from PI-sensitive ones to PI-insensitive ones when they are fed PI-containing diets.[83]

4.08.1.2.2(ii) Amylase inhibitors Amylase inhibitors are found in the seeds of plants such as cereal grains (wheat, maize, rice, barley) and legumes (kidney beans, cowpea, adzuki beans). Amylase inhibitors inhibit amylases of insects in general and inhibit the growth of insects, and thus serve as defense proteins in both cereal grains and bean seeds.[77] Seed weevils not specialized in feeding on the kidney bean cannot grow on this bean because their amylase is inhibited by amylase inhibitor in kidney bean, but seed weevils specialized in feeding on kidney bean can feed on this bean because their digestive proteinase can digest and inactivate amylase inhibitor in kidney bean.[84] Seed weevils utilize cysteine proteinases (CPs) instead of serine proteinases as their digestive proteinase, which enables seed weevils to feed on beans rich in serine proteinase inhibitors.[55]

4.08.1.2.2(iii) Proteinase Recently, proteinases have become recognized as plant defense proteins. In a maize line resistant to a lepidopteran pest, *Spodoptera frugiperda*, a unique 33-kDa cysteine proteinase, Mir1-CP, with chitin binding activity was found to accumulate in damaged foliage sites, move through vascular tissues, and confer defense on maize, *Zea mays*.[85,86] In addition to Mir1-CP in maize, it has been directly demonstrated that CPs such as papain and ficin in the latex of papaya and fig latex, respectively, inhibit the growth and are responsible for the strong lethal toxicity and defense activities of these plants against herbivorous insects.[87]

4.08.1.2.2(iv) Polyphenol oxidases and peroxidase Polyphenol oxidases (PPO), which occur in many plants together with phenolics, catalyze the oxidation of mono-, di-, and polyhydric phenols to quinones.[55] Quinones formed by these reactions are active and covalently bind to nucleophilic side chains (–SH, $–NH_2$, ═NH) of proteins and, as a result, lysine, histidine, cysteine, and methionine are lost from dietary proteins and the nutritive value of the protein is significantly reduced (**Figure 7**).[55,88] POD also catalyze the oxidation of phenolics and, as a result, oxidation products such as quinone and quinone methides are formed. These oxidation products also covalently bind to side chains of amino acids such as lysine and cysteine52 (**Figure 7**).[55]

4.08.1.2.2(v) Lipoxygenase Lipoxygenases (LOX), which are widely distributed in plant species and abundant in legume seeds such as soybean, oxidize polyunsaturated fatty acid containing a *cis*, *cis*-1,4-pentadiene moiety into conjugated dienoic hydroperoxides, which are further oxidized into epoxides, C6 aldehydes, malondialdehyde, hydroxynonenal (an α,β-unsaturated aldehyde), and jasmonic acid (JA), an important elicitor of plant defense genes (see Section 4.08.1.3.3(ii)).[55,81,89] These products bind covalently to amino acid side chains (–SH, $–NH_2$,═NH) and exert toxicity to insects including caterpillars and planthoppers.[55,89–92]

4.08.1.2.2(vi) Lectins Lectins are proteins that bind to specific sugars and are found in a wide variety of plant species, but are abundant in legume seeds.[93–96] Typical lectins have multiple binding sites in each molecule, and thus they have cell agglutinating property, but some lectins have single binding site. Lectins from kidney bean seeds (Phaseolus hemagglutinin (PHA)),[97] lectin from wheat germ (wheat germ agglutinin (WGA)),[98]

and lectin from snowdrop, *Galanthus nivalis* (snowdrop lectin, GNA),[92] are the three lectins whose chemical characters and toxicity against insects have been intensively studied.[93,95,96] PHAs specifically bind to α-GalNAc and GNA specifically binds to mannose. WGA specifically binds to GlcNAc β(1,4) GlcNAc and chitin.[93,96] Expression of GNA in a transgenic tobacco plant resulted in added protection against aphids, which suggested that plant lectins could be useful in the development of crops tolerant to pests.[99] PHAs and GNA bind to midgut epithelia and the binding is supposed to be the cause of toxicity of lectin against insects; however, the detailed mechanisms of toxicities are not clear.[93,95,96] WGA is supposed to exert its toxicity by binding to or by preventing the formation of peritrophic membrane in midgut lumen rich in chitin;[100] however, the detailed mechanism of toxicity is unknown.

4.08.1.2.2(vii) Jasmonic acid-inducible proteins: Arginase, threonine deaminase, and vegetative storage proteins Recently, several proteins induced by JA, a plant hormone that plays important roles in the induction of many plant defense responses and defense genes[81] (see Section 4.08.1.3.3(ii)), namely arginase, threonine deaminase,[101] and vegetative storage protein (VSP),[102] were proven to be plant defense proteins against insect herbivores. In tomato, *Solanum lycopersicum*, JA-inducible arginase and threonine deaminase degraded arginine and threonine, both of which are necessary for insect growth, and the tomato plant that overexpresses arginase was more resistant to *M. sexta* larvae[101] (**Figure 13**). VSPs, which are widely distributed in plants such as soybean and *A. thaliana*, and are shown to be induced by JA, are found to have an acidic phosphatase activity and have toxic effects to insects such as bruchid beetles and dipteran insects that have acid gut lumen.[102]

4.08.1.2.3 Temporally and spatially restricted distribution of defense substances

When we attempt to extract defense substances, sometimes we assume that the substances are ubiquitously present in plant tissues. However, recent studies show that quite often this is not the case. Without being aware of this fact, we might fail to detect and extract defense substances. Here, three types of such defense systems are introduced.

4.08.1.2.3(i) Enzymatic activation of precursors, compartmentation, and synergism of defense factors Some defense substances are kept in plants as precursors that do not have biological activities (or defense activities). The defense substances that are even hazardous and toxic to plants themselves are often kept in plant tissues as stable and safe precursors. The enzymes that convert or activate the precursors are

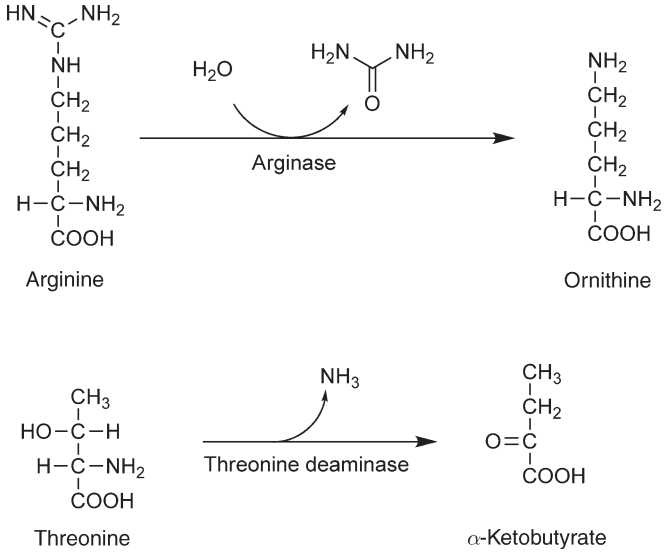


Figure 13 Degradation of amino acids by arginase and threonine deaminase, defense proteins induced in tomato leaves after insect herbivory.

kept in compartments separate from precursors. When plant tissues are damaged and the compartments are broken, activating enzymes come in contact with the precursors and activate them to bioactive forms.

Such compartmentation and enzymatic activation of precursors are commonly observed in several different groups of secondary metabolites,[103] which includes the activation of cyanide glycosides by β-glucosidase and hydroxynitrile lyase that produce toxic hydrogen cyanide in many plants such as sorghum, clover, and almond[34,35,37–40] (**Figure 4**), the activation of glucosinolates by thioglucosidase that produce toxic isothiocyanates or nitriles in plants belonging to the order Brassicales[43–45] (**Figure 5**), the activation of *O*-coumaric acid by β-glucosidase that produces coumarin in white melilot (*Meliotus alba*),[103] the activation of phenolics by PPO that produce highly reactive quinones in many plants[55] (**Figure 7**), and the activation of an iridoid glycoside, oleuropein, by β-glucosidase[73] (**Figure 12**).

Not only defense secondary metabolites but also defense proteins may exist as precursors. For example, papain, a cysteine proteinase that exists in papaya latex and recently found to have strong defense activity, is kept in laticifer as an inactive precursor and within 2 min after wounding, it becomes active.[104,105]

To obtain precursors in stable form, preheating leaves in steam before extraction is essential to inactivate the activating enzyme; however, in order to detect or reproduce bioactivity, activation of precursor by activating enzyme is needed.[73,106]

In compartmentation systems (or precursor activation systems), precursors and activating enzymes work synergistically to exert plant defense; however, other kinds of synergism were observed. For example, chemicals that enhance the toxicity of furanocoumarin by inhibiting detoxifying enzyme of insects (i.e., cytochrome P-450 monooxygenase) coexist with furanocoumarin in plants.[53]

4.08.1.2.3(ii) Plant tissues specialized in defense and localization of defensive substances Defense substances are not always distributed evenly throughout plant tissues. In practice, there are some tissues specialized in defense against insects in which the defense substances are very abundant, whereas in other tissues the defense substances are almost absent. Glandular trichomes and latex (laticifer) are examples of such defense-specialized tissues.

Glandular trichomes are typically hair-shaped glands (some are peltate) on plant surface that exude solution containing defense substances.[103,107,108] Some glandular trichomes constantly exude solution, whereas others contain solution and rupture when insects contact. The glandular trichomes are common in families such as Solanaceae, Labiatae, and Asteraceae. The exudates from trichomes on tobacco leaves contain alkaloids such as nicotine and anabasine, and are toxic to aphids and tobacco hornworm *M. sexta*.[108,109] The resistance of wild tomato, *Lycopersicum hirsutum*, to lepidopteran larvae is due to 2-tridecanone and 2-undecanone, both of which are contact toxins to lepidopteran larvae and which are contained in the exudates of glandular trichomes.[107,108,110,111] Similarly, the exudates of peltate glandular trichomes in mint, *Mentha* × *piperita*, contain essential oil consisting primarily of *p*-menthane-rich monoterpenes such as menthol, which are toxic to insects.[45,112] In both tomato and mint cases, the localization, characterization, and/or genes of synthetic enzymes in trichomes have been studied in detail.[111,112]

Plant latex is a sap that is exuded from damaged vein. It is kept in a tissue specialized in exuding latex called laticifer and is kept inside laticifer cells. It works as an efficient defense system against herbivorous insects.[113–115] Resin, which is kept outside the cells, and exudate, which is transparent, and the contents of oil duct exudates are similar to latex in appearance and biological functions. Latex, exudates, and resin are found in more than 35 000 species of plants.[116] Latex has been shown to contain a wide variety of bioactive substances, both secondary metabolites and proteins, in condensed form.[113,116] Some of the latex substances have been shown to be toxic to insects, such as cardenolides in milkweed latex,[66] but the toxicities of latex ingredients against insects have not been tested in most latex-exuding plants. However, recently, more and more latex ingredients have been proven to have toxicity against insects and serve as defense against insect herbivores. The ingredients of plant latex shown to have defense activities are as follows.[113,116] Cardenolides or cardiac glycosides, a group of glycosylated steroids that are inhibitors of $Na^+ - K^+$ ATPase of animals, exist in high concentrations in the latex of Asclepidaceae and Apocynaceae[66,67] and are highly toxic to animals including insects and serve as plant defense. Morphine and other alkaloids in the latex of poppy (*Papaver somniferum*) and plants of poppy families, whose synthetic mechanism and localization have been recently elucidated in detail,[117] are toxic to animals. Furanocoumarins such as xanthotoxin abundant in oil duct exudates of wild

parsnip *Pastinaca sativa*[118] are toxic to insects in general. Recently, high titer of CPs such as papain and ficin in papaya and fig latex, respectively, are experimentally proven to be defense proteins that are highly toxic to herbivorous insects.[87] As CP and other types of proteinases are often found in plant latex such as latex of Asclepidaceae species,[119] those proteinases could also function as plant defense. Latex that exudes from mulberry leaves, which have been used as a suitable host plant of the silkworm *Bombyx mori*, is recently found to function as effective defense to nonadapted insects because of latex ingredients toxic to nonadapted insects, namely high concentrations of sugar-mimic alkaloids such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) and DNJ (**Figure 1**), which altogether make up 2.5% of wet latex and 18% of dried latex, and a novel unidentified defense protein.[22] Latex-borne defense is unique in that even though the total amount of toxin that a latex-exuding plant individual contains is small, and thus the average concentration of toxin is low, for herbivorous insects small in size, a large amount of condensed toxin that moved through the laticifer rushes out at the very point of insect damage immediately after the damage. This means that the amount of toxin that the insects encounter and the defense effect of toxins in latex on insects would be much greater than those estimated from average concentration. For example, the average concentrations of sugar-mimic alkaloids in mulberry leaves are as low as 0.1–0.01% of leaf dry weight; however, herbivores feeding on mulberry latex would encounter latex containing 2.5% wet weight and 18% dry weight of sugar-mimic alkaloids.[22] Similarly, the average titer of papain in papaya leaves is much lower than the lethal dose, and the titer of papain in latex that insects encounter is much higher than the lethal dose.[87]

It is clear from the above discussion that if we do not know about the localization of defense substances in specialized tissues like latex and glandular trichomes, adopt extraction methods disregarding the localization, and rely on average concentration, we may underestimate or may even overlook the biological effects and the existence of defense substances in defense tissues. On the contrary, the plant substances found in such defense-specialized tissues are very likely to have roles as defense substances.

4.08.1.2.3(iii) Induction of defense substance The induction of defense substance (i.e., increase in the amount of defense substance in response to herbivory) was first reported in 1970s, where PIs were found to be induced in plant leaves after wounding and herbivory.[79] At present, induction of defense substances has been reported in more than 100 plant species belonging to 34 families,[81] and the substances reported to be induced include defense proteins such as PIs,[79] cysteine proteinases (Mir1-CP),[85,86] lipoxygenase,[91,120] and secondary metabolites involved in defense such as tannins,[121] phenolics,[121] tobacco alkaloids (nicotines),[122,123] glucosinolates,[124] and phytoecdysteroids.[65] Now, induction of defense substances is considered a common phenomenon. The extent of induction differs among each plant species and even within the same species. For example, nicotine in tobacco leaves increases after herbivory only twofold the original concentration before herbivory[123] and PIs are practically absent from undamaged tobacco leaves and increase to high titer after induction.[82]

It is generally believed that in condition where herbivory is regularly expected, constitutive defense is more adaptive, whereas in condition where herbivory is expected only once in a while, it is better for plants to produce defense substances when plants are damaged by herbivory, and when there are no herbivores, it is better to invest resource to plant growth rather than defense. This idea is supported by field experiments using plant disabled to induce defense substance by gene modification techniques, and comparing seed production between plant deficient in induction and the control type.[123]

Induced defense reaction is also triggered by oviposition. When rice hoppers lay eggs on rice blades, exudates containing benzyl benzoate exude around the eggs and kill them.[125] Induction of defense substances triggered by insect herbivory is partly reproduced by mechanical damage[126] or application of insect saliva to the leaves.[127] Even though a single artificial damage using razor blade or pattern wheel may induce defense substance, it was recently found that continuous multiple damages better mimic the damage of insects and cause responses that are very close to real herbivory, and such continuous multiple damages and other types of damage, different in magnitude, speed, and patterns, can be made using a mechanical caterpillar, MecWorm that has been designed in a computer-programmed manner to very closely resemble the herbivore in damages caused to leaves.[128] In many plant species, insect damages induce a plant hormone, JA, that mediate the defense responses in plants, including induction of many defense substances (see Section 4.08.1.3.3(ii)).[81,126] Spraying JA on plants can induce defense substances that are induced by insect feeding.[65,81,126]

Recently, using model plants such as *A. thaliana*, tobacco, tomato, and maize, the profiles of gene induction after treatment such as insect feeding, JA application, and mechanical damage were intensively analyzed using gene technologies such as microarray techniques.[129–131] Some of the induced gene products (proteins) may be involved in signal transduction, others in the synthesis of secondary metabolites involved in defense, and the rest may work as defense proteins. In practice, gene products of two genes that had been known to be induced by JA in tomato, namely arginase and threonine deaminase, were recently proven to function as defense proteins by degrading amino acids in diets and by decreasing the nutritive value[101] (**Figure 13**). Similarly, it is likely in future that more novel defense proteins or proteins involved in the synthesis of secondary metabolites involved in defense would be found among the products of such genes induced by herbivory, mechanical damages, saliva treatment, or JA treatment.

4.08.1.3 Indirect Defense of Plants against Herbivores

Indirect defense of plants against herbivores is another type of defense that involves all the ways by which plants enhance the effectiveness of natural enemies of the herbivores. There are three types of indirect defense of plants against herbivores: (1) offering shelters to carnivores, (2) offering alternative foods to carnivores, and (3) attracting carnivores by emitting the so-called 'herbivore-induced plant volatiles (HIPVs)'. Because the focus of this chapter is on the role of chemicals in plant defense, the second and the third type of indirect defense will be discussed here.

4.08.1.3.1 Offering food to carnivores

Secretion of extrafloral nectar (EFN) is shown to act as an indirect defense against herbivores. EFN is used by carnivorous natural enemies such as ants. Some plant species, including lima bean plants, increase EFN in response to herbivory.[132–135] Further, Heil[135] reported that the production of EFN by lima bean plants increased after the exogenous application of JA, which is an important plant hormone regulating a defense signaling pathway against herbivores and pathogens. The components of EFN have been reported in several plant species. For example, Choh *et al.*[136] reported EFN of lima bean plants contained fructose, glucose, and sucrose as major sugars. Interestingly, *T. urticae*-infested lima bean plants contained smaller amounts of sucrose and larger amounts of fructose in EFN than uninfested plants. Whereas EFN provides fuel for survival and search, plants can also provide predatory arthropods with alternative food containing nutrients (amino acids and lipids) required for development and reproduction. Central American Acacia trees stand out as a landmark example.[137] Apart from providing nesting sites (in enlarged, hollow stipular thorns) to certain ants and nectar from large foliar nectaries, they also produce protein- and lipid-rich organs called Beltian bodies on the leaf pinnules. These food bodies are eagerly harvested by foraging ants and fed to their larvae. The ants in turn kill insect herbivores, repel mammalian herbivores, and destroy plants interfering with the Acacia tree. A major question is how plants benefit from producing such nutritive foods, even when they cannot prevent organisms harmful to the plant from using it. For example, protein-rich pollen of several plants can be utilized by many predatory mites (e.g., *Iphiseius degenerans*), but also by the herbivorous arthropods they prey on (e.g., Western flower thrips, *Frankliniella occidentalis*). Despite the danger of herbivores reaping the benefits, plants still profit because any surplus prey boosts predator populations and because pollen are usually provided locally, which allows predators to occupy the site and repel herbivores. Thus, by the distribution of pollen over the plant, plants influence the degree to which predatory mites can monopolize pollen as an alternative food source at the expense of the herbivore. It is an entirely open question how plant-provided foods affect the motivation of predators to capture herbivores. Nutritive deficiencies in the alternative foods could stimulate the predators to maintain a predatory lifestyle.

4.08.1.3.2 Attracting carnivorous natural enemies by using herbivore-induced plant volatiles

Plants respond to herbivory by producing volatiles that in turn attract carnivorous natural enemies of the herbivores. These volatiles are produced by the plants as a specific response to herbivore damage or mainly as a result of mechanical damage. These so-called HIPVs attract carnivores, which in turn reduce the damage caused by herbivorous arthropods. These chemical alarm calls thus represent an example of induced indirect

defense of the plant against herbivores. HIPVs may vary quantitatively and/or qualitatively depending on herbivore species, plant species, and growth conditions of plants and attacking herbivores,[1,2,138] suggesting that HIPVs may convey information on the status of damage of plants.

4.08.1.3.2(i) Tritrophic interactions of plant–spider mite–carnivorous natural enemy Spider mites (Tetranychidae) are polyphagous herbivores that have a severe impact on plants due to their explosive population dynamics in the absence of their carnivorous natural enemies such as predatory mites and predatory insects. In many plant–spider mite–carnivorous natural enemy interactions, infested plants attract natural enemies by producing a blend of volatiles – several terpenoids, phenolics, green leaf volatiles – that differ from the volatiles emitted from intact plants. A well-studied example is a tritrophic system consisting of lima bean plants (*Phaseolus lunatus*), herbivorous two-spotted spider mites (*Tetranychus urticae*), and predatory mites (*Phytoseiulus persimilis*).[139] In this system, infested lima bean leaves emitted more than 100 *T. urticae*-induced volatiles. Among them, linalool, (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and methyl salicylate attract the specialist predatory mite *P. persimilis*[140] (**Figure 14**). The generalist predatory mite *Neoseiulus californicus* also preferred volatiles from lima bean leaves infested with *T. urticae* than from intact lima bean leaves, but this predator was attracted by other volatiles such as linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate[141] (**Figure 14**). The predatory mite *N. womersleyi* is attracted only to previously experienced plant volatiles and not to volatiles they have not experienced.[142] The predatory mites reared on *Tetranychus kanzawai*-infested tea leaves showed significant preference for a mixture of three synthetic compounds (mimics of the *T. kanzawai*-induced tea leaves volatiles: (*E*)-β-ocimene, DMNT, and (*E,E*)-α-farnesene)[142] (**Figure 14**). However, mixtures lacking any of these compounds did not attract the predatory mites. Likewise, *N. womersleyi* reared on *T. urticae*-infested kidney bean plants showed a significant preference for a mixture of four synthetic compounds (mimic of the *T. urticae*-induced kidney bean plant volatiles: DMNT, methyl salicylate, β-caryophyllene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene)[142] (**Figure 14**). The lack of any of the four compounds resulted in no attraction.

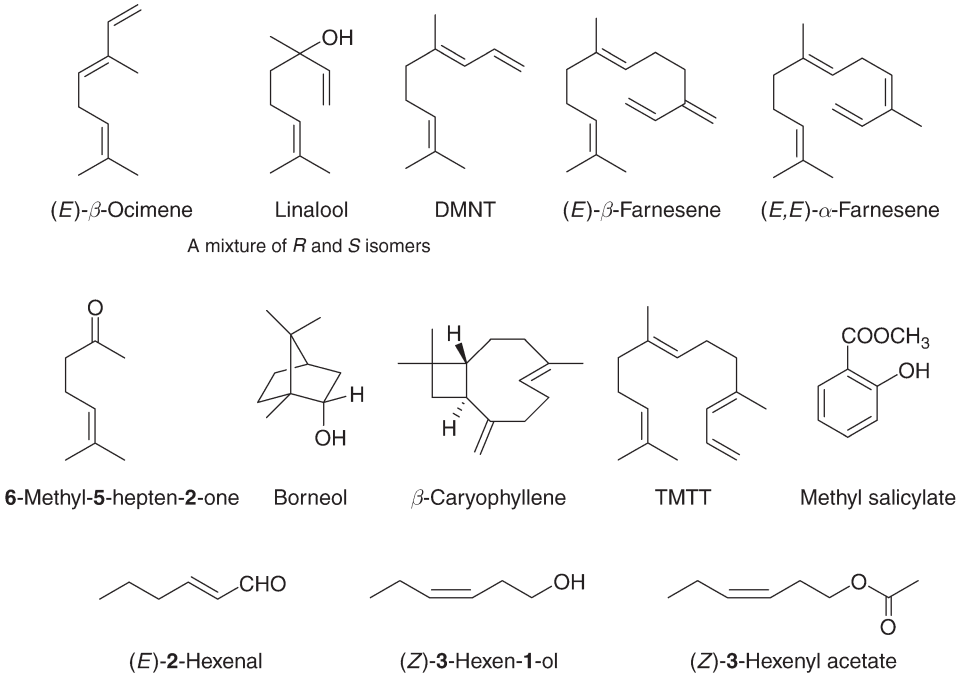


Figure 14 Volatile compounds that attract carnivorous natural enemies of herbivores.

4.08.1.3.2(ii) Tritrophic interaction of plant–herbivorous insects–parasitoid The volatiles emitted from plants infested with caterpillars and aphids can also attract parasitic wasps of the herbivore. For example, maize plants infested with the noctuid larvae *Mythimna separata* emit volatiles that attract a specialist parasitic wasp *Cotesia kariyai* when volatiles from intact plants or artificially damaged plants are the alternative. These attractive volatiles include terpenoids, green leaf volatiles, indole, oxime, and nitriles.[143] The composition of the volatiles is specific for the larval stage damaging the plant. Among them, a blend of four chemicals, geranyl acetate, β-caryophyllene, (*E*)-α-farnesene, and indole, elicited a response in naive *C. kariyai*, but making them to learn to associate these volatiles with a reward did not enhance this response.[144] A blend of five chemicals, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, β-myrcene, and linalool, which are known to be released not only from plants infested with the host larvae, but also from artificially damaged plants or undamaged ones, elicited little response in naive wasps, but making them to learn to associate these volatiles and a reward significantly enhanced the wasp's response.[144] Both the host-induced and nonspecific volatile compounds appear to be important for *C. kariyai* females in host location. A tritrophic system of the parasitoids *Cotesia marginiventris*, host larvae *Spodoptera* spp. and maize plants is also a well-studied system (see D'Alessandro *et al.*[145] and citation therein).

Cabbage plants infested with cabbage white butterfly larvae *P. rapae* emit volatiles that attract a parasitic wasp *Cotesia glomerata.*[146] In this system, the attraction is not specific; the wasp is also attracted to either artificially damaged cabbage plants or cabbage plants infested with nonhost larvae (*P. xylostella*).[146] In fact, *Cotesia glomerata* is attracted to products of the lipoxygenase pathway, that is, to green leaf volatiles such as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl acetate (**Figure 14**), that are induced in response to mechanical damage of leaf tissue.[147]

Psylla-infested pear trees attract anthocorid predators by release of at least two volatiles, (*E*,*E*)-α-farnesene and methyl salicylate.[148] A similar example exists for whiteflies (*Trialeurodes vaporariorum*). They induce four *de novo* emitted volatiles in beans and three of these elicit oriented flight and landing of *Encarsia formosa* when offered in pure form, most effective being a mixture of (*Z*)-3-hexen-1-ol and 3-octanone.[149] Plants infested with aphids also emit induced volatiles that attract parasitic wasps. For example, *Aphidius ervi* females were attracted to volatiles emitted from broad bean plants infested with host aphid *Acyrthosiphon pisum*, but not to those from plants infested with nonhost aphid *Aphis fabaei.*[150] 6-Methyl-5-hepten-2-one, linalool, (*Z*)-3-hexenyla acetate, (*Z*)-3-hexenol, (*E*)-β-ocimene, and (*E*)-β-farnesene separately attract the wasps[151] (**Figure 14**). 6-Methyl-5-hepten-2-one is most attractive and is found in the headspace of host-infested plants but not in that of nonhost-infested plants.[151] An aphid parasitoid *Diaeretiella parae* female is attracted to 3-benzyl isothiocyanate of host plant origin.[152] Some parasitic flies are also attracted to plant volatiles. Borneol attracts a tachinid fly *Cyzenis albicans* in the field[153] (**Figure 14**). A parasitic fly *Exorista japonica* is attracted to maize plants infested with common armyworms (*Mythimna separata*),[154] but the chemical nature of the attractants is now under investigation.

4.08.1.3.2(iii) HIPVs affect neighboring plant's indirect defense In response to volatiles emitted from herbivore-infested plants (HIPVs), neighboring intact plants enhance either their direct defense (i.e., becoming a less suitable resource for herbivores[155–158]) or their indirect defense (i.e., attracting carnivorous natural enemies of herbivores[159–162]). Such responses of plants prior to biotic stress are called priming. A well-studied example of the latter case is a tritrophic system consisting of lima bean plants (*Phaseolus lunatus*), herbivorous two-spotted spider mites (*Tetranychus urticae*), and predatory mites (*Phytoseiulus persimilis*). Lima bean plants infested with *T. urticae* emit HIPVs that in turn attract *P. persimilis.*[159] When intact lima bean plants are exposed to volatiles from conspecific plants infested with *T. urticae*, they become more attractive to *P. persimilis.*[159] This increased attractiveness was explained by the fact that uninfested lima bean leaves that were exposed to volatiles from infested conspecific leaves could adsorb the volatiles and re-emit them.[161] Choh *et al.*[136] further reported that HIPV-exposed intact plants produced more EFN compared to control plants (plants exposed to volatiles from intact plants). It remains unanswered how the mechanisms involved in the production of components of EFN differ between infested plants and HIPV-exposed plants.

Bate and Rothstein[163] showed that (*E*)-2-hexenal induced several defense-related genes such as *chalcone synthase*, *AOS*, and *lipoxygenase 2* (*LOX2*) in *A. thaliana.* Arimura *et al.*[164] also showed that (*E*)-2-hexenal or

(*Z*)-3-hexenol induced genes encoding basic pathogenesis-related proteins (PRs), LOX, or phenylalanine ammonia lyase in lima beans. In addition, it has been reported that (*E*)-2-hexenal induced phytoalexin accumulation in cotton plants (*Gossypium hirsutum* L.). Recently, Gomi *et al.*[165] reported that (*E*)-2-hexenal induced genes encoding AOS, LOX, and HPL, and (*E*)-2-hexenal enhanced resistance of citrus tree (*Citrus jambhiri*) against *Alternaria alternata*. Vancanneyt *et al.*[166] also reported that antisense-mediated deletion of HPL in transgenic potato (*Solanum tuberosum* cv. Desiree) led to an increase in aphid performance. These findings suggest that C6-aldehydes function as signals to initiate defense responses of undamaged plants.

4.08.1.3.2(iv) Do entomopathogens and non-arthropod insectivores use HIPVs as a signal? Predatory arthropods (phytoseiid mites and heteropteran bugs) and parasitoid wasps have been best explored for their responses to HIPVs, whereas other classes of natural enemies have received little attention. Some major advances have been achieved recently with respect to entomopathogens. Van Tol *et al.*[167] were the first to show that indirect plant defenses also operate underground. They found that the roots of a coniferous plant (*Thuja occidentalis*) release chemicals upon attack by vine weevil larvae (*Otiorhynchus sulcatus*) and that these chemicals attract nematodes that parasitize weevils (*Heterorhabditis megidis*). Hountondji *et al.*[168] were the first to show that volatiles emanating from cassava plants infested with green mites (*Mononychellus tanajoa*) trigger production of conidia, the infectious stage, in different isolates of a mite-pathogenic fungus (*Neozygites tanajoae*), whereas volatiles from clean plants suppress conidiation. These opposing effects make sense in that the entomopathogenic fungus tunes the release of conidia, the stage most vulnerable to environmental conditions, to herbivore-induced plant chemicals that signal the presence of hosts.

4.08.1.3.3 Mechanisms involved in the production of herbivore-induced plant volatiles

There are several lines of evidence for an active role of the plant in releasing HIPVs: (1) whereas many natural enemies are attracted to plants harboring herbivores as prey, herbivorous arthropods and some of the products they deposit on the plant (e.g., feces, silk) can be eliminated as the source of volatiles found in blends of HIPV;[169] (2) the compounds identified in blends of HIPV have been reported to occur in plants;[140,148,151,170] (3) pathways for biosynthesis of compounds in blends of HIPV exist in plants and are shown to be inducible by herbivory;[170–172] (4) blends of HIPV are not only emanated from the leaf under attack by the herbivore, but are also systemically induced by the plant;[173–175] (5) application of the phytohormone JA to wild-type plants or defense-signaling mutants (e.g., JA) leads to induction of volatiles similar to HIPV;[176–181] (6) herbivore-induced gene expression patterns in plants are similar to those mediated by jasmonates;[181–184] (7) elicitors of HIPV synthesis in plants have been found in the regurgitate/saliva of herbivores (*N*-(17-hydroxylinolenoyl)-L-glutamine or volicitin) (**Figure 15**),[185,186] β-glucosidase,[127,177] and inceptin ($^{+}$ICDINGVCVDA^{-}).[187]

4.08.1.3.3(i) Insect factors affecting the production of HIPVs One of the striking features of HIPV as a means to attract natural enemies is that there are usually several compounds involved and that these mixtures of volatiles contain a great deal of specific information: the plant provides predators with information that is sufficiently specific to allow discrimination by olfaction. This hypothesis was confirmed by several studies.[146,174,188–190] For example, De Moraes *et al.*[189] carried out a study on olfactory responses of the parasitoid *Cardiochiles nigriceps* to three species of host plants (tobacco, maize, and cotton) that were attacked by two closely related herbivore species, the tobacco budworm (*Heliothis virescens*) and the corn earworm (*H. zea*). These authors found that tobacco, cotton, and maize plants each released distinct volatile blends in response to damage by the two herbivore species, and that the parasitoid exploits these differences in odor blends to distinguish infestation by its host *H. virescens* from that by the nonhost *H. zea*.

Figure 15 Volicitin from the oral secretion of *Spodoptera exigua* larvae.

How do plants manage to respond differently to different herbivore species? One of the possible cues is in the oral secretion of herbivores. In fact, several classes of elicitors of insect origin have been isolated. For example, volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine) (**Figure 15**) has been shown to induce the production of HIPVs in maize when applied to wounded parts.[185] Volicitin is, however, not generally active. Leaves of lima bean, for example, do not respond to volicitin with the induction of volatile emission.[190] Other fatty acid conjugates have been isolated from the regurgitate of several caterpillars.[191,192] For example, *N*-linolenoyl-glutamine in the regurgitate of the tobacco hornworm *M. sexta* was characterized as a potential elicitor of volatile emission in tobacco plants.[189]

The other elicitor of insect origin that induces the production of HIPVs is β-glucosidase present in the oral secretion of cabbage white butterfly larvae *Pieris brassicae*.[127] Cabbage plants infested with *P. brassicae* larvae attract a parasitic wasp *C. glomerata*. Although this plant emits the same volatiles as those emitted when damaged mechanically, it does so in larger quantities and for a longer time. In this system, major volatiles involved are fatty acid derivatives. The elicitor β-glucosidase also induces the production of homoterpenes ((*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) in lima bean plants and maize.[176] Recently, a disulfide-bridged peptide called inceptin ($^{+}$ICDINGVCVDA^{-}) was isolated from the oral secretion of *Spodoptera flugiperda* larvae.[187] Inceptin promotes ethylene production by cowpea and triggers an increase in the defense-related phytohormone salicylic acid and JA.

Although elicitors from insect oral secretions have received special attention, the quality and quantity of HIPVs may also be affected by insect feeding behavior such as continuous or interrupted leaf chewing, phloem sucking, and even egg deposition.[193,194] Recently, it was shown that continuous mechanical wounding was sufficient to induce local as well as systemic emission of volatiles that are emitted as HIPVs.[195] Thus, the role of insect elicitors in HIPV production still remains largely an open question.

4.08.1.3.3(ii) Signaling pathways involved in the production of HIPVs in plants The oxylipin pathway in plants (**Figure 16**) is known to be involved in the production of HIPVs. In the oxylipin pathway, an LOX generates either 9- or 13-hydroperoxide from unsaturated fatty acids such as linoleic and linolenic acids.[196] It serves as a substrate for two main pathways in the oxylipin metabolism. One is the allene oxide synthase (AOS) pathway, which leads to the formation of JA and methyl jasmonate (MeJA) (**Figure 16**). JA and MeJA play essential roles both in the responses against biotic/abiotic stresses and in plant development.[197] Further, JA and MeJA are involved in the production of compounds regarded as HIPVs. Hopke *et al.*[176] reported that most of *T. urticae*-induced volatiles could be triggered by treatment of lima bean plants with solutions of JA. Dicke *et al.*[198] also reported that damage by *T. urticae* and application of JA had similar, although not identical, effects on the composition of induced volatiles in lima bean leaves and the attraction of a carnivore (*Phytoseiulus persimilis*). Ozawa *et al.*[177] reported that in lima bean leaves, the JA-related signaling pathway is involved in the production of volatiles induced by armyworms (*M. separata* and *S. exigua*). Furthermore, in corn plants (*Z. mays*), JA triggers the emission of all volatiles that are known to be emitted in response to damage by *S. exigua*.[176]

JA and ethylene act synergistically in response to wounding.[199] Arimura *et al.*[200] found that lima bean plants infested with two-spotted spider mites (*T. urticae*) showed emission of ethylene. Synergistic effects of ethylene on JA-induced volatile production in lima bean leaves are reported.[201]

Another pathway is the fatty acid hydroperoxidase lyase (HPL) pathway. This pathway produces C6-aldehydes and C12-oxo acids.[202] C6-volatiles, including (*E*)-2-hexenal, (*Z*)-3-hexenal, hexanal, as well as their corresponding alcohols or esters, are produced from mechanically wounded plant tissue.[197,203] C6-aldehydes are also formed during hypersensitive response to infection by bacterial pathogens, after insect feeding, and after exogenous application of JA.[204,205] As already shown in this chapter, some of C6-volatiles are known to be attractants of parasitoids and predators.

Salicylic acid (SA) is an endogenous signal implicated in eliciting plant resistance. SA is known to act as a signal for systemic acquired resistance (SAR) in pathogen-infected plants.[206] SA levels in resistant tobacco increased in both the tobacco mosaic virus (TMV)-inoculated and uninoculated leaves of the same plant; the rise in SA levels preceded the induction of PR (Pathogenesis Related) genes in the uninoculated leaves. Exogenously supplied SA also induces the same genes as those that are activated systemically upon TMV infection.[207] Ozawa *et al.*[177] showed that gaseous treatment of MeSA induces some of HIPVs in lima bean leaves. They suggested that both SA signaling pathway and JA signaling pathway are involved in the production of *T. urticae*-induced volatiles.

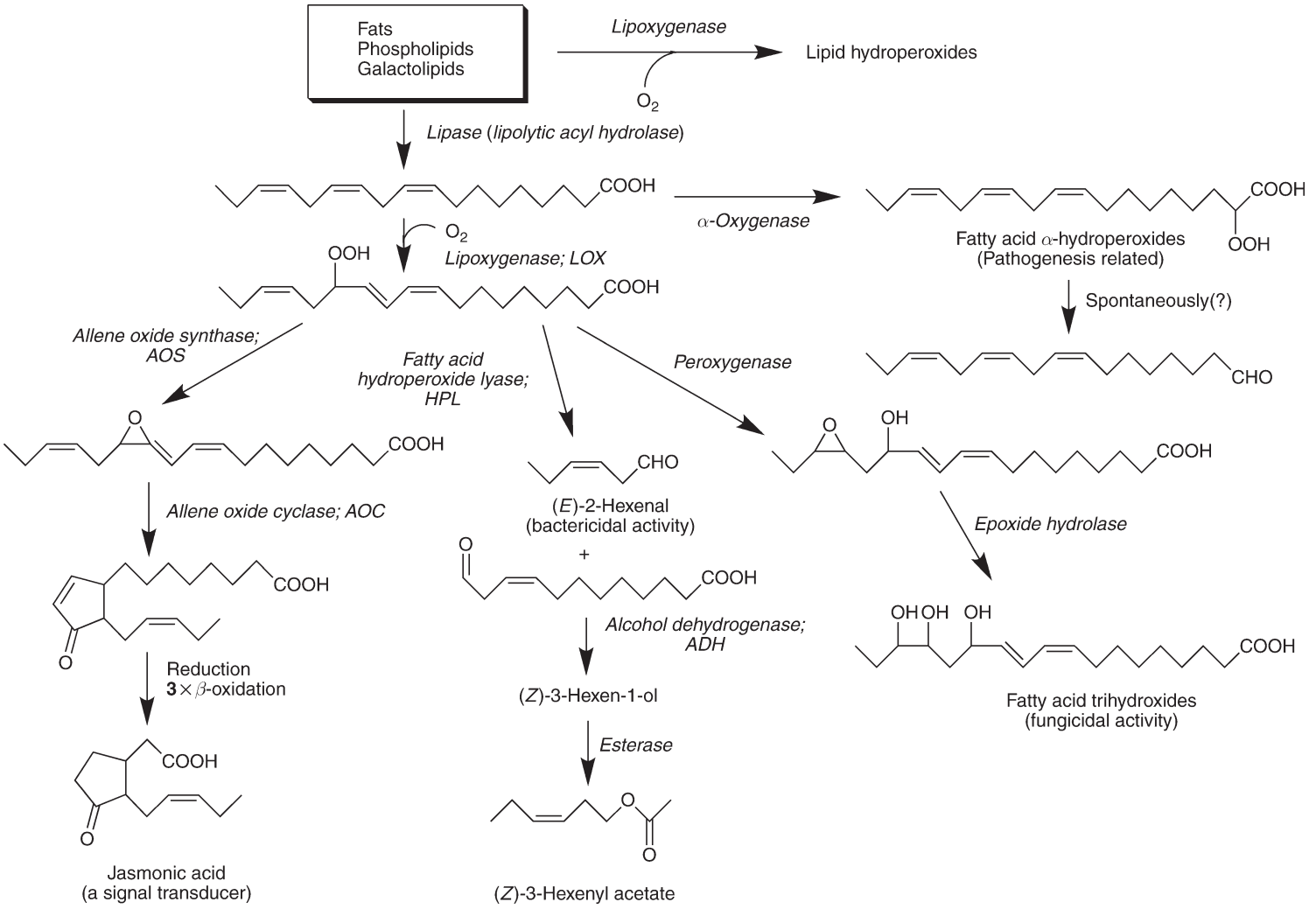


Figure 16 Phytooxylipin pathway.

4.08.1.3.3(iii) Cross talks Ozawa *et al.*[177] observed that the *T. urticae*-specific blend of HIPV from lima bean plants is different from that induced by exogenous JA. They discussed the possibility that this specificity arises from the interaction between JA and SA signaling pathways in *T. urticae*-infested leaves. In general, the upregulation of SA biosynthesis suppresses the production of JA, which is required for the general stimulation of induced volatile biosynthesis. Thus, the intensity of upregulation of SA biosynthesis might cause the blend of HIPVs in *T. urticae*-infested lima bean leaves to be unique. The cross talk between JA biosynthesis and SA biosynthesis caused by herbivory could be one of the important mechanisms for the production of herbivore-specific blend of HIPV. Specificity of HIPV blends arises especially from cross talk and feedback between different signaling pathways.

4.08.1.4 Coordination of Direct and Indirect Defense Responses

Effective plant defense requires coordination of direct and indirect defenses such that they do not interfere or even act synergistically. A relevant example comes from studies by Baldwin,[208] who wondered why the nicotine defense of wild tobacco is downregulated by an ethylene burst following attack by young tobacco hornworm larvae. He formulated three – as yet untested – hypotheses to explain this.

First, the hornworm larvae may suppress the plant's nicotine response or they may reduce their food intake and therefore run less risk to alert the plant's nicotine response.

Second, the tobacco plant may improve the impact of its indirect plant defenses by reducing possibilities for hornworm larvae to sequester nicotine as a defense against predators and parasitoids, and by slowing down hornworm growth (through production of digestion inhibitors), thereby causing prolonged exposure of hornworm larvae to their predators. In this way, the plant saves fitness costs from investment in nicotine defense and gains more protection from indirect defense.[209]

Third, the plant may suppress the nicotine response until the hornworm larvae reach a size (i.e., fourth or fifth instar) where they impose a serious death risk to the tobacco plant and then switch to produce nicotine to make these large larvae move to their neighboring competitors.[210]

These three hypotheses – herbivore stealth, indirect defense optimization, and herbivores as allies in plant competition – do not necessarily exclude each other. If the plant first chooses to optimize indirect defense against young hornworm larvae, it may switch to direct defense by the time the hornworm larvae are large enough, thereby deterring the larvae and imposing immediate and present danger to neighboring (=competing) plants. This defense scenario is not likely to be general because the best scenario depends on the efficacy of alternative defenses. There are examples of plants switching on direct defenses in response to feeding by caterpillars even though this reduces the suitability of the caterpillars as hosts for parasitic wasps.[211,212]

4.08.2 Antimicrobial Chemical Defense

4.08.2.1 Introduction

Higher plants produce a remarkably diverse array of secondary metabolites that are involved in defense against pathogens,[4] although antimicrobial proteins such as PRs[213] are also involved in defense. Antimicrobial secondary metabolites can be preformed in the plant, the so-called 'constitutive antimicrobial compounds', or they are induced after infection through *de novo* enzyme synthesis, the 'phytoalexins'.[214] It is often difficult to determine whether compounds are phytoalexins or constitutive antimicrobial compounds, as the distinction between them is not always clear. In addition, some compounds are phytoalexins in one organ and constitutive in another organ of the same plant species. Some of these secondary metabolites are implicated in defense against not only microbial attack, but also herbivore/animal predation. Several books and reviews on constitutive antimicrobial compounds and/or phytoalexins have been published.[4,214–219] This section will focus mainly on the chemical structures and distribution of constitutive antimicrobial compounds and phytoalexins in higher plants. In addition, recent findings from biosynthetic studies of antimicrobial compounds will be summarized.

4.08.2.2 Constitutive Chemical Defense

The first chemical barrier to microbial infection is the constitutive presence of antimicrobial substances that occur in sufficient concentrations to represent a barrier to infection. The constitutive antimicrobial compounds are largely divided into two groups, preinfection plant metabolites, which are normally present in concentrations high enough to inhibit the growth of pathogens, and antimicrobial metabolites produced by plants in response to infection, but whose formation does not involve *de novo* biosynthetic enzyme synthesis. The latter metabolites are normally present in plants in an inactive, bound form, but are converted into the active form after infection by means of a short and simple biochemical reaction such as enzymatic hydrolysis. VanEtten *et al.*[220] suggested that all these constitutive antimicrobial compounds be called 'phytoanticipins', which can be defined as low-molecular-weight antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from pre-existing constituents. The structurally diverse array of constitutive antimicrobial compounds can be classified into several groups such as terpenoids, nitrogen-containing compounds, and aromatics.

Terpenoids: In *Nicotiana* spp. (Solanaceae), the diterpenoids sclareol, episclareol, and 2-ketoepimanool (**Figure 17**) on the leaf surface have been identified as constitutive antimicrobial compounds.[221] Another series of diterpenoids, α- and β-4,8,13-duvatriene-1,3-diol (**Figure 17**), have been identified from *N. tabacum* leaves, where they appear to play a role in resistance to blue mold.[214,222] Diterpenoid resin acids such as 7-ketodehydroabietic acid and 7-hydroxyabietic acid have been identified from the needles of *Pinus radiata* (Pinaceae) (**Figure 17**). These compounds inhibit both spore germination and mycelial growth of a pine pathogen.[214] The diterpenoid momilactones A and B accumulate in rice seeds,[223] although they are induced in rice leaves as phytoalexins,[224] as described in Section 4.08.2.3. In rice, the antimicrobial diterpenoid oryzalide A (**Figure 17**) and related compounds have been identified in healthy rice leaves, but were found to further increase after inoculation with the pathogen *Xanthomonas campestris* pv. oryzae.[225] The constitutive antimicrobial triterpenoids cucurbitacin I (**Figure 17**) and meliacins have been identified in *Ecballium elaterium* (Cucurbitaceae) and *Chisocheton paniculatus* (Meliaceae), respectively.[214]

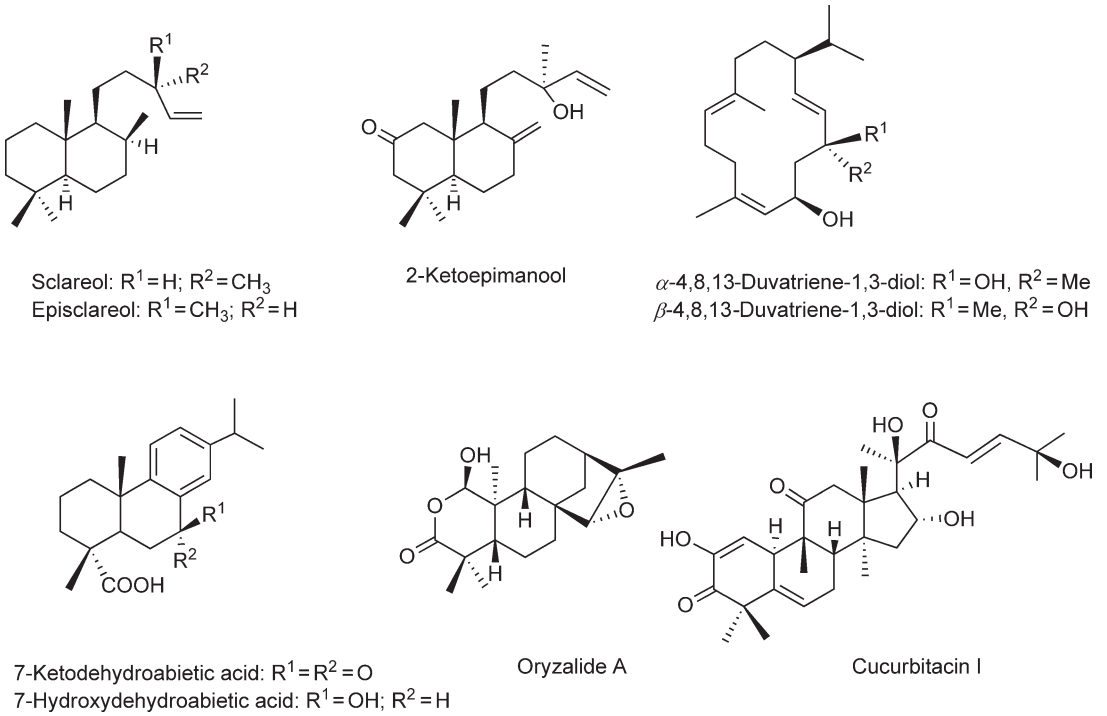


Figure 17 Structures of some constitutive antimicrobial terpenoids.

Saponins are an important source of constitutive antimicrobial triterpenoids. The antimicrobial activity is generally correlated with the sugar moiety glycosylated to the 3-hydroxyl of the triterpenoids. The ivy *Hedera helix* (Araliaceae) produces two related saponins, hederasaponins B and C, which are stored in the cell vacuoles. When ivy leaves are damaged by pathogen infection, these two saponins undergo partial hydrolysis, with loss of the sugars attached to the 26-carboxylic acid group, to yield α- and β-hedrins, which are highly toxic to pathogens. Further loss of sugar at the three positions of α- and β-hedrin results in sapogenins, which are completely inactive (**Figure 18**).[214] In addition to the ivy saponins, triterpenoid saponins have been identified from many species, including oat (*Avena sativa*), *Dolichos kilimandscharicus* (Leguminosae), *Rapanea melanophloeos* (Myrsinaceae), *Primula sieboldii* (Primulaceae), and *Camellia japonica* (Theaceae).[214]

Nitrogen-containing compounds: Many alkaloids showing activity against human pathogens have been identified. Although it remains almost unknown whether these alkaloids play a role in defense of the host plant against pathogens, some show activity against plant pathogens: for example, the indole alkaloid gramine in barley leaves (*Hordeum vulgare*) (**Figure 19**) and quinolizidine alkaloids in Leguminosae species.[214]

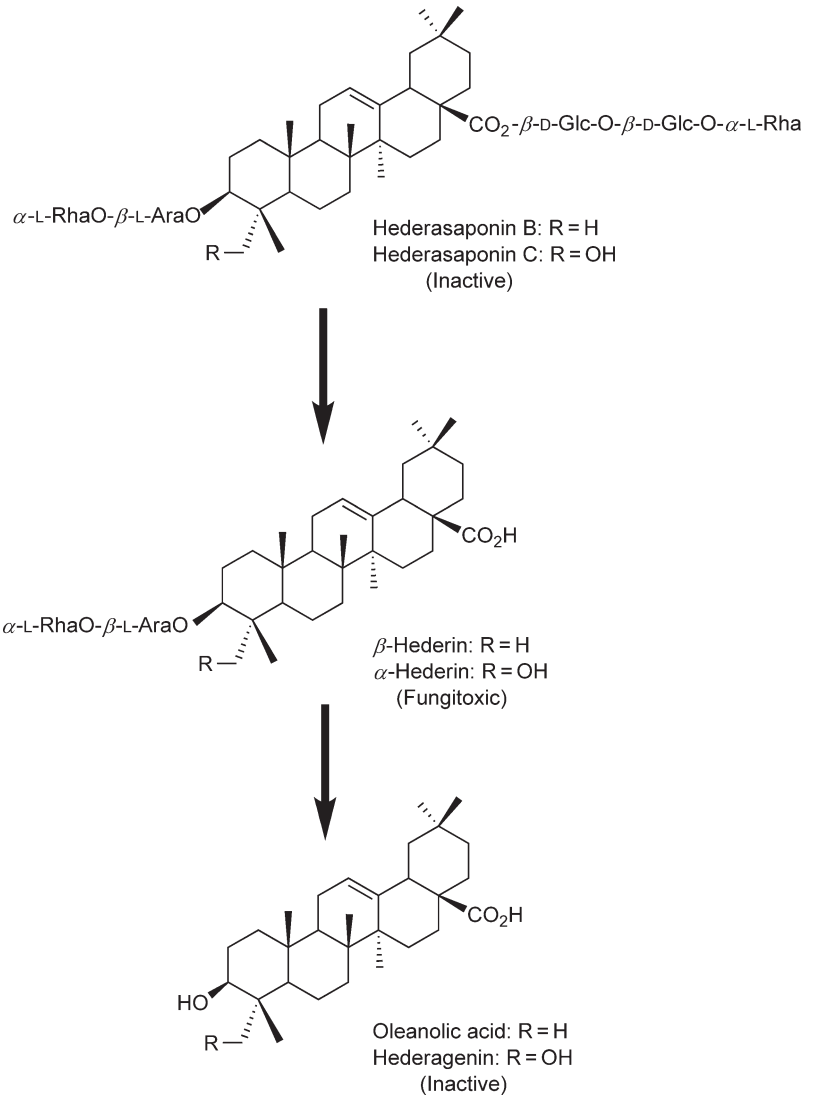


Figure 18 Release of sugars by hydrolysis from the hederasaponins B and C.

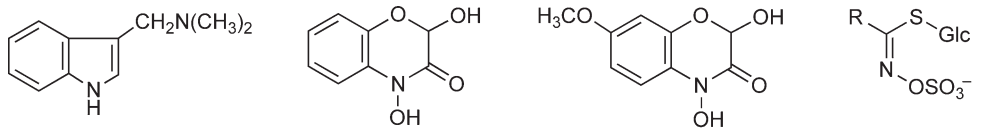


Figure 19 Structures of some *N*-containing, constitutive antimicrobial compounds.

The cyclic hydroxamic acid 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its precursor 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) (**Figure 19**) form part of the defense against microbial pathogens and insects in the Gramineae. DIBOA is the main hydroxamic acid in rye (*Secale cereale*), whereas DIMBOA is the predominant form in wheat (*Triticum aestivum*) and maize (*Z. mays*).[226] The hydroxamic acids, while not present in the seeds of cereals, appear upon germination in maize, wheat, and rye. Their levels increase with age to reach a maximum a few days after germination in maize and wheat, and are higher in the stem than in the leaf tissue.[227] Five genes, *Bx1–Bx5*, that are involved in DIBOA biosynthesis are localized to a 6-cM region on the short arm of chromosome 4 in maize (**Figure 20**).[226]

Glucosinolates (**Figure 19**) are nitrogen- and sulfur-containing anionic natural products that have been reported almost exclusively from the order Brassicales, containing Brassicaceae. They produce toxic products such as isothiocyanates, thiocyanates, and nitriles upon hydrolysis by endogenous myrosinases, as described in Section 4.08.1.2. In some cases, resistance to pathogens is associated with glucosinolate content, but some pathogens do not cause enough cell damage to activate the glucosinolate–myrosinase system.[228] Glucosinolates are biosynthesized from aliphatic or aromatic amino acids. In *A. thaliana*, indole glucosinolates are biosynthesized from tryptophan via indole-3-acetaldoxime, which is also a biosynthetic intermediate of the phytoalexin camalexin.[229]

Cyanogenic glycosides, which are widely distributed in higher plants, are a bound form of toxic hydrogen cyanide, which is released from the glucoside following enzyme hydrolysis.[214]

Aromatics: Several flavonoids, including pinocembrin in *Populus deltoides*, sakuranetin in black currant bushes (*Ribes nigrum*, Grossulariaceae),[230] and 6-isopentenylnaringenin in the hop plant (*Humulus lupulus*), are present on the leaf surface and exhibit antimicrobial activity (**Figure 21**). The flavanone sakuranetin occurs constitutively in the leaves of black currant, but is induced as a phytoalexin in rice leaves (*Oryza sativa*, Gramineae), as described in Section 4.08.2.3. Flavan-4-ols occur in grains and leaves of *Sorghum*, and a positive correlation has been found between flavan-4-ols and disease resistance.[214] The isopentenyl isoflavonoids luteone and wighteone (**Figure 21**) occur on the leaf surface of *Lupinus* species (Leguminosae).[214] Stilbene glucosides such as astringin (5,3′,4′-trihydroxystilbene-3β-D-glucoside) and rhaponticin (5,3′-dihydroxy-4′-methoxystilbene-3β-D-glucoside) occur in the bark of sitka spruce (*Picea sitchensis*, Pinaceae) and are antimicrobial themselves,

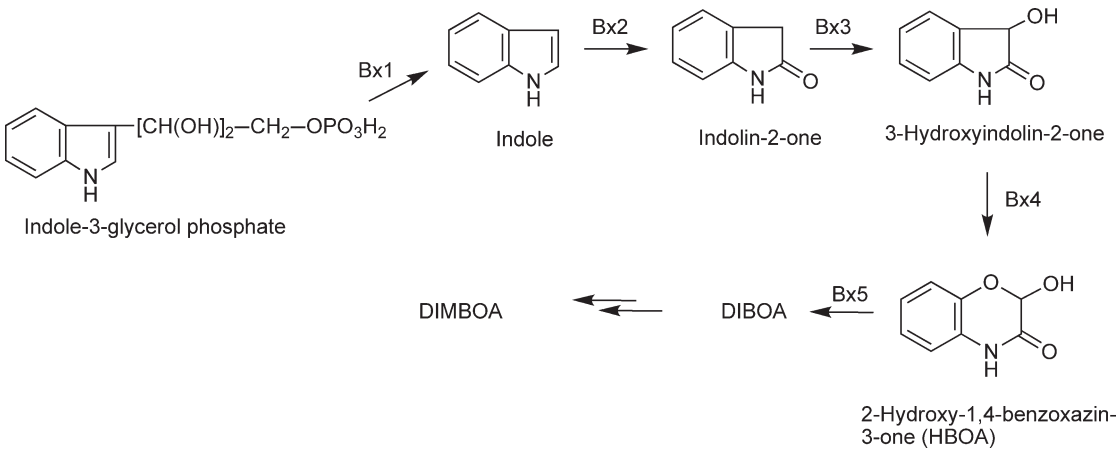


Figure 20 Biosynthetic pathway of DIMBOA in *Zea mays*.

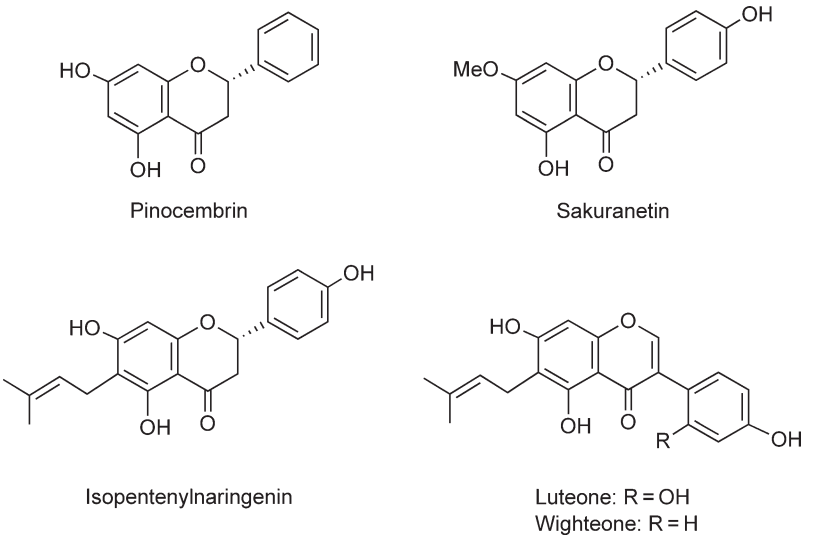


Figure 21 Structures of some constitutive antimicrobial flavonoids.

while their aglycones have much higher activity.[231] In the Rosaceae, the majority of plants are devoid of phytoalexins; instead, many contain catechin-like phenolic compounds with antimicrobial activity.[214]

Others: Alkadienals and a number of epoxy and hydroxyl linoleic and linolenic acids have been identified from wheat and rice leaves, respectively.[214]

Acetylenic compounds such as safynol are constitutive in some species of Compositae and Umbelliferae, and are phytoalexins in other species (see Section 4.08.2.3). Antimicrobial long-chain alcohols, some of which are acetylenic, have been identified in the skin of immature avocado fruits (*Persea americana*, Lauraceae).[214]

4.08.2.3 Phytoalexins

The term 'phytoalexin' was originally coined by K. O. Muller for plant-formed antibiotics that are synthesized *de novo* after the plant tissue is exposed to microbial infection.[232] A more recent consensus definition has gained general acceptance: phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized by and accumulated in plants after exposure to microorganisms.[220,233] Production of phytoalexins is also induced by abiotic elicitors, including heavy metals and biosurfactants, biotic elicitors such as pathogen-derived constituents (the so-called pathogen-associated molecular patterns), and ultraviolet (UV) light.

The pterocarpan pisatin was first isolated and characterized as a phytoalexin in *Pisum sativum* in the early 1960s.[234,235] Since then, structurally diverse phytoalexins have been reported from more than 30 families of higher plants. Much progress is also being made in studies on phytoalexin biosynthesis based on genomic information.

Terpenoids: The sesquiterpenoid capsidiol has been identified as a major phytoalexin from several Solanaceae species, including *Nicotiana tabacum* and *Capsicum annuum*[236,237] (**Figure 22**). The sesquiterpene hydrocarbon 5-*epi*-aristolophene, a biosynthetic precursor of capsidiol, is biosynthesized from farnesyl diphosphate by the action of 5-*epi*-aristolophene synthase (EAS) and is sequentially hydroxylated at C1 and C3 to form capsidiol.[238] Two EAS genes (*EAS1* and *EAS2*) and a P-450 gene encoding 5-*epi*-aristolophene-1,3-dihydroxylase (CYP71D) were functionally identified from cDNA libraries prepared from elicitor-induced tobacco cells, with both *EAS* and *CYP71D* being elicitor-inducible.[237,239] In potato (*S. tuberosum*), production of sesquiterpenoid phytoalexins, including rishitin, is induced in response to elicitor treatments and pathogen infection.[236] In pathogen-infected sweet potato (*Ipomoea batatas*) root tissue, various furanosesquiterpene phytoalexins, including ipomeamarone, have been identified[240] (**Figure 22**). The sesquiterpenoid phytoalexin lettucenin A was reported from lettuce, *Lactuca sativa* (Compositae) (**Figure 22**).[214]

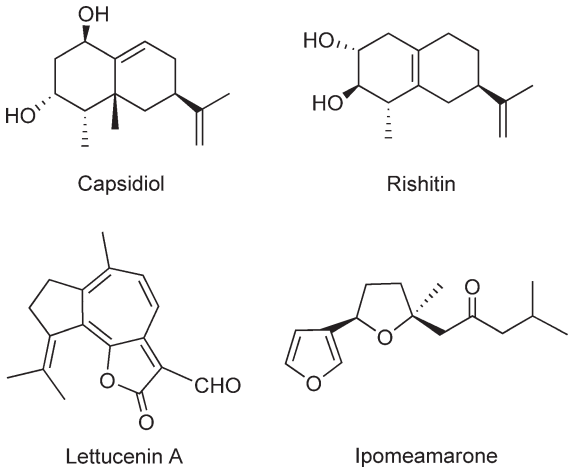


Figure 22 Structures of some sesquiterpenoid phytoalexins.

In *Gossypium* spp. (Malvaceae), sesquiterpenoid phytoalexins identified so far are cadinene derivatives that are biosynthesized from δ-cadinene.[241] CAD1-A and CAD1-C were functionally identified as sesquiterpene cyclases that catalyze the conversion of farnesyl diphosphate into δ-cadinene[242,243] (**Figure 23**). GaWRKY1 is a transcription factor involved in elicitor-inducible *CAD1-A* expression.[241] Gossypol, a major phytoalexin in *G. arboreum*, is likely to be biosynthesized via 8-hydroxy-δ-cadinene (**Figure 23**).[244] CYP706B1 was functionally identified as a cytochrome P-450 enzyme that catalyzes the conversion of δ-cadinene into 8-hydroxy-δ-cadinene, with *CYP706B1* being highly elicitor-inducible in suspension-cultured cotton cells.[244]

In rice (*Oryza sativa*), 15 compounds have been identified as phytoalexins in leaves infected with the blast fungus *Magnaporthe grisea* or on UV irradiation. Except for the flavanone sakuranetin (**Figure 21**), they are all diterpenoids. The rice diterpenoid phytoalexins are classified into four groups based on their basic carbon frameworks: phytocassanes A–E,[245–247] oryzalexins A–F,[248–250] momilactones A and B,[223,224] and oryzalexin S (**Figure 24**).[251] By analogy with known biosynthetic pathways of polycyclic diterpenes such as

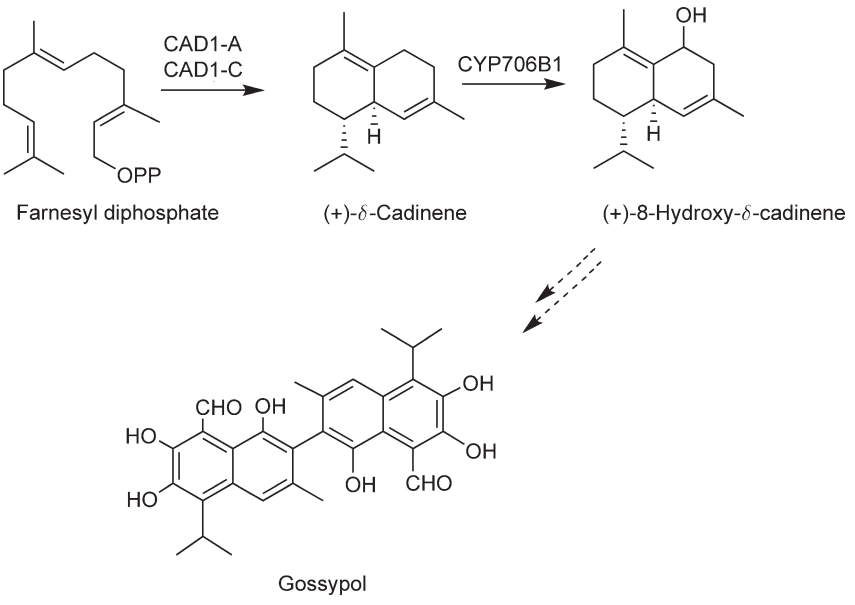


Figure 23 Putative biosynthetic pathway of a sesquiterpenoid phytoalexin, gossypol.

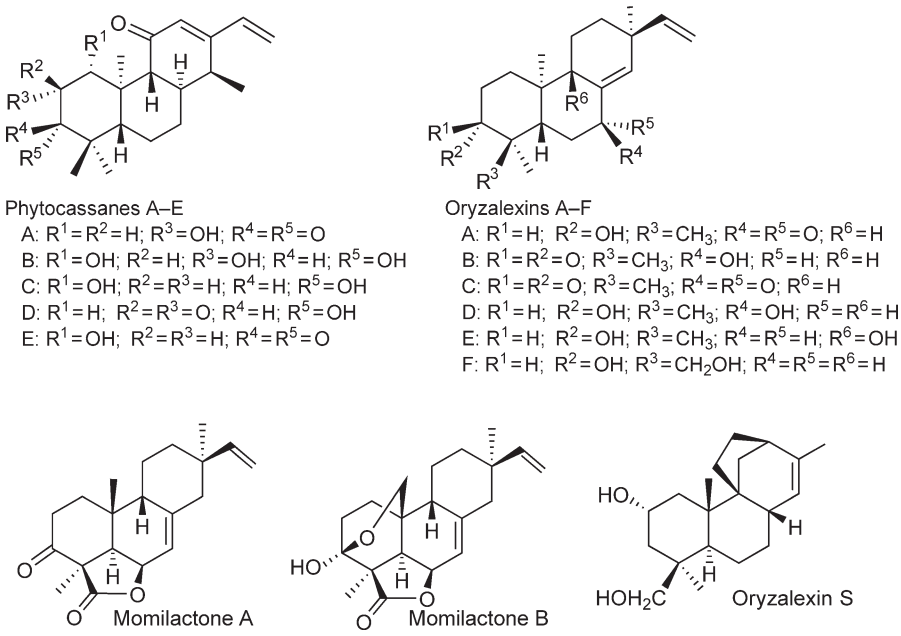


Figure 24 Structures of rice diterpenoid phytoalexins.

gibberellins,[252] the common precursor of these molecules, geranylgeranyl diphosphate (GGDP), is postulated to be sequentially cyclized via *ent*-copalyl diphosphate (*ent*-CDP) to *ent*-cassa-12,15-diene and *ent*-sandaracopimaradiene, leading to phytocassanes A–E and oryzalexins A–F. GGDP is also cyclized via *syn*-CDP to 9β*H*-pimara-7, 15-diene and stemar-13-ene, leading to momilactones A and B and oryzalexin S. By utilizing the information from the rice genome database that was recently opened to the public, six diterpene cyclases have been demonstrated to be involved in the conversion of GGDP into the four diterpene hydrocarbon precursors via *ent*- or *syn*-CDP. OsCPS2 (OsCyc2) and OsCPS4 (OsCyc1) catalyze the conversion of GGDP into *ent*-CDP and *syn*-CDP,[253,254] and OsKSL7 (OsDTC1), OsKSL10 (OsKS10), OsKSL4 (OsKS4), and OsKSL8 (OsDTC2) catalyze the conversion of *ent*-CDP or *syn*-CDP into the four diterpene hydrocarbons *ent*-cassa-12,15-diene, *ent*-sandaracopimaradiene, 9β*H*-pimara-7,15-diene, and stemar-13-ene, respectively (**Figure 25**).[255–258] These diterpene cyclase genes have also been shown to be induced by irradiation of rice leaves with UV light and by treatment of suspension-cultured rice cells with a chitin oligosaccharide elicitor, with the accumulation of all of their mRNAs peaking at 4–8 h after elicitor treatment.[255,257,259] It should be noted that *OsCPS1* encodes an *ent*-CDP synthase involved in the biosynthesis of GAs, and that expression of the *OsCPS1* transcript was not induced either by UV light in the rice leaves[253] or by a chitin oligosaccharide elicitor in suspension-cultured rice cells.[254]

It has been suggested that cytochrome P-450s are involved in the downstream oxidation of diterpene hydrocarbons, leading to the bioactive phytoalexins, by analogy to known biosynthetic pathways for the diterpenoid gibberellins.[258] On chromosome 2 of the rice genome, *OsCPS2* and *OsKSL7* are closely located. Similarly, on chromosome 4, *OsKSL4* is located near *OsCPS4*. In addition, several chitin oligosaccharide-inducible P-450s have been found near the cyclase genes on chromosomes 2 and 4.[256] In fact, it was shown that a 168-kb gene cluster on chromosome 4 encodes, in addition to OsCPS4 and OsKSL4, momilactone A synthase (a dehydrogenase named OsMAS) and two cytochrome P-450s, either or both of which are involved in momilactone biosynthesis.[260] These results suggest that phytocassane and momilactone biosynthesis genes are clustered on chromosomes 2 and 4, respectively. Phytocassanes and momilactones are major representatives of four distinct types of diterpenoid phytoalexins in rice. Although the biological significance of gene clusters in the synthesis of secondary metabolites is not clear, such gene clusters might contribute to efficient coordinated expression of the genes after elicitation, followed by production of high levels of diterpenoid phytoalexins.

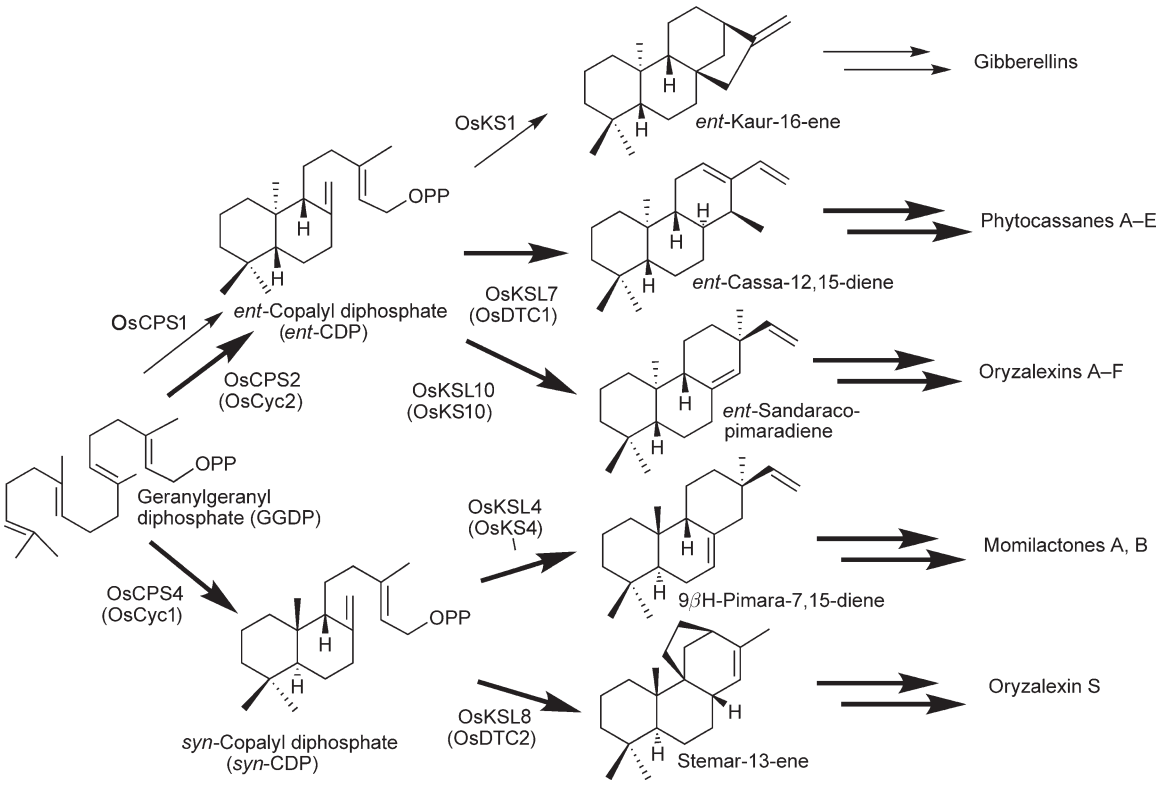


Figure 25 Biosynthetic pathways of gibberellins and diterpenoid phytoallexins in rice.

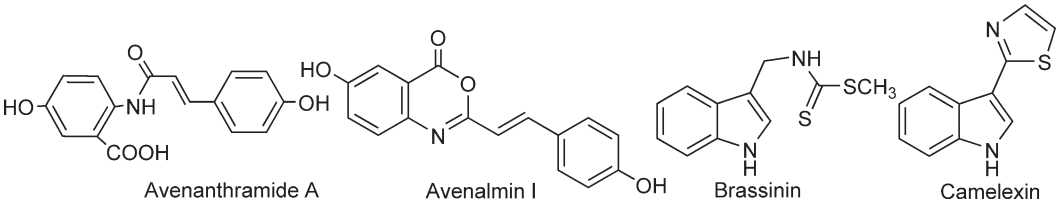


Figure 26 Structures of some *N*-containing phytoalexins.

Nitrogen-containing compounds: Anthranilate derivatives, such as avenanthramides, and avenalmin I, II, and III have been reported in *A. sativa* (Gramineae) (**Figure 26**).[261,262]

In the Brassicaceae, sulfur-containing indole phytoalexins such as brassinin, camalexin, and their derivatives have been identified (**Figure 26**).[214] Within this family, *A. thaliana* is a useful plant to investigate biological roles and regulatory mechanisms of phytoalexins, because the plant appears to produce only camalexin. In addition, the *Arabidopsis* genome database is open to the public, and useful genetic tools such as mutant lines and gene chips are readily available for *A. thaliana*.

Camalexin was originally isolated from the leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae*.[263] Infection of *A. thaliana* leaves with both biotrophic and necrotrophic pathogens induces camalexin production.[229] Camalexin originates from tryptophan via indole-3-acetaldoxime (IAOx) and (*S*)-dihydrocamalexic acid (**Figure 27**). IAOx is also an intermediate of the indole glucosinolates that are constitutively formed in *A. thaliana* and the auxin indole-3-acetic acid (IAA). Synthesis of IAOx from tryptophan is catalyzed by P-450 enzymes CYP79B2 and CYP79B3.[264] Expression of the *CYP79B2* transcript was elicitor (silver nitrate)-inducible, but that of the *CYP79B3* transcript was not elicitor-inducible. A *cyp79B2/cyp79B3* double knockout mutant was shown to be devoid of both camalexin and indole glucosinolates, and could only synthesize reduced levels of IAA.[265] The camalexin-deficient mutants *pad1–pad5* were isolated[266,267]

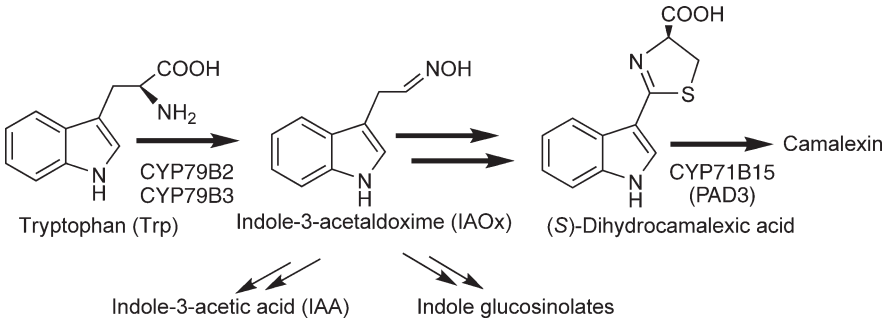


Figure 27 Biosynthetic pathway of camalexin.

and *PAD3* was shown to encode the P-450 enzyme CYP71B15, which catalyzes the last step in camalexin biosynthesis, the conversion of (*S*)-dihydrocamalexic acid into camalexin.[268] *PAD2* was shown to encode γ-glutamylcysteine synthetase 1 involved in glutathione biosynthesis,[269] suggesting the involvement of glutathione in camalexin biosynthesis, either as a regulatory component or as a biosynthetic precursor of the thiazole ring moiety of camalexin. *PAD4* encodes a lipase-like protein[270] that acts upstream from SA to affect expression of the pathogenesis-related protein gene *PR-1* and camalexin synthesis.[271] The cytochrome P-450 genes *CYP79B2* and *CYP79B15* (*PAD3*) involved in camalexin biosynthesis and the tryptophan biosynthetic gene *ASA1* (anthranilate synthase) are transcriptionally induced by *P. syringe* infection. Camalexin synthesis and the induction of *CYP79B2*, *PAD3*, and *ASA1* were strictly colocalized with the infection site of *Alternaria alternata*, a potent camalexin inducer.[272]

Aromatics: In the Leguminosae, most phytoalexins are isoflavonoids. These phytoalexins have been identified mainly in the leaves of herbaceous members of the Leguminosae, and include pisatin, kievitone, and glyceollin I in *Pisum sativum*, *Phaseolus vulgaris*, and *Glycine max*, respectively (**Figure 28**). Pisatin is biosynthesized from 6a-hydroxymaackian by 6a-hydroxymaackian 3-*O*-methyltransferase (HMM). Wu *et al.*[273] reported isolation and characterization of two HMM cDNA clones (pHMM1 and pHMM2) created from RNA obtained from pathogen-infected pea tissue; the deduced amino acid sequences of HMM1 and HMM2 were highly homologous to each other.

Gramineae species also produce flavonoid phytoalexins. The flavanone sakuranetin (**Figure 21**) is a major phytoalexin in rice leaves,[274] although this compound is constitutive in black currant leaves, as described in Section 4.08.2.2. *S. bicolor* produces 3-deoxyanthocyanidins such as luteolinidin and apigeninidin (**Figure 29**) in response to fungal infection.[275]

In carrot, *Daucus carota* (Umbelliferae), 6-methoxymellein was identified as a phytoalexin (**Figure 29**). It is biosynthesized by 6-hydroxymellein-*O*-methyltransferase from 6-hydroxymellein, which is biosynthesized from 1 mol of acetyl coenzyme A (CoA) and 4 mol of malonyl-CoA by a polyketide biosynthetic enzyme, 6-hydroxymellein hydroxylase.[276,277]

Hydroxystilbenes such as resveratrol (**Figure 29**) have been identified in several unrelated families, including the grapevine *Vitis vinifera* (Vitaceae), peanut *Arachis hypogaea* (Leguminosae), *Veratrum grandifolia*

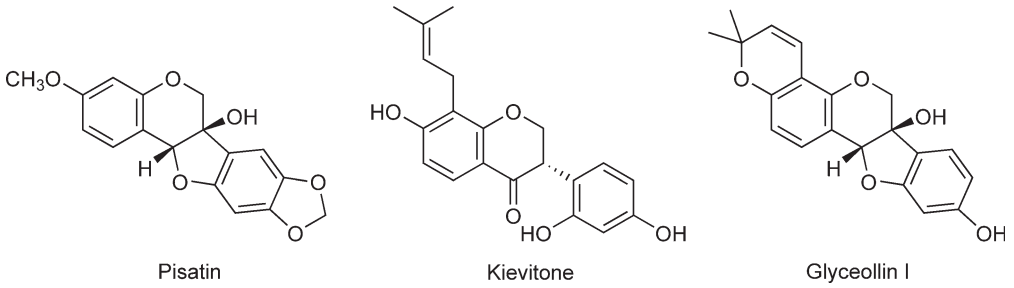


Figure 28 Structures of some isoflavonoid phytoalexins.

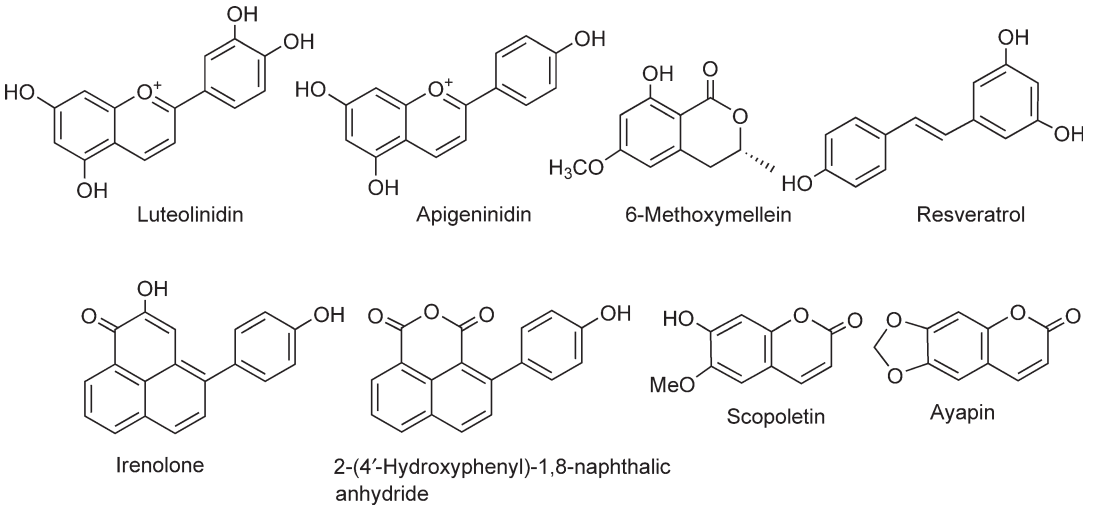


Figure 29 Structures of some typical aromatic phytoalexins.

(Liliaceae), and *Festuca versuta* (Gramineae). Constitutive overexpression of a grapevine stilbene synthase gene in tobacco and alfalfa resulted in increased resistance to pathogens.[219,278]

In the banana *Musa acuminata* (Musaceae), a series of novel phenalenone-type compounds, including irenolone and 2-(4′-hydroxyphenyl)-1,8-naphthalenedicarboxylic anhydride, have been identified (**Figure 29**).[279,280] In the Compositae, acetophenone derivatives in yacon (*Polymnia sonchifolia*)[214] and the coumarins scopoletin and ayapin in sunflower (*Helianthus annuus*)[281] have been reported (**Figure 29**).

Others: In the Compositae, acetylenic compounds such as safynol and dehydrosafynol in safflower (*Carthamus tinctorius*)[282] and (*E*)- and (*Z*)-mycosinol in corn marigold (*Coleostephus myconis*)[283] have been reported (**Figure 30**).

4.08.2.4 Chemical Defenses of Plants

Antimicrobial plant metabolites that function as chemical barriers against pathogens are divided into constitutive compounds and phytoalexins. These two types of chemical barriers are likely to cooperatively function as defense systems against pathogens, together with other defenses. Although induction of phytoalexin production under stressed conditions, including pathogen infection, is general within the flowering plants, and a variety of compounds have been identified as phytoalexins from over 30 families, there are also many plants in which phytoalexin production is not induced. For example, a survey of Rosaceae leaf tissue indicated a relatively low frequency of phytoalexins, with no more than 15% of species forming phytoalexins. Constitutive barriers might be well developed in such leaves.[214] In the Cucurbitaceae, phytoalexins have not been reported, but these plants appear to respond to pathogen infection by SAR, in which the resistance is provided by antimicrobial proteins, including PRs,[216] although SAR is also a general defense mechanism throughout the higher plants.

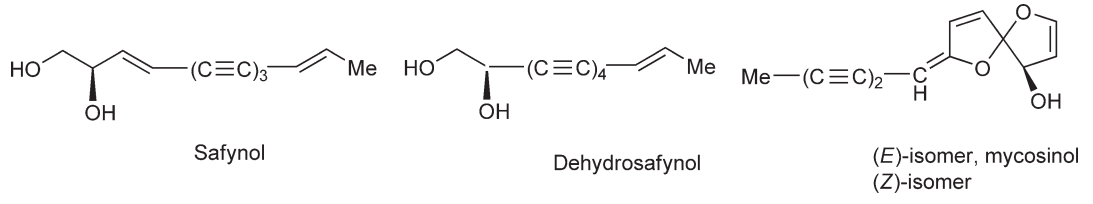


Figure 30 Structures of some acetylenic phytoalexins.

Several model plants, including *O. sativa*, *A. thaliana*, *Z. mays*, *G. max*, and *Medicago truncatula*, for which genome information is currently available,[219] are a rich source of antimicrobial metabolites. Genetic and reverse genetic approaches are providing evidence for the biological importance of antimicrobial compounds in host defense mechanisms.

4.08.3 Phytotoxins

4.08.3.1 Introduction

Microbial pathogenesis in plants is an intricate developmental process requiring biological components found in most microorganisms, as well as factors that are unique to microbial species that participate in particular microorganism–plant interactions. Toxic substances isolated from plant pathogens are often called phytotoxins. In many cases, it is known that phytotoxins play an important role in disease development causing chlorosis, necrosis, or wilting.[4,284] Because of agricultural importance, crop–phytopathogenic microbe interaction is studied extensively and is one of the well-studied examples of plant–microbe relationship. In some cases, phytotoxins also participate in particular microorganism–plant interactions. This type of phytotoxin is called host-specific or host-selective toxin (HST)[285] to distinguish from host nonselective toxin. HST reproduces the symptom of disease and the ability to produce phytotoxins is strongly related to virulence. The phytotoxins are usually isolated from fermentation media with the guidance of appropriate bioassay using host plants. In the last decade, significant progress has been made in elucidating phytotoxin biosynthetic genes, which are usually clustered on chromosomes. The study on biosynthetic gene cluster of phytotoxins provides information on transcriptional regulation of gene expression, transport of pathway products, and self-resistance mechanism.

Similar to other natural products, phytotoxins are classified on the basis of their structural types considering their biosynthetic pathways. Among various phytotoxin families, polyketides including aromatic and reduced polyketides, peptides including diketopiperazines, and terpenes are most frequently described in the literature. In this section, representative phytotoxins that are well-characterized biosynthetically and physiologically will be introduced.

4.08.3.2 Polyketides

Fungal polyketide synthase (PKS)[286] is a large protein and consists of a single set of module containing a keto synthase (KS), an acyltransferase (AT), a thioesterase/Claisen cyclase (TE/CYC), and two acyl carrier protein (ACP) domains. This enzyme catalyzes the condensation of malonyl CoA and enzyme-bound acyl group to afford complex fatty acid possessing methyl substituent and various functional groups. Interestingly, fungal type-I PKS produces both aromatic and reduced polyketides contrary to bacterial PKSs, which are divided into type-I and type-II for reduced and aromatic ones.

Cochliobolus heterostrophus (*Helminthosporium maydis*) race T, the causal agent of southern corn leaf blight, produces polyketol of a long carbon chain polyketide (C41) T-toxin (or HMT-toxin).[287] High virulence of this fungus on T-cytoplasm maize is responsible for the production of host-selective toxin T-toxin. Production of T-toxin requires PKS1 and a decarboxylase DEC1.[288] Recently, the second PKS gene (PKS2) has been identified. It is speculated that rather long backbone of T-toxin is constructed by two separate PKSs using one of the products as a starter unit of the second PKS (**Figure 31**).

Cercosporin[289] is a light-activated, nonhost-selective toxin produced by many *Cercospora* fungal species. The dimeric perylenequinone structure indicates that cercosporin is a typical aromatic polyketide biosynthesized by iterative type-I PKS.[290] This toxin acts as a photosensitizer that activates molecular oxygen in the presence of light,[289] and the resultant reactive oxygen species causes oxidative damage to cells. Studies on toxin-deficient mutants and on the involvement of light in symptom development have demonstrated the importance of this toxin in diseases by *Cercospora* pathogen. Interestingly, light is the primary signal to trigger cercosporin biosynthesis.

Another fungal pathogen *Alternaria* produces a variety of nonhost-selective phytotoxins[291] such as aromatic polyketides (alternariol, zinniol) and reduced polyketides (alternaric acid, solanapyrones). Alternaric acid[292] isolated from *Alternaria solani* shows unique physiological effects on the hypersensitive cell death of potato

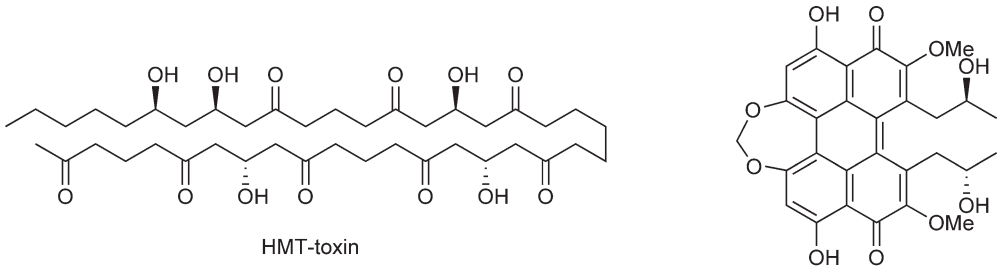


Figure 31 Structures of polyketide

cells.[293] The causal fungi of potato early blight *Alternaria solani* produce solanapyrones,[294] which are also known as specific inhibitors of DNA polymerase β.[295] Solanapyrone is the first demonstrated example biosynthesized via enzymatic Diels–Alder reaction.[296] Recently, a biosynthetic gene cluster of solanapyrones has been identified[297](**Figure 32**).

In addition to those host nonselective toxins, *Alternaria* pathogens produce a number of host-selective toxins such as AK-toxin, AF-toxin, and ACT-toxin.[298] 9,10-Epoxy-8-hydroxy-9-methyldecatrienoic acid, the common backbone of these toxins, was biosynthesized via the polyketide pathway. Genetic approach has allowed the identification of a gene cluster for AF-toxin biosynthesis.[299]

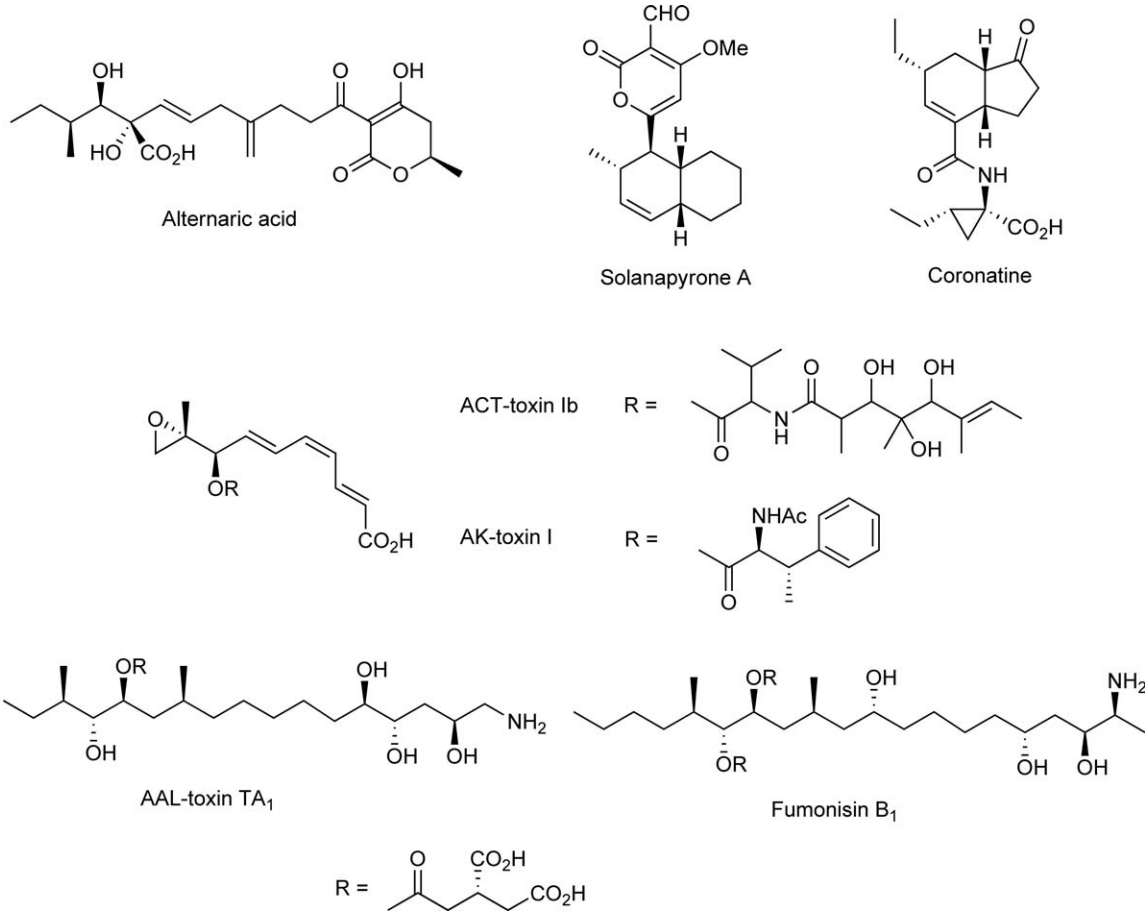


Figure 32 Structures of polyketide phytotoxins (continued).

Host-selective sphinganine-like phytotoxin AAL-toxin[298] produced by *Alternaria alternata* f. sp. *lycopersici*, a causal fungus of tomato stem canker disease, reproduces symptoms similar to those of the disease for susceptible genotype of tomato leaf in concentrations less than 10 ng ml^{-1}.[300] Interestingly, AAL-toxin and its structural analogue fumonisin, which is known as a mycotoxin, are phytotoxic to tomato and are cytotoxic to cultured mammalian cells due to apoptosis induced by inhibition of ceramide synthase.[301] Similar overlapping toxicity between plants and animals has been reported in the case of phytotoxins (and also mycotoxin) such as tricothecenes[302] and cytochalasins.[303]

Nonhost-selective toxin coronatine was originally isolated as a chlorosis-inducing factor from Italian ryegrass infected with *Pseudomonas syringae* pv. *atropurpurea*[304] and contributes to virulence in several host–pathogen interactions.[305] Later, it was found that coronatine showed very similar, but stronger, activities to that of the phytohormone JA.[306] Its potency of biological activities is attributed to fixing the *cis* configuration of cyclopentane ring, which is easy to isomerize *trans* in the case of JA.

The biosynthetic pathway of coronatine has been extensively studied. The coronafacic acid moiety is proposed to be constructed by bacterial modular type-I PKS via 5-oxocyclopentene carboxylic acid. A recent study clarified the detailed mechanism of the formation of highly unusual cyclopropane amino acid via D-3-chloroalloisoleucine (enzyme-bound form).[307]

4.08.3.3 Nonribosomal Peptides

Fungi and bacteria produce various phytotoxic cyclic peptides using nonribosomal peptide synthetase (NRPS).[308] Similar to modular organization of PKS, NRPS consists of several modules containing condensation (C), adenylation (A), and thiolation (T) domains.

Alternaria alternata apple pathotype (previously described as *A. mali* Roberts) causes *Alternaria* blotch of susceptible apple cultivars through production of a cyclic peptide host-selective toxin, AM-toxin, whose complete structure has been determined for the first time among host-specific toxins.[298] Disruption of AM-toxin synthetase (AMT) gene resulted in toxin-minus mutants, which were also unable to cause disease symptoms in susceptible apple cultivars, indicating that AMT is a primary determinant of virulence and specificity in the *A. alternata* apple pathotype[309] (**Figure 33**).

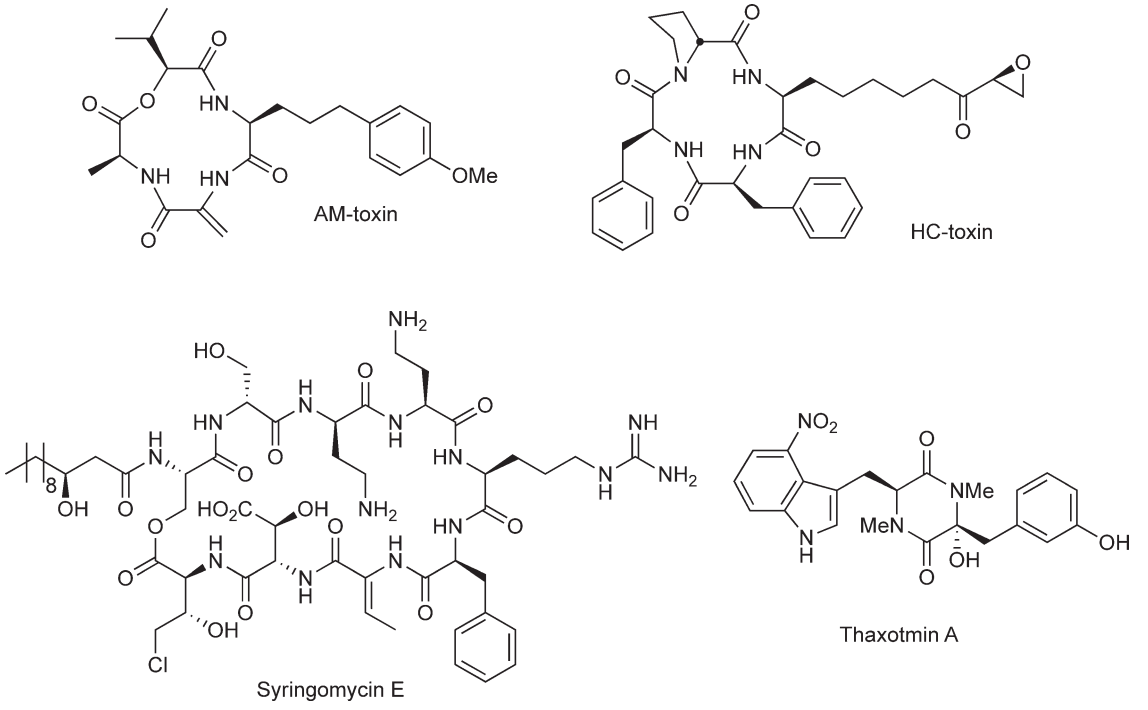


Figure 33 Structures of nonribosomal peptide phytotoxins.

Maize *Helminthosporium* leaf spot is caused by *Cochliobolus carbonum* race 1, a fungal pathogen whose infection is dependent on the production of a cyclic depsipeptide HC-toxin. HC-toxin consists of a family of four related compounds the most abundant of which is a cyclic depsitetrapeptide. In the backbone biosynthesis of HC-toxin, NRPS (HTS1), encoding a large peptide synthetase of about 574 kDa, and PKS (TOXC) are required, which are responsible for depsipeptide formation and biosynthesis of unusual amino acid, respectively.[310] Nontoxigenic mutants that are avirulent for maize lines susceptible to the wild type are obtained only if both copies are disrupted.[311]

Pseudomonas syringae parasitizes a wide variety of plant species and is divided into more than 50 different pathovars (pv.) based on host preference.[312] In many *P. syringae* pv., nonhost-selective phytotoxins are produced, which induce chlorotic and necrotic symptoms in various host plants. For example, strain B301D (a pathogen of pear) of *P. syringae* pv. *syringae* produced cyclic depsipeptide syringomycin,[313] whereas strain SY12 (lilac blights) produced structurally related analogue syringostatin.[314] Their roles in disease development are extensively studied. Halogenation at the aliphatic carbon is a rare transformation in the biosynthesis of natural products. The detailed mechanism of halogenation with *syrB1* and *syrB2* in the biosynthetic gene cluster of syringomycin E has been elucidated.[315]

Streptomyces pathogens are quite rare in plant diseases. Thaxtomin A from *Streptomyces scabies* causes scab disease of potato, which is characterized by conspicuous corky lesions on tubers. Molecular genetic investigation has revealed that thaxtomins are biosynthesized by nonribosomal peptide synthetases (*txtAB*) that condense modified L-phenylalanyl and L-4-nitrotryptophanyl units to form a 2,5-dioxopiperazine skeleton.[316] Disruption of *txtA* results in the formation of nonpathogenic strain. This toxin is shown to affect the movement of calcium ions and protons across the plasma membrane and also inhibit cellulose biosynthesis.[317]

4.08.3.4 Terpenoids

Terpenoid phytotoxins, including diverse fungal diterpenoids and sesquiterpenoids, are produced by many phytopathogenic fungi. In general, the biosynthesis of terpenes starts with the formation of the molecular skeleton with terpene cyclase to afford cyclic hydrocarbon, which is usually hydroxylated and subsequently modified by alkylation, acylation, and glycosylation. Some of these phytotoxins show useful biological activity, and are used as plant growth regulators in agriculture and as biochemical agents for plant and cell physiology (**Figure 34**).

Diterpene glucoside fusicoccin is produced by *Phomopsis* (*Fusicoccum*) *amygdali* as a principal toxin implicated in the wilting disease of almond and peach in Italy, and a fusicoccin-producing *P. amygdali* Niigata 2 is newly found in a peach *Fusicoccum* canker fungus in Japan. Fusicoccin A shows potent phytohormone-like activities and is used as a biochemical agent for plant physiology. It permanently activates plasma membrane H^+-ATPase in all higher plants and its mode of action is investigated by X-ray crystallographic analysis of the ternary complex of a plant 14-3-3 adapter protein, fusicoccin, and a synthetic phosphopeptide of the C-terminus of H^+-ATPase.[318] Current biosynthetic studies of fusicoccin aglycone indicate the presence of the genuine biosynthetic tricyclic hydrocarbon intermediate (+)-fusicocca-2,10(14)-diene in the mycelia of

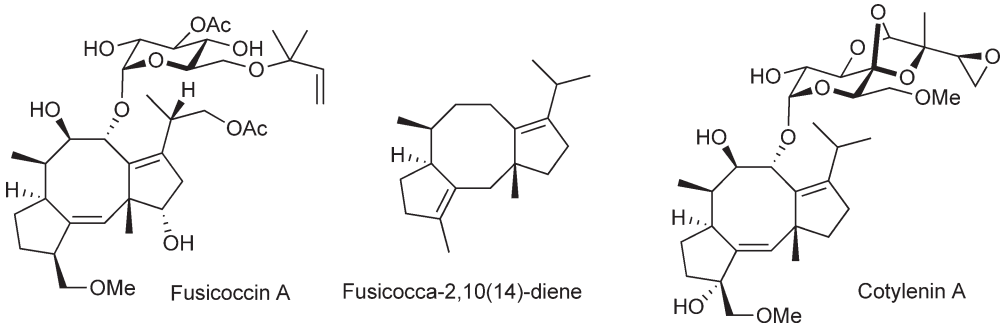


Figure 34 Structures of terpenoid phytotoxins.

P. amygdali Niigata 2.[319] Cloning of fusicoccadiene synthase gene and its expression allowed elucidation of the highly unusual multistep conversion of C_5 isoprene units into fusicoccadiene, showing that fusicoccadiene synthase possesses both prenyltransferase and terpene cyclase activities.[320] Fusicoccin biosynthetic gene cluster has been identified by chromosomal walking. In connection with structure and bioactivities of fusicoccin, its sole congener cotylenin A, a plant growth regulator isolated from a fungus *Cladosporium* sp. 501-7W, is originally characterized as a potent differentiation-inducing substance in mammalian cells and an antitumor agent against human lung carcinoma cells.[321]

The plant hormones gibberellins $GA_{1/3}$ and GA_4 are produced by *Gibberella fujikuroi*, a Bakanae disease fungus of rice, and *Sphaceloma manihoticola*, a super-elongation disease fungus of cassava, respectively. Fungal gibberellins GA_3 and GA_4 are used as plant growth regulators for horticultural production. The biosynthesis of gibberellins in *G. fujikuroi* is determined at the molecular level; biosynthetic enzymes responsible for GA_1 formation include only *ent*-kaurene synthase and four cytochrome P-450 enzymes.[322] This pathway is totally different from the corresponding plant counterpart. Another gibberellin-producing fungus *Phaeosphaeria* sp. L487, known as one of the phytopathogenic fungi, produces GA_1 in plant-like gibberellin biosynthetic pathway through GA_9 and $GA_{4/20}$. Its *ent*-kaurene synthase in the GA_1 biosynthesis catalyzes the formation of *ent*-kaurene from GGDP through *ent*-copalyl diphosphate[323] (**Figure 35**).

Aphidicolin, a well-known biochemical agent that functions as a specific inhibitor of DNA polymerase α, is produced by *Phoma betae*, a fungal pathogen of beet. Its biosynthetic precursors, including aphidicolan-16β-ol, were elucidated by treatment of *P. betae* with cytochrome P-450 inhibitors.[324] From this fungus, a cDNA encoding aphidicolan-16β-ol synthase was cloned, and its recombinant fusion protein was found to catalyze the direct formation of 16β-ol from GGDP through *syn*-copalyl diphosphate.[325] Furthermore, chromosomal walking adjacent to the aphidicolol synthase gene allowed to identify the aphidicolin biosynthetic gene cluster.

Diterpene phytotoxins sphaeropsidins A–F, tri- and tetracyclic unrearranged pimarane skeleton, are isolated from *Sphaeropsis sapinea*, a fungus that causes a canker disease of Italian cypress. Sphaeropsidin A is the major toxic substance showing nonhost-selective phytotoxic activity[326] (**Figure 36**).

Trichothecene phytotoxins such as deoxynivaenol are produced by some phytopathogenic species of *Fusarium*. Biosynthetic studies on this phytotoxin show that a bicyclic hydrocarbon intermediate trichodiene, formed from FDP, and the subsequent oxidations with a series of cytochrome P-450s such as Tri4 give isotrichodiol, isotrichotriol, and deoxynivaenol.[302] The gene cluster responsible for trichothecene biosynthesis was found in *Fusarium* and *Myrothecium* fungi.

Another phytohormone abscisic acid is produced by phytopathogenic fungi *Cercospora cruenta*, *C. cruenta*, and *Botrytis cinerea*. These fungi biosynthesize abscisic acid by oxidation of ionylideneethane with molecular oxygen following cyclization of allofarnesene.[327] This direct pathway via ionylideneethane and subsequent ionylideneethanol is common among abscisic acid-producing fungi.

Sorokinianin, an unusual sesquiterpenoid, is isolated from *Bipolaris sorokiniana*, a fungal phytopathogen that causes spot blotch or foot and root rot diseases in wheat, barley, and oat. It is biosynthesized from phytotoxic prehelminthosporol and C_3 unit derived from oxaloacetic acid.[328] Prehelminthosporol itself was isolated as a phytotoxin of *Helminthosporium sativum*. Sorokinianin is more phytotoxic than prehelminthosporol in inhibiting the germination of barley seeds.

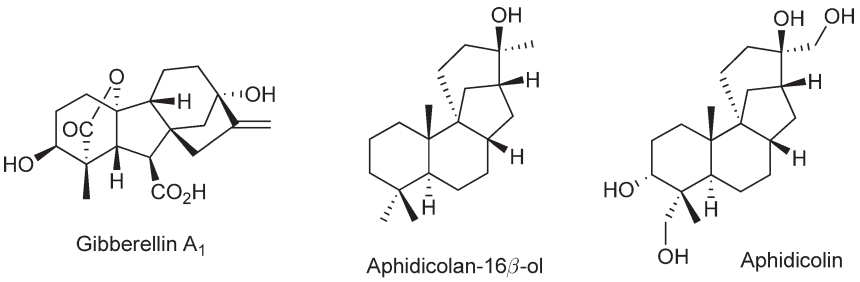


Figure 35 Structures of terpenoid phytotoxins (continued).

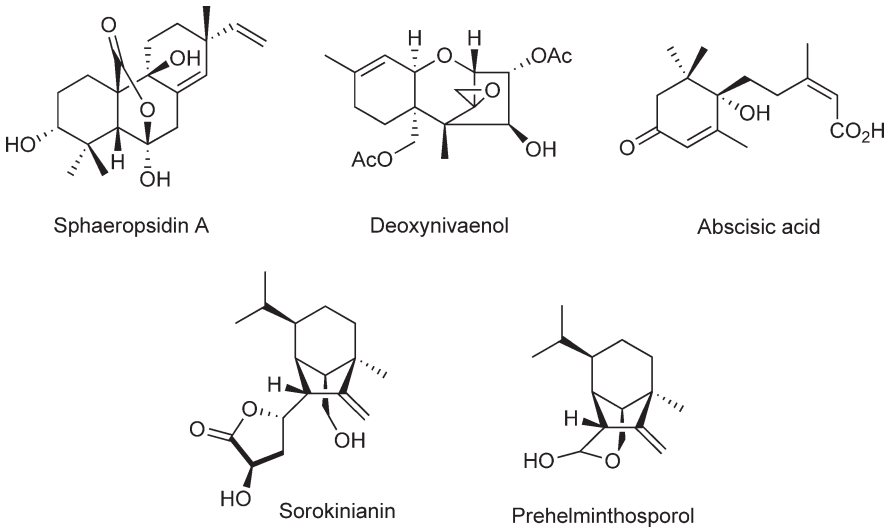


Figure 36 Structures of terpenoid phytotoxins (continued).

4.08.3.5 Macromolecular Phytotoxins

Various phytopathogenic microorganisms produce glycoproteins and polysaccharides that have been implicated as phytotoxic agents. For example, *Pseudomonas syringae* pv. *glycinea* produces exopolysaccharide alginate, a copolymer of D-mannuronic acid and L-glucuronic acid, which is strongly correlated with virulence in host plants.[329] In the case of *Verticillium* wilt, it is suggested that protein–lipopolysaccharide and glycoprotein are involved in the production of disease symptoms in susceptible host plants.[330]

Stereum purpureum, the causal fungus of apple silver leaf disease, produces three endopolygalacturonases (EndoPG Ia, Ib, and Ic), which are fully characterized. EndoPGs, present at a concentration of about 50 ng in an infected leaf, degrade the pectin in leaves and cause the silver leaf symptoms. EndoPG Ia contains 379 amino acid residues in the amino acid sequence excluding the signal peptide and has two N-binding sugar chains with $Man_5GlacNAc_2$. Its glycosylated asparagines are Asn92 and 161, and three disulfide bridges are at Cys3–Cys17, Cys175–Cys191, and Cys300–Cys303.[331] Three crystal structures of EndoPG Ia (the unliganded EndoPG Ia and the binary and ternary complexes of EndoPG Ia with galacturonate) are determined in native and two galacturonic acid complex states by X-ray crystallography and, consequently, the catalytic mechanism of EndoPG Ia is elucidated.[332]

4.08.3.6 Perspectives

Completion of genomic analysis of several plant pathogens *Magnaporthe grisea* (rice blast), *Gibberella zeae* (head blight in wheat and barley), and *Streptomyces scabies*, causing potato scab, allowed us to identify genes for phytotoxin biosynthesis and to study disease development in detail at molecular level. It has been found that rice blast fungus produces some polyketide–nonribosomal peptide hybrid molecules during infection. The dynamic action and role of phytotoxins will be elucidated by transcriptome and metabolome analysis. Comparative genetic study in usual and pathogenic strains will provide information on how pathogenicity is acquired and developed. These studies on various types of diseases give a general idea on the strategy of microorganisms in disease development, and also the solution to prevent their infection.

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Biographical Sketches

Hisakazu Yamane is a Professor at Biotechnology Research Center, University of Tokyo. He received his Ph.D. in 1975 in agricultural chemistry from the University of Tokyo. He joined the Department of Agricultural Chemistry at the University of Tokyo as an Assistant Professor in 1974 and then moved to Biotechnology Research Center, the University of Tokyo as an Associate Professor in 1993 and was promoted as Professor in 1998. His present research focuses on elucidation of molecular mechanisms to activate disease resistance in higher plants.

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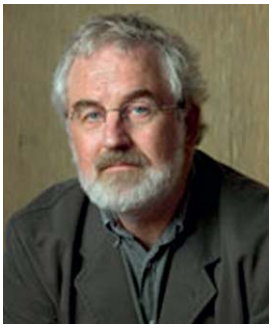

Maurice W. Sabelis is a Professor at the Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands. He received his Ph.D. degree in theoretical production ecology and entomology in 1981 from the Agricultural University of Wageningen. After his Ph.D., he was an Associate Professor for 4 years in the department of Animal Ecology of Agricultural University of Wageningen and also held that position for 4 years at the University of Leiden. Starting from 1988 he held a chair in population biology at the University of Amsterdam. In 2006, he was selected to be a KNAW professor in the service of the Dutch Academy of Sciences. His current research interest concerns the evolutionary and ecological dynamics of tritrophic systems.

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Takeshi Sassa is a Professor Emeritus at the Faculty of Agriculture, Yamagata University. He received his Ph.D. (1969) in agricultural chemistry from Nagoya University. He spent 3 years at Nagoya University, working on bioactive fungal metabolites from 1967, and moved as an Associate Professor to Faculty of Agriculture, Yamagata University in 1970. He worked as a Visiting Researcher with Professor R. P. Pharis at Calgary University in 1975. He received an award for encouragement of young scientists for the study of plant growth substances produced by fungi from the Japan Society of Bioscience, Biotechnology, and Agrochemistry in 1975, and the Japanese Society for Chemical Regulation of Plants Award in 1993. In 1977, he was promoted as Professor at the same faculty. He retired in 2005, and then spent 2 years as a Visiting Professor at the same faculty to study the biosynthesis, including cyclases, of fungal diterpenoids. His present research focuses on creation of a differentiation-inducing anticancer substance by chemical modification of fungal diterpene glucosides, cotylenin, and fusicoccin.

Hideaki Oikawa is a Professor at the Division of Chemistry and the Graduate School of Science, Hokkaido University. He received his Ph.D. in 1984 in organic chemistry from Hokkaido University. He worked as a postdoctoral fellow with Professor D. E. Cane at Brown University from 1984 and with Dr. K. Isono at RIKEN in Japan from 1985. He joined the Department of Agriculture, Hokkaido University as an Assistant Professor in 1986 and was promoted as Associate Professor in 1999. He attained the present position in 2003. He received an award for encouragement of young scientists for the study of biosynthesis of natural products involving Diels–Alder reaction from the Japan Society of Bioscience, Biotechnology, and Agrochemistry in 1993, and the Chemical Society of Japan Award for Creative Work in 2004. His present research focuses on engineering of biosynthesis of natural products, combinatorial biochemistry, and reaction mechanism of C–C bond forming enzymes.

4.09 Chemical Defense and Toxins of Lower Terrestrial and Freshwater Animals

Konrad Dettner, University of Bayreuth, Bayreuth, Germany

4.09.1 Introduction

Terrestrial and freshwater animals possess various mechanisms for defense against predatory or pathogenic organisms.[1,2] In all animals, defensive substances may be present in tissues, the blood (nonglandular secretion), and exocrine eversible or noneversible glands. Occasionally, such compounds are found in regurgitants or enteric discharges and defecations.[3] Certainly, all developmental stages from eggs to adults,[4] and also from monocellular to multicellular organisms, that is, from protozoans to vertebrates, may be chemically protected. Many of these natural products represent simple, widely distributed chemicals; however, there are also various unique products,[5] frequently restricted to special taxa. The glands may represent oozing, spraying, reactor, or tracheal glands.[3] It is also of interest to investigate whether animals manufacture their own toxic or distasteful compounds (intrinsic origin) or whether behavior-modifying chemicals are acquired from host plants, other animals, bacteria, or fungi (extrinsic

origin). Considerable biosynthetic knowledge is especially available in insects,[6] while data from other animals are less available or missing. Usually, defensive compounds may exert their effect via foul smell (repellents) or bad taste (deterrent). Very often, defensive compounds may induce cleaning behavior in the aggressor, giving the prey time to escape. There are a number of irritant compounds that may also induce pain. Examples include nonspecific toxicants, or hot secretions. Especially, urticating hairs and delivery of venom by injectable means, for example, stings or fangs, are widely observed. Venoms are usually complex aqueous mixtures of proteins (enzymes), peptides, carbohydrates, nucleosides, biogenic amines, amino acids, lipids, and metallic cations. Many of these toxins represent presynaptic and postsynaptic neurotoxins, cytotoxins, myotoxins, and cardiotoxins. These poisons may act immediately or may show delayed effects (e.g., emetics, vesicants). Certain animals may secrete sticky components that harden like glues and incapacitate attackers.[3] The chemistry of these sticky, slimy, or resinous fluids is often not well known.

This chapter focuses on low molecular defensive compounds, typical products of secondary metabolism; however, it also covers principal data on high-molecular, behavior-modifying chemicals. Taxonomically, this compilation covers all protozoan and metazoan taxa apart from Deuterostomia (especially vertebrates) and Insecta. Allelochemicals of insects and vertebrates are not included as these have been recently reviewed,[7,8] and the actual data will be published elsewhere. Whenever possible, actual reviews are cited and updated. Chemical structures of chiral compounds depicted here reflect the relative or absolute configurations of the substances as far as they are known today. Concerning a wide array of organisms, there exist actual surveys of chemical defenses of insects,[3,11–15] arthropods,[2,3,4–7,9,10–16] or animals in general.[13–15] However, until now, many taxonomically lower animal taxa have never been reviewed with respect to chemical defense. Therefore, here such phenomena are cited in selected taxa even if no or scarce chemical data on the relevant natural compounds are available. Various reviews approach venoms and toxins of animals more generally,[15–21] and sometimes may include low molecular defensive compounds.[16] Taxonomic data are especially taken from Dettner and Peters,[22] Storch and Welsch,[23] Westheide and Rieger,[24] and Resh and Cardé.[25] Chemicals of extrinsic origin are not dealt with in detail; however, they may be of special interest if they are chemically modified during metabolic processes.

4.09.2 Alveolata

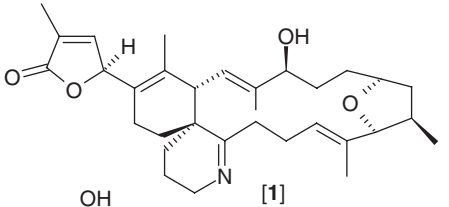
Alveolata include the former unicellular Dinoflagellata, Ciliophora (Ciliata), and Apicomplexa (Sporozoa). It is interesting to note that toxic species within unicellular eukaryotes seem to be restricted to representatives of Alveolata. Among 2500 species of Dinoflagellata, several mostly photosynthetic and marine species produce or accumulate toxins.[26] Only representatives of the freshwater and marine genus *Gymnodinium* are known to produce the pentacyclic imine gymnodimine (**1**),[27] which is moderately toxic (LD_{50}: intraperitoneal injection, mouse, 96 mg kg^{-1}).

Some heterotrich ciliates possess specialized exocytotic organelles, the extrusomes. Upon molestation, these structures may discharge material to the cell surface. Especially in ciliates, there are haptocysts (with toxic enzymes), mucocysts (with a protective coat), trichocysts (spindle-shaped bodies with paracrystalline matrix), and toxicysts (tubular structures).[28] Recently, it was discovered that the blue and red pigments stentorin (**2**) and blepharismin (**3**), two polyketides from the exocytotic organelles of *Stentor* and *Blepharisma*, primarily act as chemical defense of these Ciliophora against small predators.[29] The same function was ascribed to climacostol (**4**, 5-(*Z*)-non-2-enylresorcinol) and the two congeners of climacostol (**5**, 5-(*Z*,*Z*)-undeca-2,5-dienylresorcinol; **6**, 5-(*Z*,*Z*,*Z*)-undeca-2,5,8-trienylresorcinol), three colorless lipids isolated from the heterotrich ciliate *Climacostomum*.[30]

In addition, spirostomin, spiro[(2.5-dimethyl-5,6,7,8-tetrahydronaphthalene-1,4-dione)-8,6′-(pyrane-2′,5′-dione)], was identified from the ciliate *Spirostomum teres*.[31] It exhibits toxic activities against predatory ciliates.

As shown by bioassays, the karyorelictid ciliate *Loxodes striatus* may release toxin-containing yellow-brown extrusomes, which repel predators such as *Dileptus* (Ciliata) and *Stenostomum* (Turbellaria).[32]

Other high molecular toxins were recorded from the Apicomplexa (Sporozoa). A protein–hyaluronic acid complex (toxotoxin) was described from peritoneal exudates of mice, which is probably produced by hosts in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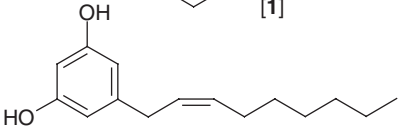
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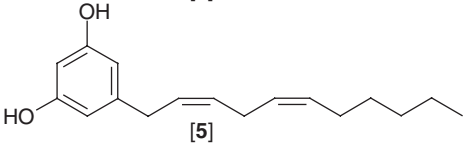
[**4**]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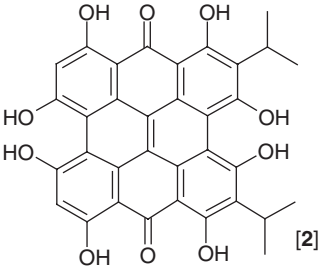
[**5**]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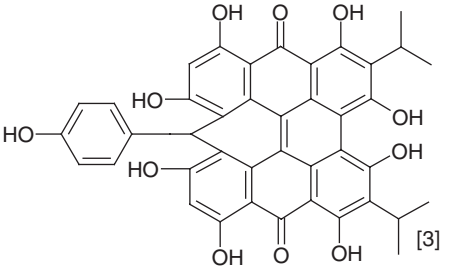
OH

[**2**]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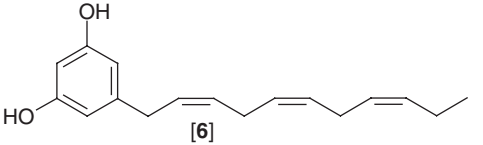
[3]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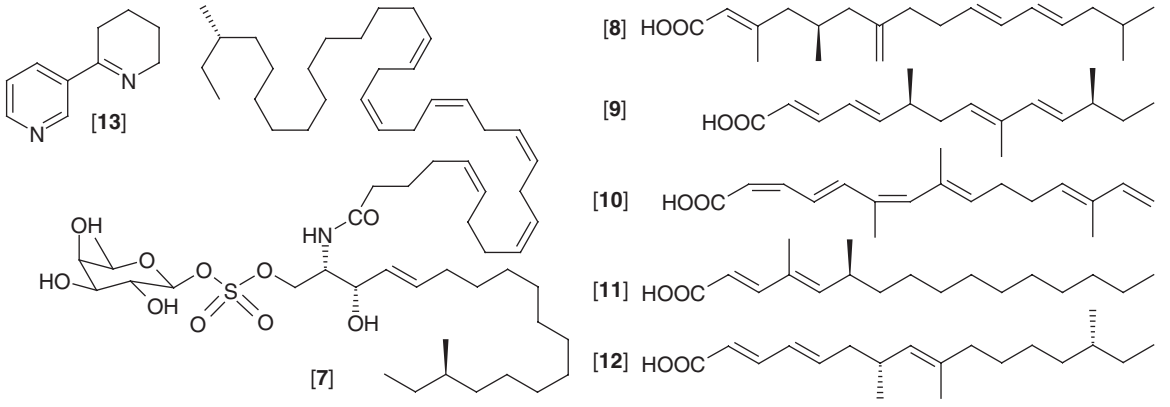
[**6**]

response to coccid infections.[33] Moreover, there are known toxic *Toxoplasma* lysates, such as toxoplasmine, extracts from the cysts of *Sarcocystis* (Sarcocystin), or an *Eimeria* toxin.[34] A further toxofactor, a glycoprotein (molecular mass (MM) 50 000–100 000), was found to be associated with *Toxoplasma gondii*.[35]



4.09.3 Porifera

The sessile sponges (10 000 species), especially marine species[36] and also dry powders of freshwater species,[37] may contain a large array of bioactive molecules and may be even used as pharmaceuticals. Also freshwater species of the family Spongillidae contain more than 100 novel unusual and rare fatty acids, lipids, and sterols.[38] From the freshwater species *Ephydatia syriaca*, syriacin (**7**) was isolated. It is a novel unusual sulfated ceramide glycoside containing a branched long-chain fatty acid, that is, (all *Z*)-34*S*-methylhexatriaconta-5,9,12,15,18,21-hexaenoic acid.[39] Syriacin showed a distinct antifeeding activity against goldfish. Other freshwater species of the genera *Ephydatia*, *Nudospongilla*, and *Cortispongilla* produce multibranched polyunsaturated and long-chain fatty acids (**8**–**12**) that are active against Gram-positive bacteria and have been proved to be toxic against the shrimp *Artemia salina*.[40] Among these compounds, **9** shows an unusual carbon skeleton as the 6,9-methyl branching includes two unsubstituted carbons, which is not in line with a typical biosynthetic scheme involving acetate and propanoate. Finally, a *Lubomirskia* species from the Lake Baikal and its symbiotic dinoflagellates, which are related to *Gymnodinium sanguineum* (see gymnodimine (**1**)), contain the polyether toxin okadaic acid. In the sponge, this strong protein phosphatase 2A inhibitor is present in the free form as well as in a protein-bound state. The authors suppose that the toxin may contribute to the cold resistance of the sponge.[41]

4.09.4 Cnidaria

Just as Porifera, the sessile, predatory, and often soft-bodied Cnidaria (9200 species) depend on offensive and defensive allomones for prey capture and survival. This is also true for the small group of freshwater species belonging to Hydrina (Capitata). The nematocyst venom of *Hydra vulgaris* has been reported to exhibit strong neurotoxic and hemolytic activities and a phospholipatic activity similar to snake venoms.[42] Venom fractionation revealed the presence of a high-molecular-weight (100–200 kDa) toxic cytolysin (a pore-forming substance), a toxic phospholipase, and a 30–100 kDa neurotoxin causing paralysis and death in *Drosophila*. By a bioinformatic approach in *Hydra magnipapillata*, orthologues of cnidarian phospholipase A2 (PLA2) toxins and cytolysins were found, which belong to the actinoporin family. *Hydra magnipapillata* also expresses proteins similar to elapid-like (elapid PLA2s) and *Conus*-like phospholipases (Conus PLA2, conodipine-M), CRISP proteins (cysteine-rich secretory protein: wasp venom antigen 5), prokineticin-like polypeptides, and toxic deoxyribonucleases.[42] In contrast, short-chain neurotoxins affecting sodium and potassium conductance were found to be absent in *H. magnipapillata*.[42] From noncnidocystic origin, a paralysis-inducing neurotoxin was isolated from *Chlorohydra viridissima*.[43] The compound showed cytotoxic activities against insect cells but not against mammalian tissue.

It was also reported that polyps of the freshwater jelly-fish *Craspedacusta* produce toxins which may damage larvae of freshwater fishes and amphibians.

4.09.5 Platyhelminthes (Flatworms)

Flatworms with a total of about 22 500 species distributed worldwide (75% of the marine, limnic, or terrestrial species are parasitic) are chemically poorly studied but use interesting means for chemical defense. Many species show active and passive toxicities, and planarian extracts usually cause severe reactions when they are applied intraperitoneally into vertebrates.[44] Epidermal and subepidermal gland cells of flatworms produce various types of ovoid solid gland secretions (rhabdoids), which are especially typical for representatives of Rhabditophora. These solid proteinaceous secretions are characterized by an enormous swelling and sorption capacity.[45] The material protects against bacteria and fungi, may close wounds, and represents a deterrent against fish.[46] In addition, the multiciliar epidermis of many species together with frontal glands may produce large amounts of mucus (for planarian locomotion or fixation of prey) or may generate mucus, which is formed from rhabdites.[47] Few species may penetrate with their male sexual organs into prey and paralyze them with secretions produced by associated toxin glands.[44] From predatory planarians (e.g., *Mesostoma*), both 'mucus-trapping' of prey[48] and release of a chemically unknown neurotoxin into the water for catching prey are known.[49] Finally, *Microstomum* species may take up toxic nematocysts from *Hydra* polyps, which are subsequently used as defense against aggressors.[44]

4.09.6 Nemathelminthes: Nematoda

This large group of economically important animals includes more than 15 000 species found in different environments such as freshwater and soil. Basic data on the bionomy or chemical ecology of this group are rare; however, there are predatory species (e.g., *Aphelenchoides*) that may kill their prey by injecting unknown toxic and proteolytic secretions from esophageal glands with the help of stylets or teeth.[50] In addition, *Ascaris* species rely on an internal peptide-based antibacterial system to destroy Gram-negative (linear peptide cecropin P1) or Gram-positive (cysteine-rich ASABF peptides) bacteria.[51] As was shown in *Caenorhabditis elegans* with the presence of p38 mitogen-activated protein (MAP) kinase pathway, a conserved innate immune defense system against pathogens exists.[52]

Because of the increased internal pressure ('Hydroskelett'), dissected mawworms emit malodorous compounds, which are present in both perienteric fluid and tissues of these nematodes. During dissection, these volatiles may evoke pruritus, inflammation of the eye, and vomiting in humans. In *Ascaris lumbricoides*, and also in other species such as *Parascaris equorum*, formic, acetic, propionic, *n*-butyric, 2-methylbutanoic, and caproic acids (C_5- and C_6-acids: main compounds) were found along with unidentified C_5-α,β unsaturated acids.[53] These acids are probably derived from mawworm metabolism and are not formed by microbial activity.

4.09.7 Rotatoria (Rotifers)

Very small-sized rotifers dominate in various freshwater habitats. As a defense against predators, many species possess an intrasyncytial covering that may be either flexible or rigid and hard. Uniquely, colonies of the freshwater colonial rotifer *Sinantherina socialis* (Monogononta, Flosculariidae) have been shown to be unpalatable to various zooplantivorous fish[54] and aquatic invertebrates (dragonfly and damselfly larvae, notonectids, *Hydra*).[55] Actually, the origin and chemistry of these deterrents are unknown.

4.09.8 Nemertini

Nemertini worms, which are mainly found in the sea and also occur on land and in freshwater habitats, comprise about 1100 mostly predatory species. Various toxins are found in integumentary tissues (especially pseudocnidia and rhabdoids) and glandular epithelia that are associated with the stylet and proboscis. Known toxins are neurotoxic pyridyl toxins such as anabaseine (**17**), 2,3′-bipyridyl, and nemertelline, the last of which is

made up of four pyridine subunits.[56] However, toxic peptides such as *Cerebratulus* toxins A II, A III, B II, B IV as well as cytolysins, amphiporine, and nemertine have also been found. It is suggested that protein toxins serve as chemical defense against predators, whereas pyridine alkaloid toxins represent both offensive and defensive toxins.

4.09.9 Mollusca

In order to defend against invertebrate or vertebrate predators, land and freshwater snails usually produce large quantities of viscous mucus. The solids in the mucus are slightly more concentrated than the blood. In *Oxcychilus alliarius* the mucus consisted of 6.8% solids composed of inorganics (8%), proteins (77%), and carbohydrates (15%; e.g., fructose, glucose, galactose, glucosamine, galactosamine).[57] The viscosity of the mucus is variable and is highly dependent on divalent-ion content of the slime, which is important on molestation. If an enemy pierces the body wall of certain slug species, crystal-laden integumental cells void their white contents into the slime, which coagulates.[3] Specific 15 kDa glue proteins may cross-link other proteins in the gel, resulting in rapid precipitation as soon as substantial quantities of zinc (46–189 ppm) and/or lower amounts of iron, copper, and manganese are added.[58] On repeating the experiment by adding a chelator, the viscosity of the secretion was found to be very low. It is also remarkable that layers of mucus that were originally deposited as trails may be incorporated into a new thinner slime trail by another trail-following slug individual, which represents an effective mechanism of energy saving.[59]

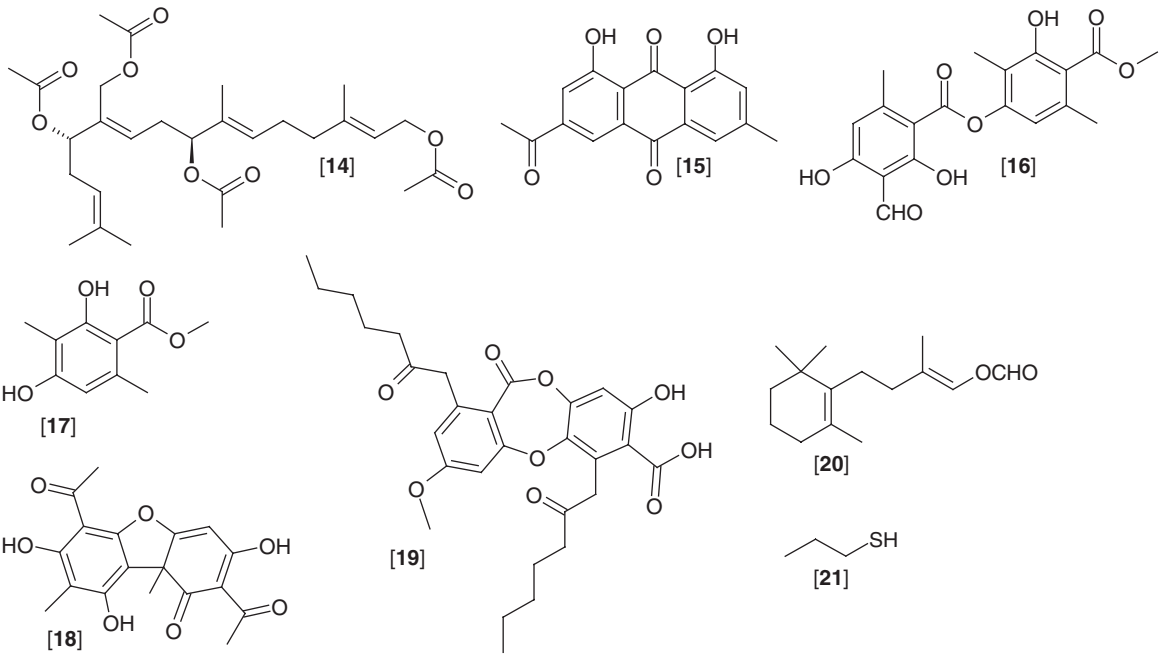
As compared to marine snails where toxins and defensive compounds are abundant, chemical defence in terrestrial snails seems to be rare. The eggs of terrestrial *Arion* snails were found to contain the first-characterized more complex diterpene miriamin (**14**).[60] This polyoxygenated geranylgeraniol derivative was shown to be an antifeedant against beetles. Few other similar miriamin analogues were identified from Asteraceae, where analogues occur as free alcohols. The authors suggest these compounds to be biosynthesized *de novo* by the slugs.

Chemically unknown tissue toxins from internal organs that may kill *Pterostichus* carabids are uniquely known from the milacid slug *Tandonia budapestensis*,[61] which shows an aposematic coloration (orange dorsal line). Moreover, *Balea*, *Chondrina*, and *Helicigona* species sequester the anthraquinone parietin (**15**) and the depside atranorin (**16**) (and degradation products; **17**) from lichens they feed upon and may transfer these compounds into eggs and neonates.[62] Other lichen metabolites such as (+)-usnic acid (**18**) and α-collatolic acid (**19**) were not found in the snails' body but appeared in their feces. These data, along with feeding experiments with *Arion lusitanicum* and various alkaloids such as sparteine, lupanine, quinidine, and atropine indicated that slugs can manage very well with toxic compounds and have a higher tolerance against such alkaloids than vertebrates.[63]

Obviously, further species of the genera *Pomacea* (freshwater) and *Theba* (terrestrial, Mediterranean, bitter-tasting[61]) contain unknown toxic compounds or deplete luminant slime as was observed in the freshwater snail *Latia neritoides* (Lymnaeidae, Basommatophora) from New Zealand.[64] When touched, the latter species secretes the sticky luminescent slime, which repels predators and releases cleaning behavior.[65] The bioluminescence involves a 178 kDa luciferase, the formiate of the enol of a nor-sesquiterpene aldehyde (**20**, (*E*)-2-methyl-4-(2,6,6-trimethylcyclohexenyl)but-1-enylformate) and a protein. A catalytic role was suggested for this purple protein, which has to be confirmed. The emitter was suggested to be a protein-bound flavin.[66] *Dyakia striata* represents the only land mollusk known to be luminescent. The chemically unknown secretions are produced from gland cells situated below the mucous gland in the anterior part of the foot. However, the luminous reaction does not take place upon disturbance.[65]

Aposematically colored eggs of *Pomacea* are rejected by various predators from fish to birds.[67] Finally, it is known that southern African carnivorous slugs of Chlamydephoridae (hunter slugs) killed or immobilized earthworms, other snails, or diplopods by transferring a chemically unknown toxin from the anterior pedal mucous gland.[68] Upon molestation, the garlic snail *Oxychilus alliarius* produces a distinct garlic-like odor, which is due to 1-propanethiol (**21**), and deters hedgehogs.[69] The compounds are produced at the right side of the mantle close to the pneumostome. The secretions represent proteinaceous material enriched with sulfur compounds. During isotope-labeling experiments, ^{35}S was incorporated into the tissue of the odor gland (two cell types), which discharges its secretion into a groove of the pneumostome. The related species, *Hyalinia*

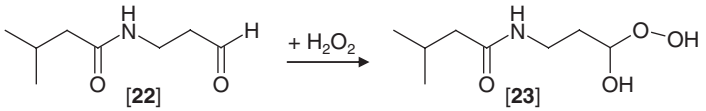
cheliella, was also described as odorous. Furthermore, mantle glands, which possibly secrete defensive secretions, are found in representatives of the genera *Cassidula*, *Oncidielle*, and *Carychium tridentatum.*



4.09.10 Annelida: Clitellata: Oligochaeta

Several species of earthworms (4000 species) are unpalatable or exhibit an unpleasant smell. In these species, between segments 5 and 15, dorsal pores are found through which worms such as *Eisenia foetida* may actively deplete malodorous coelomic fluid to defend against aggressors. Coelomic fluid together with coelomocytes can also be experimentally obtained through these pores by electric stimulation of the worms.[70] In certain species such as *Pheretima ophiodes*, *Megascolides australis*, and *Didymogaster sylvaticus*, this fluid can be maximally shot up to 1 m or more.[71] It has been shown that the coelomic fluid especially of *E. foetida* is toxic to vertebrates but not to invertebrates.[72] This toxicity is partly due to a 41 kDa protein called lysenin, which binds specifically to sphingomyelin.[73] Apart from lysenin, a lysenin-related protein and the cytolytic eiseniapore (38 kDa[74]) and two other related 40 and 45 kDa hemolytic and antibacterial hemolysins called fetidins could be recorded from *E. foetida*.[75,76] Moreover, this species contains an antibacterial peptide, OEP3121, with an MM of 510.8 Da (sequence: ACSAG) in its hemolymph.[77]

It is remarkable that various representatives of 5 families (terrestrial species: Acanthodrilidae Magascolecidae, Lumbricidae, Octochaetidae; freshwater species: Enchytraeidae) out of 32 families include luminescent species. In response to a mechanical stimulation, most of these species become luminous during exuding coelomic fluid and its free cells through dorsal pores.[78,79] In *Diplocardia* earthworms, a copper-dependent oligomeric luciferase of 300 kDa acts as a catalyst in a bioluminescent reaction involving the degradation of 3-(isovalerylamino)-1-hydroxy-1-propyl hydroperoxide (**23**). This substrate is formed spontaneously on addition of hydrogen peroxide to *Diplocardia* luciferin (3-(isovalerylamino)propanal) (**22**).[79] Both in *Fridericia heliota* and in the genus *Henlea* there was detected a luciferin-luciferase reaction which is not H_2O_2–dependent and does neither cross react with *Diplocardia* luciferin nor with reaction components from the firefly *Photinus pyralis*.[79] In *Fridericia* luciferin is a stable compound (0.5–0.7 kDa) whereas it is unstable in *Henlea*. The *Fridericia*- luciferase is a dimer of about 60 kDa (requiring O_2, ATP and Mg^{2+}), the *Henlea*-luciferase is a homodimer of 72 kDa, which requires O_2 and Ca^{2+} as activators.[79]



Whether carnivorous earthworms such as *Agastrodrilus dominicae* use toxins to kill other species or conspecifics is yet to be determined.[71] As a whole, chemical defense mechanisms of terrestrial and freshwater earthworms have been poorly studied.

4.09.11 Annelida: Clitellata: Hirudinea

Most of the 600 species of leeches are predatory or ectoparasites; few of them are used in medicine and veterinary medicine.[80,81] In order to paralyze or consume an invertebrate or vertebrate prey, leeches inject various gland constituents from salivary glands into their victims but they are also able to conserve ingested vertebrate blood by antibiotics and may concentrate it. From *Hirudo medicinalis* and *H. verbena*, about 40 different salivary gland constituents are known.

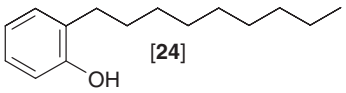
Hirudin, a peptide consisting of 65 amino acids from *H. medicinalis*, is known to inhibit blood coagulation by binding to thrombin just as hementin from *Haementaria ghilianii*, which decomposes fibrinogen and fibrin.[81,82] In contrast, factor Xa inhibitor inhibits the activity of coagulation factor IXa and forms equimolar complexes. Calin, another peptide, inhibits both collagen-mediated platelet aggregation and binding of Willebrand factor to collagen. Moreover, destabilase may dissolve fibrin and show thrombolytic effects, whereas hirustasin inhibits kallikrein, trypsin, chymotrypsin, and neutropholic cathepsin G. Bdellins and eglins, among other inhibitory functions, are anti-inflammatory. Hyaluronidase increases interstitial viscosity, acts as a 'spreading factor' and tryptase inhibitor, and inhibits proteolytic decomposition of host mast cells. In addition, salivary secretions of *H. medicinalis* contain complement and carboxypeptidase inhibitors, histamine-like substances, and acetylcholine as vasodilators and anesthetic substances.

Interestingly, the slime of *Glossiphonia* and other leeches may evoke a remarkable defensive behavior in freshwater snails of the genus *Physa*.[83] It is remarkable that salivary glands in Gnathobdelliformes open at the teeth surface, whereas in Rhynchobdelliformes salivary glands are associated with the base of the extrusible proboscis.

4.09.12 Arthropoda: Onychophora

Onychophoran species (180 species) produce a sticky secretion in their large paired slime glands for entangling of prey and also for self-defense. The fluid material is rapidly ejected (maximally 30 cm distance) from a pair of oral papillae, is denatured by the air, and develops increasingly sticky whitish threads.[84] In the laboratory, *Peripatopsis* may deplete its secretion 10 times per 2 min and afterward needs about 20 min in order to regenerate.[84] Only after 5–6 weeks, young onychophorids are capable of ejecting secretion.

The onychophorid secretion consists of threads of varying diameter and represents a composite material containing protein, sugar, lipid, and a surfactant.[85] On the hydrofuge tuberculate surface of onychophorids, the secretion quickly decomposes and is eliminated. In a *Peripatopsis* species,[86] the secretion is stored in a watery glycine/glutaminic acid buffer and consists of 84% water and 16% proteine. Mainly glycine (41%), glutamic acid (11%), aspartic acid (2.7%), and lysine (1.3%) could be recorded as free amino acids. In an *Euperipatoides* species, the amino acid composition (mainly glycine: 27.25%; proline: 13%; lysine: 6.9%) of the slime (90% water) suggests the presence of collagen or collagen-like compounds. About 1.3% of the dry weight of the slime gland contains sugars, which is due to the *O*-glycosylation proteins especially as *N*-galactosamine.[85] Moreover, the slime contains small amounts of C_{18} fatty acids and several isomers of nonylphenol as surfactants with 2-nonylphenol (**24**) as the main constituent.[85] As nonylphenols are widespread environmental pollutants, it remains to be checked whether the identified group of isomers represent true natural products. Probably, lipids prevent the slime gland secretions from adhering to the interior wall of the gland tissue.



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4.09.13 Arthropoda: Tardigrada

The very small aquatic and terrestrial tardigrades with about 700 species possess stylets behind their mound opening, which are associated with a large gland. It is not exactly known whether those predatory species feeding on Rotatoria, nematodes, enchytraeids, or other tardigrades secrete either digestion enzymes or toxins that may immobilize the prey.[24]

4.09.14 Arthropoda: Crustacea, Ostracoda

Ostracoda represent small, often planktonic, crustaceans from marine, brackish, and freshwaters and comprise 62 000 fossil and recent species. Despite the fact that apparently no toxic species exist, some of them show unusual abilities in that they may survive the gut passage of predatory fish. Among the freshwater species, 26% of experimental specimens belonging to the ostracod *Cypriodopsis vidua* traversed the gut of bluegill sunfish apparently unharmed,[87] which might be due to mechanical defense. *Vargula* (*Cypridina*) *hilgendorfii* and also other mainly marine species from the genera *Cypridina* and *Pyrocypris* may produce luminescent signals to surprise and deter predators. The *Cypridina* luciferin, 2-[3-[2-[(2*S*)-but-2-yl]-6-(1*H*-indol-3-yl)-3-oxo-7*H*-imidazo[2,1-*c*]pyrazin-8-yl]propyl]guanidine, comprises moieties of tryptophan, arginine, and isoleucine, and is enzymatically oxidized to an oxyluciferin. Both enzyme and substrate are ejected into the water from a gland localized in the upper lip. In contrast to fireflies, there is no need for ATP or other substances.[88] The same luciferin is also trophically transferred into certain fish species.[88]

4.09.15 Arthropoda: Crustacea, Decapoda

Among this large crustacean group, comprising 18 000 species, various defense mechanisms exist ranging from autotomy of legs or pincers, to mechanical defense such as hard carapaces and spines. Chemical defense seems very rare. In Japan (Lake Suwa) and India (Chilka Lake), the freshwater shrimp *Xiphocaridina* (*Paratya*) *compressa* and the brackish water species *Pennaeus indicus* produce bioluminescence due to infection by bacteria.[88]

4.09.16 Arthropoda: Crustacea, Amphipoda

The more than 6000 species show various mechanisms of defense against predators. In marine species, amphipod luminescence always seems to be a defensive response.[88] In the freshwater species *Hyalella azteca*, a bioluminescence caused by bacteria was reported.[89]

4.09.17 Arthropoda: Crustacea, Isopoda

Upon molestation, many woodlice (Isopoda; more than 10 000 species) may discharge a malodorous sticky fluid (in *Oniscus asellus*: amount 48–54 μg per individual) from the uropods, where the material is produced by internal gland cells. The fluid material is collected within the uropod grooves and forms a small droplet at the uropod tips, which may be pulled into long threads.[90,91] The proteinaceous secretion is unusually rich in glycine (21%) and proline (14%) and coagulates because of a polymerization process. The maximal molecular weight of the protein amounts to 13 500. The low level of hydroxyproline (0.5–1%), cysteine (0.5%), and

valine (3%) indicates that the isopod material is not related to collagen-like, mollusk adhesive-like, or elastin-like proteins.[90] The secretion although not toxic evidently represents a deterrent to various ants and is also directed against spiders.[92]

Additional glands, opening laterally of tergal plates, also discharge upon molestation. The chemically unknown secretion is said to have deterrent activities against spiders.[90]

4.09.18 Arthropoda: Chelicerata, Scorpiones

Toxins of scorpions (about 1300 species worldwide) are produced by a pair of glands that are covered by muscles and are found in the telson (bulbous vesicle and needle-like oculeus), that is, the abdominal tip. Both exit ducts of the venom glands open via two apertures before the tip of the oculeus.[93,94] Scorpions use their venoms either when hunting and seizing prey or when they are threatened. Envenomation by scorpions can be usually effected by stinging and in a few cases (species with large tail segments and large venom vesicles such as *Hadrurus* and *Parabuthus*, respectively) by squirting (emission of a fine spray for distances of up to 1 m is possible). If these venoms enter the eye or open lesions, the consequences might be similar to those caused by cobra envenomation by spitting.[95] The milky secretions of these selected species may act as allergens and smell like mustard.[94]

Scorpionid secretions represent a mixture of neurotoxic polypeptide toxins, proteolytic and hemolytic enzymes (phospholipases A, acetylcholinesterases, ribonucleases, hyaluronidases), and biogenic amines (serotonin, tryptamine, histamine). The polypeptide toxins (the so-called scorpamines) contain fewer than 40 or 60–76 mostly alkaline and aromatic amino acids stabilized by four disulfide bridges.[20,96]

Neurotoxins of scorpions especially represent ion channel toxins that mainly affect sodium and potassium channels. Several compounds represent neurotoxins that are directed selectively against insects.[97] Na^+ channel-specific α-, β-, and γ-toxins are composed of 58–76 amino acids and contain four stabilizing disulfide bridges.[98] The well-studied K^+ channel-specific toxins (divided into at least nine distinct peptide subfamilies) bind to the extracellular face of the channel and comprise 29–39 amino acids stabilized by 3–4 disulfide bridges.[99] Various Ca^{2+} channel scorpion toxins, antimicrobial peptides, and short insectotoxins active on Cl^- channels have been found.[96]

4.09.19 Arthropoda: Chelicerata, Pseudoscorpiones

Members of most families of predaceous Pseudoscorpiones (more than 3000 species) possess poison glands located in one or both chelal fingers.[100] The secretion, which is present in most members of Chelonethi, is used for anesthetizing or killing larger prey organisms such as arachnids or insects. It acts very fast, and also in humans painful effects may be observed.[101] No detailed chemical data exist on the secretions; however, components of the venom of *Paratemnus elongates* have been found to act in the binding and dynamics of transmitters L-glutamate and GABA, which was investigated at synaptosomal membranes of rat cerebral cortex.[102] Therefore, these venoms can serve as tools and new drug models for understanding neurotransmissions.

4.09.20 Arthropoda: Chelicerata, Acari (Mites)

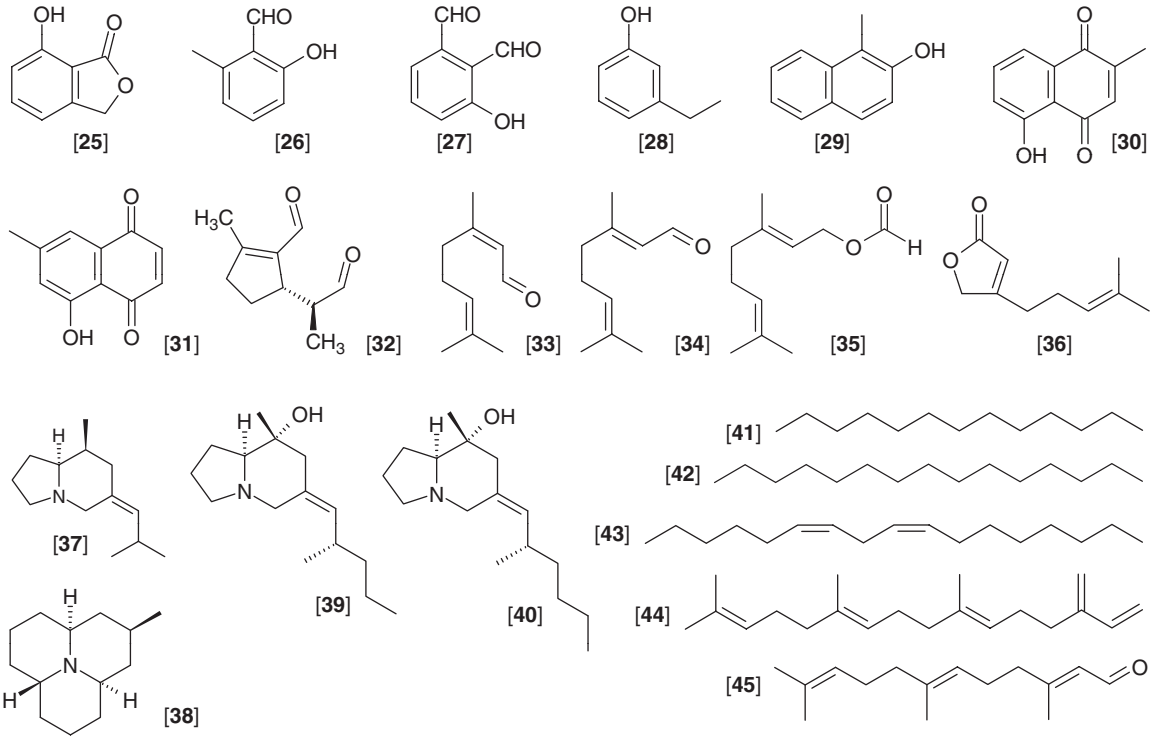
With about 400 families and 50 000 species (probably more than 100 000), Acari represent economically and medically the most important Chelicerata, which are found in almost all habitats.

As can be expected by their varying biology, mites show manifold mechanisms to avoid predation, such as jumping away, shedding of legs (autotomy), curling legs against body (thanatosis), armored cuticles (e.g., beetle and armored mites), withdrawal of legs under armored cuticle shields (e.g., box mites), erection of spiny long setae, and covering of integuments with wax, soil particles, or detritus.[103] In addition, many mite species are aposematically colored or possess exocrine glands with allomones, alarm pheromones,[103] and may also contain aggregation or sex pheromones or compounds with antifungal or bactericidal activities.

Obviously, chemical defense is widespread in Acari; however, due to their small size, chemistry and biological significance of the secretions are poorly known.

Acari are divided into Anactinotrichida (Parasitiformes in a wider sense) and Actinotrichida (Acariformes).

Among Anactinotrichida, defensive compounds, toxins, and antibacterial agents are found in representatives of Holothyrida, Ixodida, and Gamasida. In Holothyrida (Tetrastigmata), nymphs of *Allothyrus* mites produce defensive droplets from gland openings when molested.[104] In Ixodida (Metastigmata; ticks), certain species are known to transmit pathogens, but in addition it is found that during blood meals neurotoxins from salivary glands of more than 80 (of 870) ixodid species worldwide may be transferred into vertebrates (e.g., humans, cows, sheep, birds) and cause tick paralysis. Worldwide, thousands of animals (cows and sheep) are killed, and interference with neuromuscular transmission seems to represent the primary target of these toxins. At first a 40–80 kDa protein fraction from *Ixodes holocyclus* seemed to contain the toxin called holocyclotoxin.[105] Later, a 6 kDa toxin with 50 amino acids was isolated from the same species and was found to be homologous to scorpion and spider toxins. It is suggested that one of several closely related toxins alone may cause the paralysis syndrome. According to Jones,[106] another red-colored, water-soluble toxin from the tick's cuticle may cause respiratory paralysis in mice. Recently, further low-molecular-mass paralysis toxins could be isolated such as an 11 kDa toxin from *Argas*[107] or four tick salivary gland proteins (TSGPs) from *Ornithodoros*, which were assigned to the protein family of lipocalins.[108]



It was furthermore suggested that some metastriate ticks produce gland secretions that protect them from ants.[103]

In *Ornithodoros* ticks, the peptide defensin A and three isoforms of defensin were recorded to be secreted into the midgut lumen and, therefore, provide midgut defense.[109]

With respect to chemical defense, most species of the Gamasida or Mesostigmata seem to be nonsecreting. However, Sakata *et al.* [110] could identify the two hydroxymethyl naphthoquinones plumbagin (**30**) (5-hydroxy-2-methyl-1,4-naphthoquinone) and 7-methyljuglone (**31**) (5-hydroxy-7-methyl-1,4-naphthoquinone) in *Uroactinia hirschmanni*, which has several exocrine glands.

Among Actinotrichida (Acariformes), defensive compounds and growth regulators were recorded from Actinedida, Oribatida, and Acaridida.

Eriophyoid mites from Actinedida (Trombidiformes) are reported to produce unknown chemicals in their salivary glands with plant growth regulatory effects. These activities were tested by using a wheat coleoptile and an excised cotyledon growth test, which are used for recording indole-3-acetic acid (IAA)-like and cytokinin-like

activities.[111] In certain dytiscid water beetles, IAA and other growth-promoting substances with weak fungicidal and bactericidal activities are present in typical defensive glands such as the pygidial glands.[112]

Many mite species are aposematically colored but especially in water mites (Hydrachnidia), which are usually avoided by fish (only occasionally found in fish stomachs) and many invertebrate predators, a foul taste (secretion probably from dorsal skin glands) may be correlated with bright colors especially red.[103]

Apart from some primitive oribatid groups (Palaeosomata, Enarthronota), all the remaining (the so-called glandulate oribatids) representatives of Oribatida (Cryptostigmata: Parhyposomata, Mixonomata, Desmonomata, Brachypylina) and Acaridida (Astigmata) are characterized by opisthonotal or oil glands, which represent cuticle-lined exocrine glands that open laterally through pores on the opisthonotum.[113] It is remarkable that the secretions of these mites, which are used for defense or as alarm pheromones, have only recently been investigated. The following were identified: several alkanes such as tridecane (**41**), pentadecane (**42**), heptadecane, alkenes and alkadienes such as 1-tridecene, pentadecene, (*Z*)-8-heptadecene, (*Z*,*Z*)-6,9-heptadecadiene (**43**),[114] and also 3-ethylphenol (**28**),[115] 1-methyl-2-naphthol (**29**),[115] γ-acaridial (2-formyl-3-hydroxybenzaldehyde) (**27**), neral (**33**), geranial (**34**),[115] (*E*,*E*) (**45**)- and (*Z*,*E*)-farnesal, geranial,[116] 9,17-octadecadienal[117] (tentatively), geranyl formate (**35**),[118] neryl formate, 1,8-cineole,[119] (3*S*,8*S*)-chrysomelidial (**32**),[117] the diterpene β-springene (**44**) (tentatively),[117] and 2-hydroxy-6-methylbenzaldehyde (**26**).[120]

It is remarkable that the oribatid mite *Scheloribates azumaensis* was found to contain several alkaloids that are also present in the skins of dendrobatid poison frogs.[121] Pumiliotoxin 237A (8-hydroxy-8-methyl-6-(2′-methylpentylidene)-1-azabicyclo [4.3.0] nonane, **39**), pumiliotoxin 251D (8-hydroxy-8-methyl-6-(2′-methylhexylidene)-1-azabicyclo [4.3.0] nonane, **40**), deoxypumiliotoxin 193H (**37**), a 6,8-diethyl-5-propenylindolizidine, and a 1-ethyl-4-pentenylquinolizidine could be identified. Moreover, precoccinelline 193C (**38**) and another coccinelline-type alkaloid were found.

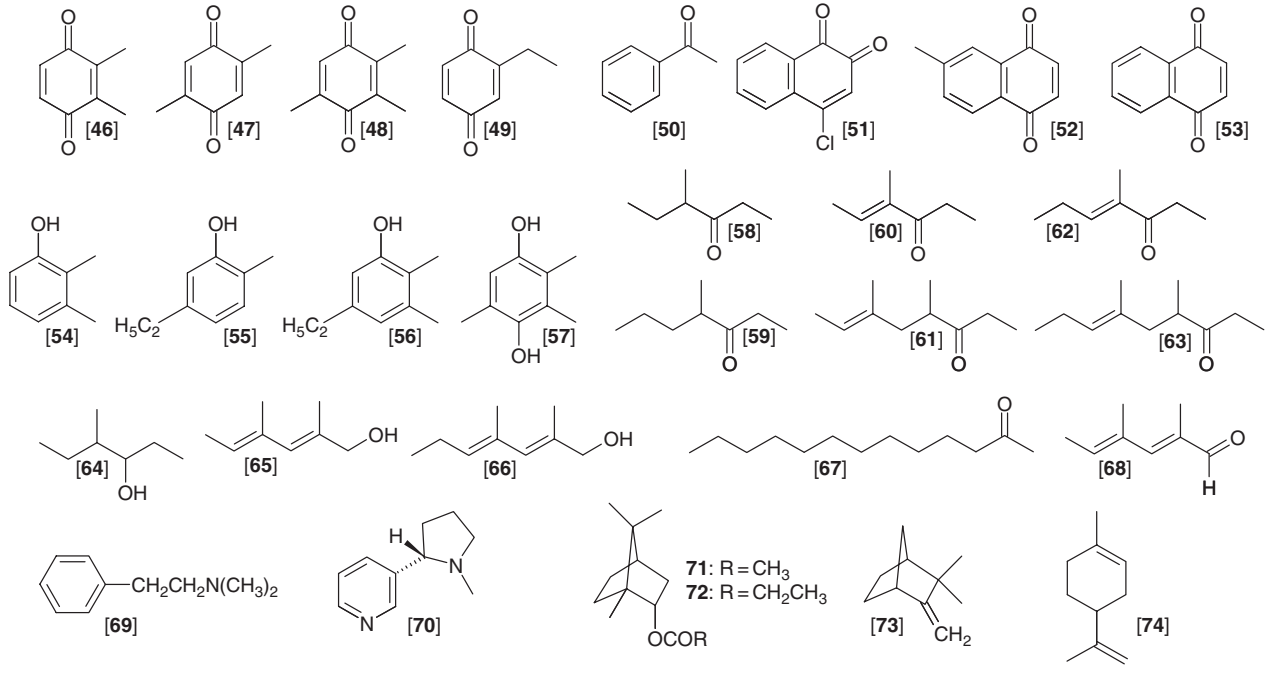
Apart from laterally discharged aggressive compounds from opisthonotal glands, some oribatid mites produce a sticky substance that deters ant attacks.[103]

As in most species of Oribatida, all representatives of Acaridida (Astigmata) apart from some parasitic groups are characterized by opisthonotal glands. Here they produce a number of natural compounds that may function as alarm, aggregation, or sex pheromones or as allomones.[122] In addition to many oribatid compounds, a new salicyl lactone was detected from an *Oulenzia* species and was assigned to be 7-hydroxyphthalide (7-hydroxy-3*H*-isobenzofuran-1-one, **25**).[123] From the genera *Schwiebea* and *Rhizoglyphus*, 3-(4-methyl-3-pentenyl)-2-(5*H*)-furanone (**36**; α,α-acariolide) and 4-(4-methyl-3-pentenyl)-2-5*H*-furanone (α,β-acariolide) could be identified,[124] whereas the opisthonotal secretion of the genus *Dermatophagoides* contained 2-formyl-3-hydroxybenzyl formate.[125]

Because of the enormous biomass of mites such as oribatids, their peculiar natural products might be more abundant in unrelated animals of terrestrial ecosystems.[126] So, the chemistry of skin alkaloids in poisonous frogs often reflects their recent arthropod diet such as oribatids and ants.[126] Moreover, several ants might be specialized on certain oribatid mites,[127] whereas certain mites often prefer food such as pigmented fungal hyphae.[126] Since alkaloids in scheloribatid mites are not present in larvae but found only in adults,[121] the compounds might be either biosynthesized by adult mites or sequestered by adult mites from their fungal food.

4.09.21 Arthropoda: Chelicerata, Opiliones

Opilionids or harvestmen represent more than 7000 species and are divided into Cyphophthalmi, Laniatores, and Palpatores (Dyspnoi + Eupnoi). They are characterized by various defense mechanisms such as cryptic and aposematic coloration (e.g., certain Gonyleptidae), death-feigning (thanatosis), body vibrating (bobbing), painful pinching, autotomy of a leg (which may then twitch rhythmically) between trochanter and femur when disturbed, gregariousness, or running for escape.[128] Remarkably, autotomized legs cannot be regenerated. When all these evasive mechanisms fail, all species may utilize chemical defenses and, therefore, possess paired homologous defensive glands (scent, odoriferous, repugnatory, stink glands). These so-called ozopores open anterolaterally of the prosoma and may emit various more or less volatile compounds. It is interesting to note that a *Parampheles* species shows orange markings at the defensive gland openings and in contrast emits a translucent defensive secretion devoid of quinones.[129] Reviews on chemical defenses of opilionids are published by Eisner *et al.*[128] and Gnaspini and Hara.[129]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72: R = CH2CH3
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After molestation, harvestmen deplete a clear aqueous droplet from their ventrally situated mouth and secretion droplets from gland openings or ozopores of their dorsally situated paired defensive glands.[128–130] These represent compressible sacs that are associated with various opening muscles. By characteristic grooves located on the body surface and by movements of legs, the secretions are mixed and subsequently distributed over the whole body surface. During this process, the droplets accumulate at other body areas, and the mixture becomes odorous and darkens continuously,[131] which is due to the mixing of enteric phenols with quinones from the glands. Representatives of Cyphophthalmi and Laniatores can further dab their secretions on aggressors; however, it is also known that secretion can be ejected as a spray.[130] The defensive secretions of opilionids, which often show antimicrobial activities, are directed against parasitoids and predators such as ants, other opilionids, and various vertebrates.[128,132] Also microarthropods such as spiders and isopods immediately die after contamination with the secretion.[133]

The Cyphophthalmi of the genera *Cyphophthalmus* and *Siro* revealed more than 20 compounds including saturated and unsaturated C_{11}–C_{15}-methyl ketones, for example, tridecan-2-one (**67**), four naphthoquinones (especially 1,4-naphthoquinone (**53**), 6-methyl-1,4-naphthoquinone (**52**), 4-chloro-1,2-naphthoquinone (**51**) and its 6-methyl analogue), and acetophenone (**50**).[133] Chlorinated exocrine compounds of arthropods seem to be particularly unusual, apart from chlorophenol, a sex pheromone of ticks.[133] As a whole, Cyphophthalmi are chemically more similar to Palpatores than to Laniatores.

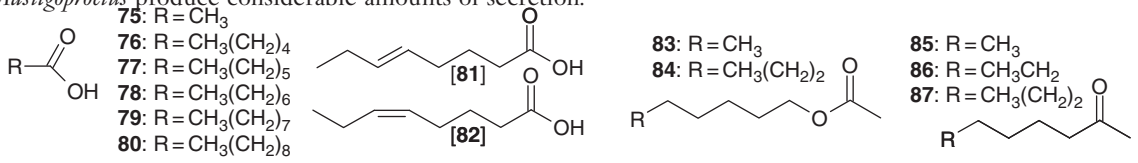
In Palpatores[128] (e.g., Phalangiinae[134]), various volatile, primarily acyclic, often branched compounds (e.g., hydrocarbons, ketones, alcohols, aldehydes) are found along with 1,4-naphthoquinones such as **52** and **53**. For example, 4-methylhexan-3-one (**58**), 4-methylheptan-3-one (**59**), *E*-4-methylhex-4-en-3-one (**60**), *E*-4-methylhept-4-en-3-one (**62**), *E*-4,6-dimethyloct-6-en-3-one (**61**), *E*-4,6-dimethylnon-6-en-3-one (**63**), 4-methylhexan-3-ol (**64**), *E,E,*-2,4-dimethylhexa-2,4-dien-1-ol (**65**), *E,E*-2,4-dimethylhepta-2,4-dien-1-ol (**66**), and *E,E*-2,4-dimethylhexadienal (**68**) were recorded. Most of these volatiles have been found in the genera *Leiobunum*, *Hadrobunus*, and *Phalangium*.[128]

In Laniatores,[128] the defensive secretions are characterized by alkylated 1,4-benzoquinones and phenols. Some of these unusually alkylated quinones occur more restrictedly. In Gonyleptidae, the defensive secretions were comparatively characterized.[135] Important defensive compounds in this and related families are 2-ethyl-1,4-benzoquinone (**49**), 2,3-dimethyl-1,4-benzoquinone (**46**), 2,5-dimethyl-1,4-benzoquinone (**47**), and 2,3,5-trimethyl-1,4-benzoquinone (**48**), 2,3-dimethylphenol (**54**), 2-methyl-5-ethylphenol (**55**), 2,3-dimethyl-5-ethylphenol (**56**), and 2,3,5-trimethyl-1,4-hydroquinone (**57**).[135,136] Some laniatorids belonging to Travunioidea of the genus *Sclerobunus* secrete unusual terpenes such as bornyl acetate (**71**) and bornyl propionate (**72**), camphene (**73**), limonene (**74**) together with N-containing compounds (nicotine (**70**)), and *N,N*-dimethyl-β-phenylethylamine (**69**).[137] Few Laniatores quinones have a limited distribution in insects; in contrast, ketones and naphthoquinones are absent in Laniatores and probably have been lost during evolution.[133]

4.09.22 Arthropoda: Chelicerata, Pedipalpi

Pedipalpi with more than 470 species worldwide are nonpoisonous. Representatives of Amblypygi are devoid of anal defensive glands, whereas Thelyphonida and Schizomida (both groups formerly called Uropygi) possess a pair of large anal glands in the posterior opisthosoma, which open at the end of a postabdominal knob that usually forms the base of the flagellum. Upon contact stimulation, a defensive spray may be discharged (up to 80 cm) by many subsequent ejections (maximal number of discharges: 19) and exactly aimed toward aggressors. Typically, acetic acid (**75**) represents the main constituent (45–98%) of all six species from three thelyphonid genera (*Mastigoproctus*, *Typopeltis*, *Thelyphonus*) hitherto analyzed. The watery secretion (11–26% water) may also contain minor amounts of further saturated C_6- to C_{10}-acids (**76–80**) and monounsaturated (*E*)- and (*Z*)-5-octenoic acids (**81,82**). Further constituents such as 1-octanol, hexyl and octyl acetate (**83,84**; *Telyphonus linganus*),[138] and C_7–C_9 2-ketones (**85–87**; *Typopeltis guangxiensis*)[139] are restricted to certain species.[140] In other species with aberrant odors, probably additional compounds may be detected.[141] The concentrations of the constituents of the defensive secretions vary individually; however, there are no age or sex differences between individuals. Only first-stage larvae of *Thelyphonus caudatus* seem to be devoid of functional defensive glands,[142] whereas first instars of

Mastigoproctus produce considerable amounts of secretion.[141]



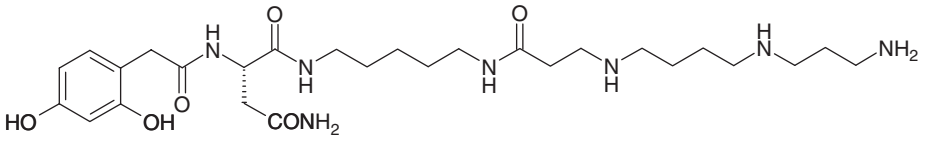
Obviously, also the small-sized and blind microwhip scorpions (Schizomida) are able to secrete an acidic spray from their abdominal tip. The defensive secretion of Thelyphonida is maximally effective on sensitive respiratory, visual, and sensory systems of arthropod and vertebrate predators.[140] In *Mastigoproctus*, the presence of caprylic acid promotes the acetic acid-containing spray over the cuticle and increases its permeability through the cuticular barriers of target organisms.[142] Similar effects are probably achieved by the addition of acetic acid with other wetting agents such as 2-ketones,[139] and hexyl and octyl acetates.[140] In humans, natural sprays of *Typopeltis crucifer* evoked both a painful sensation (lasting for a short time) and a corneal opacity, which disappeared after 4 days.[143]

4.09.23 Arthropoda: Chelicerata, Araneae

Apart from representatives of Mesothelae (90 species), the remaining Opisthothelae (about 40 000 species) altogether possess hollow fangs with associated openings of efferent ducts from venom glands and can inject venoms to paralyze and kill their prey, to preingest the intended meals by digestive fluids, or to protect themselves. The venom glands, which are located in chelicerae (the fang bases) and cephalothorax, were secondarily reduced in Uloboridae and certain Liphistiidae.[144] In addition, the abdomen of many species of Araneae contains different kinds of silk glands, each producing a different kind of silk for different purposes (e.g., to modify habitats, catch prey, protect adults and their young). Sometimes, webs are constructed that bear droplets of adhesive. To maintain certain characteristics of webs, spiders impregnate their webs with KNO_3 (against denaturation), KH_2PO_4 (bactericidal), and 2-pyrrolidone (hygroscopical to prevent dry out).[145] Because of the adhesiveness of spider silk, many insects evolved as defense detachable hairs, scales, or waxy powder and, therefore, are protected against entrapment.[146]

In a few species, additional defense strategies are known. Representatives of *Mastophora* and *Cyrtarachne* emit a distinct odor, probably from regurgitant, when they are molested.[147] In bird spiders (Theraphosidae), urticating hairs are located at the hind body. These hairs or setae obviously act mechanically and can be lost and shot toward aggressors where they may irritate skin, conjunctivae, and other mucous membranes.[16,18–20] Other species may even show leg autotomy, which is effective against scorpions. Representatives of 'spitting spiders' (Scytodidae) are unusual in ejecting two streams of sticky silk over their prey, which is glued to the substrate.[144] Obviously, the enlarged venom gland produces both silk and poisonous components. Whereas scytodids spit to capture their prey, especially females of *Peucetia viridans* (Oxyopidae) spray their bitter-tasting, eye-irritating, and cooling secretions from their venomous glands as a kind of defensive behavior.[148]

Until now, spider venoms have been studied in only 0.1% of the known species. Apart from Uloboridae, all spiders are, strictly speaking, poisonous. However, concerning humans, only few spider species (e.g., from the genera *Atrax*, *Cheiracanthium*, *Harpactirella*, *Latrodectus*, *Loxosceles*, *Mastophora*, *Phoneutria*, and *Trechona*) can penetrate the human skin and may cause medically significant envenomations. Medical aspects of spider bites are discussed by Vetter and Isbister,[149] while many publications deal with poisonous spiders.[16,20,93] Spider venoms represent complex mixtures of biologically active and inactive substances and contain proteins, polypeptides (more than 3000 Da), polyamine neurotoxins (under 1000 Da), enzymes, nucleotides, amino acids, monoamines, and inorganic salts.[150,151] To paralyze the prey, many spider toxins affect the nervous system. Consequently, toxins can be classified according to their mode of action affecting glutamatergic transmission (glutamate receptor antagonists and inhibitors of glutamate uptake into synaptosomes) and calcium, sodium, potassium, and chloride channels, or as toxins that either stimulate transmitter release or block postsynaptic cholinergic receptors. It is interesting that these venoms represent an important source of molecules for the design of novel pharmaceutical drugs[152] or compounds used for insect control.[153]



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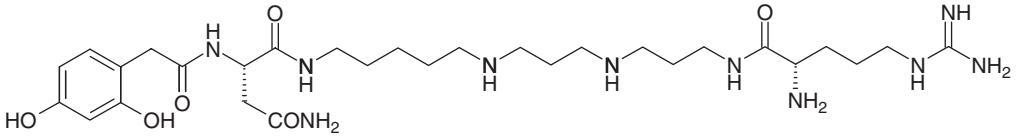

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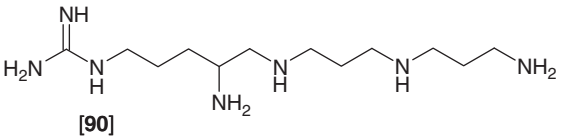
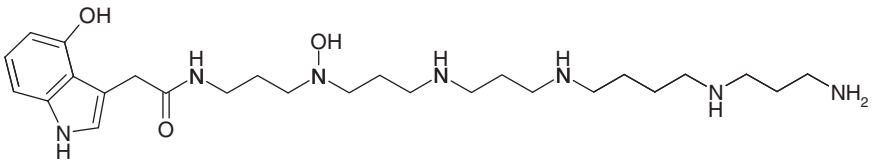


[**90**]



[**91**]

The polyamine toxins,[154,155] some of which are also found in *Philanthus* wasps, possess a linear α,ω-diamino polyazaalkane backbone, modified at one end mostly with an aromatic acyl group (see **88**,**89**), which is separated from the polyamine backbone by one or several α-amino acid moieties. Many polyamine toxins are further modified at the tail of the polyamine backbone with an additional basic amino acid fragment (see **89**). There are known structures of argiopine (or ARG 636) (**89**) from the Araneidae genus *Argiope*, JSTX-3 (**88**) from Araneidae (*Nephila*), AG 505 (or HO 505, or AGEL 505) (**91**) from the Agelenidae genera *Agelenopsis* and *Hololena*, and FTX-3.3 (**90**) from the genus *Agelenopsis*.

Spider venoms may also contain various peptides that do not represent neurotoxins but are insecticides and bactericides. Venomous secretions of spiders also contain various enzymes such as hyaluronidase, phosphodiesterase, alkaline phosphatase, esterase, ATPase, sphingomyelinase D, kininase (endopeptidase), collagenase, peptide isomerase, phospholipase A, and proteases. Finally, spider toxins may contain various low-molecular-weight compounds such as biogenic amines (5-hydroxytryptamine (**142**) or 5-methoxytryptamine, histamine, noradrenaline), free amino acids (especially γ-aminobutyric, glutamic, and aspartic acids, taurine), and inorganic salts. Furthermore, there may be found nucleotides ATP, ADP, AMP, purine derivatives adenosine, guanosine, 2,4,6-trihydroxypurine, citric acid, free polyamines spermine, spermidine, cadaverine, putrescine, glucose, lactic and phosphoric acid, glycerol, and urea.

4.09.24 Arthropoda: Myriapoda: Opisthogoneata (Centipedes)

Representatives of centipedes (Chilopoda: Scutigeromorpha + Pleurostigmophora; about 3000 species) show various defense mechanisms ranging from stridulation, autotomy of legs, thanatosis to mechanical defense via hind legs. Also there have reports of chemical defenses through forcipular glands and various glands that are situated on different parts of the body surface.

Typically, venom glands are situated within each forcipule but may also extend far into the body cavity (e.g., *Chaetechelyne*). The duct of each gland opens at the inner side of the apical segment of each forcipule. The secretions primarily serve for immobilizing and killing the prey (arthropods to small vertebrates) or for self-defense upon attack.[156] There are several records of human bite and envenomation by centipedes.[156,158]

Only few data are available on the chemistry of the centipede venom from the large forcipular glands because it is difficult to obtain significant amounts of secretion. Various enzymes such as an esterase, acid and alkaline phosphatases, and amino acid naphthylamidase (*Scolopendra morsitans*) have been reported.[157,158] In addition, a 60 kDa acidic and heat-labile protein called toxin-S was isolated from *Scolopendra subspinipes*.[158] In *S. morsitans*, lipoproteins, phospholipids, cholesterol, fatty acids, triglycerides, cholesterol esters, and squalene were detected.[157] Obviously, the *Scolopendra* toxins act against both the insect nervous system and the vertebrate autonomic nervous system.[12] Apart from coagulant and anticoagulant venoms, in centipedes there are pain-producing biogenic amines 5-hydroxytryptamine and histamine.[158] However, the secretions of *Scutigera* species (Scutigeromorpha) causing painful bites have not yet been analyzed.[12]

Apart from forcipular glands, true defensive glands are located on the sternites of representatives of Geophilomorpha.[156] These unicellular glands open through porous plates and produce a sticky, proteinaceous secretion with an often characteristic smell.[12,156] In *Geophilus vittatus* and *Orphnaeus brasilianus*,[12] production of the cyanogenic compounds mandelonitrile and benzoyl cyanide was shown. When the secretion is ejected, both precursors break down into benzaldehyde and benzoic acid by liberating hydrogen cyanide.[159] Hydrogen cyanide was also detected in the genera *Pachymerium*[160] and *Strigamia*.[159] The secretion is used against ants and spiders but females also deplete secretion upon molestation or when they guard their eggs.[12] In *Henia vesuviana*, another geophilomorph, the proteinaceous glue hardens within a few seconds of exposure to air[161] and contains 12 and 130 kDa proteins[162] (no cyanide), which may physically immobilize the attacking predators such as *Staphylinus* beetles.

Probably homologous glands are also present in the Scolopendromorpha; however, they are not only localized on sternites but also occur on pleurites, tergites, and legs. In *Asanada* species from Sri Lanka, the defensive secretion contained hydrogen cyanide, a protein, and an unknown carbonyl compound.[163] Also the secretion of another scolopendromorph *Cormocephalus nitidus* is characterized by a fetid odor.[156]

Further types of defensive glands located in the last two pairs of legs are recorded from stone centipedes (*Lithobius*). The secretion contains a sticky material of unknown chemistry, which serves to entangle enemies.[12]

Several representatives of Geophilomorpha (e.g., *Orya*, *Geophilus* Scolioplanes) and Scolopendromorpha (e.g., *Otostigmus*) produce sticky secretions that are characterized by strong fetid and/or pleasant odors[156] and sometimes are luminescent too. In *Orphnaeus*, the ventral gland secretion emits a faint blue-green glow for some seconds after emission.[12] In *Orphnaeus* bioluminescent slime, maxima were found at 510 and 480 nm. Moreover, the reaction required a luciferin, a luciferase, unusual low pH, and it was shown that oxygen interacted with only one of the components allowing for anaerobic light emission.[164] Since the vesicant secretions of these centipedes deter both mammals and predatory arthropods, they may represent a true defensive secretion.[156]

4.09.25 Arthropoda: Myriapoda: Progoneata: Diplopoda (Millipedes) and Symphyla

Together with Pauropoda and Diplopoda, representatives of Symphyla comprise the taxon Progoneata. The soil-dwelling predatory Symphyla (160 species) resemble centipedes and possess large spinnerets at the posterior body parts. Upon molestation, ducts of spinning glands emit sticky threads, which may entangle the mouth parts of all kinds of aggressors.

Herbivorous to saprophagous millipedes, which comprise about 13 000 species worldwide (probably 80 000 Myriapoda), lack poisonous fangs and do not bite. Usually, they roll into a defensive ball or spiral, and many species emit highly toxic or foul-smelling compounds. With the exception of five orders Polyxenida, Sphaerotherida, Glomeridesmida, Chordeumatida, and Siphoniulida, representatives of the remaining sometimes even aposematically colored 10 Diplopoda taxa produce defensive secretion in serially arranged defensive glands.[165,166]

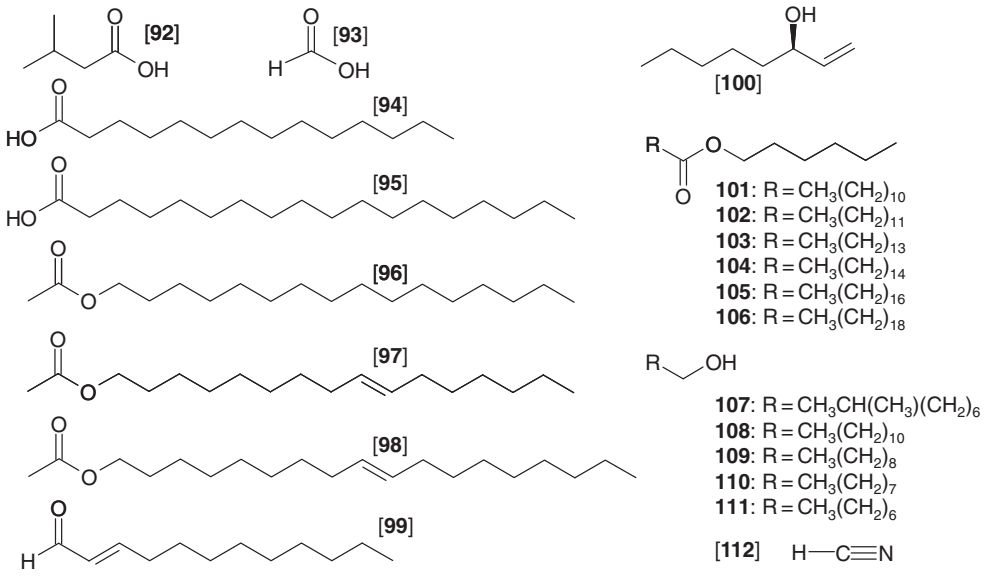
The basally arranged noncalcareous Polyxenida (Penicillata; bristle millipedes) lack defensive glands and instead project hooked bristles against attackers such as ants.[12] Similar to modified larval hairs of dermestid beetles, predators are thus effectively entangled.

Among Pentazonia, which can coil into a sphere or 'pill', Sphaerotherida and Glomeridesmida lack defensive glands, whereas Glomerida (*Glomeris*, *Loboglomeris*) have eight pairs of mid-dorsally evacuating defensive glands, which contain a bitter-tasting, sticky, proteinaceous and colorless secretion. The glandular material contained

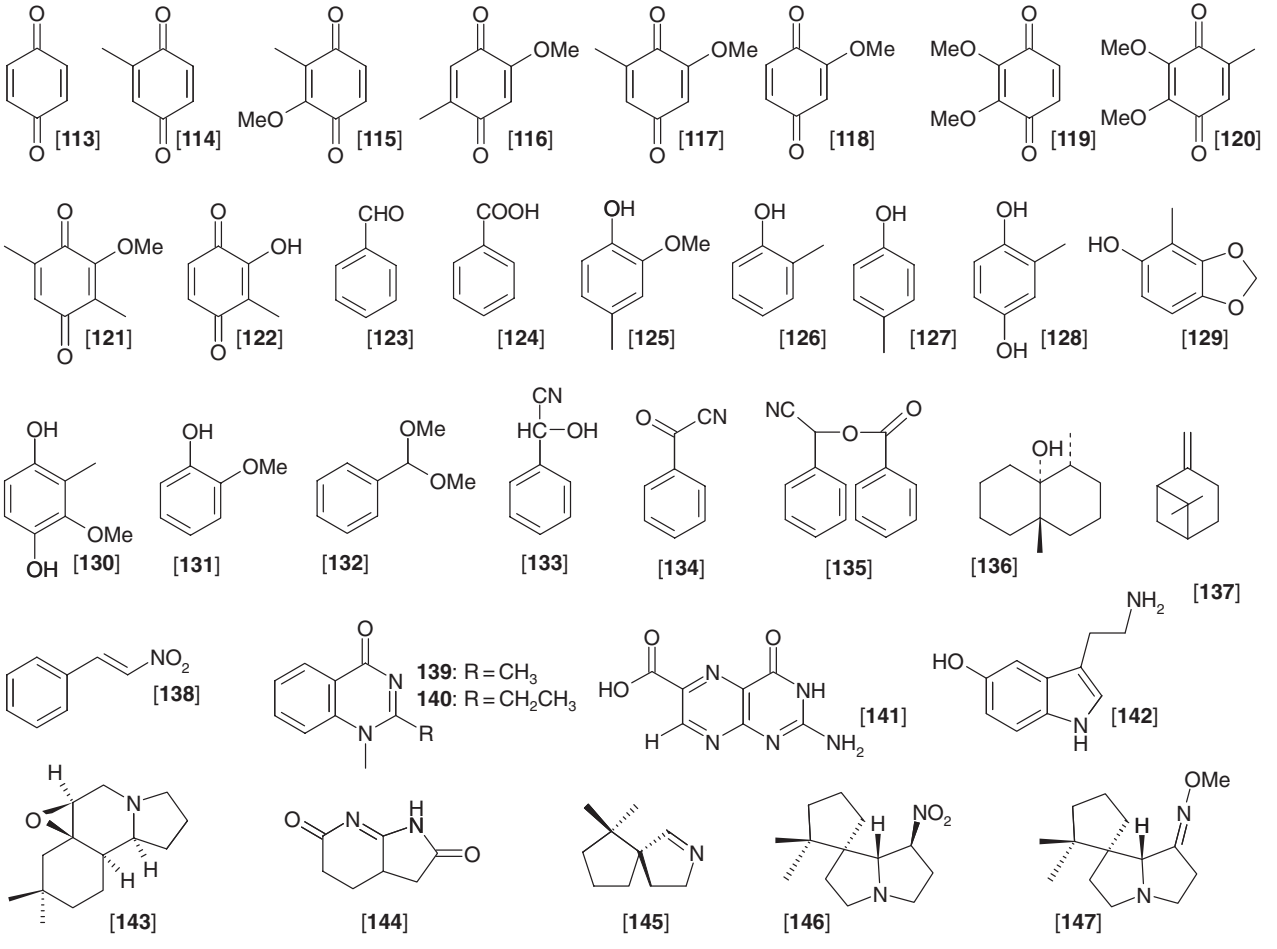
the quinazolinone alkaloids 1,2-dimethyl-4-quinazolone (glomerin, **139**) and 1-methyl-2-ethyl-4-quinazolone (homoglomerin, **140**), which are unusual for animals[12,167] and may deter and paralyze spiders, ants, carabid beetles, and vertebrates such as mice, birds, and toads. This contrasts with the large armored pill millipedes of the genus *Sphaerotherium*, which are devoid of defensive secretions. *Mungos* hurl these millipedes against a rock and subsequently smash them.[168] These Glomerida alkaloids resemble quinazoline alkaloids such as arborine (2-benzyl-1-methylquinazol-4-one), recorded from Indian medicinal plants. Both **139** and **140** are produced from anthranilic acid as was shown by feeding glomerids with labeled precursors.[165]

Colobognatha, the neighbor group of Pentazonia, include chemically defended taxa with paired laterally arranged defensive glands in the order Polyzoniida. *Polyzonium rosalbum* emits a sticky whitish defensive fluid with a strong odor. The fluid consists of two spirocyclic terpene alkaloids, (+)-polyzonimine (6,6-dimethyl-2-azaspiro[4.4]non-1-ene, **145**) and the related tricyclic (+)-nitropolyzonamine (2′,2′-dimethyl-6-nitrospiro-{1-azabicyclo[3.3.0]octane-4,1′-cyclopentane}, **146**). Both compounds, which contain a 2-azaspiro[4.4]nonane system, represent ant deterrents and repellents.[12] Enantiomerically pure **145** and **146** were synthesized by asymmetrical Michael addition of the enamine derived from 2,2-dimethylcyclopentanecarboxaldehyde and (*S*)-prolinol methyl ether to nitroethylene.[169] Another polyzoniid species of the genus *Buzonium* secretes the interesting tetracyclic alkaloid buzonamine (**143**), an epoxy group, and a tertiary nitrogen.[170] Apart from this ant repellent, the secretion contains limonene (**74**) and β-pinene (**137**). From a further polyzoniid species *Rhinotus purpureus*, the spiropyrrolizidine *O*-methyloxime was isolated (**147**).[171] Because traces of this compound were also detected in skin extracts of sympatric poison frog *Dendrobates pumilio*, a dietary source of this alkaloid was supposed.

In *Abacion magnum*, a representative of the neighbor group Nematophora (Callipodida), the defensive secretion contained *p*-cresol (**127**).[165]



Several papers concerning the defensive secretions of Polydesmida (another neighbor group, also called Merocheta) have been published. Polydesmida possess segmentally arranged special reactor glands characterized by a reservoir, a smaller vestibule, and an opening valve between both compartments.[165] *Leonardesmus injucundus* secretes *p*-cresol (**127**)[172] and represents a primitive polydesmid, closely related to the callipodid *Abacion*. Most other representatives of polydesmid taxa[165,173,174] produce mandelonitrile (**133**), the precursor of benzaldehyde (**123**), and hydrogen cyanide (**112**). Other polydesmid defensive compounds are benzoyl cyanide (**134**), mandelonitrile benzoate (**135**), 2-methoxyphenol (guaiacol, **131**), phenol, benzoic acid (**124**), ethyl benzoate, formic acid (**93**), acetic acid (**75**), 3-methylbutanoic acid (**92**), 2-methylbutanoic acid, myristic acid (**94**), and stearic acid (**95**). Erratically distributed polydesmid compounds are benzaldehyde dimethyl acetal (**132**) and 2-methoxy-4-methylphenol (creosol, **125**) in *Chamberlinius*[175] and *Oxidus*;[176] 1-octen-3-ol (**100**) and geosmin (**136**) in *Niponia*;[177] and (1*E*)- (**138**) and (1*Z*)-2-nitroethenylbenzene (*E*/*Z* ratio: 56:1; 2–3 µg per millipede) in *Eucondylodesmus*.[178]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139: R = CH3
140: R = CH2CH3
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The polydesmid secretions represent effective repellents against ants, lizards, and birds but compounds such as **124**, **134**, and **138** also inhibit mycelial growth and spore germination.[179] In addition, **138** has antibacterial and insecticidal properties.[178] Quantitative differences were recorded in developing female polydesmids, when titers of methyl benzoate and **131** were compared, which indicates that the compounds may also have certain physiological functions related to reproduction and development.[175] Moreover, in another species, males contained twice as much **123** and **133** compounds as females.[180] Finally, Ômura *et al.*[177] suggested that 1-octen-3-ol, which is a typical mushroom volatile, might also act as an alarm pheromone. It is interesting to note that L-phenylalanine is used as a precursor for both 2-nitroethenylbenzene[178] and mandelonitrile,[165] which was proved by using the labeled precursor [2-^{14}C]phenylalanine and α,β,β,2,3,4,5,6-d_8-L-phenylalanine, respectively. Moreover, by using ^{14}C-labeled precursors it was shown that phenol and guaiacol (**131**) are derived from tyrosine, whereas H^{14}CN is detoxified and converted primarily to thiocyanate by rhodanase with minor conversion to β-cyanoalanine and asparagine.[181]

Most chemically studied millipedes belong to the Juliformia with Julida, Spirobola, and Spirostreptida.[165] Segmentally arranged glands represent spherical sacs with efferent ducts and opening muscles near the outer orifice.[165] The secretions of the three orders are characterized primarily by *p*-benzoquinones such as 2-methyl-1,4-benzoquinone (**114**), 2-methyl-3-methoxy-1,4-benzoquinone (**115**), 1,4-benzoquinone (**113**), 2,3-dimethoxy-1,4-benzoquinone (**119**), 5-methyl-2,3-dimethoxy-1,4-benzoquinone (**120**), 2-methyl-1,4-hydroquinone (**128**), and 2-methyl-3-methoxy-1,4-hydroquinone (**130**). In a few species, *o*-cresol (**126**), hexadecyl acetate (**96**), 9-hexadecenyl acetate (**97**), 9-octadecenyl acetate (**98**), and (*E*2)-dodecenal (**99**) could be detected. Further defensive compounds that are erratically distributed in Spirobolida are 2-ethyl-1,4-benzoquinone (**49**), 2-hydroxy-3-methyl-1,4-benzoquinone (**122**), hydroquinone, 2-methoxy-3,6-dimethyl-1,4-benzoquinone (**121**), 2,3-dimethoxyhydroquinone, 2-methyl-3,4-methylenedioxyphenol (**129**), 2,3-dimethoxy-5-methylhydroquinone in *Acladocricus*[182] and some *Floridobolus* species.[183] The neotropical spirobolid *Rhinocricus padbergi* is unusual in secreting the alkaloid 3,3*a*,4,5-tetrahydro-1*H*-pyrrolo-[2,3-*b*]pyridine-2,6-dione (**144**), together with **114** and linear hydrocarbons from C_{21} (heneicosane) to C_{29} (nonacosane).[184] New constituents in the spirostreptid *Telodeinopus aoutii* are 2-methoxy-1,4-benzoquinone (**118**) and naphthoquinone (**53**). In a harpagophorid species, the presence of 2-methoxyhydroquinone is worth mentioning.[185] Apart from stereotypic quinones and hydroquinones, several julid species of the genera *Julus*, *Leptoiulus*, *Ommatoiulus*, *Tachypodoiulus*, *Enantiulus*, and *Cylindroiulus* contained 2-methoxy-5-methyl-1,4-benzoquinone (**116**), 2-methoxy-6-methyl-1,4-benzoquinone (**117**), a homologous series of hexyl esters ranging from dodecanoic acid hexyl ester (**101**), tridecanoic acid hexyl ester (**102**), tetradecanoic acid hexyl ester, pentadecanoic acid hexyl ester (**103**), hexadecanoic acid hexyl ester (**104**), octadecanoic acid hexyl ester (**105**) to eicosanoic acid hexyl ester (**106**).[186] *Cylindroiulus caeruleocinctus* exclusively shows *n*-alkanols comprising 1-octanol (**111**), 1-nonanol (**110**), 1-decanol (**109**), 1-dodecanol (**108**), and 8-methyl-1-nonanol (**107**).[186]

Various data exist on the biological significance of diplopod defensive chemicals. Compounds such as **114**, **115**, **123**, **124**, **134**, and **135** are toxic to fungi,[187] nematodes, and bacteria.[188] It was also suggested that, similar to opilionid defensive secretions, minor components such as **47** and **48** contribute much more to the antibiotic activity of the whole secretion than the main constituent **46**.[186] As demonstrated in *Ommatoiulus sabulosus*, its defensive secretions are repulsive to vertebrates, which exhibit an avoidance behavior.[189] Quinazolinones from *Glomeris* can induce a significant spider sedation.[190] In addition, certain vertebrates such as capuchin monkeys frequently use diplopods and their secretions to deter mosquitoes and ticks.[191] Moreover, diplopod defensive compounds such as **114** and **115** attract certain necrophagous dung beetles, which normally feed on freshly dead millipedes.[192]

Millipedes may show bioluminescence after molestation. The luminescent system of *Luminodesmus sequoiae* (now *Motyxia sequoiae*) is activated by ATP, magnesium, and molecular oxygen and involves a 104 kDa luciferase.[193] Although the details of the mechanisms are unknown, it was concluded that 7,8-dihydropterin-6-carboxylic acid (**141**) is the light emitter.[141,194]

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Biographical Sketch

Professor Dr. Konrad Dettner studied biology and chemistry at the universities of Stuttgart and Stuttgart-Hohenheim where he got his Ph.D. degree in 1977. After a postdoctoral time at RWTH Aachen in 1985, he received a habilitation degree and venia legendi, and in 1986 he was appointed as full professor of animal ecology at the University of Bayreuth. His main research areas are the chemical ecology, especially the investigation of defensive compounds, of insects, the role of endosymbiotic bacteria of arthropods, and the biology of freshwater beetles.

4.10 Toxins of Microorganisms

Shohei Sakuda, University of Tokyo, Tokyo, Japan

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4.10.1 Introduction

Microorganisms produce a variety of secondary metabolites with toxicity. This chapter focuses on such microbial toxins with low molecular weight. Definition of the term 'toxicity' is a little difficult. On the one hand, it means the biological activity that causes disease in mammals, especially in human beings. In this sense, toxicity is shown to be absolutely poisonous to human beings. On the other hand, in a broader sense, toxicity is used as the growth-inhibiting activity targeting any organisms. In this sense, toxicity coupled with selectivity is sometimes useful for us to develop medicines or pesticides. For example, novel compounds with selective toxicity toward insects, plants, and microbes are good lead compounds for developing effective insecticides, herbicides, and antibiotics, respectively. As we have obtained many antibiotics and other biologically active compounds from microorganisms, a vast number of microbial secondary metabolites belong to toxins in the broad sense of the latter. It is very difficult to know the physiological role of a bioactive secondary metabolite produced by a microorganism.[1] For example, the role of antibiotic production is presumable but has not been proved. As a rare case, pathogenic fungi, which infect host organisms such as plants or insects, may produce secondary metabolites with selective toxicity toward their hosts.

Microbial toxic secondary metabolites have been studied mainly from the following three viewpoints: (1) they contain very useful lead compounds for developing medicines and pesticides; (2) insect or plant pathogenic fungi produce compounds toxic to their hosts for their infection; and (3) some fungal secondary metabolites are toxic to humans and livestock, and their contamination with food and feed is a serious problem for human and animal health. In the first case, many screening works search for bioactive compounds among secondary metabolites of fungi and bacteria that have afforded many useful compounds including antibiotics and pesticides. In the second case, many toxins produced by insect or plant pathogenic fungi have been identified. They are important as key compounds in the physiological study on pathogens and hosts.[2] Finally, mycotoxin contamination in agricultural products and mushroom poisoning are found to be severe problems.

Since a detailed review of antibiotics is beyond the scope of this chapter, we focus on the recent works on microbial secondary metabolites toxic to animals. In addition, there are many reviews on herbicides produced by microbes[3–5] and toxins of plant pathogenic fungi.[6,7]

In the first part of this chapter, we deal with insecticides including miticides and nematocides, which include very useful compounds such as avermectins and milbemycins, produced by bacteria and fungi. We list out microbial insecticides of importance and review the works mainly on the mode of action and biosynthesis of each metabolite. In the next part, major mycotoxins are listed and recent topics on them, especially on their biosynthesis, are described. Since contamination of two major mycotoxin groups, aflatoxins (AFs) and trichothecenes, in food and feed is a worldwide problem, they are treated in detail in the last part of this chapter. Recent studies on their biosynthesis, regulatory mechanism for their production, and inhibitors of their production are described.

4.10.2 Insecticides from Microorganisms

Many insecticidal compounds have been found in bacterial and fungal metabolites. It is notable that screening works search for insecticidal compounds among microbial metabolites succeeded in the discovery of several practically useful compounds. Especially, avermectin and its derivatives are greatly contributing to human and animal lives.[8] Entomopathogenic fungi produce specific toxins for their infection into the host insects. Some of the fungal toxins or their producers are practically useful for control of crop pests.[9] In this section, insecticidal metabolites of bacteria and fungi are described.

4.10.2.1 Insecticidal Metabolites of Bacteria

Piericidins are the first compounds obtained by the screening search for insecticidal natural products among microbial metabolites.[10] They were isolated from *Streptomyces mobaraensis* in 1963,[11] and many piericidin derivatives have been found in microbial metabolites until now.[12] Piericidins are not used as insecticides practically, but are important biological reagents because they have specific inhibitory activity toward the mitochondrial electron transport chain protein nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase (complex I).[13] Piericidin A_1 (**1** in **Figure 1**) is biosynthesized as a polyketide,[14] but genes responsible for its biosynthesis are not yet identified. Total synthesis of piericidins A_1 (**1**) was reported recently.[15]

Aureothin (**2** in **Figure 1**), a nitro compound from *Streptomyces thioluteus*,[16] was shown to have pesticidal activity against the bean weevil[17] by inhibiting mitochondrial respiratory complex II.[18] Biosynthetic pathway of aureothin (**2**) has been clarified as shown in **Figure 2** by analyzing its biosynthetic genes. *p*-Nitrobenzoate is formed from *p*-aminobenzoate by an *N*-oxygenase encoded by *aurF*.[19] The type I polyketide synthase (PKS) encoded by *aurABC* produces a polyketide chain by using *p*-nitrobenzoate as the starter unit.[20] Interestingly, iteration in which one module catalyzes two successive cycles of chain extension occurs in the PKS reaction.[21] After O-methylation by a methyltransferase encoded by *aurI*, the tetrahydrofuran ring is formed by a monooxygenase encoded by *aurH* to produce aureothin (**2**).[22]

From 1970s, screening studies to obtain pesticides among microbial metabolites were actively carried out, which led to the discovery of useful compounds including tetranactin, avermectins, milbemycins, and spinosyn. Tetranactin (**3** in **Figure 1**) was isolated from *Streptomyces aureus*[23] as an ionophore antibiotic and has been used as an effective miticide. Biosynthetic gene cluster of nonactin (**4**), a congener of tetranactin, was recently characterized, which showed that type II PKS was involved in its biosynthesis[24] and that a resistant gene encoding a hydrolase that hydrolyzes nonactin stereospecifically was present in the gene cluster.[25]

Many excellent reviews on avermectins[26–28] and milbemycins[29,30] have been already published.[31,32] Avermectins and milbemycins are metabolites of *Streptomyces* species. It was a great success that useful avermectin derivatives with selective biological activity were obtained. Abamectin (**5** in **Figure 1**), emamectin (**6**), eprinomectin (**7**), and ivermectin (**8**) have been used as miticide, insecticide, endectocide, and parasiticide, respectively.[33] In the case of milbemycins, milbemycin D (**9** in **Figure 3**), milbemycin oxime (**10**), and moxidectin (**11**) are used for animal health. Glutamate-gated chloride channels are accepted as the molecular

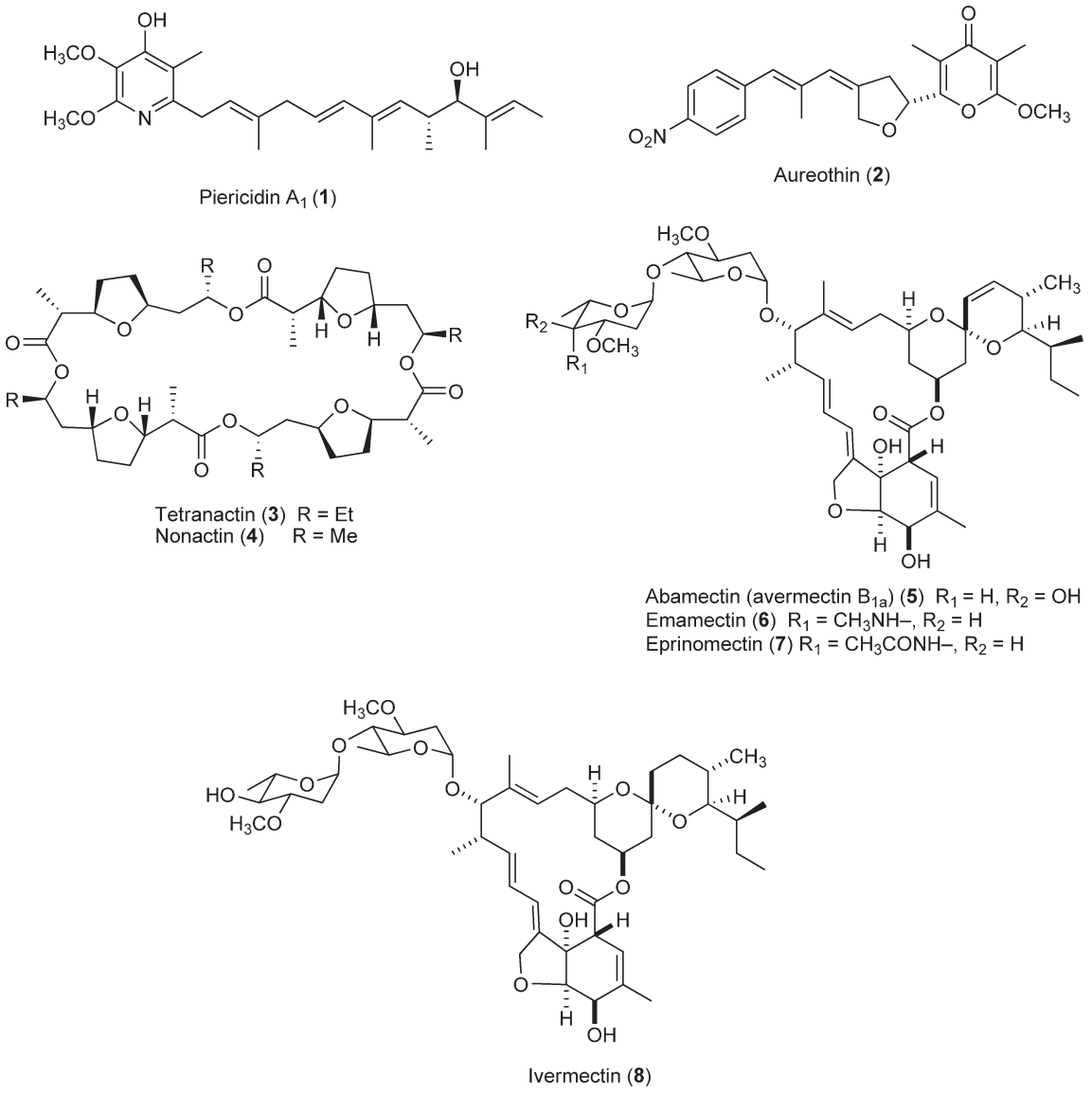


Figure 1 Insecticidal metabolites of bacteria (I).

targets of avermectins and milbemycins.[34] Biosynthetic studies on avermectin afforded not only a lot of basic information on biosynthesis of typical macrolide compound but also practically useful derivatives obtained by biosynthetic engineering technology.[35,36]

Spinosyns were isolated by screening using a mosquito larvicide bioassay from a new actinomycetes species, *Saccharospora spinosa*.[37,38] Spinosyns A (**12** in **Figure 3**) and D (**13**) with a tetracyclic lactone structure are main active components[39] and are now used as practical insecticides. They activated nicotinic acetylcholine receptors and disrupted the function of γ-aminobutyric acid (GABA) receptors, but they did not interact directly with known nicotinic or GABA receptor binding sites.[40,41] Biosynthetic genes of spinosyn, which are located in a cluster of 74 kb, involved type I PKS.[42] Biosynthetic engineering study on the spinosyn PKS has been attempted to produce new derivatives.[43]

Macrolide compounds with a hygrolide skeleton such as L-681,110 B_1 (**14** in **Figure 3**),[44] leucanicidin (**15**),[45] and bafilomycin A_1 (**16**)[46] produced by *Streptomyces* species were shown to have insecticidal activity by inhibiting H^+-ATPase.[47] They are useful as biological reagents.

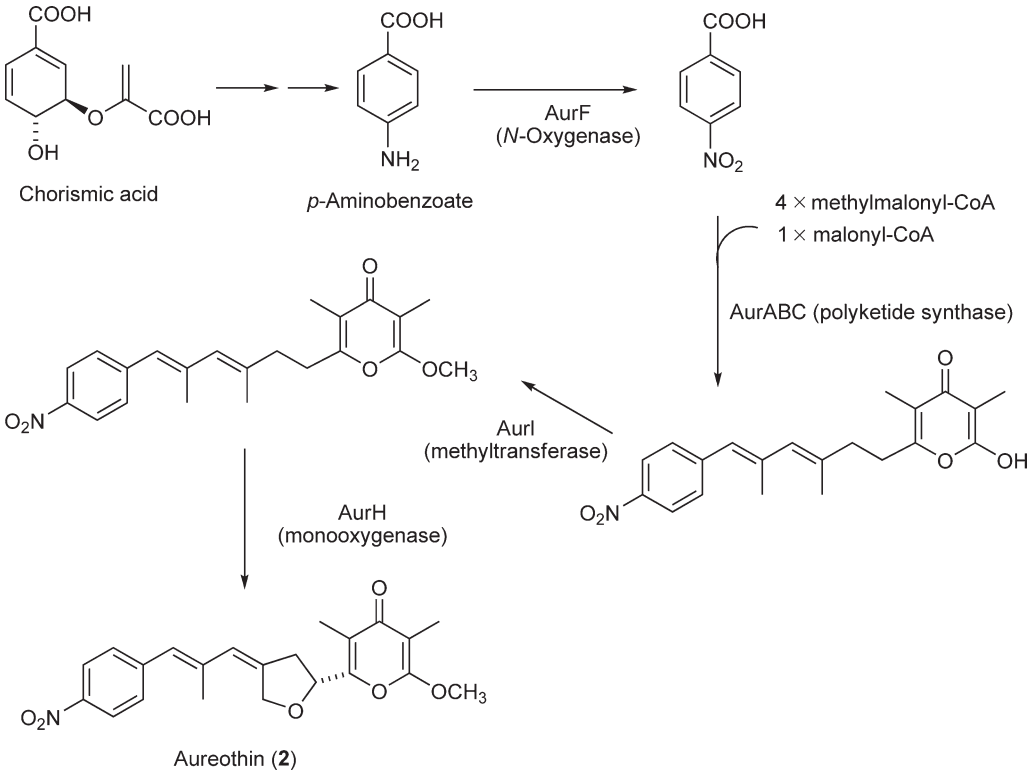
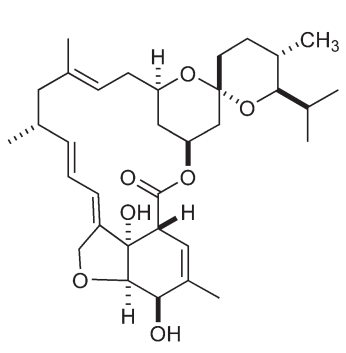


Figure 2 Biosynthesis of aureothin.

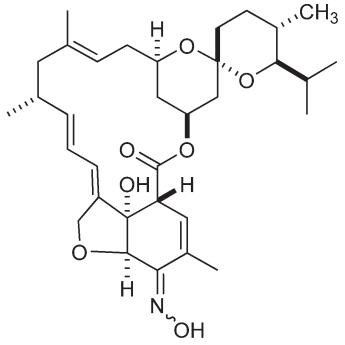
Chitin is a main constituent of insect cuticle and its turnover is necessary for insect growth. Since chitin is not present in mammals, disruptants of chitin metabolism are ideal insect growth regulators with high selectivity. Nikkomycin Z (**17** in **Figure 4**), an inhibitor of chitin synthase, which is a key enzyme for chitin synthesis, shows insecticidal activity and is practically used as a miticide.[48] Nikkomycin Z is also effective as a fungicide similar to the cases of other chitin synthase inhibitors such as polyoxins.[49,50] It is known that nikkomycin Z inhibits insect chitin synthase more strongly than other inhibitors.[51] Nikkomycin biosynthetic genes have been cloned and some of them were analyzed by gene disruption experiments to clarify their roles in the biosynthetic pathway.[52–54] On the other hand, allosamidin (**18**), an inhibitor of chitinase, which is a key enzyme for chitin degradation,[55] also shows insecticidal and miticidal activity.[56,57] Chitinases are present not only in chitin-containing organisms but also in non-chitin-containing ones including mammals. Since it was recently shown that allosamidin is effective for decreasing asthmatic Th2 inflammation by inhibiting a mammalian chitinase,[58] side effects of a chitinase inhibitor toward non-chitin-containing organisms should be considered. The cyclopentane ring of allosamidin is biosynthesized by the C–C bond formation between C-1 and C-5 of glucosamine.[59]

4.10.2.2 Insecticidal Metabolites of Fungi

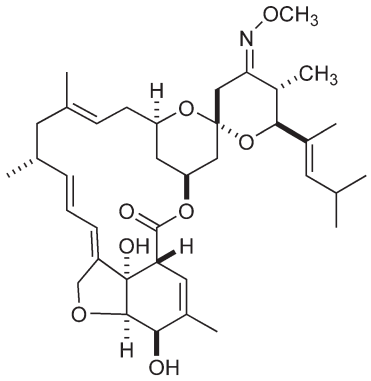
Studies on insecticidal secondary metabolites produced by entomopathogenic fungi were actively carried out from 1960s, and several peptide compounds, such as destruxins, beauvericins, bassinolide, and efrapeptins, were isolated. Destruxins were the first toxins and have been well characterized. Destruxins A (**19** in **Figure 5**) and B (**20**) were found in the metabolites of the silkworm pathogenic fungus *Oospora destructor*, later renamed *Metarrhizium anisopliae*,[60] and their structures were determined as cyclic hexadepsipeptides composed of an α-hydroxy acid and five amino acid residues.[61] Until now, many destruxin congeners were isolated from *M. anisopliae* and several other fungi.[62] Destruxins disrupted calcium balance in insect cells[63] and inhibited



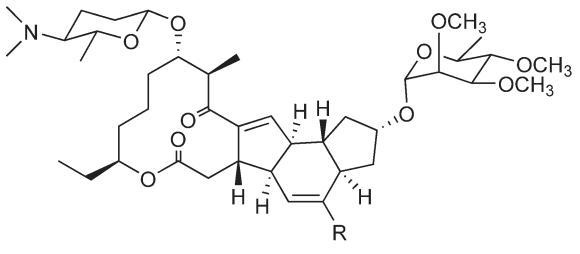
Milbemycin D (**9**)



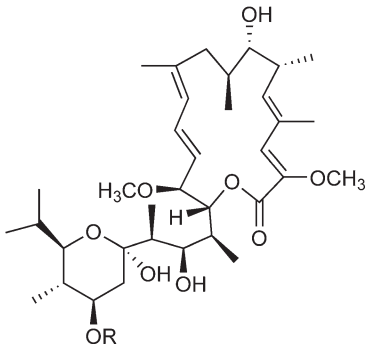
Milbemycin oxime (**10**)



Moxidectin (**11**)



Spinosyn A (**12**) R = H
Spinosyn D (**13**) R = CH_3



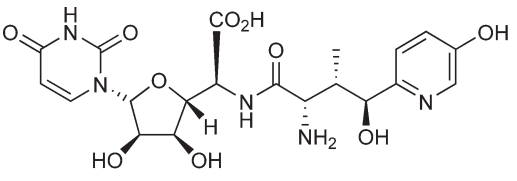
L-681,110 B_1 (**14**) R = CH_3
Leucanicidin (**15**) R = 2-*O*-methyl-α-L-rhamnopyranosyl
Bafilomycin A_1 (**16**) R = H

Figure 3 Insecticidal metabolites of bacteria (II).

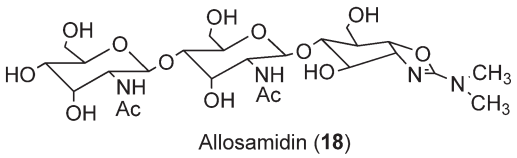
vascular-type ATPase.[64,65] A gene encoding a nonribosomal peptide synthase was obtained from *M. anisopliae*,[66] but it is not confirmed that the enzyme is involved in destruxin biosynthesis. Destruxins are not used as insecticides, but its producer, *M. anisopliae*, is practically useful to control pest as a biological control agent.

Beauvericin (**21** in **Figure 5**), a cyclic hexadepsipeptide, was isolated from *Bauveria bassiana* as a toxic ionophore.[67] Bassianolide (**22**), a cyclic octadepsipeptide, was also isolated from the same entomopathogenic fungi as an insecticidal compound.[68] *Bauveria bassiana* is now used as an important fungal biological control agent in agriculture widely.[69,70] Expressed sequence tag (EST) libraries from *B. bassiana* were analyzed, but biosynthetic genes responsible for insecticidal secondary metabolites such as beauvericin (**21**), bassianolide (**22**), or oosporein (**23**) have not been identified.[71]

Efrapeptins such as efrapeptin D (**24** in **Figure 5**) were isolated from the entomopathogenic fungus *Tolypocladium niveum*. They have a unique linear peptide structure with a bicyclic amine moiety at their C-terminus and show insect toxicity by inhibiting mitochondrial ATPase.[72]

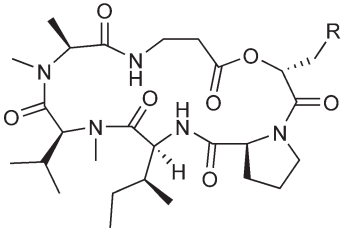


Nikkomycin Z (**17**)

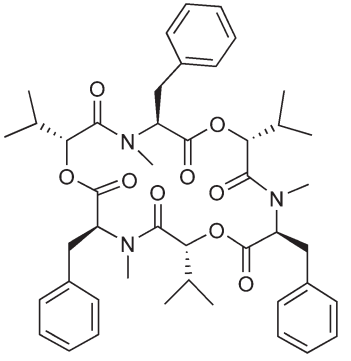


Allosamidin (**18**)

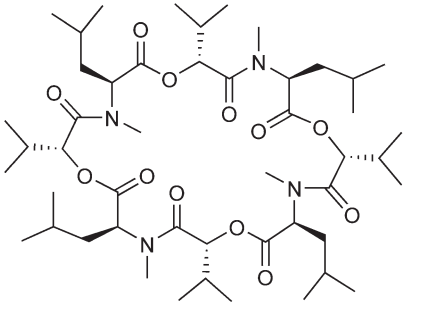
Figure 4 Nikkomycin Z and allosamidin.



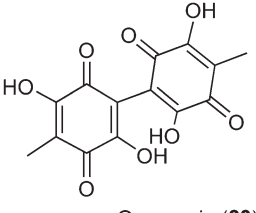
Destruxin A (**19**) R = –CH=CH_2
Destruxin B (**20**) R = $CH(CH_3)_2$



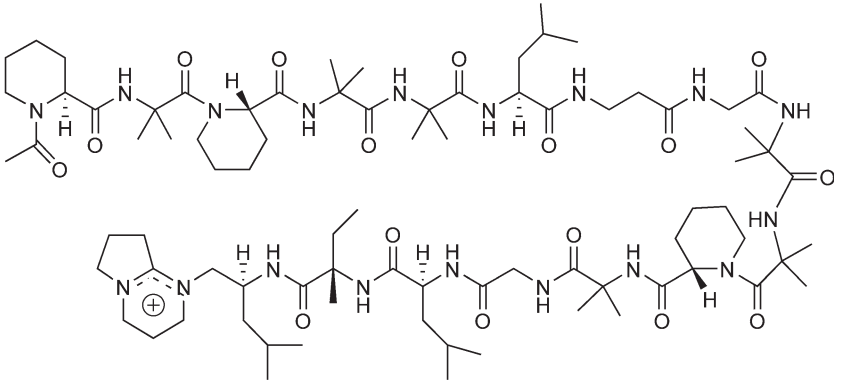
Beauvericin (**21**)



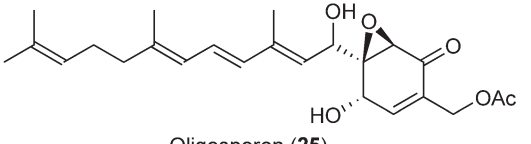
Bassianolide (**22**)



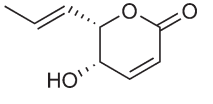
Oosporein (**23**)



Efrapeptin D (**24**)



Oligosporon (**25**)

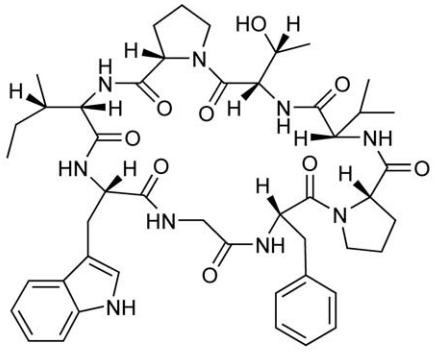


Phomalactone (**26**)

Figure 5 Insecticidal metabolites of entomopathogenic fungi.

Some nematocidal compounds were found in metabolites of the nematophagous fungi. Oligosporon (**25** in **Figure 5**) was obtained from the culture of the nematode-trapping fungus *Arthrobotrys oligospora* and exhibits nematocidal activity toward *Haemonchus controtus*.[73] Phomalactone (**26**) was first isolated as a bioactivity-unknown metabolite from a fungus. Its nematocidal activity toward *Meloidogyne incognita* and production by a plant nematode egg-parasitic fungus, *Pochonia chlamydospora*, were recently found.[74]

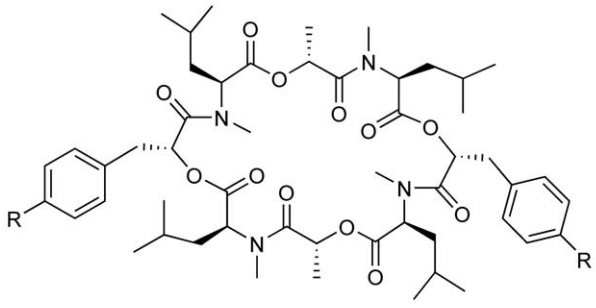
Many other insecticidal compounds have been found in metabolites of a variety of fungi.[75–78] They can be tentatively classified into three groups, peptides, alkaloids, and others, according to their structural features. Shearamide A (**27** in **Figure 6**), omphalotin A (**28**), and PF1022A (**29**) have cyclic peptide structures similar to that of toxins from entomopathogenic fungi mentioned above, which may be biosynthesized by a nonribosomal peptide synthetase. Shearamide A (**27**) was isolated from *Eupenicillium shearii* as a metabolite with insecticidal activity toward the corn earworm *Helicoverpa zea*[79] and showed that it was mainly present in the ascostromata of the fungus. Omphalotin A (**28**), a cyclododecapeptide, was isolated from the mycelia of *Omphalotus olearius*[80] and exhibited strong nematocidal activity against *M. incognita*.[81] PF1022A (**29**) was isolated from the filamentous fungus *Rosellinia* sp. as a nematocide.[82] It is a cyclic octadepsipeptide whose structure is similar to that of bassianolide (**22**). Its semisynthetic derivative, emodepside (**30**), with a morpholine ring at each of the para position of two phenyl groups is known as a very useful anthelmintic agent.[83] The target of PF1022A was



Shearamide A (**27**)

Cyclo(*N*-methylglycyl-L-tryptophyl-*N*-methyl-L-valyl-L-isoleucyl-*N*-methyl-L-valyl-*N*-methyl-L-valyl-*N*-methylglycyl-*N*-methyl-L-valyl-*N*-methyl-L-isoleucyl-*N*-methylglycyl-L-valyl-*N*-methyl-L-isoleucyl)

Omphalotin A (**28**)



PF1022A (**29**) R = H
Emodepside (**30**) R = morpholino
PF1022A-220 (**31**) R = NO_2
PF1022A-260 (**32**) R = NH_2

Figure 6 Insecticidal peptides from fungi.

identified as HC-110R, which is a transmembrane receptor concerning Ca^{2+} influx.[84] Genes encoding a nonribosomal peptide synthetase for PF1022A biosynthesis have been obtained.[85] Excellent work to prepare useful PF1022A derivatives (PF1022-220 (**31**), PF1022-260 (**32**)) with nitro or amino groups at the para position of the benzene rings was carried out by using biosynthetic engineering method.[86]

Many insecticidal alkaloids were isolated from fungal metabolites. Asperparaline A (aspergillimide; **33** in **Figure 7**) was isolated from *Aspergillus* sp. as a metabolite exhibiting paralytic activity against silkworm[87] and anthelmintic activity.[88] Biosynthesis of asperparaline A was studied by feeding experiments with labeled precursors, demonstrating that it was biosynthesized from tryptophan, isoleucine, and isoprene derivative molecules.[89] Okaramines were isolated by a screening search for an insecticidal compound among fungal metabolites. They were produced by *Penicillium simplicissimum* AK-40 when the fungus was cultured in a solid medium with bean curd lees 'okara'.[90] Okaramine A (**34**) exhibited strong insecticidal activity toward silkworm larva. Alantrypinone (**35**) and serantrypinone (**36**) were first isolated as fungal alkaloids produced by *Penicillium thymicola*.[91,92] They were later shown to have binding activity with the GABA receptor of housefly head membrane and insecticidal activity toward *Myzus persicae*.[93] Loline alkaloids are insecticidal metabolites produced by the grass-endophytic fungus *Neotyphodium uncinatum*. Biosynthesis of loline alkaloids was studied by feeding experiments of a variety of synthetic labeled precursors in the *N*-formylloline (**37** in **Figure 8**) producing fungus, and the biosynthetic pathway, in which *N*-(3-amino-3-carboxypropyl)proline (**38**) is involved as an intermediate (**Figure 8**), was clarified.[94] Genes responsible for biosynthesis of loline alkaloids have been obtained as a cluster from *N. uncinatum*.[95]

Figure 9 lists other fungal insecticidal metabolites with miscellaneous structural types. Nodulisporic acid A (**39**) was found in the metabolites of *Nodulisporium* sp. and exhibited potent insecticidal activity by modulating an invertebrate-specific glutamate-gated ion channel.[96,97] It has a complicated indole terpene structure and studies on its biosynthesis with labeled precursors showed that its biosynthetic pathway is different from other structurally related compounds since anthranilic acid was incorporated into the compound instead of tryptophan.[98] Cochlioquinone A (**40**), a metabolite of *Helminthosporium sativum*, was shown to have a nematocidal activity.[99] Chaetochalasin A (**41**) was isolated as an antiinsectan from *Chaetomium brasiliense*.[100] Benzofuran derivative (**42**)[101] and compound **43**[102] were isolated from an unidentified endophytic fungus and a conifer endophyte strain of *Canoplea elegantula*, respectively. They exhibited toxicity to the larvae of spruce budworm *Christoneura fumiferana*. NK374200 (**44**) was isolated from the culture broth of *Talaromyces* sp. as a bioactive

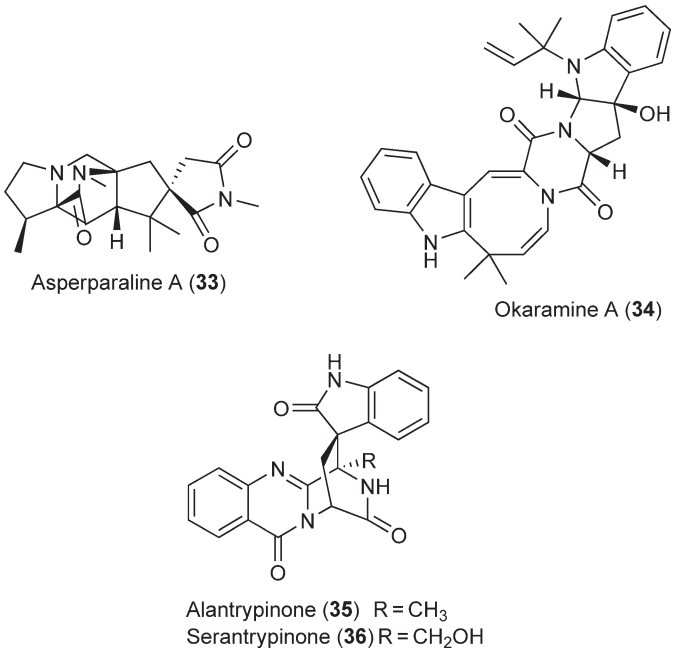


Figure 7 Insecticidal alkaloids from fungi.

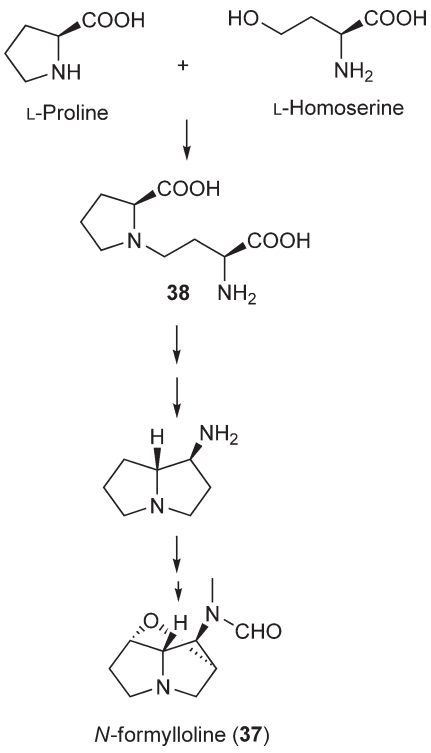


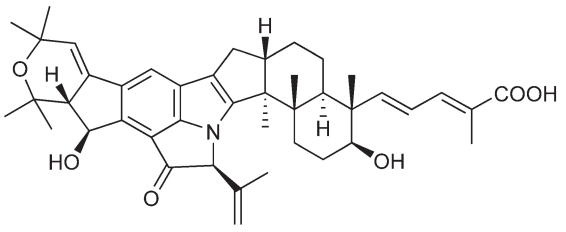
Figure 8 Biosynthesis of loline.

metabolite showing mosquito larvicidal activity.[103] Arisugacin (**45**), a metabolite of *Penicillium* sp. FO-4259, showed insecticidal activity by selective acetylcholinesterase inhibition.[104] Xanthonol (**46**) was recently isolated from a nonsporulating fungus and showed insecticidal and anthelmintic activities.[105] Brevioxime (**47**) was isolated from *Penicillium brevicompactum* as a metabolite with anti-JH hormone activity.[106] It was active against *Oncopeltus fasciatus* nymphs probably by inhibiting JH biosynthesis.[107]

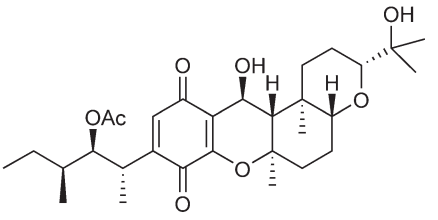
4.10.3 Mycotoxins

The term 'mycotoxicosis' means a disease caused by a fungal toxin.[108] The word 'mycotoxin' was derived from the term.[109] Mycotoxins are fungal secondary metabolites with a low molecular weight that cause mycotoxicosis. High-molecular-weight toxins such as toxic proteins produced by fungi are not included in mycotoxins. Usually, mycotoxicosis does not include mushroom poisoning, because mycotoxicosis is a disease passively caused by mycotoxins contaminated in food and feed, whereas mushroom poisoning results from intentional consumption of fungal fruiting bodies as food. Therefore, mushroom toxins are not called as mycotoxins. In this chapter, we do not deal with mushroom toxins in detail. There are many recent reviews concerning overall mushroom toxins,[110–112] psilocybin (**48** in **Figure 10**, the major psychoactive alkaloid),[113] agaritine (**49**, a toxic aryldiazonium ion-producing compound)[114] and its biosynthesis,[115] α-amanitin (**50**, an inhibitor of RNA polymerase),[116–118] ibotenic acid, (**51**) and muscimol (**52**) (active compounds connected with mysticism),[119] illudin S (**53**, DNA-interactive agent)[120] and its derivative useful as an anticancer drug.[121]

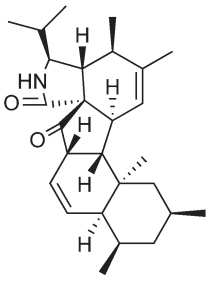
Approximately 1200 secondary fungal metabolites were known in 1978,[122] and the number at present has reached to more than 6000.[109] Approximately 10% of the 6000 metabolites are classified as mycotoxins. The number of secondary metabolites yet to be discovered may be quite large since it is estimated that we know around 5% fungal species out of the world's total fungal species.[123,109] Therefore, the number of mycotoxins will probably increase in future.



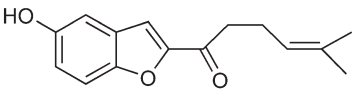
Nodulisporic acid A (**39**)



Cochlioquinone (**40**)



Chaetochalasin A (**41**)



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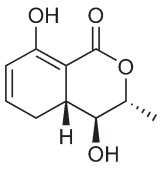

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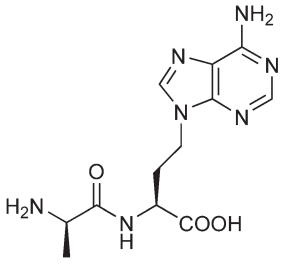
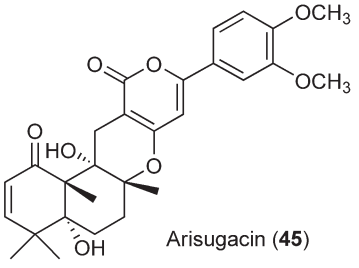
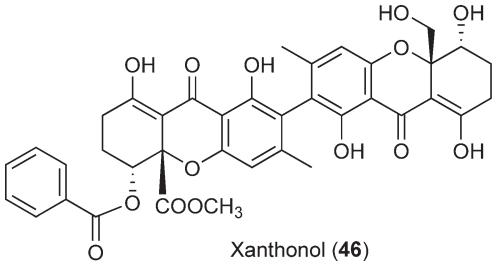


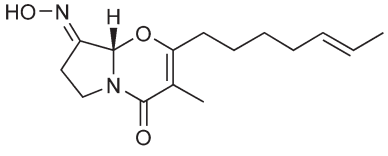
NK374200 (**44**)



Arisugacin (**45**)



Xanthonol (**46**)



Brevioxime (**47**)

Figure 9 Insecticidal metabolites of fungi.

The mycotoxins with the greatest potential risk to human and animal health as food and feed contaminants are AFs, trichothecenes, fumonisins, zearalenone, ochratoxin A, and ergot alkaloids.[109] Other mycotoxins such as cyclopiazonic acid, sterigmatocystin, gliotoxin, citrinin, penitrems, patulin, fusarin C, penicillic acid, and PR toxin have also high potential risk because of their frequency of occurrence in commodities. In this section, we describe recent topics mainly on the mode of action and biosynthesis of these mycotoxins. Since contamination of AFs and trichothecenes in agricultural products are worldwide serious problems from the viewpoints of food safety and economic loss, the two mycotoxin groups are described in detail.

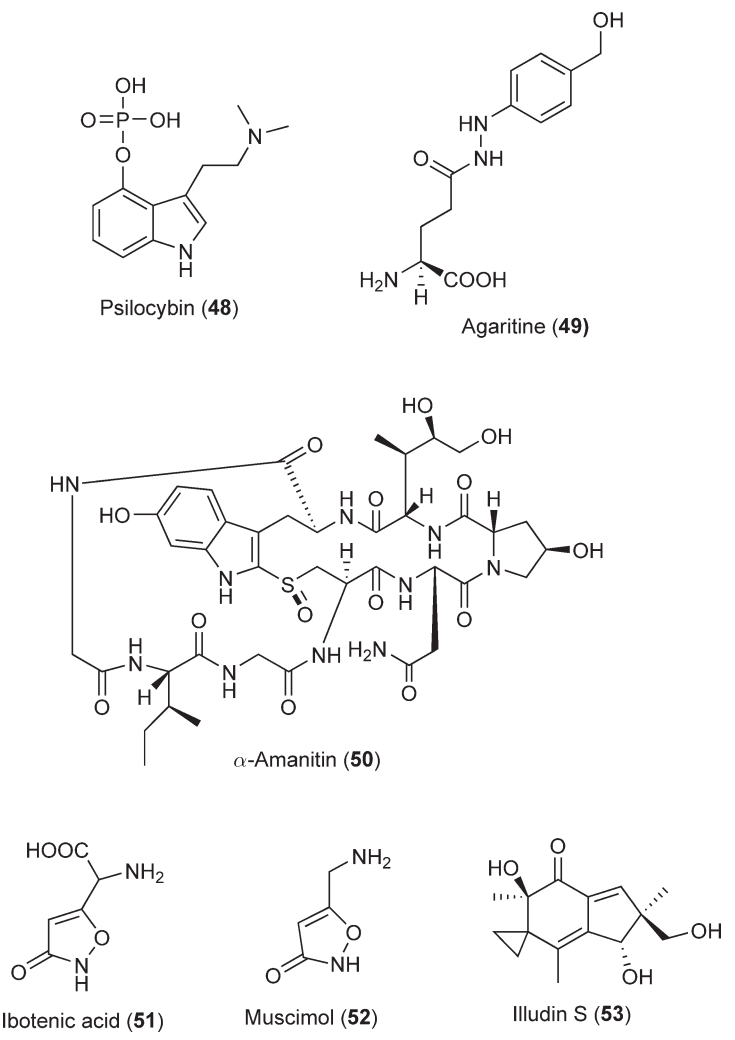


Figure 10 Mushroom toxins.

4.10.3.1 Ochratoxins, Fumonisins, Zearalenone, and Ergot Alkaloids

Ochratoxins are metabolites produced by *Aspergillus ochraceus* and some *Penicillium* species such as *P. verrucosum*.[124] It exhibits nephrotoxic effects and potent carcinogenic activity in rodents.[109] DNA adduct formation by ochratoxin A (**54** in **Figure 11**) has been suggested to be involved in a mechanism of ochratoxin A-induced tumor formation, and it was recently shown that C8-ochratoxin A–deoxyguanosine adduct (**55**) was produced when ochratoxin A reacted with deoxyguanosine in the presence of Fe(II) or HRP/H_2O_2.[125] However, this DNA–ochratoxin A adduct formation has not been confirmed in other *in vivo* experimental systems.[126] With respect to ochratoxin A biosynthesis, ochratoxin α (**56** in **Figure 11**) was shown as its biosynthetic precursor, but labeled mellein (**57**) was not incorporated into ochratoxin A.[127] Genes encoding PKS responsible for ochratoxin A biosynthesis in *A. ochraceus*[128] and *Penicillium nordicum*[129] have been cloned. Genes encoding a nonribosomal peptide synthase for the coupling of ochratoxin α and phenylalanine moiety and a chloroperoxidase for chlorination have also been obtained from *P. nordicum*.[130]

Fumonisins are mycotoxins produced by *Fusarium verticillioides*. Fumonisin B_1 (**58** in **Figure 12**) is toxic and carcinogenic to rodents and suggested to cause esophageal cancer. It has cancer-promoting activity in rats and is categorized as a factor possibly carcinogenic to humans.[131] Fumonisin B_1 is a strong inhibitor of ceramide synthase (sphinganine *N*-acyltransferase).[132,133] Inhibition of the enzyme causes blockades in ceramide

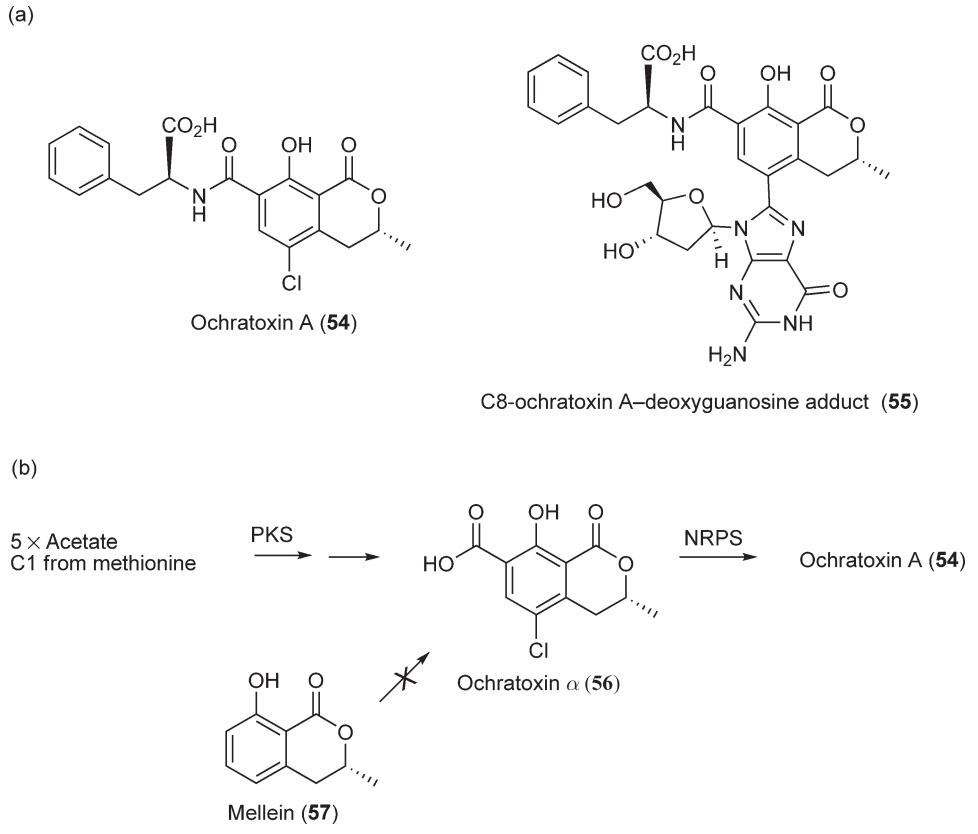


Figure 11 Structures of ochratoxin A and C8-ochratoxin A–deoxyguanosine adduct (a) and biosynthesis of ochratoxin A (b).

synthesis, leading to accumulation of ceramide substrates, sphinganine and sphingosine, and decrease of sphingolipids. This disruption of sphingolipid metabolism by fumonisin B_1 may affect apoptosis, cell cycle, and other cell functions, causing a variety of diseases in animals.[130] It was recently shown that fumonisin B_1 is a possible risk factor for birth defects, especially human neural tube defects.[134] Biosynthesis of fumonisin B_1 has been studied actively.[135,136] Fifteen genes responsible for fumonisin biosynthesis are present in a cluster form in *F. verticillioides*.[137] Roles of most of the biosynthetic enzymes encoded by the genes have been clarified as shown in **Figure 12**.[138] The nonaketide (**59**) with two methyl groups is first produced by PKS (FUM1) and condensation of the CoA derivative of the polyketide (**60**) and L-alanine produces the amine (**61**). Oxidation and reduction of the ketone, followed by two times oxidation, affords the triol (**62**). The hydroxyl groups of the triol (**62**) are esterified with the CoA derivative of the tricarboxylic acid to afford compound **63**, and final oxidation produces fumonisin B_1. The tricarboxylic acid precursor for the esterification is suggested to be biosynthesized by reduction of citrate.

Zearalenone (**64** in **Figure 13**) is produced by *Fusarium* species such as *F. graminearum*. It is implicated in reproductive disorders of farm animals and occasionally in hyperestrogenic syndromes in humans.[139,140] Zearalenone is an estrogen antagonist causing estrogenic responses in mammals by binding to estrogen receptors.[141] Zearalenone also shows carcinogenicity, genotoxicity, and immunotoxicity, but molecular mechanism of these toxicity derived from zearalenone has not been clarified. PKS genes responsible for zearalenone biosynthesis have been identified in *F. graminearum* by gene disruption experiments.[142] Two PKS genes (*zea1* and *zea2*) are necessary for the production of zearalenone. Interestingly, ZEA2 possesses the domains of β-ketoacyl reductase, dehydrotase, and enoyl reductase as observed normally in PKSs, but ZEA1 lacks them, indicating that the keto groups of the polyketide chain produced by ZEA1 remains intact. Biosynthesis of zearalenone is speculated as shown in **Figure 13**. A hexaketide is first biosynthesized by

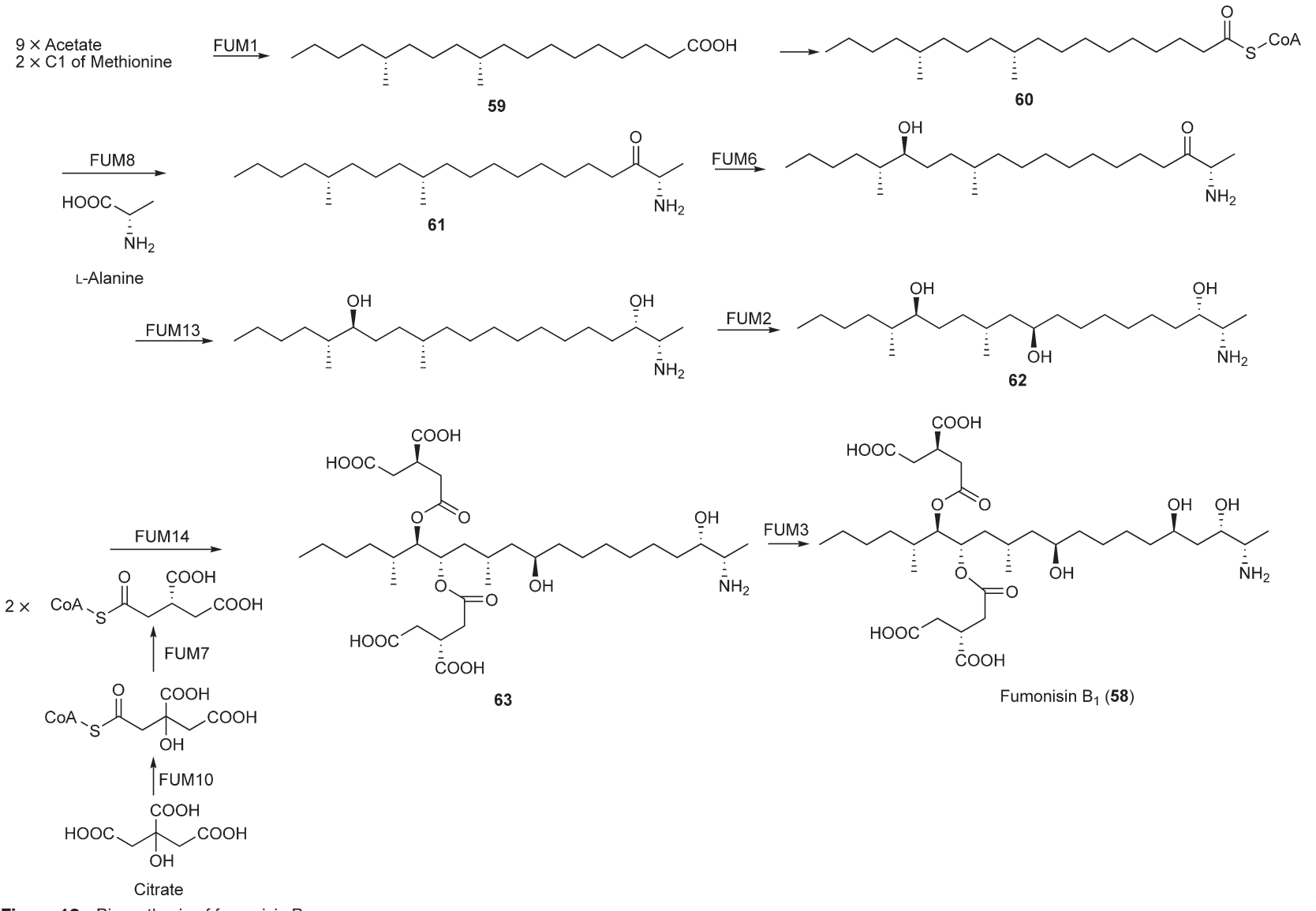


Figure 12 Biosynthesis of fumonisin B_1.

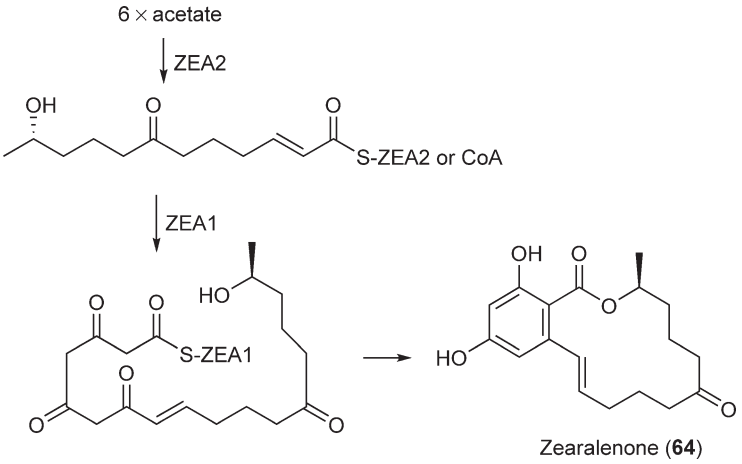


Figure 13 Biosynthesis of zearalenone.

ZEA2. ZEA1 produces a nonaketide by using the hexaketide as a starter unit. The nonaketide is easily converted into zearalenone by aromatization and lactonization.

Ergot alkaloids are produced by several species of *Claviceps*. They are derivatives of the tetracyclic ergoline skeleton and structurally divided into two main groups, the clavines such as agroclavine (**65** in **Figure 14**) and lysergic acid derivatives such as ergotamine (**66**). Their structures are homologous to neurotransmitters such as dopamine, noradrenaline, or serotonine and they can interact with receptor sites of such biogenic amines. Therefore, ergot alkaloids exhibit a broad spectrum of toxicological and pharmacological actions on central, neurohumoral, and peripheral nervous systems.[143] Ergotism is the oldest recognized mycotoxicoses of humans. The disease became epidemic in the Middle Ages, where it was known as St. Anthony's fire, but occurrence of the disease has declined now.[109] Ergotism results from consumption of products made with grains contaminated with ergot alkaloids. The two manifestations of ergotism that are known are gangrenous and convulsive forms.. The former form of ergotism is caused by the action of ergotamine group alkaloids associated with wheat and rye. The clavine group alkaloids involved in the ergot of pearl millet are suggested to be responsible for the latter form of ergotism. Gene cluster for ergot alkaloid biosynthesis has been clarified in each of *Claviceps fusiformis*[144] and *Claviceps purpurea*,[145] and the biosynthetic pathway of ergotamine has been confirmed as shown in **Figure 14**. Dimethylallyltryptophan (**67**) formed by a coupling of dimethylallyl pyrophosphate and tryptophan, which is catalyzed by a dimethylallyltryptophan synthase, is the first product in the pathway. *N*-methylation of the intermediate and subsequent conversion reactions form agroclavine (**65**). The two-step oxidation of agroclavine produces paspallic acid (**69**), which isomerizes to D-lysergic acid (**70**). Two non-ribosomal peptide synthases catalyze the formation of D-lysergyl tripeptide lactam (**71**), and subsequent oxidation and cyclization afford ergotamine (**66**). The gene encoding a cytochrome P-450 monooxygenase, which may catalyze the two successive steps from agroclavine to elymoclavine (**68**) and from elymoclavine to paspallic acid, has been identified.[146]

4.10.3.2 Miscellaneous Mycotoxins

Patulin (**72** in **Figure 15**) is produced by a number of species of *Penicillium* and *Aspergillus*. Among the patulin-producing *Penicillium* species, *Penicillium expansum* is most important because it associates with spoilage and mycotoxin production in apples and apple juices. Contamination of patulin in apple juices is now a significant problem.[147] Patulin shows cytotoxic activity, which is suggested to be caused by forming covalent adducts between patulin and cellular thiols.[148] The complicated biosynthetic pathway of patulin has been confirmed as shown in **Figure 15**,[149] but there is still little information on the genes encoding the patulin biosynthetic enzymes. Only two genes encoding 6-methylsalicylic acid synthase and isoepoxydone dehydrogenase have been identified in *Penicillium urticae* and *P. expansum*.[150] The former enzyme is a Type I PKS that produces

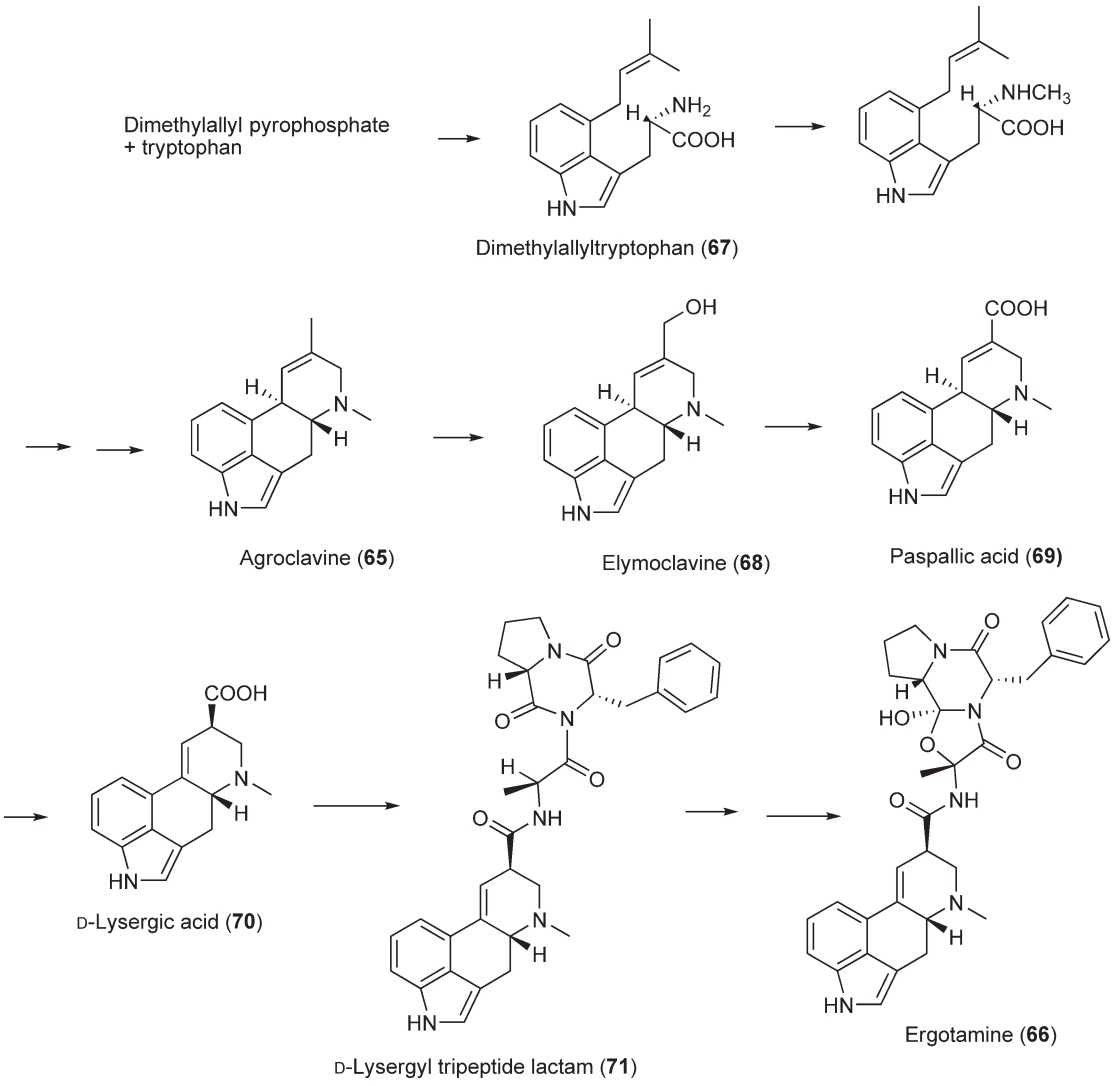


Figure 14 Biosynthesis of ergotamine.

6-methylsalicylic acid (**73**) from four acetate molecules. The latter one catalyzes the reaction from isoepoxydone (**74**) to phyllostine (**75**). Two putative cytochrome P-450 monooxygenase genes, which may be connected with the patulin biosynthesis, were also obtained from *P. expansum*.[150]

Gliotoxin (**76** in **Figure 16**) is a highly immunosuppressive compound produced by a variety of fungi.[151] *Aspergillus fumigatus*, a producer of gliotoxin, is a pathogenic fungus causing the respiratory disease known as aspergillosis. Since gliotoxin production is detected in infected animal tissues, it is thought that there is a relationship between gliotoxin production by the fungus and pathogenesis of aspergillosis. The gliotoxin biosynthetic gene cluster was found in the genome of *A. fumigatus*.[152] Among the biosynthetic genes, the gene encoding a nonribosomal peptide synthase was shown to be necessary for gliotoxin biosynthesis by a gene disruption experiment.[153,154] A nuclear protein, LaeA, is known as a regulator for the production of various secondary metabolites including gliotoxin in *A. fumigatus*. It is thought that LaeA is a key factor for the virulence of the fungus.[155]

Cyclopiazonic acid (**77** in **Figure 16**) is produced by several species of *Aspergillus* and *Penicillium*, and its contamination has been found in a variety of agricultural products. Since *Aspergillus flavus* often produces AF and cyclopiazonic acid concurrently, it is speculated that mycotoxicosis caused by cyclopiazonic acid may be

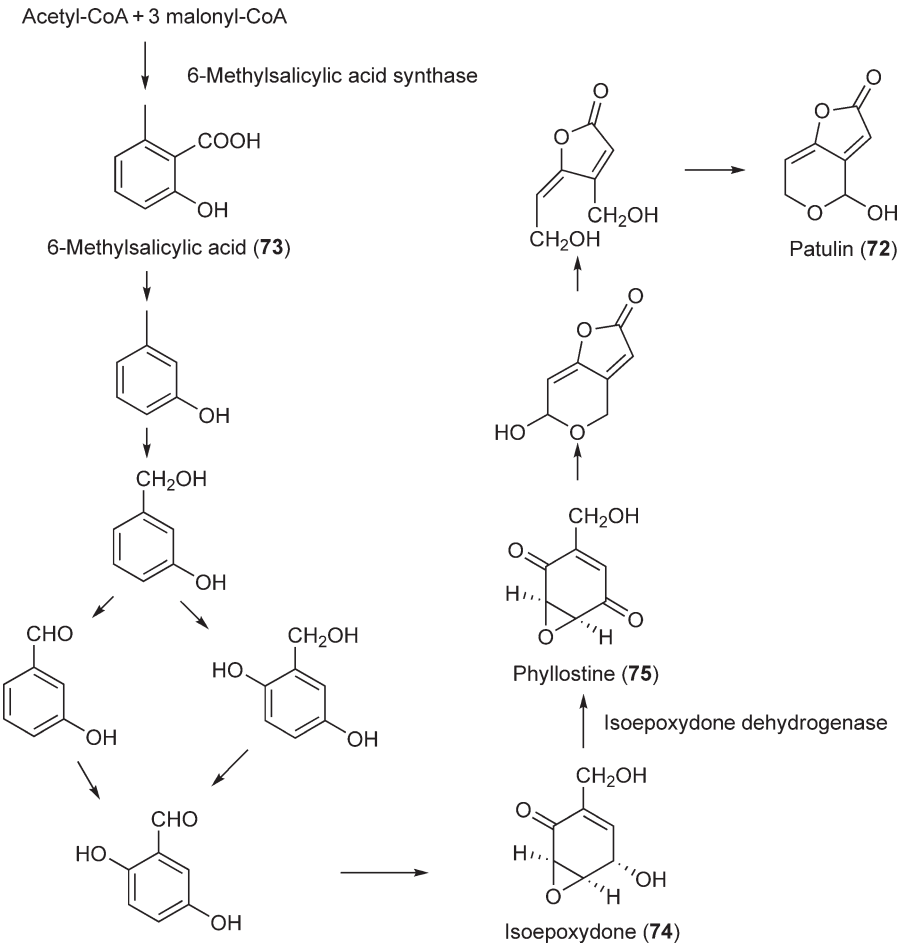


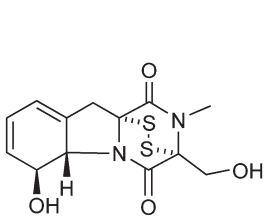
Figure 15 Biosynthesis of patulin.

disguised in the presence of aflatoxicosis.[156] Cyclopiazonic acid is known as a potent inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-activated ATPase. The indole-tetramic acid skeleton of cyclopiazonic acid is biosynthesized from tryptophan, mevalonate, and two molecules of acetate, but its biosynthetic genes are not obtained yet. VeA, a global regulatory protein controlling AF production and sclerotial formation in *A. flavus*, was also shown to regulate cyclopiazonic acid production by the fungus.[157]

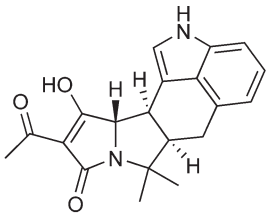
Citrinin contamination in food is caused mainly by the infection of *Penicillium citrinum* and *P. verrucosum* although a variety of fungi can produce citrinin.[158,159] Citrinin (**78** in **Figure 16**) is known as a nephrotoxin.[160,161] Since *P. verrucosum* often produces ochratoxin A, which also has a nephrotoxic effect, concurrently, citrinin may interact synergistically with ochratoxin A.[162] *Monascus purpureus*, a producer of a useful red pigment, produces citrinin as an undesired side product. The gene encoding PKS for citirinin biosynthesis in *M. purpureus* has been clarified.[163]

Citreoviridin (**79**), luteoskyrin (**80**), and cyclochlorotine (**81**) are historical mycotoxins studied in Japan. Citreoviridin was isolated as a toxin from *Penicillium citreoviride* associated with a disease called cardiac beriberi or 'shoshin kakke'.[164] Luteoskyrin and cyclochlorotine were isolated from *Penicillium islandicum*, which was infected into toxic yellowed rice.[165] Genes responsible for biosynthesis of these compounds are not obtained.

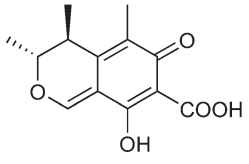
Penicillic acid (**82** in **Figure 17**) is a toxic compound produced by many *Penicillum* and some *Aspergillus* species.[166] It is biosynthesized from orsellinic acid through the pathway shown in **Figure 17**.[167] A candidate of a gene encoding a PKS for penicillic acid biosynthesis was obtained from *A. ochraceus*.[168]



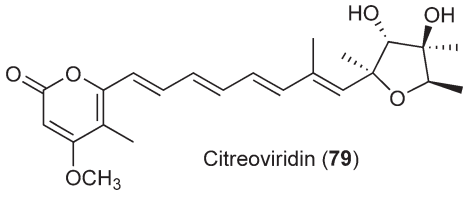
Gliotoxin (**76**)



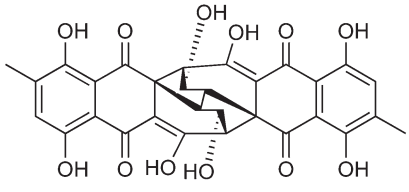
Cyclopiazonic acid (**77**)



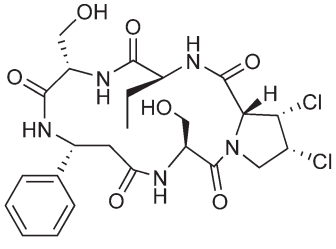
Citrinin (**78**)



Citreoviridin (**79**)

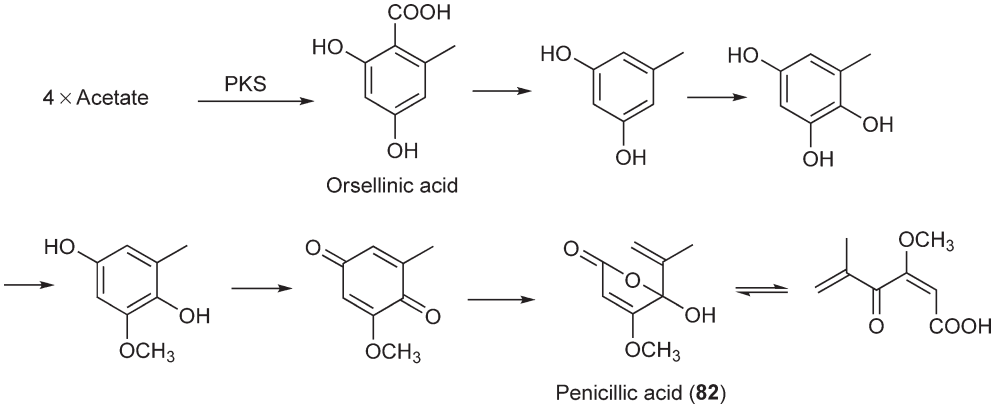


Luteoskyrin (**80**)



Cyclochlorotine (**81**)

Figure 16 Structures of gliotoxin, cyclopiazonic acid, citrinin, citreoviridin, luteoskyrin, and cyclochlorotine.



Penicillic acid (**82**)

Figure 17 Biosynthesis of penicillic acid.

Phomopsin A (**83** in **Figure 18**) was isolated from *Phomopsis leptostromiformis* as the hepatotoxic metabolite responsible for lupinosis, which is a disease in animals caused by ingestion of *Lupinus* species infected with the fungus.[169] *Penicillium* species produce other significant mycotoxins such as penitrem A (**84**), PR toxin (**85**), and rubratoxin B (**86**).[170] Fusarin C (**87**) and fusaproliferin (**88**) are known as mycotoxins produced by *Fusarium*

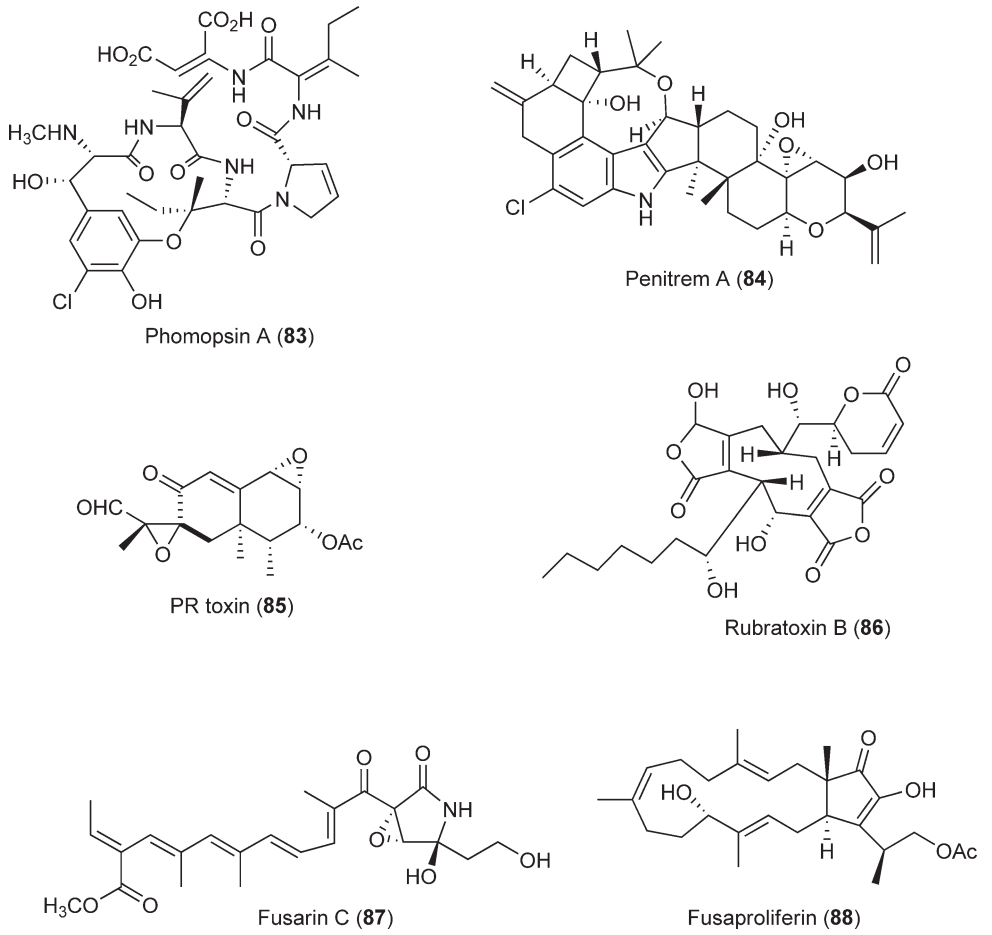


Figure 18 Structures of phomopsin A, penitrem A, PR toxin, rubratoxin B, fusarin C, and fusaproliferin.

species. A gene encoding a PKS fused with an unusual nonribosomal peptide synthase module responsible for biosynthesis of fusarin C was obtained.[171] Fusaproliferin was discovered as a toxic metabolite of *Penicillium proliferatum* in 1995,[172] but it has become an important mycotoxin because its contamination in corn samples has been detected in many countries.[173]

4.10.3.3 Aflatoxins

AFs are produced primarily by *A. flavus* and *Aspergillus parasiticus*. Two other *Aspergillus* species, *A. nomius* and *A. pseudotamarii*,[174] can also produce AFs. *Aspergillus oryzae* and *Aspergillus sojae*, important strains used in the fermentation industry, are known as ancestors of *A. flavus* and *A. parasiticus*, respectively. It has been clearly proved that they never produce AFs by analysis of their AF biosynthetic genes.[175,176] Aflatoxins B_1 (**89** in **Figure 19**) and B_2 (**90**) (AFB_1 and AFB_2, B series AFs), and G_1 (**91**) and G_2 (**92**) (AFG_1 and AFG_2, G series AFs) are known as natural products. *A. parasiticus* produces both series AFs, while *A. flavus* produces only B series AFs due to lack of the biosynthetic enzymes for production of G series AFs. Aflatoxins M_1 (**93**) and M_2 (**94**) (AFM_1 and AFM_2) are metabolic products of AFB_1 and AFB_2, respectively, which were first isolated from the milk of lactating animals fed AF-contaminated feeds.[177] Contamination of these six AFs in foods and feeds is observed.

Aflatoxins show strong acute toxicity, that has caused serious acute hepatitis in humans.[178] Recent outbreaks of acute aflatoxicosis in Kenya in 2004, 2005, and 2006, which were caused by AF contamination in maize, affected 317, 75, and 51 persons and approximately 41% of those died.[179] The strength for the acute toxicity of

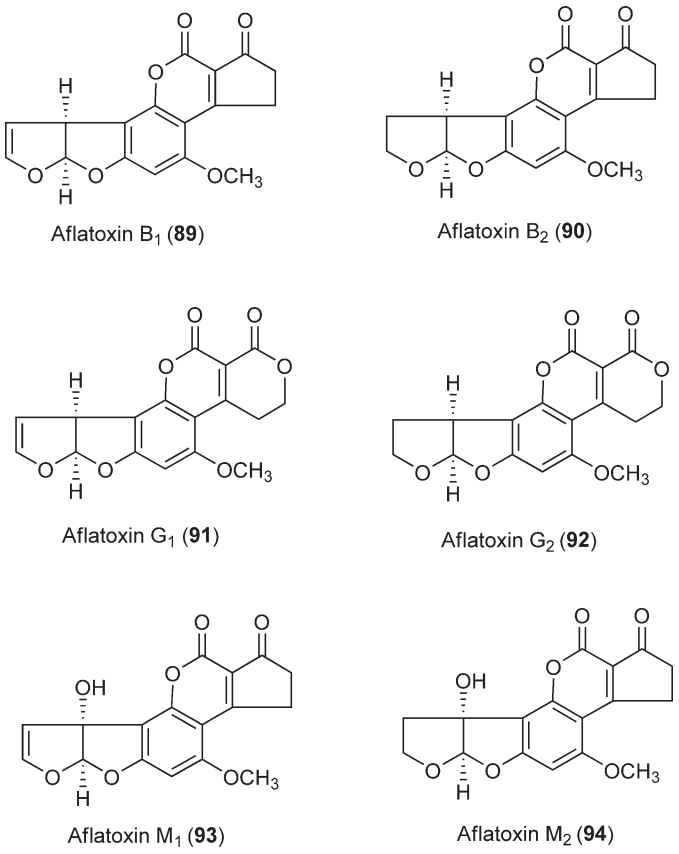


Figure 19 Structures of aflatoxins.

natural AFs is given in the following order: $AFB_1 > AFG_1 > AFB_2 > AFG_2$.[177] AFB_1 has the strongest carcinogenic activity in known natural products. The strength of the carcinogenicity toward rainbow trout or rats has been determined in the order: $AFB_1 > AFM_1 > AFG_1$.[180] AFB_2 and AFG_2 did not show carcinogenic activity toward such organisms.[179] Several epidemiological studies in localities with a high incidence of liver cancer have strongly suggested the association of AF with hepatocellular carcinoma.[181] The molecular mechanism of the carcinogenic activity of AFB_1 has been proposed as shown in **Figure 20**. The stereospecific oxidation of the C8–C9 double bond of AFB_1 occurs by the action of a cytochrome P-450 monooxygenase in liver to afford the epoxide (**95**). The epoxide can bind to a guanine residue of DNA, forming a DNA–AF adduct.[182] The DNA adduct may mutate an important gene such as *p53*, a tumor suppressor gene, which leads to cancer.[183] This mechanism matches the fact that AFB_2 or AFG_2 showed no carcinogenic activity, but such AFs without a double bond at C8–C9 still have strong mutagenicity in addition to the above-mentioned acute toxicity.[180] Therefore, other unknown mechanisms to explain biological activities of AFs may be present.

It is very difficult to answer the question why an AF-producing fungus produces AF or what is the physiological role of AF in its producer. AF production may not be necessary for the fungal growth and infection into host plants because *A. parasiticus* or *A. flavus* without the ability of AF production can do them normally. A hypothesis that AFs act as an antifeedant toward animals for protecting host plants from their attack does not fit with the fact that animals cannot discriminate between feeds contaminated with and without AFs. Antibiotic activity of AFs is not so strong, but AFs shows relatively strong insecticidal activity,[184] which might be a clue to clarify a physiological role of AFs.

Aflatoxin-producing fungi are cosmopolitan organisms that are able to contaminate a wide range of natural substrates including cereal grains, oil seeds, cottonseed, etc. Owing to their airborne propagules, they can be easily distributed from their natural ecological niches to susceptible plants and crops all over the world. This AF contamination in crops is a serious problem from the viewpoint of not only public health but also economic

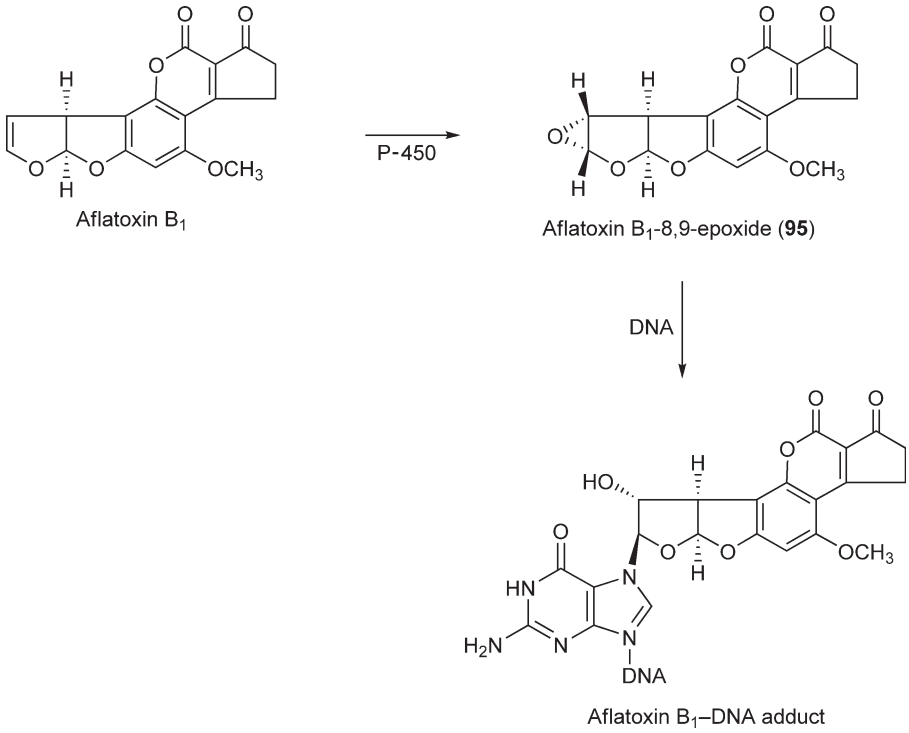


Figure 20 Formation of aflatoxin–DNA adduct.

loss.[185,186] However, it is difficult to resolve the problem due to lack of an effective method to control AF production. Since studies on mechanism of AF biosynthesis and its regulation are very important as a basic research to develop a useful method to protect food and feed from AF contamination, we review recent information on them. Practical methods and new attempts to regulate AF production and contamination are also described.

4.10.3.3.1 Biosynthesis of aflatoxins

Biosynthesis of AFs has been extensively studied and many excellent reviews on it have been written constantly.[187,188] Although a few ambiguous steps still remain, intermediates involved in the biosynthetic pathway and genes encoding biosynthetic enzymes have been clarified as shown in **Figures 21** and **22**. Biosynthetic genes are clustered in a 70 kDa DNA region in *A. parasiticus* or *A. flavus*.[187] The names with the three-letter code '*afl*' are used to represent the 25 genes (*aflA–Y*) in the figures. The renaming was proposed in 2004 by Yu *et al.*[187] The AF biosynthetic pathway is one of the most complicated ones observed in the biosynthesis of natural products. Aflatoxin is a polyketide compound derived from ten acetate molecules. A PKS encoded by *aflC* (original name: *pksA*) may form hexanoyltetrahydroxyanthrone (**96** in **Figure 21**) by using hexanoyl CoA as a starter unit. The hexanoyl CoA itself is biosynthesized by fatty acid synthases α and β subunits encoded by *aflA* (*fas-1*) and *aflB* (*fas-2*). Hexanoyltetrahydroxyanthrone (**96**) has not been isolated as an intermediate, but it is oxidized spontaneously or by an unknown enzyme to produce norsolorinic acid (**97**) with an anthraquinone skeleton. Norsolorinic acid (**97**) is then reduced stereospecifically to afford averantin (**98**). This reaction is catalyzed by a reductase encoded by *aflD* (*nor-1*). But, another reductase encoded by *aflE* (*norA*) can also catalyze the conversion. Furthermore, a gene, *aflF* (*norB*), encoding a protein homologous to AflE is also present in the gene cluster. Although the reductase encoded by *aflD* seems to be mainly responsible for this reaction, further studies may be necessary to clarify the function of each of the three genes (*aflD*, *aflE*, and *aflF*). It is unknown why these three genes are present for the simple reductive step. Hydroxylation of averantin (**98**) by a cytochrome P-450 monooxygenase encoded by *aflG* (*avnA*) produces 5′-hydroxyaverantin (**99**). Since this reaction is not stereospecific, diastereomers are produced. Both the diastereomers produced are oxidized to 5′-oxoaverantin (**100**) by an alcohol dehydrogenase encoded by *aflH* (*adhA*). 5′-Oxoaverantin (**100**) is next converted into averufin (**101**) by a cyclase encoded by *aflK* (*vbs*). Averufin (**101**) is oxidized by a cytochrome

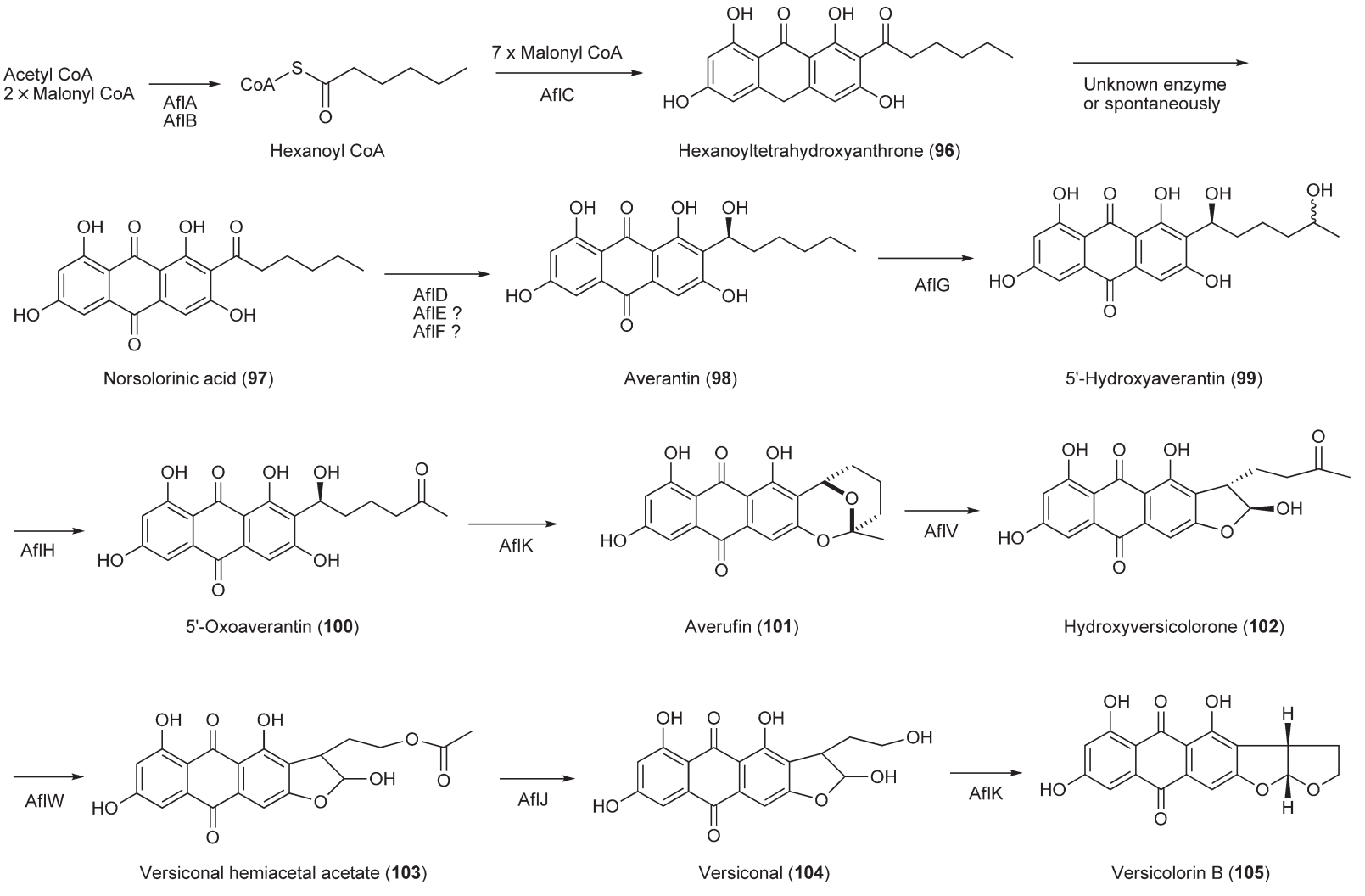


Figure 21 Aflatoxin biosynthetic pathway from hexanoyl CoA to versicolorin B.

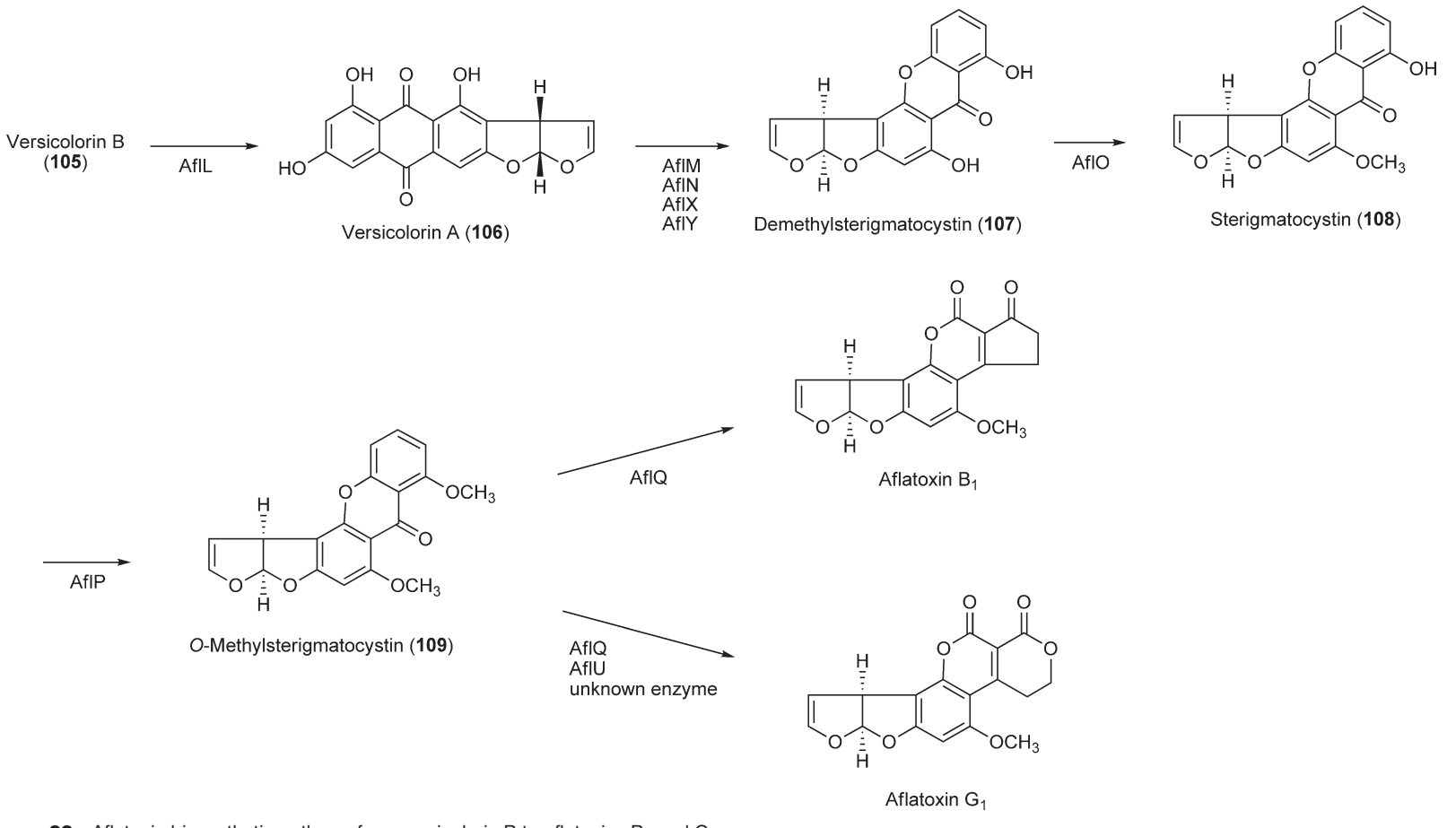


Figure 22 Aflatoxin biosynthetic pathway from versicolorin B to aflatoxins B_1 and G_1.

P-450 monooxygenase encoded by *aflV* (*cypX*) to produce hydroxyversicolorone (**102**), which is further oxidized to versiconal hemiacetal acetate (**103**) by a monooxygenase encoded by *aflW* (*moxY*). The latter conversion from hydroxyversicolorone (**102**) into versiconal hemiacetal acetate (**103**) is a Baeyer–Villiger reaction.[189] Versiconal hemiacetal acetate (**103**) is hydrolyzed by an esterase encoded by *aflJ* (*estA*) to afford versiconal (**104**),[190] which is converted into versicolorin B (**105**) by a cyclase. It is very interesting that the cyclase catalyzing the conversion from versiconal (**104**) into versicolorin B (**105**) is the same enzyme encoded by *aflK* as that catalyzing the above mentioned reaction from 5′-oxoaverantin (**100**) to averufin (**101**).[191] Versicolorin B (**105**) is converted by a desaturase encoded by *aflL* (*verB*) to produce versicolorin A (**106** in **Figure 22**). AFB_1 and AFG_1 are biosynthesized from versicolorin A (**106**), whereas this desaturase reaction is not involved in the biosynthesis of AFB_2 and AFG_2. The next reaction from versicolorin A (**106**) to demethylsterigmatocystin (**107**) is complicated, with which at least four proteins encoded by *aflM* (*ver-1*), *aflN* (*verA*), *aflY* (*hypA*), and *aflX* (*ordB*) are involved.[192,193] The reaction mechanism has not been clarified, but oxidation–reduction–oxidation mechanism is proposed as shown in **Figure 23**.[194] Versicolorin A (**106**) is oxidized by a cytochrome P-450 monooxygenase encoded by *aflN* to produce an epoxide (**110** in **Figure 23**), which is converted by an oxidoreductase encoded by *aflX* to compound **111**. Compound **111** is reduced by a dehydrogenase encoded by *aflM* to compound **112**, which is converted into demethylsterigmatocystin (**107**) by a protein encoded by *aflY* through a Baeyer–Villiger reaction. Demethylsterigmatocystin (**107**) is methylated twice by methyltransferases encoded by *aflO* (*dmtA*) and *aflP* (*omtA*), successively, to produce sterigmatocystin (**108**) and *O*-methylsterigmatocystin (**109**). *O*-Methylsterigmatocystin is converted into AFB_1 by an oxidoreductase encoded by *aflQ* (*ordA*). AFG_1 is also produced from *O*-methylsterigmatocystin (**109**), but at least three proteins, the same oxidoreductase encoded *aflQ*, a cytochrome P-450 monooxygenase encoded by *aflU* (*cypA*), and an unknown membrane-bound protein, are necessary for the conversion from *O*-methylsterigmatocystin (**109**) to AFG_1.[195] The molecular mechanism for the production of AFB_1 and AFG_1 from *O*-methylsterigmatocystin (**109**) is not clear. AFB_2 and AFG_2 are biosynthesized from versicolorin B (**105**) through dihydrodemethylsterigmatocystin (**113** in **Figure 24**), dihydrostigmatocystin (**114**), and dihydro-*O*-methylsterigmatocystin (**115**) by the same enzymes involved in the biosynthesis of AFB_1 and AFG_1.

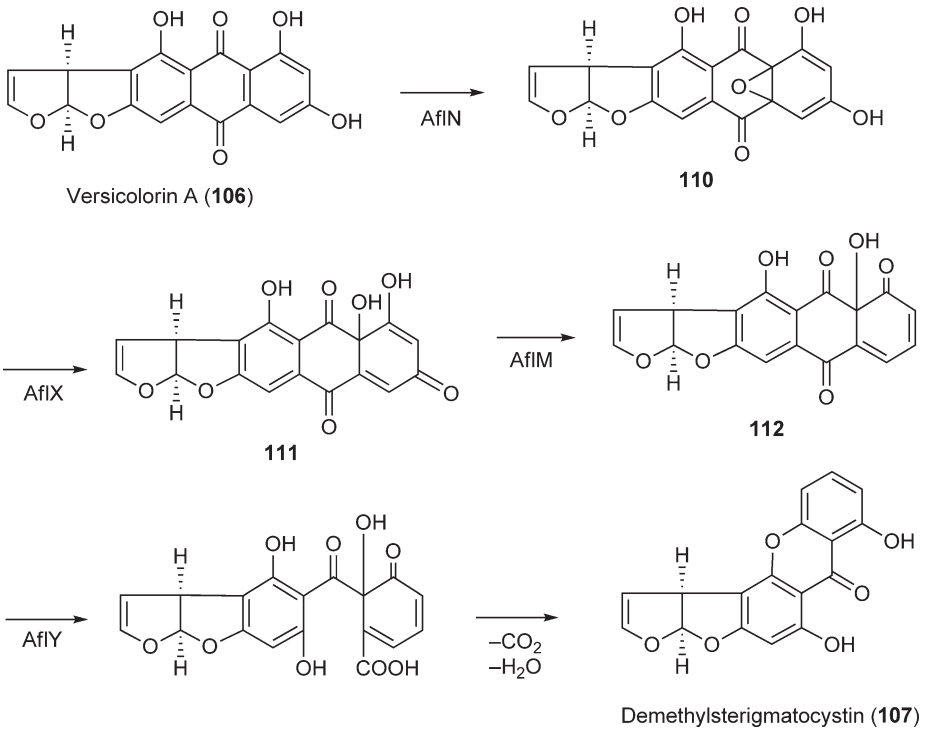


Figure 23 Putative biosynthetic mechanism from versicolorin A to demethylsterigmatocystin.

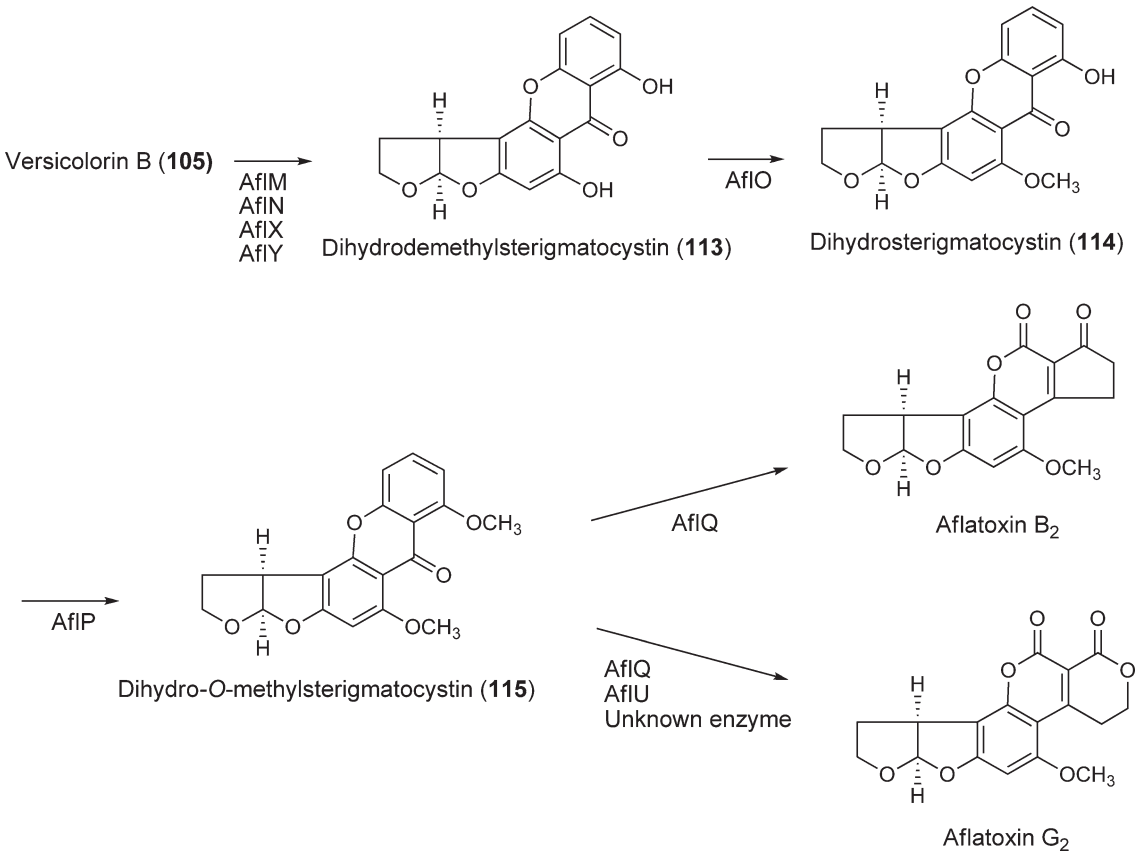


Figure 24 Aflatoxin biosynthetic pathway from versicolorin B to aflatoxins B_2 and G_2.

Among the 25 genes involved in the AF biosynthetic cluster, studies on 21 genes are described above. Functions of the remaining four genes, *aflR* (*aflR*), *aflS* (*aflJ*), *aflI* (*avfA*), and *aflT* (*aflT*), also have been studied. An oxidase encoded by *aflI* is suggested to be concerned with the steps from averufin (**101**) to versiconal hemiacetal acetate (**103**),[196] but its role is not still clear. The gene *aflT* encodes a transporter, suggesting that it has a role in the AF secretion, but its role is not confirmed.[197] Two proteins encoded by *aflR* and *aflS* are important for regulation of the expression of AF biosynthetic enzymes, which are described in detail in the next section.

4.10.3.3.2 Regulatory mechanism of aflatoxin production

Aflatoxins are produced as secondary metabolites by fungi. Secondary metabolism is regulated by a signal from primary metabolism, but it is not easy to clarify a regulatory mechanism of the change from primary metabolism to secondary one. In the case of AF production, there are the following three important clues to investigate the regulatory mechanism leading to the start of AF biosynthesis[198,199]: (1) regulatory proteins which control expression of AF biosynthetic enzymes are present in the gene cluster of AF biosynthesis, (2) some environmental and nutritional factors strongly affect AF productivity, and (3) fungal development such as conidiation often links with AF production.

First, a protein encoded by *aflR* is known as the key regulator for AF biosynthesis.[190,198] AflR protein is a positive-acting transcription factor, which binds to a specific recognition site in the promoter regions of most of *afl* genes encoding AF biosynthetic enzymes, and can provoke their expression. Loss of AflR protein causes complete inhibition of transcription of *afl* genes, indicating that expression of AflR is the initial switch that can start the secondary metabolism. The signaling pathway or regulatory system in the primary metabolite, which leads to *aflR* expression has not been clarified well. The presence of putative binding sites of PacC and AreA,

transcriptional factors for pH control and nitrogen utilization, respectively, in the promoter region of *aflR* may coincide with the facts of regulation of AF production by pH and nitrogen source later mentioned. On the other hand, *aflS* is known as another regulatory gene present in the AF biosynthetic gene cluster. It is suggested that AflS protein binds to the C-terminal region of AflR and acts as a transcriptional enhancer or a coactivator of AflR.[198]

Second, carbon and nitrogen sources, pH, temperature, and water activity are important nutritional and environmental factors for AF production.[199] It is known that AF production is induced by glucose or sucrose, but not induced by peptone or lactose, suggesting that a catabolite repression is involved in AF production. AF production is strongly suppressed by the presence of nitrate or under an alkaline condition. AF contamination in crops is observed only in tropical and semitropical areas with optimal environmental conditions for AF production by fungi.

It is known that there is a relationship between conidiation and mycotoxin production in *Aspergillus* species.[200] For example, many of the fluffy mutant strains of *Aspergillus nidulans* that are unable to form conidia cannot produce sterigmatocystin, an intermediate involved in the late steps of AF biosynthetic pathway (**Figure 22**).[201] Since genes responsible for sterigmatocystin biosynthesis in *A. nidulans* are homologs of the corresponding ones for AF biosynthesis in *A. flavus* or *A. parasiticus*, production mechanism of sterigmatocystin by *A. nidulans* has been studied as a model system. From the analysis of a mutant defective in both conidiation and sterigmatocystin production, a G-protein/cAMP/protein kinase A signaling cascade, which regulates both asexual sporulation and sterigmatocystin production, was found in *A. nidulans*.[202] In the cascade, hydrolysis of GTP bound to the FadA protein, the α-subunit of the G-protein, is stimulated by a signal of FlbA, a regulator of G-protein signaling-type protein, leading to an increase in the cAMP levels. The cAMP may act as a second messenger to activate a protein kinase A that may modulate the activity of AflR protein to start sterigmatocystin production. It was shown that a similar cascade present in *A. flavus* and *A. parasiticus* regulates AF production.[203] The *veA* gene encoding a transcriptional regulator controls both sterigmatocystin production and sexual development of *A. nidulans*.[204] In *A. parasiticus*, the *veA* gene is required for both AF production and sclerotia formation.[205] LaeA protein is present in *Aspergillus* species broadly and may regulate production of a variety of secondary metabolites such as sterigmatocystin or lovastatin,[206] but it is not clear if LaeA regulates AF production.

Regulatory mechanism for AF production described above is summarized in **Figure 25**. It is the key point how the expression of AflR protein is regulated. AflR protein expressed induces expression of genes responsible for AF biosynthesis present in the AF gene cluster to start AF production. Some environmental and nutritional factors may affect AflR expression, but the signaling pathway from an outside signal for the gene expression is still a black box. A G-protein cascade and a regulation by VeA protein also affect AflR expression, but they are not specific for AF production and only a small part of the cascade or regulation is known. To clarify the whole regulatory mechanism for AF production, it may be necessary to obtain more information on many molecules that are involved in the regulation by using a new method such as a microarray technology.[207]

4.10.3.3.3 Regulation of aflatoxin production and contamination

Several decontamination and detoxification strategies for AFs have been attempted to resolve the AF contamination problem. Among them, nontoxigenic strains and AF-binding agents are practically used. Nontoxigenic strains that do not produce AFs can decrease AFs in crops by competing with toxic strains.[208] Some binding agents such as calcium montmorillonite clay can decrease the AF uptake by animals involved in the diet.[109] Chemical detoxification methods by ammoniation or ozonization are also known, but they are not useful because they strongly reduce food quality.[109]

Use of antifungal agents may be one of the effective methods to prevent AF contamination. However, strong fungicides are sometimes toxic to mammals, and resistant strains may spread rapidly as known in the case of use of antibiotics. Since AF production is not necessary for its producing fungus, specific inhibitors of AF production with no fungicidal activity may be useful to prevent foods and feeds from AF contamination without incurring rapid spread of resistant strains.

Until now, many substances including pesticides, extracts of plants, and microbial metabolites have been bioassayed to find inhibitory activity on AF production. Organophosphorus insecticides with cholinesterase inhibitory activity, such as dichlorvos (**116** in **Figure 26**), can inhibit AF production by inhibiting the esterase

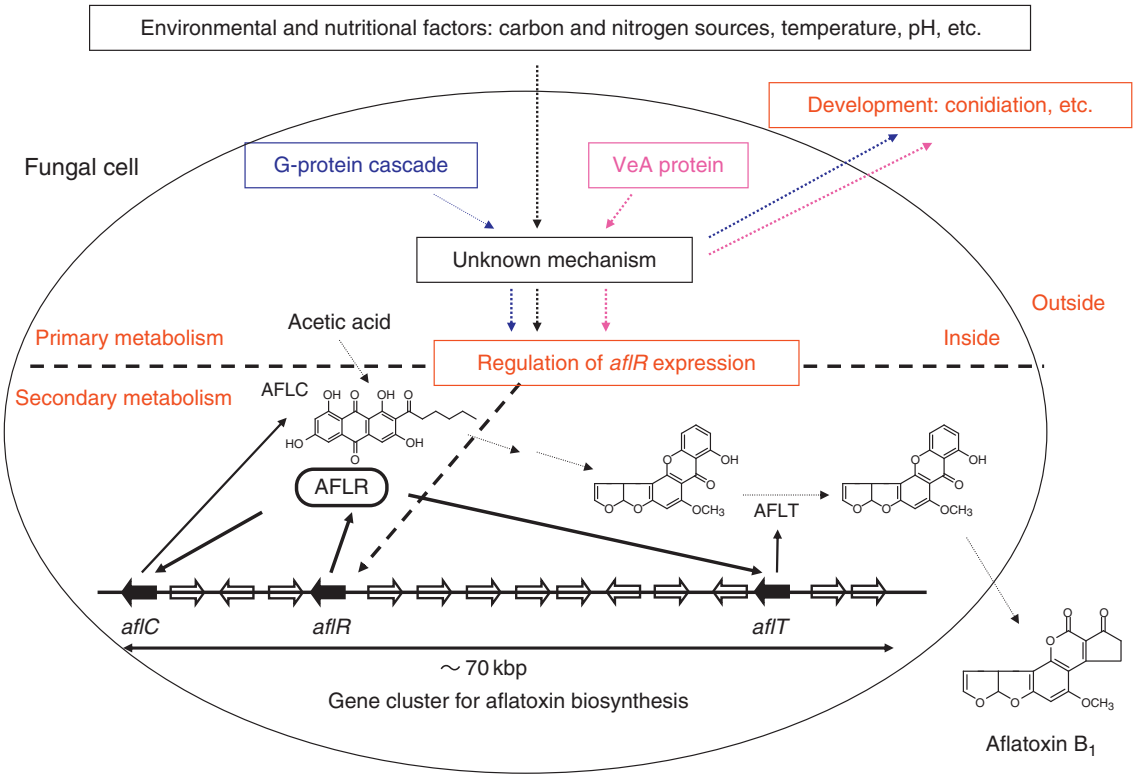
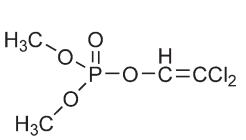
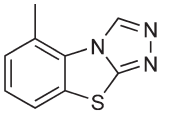


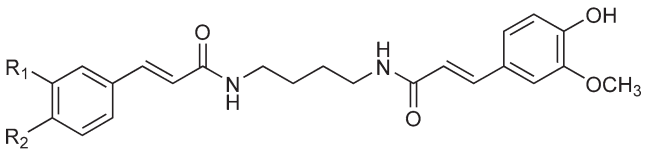
Figure 25 Regulatory systems for aflatoxin production.



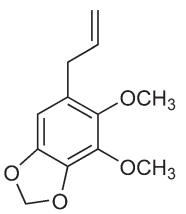
Dichlorvos (**116**)



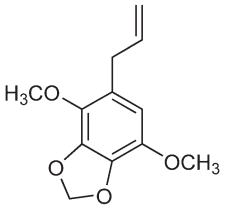
Tricyclazole (**117**)



Diferuloylputrescine (**118**) $R_1 = OCH_3$, $R_2 = OH$
p-Coumaroylferuloylputrescine (**119**) $R_1 = H$, $R_2 = OH$



Dillapiol (**120**)



Apiol (**121**)

Figure 26 Aflatoxin production inhibitors from pesticides and plants.

catalyzing the biosynthetic step of conversion from versiconal hemiacetal acetate (**103**) into versiconal (**104**) (**Figure 21**).[209] Some inhibitors of pentaketide-derived melanin biosynthesis in fungi, such as tricyclazole (**117**), show AF production–inhibitory activity.[210] They can inhibit the reductase encoded by *aflM* involved in the conversion step from versicolorin A (**106**) into demethylsterigmatocystin (**107**) (**Figure 22**). As constituents of plants, anthocyanins,[211] gallic acid,[212] diferuloylputrescine (**118**), and *p*-coumaroylferuloylputrescine (**119**),[213] are known as AF production inhibitors. Dillapiol (**120**) and apiol (**121**) exhibit specific inhibition toward AFG_1 production of *A. parasiticus* without inhibiting AFB_1 production and fungal growth.[214] They may probably inhibit the cytochrome P-450 monooxygenase encoded by *aflU* (**Figure 22**).

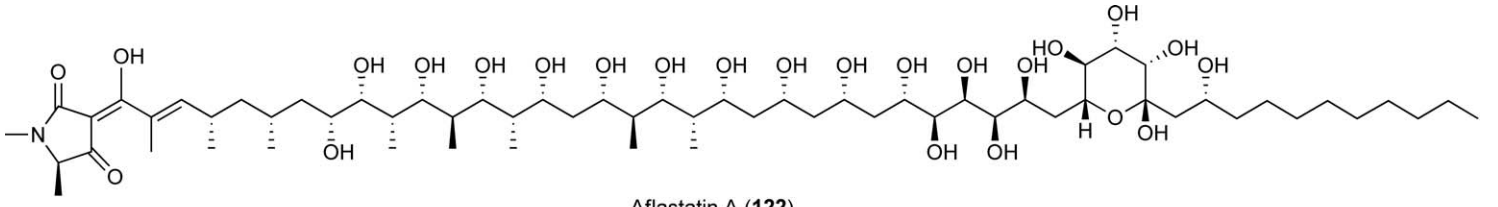
Aflastatins, blasticidin A, and dioctatin A are known as specific AF production inhibitors of microbial origin. They are all *Streptomyces* metabolites. Aflastatin A (**122** in **Figure 27**) is the first compound found by screening search for useful AF production inhibitors among microbial metabolites.[215] It is a unique tetramic acid derivative with a highly oxygenated long alkyl side chain.[216] The absolute stereochemistry of aflastatin A proposed previously[217] was recently revised partly.[218] It strongly inhibits production of not only AF but also other polyketide compounds including patulin or pentaketide-derived melanin.[215,219] Since aflastatin A reduces the mRNA levels of *aflR* and affects carbon metabolism of the fungi, its molecular target may be present in the early regulatory system before AflR expression for AF production.[220,221] Blasticidin A (**123**) was found as an antibiotic[222] and recently rediscovered as an AF production inhibitor.[223] Its structure including the absolute stereochemistry is very similar to that of aflastatin A.[218] Since biological activities of blasticidin A on AF production, *aflR* expression, and carbon metabolism are the same as those of aflastatin A, the target molecule of blasticidin A may be identical with that of aflastatin A.[220] Both aflastatin A and blasticidin A do not affect conidiation of *A. parasiticus*. Dioctatin A (**124**) was isolated as an inhibitor of human dipeptidyl peptidase II and recently shown to have a specific AF production–inhibitory activity.[224] Dioctatin A reduces the *aflR* mRNA level and inhibits not only AF production but also conidiation of *A. parasiticus*. On the other hand, it strongly activates the production of kojic acid by the fungus. Therefore, dioctatin A may have pleiotropic effects on regulatory mechanisms of fungal secondary metabolite production and differentiation. Studies on the mode of actions of specific AF production inhibitors including aflastatin A, blasticidin A, and dioctatin A are very important not only for developing new effective AF inhibitors but also to better characterize regulatory mechanisms of secondary metabolite production in fungi.

4.10.3.4 Trichothecenes

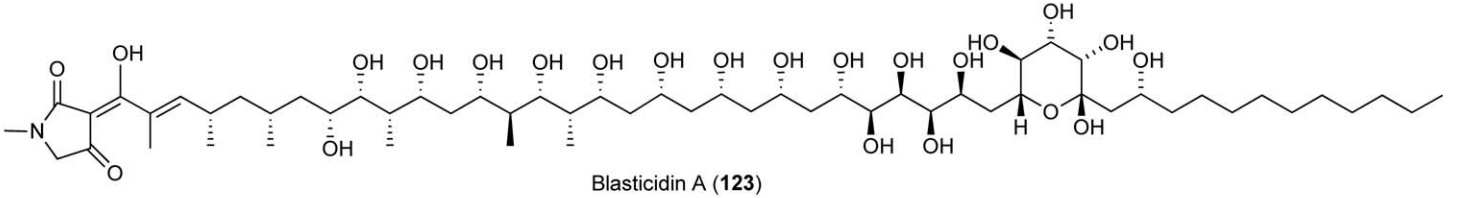
Trichothecenes are toxic secondary metabolites of *Fusarium*, *Trichothecium*, *Myrothecium*, and other fungal genera.[225,226] They have a common trichothecene skeleton, 12,13-epoxytrichothec-9-ene (EPT), comprising of A-ring, B-ring, and C-ring (see **Figure 28**). Their structural diversity arises by combinations of functional groups attached to the skeleton. Typical examples of major known trichothecenes include deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), 4,15-diacetylnivalenol (4,15-diANIV), and T-2 toxin, whose structures are illustrated in **Figure 28**. However, substitutions with arbitrary combinations of functional groups are not allowed in constructing the structures of naturally occurring trichothecenes, and the producing fungal strains show selective structural diversity. For example, there are five positions for the attachment of substituents in *Fusarium* trichothecenes (i.e., $2 \times 3 \times 3 \times 2 \times 4 = 144$ possible combinations; see **Figure 28**), but only less than 10 chemotypes are known as naturally occurring end products of extant *Fusarium* species.

Trichothecenes inhibit protein translation in eukaryotes and pose serious threats to animal health and food safety.[227] Among this group of mycotoxin, DON (also known as vomitoxin) is the most major compound that frequently occurs in cereals. It causes vomiting in exposed animals literally, and also other toxicoses including diarrhea, dermatitis, immunosuppression, and hemorrhagic septicemia, with extremely high dose ultimately resulting in death.[228] For detoxification of DON and other trichothecenes in feeds, Biomin GmbH commercialized a ruminal bacterial strain as feed additives for irreversible de-epoxidation of the toxic 12,13-epoxy ring although the responsible detoxification system remains to be characterized in detail.[229]

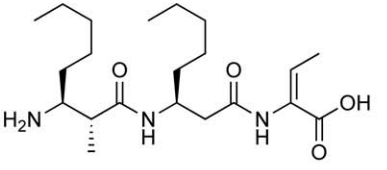
In addition to their impacts as mycotoxins, trichothecenes are also known as phytotoxins for the toxin-producing phytopathogenic fungi.[230] Above all, *Fusarium* species, such as *F. graminearum* and *F. culmorum*, are the problematic fungal species producing trichothecenes because they are the causal pathogens of *Fusarium* head



Aflastatin A (**122**)

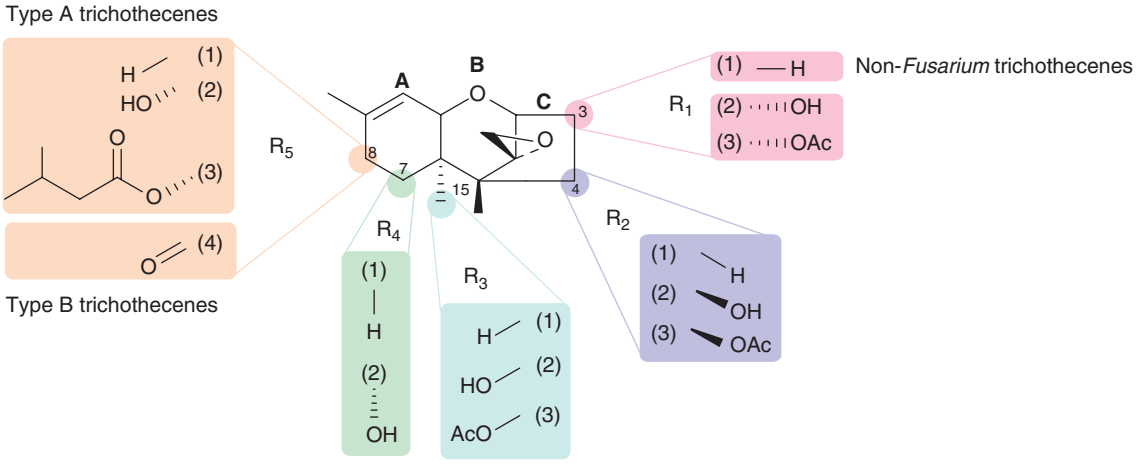


Blasticidin A (**123**)



Dioctatin A (**124**)

Figure 27 Aflatoxin production inhibitors from microorganisms.



	R_1	R_2	R_3	R_4	R_5
DON (deoxynivalenol)	(2)	(1)	(2)	(2)	(4)
3-ADON (3-acetyldeoxynivalenol)	(3)	(1)	(2)	(2)	(4)
15-ADON (15-acetyldeoxynivalenol)	(2)	(1)	(3)	(2)	(4)
NIV (nivalenol)	(2)	(2)	(2)	(2)	(4)
4-ANIV (4-acetylnivalenol)	(2)	(3)	(2)	(2)	(4)
4,15-diANIV (4,15-diacetylnivalenol)	(2)	(3)	(3)	(2)	(4)
T-2 toxin	(2)	(3)	(3)	(1)	(3)
NEO (neosolaniol)	(2)	(3)	(3)	(1)	(2)
4,15-DAS (4,15-diacetoxynivalenol)	(2)	(3)	(3)	(1)	(1)
CAL (calonectrin)	(3)	(1)	(3)	(1)	(1)
ITD (isotrichodermin)	(3)	(1)	(1)	(1)	(1)

Figure 28 Structures of *Fusarium* trichothecenes (including trichothecene intermediates isolated as natural products). The side chains (e.g., hydroxyl, acetyl, keto) are added to the trichothecene skeleton at C-3 (R_1), C-4 (R_2), C-15 (R_3), C-7 (R_4), and C-8 (R_5), which are shaded in colors. While type A trichothecene has a single bond at C-8, type B trichothecene has a keto at C-8. Three rings that constitute the trichothecene skeleton [EPT; $R_1 = (1)$, $R_2 = (1)$, $R_3 = (1)$, $R_4 = (1)$, $R_5 = (1)$] are labeled A, B, and C in bold. It should be noted that EPT is not a *Fusarium* trichothecene.

blight, a devastating disease of wheat, barley, and other important cereal crops.[231] For this reason, *Fusarium* trichothecenes have been used to examine their roles during infection of the toxin-producing fungal pathogen to host plants,[232,233] where they seem to provoke different biochemical reactions depending on their chemotypes.[234]

Evolutionary analyses of the trichothecene biosynthesis genes (*Tri* genes) in the *Tri5* gene cluster suggested that selectively optimal trichothecene chemotype varies by environment or changes overtime due to spatial or temporal heterogeneity in selective pressure.[235] Indeed, analyses of virulence of *F. graminearum* strains demonstrated that the chemotype differences affect host range or fitness to host plants.[236] In view of the ecological significance of the chemotypes, *Fusarium* trichothecenes are an attractive model to study the genetic and biochemical mechanisms that generated the selective structural diversity for a group of secondary metabolites.

Classified into the chemical group sesquiterpenes, trichothecene skeleton is constructed from farnesyl pyrophosphate (FPP) with two rounds of cyclization.[237–239] Isotrichodiol (ITdiol)[240] is a branching-point intermediate of *Fusarium* trichothecenes and non-*Fusarium* trichothecenes that precedes the second cyclization. In this section, we summarize outlines of trichothecene biosynthesis focusing on those of *Fusarium* species.

4.10.3.4.1 A common pathway of trichothecene biosynthesis: From FPP to ITdiol

Trichodiene (TDN) (**125**; **Figure 29**) is the first stable biosynthetic intermediate identified as a natural metabolite of trichothecin-producing fungus, *Trichothecium roseum*.[241–243] Using this fungal species, Cane *et al.*[244,245] elegantly elucidated enzymatic cyclization of all-*trans*-FPP to TDN. The enzyme TDN synthase responsible for this cyclization was purified from *Fusarium sporotrichioides*[246] and the encoding gene *Tri5* (formerly *Tox5*) was subsequently isolated.[247]

In both *Fusarium* and non-*Fusarium* species that produce trichothecenes, the resulting alicyclic hydrocarbon TDN undergoes sequential oxygenations as follows (**Figure 29**): C-2 hydroxylation [2α-hydroxytrichodiene

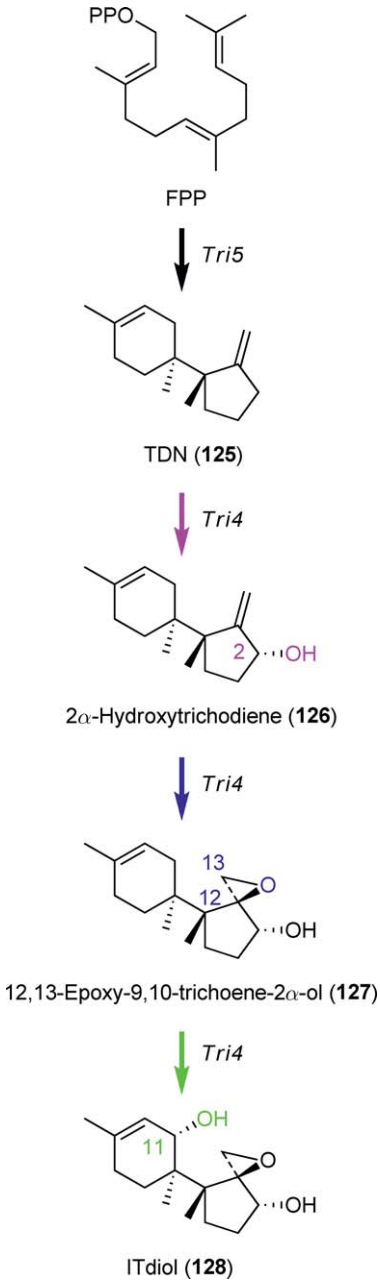


Figure 29 A common pathway of trichothecene biosynthesis. FPP is cyclized to TDN (**125**) by a cyclase encoded by *Tri5*. In the biosynthesis of both *Fusarium* trichothecenes and non-*Fusarium* trichothecenes, three oxygenations catalyzed by a CYP encoded by *Tri4* follow to yield ITdiol (**128**).

(**126**)][248] → C-12,13 epoxidation [12,13-epoxy-9,10-trichoene-2α-ol (**127**)][249] → C-11 hydroxylation [ITdiol (**128**)].[240,250] This common trichothecene pathway is catalyzed by a cytochrome P-450 monooxygenase (CYP) encoded by *Tri4*.[251–254] Trichothecene biosynthesis is known to be inhibited by some CYP inhibitors, such as xanthotoxin and flavones.[255] These plant shikimate aromatics proved to inhibit oxygenation of trichodiene-11-one,[256] a TDN (**125**) analogue, by *Saccharomyces cerevisiae* expressing *FgTri4* (our unpublished results). Since the trichothecene skeleton is not formed without these oxygenation steps, TRI4 could be a good target with which one can develop more specific inhibitors of the toxin biosynthesis.

A multifunctional CYP that participates in secondary metabolism is also known in other fungal species. For example, *Fusarium fujikuroi* possesses *P450-1* that codes an enzyme with broad substrate specificity acting on separate carbons of precursors in gibberellin biosynthesis:[257] *Phoma betae* also appears to use a similar type of a CYP gene in aphidicolin biosynthesis.[258] In the case of *Fusarium Tri4*, the encoded enzyme can catalyze an additional oxygenation at C-3 (see Section 4.10.3.4.2). To date, no known multifunctional CYP acts on greater than three different carbons of precursors other than that encoded by *Fusarium Tri4*.

4.10.3.4.2 Biosynthesis of Fusarium trichothecenes

In the biosynthesis of *Fusarium* trichothecenes, isotrichotriol (**129**)[259] is the final product of oxygenation reactions by TRI4 enzyme (**Figure 30**). This tetraoxygenated biosynthetic intermediate undergoes acid-catalyzed spontaneous cyclization to give isotrichodermol (**130**), the first intermediate with a toxic trichothecene skeleton, by intramolecular attack of the C-2 hydroxyl to C-11.[261] So far, enzymes that catalyze this second cyclization have not been identified. Rather, it seems plausible that a locally high concentration of isotrichotriol (**129**) on the ER membrane significantly accelerates this nonenzymatic cyclization in the biosynthesis of trichothecenes by *Fusarium* species. Taken together, this implies that only two genes, *Tri5* and *Tri4*, are needed to build up the trichothecene skeleton with an additional hydroxyl at C-3.[254]

Trichothecene 3-*O*-acetyltransferase encoded by a noncluster gene, *Tri101*, readily converts isotrichodermol (**130**) into isotrichodermin (ITD, **131**) (**Figure 30**).[262,263] This acetylation step is important for the self-protection of trichothecene-producing *Fusarium* species. The C-3 acetyl is also essential for subsequent biosynthetic steps to proceed. Indeed, the *Tri101*$^-$-targeted gene disruption mutant of *F. sporotrichioides* could not metabolize 3-hydroxytrichothecenes, but efficiently converted exogenously added ITD (**131**) and other C-3 acetylated intermediates into T-2 toxin.[264] After ITD (**131**), the biosynthetic pathways are different between type A and type B trichothecenes.

4.10.3.4.2(i) Type A trichothecenes T-2 toxin is an extensively studied model type A trichothecene with maximum number of biosynthetic steps among this group of trichothecenes. In the biosynthetic pathway to T-2 toxin (see **Figure 31**), C-15 of ITD (**131**) is oxygenated to 15-deacetylcalonectrin (15-deCAL; **132a**) by a CYP encoded by *Tri11*.[265] The *FsTri11*$^-$-targeted gene disruption mutant of *F. sporotrichioides* mainly accumulates ITD (**131**), but also produces small amounts of 8-hydroxyisotrichodermin (8-HIT; **132b**) and 8-hydroxyisotrichodermol.[266] 8-Hydroxyisotrichodermin (**132b**) and 8-hydroxyisotrichodermol were not converted into T-2 toxin when fed to a *FsTri4*$^-$ mutant of *F. sporotrichioides* although the later compound was completely converted into the former (**132b**).[266] This indicates that these 8-hydroxytrichothecenes are shunt metabolites and the C-15 oxygenation step must follow immediately after the formation of ITD (**131**) in the biosynthesis of type A trichothecenes (**Figure 31**).

After formation of 15-deCAL (**132a**), the C-15 hydroxyl is acetylated by an acetyltransferase encoded by *FsTri3*.[267] The resulting intermediate, calonectrin (CAL, **133**), serves as a substrate of a CYP responsible for C-8 hydroxylation (encoded by *FsTri1*)[268] or C-4 hydroxylation (encoded by *FsTri13*)[269] in the biosynthesis of T-2 toxin. Owing to the broad substrate specificities of FsTRI1 and FsTR13 enzymes, several biosynthetic routes operate after CAL (**133**) along the metabolic grids rather than a single pathway (**Figure 31**). On the biosynthetic grids, two transferases encoded by *FsTri7*[270] and *FsTri16*[271] participate in the transfer of C-4 acetyl and C-8 isovaleroxy groups to the T-2 toxin intermediates. Finally, the resulting product of the biosynthetic grids, 3-acetylT-2 toxin, is subject to deacetylation at C-3 by an esterase encoded by *FsTri8*.[272]

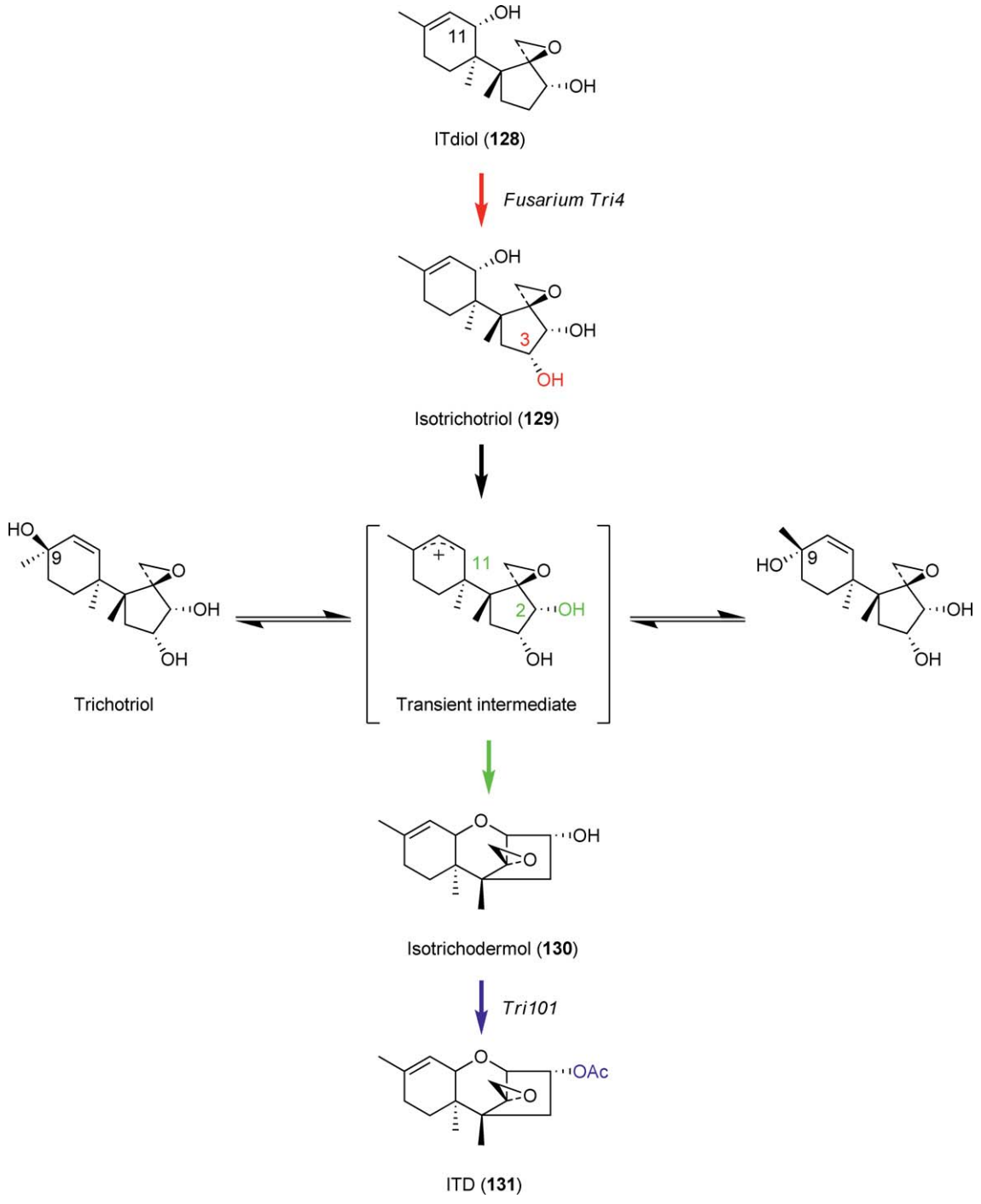


Figure 30 Biosynthesis of *Fusarium* trichothecenes. In trichothecene-producing *Fusarium* species, one additional oxygen is added to ITdiol (**128**) to give isotrichotriol (**129**). Cyclization of isotrichotriol (**129**) to isotrichodermol (**130**) under acidic condition assumes a transient intermediate, which can also undergo isomerization to trichotriol[260] and its 9α-epimer.[261] Acetylation at C-3 of isotrichodermol (**130**) to ITD (**131**) is an essential step for the biosynthesis of *Fusarium* trichothecenes.

4.10.3.4.2(ii) Type B trichothecenes In the biosynthesis of type B trichothecenes, three biosynthetic routes to the next oxygenation step operate after ITD (**131**) (see **Figure 32**). This pathway diversification is attributed to the function of a key hydroxylase encoded by *FgTri1*,[273] which is similar to but distinct from its homologue *FsTri1*. FgTRI1 enzyme is a multifunctional CYP that oxygenates both C-7 and C-8 of ITD (**131**) and CAL

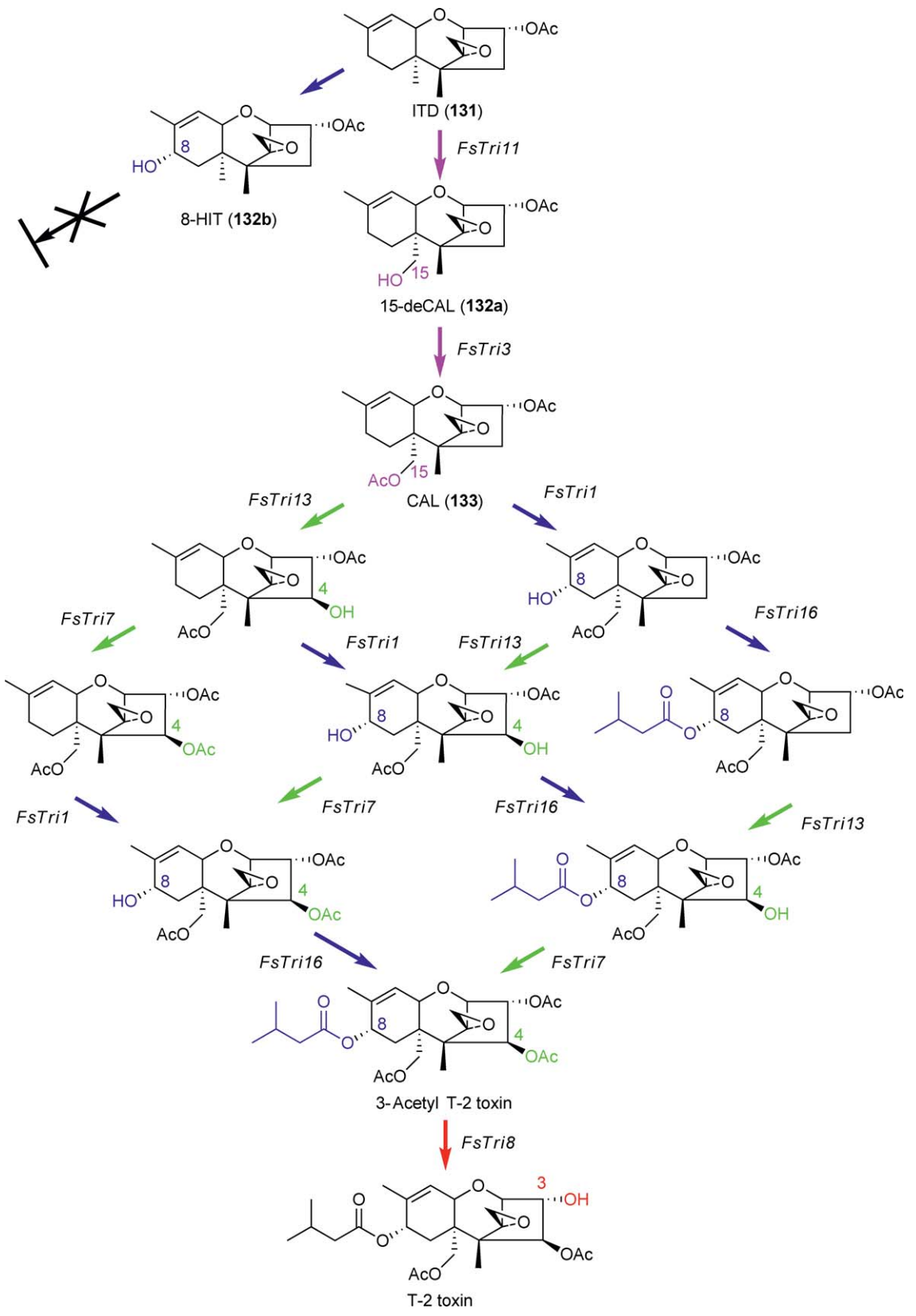


Figure 31 Biosynthesis of type A trichothecenes. ITD (**131**) is metabolized to T-2 toxin along the biosynthetic grids. Either C-8 hydroxylation or C-4 hydroxylation of CAL (**133**) may occur.

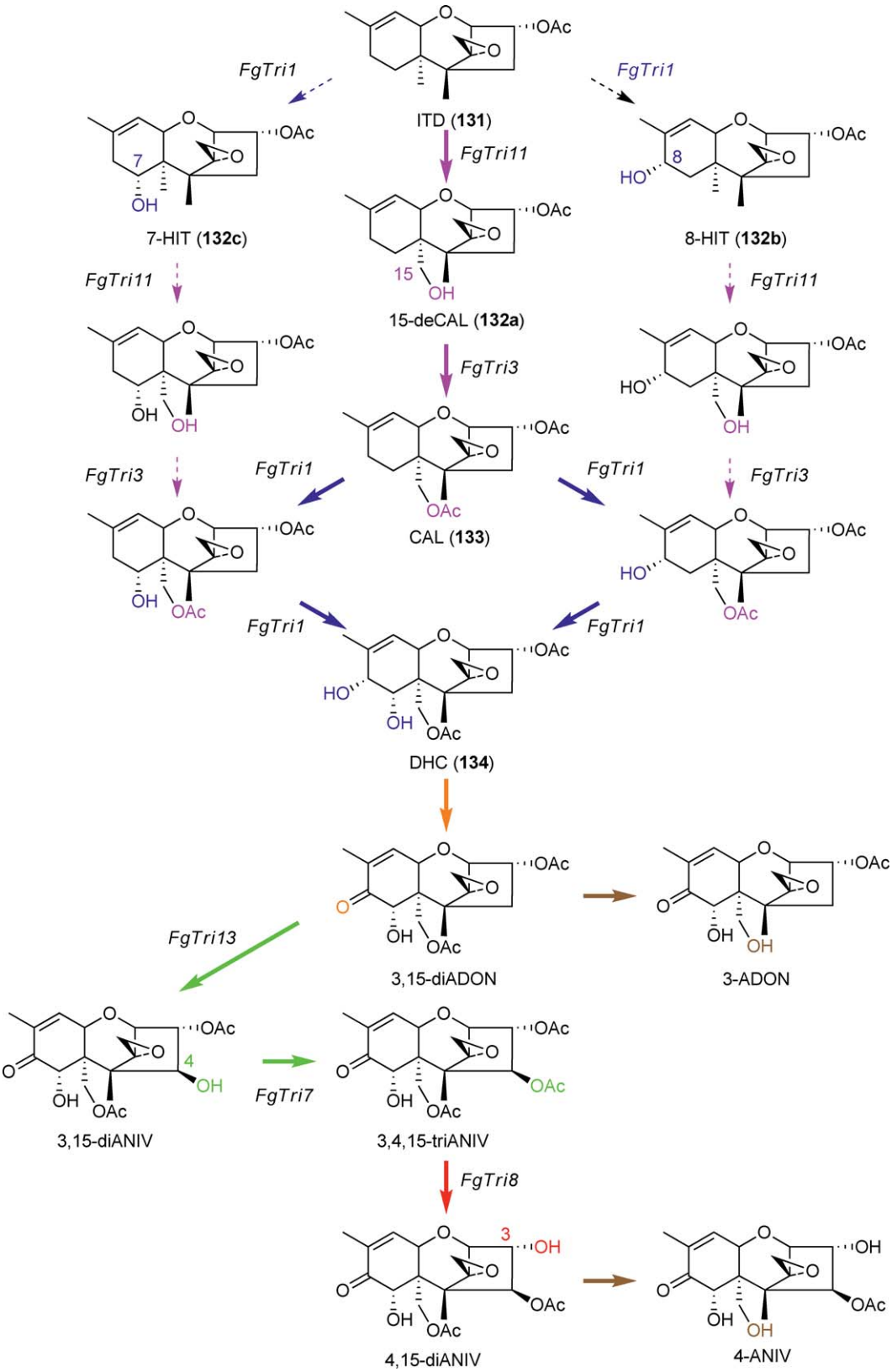


Figure 32 Biosynthesis of type B trichothecenes. ITD (**131**) is metabolized to 3-ADON (3-ADON-producing strain) or 4-ANIV (4-ANIV-producing strain) along the biosynthetic grids. In this tentative model, C-7/C-8 hydroxylation of CAL (**133**) must precede C-4 hydroxylation.

(133).[274] In contrast to T-2 toxin biosynthesis, 8-HIT (132b) is not a shunt metabolite of type B trichothecenes; both 8-HIT (132b) and 7-hydroxyisotrichodermin (7-HIT; 132c) were efficiently converted into 3-acetyldeoxynivalenol (3-ADON) along the metabolic grids in a 3-ADON-producing strain (Figure 32).[275] Nevertheless, a major pathway from ITD (131) to 3-ADON should involve 15-deCAL (132a) and CAL (133), because these intermediates showed significant incorporation characteristics of their [^{14}C] label into 3-ADON than those of 8-HIT (132b) and 7-HIT (132c). On the biosynthetic grids to 7,8-dihydroxycalonectrin (DHC, 134), enzymes encoded by *FgTri11* and *FgTri3* catalyze C-15 oxygenation and C-15 acetylation, respectively. This C-15 acetylation step is essential for the biosynthesis of trichothecenes with a hydroxyl at C-15 (e.g., DON, NIV, 3-ADON), suggesting the occurrence of a later deacetylation step in the biosynthesis.

The biosynthetic pathways after DHC (134) are not currently well understood. For the study of type B trichothecene biosynthesis, 4-acetylnivalenol (4-ANIV)-producing strains are an attractive model because other chemotype strains could have arisen (e.g., DON, 3-ADON, 15-ADON, NIV) via the inactivation of appropriate pathway *Tri* genes in evolution. A series of our preliminary investigations suggested that oxygenations of A-ring by FgTRI1 enzyme must precede oxygenation at C-4 and that C-3 deacetylation must precede C-15 deacetylation in the biosynthesis of 4-ANIV. A tentative biosynthetic pathway of 4-ANIV is proposed as illustrated in Figure 32.

4.10.3.4.3 *Biosynthesis of non-*Fusarium *trichothecenes*

In fungal species such as *Myrothecium roridum* and *T. roseum*, ITdiol (128) is subsequently cyclized to EPT, a minimum trichothecene skeleton also given a specific compound name 'trichothecene' (Figure 33). EPT is a precursor specific to non-*Fusarium* trichothecenes because it is not metabolized to *Fusarium* trichothecenes (that have an acetyl or a hydroxyl at C-3) by trichothecene-producing *Fusarium* species.[276] This implies that the determinant of *Fusarium* trichothecene and non-*Fusarium* trichothecene is the functional difference of *Tri4* that functions between the first and second cyclizations in the biosynthetic pathways.

In contrast to the *Fusarium* species, the biosynthesis mechanisms after the formation of the trichothecene skeleton are poorly understood for the toxin-producing non-*Fusarium* species. So far, only four genes have been isolated and characterized from non-*Fusarium* fungal species that produce trichothecenes, *T. roseum TrTri4*[254] and *M. roridum MrTri4*, *MrTri5*, and *MrTri6*.[252] Among these genes, functions were demonstrated for *TrTri4* and *MrTri4* by heterologous expression of the genes in *S. cerevisiae*[254] and *F. sporotrichioides* (*FsTri4*$^-$ mutant),[252] respectively.

4.10.3.4.4 *Clustering and nonclustering of trichothecene biosynthesis genes in* Fusarium *species*

Similar to the bacterial antibiotic biosynthesis genes, genes that specifically contribute to fungal secondary metabolism are often found in gene clusters. Examples include the gibberellin biosynthesis gene cluster of *F. fujikuroi*,[257] the T-toxin biosynthesis gene cluster of *Cochliobolus heterostrophus*,[277] the HC-toxin biosynthesis gene cluster of *Cochliobolus carbonum*,[278] and the aphidicolin biosynthesis gene cluster of *P. betae*.[258] In the case of trichothecene-producing *Fusarium* species, most but not all of the *Tri* genes are found in the *Tri5* gene cluster.[279] Two additional *Tri* genes are found in the *Tri1–Tri16* two-gene cluster,[268] and *Tri101* is separated alone from all other *Tri* genes.[263] *Tri* genes encoding trichothecene C-15 deacetylase and DHC C-8 oxidoreductase are necessary for the biosynthesis of some type B trichothecenes such as 3-ADON and 4-ANIV, but yet remain to be cloned and characterized. These genes also appear to exist separated from other *Tri* genes in the fungal genome.

4.10.3.4.4(i) *Pathway, transport, and regulatory genes for the biosynthesis of* Fusarium *trichothecenes in the* Tri5 *gene cluster*

In the *Tri5* gene cluster of T-2 toxin- and 4-ANIV-producing strains, there are 12 *Tri* genes, *Tri8*, *Tri7*, *Tri3*, *Tri4*, *Tri6*, *Tri5*, *Tri10*, *Tri9*, *Tri11*, *Tri12*, *Tri13*, and *Tri14*, which are upregulated with the onset of trichothecene biosynthesis (Figure 34). Among these cluster genes, *Tri9* (encoding a peptide of 43 amino acid residues) was identified by comparative analysis of *F. graminearum* and *F. sporotrichioides* cDNAs,[270] but the contribution of *Tri9* to the biosynthesis is not clear. Although designated as a *Tri* gene, this gene may not meet the criteria of functional trichothecene genes. *Tri14* is not needed for the production of trichothecenes on media, but the gene was reportedly needed for high virulence and for the toxin production during pathogenesis in wheat tissues.[280]

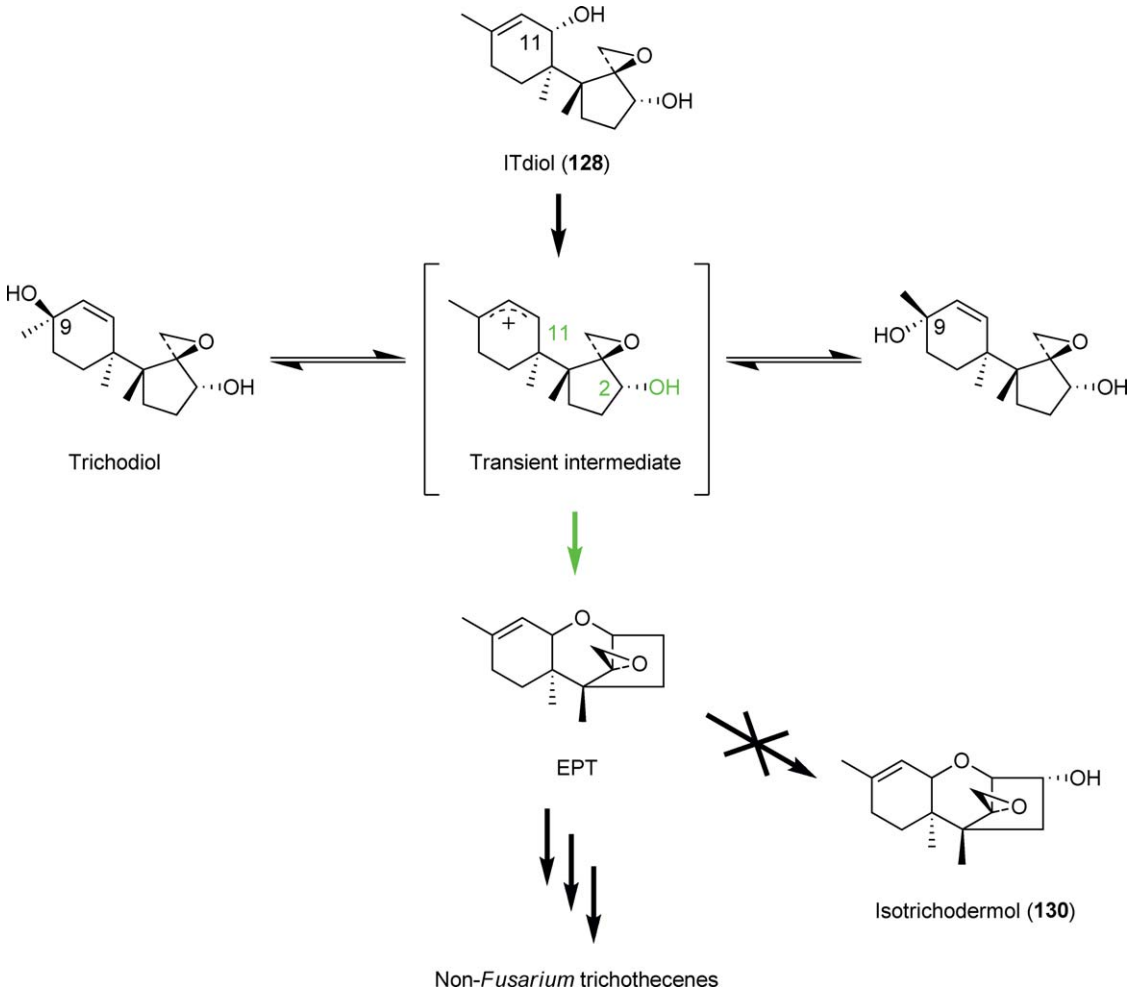


Figure 33 Biosynthesis of non-*Fusarium* trichothecenes. Cyclization of ITdiol (**128**) to EPT under acidic condition assumes a transient intermediate, as is the case with the cyclization of isotrichotriol (**129**).

Among the remaining 10 *Tri* genes, seven genes are pathway genes whose functions were determined by targeted gene disruption experiments. These include *Tri8* (encoding trichothecene C-3 deacetylase),[272] *Tri7* (encoding 3-acetyltrichothecene 4-*O*-acetyltransferase),[270] *Tri3* (encoding 3-acetyltrichothecene 15-*O*-acetyltransferase),[267] *Tri4* (encoding a multifunctional oxygenase responsible for the conversion of TDN into isotrichotriol; CYP),[251,253,254] *Tri5* (encoding TDN synthase),[247] *Tri11* (encoding ITD C-15 hydroxylase; CYP),[265] and *Tri13* (3-acetyltrichothecene C-4 hydroxylase; CYP)[269,281] as mentioned in Sections 4.10.3.4.1 and 4.10.3.4.2. In addition to these pathway *Tri* genes, the *Tri5* gene cluster contains two regulatory genes, *Tri6* and *Tri10*, and a transporter gene *Tri12*.

Tri6 encodes a zinc-finger transcription factor with an acidic activation domain in the N-terminal half of the protein and three regions showing some similarity to known Cys_2His_2 zinc-finger motifs at the C-terminus.[282] This positive regulator of *Tri* gene expression is essential for the production of trichothecenes. TRI6 binds to the consensus sequences of YNAGGCC,[283] which is found in the promoter regions of *Tri* genes, basically except that of *Tri6* and *Tri10*. *Tri10* encodes a novel type of regulatory protein essential for production of trichothecenes.[284] *Tri10* is involved not only in the *Tri6*-mediated activation of *Tri* genes, but also in the activation of genes encoding enzymes for FPP synthesis (in the isoprenoid primary metabolic pathway) and unrelated genes designated *Ibt* (influenced by *Tri10; ten*).[285] *Tri12* encodes a trichothecene efflux pump showing high similarity to the major

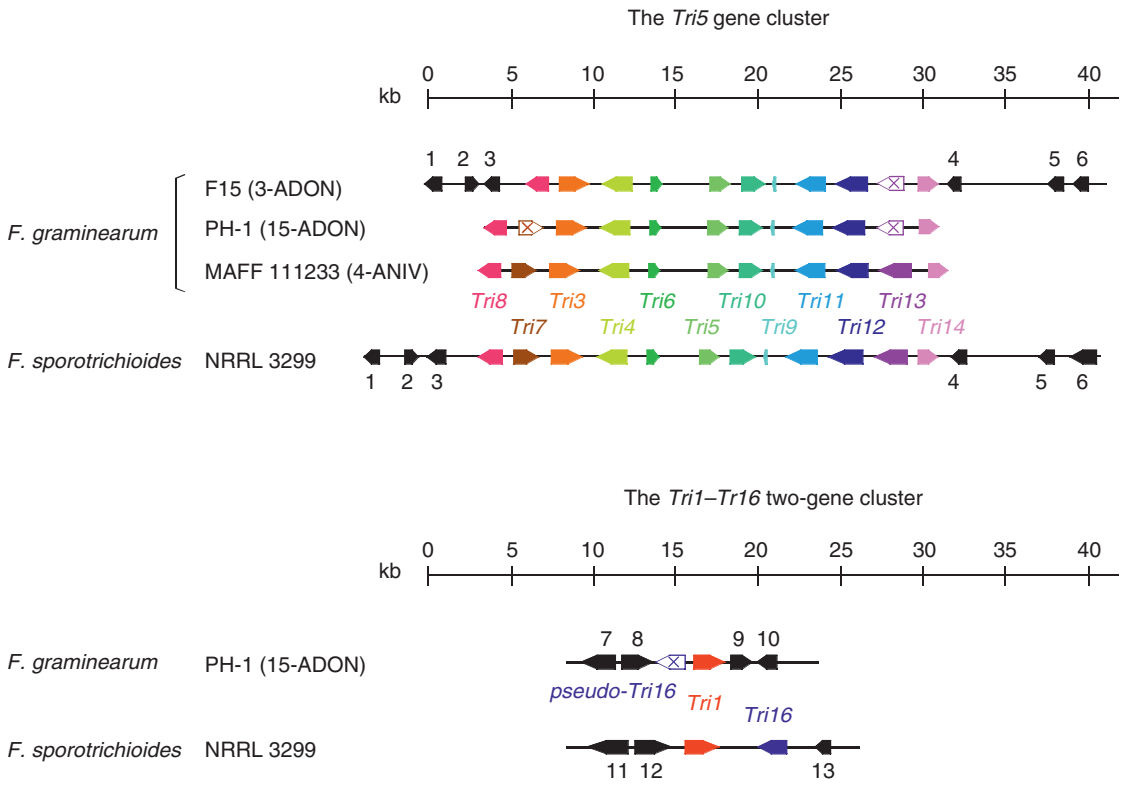


Figure 34 Trichothecene biosynthesis gene clusters of *F. sporotrichioides* and different chemotype strains of *F. graminearum*. *Tri* genes with transcribed directions are illustrated in colored pointed boxes. 'X' in the boxes denotes inactivation of the genes. Gene 1, β-1,3-glucosidase precursor; gene 2, putative esterase; gene 3, putative tyrosinase; gene 4, putative polysaccharide deacetylase; gene 5, 3-hydroxyacyl CoA dehydrogenase; gene 6, NADH-cytochrome b5 reductase; gene 7, orfA (GAL4-like transcription factor); gene 8, orfB (putative sugar transporter); gene 9, orfC (unknown); gene 10, orfD (WW domain-containing oxidoreductase); gene 11, orf2 (putative membrane protein); gene 12, GAL4-like protein; gene 13, orf1 (unknown).

facilitator superfamily transporters.[286] Disruption of *F. sporotrichioides FsTri12* results in significantly reduced production of T-2 toxin and increased sensitivity to trichothecenes added to the medium.

The *Tri5* gene cluster is found in a region of synteny (or colinearity) between *F. sporotrichioides* and *F. graminearum* (**Figure 34**).[279,287] However, when comparison was made between trichothecene-producing and nonproducing *Gibberella* species, nonbiosynthesis genes (e.g., house-keeping genes) surrounding the *Tri5* gene cluster are not syntenic to each other.

4.10.3.4.4(ii) Pathway genes for the biosynthesis of* Fusarium *trichothecenes in the* Tri1–Tri16 *two-gene cluster *Tri1*[268,273,288] and *Tri16*[271] are located in the *Tri1–Tri16* two-gene cluster (**Figure 34**) although the latter gene is inactivated in the genome of the type B trichothecene producers. As mentioned in Section 4.10.3.4.2, the function and substrate specificity of a CYP encoded by *Tri1* differ significantly between *F. sporotrichioides* and *F. graminearum*; while *FsTri1* encodes 3-acetyltrichothecene C-8 hydroxylase, *FgTri1* codes for CAL C-7/C-8 hydroxylase. FgTRI1 appears to be unable to act on trichothecenes with a hydroxyl or an acetyl at C-4. *FsTri16* encodes an acyltransferase that catalyzes addition of isovaleroxyl at C-8 of 8-hydroxytrichothecenes.[271]

In contrast to the *Tri5* gene cluster, the regions surrounding the *Tri1–Tri16* two-gene cluster are not syntenic between *F. sporotrichioides* and *F. graminearum*.[273,288] Indeed, *Tri1* is much more divergent between these *Fusarium* species (e.g., 59% identity between FsTRI1 and FgTRI1) compared to the diversity between the same *Tri* genes in the *Tri5* gene cluster (e.g., 90% total protein sequence identity between FsTRI11 and FgTRI11). This suggests that *Tri1* and *Tri16* have a longer evolutionary history than the *Tri* genes in the *Tri5* gene cluster

or experienced more robust evolutionary events. During the evolutionary process, these two-gene cluster genes appear to have evolved in different ways to type A and type B trichothecene producers: perhaps under unknown selective pressures, *FgTri1* evolved to code for a CYP with limited substrate specificity and additional function, which in turn resulted in altered (i.e., limited) substrate specificity of FgTRI13 in order not to compete with FgTRI1 for CAL (**133**) in the biosynthetic pathway. Alternatively, limited substrate specificity of FgTRI13 may have altered enzymatic properties of FgTRI1 (i.e., evolved new function), but this possibility seems less likely than the former.

4.10.3.4.4(iii) Pathway and self-protection gene* Tri101 *that resides outside of the* Tri *gene clusters As mentioned in Section 4.10.3.4.2, all the clustered *Tri* genes (in the *Tri5* gene cluster and the *Tri1–Tri16* two-gene cluster) are not sufficient for the biosynthesis of trichothecenes other than isotrichodermol (**130**). A key pathway gene missing from the gene clusters, *Tri101* encoding trichothecene 3-*O*-acetyltransferase,[262] is essential for pathway *Tri* genes (except *Tri4* and *Tri5*) to participate in trichothecene biosynthesis.[264] This gene, also known as a gene for self-protection, is located between the phosphate permease (*pho5*) and UTP-ammonia ligase (*ura7*) genes.[263] Despite the isolated occurrence of *Tri101* in the genome, a TRI6-binding sequence is found in the promoter region of *Tri101* in both *F. graminearum* and *F. sporotrichioides*. Consistent with this structural feature, the expression of *Tri101* is under the regulatory control of *Tri10* and *Tri6*.[284]

Interestingly, trichothecene-non-producing *Gibberella* species carry a dysfunctional copy of *Tri101* between *pho5* and *ura7*,[289] and a functional homologue of *Tri101*, designated *Tri201* (70% nucleotide sequence identical with *Tri101* of trichothecene-producing fusaria), between a putative β-galactosidase gene and a homologue of a *Drosophila* hypothetical protein gene. These genes are directly flanking each other in the genome of *F. graminearum*,[290] suggesting that *Tri201* is a paralogue of *Tri101* generated by gene duplication during evolutionary process. Further studies resulted in the functional identification of trichothecene 3-*O*-acetyltransferase genes in *Fusarium decemcellulare* (teleomorph genus; *Albonectria*), *Fusarium solani* (teleomorph genus; *Neocosmospora*), *Magnaporthe grisea*, and *Saccharomyces cerevisiae*, designated *TAT* (*Fusarium* and *Magnaporthe*)[290] or *AYT1* (*S. cerevisiae*).[291] The phylogeny of the 3-*O*-acetyltransferase (TRI101, TRI201, TAT) was mostly concordant with the rDNA phylogeny of these ascomycetous fungi.[290] This indicates a different evolutionary origin of *Tri101* from other *Tri* genes in the *Tri5* gene cluster, which were grouped into phylogenetically distinct lineages according to the chemotypes.[235]

4.10.3.4.5 Summary and perspectives

Outlines of trichothecene biosynthetic pathways were determined with *Fusarium* trichothecenes and most of the *Tri* genes needed for the biosynthesis were isolated. Briefly, first cyclization, four consecutive oxygenations, and second cyclization build up the skeleton of *Fusarium* trichothecenes and subsequent systematic acetylations, oxygenations, esterification, and/or deacetylations determine their chemotypes. Regardless of the chemotypes at C-3 and C-15, these two positions must once be acetylated along the biosynthetic pathways to the final product of trichothecenes.

For the biosynthesis of a model type A trichothecene T-2 toxin, all the necessary *Tri* genes were already identified.[226] However, there still remain two *Tri* genes as-yet-unidentified for the biosynthesis of 4-ANIV, a model type B trichothecene. These include genes encoding DHC C-8 oxidoreductase, whose activity was also detected from *F. verticillioides* (teleomorph genus; *Gibberella*), which does not produce trichothecenes,[274] and trichothecene C-15 deacetylase,[292] whose distribution and substrate specificity are not well examined.

The relative toxicity of *Fusarium* trichothecenes is determined by the pattern of oxygenation, acetylation, and/or esterification, which also affects host range or fitness of the producing strains to host plants. Hence, it is important to understand the genetic and biochemical mechanisms that generate the different substitution patterns of trichothecene side chains at the late stages of the biosynthesis. Detailed study of the late steps differentiating the type B trichothecene chemotypes is further needed to understand the mechanisms generating the selective structural diversity for this group of important secondary metabolites.

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Biographical Sketches

Shohei Sakuda was born at Kochi city, Japan, in 1958. He graduated from the Department of Agricultural Chemistry, University of Tokyo, in 1982. He finished the master's course from the Graduate School of Agriculture, University of Tokyo in 1984 and thereafter continued his doctoral studies. He obtained a position as an assistant professor in the Department of Fermentation Technology, Osaka University, in 1986, and his Ph.D. in agriculture from the University of Tokyo in 1988. He moved to the Department of Applied Biological Chemistry, University of Tokyo, in 1994 as an associate professor. He studied insecticidal metabolites of *Streptomyces* as the theme of his bachelor's thesis and is continuing to study bioactive secondary metabolites of microbes and plants in the research fields of bioorganic chemistry and natural products chemistry. Chitinase inhibitors, allosamidins, and aflatoxin production inhibitors, aflastatins, and blasticidin A are main topics in his recent research.

Makoto Kimura was born in Sakai, Osaka, Japan, in 1965. He graduated from the Department of Agricultural Chemistry, The University of Tokyo, in 1989, and finished the master's course from the Graduate School of Agriculture, The University of Tokyo, in 1991. In the same year, he obtained a position as a research associate in RIKEN and studied the function,

evolution, and application of fungal antibiotic resistance genes. In 1995, he obtained his Ph.D. in agriculture from The University of Tokyo. His thesis entitled 'Studies on Blasticidin S Deaminase (BSD) from *Aspergillus terreus*' was under the supervision of Professor Haruo Seto. From September 1998 to March 2000, he visited UC Berkeley as a research fellow and studied the rice blast fungus (rice pathogen) *Magnaporthe*. In April 2000, he joined the Laboratory for Remediation Research, Environmental Plant Research Group, Plant Science Center (PSC1), RIKEN Yokohama Institute, as a research scientist, and in 2003, he was appointed as a deputy team leader. In 2005, he was promoted to research unit leader of Plant & Microbial Metabolic Engineering Research Unit, DRI, RIKEN. He has been studying the interaction between wheat and fungal pathogen *Fusarium graminearum*, and recently, his major research interests are on metabolic engineering of plants and microorganisms.

4.11 Terrestrial Natural Products as Antifeedants

Falko P. Drijfhout and E. David Morgan, Keele University, Staffordshire, UK

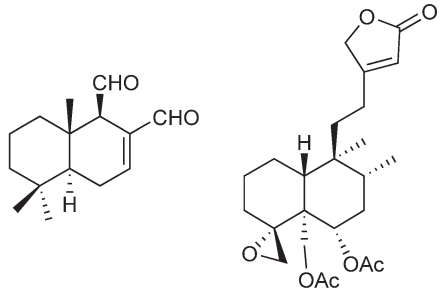
4.11.1 Introduction

The use of plant extracts to treat sickness or control pests dates back at least two millennia in the records of ancient China, Egypt, and Greece.[1] These were crude extracts of unknown activity. In the nineteenth and the early twentieth centuries, through advances in chemistry, these became better defined plant extracts such as derris, pyrethrum or

nicotine.[2] In the 1940s there was an abrupt change of direction with the introduction of new synthetic pesticides. Interest in natural products temporarily ceased, until overuse of synthetics led to unforeseen problems. In order to reduce these problems, government agencies set rules for which compounds could be licensed for use and the concentrations and conditions in which pesticides could be used (see Chapter 2.02). These, often strict, regulations drove chemists back to natural materials to seek again for more acceptable and less persistent substances to control agricultural and other pests.

Plants have, in the two-and-a-half million years they have coexisted with arthropods, evolved their own defenses against pests that attack them. It is only logical that we should look to them for compounds and structures that will deter pests. The search for feeding deterrents, commonly called antifeedants, is currently a rich source of new compounds potentially useful against pests of agriculture, forestry, and horticulture.

One of the lessons learned by mistakes of the synthetic pesticides period is that we must not destroy useful insects along with the pests. A major advantage of some of these natural products is that they may be group- or species-specific. On the one hand, the drimane sesquiterpenoid (–)-polygodial (**1**) is a very effective antifeedant for aphids,[3] while many coleopterans are not affected by this compound. On the other, the ajugarins (e.g., ajugarin I, **2**) are very effective against coleopteran pests in northern Europe,[3] but are ineffective against the diamondback moth *Plutella xylostella* and have virtually no effect on aphids. The high selectivity of some natural products makes them ideal for use in modern pest management.



Polygodial (**1**) Ajugarin I (**2**)

In spite of the many studies on isolation, activity, and synthesis of natural antifeedants, the number of compounds commercially available remains low, often due to their cost of isolation, availability of the plant source, or low persistence in field conditions. To overcome these drawbacks, much research is conducted on structure–activity relationships (SAR). The rationale behind these studies is to discover the correlation between biological activity and chemical structure and to draw from that optimum structures having both the activity, stability, and selectivity for maximum feeding deterrence.[4,5] SAR are much used in drug design[6] and have been applied to insecticides.[7]

While the greatest emphasis is on insects and other arthropods and mollusks because of the enormous commercial damage they do, and the difficulties in controlling them, studies of antifeedants is not confined to them only, and a considerable body of effort has been expended in seeking ways to control the feeding of higher animals and birds.

4.11.2 Bioassays

In all studies related to the isolation and identification of biologically active compounds, suitable bioassays are essential. Without a proper bioassay, no meaningful conclusions are possible from any chemical isolation study. In the isolation of active compounds bio-guided fractionation is often used, which means that each fraction is tested for its biological activity. If the activity is lost within a fraction that fraction can be discarded, or if all activity is lost, synergism is more likely. Therefore, it is essential to design the bioassay with care, before starting the study. Several bioassays are used in the investigation of extracts for antifeedants.[8] The observed feeding deterrence can be measured in different ways. In one method, the percentage feeding deterrence is calculated as $(1-T/C) \times 100\%$ where T and C are the amounts consumed on the treated and control leaves.[9] Other studies use the term antifeeding inhibition (% AFI) or feeding inhibition (% FI), which is calculated as $[(C-T)/(C+T)] \times 100\%$, where C and T are again the amounts of control and treated leaves consumed, and has an advantage that it does not assume a function of deterrency or feeding stimulation.[10] A positive value indicates an antifeedant (with an AFI = 100%

equals maximum protection), while a negative value is the result of a phagostimulant.[10] Some researchers prefer to give an index value, in which case the final value is not multiplied by 100% and values vary between −1 and 1, with 1 corresponding to maximum antifeeding and 0 with no effect. In order to determine the concentration where 50% FI is reached, the AI_{50} (Antifeedant Index) or FI_{50} (Feeding Index) is calculated. This involves a dose–response experiment, where a series of different concentrations are used and the concentration that will result in 50% FI is calculated. The lower is the concentration, the more active is the antifeedant. Instead of using AI_{50}/FI_{50}, the term ED_{50}[11] or EC_{50} [12] (effective dose or effective concentration for 50% inhibition) is often used.

Important aspects bioassays as well as their types are discussed below. One of the main drawbacks with the bioassays is that some of them have long durations, which can delay the results. Hence, researches on new and faster bioassays are carried out. An interesting new possible bioassay involves the use of olfactory β-waves; that is, bursts of *c.*20 Hz fast waves that are elicited in the olfactory bulb and pyriform cortex in rats are observed.[13] These fast waves are also observed in voles.[14] These studies indicate that these β-waves may provide an easy means of identifying new antifeedants in small herbivores.

4.11.2.1 Choice or No-Choice Bioassays

These are two different bioassays, each revealing different information. In a choice experiment, the insects are given the option to choose between two different treatments, either the control or the extract (or pure compound), or between two different compounds. Information obtained via this type of bioassay shows that one of the treatments is preferred to the other. Or in case of the difference between control and compound, that if the control is preferred, the compound could be an antifeedant.

In a no-choice bioassay, the insect cannot choose from different treatments. Only one treatment is given. A strong antifeedant will result in no consumption of the treatment, whereas a weak antifeedant will lead to some consumption of the treatment, as the insect needs to gain nutrition.

Both these methods have their advantages and disadvantages. To compensate for this difference in choice and no-choice bioassays, the activity of compounds are expressed by three coefficients, namely, the absolute coefficient of deterrence (A) determined by the no-choice test, the relative coefficient of deterrence (R) determined via the choice test, and the total coefficient of deterrence (T), which is calculated by combining A and R.[15,16] Values of A and R are from 0 to 100. The maximum total coefficient is therefore 200. Strong antifeedants had values of 151–200.

4.11.2.2 Leaf Disk Bioassay

The leaf disk method is a conventional type of bioassay in which disks of constant area are cut from leaves of a certain plant and are coated with the extract or pure compound (in solution). These are then presented to insects in a petri dish. After a certain amount of time, the percentage of the leaf that has been consumed is estimated, visually[17] or photographically with or without software.[18] A leaf that is coated with the solvent only is used as the control. This bioassay can be used with small variations, for example, instead of leaves, an artificial diet containing sucrose, flour, or even calcium alginate[19] can be mixed with the compound.

4.11.2.3 Microassay

Small quantities of compounds in natural extracts are often a problem when these need to be evaluated in bioassays. Sometimes there is just not enough of the compound isolated to carry out the usual bioassay.[20] Microassays have been developed[10,21] to overcome this problem. Typically, a microassay is carried out on a thin-layer chromatography plate with a cellulose layer. A small droplet (1.5 µl) of the tested compound in a solvent (1–102 nmol cm^{-2}) is then added on the plate. After the solvent has evaporated a small amount (5 µl) of sucrose solution 1 mol l^{-1} is added to the place where the compound was added. In the control the same procedure was followed on a different plate, but with the solvent alone, with no compound added. These two plates are then placed in a petri dish with the test insect species. In the past, when paper chromatography was widely used, a crude plant extract was placed on the origin of the paper and then eluted into bands. The paper was freed of solvent, sprayed with sugar solution, and used directly in a bioassay to see which parts of the paper were not eaten, and therefore of interest for further examination.

4.11.2.4 Twig Bioassays

This bioassay is a variation of the leaf disk method, as not all insects will eat leaves. Insects such as the pine weevil *Hylobius abietis* feed on barks.[22] Sticks from a specific tree are wrapped in aluminum foil to prevent them from drying out. In each test twig two metal rings (5 mm diameter) are punched through the foil and into the bark. After the foil inside the ring is removed a certain amount (e.g., 100 µl) of the extract or compound to be tested is applied in one of the rings. The control (solvent) is applied to the second ring. The metal rings are removed as soon as all the solvent has evaporated.[5,22] The amount consumed by the insect can then be calculated, either by the amount of consumed bark[5] or by counting the number of feeding scars.[22]

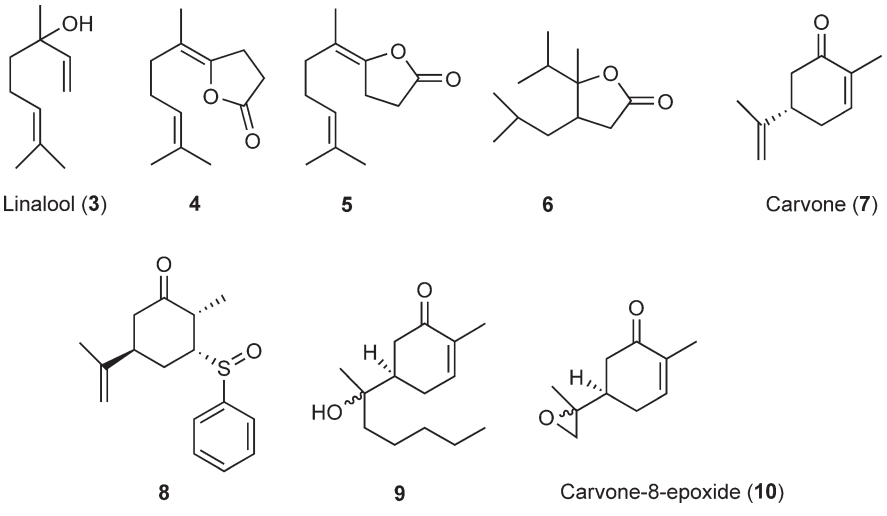
4.11.3 Plant Terpenes and Derivatives

4.11.3.1 Monoterpenes

Monoterpenes are widely distributed in plants, and certainly act to deter predators. The vapor of several monoterpenes are effective in deterring biting flies, but generally C-10 compounds are too volatile for practical use. Part of the research therefore is to find compounds that are suitably modified to make them more persistent and show strong feeding deterrence. Earlier work on monoterpenes has been reviewed.[2]

The abundant natural compound linalool (**3**) is an attractant for the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*. Two isomeric derivatives of linalool, (*Z*)-5-(1.5-dimethylhex-4-enyldiene)-dihydrofuran-2-one (**4**) and (*E*)-5-(1.5-dimethylhex-4-enyldiene)-dihydrofuran-2-one (**5**), were synthesized and were found to be moderate antifeedants against the CPB. A lactone derived from linalool strongly affected the properties of the original compound linalool, which is an attractant to the CPB. A similar effect is seen with the activity of either linalool (**3**) or 4-isobutyl-5-isopropyl-5-methyl-dihydrofuran-2-one (**6**) to aphids. Compared to linalool, the lactone reduced the total time the aphid spent on the leaf.[16]

Carvone (**7**), abundantly present in many essential oils[23] and present in trace amounts in conifer plants (Pinaceae), has antifeedant properties against the pales weevil, *Hylobius pales*,[24] and the large pine weevil, *H. abietis*,[25] as well as lepidopteran species.[26] Although various studies have confirmed the antifeedant or repellent properties of carvone, long-term protection of seedlings in the field has not been successful, probably due to the high volatility of carvone. In order to address this problem, less volatile analogues of the compound were synthesized. Twelve carvone analogues were tested, both in microassays and in twig bioassays. Although several were very active in the microassay, the results did not always correlate with the twig bioassay.[21] Of the compounds with very low volatility, the sulfoxide **8** and 9-butyl-8-hydroxy-*p*-menth-6-en-2-one (**9**) had AFI values comparable to carvone (1.00 and 0.82, respectively) and ED_{50} values of 0.15 and 0.06%, respectively, less than that of carvone ($ED_{50} = 2.6\%$).[21] The more volatile carvone-8-epoxide (**10**) induced 100% antifeeding activity at high doses (100 nmol cm^{-2}), but had an ED_{50} value of 0.52%.

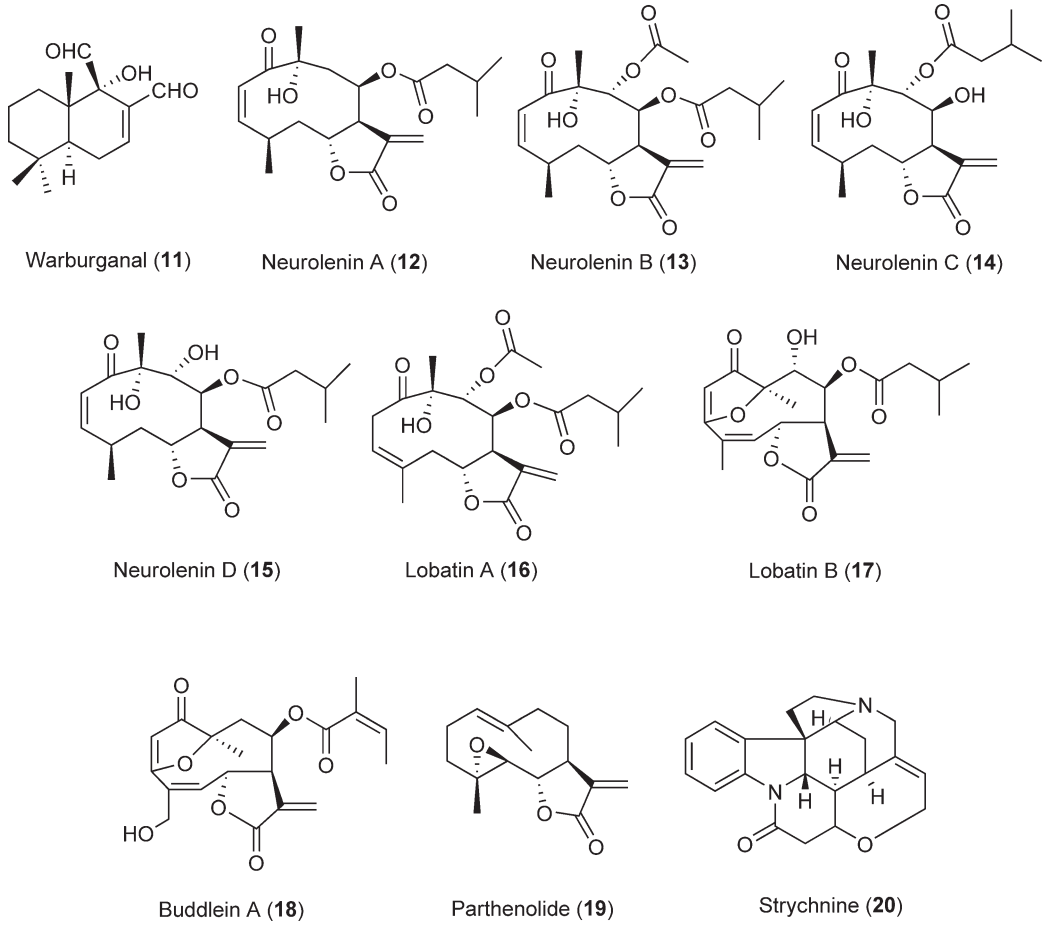


Linalool (**3**) **4** **5** **6** Carvone (**7**)

8 **9** Carvone-8-epoxide (**10**)

4.11.3.2 Sesquiterpenes

Their lower volatility makes sesquiterpenes more possible as antifeedants. A number of known sesquiterpenes, such as warburganal (**11**) and polygodial (**1**), have notable antifeedant effects on insects.[2] The sesquiterpene germacranolides, neurolenin A (**12**), neurolenin B (**13**), neurolenin C (**14**), neurolenin D (**15**), and lobatin A (**16**), and the furanoheliangolide lobatin B (**17**)[27] were isolated from the aerial parts of *Neurolaena lobata* (Asteraceae), a plant of the sunflower family from Guatemala and have been tested as antifeedants against *Spodoptera litura*. All had moderate antifeedant activity, with AFI values ranging from 41 to 52%. In the same test, buddlein A (**18**), a furanoheliangolide isolated from the herbaceous Mediterranean plant *Viguera buddleiformis* (Cannaceae),[27] and parthenolide (**19**), an epoxy-containing germacranolide from *Tanacetum parthenium* (feverfew, Asteraceae) were tested. Overall, the latter exhibited the highest antifeedant activity (77%), followed by neurolenin B (**13**) and lobatin A (**16**); both had a feeding deterrence of 52%. Strychnine (**20**), a known γ-aminobutyric acid (GABA) antagonist, had the same antifeedant activity as the most potent antifeedants, indicating that neurolenin B (**13**) and lobatin A (**16**) both act on GABA receptors, as suggested by Mullin *et al.*[28]

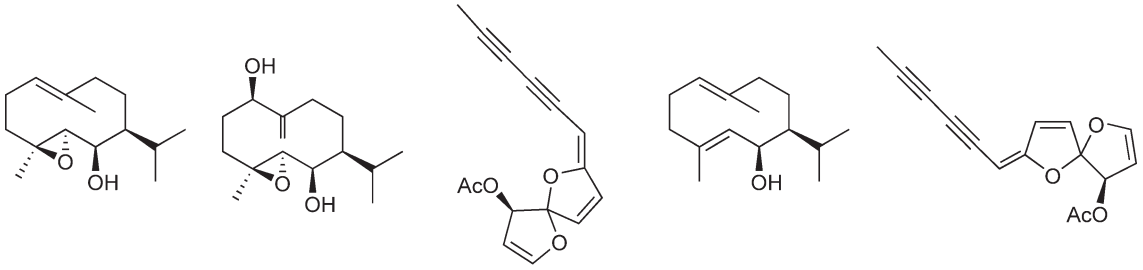


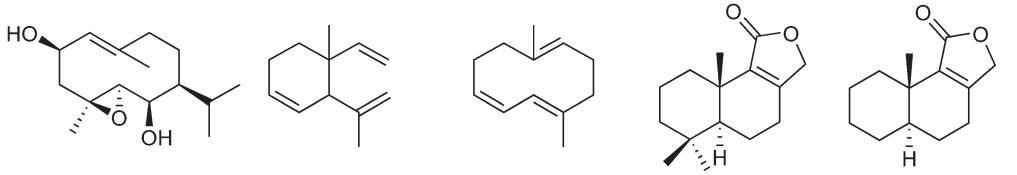
Investigation of the hexane extract of aerial parts of *Santolina rosmarinifolia* (Asteraceae) growing in southern Spain and used in folk medicine afforded several new antifeedants. The germacrenes shiromool (**21**) and 4β,5α-epoxy-7αH-germacr-10(14)-ene-1β,6β-diol (**22**) and the polyacetylene (*Z*)-4-acetoxy-7-(2,4-hexadiynylidiene)-1,6-dioxaspiro[4,4]nona-2,8-diene (**23**) all exhibited antifeedant indices of 45–46% when tested against the final stadium larvae of *Spodoptera littoralis*. Comparing the structures isolated but not found active showed that the presence of the epoxy group in **21** increased the activity, as **24**, which lacks the epoxy group, was not active. In addition, it was concluded that geometric isomerism also influenced the feeding deterrence, for **23** was active while the *E*-isomer (**25**) was not active.[29] Although compound (*E*)-4β,5α-epoxy-7αH-germacr-1(10)-ene-2β,6β-diol (**26**) was tested, no antifeedants index value was given.

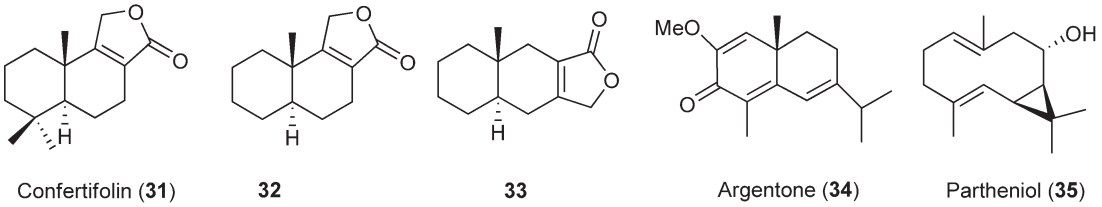
Sesquiterpenes from essential oils were suggested to have antifeedant activity as well. Geijerene (**27**) and pregeijerene (**28**), newly isolated from the essential oil of the leaves of the Ceylon satinwood tree, *Chloroxylon swietenia* (Rutaceae), exhibited a feeding deterrence effect when tested against the tobacco cutworm, *S. litura*.[30] There seem to be no synergistic effect between these compounds as the pure compounds were as active as the total oil itself. Although these compounds are able to deter feeding to 100% at concentrations of 200 $\mu g\,cm^{-2}$, it is still four times less effective than azadirachtin (**213**).

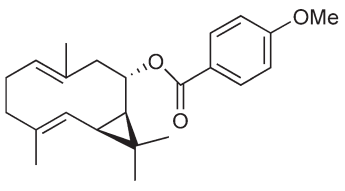
In recent years, research on antifeedants has focused more on natural antifeedants as starting points to synthesize new (non)-natural, but more effective, antifeedants. Messchendorp *et al.*[31] studied the effect of 11 synthetic drimanes on feeding inhibition. In a no-choice bioassay, only the drimanes with a lactone group **29–33** inhibited feeding by the larvae of the large white butterfly, *Pieris brassicae*. The two well-known sesquiterpene antifeedants, warburganal (**11**) and polygodial (**1**), did not have any antifeedant effect on *P. brassicae*.

Compounds from guayule resin, *Parthenium argentatum*, were found to be more effective as antifeedants than azadirachtin. Among the sesquiterpenes isolated, argentone (**34**) exhibited the strongest activity in choice tests, followed by partheniol (**35**), which had an activity similar to azadirachtin. Although the sesquiterpene ester, guayulin B (**36**), did inhibit feeding, it was only a weak antifeedant.[32]



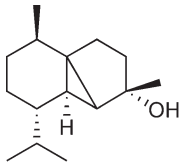




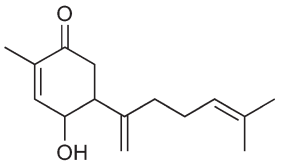


Investigation on antifeedants against a snail species that is a worldwide pest of many vegetables and several other crops, *Acusta despesta*, focused on the crude methanol extract from the Japanese cedar or Sugi, *Cryptomeria japonica* (Taxodiaceae). Two active compounds were isolated from the hexane extract (fractionated from the crude

methanol extract) and they were identified as (–)-cubebol (**37**) and (+)-2,7(14),10-bisabolatrien-1-ol-4-one (**38**). Both exhibited high antifeeding activities against *A. despesta* at 120 and 80 μg cm^{-2}, respectively.[33] The sesquiterpenol **38** was later[33] further identified as (1*S*,6*R*)-2,7(14),10-bisabolatrien-1-ol-4-one (**39**) and isolated together with **40** from *C. japonica*. However, only in combination did they elicit antifeeding activity against *Locusta migratoria*.[34] None of these were active when tested alone, indicating a synergistic effect when tested against *L. migratoria*, although this is not the case if it was tested against a snail as **38** alone was found to be active against *A. despesta*.[33]



(–)-Cubebol (**37**)



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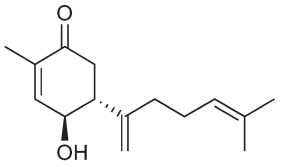

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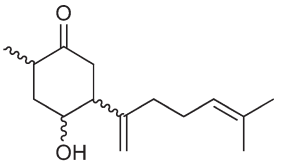
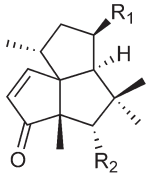
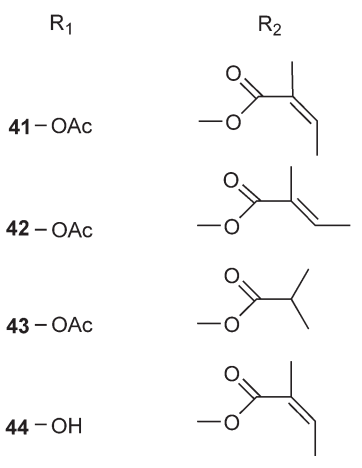
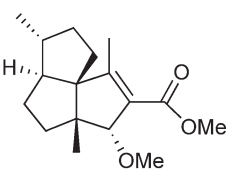


40

Silphinene sesquiterpenes, isolated from *Senecio palmensis* (Asteraceae), exhibited antifeedant activity to a range of insects, such as aphids,[35] CPB (*L. decemlineata*),[36] and *S. littoralis*.[35] The silphene 11β-acetoxy-5α-angeloyloxysilphinen-3-one (**41**), isolated from *S. palmensis*, as well as two synthetic silphinenes, 11β-hydroxy-5α-angeloyloxysilphinen-3-one (**44**) and 11β,5α-dihydroxysilphinen-3-one (**45**), both generated via chemical hydrolysis of the natural 11β-acetoxy-5α-angeloyloxysilphinen-3-one (**41**), were active as antifeedants against CPB36, yet in choice bioassays they were less effective than the natural compound. In no-choice bioassays, a similar effect was observed against *L. decemlineata*. The fact that the natural compound was more active suggests that the acetoxy substituent on C-11 is an important feature to increase feeding inhibition. In a follow-up study, six natural silphinene sesquiterpenes **41–44**, **49**, and **50** and four semisynthetic ones **45–48** (generated from the natural silphinene 11β-acetoxy-5α-angeloyloxysilphinen-3-one, **41**) were tested for their feeding inhibition against aphids and two lepidopteran species.[35] The CPB was sensitive to all the natural silphinenes and semisynthetic silphinene analogues, as well as to two GABA antagonists, thymol (**51**) and picrotoxinin (**52**), which further supports the theory that there is a shared molecular antifeedant taste chemoreception in divergent species.[37] The aphids that were tested reacted variably, with all aphids sensitive to thymol (**51**) (a GABA antagonist) and farnesol, but only the silphene 1β-acetoxy-5α-isobutyryloxysilphinen-3-one (**43**) was active against all aphid species. The aphid *Diuraphis noxia*, with the most restricted host range (wheat and barley), was the most sensitive aphid to the silphinene derivatives 1β-acetoxy-5α-angeloyloxysilphinen-3-one (**41**), 11β-acetoxy-5α-isobutyryloxysilphinen-3-one (**43**), and 11β-hydroxy-5α-angeloyloxysilphinen-3-one (**44**).[35]







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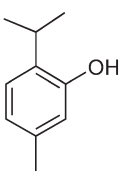
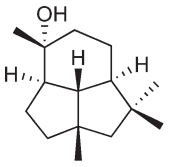
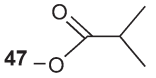


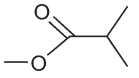


49

45 – OH —OH

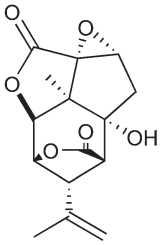
46 – OAc —OAc





48 =O =O

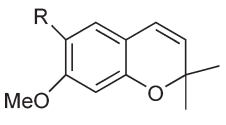
Thymol (**51**)



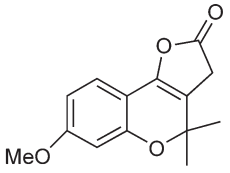
Picrotoxinin (**52**)

Aphids are also sensitive to precocenes I (**53**) and II (**54**) isolated from *Ageratinae* species.[15] Precocenes also exhibited antifeeding activity when given to neonate larvae of the earworm, *Heliothis zea*,[38] and *Rhodnius prolixus*.[39] Several derivatives of precocenes I and II were synthesized and tested against a wide variety of insects.[40] From this study, it became clear that precocenes exhibited a strong antifeedant effect against all storage pests and aphids with precocene II (**54**) (with two methoxy groups) being the more active compound. Although all derivatives exhibited antifeedant activity, the natural precocenes were more active than the derivatives. The introduction of a lactone moiety in the derivatives, as in **55**, reduced the antifeedant effect. The only derivatives exhibiting a strong antifeedant effect were the iodolactones **56** and **57**, which were more active than **53** and **54** when tested against adults of the CPB. However, when tested against the larvae the effect was similar.[40]

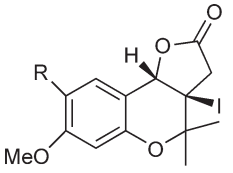
Nootkatone (**58**), a sesquiterpene ketone found in the oil of Alaskan yellow cedar, *Chamaecyparis nootkatensis*,[41] also was isolated from vetiver oil extracted from vetiver grass, *Vetiveria* (*Chrysopogon*) *zizanioides* (Poaceae), [42–44] and proved to be a very effective antifeedant at 100 $\mu g\, g^{-1}$ of sand against the Formosan subterranean termite, *Coptotermes formosanus*. It caused a high reduction in tunnelling activity, wood consumption, and survival.[43,44]



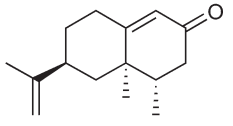
Precocene I (**53**) R = H
Precocene II (**54**) R = OMe



55



56 R = H
57 R = OMe



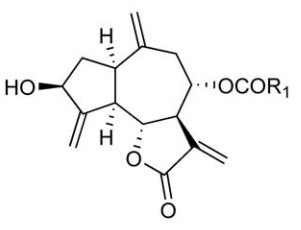
Nootkatone (**58**)

4.11.3.2.1 Sesquiterpene lactones

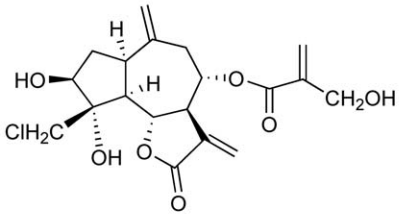
Many sesquiterpene lactones exhibited high antifeedant activity against storage pests.[45–47] *Rhaponticum pulchrum* (Asteraceae) grows in the Caucasus region, 900–1200 m above sea level. Eight different sesquiterpene lactones (guaianolides) and syringin (**61**) were isolated from it and identified.[48] Of these nine, three were found to inhibit feeding in three different coleopteran pests, *Sitophilus granarius*, *Trogoderma granarium*, and *Tribolium confusum*. These were aguerin B (**59**), chlorojanerin (**60**), and syringin (**61**). Two additional sesquiterpenes, janerin **62** and cynaropicrin (**63**), were active antifeedants specifically against *T. confusum*. However, the extract of the plant abundant in sesquiterpene lactones still inhibits feeding more than any of the pure compounds does,[48] indicating that either compounds are acting synergistically or other compounds not yet identified are stronger antifeedants than those isolated and identified. Guaianolides were also isolated from the acetone extract of the aerial parts of *Centaurea babylonica*[49] and *C. hololeuca*[50] (Asteraceae), The

antifeedant activities of these natural compounds and of the four chloro derivatives, synthesized from repin (**64**) and janerin (**62**), were tested against the larvae of *S. littoralis*.[50] The natural product cebellin J **65** and the chloro derivative chlorojanerin (**60**) exhibited significant antifeedant activity at 100 ppm, whereas at this concentration cebellin G (**66**) and 15-dechloro-15-hydroxychlorojanerin (**67**) stimulated feeding. Cebellin G (**66**) stimulated the larvae of *S. littoralis* to feed at low concentration, but deterred feeding at high concentrations. The addition of chlorine to repin (**64**) resulted in an increase in antifeedant activity.[50]

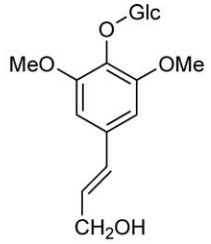
The crude extract of the achenes, leaves, and fruits of *Coriaria sinica* (Coriaceae) displayed antifeedant effects on the forest pests *Stilpnotia candida* and *Arge captive*.[51] Further investigation on the contents of the achenes of this plant led to the isolation of the sesquiterpene lactone tutin (**68**), which had low antifeedant activity, 28% feeding inhibition at 2 mg ml^{-1}.[52] However, one of the derivatives of tutin, 2-(3-methyl-2-butenoyl) tutin (**69**), was highly active when tested against the fourth instar larvae of *Mythimna separata*, giving 74.9–84.8% feeding inhibition at the same concentration. Other derivatives also exhibited higher antifeedant activity than the parent compound, but lower compared to that of **69**.



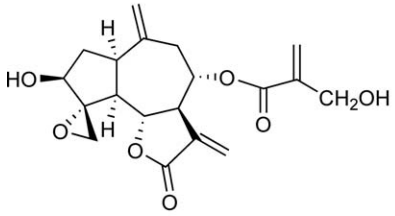
59 $R_1 = C(=CH_2)Me$
63 $R_1 = C(=CH_2)CH_2OH$



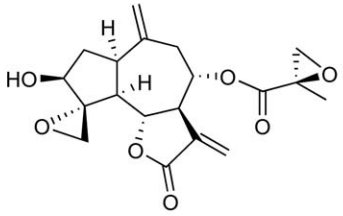
60



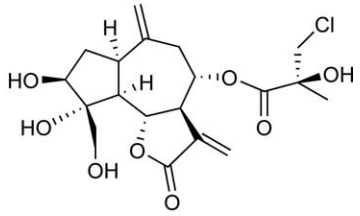
61



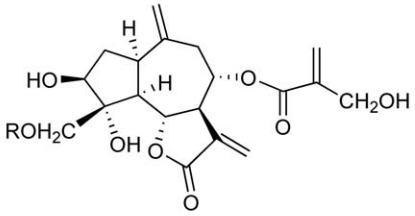
62



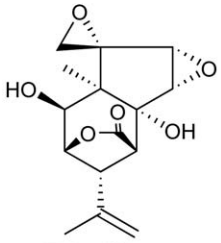
Repin (**64**)



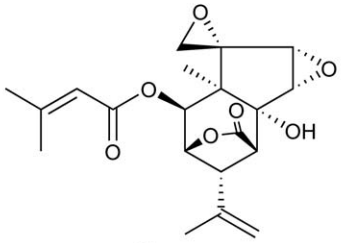
Cebellin J (**65**)



66 R = Ac
67 R = H



Tutin (**68**)



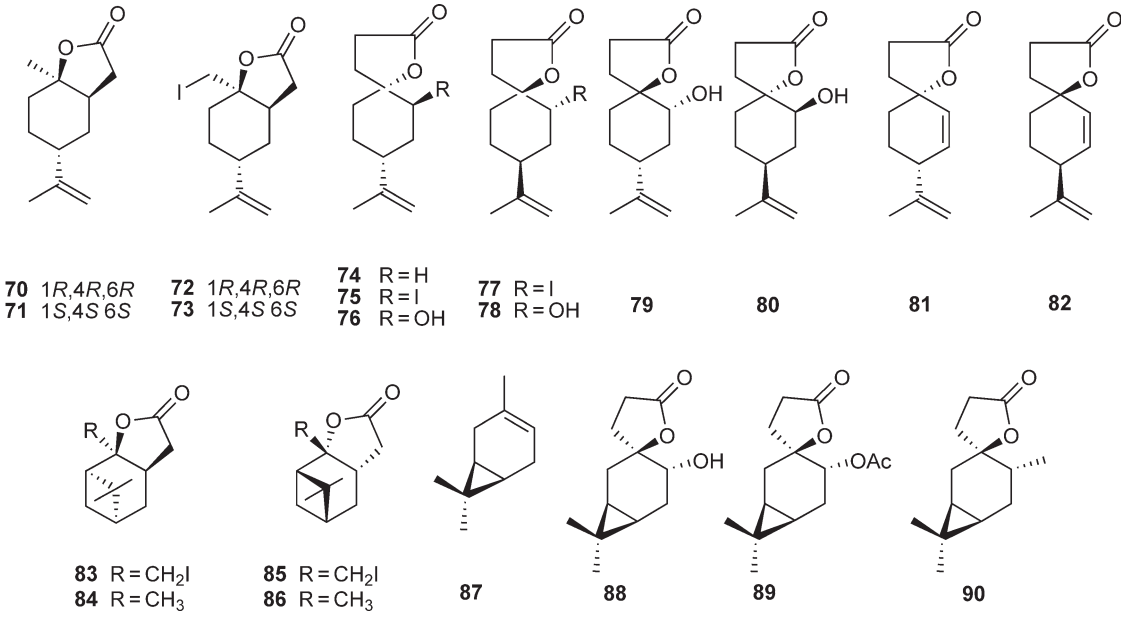
69

Owing to the large numbers of sesquiterpene lactones with antifeedant activity, Paruch *et al.*[53,54] synthesized more than 50 mono-, bi-, and tricyclic terpenoid lactones. The lactones obtained were all tested for antifeeding activity toward grain storage pests: the granary weevil beetle (*S. granarius*), the khapra beetle (*T. granarium*), and the confused flour beetle (*T. confusum*). Starting from (+)- and (−)-perillyl alcohols, via Claisen rearrangement and iodolactonization, four enantiomeric pairs of γ-lactones were obtained. Two of these semisynthetic

compounds, (1*R*,4*R*,6*R*)-(−)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one **70** and its enantiomer **71**, are very active antifeedants against all of the above tested species.[53] The lactone **71** is also active against the peach potato aphid (*Myzus persicae*).[53] The fragrance of **70** was reported to be a moderately intense, agreeable, herbaceous odor with lupine flower and parsley root notes, whereas that of the isomer **71** is faint, mushroomy, and moldy with a floral note. Two iodolactones **72** and **73** were active against adults of *S. granarius* but not against any other storage pests. Iodolactone **72** had a total coefficient of deterrence of 161.0, which is quite close to that of azadirachtin (174.3).[53]

In a second attempt, a whole range of bicyclic γ-spirolactones (synthesized from (+)- and (−)-limonene) as well as tricyclic γ-lactones with a pinane system (from (R)-(−)-myrtenol and (−)-α-pinene) were synthesized. In total, 13 semisynthetic compounds **74–86** were tested for their antifeedant activity against the three storage pests. Antifeedant activities of the semisynthetic compounds were generally not very high when tested against *S. granarius*, except for **84** and **86**, and *T. granarium*, but several were strongly antifeedant against the larvae of *T. confusum*. The iodolactones were found to be weaker antifeedants, unlike the iodolactones of precocenes, which were the only semisynthetic compounds having a strong antifeedant effect. An excellent antifeedant against *T. confusum* is compound **81**, which had an activity that is comparable to that of azadirachtin (**213**). Its enantiomer **82** was only slightly less active.[54]

A further set of terpenoid lactones of the carane type were synthesized starting from (+)-3-carene (**87**). Three of the semisynthetic lactones **88–90** were active as antifeedants, although full data are not yet published.[55]

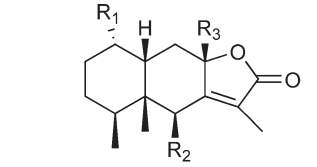


4.11.3.2.2 Eremophilanolides

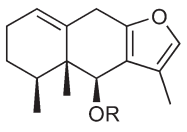
Eremophilanolides are commonly found in *Senecio* species.[56] Two new eremophilanolides were isolated from the methanolic extract of the aerial parts of *Senecio miser* (Asteraceae). The more nonpolar fraction of this methanolic extract (Fr-1 and Fr-2) contained two sesquiterpene lactones of the eremophilanolide type and was identified as 1α-acetoxy-8β-methoxy-10βH-eremophil-7(11)-en-8α,12-olide (**91**) and 1α-angeloyloxy-6β-hydroxy-8β-methoxy-10βH-eremophil-7(11)-en-8α,12-olide (**92**).[57] Eremophilanolide (**91**) proved to be a strong aphid repellent, while **92** was the most active deterrent to the CPB. None of these sesquiterpene lactones was active in no-choice tests, while fractions Fr-1 and Fr-2 were, suggesting a possible synergistic effect of these compounds, either with themselves or with 1α-angeloyloxy-8βH,10βH-eremophil-7(11)-en-8α,12-olide (**93**), which is not active alone.[57]

Joseph-Nathan and co-workers isolated several eremophilanolides from *Senecio toluccanus*, as well as several cacalolides (strictly speaking no sesquiterpenes) from *S. madagascariensis* and *Senecio barba-johannis*.[58–62] Several derivatives were also synthesized. The naturally occurring eremophilanolides from *S. toluccanus*,

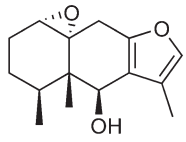
6-hydroxyeuryopsin (**94**) and 1(10)-epoxy-6-hydroxyeuryopsin (**95**) were strong antifeedants against the CPB, *L. decemlineata*. Feeding was reduced by 85.5% and 71.6%, respectively, at 50 μg cm^{-2}. The best antifeedant however was one of the derivatives, 6-acetyloxyeuryopsin (**96**), with 93.3% feeding inhibition, followed by toluccanolide A acetate (**97**), which inhibited feeding by 83.9%. Of the naturally occurring cacalolides, only 14-isovaleryloxy-1,2-dehydrocacalalol methyl ether (**98**) was an effective antifeedant. Of the derivatives, cacalol acetate (**99**) had a strong inhibition effect (73.4% feeding inhibition at 50 μg cm^{-2}). Though most of the tested compounds had an antifeedant effect on the larvae of *S. littoralis*, the effect was moderate.[56]



91 R_1 = OAc R_2 = H R_3 = OMe
92 R_1 = OAng R_2 = OH R_3 = OMe
93 R_1 = OAng R_2 = R_3 = H



94 R = H
96 R = Ac



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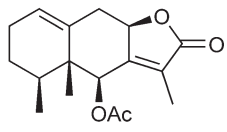
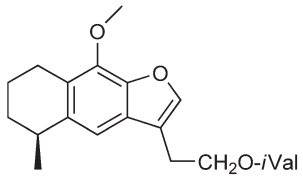
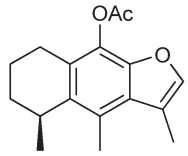


97



98



99

4.11.3.3 Diterpenes

Diterpenes are more restricted in their distribution in plants, but they contain some well-known examples of antifeedants, and deserve close examination for further examples.

4.11.3.3.1 Clerodanes

The clerodanes are a notable example of feeding deterrents[2] and further examples have been discovered recently. The natural insect antifeedant clerodin (**100**) forms the basis of the name of this type of diterpenes called the *neo*-clerodane diterpenes. *Neo*-clerodanes are compounds with a clerodane skeleton and exactly the same absolute configuration as clerodin. Those that have the opposite absolute configuration are referred to as *ent-neo*-clerodanes.

Clerodane-type secondary metabolites have been found in several hundreds of plant species from various families and in organisms from other taxonomic groups, such as fungi, bacteria, and marine sponges.[8] Especially, various genera from the plant families Labiatae and Verbenaceae have been identified as rich sources of clerodanes with antifeedant activity.[63] Species of the genus *Scutellaria* (Labiatae) produces some of the most potent clerodane antifeedants known so far.

The antifeedant activities of all clerodanes (of natural and semisynthetic origin) have been reported in an extensive review[8] covering all literature until December 2001. This review covers a total of 382 clerodanes, all tested on a variety of insect species, yet most tests are with *Spodoptera* species, of which *S. littoralis* is most often used. Other species frequently used are the CPB (*L. decemlineata*), *Helicoverpa armigera*, *P. brassicae*, and *Ostrinia furnacalis*.

Scutecyprol A (**101**) has been isolated from the aerial parts of *Scutellaria sieberti*[64] as well as from *Scutellaria rubicunda* subsp. *ribucunda* (skullcaps, placed in the Labiatae or Lamiaceae) but had no antifeeding activity against a range of insects.[65] However, in a later study,[64] 15-oxo-derivative **102** as well as several halohydrins of **101** and **102**, **103–108,** were found to be all effective antifeedants with 100% feeding inhibition at 100 ppm for *S. littoralis*. Yet all the halogenated derivatives (opening up the C-4—C-18 epoxy ring) had lower FI_{50} values

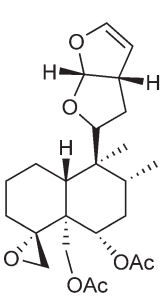
(between 33 and 48 ppm instead of 21.5 ppm for **101** and 22.5 ppm for **102**), indicating that opening of the epoxy ring decreases the activity of **100**, although they still remained potent antifeedants.

Scutecyprol B (**109**) was isolated from the aerial parts of *S. rubicunda* and found to be a strong antifeedant against several insect species,[65,66] that is, *S. littoralis*, *Spodoptera frugiperda*, *Mamestra brassicae*, and *P. brassicae*, inhibiting feeding from 75% (in *P. brassicae*) to 100% in *S. littoralis* at 100 ppm. It inhibited feeding up to 65% in *H. armigera* also at 100 ppm. Scutalbin A (**110**), from the same plant was a moderate antifeedant against these insects.[65]

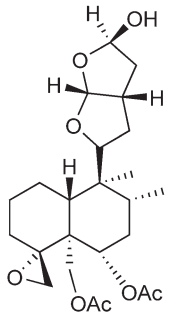
Ajuga reptans, or bugle (Lamiaceae), a common herb found throughout Europe, contains several *neo*-clerodanes, yet for those isolated earlier no feeding inhibition was observed.[67] Further examination of the ethanol extracts led to the isolation of three new *neo*-clerodanes, of which 14,15-dehydroajugareptansin (**111**) had significant antifeeding activity against *S. littoralis* at 100 ppm when 92% feeding inhibition was observed.[68] It was more effective than clerodin (74% FI), but not as effective as jodrellin B (**112**), which had 100% feeding inhibition. Three new clerodanes, named hativenes A, B, and C (**113–115**), were isolated via bio-guided fractionation from the acetone extract of *Ajuga pseudoiva* leaves.[69] All three were active antifeedants against *S. littoralis* at 10 $\mu g\,ml^{-1}$, causing 100% feeding inhibition. Even at 1 $\mu g\,ml^{-1}$, about 65% feeding inhibition was observed. Lupulin A (**116**), present in *A. pseudoiva* leaves as well as in *Ajuga lupulina*, was also an effective antifeedant with 75% feeding inhibition at 1 $\mu g\,ml^{-1}$.[69]

Two epimers, ivain IV (**117**) and 14,15-dihydroajugapitin (**118**), were isolated together for the first time from *Ajuga iva*, a traditional medicinal plant from Algeria locally known as 'chendgoura'. Both the diterpenoids were effective antifeedants against two *Spodoptera* species, *S. littoralis*[67,70] and *S. frugiperda*,[70] at 100 ppm with 14,15-dihydroajugapitin (**118**) slightly more active against *S. frugiperda*. 14,15-Dihydroajugapitin (**118**) was isolated also from *A. pseudoiva* leaves.[69]

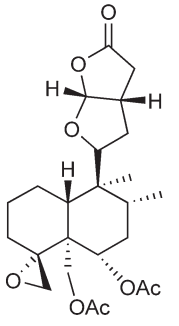
An extract of the aerial parts of *Ajuga nipponensis* was examined by using high pressure liquid chromatography (HPLC), resulting in the isolation of several known and two new *neo*-clerodanes, called ajuganipponin A (**119**) and ajuganipponin B (**120**). Although these and the other isolated *neo*-clerodanes did exhibit antifeedant activity against *S. littoralis*,[71] the activity was only moderate to low.



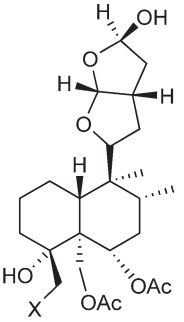
Clerodin (**100**)



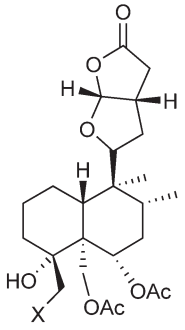
Scutecyprol A (**101**)



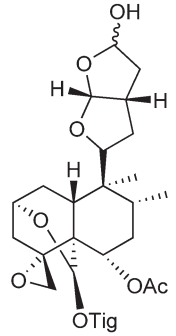
15-Oxoscutecyprol A (**102**)



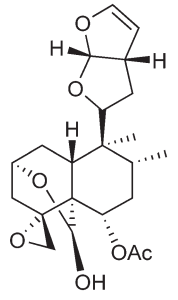
103 X = Cl
104 X = Br
105 X = I



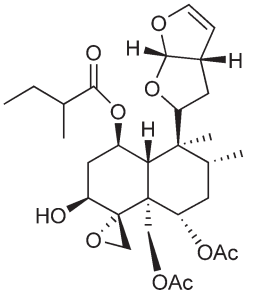
106 X = Cl
107 X = Br
108 X = I



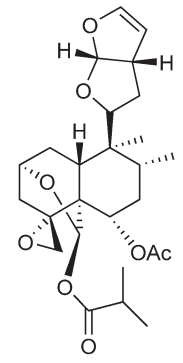
Scutecyprol B (**109**)



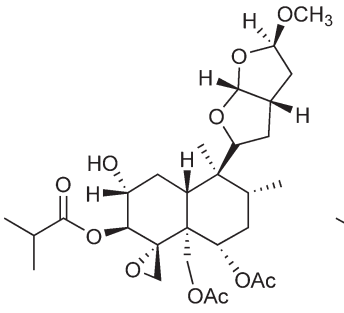
Scutalbin A (**110**)



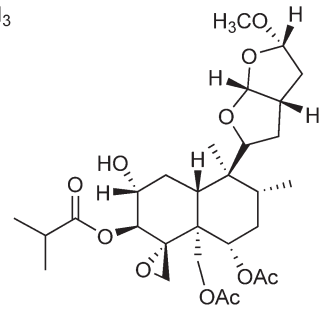
14,15-Dehydroajugareptansin (**111**)



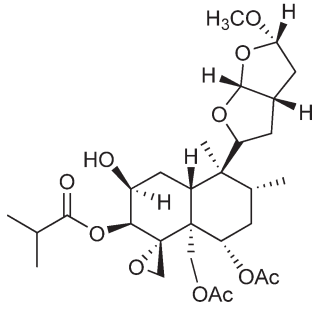
Jodrellin B (**112**)



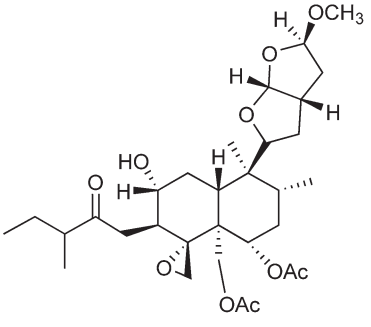
113



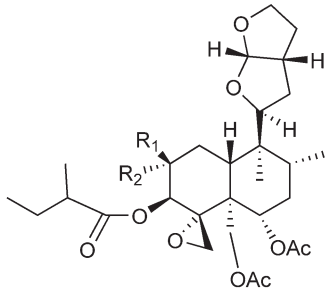
114



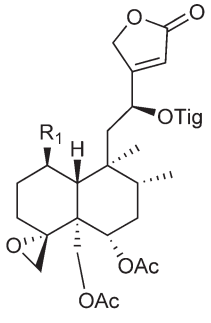
115



Lupulin A (**116**)



Ivain IV (**117**) R_1 = OH, R_2 = H
14,15-Dihydroajugapitin (**118**) R_1 = H, R_2 = OH



119 R = OAc
120 R = H

Plants of the *Teucrium* genus (Lamiaceae), known as germander, are also a rich source of diterpenoids. More than 200 diterpenoids having the *neo*-clerodane skeleton have been isolated from the aerial parts of about 80 species or subspecies.[72]

One of the major components of the acetone extracts of the aerial parts of *Teucrium massiliense*, deacetylajugarin II (**121**), a known antifeedant to several insects, was used as a starting point to create possible new antifeedants. Surprisingly, deacetylajugarin II (**121**) in itself is not active against either *Spodoptera exigua* or *L. decemlineata*, but when the 4α,18-epoxide group was replaced by a chlorohydrin as in **122** or when a carbonyl group was introduced at C-4 **123**, both compounds were active in choice antifeedant bioassays, giving data similar to that from opening of the epoxy ring in scutecyprol A;[64] larvae will however feed on it in a no-choice situation.[73] Two furoneoclerodanes, teumassilenins A and C (**124** and **125**), also isolated from *T. massiliense* were active only when tested against the CPB in both choice and no-choice bioassays. Ajugarin I (**126**) was however not active against CPB, yet it did inhibit feeding in *S. exigua* by 70% at 100 ppm.

Investigation of the contents of the acetone extract of *T. tomentosum* yielded in total six clerodanes. Although there were some slight differences, all six clerodanes **127–132** were active antifeedants against both *S. litura* and *Plutella xylostella* with feeding inhibition values between 60 and 85% at 10 $\mu g\,cm^{-2}$ and 55–75% at 5 $\mu g\,cm^{-2}$. Teuflin (**128**) was the most effective. However, compared with azadirachtin, the effectiveness is still about 10 times less.[74]

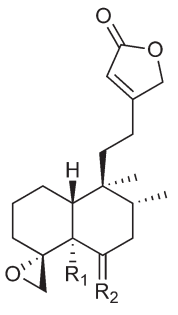
Treatment of potato leaf disks with 10 *neo*-clerodanes isolated from *Teucrium* and with 10 of their synthetic derivatives resulted in a significant antifeedant activity against *L. decemlineata*, although in choice and no-choice bioassay concentrations of 1000 ppm were used. For the most active compounds, effective concentration to inhibit 50% of the feeding (EC_{50}) ranged from 53 to 394 ppm.[75] Of the 12 most active antifeedants **128–139**,

nine are natural products (**128–132** and **136–139**), suggesting that the whole molecular structure is important for a more effective biological action. Montanin D (**130**) and 6β-hydroxytuescordin (**132**) were also found in the acetone extract of the above-ground parts of *T. arduini* and were active antifeedants against *S. littoralis* at 100 ppm. Transformation of the hydroxy group at C-6 in **130** into a carbonyl group decreased the antifeedant activity slightly.[76]

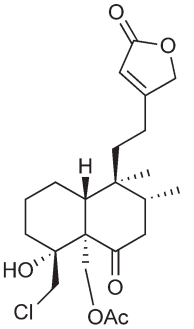
Auropolin (**140**) was isolated from the aerial parts of *Teucrium polium* (Lamiaceae).[77] Although the naturally occurring compound **140** did not reduce feeding by final stadium larvae of *S. littoralis*, the semisynthetic acetylated compound, acetyl-auropolin (**141**), reduced feeding by 57.8% at 100 ppm with an EC_{50} value (calculated via dose-dependent experiments) of 90 ppm.[77] This result shows the importance of the esterification of the hydroxy group at C-20 in auropolin.

Scutalpin B (**142**) was isolated from *Scutellaria alpina* subsp. *lambrensis* (Lamiaceae) together with scutalpin C (**143**). In choice antifeedant bioassays against *S. littoralis*, **143** was only slightly less active than jodrellin B (**112**) (97% versus 100%). Acetylation of the 11β-hydroxyl group of **143** resulted in the formation of **142** and caused a significant decrease in antifeedant activity (from 97 to 27% inhibition).[78]

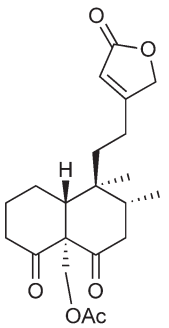
Scutegalin B (**144**), a naturally occurring *neo*-clerodane in *Scutellaria galericulata*, is a known phagostimulant for the larvae of the Egyptian cotton leafworm, *S. littoralis*. Transformation of this compound into **145** led to high antifeedant activity, which could be due to the nonesterified 19,2α-hemiacetal. When this 19,2α-hemiacetal function was changed into a 19-*O*-methyl-19,2α-acetal, as in **146**, the result was a phagostimulant.[79]



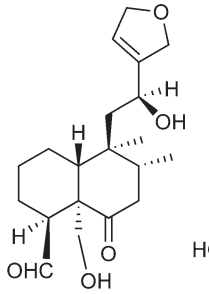
121 $R_1 = CH_2OH$, $R_2 = \alpha OH, \beta H$
126 $R_1 = CH_2OAc$ $R_2 = \alpha OAc, \beta H$



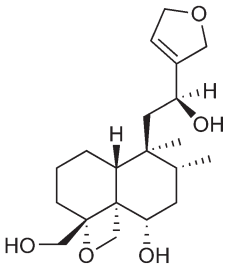
122



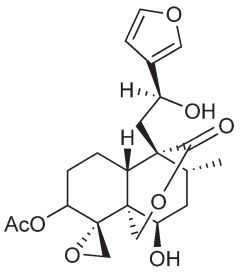
123



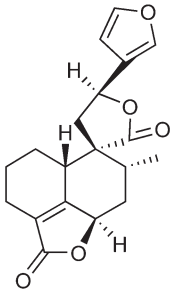
Teumassilenin A (**124**)



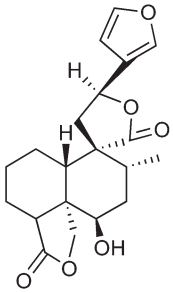
Teumassilenin C (**125**)



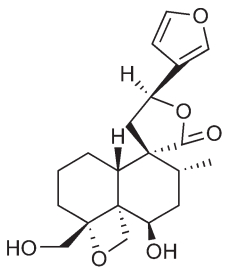
Teuctosin (**127**)



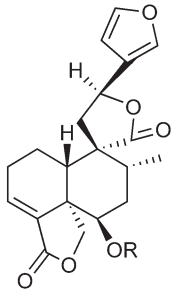
128



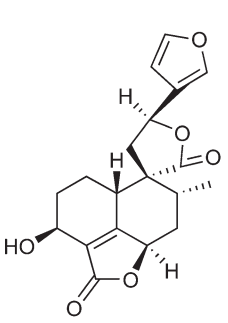
129 R = OH



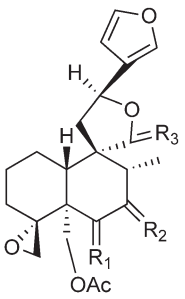
130 R = H



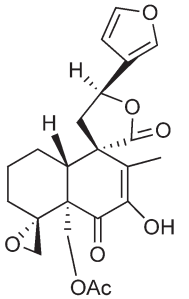
131 R = OAc
132 R = H



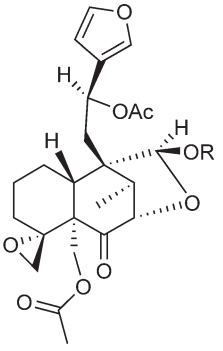
133



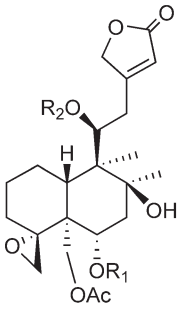
	R_1	R_2	R_3
134	O	αOH,βH	αH,βOAc
135	O	αOAc,βH	O
136	O	H,H	O
137	αOH,βH	O	αH,βOMe
138	αOH,βH	αOAc,βH	O



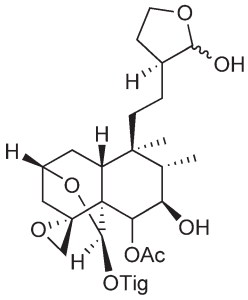
139



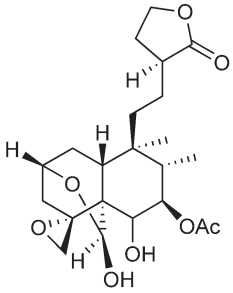
140 R = H
141 R = Ac



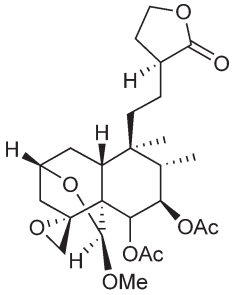
Scutalpin B (**142**) R_1 = Tig R_2 = Ac
Scutalpin C (**143**) R_1 = Tig R_2 = H



Scutegalin B (**144**)



145

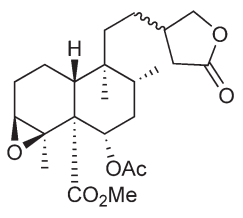


146

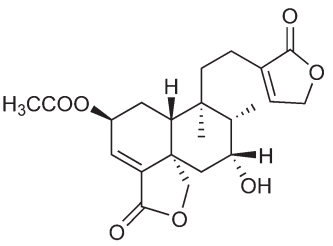
Clerodane-type diterpenoids are also found in species of *Salvia* (Labiatae), growing in Mexico. Compounds **147** and **148**, isolated from *Salvia melissodora*, **149** from *Salvia semiatrata*, **150** from *Salvia keerlii*, **151** and **152** both from *Salvia rhyacophilla*, **153** from *S. lineata*, and **154** from *Salvia tiliaefolia* all proved to be potent antifeedants against *S. littoralis*, with antifeeding index values of more than 50% at 100 ppm and all had an AI_{50} value of less than 100 mg l^{-1}.[63]

Although most studies have been performed on *Spodoptera* species,[69,80] *neo*-clerodanes are also effective antifeedants against the spotted bollworm, *Earias vitella*. Clerodendrin B (**155**), 3-epicaryoptin (**156**), 15-hydroxyepicaryoptin (**157**) were all isolated from *Clerodendron inerme*, a wild, as well as a hedge plant, from India. All exhibited good feeding inhibition (>78%) toward *S. litura* and >70% toward *E. vitella*.[81] Caryoptin (**158**), the stereoisomer of 3-epicaryoptin (**156**), was an effective antifeedant against *Henosepilachna vigintioctopunctata*, yet unlike in the case of *E. vitella*, 3-epicaryoptin and its analogues were not active, indicating that the stereochemistry at C-3 is important for antifeedant activity.[82]

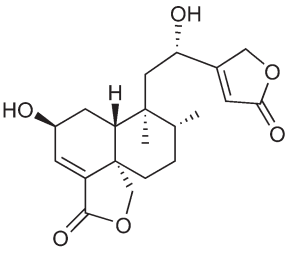
Several natural *neo*-clerodanes isolated from *Linaria saxatilis* (placed in Scrophulariaceae or Plantaginaceae) were tested alongside some semisynthetic compounds (all derived from the natural compounds) and tested against insects with divergent feeding adaptations. Of the natural compounds, *E*-isolinaridial (**159**), an α,β-unsaturated-1,4-dialdehyde, was the most active in reducing feeding by 90% in both choice and no-choice bioassays. Research has confirmed that the 4,18-epoxy group is important for activity, as the semisynthetic compounds **160** and **161** were more effective than their corresponding natural parent compound **162** and **163** against CPB. Creating a 4,18-diol group resulted in similar bioactivity.[83] All compounds tested had only moderate antifeedant activity against the aphid *M. persicae*; this suggests a different mode of action in these insects.



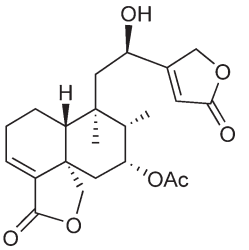
147



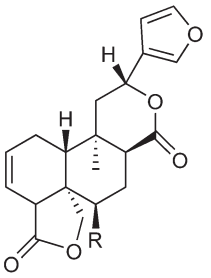
148



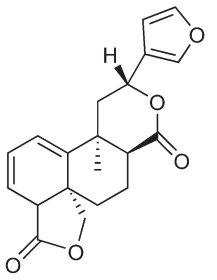
Semiatrin (**149**)



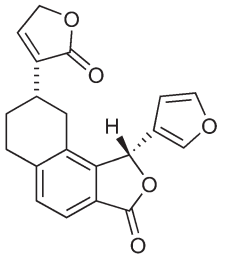
Kerlinolide (**150**)



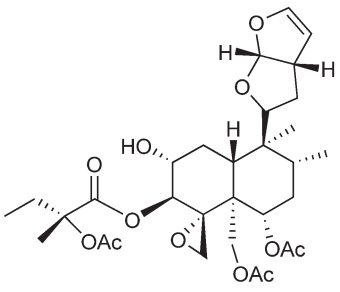
Salviarin (**151**) R = H
6β-Hydroxysalviarin (**151**) R = OH



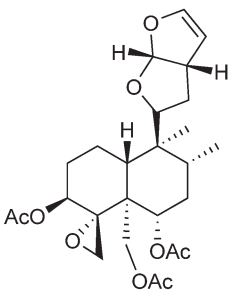
1(10)-Dehydrosalviarin (**153**)



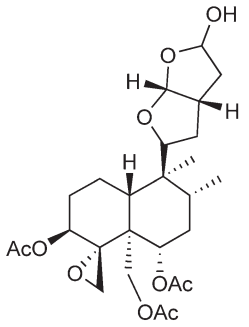
Tilifodiolide (**154**)



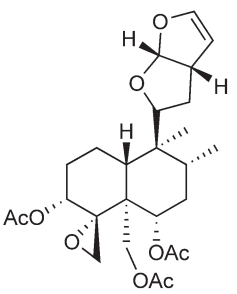
Clerodendrin B (**155**)



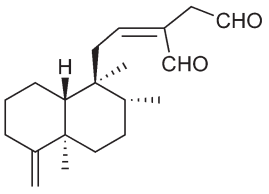
3-Epicaryoptin (**156**)



15-Hydroxyepicaryoptin (**157**)



Caryoptin (**158**)



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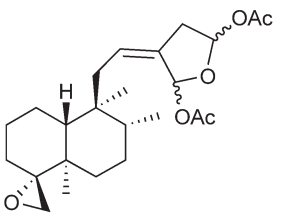

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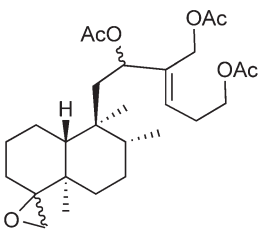

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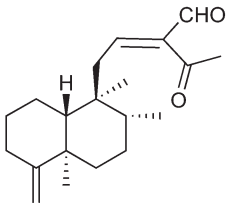
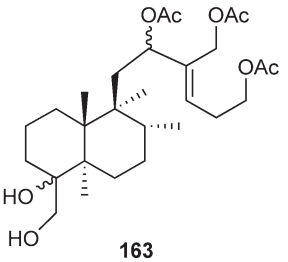


162

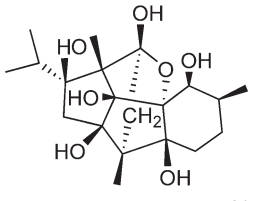


163

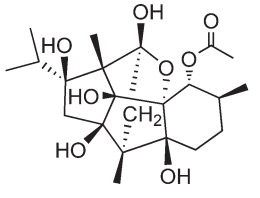
4.11.3.3.2 Ryanoids

Ryanodine is a diterpene derivative toxic to insects that has been known for some 60 years.[2] It is classified as an alkaloid although the nitrogen atom is held in a nonbasic pyrrole ring. Ryanoid diterpenes can be classified into two groups: alkaloid type (ryanodine and spiganthine types) and nonalkaloid type (ryanodol and isoryanodol types). The nonalkaloid ryanoids are isolated from *Persea indica* (Lauraceae) whereas the alkaloid-type ryanoids are isolated from *Spigelia anthelmia* (Strychnaceae).[84] When ryanoids from the nonalkaloid type were tested against *S. littoralis* and *L. decemlineata*, several exhibited antifeedant effects that were species-dependent, with *epi*-cinnzeylanol (**164**) and cinnzeylanol 1-acetate (**165**) as the most powerful antifeedants against *S. littoralis* ($<0.5\ \text{nmol cm}^{-2}$), although still not as potent as azadirachtin ($0.7 \times 10^{-6}\ \text{nmol cm}^{-2}$). Other compounds that exhibited good feeding inhibition were ryanodol 14-acetate (**166**), cinnzeylanone (**167**), and perseanol (**168**). *Leptinotarsa decemlineata* exhibited an overall lower response to the tested compounds. Compound **165** exhibited the strongest antifeeding effect with 100% feeding inhibition and an EC_{50} value of $0.01\ \text{nmol cm}^{-2}$. From the alkaloid type, only ryanodine (**169**), 2α-hydroxyryanodine (**170**), and 2,3β-epoxy-2-*epi*-ryanodine (**171**), had some antifeeding effect, yet concentrations were 0.9–$2.7\ \text{nmol cm}^{-2}$. In both the insects the nonalkaloid-type ryanoids had a stronger effect. Two of these compounds, **165** and **169**, exhibited a knockdown effect and led to paralyzed beetles.[84]

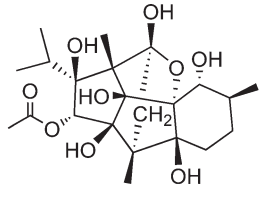
Ryanodol (**172**), cinnzeylanol (**173**), and *epi*-cinnzeylanol (**164**) isolated from *P. indica* also had antifeedant effects on *S. litura*.[85] Continuous research on the extract of aerial parts of *P. indica* led to the isolation of three additional rare isoryanodanes, indicol (**174**), vignaticol (**175**), and perseanol (**168**), all of them having antifeedant activity against *S. litura*, with perseanol being the most active.[86] Cinnzeylanine (**176**), also isolated from the aerial parts of *P. indica* and very similar to **173**, was the least effective ryanoid against *S. litura*.[85]



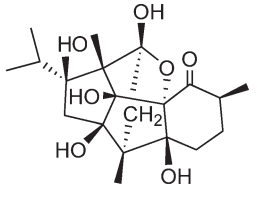
epi-Cinnzeylanol (**164**)



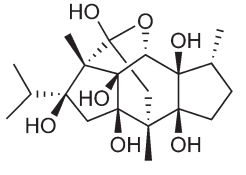
Cinnzeylanol 1-acetate (**165**)



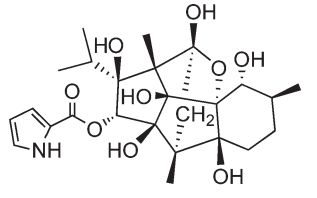
Ryanodol 14-acetate (**166**)



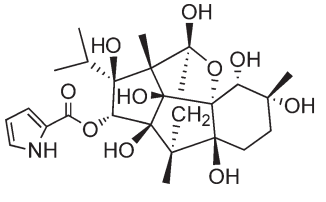
Cinnzeylanone (**167**)



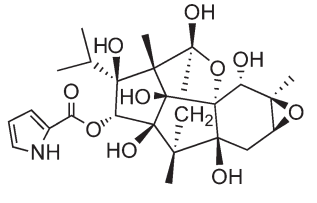
Perseanol (**168**)



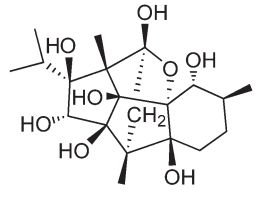
Ryanodine (**169**)



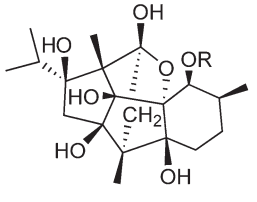
2α-Hydroxyryanodine (**170**)



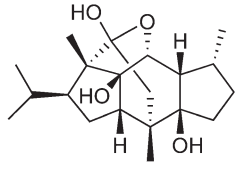
2,3β-Epoxy-2-*epi*-ryanodine (**171**)



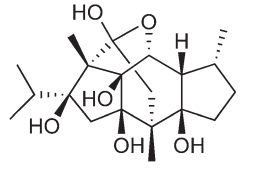
Ryanodol (**172**)



R = H Cinnzeylanol (**173**)
R = Ac Cinnzeylanine (**176**)



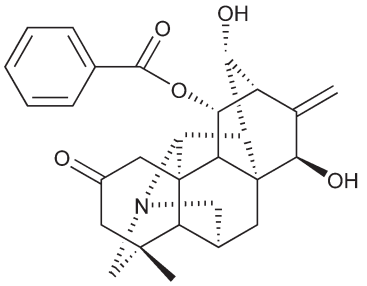
Indicol (**174**)



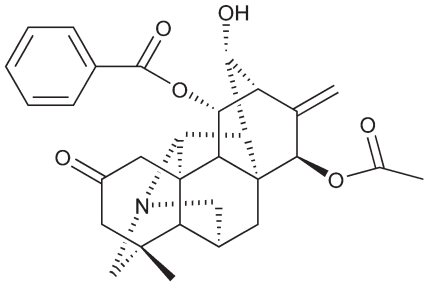
Vignaticol (**175**)

4.11.3.3.3 Diterpenoid alkaloids

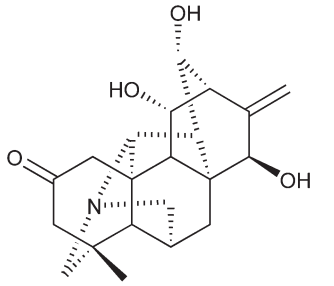
Cardiopetamine (**177**) and 15-acetylcardiopetamine (**178**) were isolated from *Delphinium cardiopetalum* (Ranunculaceae). Hydrolysis of **177** led to the amino alcohol **179**. Together with two additional seminatural products, **180** and **181**, both synthesized from cardiopetamine, they were all tested for antifeedant activity against the CPB and *S. littoralis*. Cardiopetamine (**177**) was a strong antifeedant against *S. littoralis* while 15-acetylcardiopetamine (**178**) inhibited feeding by CPB.[87] Hydroxy groups on both C-13 and C-15 are essential for antifeedant activity in *S. littoralis*. As for antifeeding activity toward CPB a C-13-hydroxyl or C-15-acetate group enhances the activity. Although a benzoate group on C-11 is not essential it enhances the biological effect on both species.



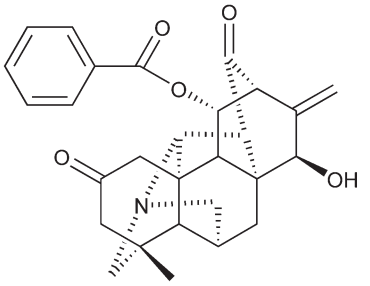
Cardiopetamine (**177**)



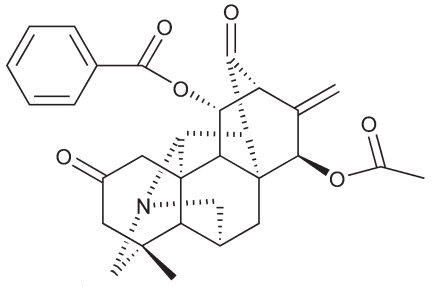
15-Acetylcardiopetamine (**178**)



Debenzoylcardiopetamine (**179**)

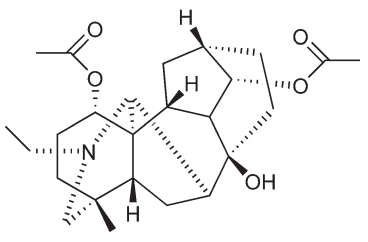


13-Oxocardiopetamine (**180**)

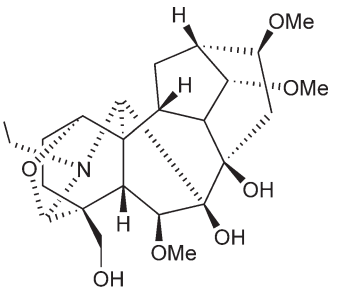


15-Acetyl-13-oxocardiopetamine (**181**)

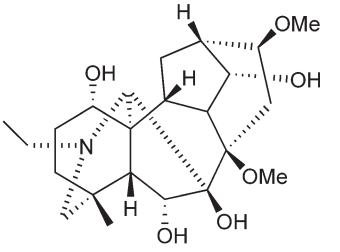
Extracts of several plants from the genera *Aconitum*, *Consolida*, and *Delphinium* (all Ranunculaceae) were obtained and led to the isolation of 40 natural norditerpenoid alkaloids. Three additional semisynthetic alkaloids were prepared as well.[88] All 43 alkaloids were tested for their antifeedant activity against *S. littoralis* and the CPB, *L. decemlineata*, to establish SAR. None of these were strong antifeedants against *S. littoralis*, but several strongly inhibited feeding by the CPB at concentrations below 1 $\mu g\,cm^{-2}$. 1,14-Diacetylcardiopetaline (**182**) and 18-hydroxy-14-*O*-methylgadesine (**183**) were the most potent, followed by 8-*O*-methylconsolarine (**184**), 14-*O*-acetyldelectinine (**185**), karakoline (**186**), cardiopetaline (**187**), 18-*O*-demethylpubescenine (**188**), 14-*O*-acetyldeltatsine (**189**), takaosamine (**190**), ajadine (**191**), and 8-*O*-methylcolumbianine (**192**).[88] SAR studies indicated that in order to be strong antifeedants against the CPB, compounds should lack a benzoyl group at C-14, as well as have oxygenated substituents at C-16. Hydroxylation at C-14 seems to increase the antifeeding activity.



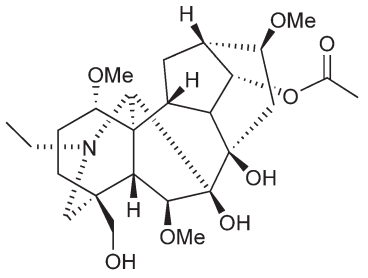
1,14-Diacetylcardiopetaline (**182**)



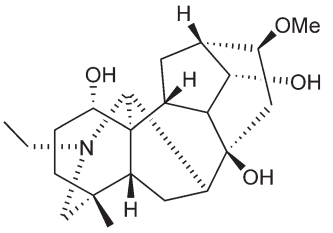
18-Hydroxy-14-*O*-methylgadesine (**183**)



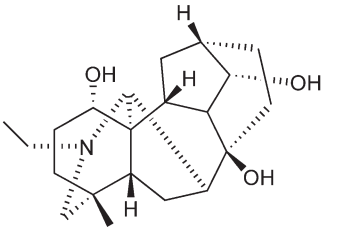
8-*O*-methylconsolarine (**184**)



14-*O*-acetyldelectinine (**185**)



Karakoline (**186**)



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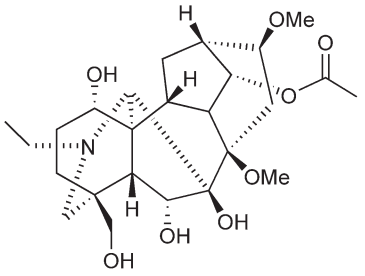
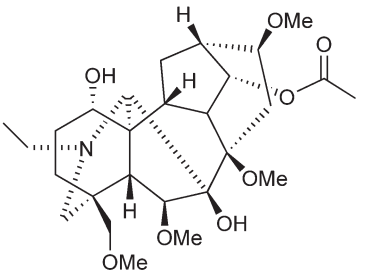
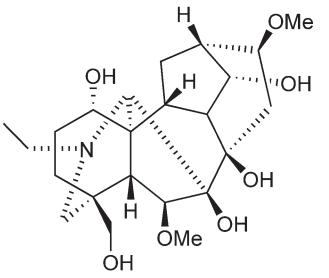


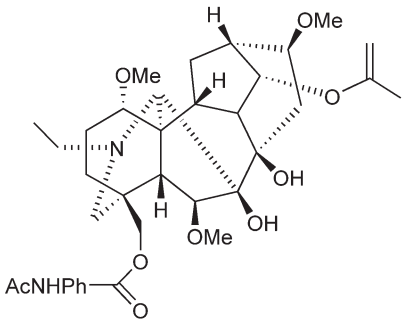
18-*O*-demethylpubescenine (**188**)



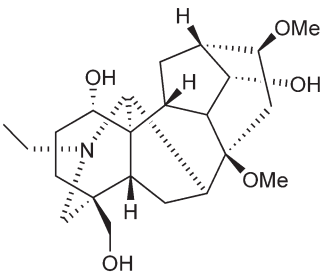
14-*O*-acetyldeltatsine (**189**)



Takaosamine (**190**)



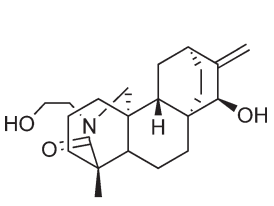
Ajadine (**191**)



8-*O*-methylcolumbianine (**192**)

Twenty-one diterpene alkaloids (16 were natural products isolated from these plants, while five were semisynthetic compounds) were also tested to establish SAR. 19-Oxodihydroatisine (**193**) was a strong antifeedant against *S. littoralis*, yet it did not affect feeding behavior in the CPB. However, the CPB responded to a larger number of compounds than *S. littoralis*, both results confirming the species dependency of these antifeedants. The rearranged form of hetisine (**194**) showed the strongest response for CPB.[89] Additionally, the norditerpenoid alkaloids had higher antifeedant effects on CPB (ranging from 0.1 to 12 $\mu g\,cm^{-2}$) compared with the effects of the diterpenoid alkaloids (ranging from 2 to 28 $\mu g\,cm^{-2}$).

Akhdarenol (**195**), α-amyrin (**196**), and the semisynthetic isopimaric acid methyl ester **197** exhibited antifeedant properties against *L. decemlineata*, with the natural compounds more active than the semisynthetic ones. None of these exhibited any antifeedant activity against *S. littoralis*.[90]



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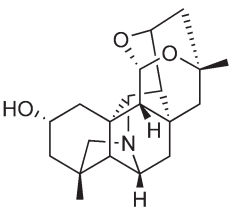

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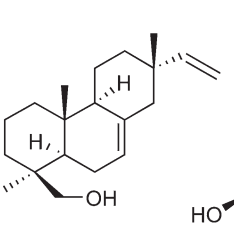

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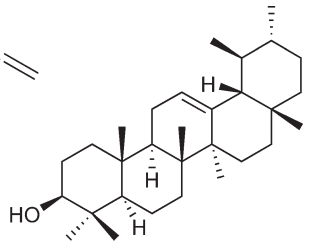

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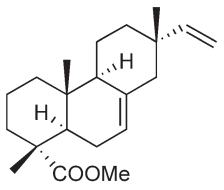
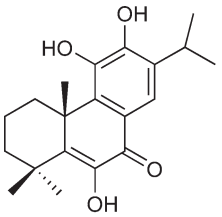


197

4.11.3.3.4 Abietanes

From the hairy roots of *Salvia broussonetia* (Lamiaceae), 14-deoxycoleon U (**198**) and demethylsalvicanol (**199**) were isolated together with other diterpenes. Of all the diterpenes isolated, 14-deoxycoleon U was a strong antifeedant against *L. decemlineata*,[12] whereas **199** exhibited only moderate activity. Several compounds similar to **198** were not active, suggesting a possible role for the unsaturation of ring B, a feature missing in 6α-hydroxydemethylcryptojaponol (**200**), which was not active. None of the isolated abietanes exhibited any antifeedant activity against *S. littoralis*.



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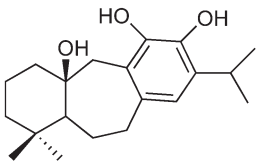

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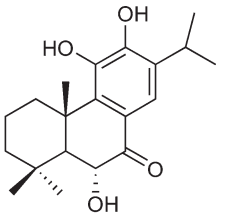


200

4.11.3.4 Triterpenes

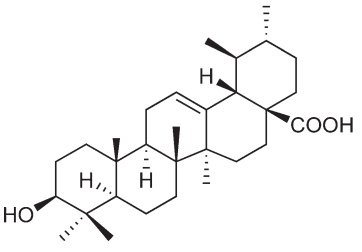
From the point of interest of antifeedants, triterpenoids can be divided into three classes, depending on the number of carbon atoms in the skeleton; there are full C-30 triterpenes, C-26 limonoids, and C-20 quassins. In addition, there are saponins, which can have either a triterpenoid or a steroid skeleton. Interesting and powerful antifeedants are found in each class.

4.11.3.4.1 Pentacyclic triterpenes

Two pentacyclic triterpenoids were isolated from *Vitex negundo*, an Indian medicinal plant of the Verbenaceae. They were identified as the known ursolic acid (**201**) and betulinic acid (**202**). After 48 h, the reduction in feeding by the larvae of the castor semilooper, *Achoea janata*, was 84.75% for **202** and 94.79% for **201**, respectively, at a dose of 10 $\mu g\,cm^{-2}$.[91]

The organic extract from the aerial part of *Junellia aspera* (also Verbenaceae) yielded several oleananes, of which daucosterol (**203**) was a strong antifeedant against adults of the rice weevil, *Sitophilus oryzae*. Oleanolic acid (**204**), which was the main secondary metabolite, was used as the starting material to prepare a series of derivatives, none of these were however active in feeding inhibition, although some were acutely toxic on ingestion.[92]

The rice weevil was also used in antifeedant bioassays testing the antifeedant activity of several triterpenoids from tropical species of the Rutales. Of the compounds isolated from *Lansium domesticum* (Meliaceae), iso-onoceratriene (**205**), 3-keto-21-hydroxyonoceradiene (**206**), onoceradienedione (**207**), lansiolic acid (**208**), and lansiolic acid A (**209**) exhibited significant antifeeding activities at 0.5% (w/w of wheat flour) using a flour disk bioassay.[93] However, crude extracts were more active than the pure compounds, which could be the results of either synergistic effects or the presence of other unidentified compounds. Three spirocaracolitones B, D, and E (**210–212**) isolated from *Ruptiliocarpon caracolito*[93] were however much more active at a concentration of 0.25% (w/w). The antifeeding properties of these spirocaracolitones were also found against the European corn borer, *Ostrinia nubilalis*.[94]



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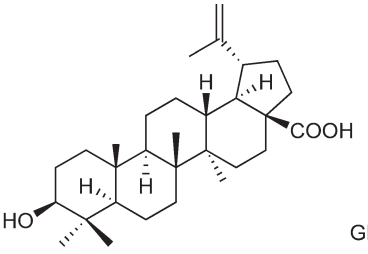

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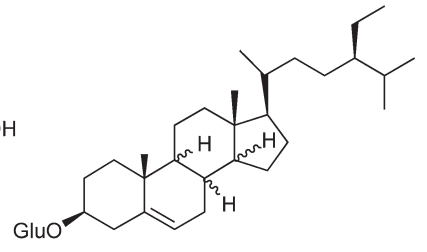
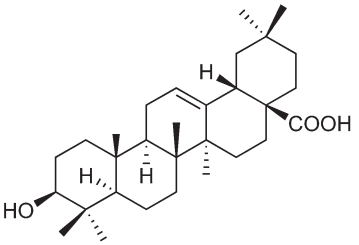
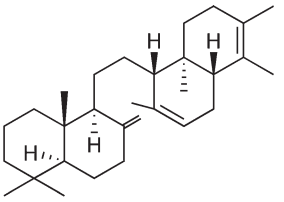


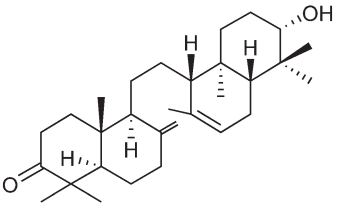
Daucosterol (**203**)



Oleanolic acid (**204**)



iso-Onoceratriene (**205**)



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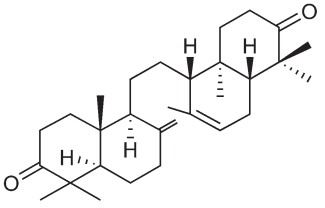
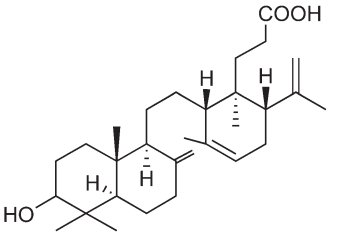
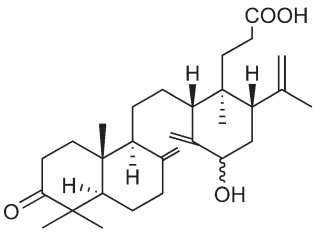


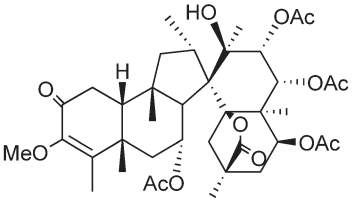
Onoceradienedione (**207**)



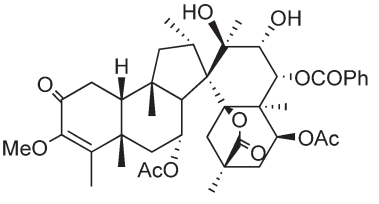
Lansolic acid (**208**)



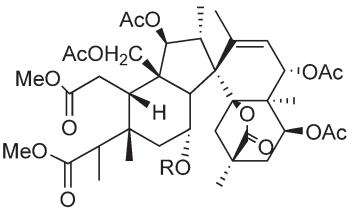
Lansolic acid A (**209**)



Spirocaracolitone B (**210**)



Spirocaracolitone D (**211**)



Spirocaracolitone E (**212**)
R = $COC(CH_3)=CHCH_3$

4.11.3.4.2 Limonoids

Limonoids are highly oxygenated triterpenes, which have lost the terminal four carbon atoms of the side chain, with the remaining side chain cyclized into a furan ring;[2] they are therefore tetranortriterpenoids. The name limonoids is derived from the first of these isolated, the bitter compound limonin, from citrus fruits and was first isolated in 1841.[2,95] Limonoids are found in leaves, bark, fruits, seeds, or kernels widely throughout the Meliaceae and Rutaceae, and less frequently in the small family Cneoraceae and *Harrisonia* sp. of the Simaroubaceae. Limonoids can be further classified by intact ring systems and those in which one of the four rings (A, B, C, or D) has been ring-opened.

The powerful antifeedant and insecticide azadirachtin (**213**), from the neem tree (*Azadirachta indica*, Meliaceae), is a highly oxidized limonoid with rings A, B, and D intact.[2] It is used as a benchmark against which all other antifeedants can be compared (*vide infra*). The total synthesis of azadirachtin has recently been achieved in 64 steps.[96] This is very unlikely to provide a synthetic source of the compound, but it does allow SAR studies to find maximum activity, and opens up the field to possible simpler synthetics modelled on it. As yet, even slight modifications of the structure tend to decrease activity. Azadirachtin (**213**) has been available commercially, particularly in the United States, but at present the cost of the seeds and the isolation procedure inhibit its wider use.

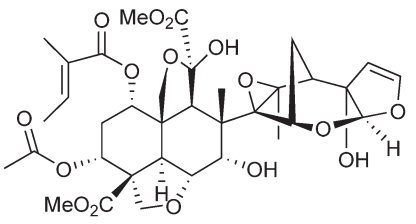
A derivative of gedunin (**214**), 6β-hydroxygedunin (**215**), previously only a synthetic compound, was recently also isolated from the Indian neem tree, *A. indica*, and was found to be an antifeedant toward the gram pod borer, *H. armigera* as well as against the Asian armyworm, with an EC_{50} of 24.1 ppm, and against *S. litura* at 21.5 ppm.[97] Although not as active an antifeedant as azadirachtin it was more powerful than gedunin (**214**). Other non-azadirachtin limonoids from the Indian neem tree such as nimbocinol, salannin (**216**), and azadiradione (**217**) also seem to have antifeedant activity against these lepidopteran pests,[98] but with an FI_{50} value at least 150 times higher than that of azadirachtin and five times more than that of 6β-hydroxygedunin (**215**).

Several derivatives of havanensin-type limonoids were also synthesized[99] and tested for their antifeedant activity against *S. littoralis* and *S. frugiperda* as well as against *L. migratoria*. The racemic β-epoxide derivative (3a-methyl-3-phenyl-hexahydro-1-oxa-cyclopropa[c]inden-4-one, **218**) was slightly more active than the α-epoxide **219**. Both were however less active than the trimethyl derivative **220**. Of all the derivatives synthesized, the deoxo β-epoxide **221** (3a-methyl-3-phenyl-octahydro-1-oxacyclopropa[c]indene) was most active toward *S. littoralis* and *S. frugiperda* with an antifeedant index of 43 and 23%, respectively at 100 ppm. Compound **220** was also a very potent antifeedant against *Locusta migratoria*, an insect for which azadirachtin is not a very potent antifeedant.

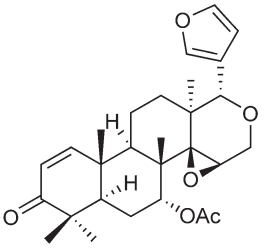
In order to find new compounds with antifeedant activity, much research is now shifted toward making simple compounds derived from natural antifeedants. Simple analogues of azadiradione and epoxyazadiradione (**222**) were made[100] and the keto-epoxide **223** was found to have a strong antifeedant activity against *S. littoralis*.[100,101] In a similar study, two racemic indenones, **224** and **225**, were found to be active as a feeding deterrent against *S. frugiperda* with an AFI of ~40% at 100 ppm.[101] Keto epoxides synthesized from the indenones, **226** and **227**, also exhibited moderate antifeedant activity when tested as racemic mixtures on *S. frugiperda*. Replacing the E-ring with a phenyl substituent had the surprising effect of creating the phagostimulant **228**, rather than an antifeedant.[101]

From the root bark of *Melia toosendan*, closely related to *Melia azedarach*, three different C-seco limonoids were isolated, all three having antifeedant activity against the larvae of *S. eridania*. Of these three, nimbolidin F (**229**) was the most active at 500 ppm while 3-*O*-acetylohchinolal (**230**) and ohchinolide C (**231**) were only active at 1000 ppm.[102]

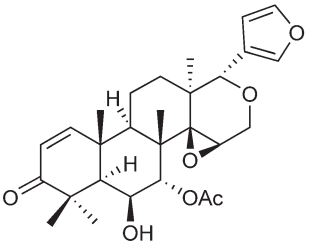
Three neoazedarachins, neoazedarachin A, B, and D, **232**–**234**, were also isolated from the same tree together with 1-*O*-acetyltrichilin (**235**) and all exhibited antifeedant activity at 400 ppm when tested on the third-instar larvae of *S. littoralis*.[103] Spirosendan (**236**) was the first spiro-limonoid isolated from the root bark of *M. toosendan*, but had only very weak antifeedant activity (active at 1000 ppm).[104]



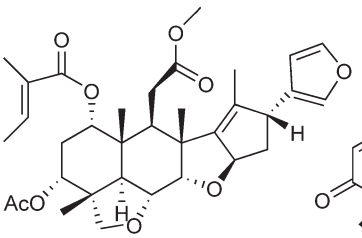
Azadirachtin (**213**)



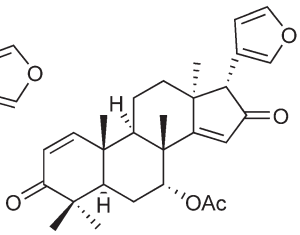
Gedunin (**214**)



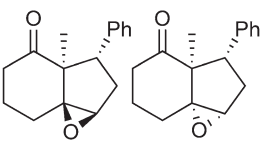
6β-Hydroxygedunin (**215**)



Salannin (**216**)



Azadiradione (**217**)



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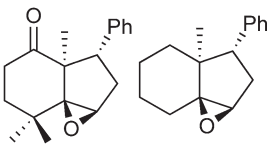

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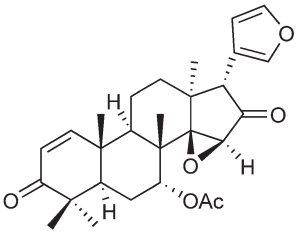
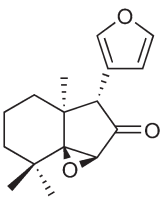


Epoxyazadiradione (**222**)



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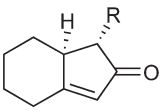
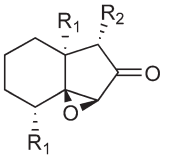
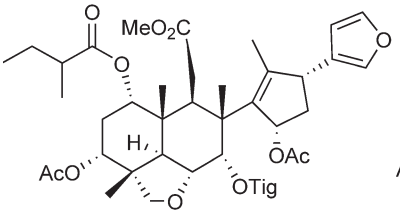


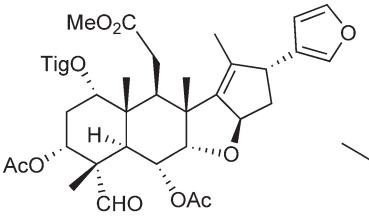
224 R = 3-Furyl
225 R = Phenyl



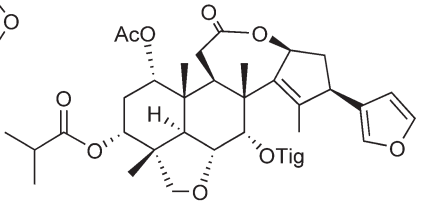
226 R_1 = H, R_2 = 3-Thienyl
227 R_1 = Me, R_2 = 3-Furyl
228 R_1 = H, R_2 = Phenyl



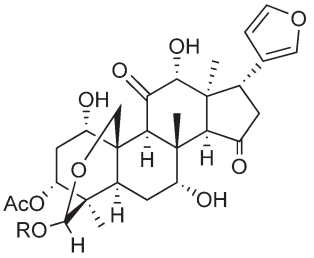
229 Nimbolidin F



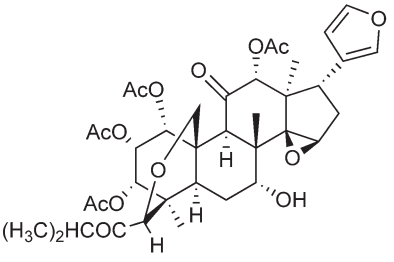
230 3-*O*-acetylhchinolal



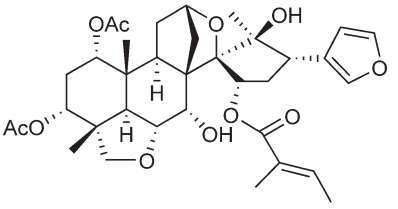
231 Ohchinolide C



232 R = COCHMeEt
233 R = $COCHMe_2$
234 R = Me (endo)

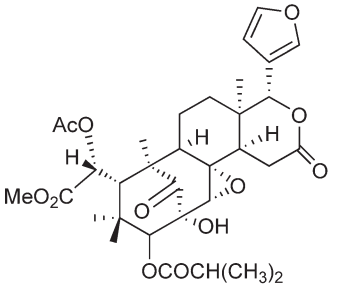


1-*O*-Acetyltrichilin (**235**)



Spirosendan (**236**)

Three humilinolides, humilinolides A–C (**237–239**) were isolated from *Swietenia humilis*.[93] Only **238** and **239** exhibited a strong feeding inhibition, with 75 and 35%, respectively, inhibition at 0.50% (w/w). Several novel A-seco limonoids were isolated from the root bark of *Croton jatrophoides* Euphorbiaceae, an east African medicinal plant locally known as 'msinduzi'. These were dumsin (**240**), zumsin (**241**),[105] dumnin (**242**), dumsenin (**243**),[106] zumketol (**244**),[20] musidunin (**245**), and musiduol (**246**).[107] All these compounds exhibited potent antifeedants activity against two lepidopteran larvae, the pink bollworm, *Pectinophora gossypiella*, and the fall armyworm, *S. frugiperda*, with EC_{50} values varying from 0.5 to 4 μg ml^{-1}. Although two additional limonoids, zumsenin (**247**) and zumsenol (**248**), were isolated from *C. jatrophoides*, they could not be tested due to the limited amount available.[20]



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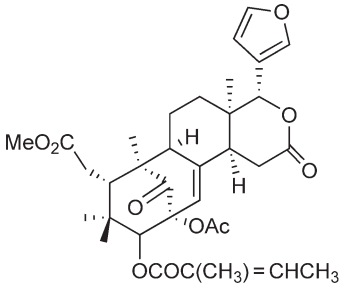

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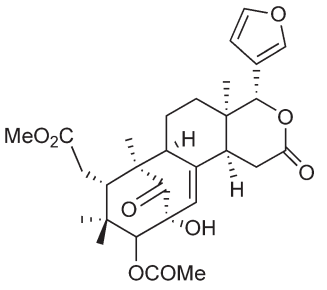
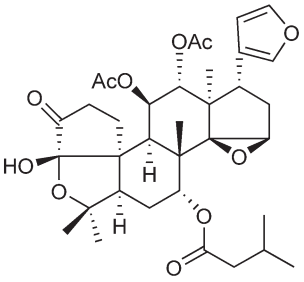
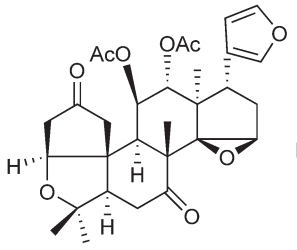


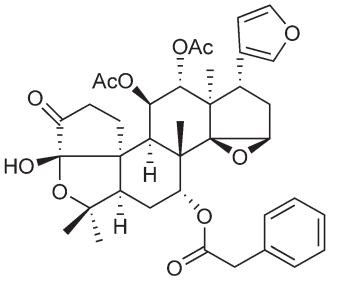
239



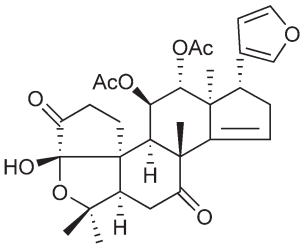
Dumsin (**240**)



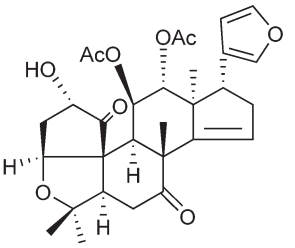
Zumsin (**241**)



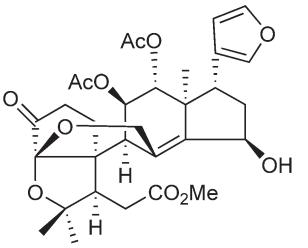
Dumnin (**242**)



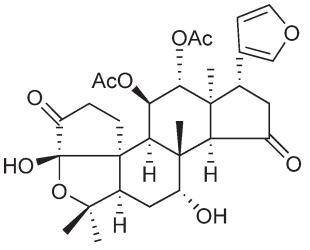
Dumsenin (**243**)



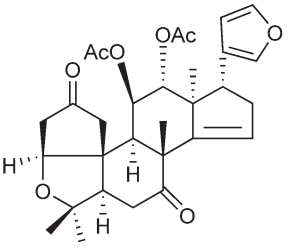
Zumketol (**244**)



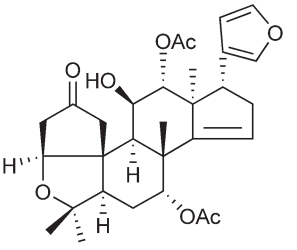
Musidunin (**245**)



Musiduol (**246**)



Zumsenin (**247**)



Zumsenol (**248**)

Other plants of the Meliaceae family are richly endowed with many other limonoids. Prieurianin (**249**) and epoxyprieurianin (**250**) were isolated from the bark of *Entandrophragma candolei* and were active as antifeedants against larvae of the gram pod borer, *H. armigera*.[108] The epoxy derivative of prieurianin (**249**) was more effective at reducing feeding by 69% at 100 ppm versus 58% feeding inhibition for the non-epoxy compound. Acetylation of these two natural products slightly increased the antifeedant activity. However, azadirachtin is about 12-fold more active than epoxyprieurianin (**250**).

Limonoids of the salannin group has also been shown to be an antifeedant against lepidopteran larvae. 3-*O*-acetyl salannol (**251**), salannol (**252**), and salannin (**216**) were all isolated from the seeds of the neem tree and tested against the gram pod borer *H. armigera* and the larvae of the tobacco armyworm, *S. litura*.[97] Although each of these three compounds exhibits antifeeding activity, 3-*O*-acetylsalannol was the most active at 64.2 ppm. There was no synergistic effect with either insect when these three salannin compounds were combined together.[109]

Extracts from Argentinian *M. azedarach* trees also exhibited antifeedants activity against the elm leaf beetle. Bio-guided fractionation of these extracts led to the isolation of a limonoid called meliartenin (**253**). It is an equilibration mixture of two tautomers, the minor tautomer can be described as 12-hydroxyamoorastatin (**254**). The name meliartenin has been given to the major tautomer **253**, which can be confusing.[110] When the tautomeric mixture was tested against *Epilachna paenulata* larvae and compared with azadirachtin and toosendanin, it was found to be as effective as these two well-known antifeedants. When tested against *S. eridania* larvae, it was slightly more effective.[111]

Five compounds similar to the tetranortriterpenoid limonin were isolated from the roots of *Trichilia pallida* (Meliaceae) with acetone. These compounds were identified as hirtin (**255**), deacetylhirtin (**256**), methyl 6-hydroxy-11β-acetoxy-12α-(2-methylpropanoyloxy)-3,7-dioxo-14β,15β-epoxy-1,5-meliacadien-29-oate (**257**), methyl 6,11β-dihydroxy-12α-(2-methylpropanoyloxy)-3,7-dioxo-14β,15β-epoxy-1,5-meliacadien-29-oate (**258**), and methyl 6-hydroxy-11β-acetoxy-12α-(2-methylbutanoyloxy)-3,7-dioxo-14β,15β-epoxy-1,5-meliacadien-29-oate (**259**).[112] These five were tested against four species of Lepidoptera (*S. littoralis*, *S. exigua*, *Heliothis virescens*, and *H. armigera*). Only **258** was active against all these four lepidopteran species at 100 ppm. Compound **256**, which also has a hydroxyl moiety rather than an acetyl moiety at C-11, exhibited antifeedant activity against *H. virescens* and *H. armigera*, but not against the *Spodoptera* species.[112] These data support earlier findings that a small change in the structure of the molecule (especially to the C-11 side chain) could drastically change bioactivity of the compound.

The root bark of *Severinia buxifolia* (Rutaceae) yielded two new limonoids called severinolide (**260**) and cycloseverinolide (**261**) as colorless plates. Of these only severinolide (**260**) was active as antifeedants against *Plutella xylostella* at a concentration of 0.0625%, reducing feeding between 21 and 40%. Also isolated from the root bark was atalantin (**262**) and cycloepiatalantin (**263**). Of these two compounds, **262** was as active as **260**. Compound **263** did reduce feeding, but only at 0.25% (**Table 1**).[113]

Table 1 Limonoids as insect antifeedants

Structure	*(Plant) source*	*Insect tested against*	*Feeding inhibition (%)*	*Concentration (ppm)*	*EC_{50} (ppm)*
214	*A. indica*	*H. armigera*			50.8
		S. litura			40.4
215	*A. indica*	*H. armigera*			24.2
		S. litura			21.5
216	*A. indica*	*H. armigera*			72.2
		S. litura			70.2
217	*A. indica*	*H. armigera*			109.6
		S. litura			102.1
218	Synthetic	*S. littoralis*	22	100	
		S. frugiperda	22	100	
219	Synthetic	*S. littoralis*	20	100	
		S. frugiperda	14	100	

(*Continued*)

Table 1 (Continued)

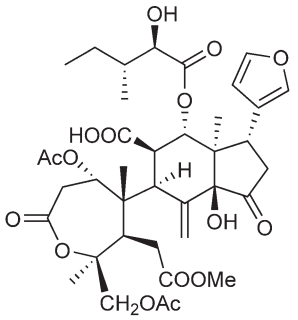
Structure	*(Plant) source*	*Insect tested against*	*Feeding inhibition (%)*	*Concentration (ppm)*	*EC_{50} (ppm)*
220	Synthetic	*S. littoralis*	43	100	
		S. frugiperda	23	100	
		L. migratoria	Not given		
221	Synthetic	*S. littoralis*	30	100	
		S. frugiperda	21	100	
222	Synthetic	*S. littoralis*	22	100	
223	Synthetic	*S. littoralis*	55	100	
224	Synthetic	*S. frugiperda*	42	100	
225	Synthetic	*S. frugiperda*	40	100	
226	Synthetic	*S. frugiperda*	36	100	
227	Synthetic	*S. frugiperda*	29	100	
228	Synthetic	*S. frugiperda*	−12[a]	100	
229	*M. azedarach*	*S. eridania*			500
230	*M. azedarach*	*S. eridania*			1000
231	*M. azedarach*	*S. eridania*			1000
232	*M. azedarach*	*S. littoralis*			400
233	*M. azedarach*	*S. littoralis*			400
234	*M. azedarach*	*S. littoralis*			400
235	*M. azedarach*	*S. littoralis*			400
236	*M. toosendan*	*S. exigua*			1000
237	*S. humilis*	*S. oryzae*	Not active	0.50%[b]	
238	*S. humilis*	*S. oryzae*	75.2	0.50%[b]	
239	*S. humilis*	*S. oryzae*	34.8	0.50%[b]	
240	*C. jatrophoides*	*P. gossypiella*			1[c]
		S. frugiperda			Not tested
241	*C. jatrophoides*	*P. gossypiella*			1[c]
		S. frugiperda			2[c]
242	*C. jatrophoides*	*P. gossypiella*			1[c]
		S. frugiperda			Not tested
243	*C. jatrophoides*	*P. gossypiella*			2[c]
		S. frugiperda			1[c]
244	*C. jatrophoides*	*P. gossypiella*			0.5[c]
		S. frugiperda			3[c]
245	*C. jatrophoides*	*P. gossypiella*			3[c]
		S. frugiperda			Not tested
246	*C. jatrophoides*	*P. gossypiella*			4[c]
		S. frugiperda			2[c]
247	*C. jatrophoides*				Not tested
248	*C. jatrophoides*				Not tested
249	*E. candolei*	*H. armigera*	57.7	100	92.2
250	*E. candolei*	*H. armigera*	69.1	100	62.7
251		*H. armigera*			64.2
		S. litura			65.6
252		*H. armigera*			79.7
		S. litura			77.4
253[d]	*M. azedarach*	*E. paenulata*	52.1	1[c]	0.8[c]
254[d]		*S. erdania*	91.2	1[c]	
255	*T. pallida*	*S. littoralis*	4	100	
		S. exigua	10	100	
		H. virescens	9	100	
		H. armigera	12	100	
256	*T. pallida*	*S. littoralis*	−14[a]	100	
		S. exigua	12	100	
		H. virescens	29	100	
		H. armigera	32	100	
257	*T. pallida*	*S. littoralis*	5	100	

(*Continued*)

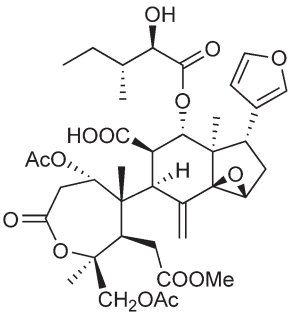
Table 1 (Continued)

Structure	*(Plant) source*	*Insect tested against*	*Feeding inhibition (%)*	*Concentration (ppm)*	*EC_{50} (ppm)*
		S. exigua	7	100	
		H. virescens	8	100	
		H. armigera	16	100	
258	*T. pallida*	*S. littoralis*	46	100	
		S. exigua	40	100	
		H. virescens	49	100	
		H. armigera	42	100	
259	*S. buxifolia*	*P. xylostella*	60–80	0.0625[b]	
260	*S. buxifolia*	*P. xylostella*	Not active		
261	*S. buxifolia*	*P. xylostella*	60–80	0.0625[b]	
262	*S. buxifolia*	*P. xylostella*	60–80	0.25	

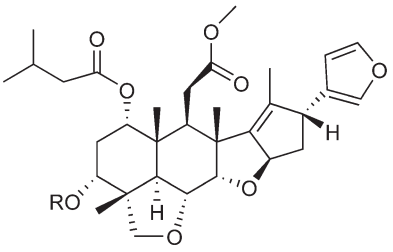
[a] Phagostimulant
[b] Concentration in % (w/w)
[c] Concentration in μg cm^{-2}
[d] Tested as tautomeric mixture



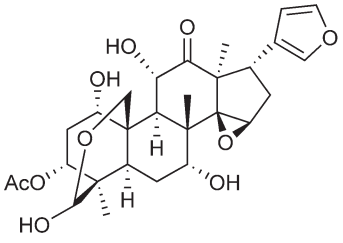
Prieurianin (**249**)



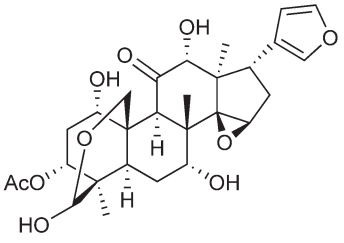
Epoxyprieurianin (**250**)



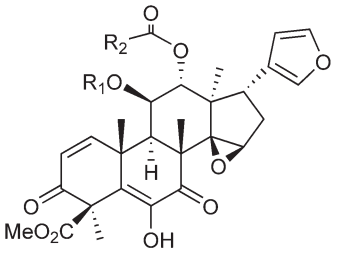
3-*O*-Acetyl salannol (**251**) R = Ac
Salannol (**252**) R = H



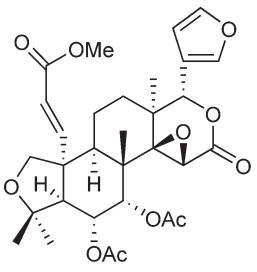
Meliartenin (**253**)



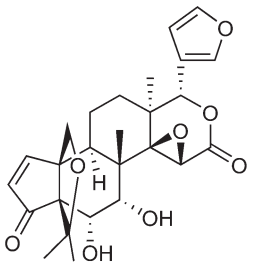
12-Hydroxyamoorastatin (**254**)



		R_1	R_2
255	Hirtin	Ac	CH_2CH_3
256	Deacetylhirtin	H	CH_2CH_3
257		Ac	$CH(CH_3)CH_3$
258		H	$CH(CH_3)CH_3$
259		Ac	$CH(CH_3)CH_2CH_3$



260



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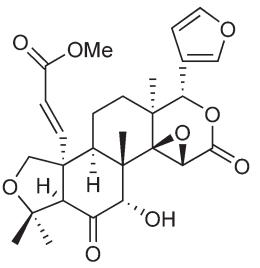

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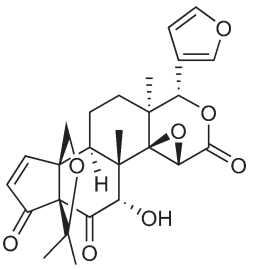


263

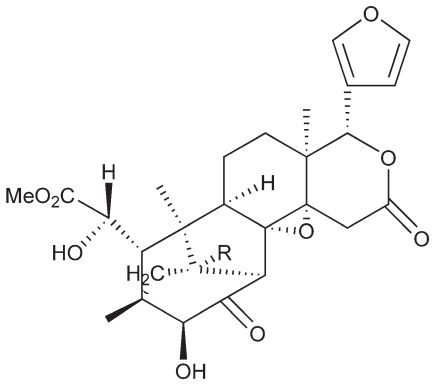
4.11.3.4.3 Phragmalins

Khaya senegalensis, also from the Meliaceae, is used as a popular traditional medicine in Africa. From the bark of this tree, several phragmalins, B,D-seco-limonoids, have been isolated and identified as antifeedants against *S. littoralis*. The phragmalin khayanolides A–E (**264–268**) were all tested[114–117] against *S. littoralis* in a leaf disk bioassay. Among the khayanolides, khayanolide E (**268**) was the most potent, active at 100 ppm,[116] with 50 ppm corresponding to 1 μg leaf cm^{-2} followed by khayanolide A (**264**), which was active at 300 ppm. Khayanolide C (**266**) exhibited moderate activity at 500 ppm.[102] Khayanolides B (**265**) and D (**267**) were active only at a concentration of 1000 ppm. Two other B,D-seco-limonoids, khayalactol (**269**) and 1-*O*-acetylkhayanolide B (**270**), were also isolated from *K. senegalensis* and were potent antifeedants against *S. littoralis* at 300 ppm.[118]

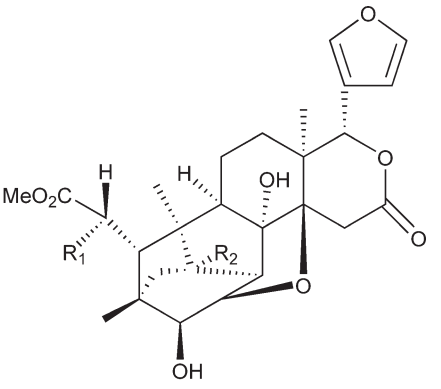
Also isolated from the Egyptian *K. senegalensis* was a limonoid glucoside named khayanoside (**271**), yet this compound exhibited only weak antifeedant activity.[116] From the ether extract of the bark of the same tree, two mexicanolide-type limonoids, seneganolide (**272**) and khayalactol (**269**), were isolated. Seneganolide was active in bioassays at 300 ppm against *S. littoralis*.[119] Further investigation of the acetone extract of the stem bark of *K. senegalensis* yielded two additional mexicanolides, khayanone (**273**) and 2-hydroxyseneganolide **274** as well as 1-*O*-acetylkhayanolide A (**275**).[120] In bioassays against *S. littoralis*, 1-*O*-acetylkhayanolide A was the most potent at 100 ppm,[117] with the other two active at 300 and 200 ppm, respectively, making them still weaker than azadirachtin and other meliacarpinins with activities in the 10–50 ppm range.

Extraction of the leaves of *Cedrela odorata* with methanol yielded four mexicanolides, cedrodorin (**276**), 6-acetoxycedrodorin (**277**), 6-deoxy-9α-hydroxycedrodorin (**278**), and 9α-hydroxycedrodorin (**279**). Rejection of *C. odorata* leaves by the weevil *Exopthalmus jekelianus* was found to be correlated with the presence of cedrodorin (**276**), 6-acetoxycedrodorin (**277**) and 6-deoxy-9α-hydroxycedrodorin (**278**) but not 9α-hydroxycedrodorin (**279**).[121] The concentration of these four mexicanolides varied with genotype, but the active compounds were always more abundant than the inactive ones.

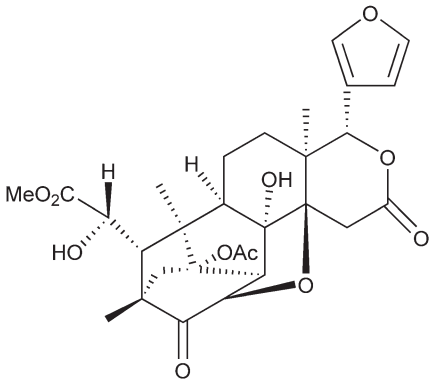
The diethyl ether extract from the root bark of *Chukrasia tabularis* (Meliaceae) yielded six new phragmalins named tabulalin (**280**) possessing an α,β-unsaturated lactone structure and tabulalides A–E (**281–285**), having novel 19-oxygenated structures. These tabulalides can be further divided into two groups, namely, tabulalides A (**281**) and B (**282**) with a C-7/C-19 lactone bridge and the tabulalides C–E **283–285** with a 19-acetoxy function. These new phragmalins all have comparable antifeedant activity against the third-instar larvae of *S. littoralis* similar to the khayanolides, with tabulalin (**280**) and tabulalide D (**284**) active at 500 ppm, corresponding to 10 μg leaf cm^{-2}. Tabulalides A, B, and E were active at only 1000 ppm, while no activity was observed for tabulalide C (**283**).[122]



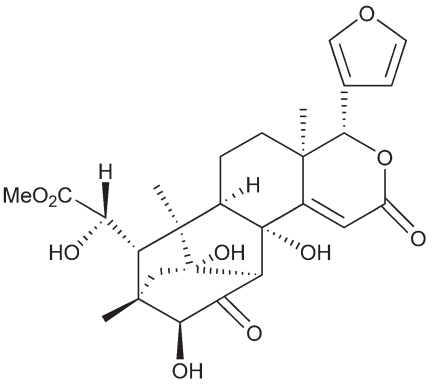
Khayanolide A (**264**) R = OH
1-*O*-Acetylkhayanolide A (**275**) R = OAc



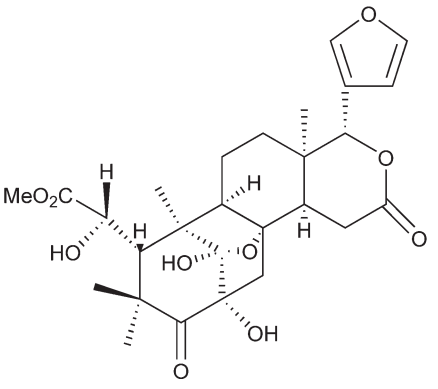
Khayanolide B (**265**) R_1 = OH R_2 = OH
Khayanolide E (**268**) R_1 = H R_2 = H
1-*O*-Acetylkhayanolide B (**270**) R_1 = OH R_2 = OAc



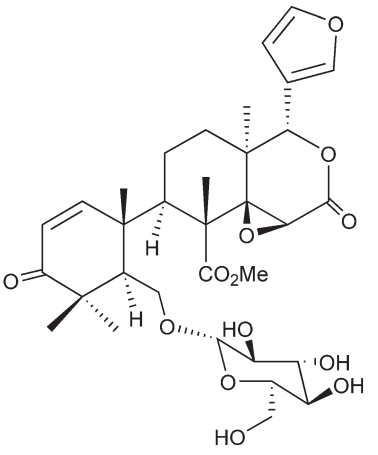
Khayanolide D (**267**)



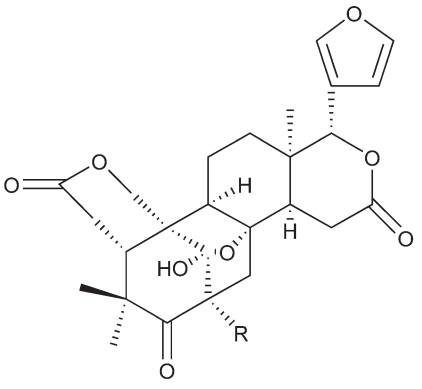
Khayanolide C (**266**)



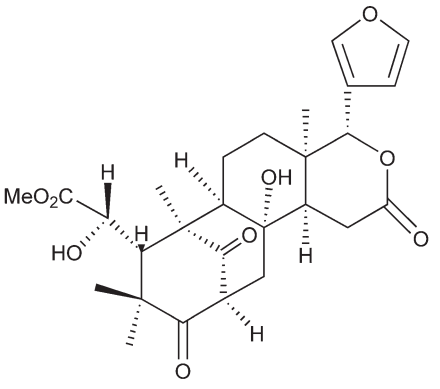
Khayalactol (**269**)



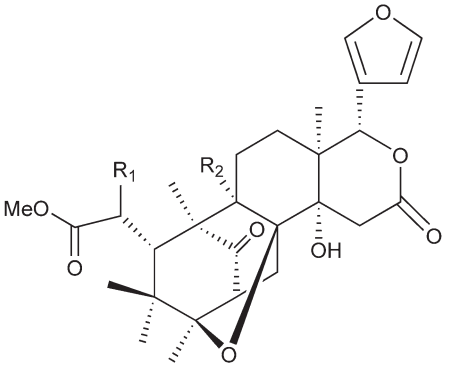
Khayanoside (**271**)



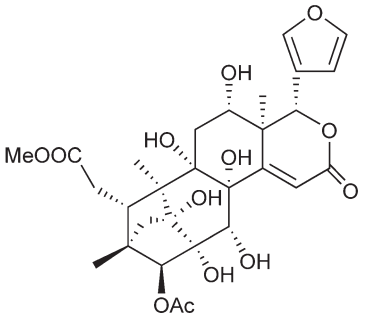
Seneganolide (**272**) R = H
2-Hydroxyseneganolide (**274**) R = OH



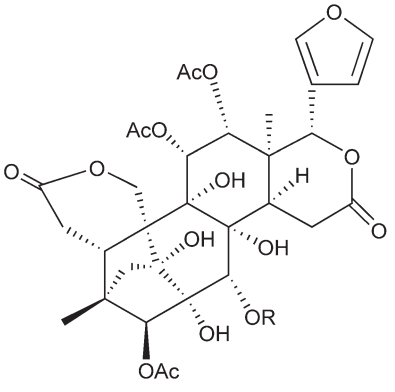
Khayanone (**273**)



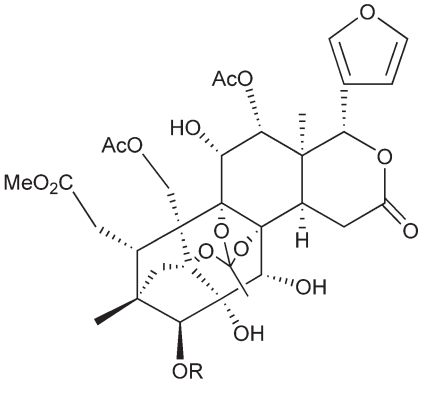
Cedrodorin (**276**) R_1 = OH, R_2 = H
6-Acetoxycedrodorin (**277**) R_1 = OAc, R_2 = H
6-deoxy-9α-hydroxycedrodorin (**278**) R_1 = H, R_2 = OH
9α-hydroxycedrodorin (**279**) R_1 = R_2 = OH



Tabulalin (**280**)



Tabulalide A (**281**) R = Ac
Tabulalide B (**282**) R = COEt

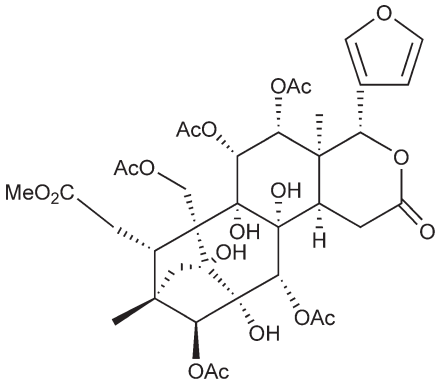


Tabulalide C (**283**) R = H
Tabulalide D (**284**) R = Ac

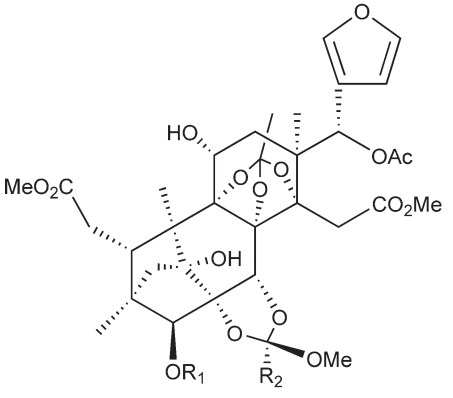
The first isolation of phragmalins from *Swietenia mahogany* (Meliaceae) revealed five new phragmalins called swietenialides A–E (**286–290**). All these are ring-D-opened phragmalin-type limonoids, yet all are only weak antifeedants against the third-instar larvae of *S. littoralis*.[123]

The Chinese mangrove, *Xylocarpus granatum* (Meliaceae), contains a large number of 8,9,30-phragmalin orthoesters, called xyloccensins.[124] These were isolated from either the seeds or the stem bark of the Chinese mangrove. In total 25 xyloccensins have been isolated, xyloccensins A–V, Y, Z1, and Z2.[124,125] Tested with a conventional leaf disk method against the third-instar larvae of *M. separata* (Walker), only two (xyloccensins P (**291**) and Q (**292**)) were active as antifeedants at a concentration of 500 ppm.[124]

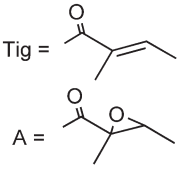
The acetone extract of the seeds of *Trichillia havanensis* contains large quantities of azadirone. Further research[126] showed that the azadirone fraction contains an additional compound in minute quantities. This compound was identified as 1β,2β;21,23-diepoxy-7α-hydroxy-24,25,26,27-tetranor-apotirucalla-14,20,22-trien-3-one (**293**) and was found to be equally active against the fourth-instar larvae of the CPB, *L. decemlineata*, as azadirone and both reduced feeding at 300 and 500 ppm.

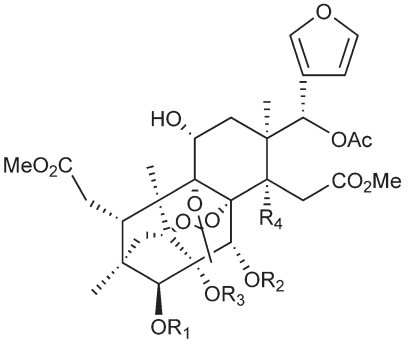


Tabulalide E (**285**)

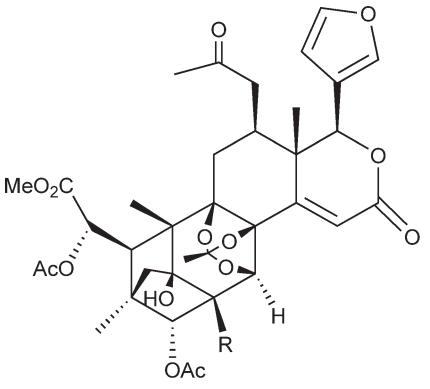


Swietenialide A (**286**) R_1 = Tig R_2 = CH_3
Swietenialide B (**287**) R_1 = Tig R_2 = CH_2CH_3
Swietenialide C (**288**) R_1 = A R_2 = CH_3

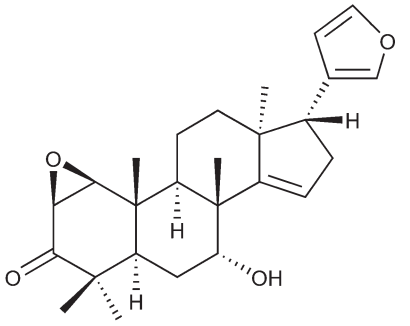




Swietenialide D (**289**) R_1 = A, R_2 = COEt, R_3 = R_4 = H
Swietenialide E (**290**) R_1 = A, R_2 = R_3 = Ac, R_4 = OH



291 R = OAc
292 R = OH



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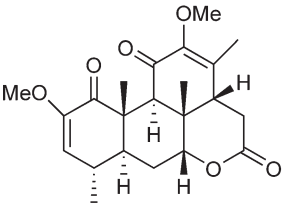
4.11.3.4.4 Quassinoids

For a century, powdered quassin wood of *Quassia amara* from South America or *Acrasma excelsa* from Jamaica (both Simaroubaceae) was a commercial insecticide. Both contain quassin (**294**) and related compounds.[2] The quassinoid glaucarubolone glucoside (**295**), previously reported as a potential fungicide,[127] was isolated from the crucifixion thorn *Castela emoryi* (Simaroubaceae) and found to have the same level of feeding deterrence toward the eastern subterranean termite, *Reticulitermes flavipes*, ED_{50} of 540 ppm, as azadirachtin with 587 ppm.[32]

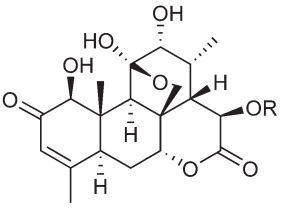
From the seeds and bark of *Samadera indica* (Simaroubaceae), indaquassin C (**296**) was isolated together with three additional quassinoids. Only **296**, present in 0.006% in seed kernels, exhibited moderate antifeedant activity toward *S. litura*, with 1 μg leaf cm^{-2}, resulting in 48.9% feeding inhibition.[128]

4.11.3.4.5 Cucurbitacins

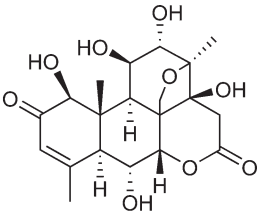
The cucurbitacins are the bitter principles of Cucurbitaceae, but are also found in five other plant families. Momordicine II (**297**), a mono-glucoside, was isolated from the leaves of bitter gourd, *Momordica charantia* (Cucurbitaceae), together with a di-glucoside and was identified as the 7-*O*-β-glucopyranoside of momordicine II (**298**). Momordicine II was a strong antifeedant against the armyworm *Pseudoletia separata*, causing a 45–60% reduction in feeding at concentrations of 0.1 and 0.5%. Momordicine II only exhibited a strong feeding inhibition at 2.5% when tested against *S. litura*. Overall, the di-glucoside **298** was a weak antifeedant against both the armyworms.[129,130]



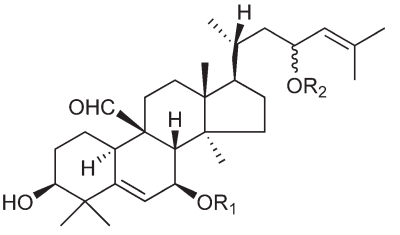
Quassin (**294**)



R = D-Glucose (**295**)



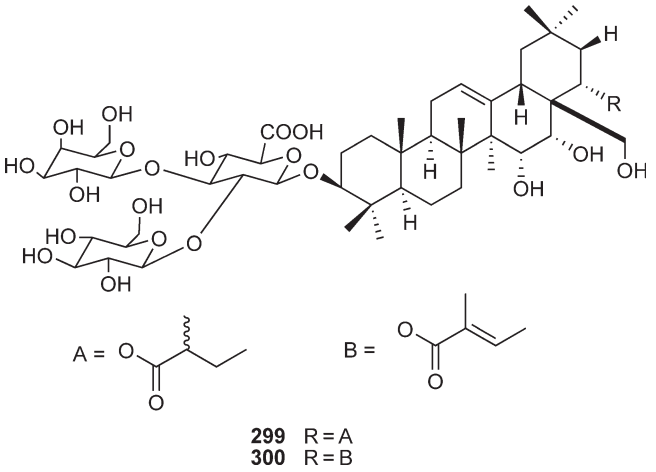
Indaquassin C (**296**)



Momordicine II (**297**) R_1 = H, R_2 = glc
298 $R_1 = R_2$ = glc

4.11.3.5 Saponins

Barringtonia asiatica (Lecythidaceae) grows extensively in coastal regions of tropical Asia and the Pacific, and it is also known as the 'fish killer tree', since ground seeds caused piscicidal activity when thrown into the water. Two major triterpenoid saponins have been isolated from the methanol extract of the seeds and these were identified as 3-*O*-{[β-D-galactopyranosyl(1→3)-β-D-glucopyranosyl(1→2)]-β-D-glucurono-pyranosyloxy}-22-*O*-(2-methylbutyroyloxy)-15,16,28-trihydroxy-(3β,15α,16α,22α)-olean-12-ene (**299**) and 3-*O*-{[β-D-galactopyranosyl(1→3)-β-D-glucopyranosyl(1→2)]-β-D-glucuronopyranosyloxy}-22-*O*-[2(*E*)-methyl-2-butenyloyloxy]-15,16,28-trihydroxy-(3β,15α,16α,22α)-olean-12-ene (**300**). Both exhibited antifeedant activity toward *Epilachna* larvae[131] with 100% feeding inhibition at 1000 ppm. The saponin containing the tiglate moiety (**300**) was more active than the saponin containing 2-methylbutyrate (**299**) at 500 ppm, with 63% inhibition compared with 54%. At 100 ppm only **300** was active.



4.11.4 Alkaloids

The enormous number and structural variety of plant alkaloids would lead one to expect them to be a rich hunting ground for antifeedants. Nevertheless, they seem to be relatively neglected compared to other groups. Their often high mammalian toxicity may be the reason.

The alkaloid dihydropinidine (**301**) is found in the needles of *Picea pungens* and the bark of *P. sitchensis*,[132] although the absolute configuration was not reported. After synthesis of two of the possible configurations (2*S*,6*R*)-dihydropinidine (**302**) and (2*R*,6*S*)-dihydropinidine (**303**), both the compounds were tested for antifeedant activity as their hydrochlorides[133] and both were strong antifeedants against *H. abietis*.

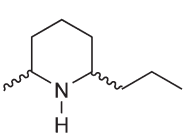
4.11.4.1 Pyrrolizidine Alkaloids

The more polar fraction of the methanolic extract of the aerial parts of *Senecio miser* deterred feeding of the CPB. Two pyrrolizidine alkaloids, **304** and **305**, were isolated and both had the same level of antifeeding activity against CPB.[57]

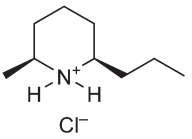
4.11.4.2 Glycoalkaloids

α-Solanine (**306**) from the potato, *Solanum tuberosum*, and the tomato *Lycopersicon esculentum* (both Solanaceae) and α-chaconine (**307**), also from the potato and other *Solanum* species, are known antifeedants toward snails, but recent tests showed that these two glycoalkaloids act synergistically. Tested alone, both compounds deterred feeding of the test snail, *Helix aspersa*, with chaconine (**307**) being more effective than solanine

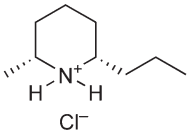
(**306**). But when they were tested as a mixture, the inhibition increased significantly more than that of each compound on its own.[134] At 0.2 mmol l^{-1}, chaconine inhibited feeding by 30% whereas 0.2 mmol l^{-1} of solanine did not affect feeding at all. Yet a mixture of solanine and chaconine, both at 0.2 m mol l^{-1} inhibited feeding by 60%. It is worth noticing that when the mixture was diluted and tested against the extract of the peel of the potato variety Home Guard, the 10 times diluted peel extract was still active as antifeedant, while the authentic glycoalkaloid mixture was not active at this dilution. This gives rise to the question whether other glycoalkaloids maybe present and also working synergistically.



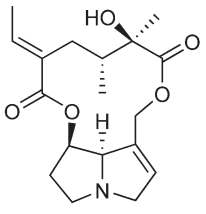
Dihydropininine (**301**)
(racemic mixture)



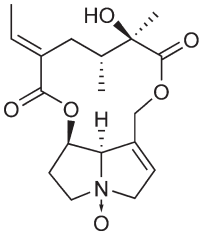
(2*S*,6*R*)-Dihydropinidine
hydrochloride (**302**)



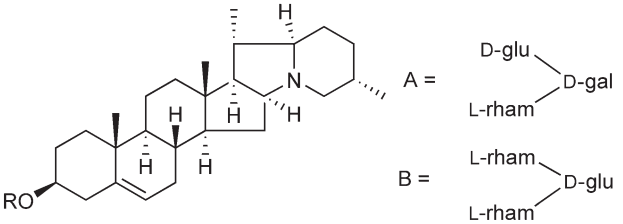
(2*R*,6*S*)-Dihydropinidine
hydrochloride (**303**)



Integerrimine (**304**)



Integerrimine N-oxide (**305**)



α-Solanine (**306**) R = A
α-Chaconine (**307**) R = B

4.11.5 Phenylpropanoids

Phenylpropanoids have an aromatic ring with a three-carbon substituent. Caffeic acid (**308**) and eugenol (**309**) are known examples of this class of compounds. Phenylpropanoids are formed via the shikimic acid biosynthetic pathway via phenylalanine or tyrosine with cinnamic acid as an important intermediate. Phenylpropanoids are a diverse group of secondary plant compounds and include the flavonoids (plant-derived dyes), lignin, coumarins, and many small phenolic molecules. They are known to act as feeding deterrents, contributing bitter or astringent properties to plants such as lemons and tea.

The pine weevil *H. abietis* is a serious pest of conifer seedlings. It has been observed that the pine weevil feeds less on the bark of *Pinus contorta* and more on *Pinus sylvestris*. This led to investigation of the bark of *P. contorta* for the presence of antifeedants. The ethyl acetate fraction of the methanol extract contained ethyl *trans*-cinnamate (**310**) as well as ethyl 2,3-dibromo-3-phenylpropanoate (**311**). Both the compounds exhibited high antifeedant activity at 50 mmol l^{-1} level; even after 24 h, the feeding inhibition for **310** and **311** was still 68 and 39%, respectively.[22] Ethyl cinnamate, which was absent from the extracts of *P. sylvestris*, is also found in plants from the Zingiberaceae[135] as well as in *Artemisia pallens*,[136] yet has not been identified as an antifeedant. Safrol (**312**), a presumed biogenetic precursor of lignans, was an active antifeedant against several storage pests, except for *T. confusum* adults.[137]

4.11.5.1 Lignans

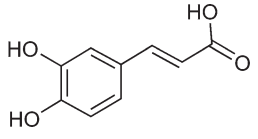
It was found that root bark, containing egg cavities with feces, were avoided by the pine weevil *H. abietis*, so its feces were investigated in an attempt to find ways to control the weevil. After fractionation by medium-pressure liquid chromatography (MPLC) and analysis with gas chromatography–mass spectroscopy (GC–MS)

and pyrolysis GC–MS, several compounds originating from lignin were identified as potent antifeedants, including *trans*-Anethole (**313**), 1,4-dimethoxybenzene (**314**), 1-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane (**315**), 1,4-dihydroxybenzene (**316**), and eugenol (**309**).[138] All of these lignans had strong antifeeding properties even after 24 h with AFI values of >31%. 1-(4-Hydroxy-3-methoxyphenyl)-3-methoxypropane (**315**) exhibited a feeding inhibition of 76% at 24 h after application. *Trans*-anethole (**313**) is also found in the anise plant *Pimpinella anisum* (Umbelliferae) and is toxic to *S. litura*[139] as well as to several beetles,[140] weevils,[141] mosquitoes, and moths.[142]

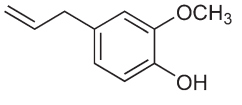
In order to find the best candidate for practical applications to protect conifer seedling against the pine weevil, a set of 55 benzoic acid derivatives were tested for their antifeedant activity.[5] This study centered on natural oxygenated aromatic compounds found in the bark of some conifers. It yielded five new highly effective antifeedants, methyl 2,4-dimethoxybenzoate (**317**), isopropyl 2,4-dimethoxybenzoate (**318**), methyl 2-hydroxy-3-methoxybenzoate (**319**), methyl (3,5-dimethoxyphenyl)acetate (**320**), methyl (2,5-dimethoxyphenyl)acetate (**321**), and methyl 3,5-dimethoxybenzoate (**322**). All these compounds reduced feeding by more than 77% at the application of 100 µl of a 50 mmol l^{-1} solution.

Yatein (**323**), isolated from the heartwood of the endangered Pacific island tree, *Libocedrus yateensis* (Cupressaceae), and cubebin (**324**), isolated from seeds of *Piper cubeba* (Piperaceae), were found to be strong antifeedants against several storage pests, *S. granarius*, *T. confusum*, and *Trigoderam granarium*. The semisynthetic hinokinin (**325**), synthesized from cubebin, was also a strong antifeedant against all the three storage pests.[137]

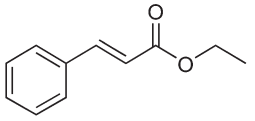
Two norlignans were isolated from the ethyl acetate fraction and found to be even more active. Sequirin-C (**326**) and agatharesinol (**327**) were very strong antifeedants against a snail species, *A. despesta*, deterring feeding by 91% at 30 µg cm^{-2} and by 90% at 40 µg cm^{-2}, respectively.[143]



Caffeic acid (**308**)



Eugenol (**309**)



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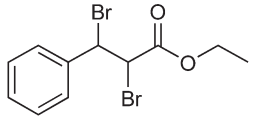

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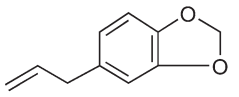
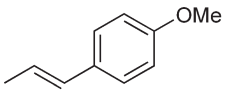
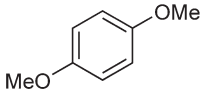


Safrol (**312**)



Trans-anethole (**313**)



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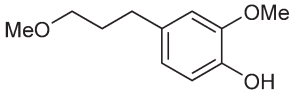

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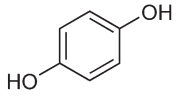

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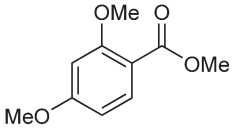

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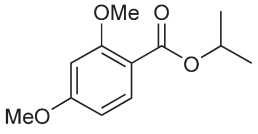

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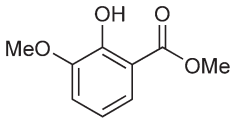

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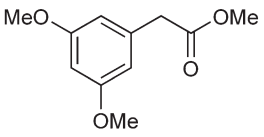

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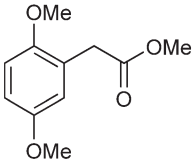

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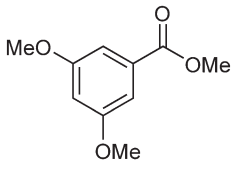

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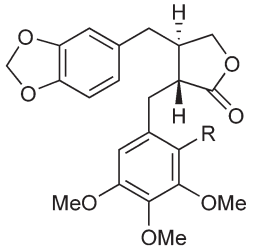

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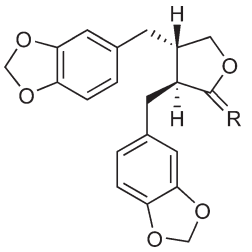
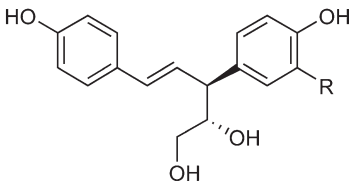


324 R = αH, βOH
325 R = O



Sequirin-C (**326**) R = OH
Agatharesinol (**327**) R = H

4.11.5.2 Furanocoumarins

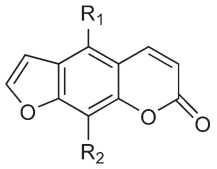
Earlier research had shown that the fruits of *Tetradium daniellii* (Rutaceae) contain several furanocoumarins. In the search for compounds with antiinsect properties, the chemistry of the fruits were investigated further to show that at least four of the compounds were insect feeding inhibitors. The compounds xanthotoxin (8-methoxypsoralen; **328**), bergapten (5-methoxypsoralen; **329**), also found in the aerial parts of *Pilocarpus goudotianus* (Rutaceae)[144] and isopimpinellin (5,8-dimethoxypsoralen; **330**) and bergamottin (5-geranyloxypsoralen; **331**), isolated from the fruits of *T. danielli*,[145] inhibited feeding by 92–100% at 1 mmol l^{-1} when tested against *S. littoralis* and *H. virescens*. Xanthotoxin (**328**) was also very effective against *Trichoplusia ni*.[146] Imperatorin (**332**), isolated from *P. goudotianus*, as well as angelicin (**333**) and psoralen (**334**), from *Psoralea glandulosa* (Leguminosae), all inhibited feeding of *S. littoralis*, yet were not as effective as xanthotoxin and bergapten.[144] Testing several of these compounds as binary mixtures indicated that there is synergism when at least 40–75% imperatorin (**332**) or 20–80% angelicin (**333**) is present.[144]

4.11.5.3 Coumarins and Quinones

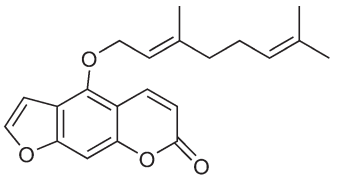
Plants of the genus *Cyperus* or sedges (Cyperaceae) include some common weeds found in upland and paddy fields in temperate to tropical regions. Two of these species, *Cyperus rotundus* and *Cyperus scariosus*, are used in traditional folk medicine. In addition, the fact that pests in these areas do not affect Cyperaceae suggests that they contain antifeedants. A study by Morimoto *et al.*[147] showed that several extracts of plants from the genus *Cyperus* were feeding inhibitors. The hexane extract from the basal stem of *Cyperus nipponicus* afforded large amounts of cyperaquinone (**335**) and its precursor remirol (**336**). Both exhibited good antifeedant properties against *S. litura*. Scabequinone (**337**) isolated from *C. distans* was an even stronger antifeedant with an ED_{50} 100 times lower.[147]

Coumaran (2,3-dihydrobenzofuran; **338**), a secondary metabolite also from *C. nipponicus*, inhibits feeding of phytophagous insects.[11] In the quest for finding additional antifeedants against *S. litura*, benzofuran derivatives were tested in a feeding bioassay. Natural remirol (**336**) and euparin (**339**), the latter isolated from the roots of *Eupatorium chinese*, inhibited feeding significantly, but the synthetic derivative, 7-acetyl-2-isopropenyl-4,6-dimethoxy-2,3-dihydrobenzofuran (**340**), had an ED_{50} value of 1.3 μg cm^{-2} against *S. litura*, that is among the most active antifeedants yet recorded.[11] Aurone (**341**), a pigment isolated from the heartwood of *Pterocarpus marsupium* (Fabaceae),[148] was found to be a strong antifeedant against the common cutworm, *S. litura*, with an ED_{50} of 0.12 μmol cm^{-2}.[149] Several derivatives of aurone exhibited antifeedant activity as well.[149]

The major constituent in the extract of madder, *Rubia tinctorum* (Rubiaceae), was identified as lucidin-3-*O*-primeveroside (**342**), a commonly used food pigment. It exhibited antifeedant activity against the carpet beetle, *Attagenus japonicus*.[150] This opens the window for using dyes from either *R. akane* or *R. tinctorum* to protect textile against these textile pests.



	R_1	R_2
328	H	OMe
329	OMe	H
330	OMe	OMe
334	H	H



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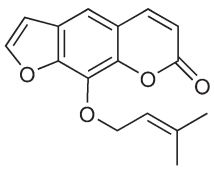

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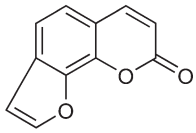
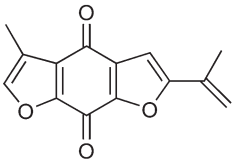
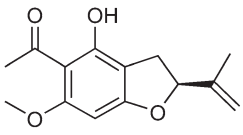


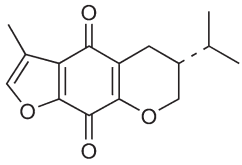
Angelicin (**333**)



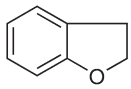
Cyperaquinone (**335**)



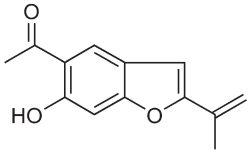
Remirol (**336**)



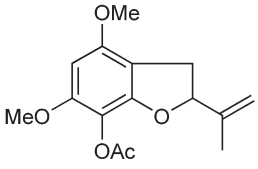
Scabequinone (**337**)



Coumaran (**338**)



Euparin (**339**)



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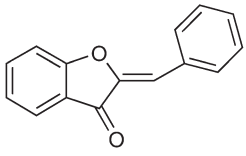

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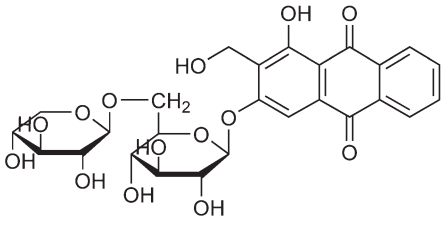


Lucidin-3-*O*-primeveroside (**342**)

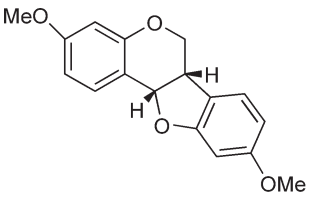
4.11.5.4 Flavonoids

Flavonoids are widely distributed in plants, and it is assumed that they are related to the resistance toward attacks by insects and fungi in several plant species. Timber from the large deciduous tree, *Pterocarpus macrocarpus* (Fabaceae), is used in making furniture and in construction of buildings, because it is resistant to termite attack. The dichloromethane extract from the heartwood of this tree yielded three pterocarpans, (–)-homopterocarpin **343**, (–)-pterocarpin **344**, and (–)-hydroxyhomopterocarpin (**345**). These three natural compounds as well as four additional semisynthetic compounds (**346–349**) were all found to be good antifeedants against the subterranean termite *Reticulitermes speratus*.[151] However, only the natural flavonoids inhibited feeding of the larvae of *S. litura*.

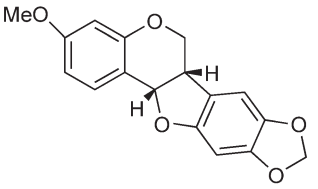
Several flavonoids were isolated from either the wood of the Japanese larch *Larix leptolepis* (Pinaceae) or from the wood of *Prunus* species.[152] In total, 14 flavonoids were tested for antifeedant activity against *C. formosanus*. Although all flavonoids did exhibit antifeeding behavior, there were large variations. Three compounds,

quercetin (**350**), taxifolin (**351**), and naringenin (**352**), were strong antifeedants, whereas others exhibited moderate antifeedant activity. Some conclusions could be drawn from these results in that the hydroxyl groups at C-5 and C-7 in ring A are important, as well as the presence for a carbonyl group at C-4 in the pyran ring. In addition, 3′,4′-dihydroxylated B-rings exhibited higher activity than those with 4′-hydroxylated B-rings.

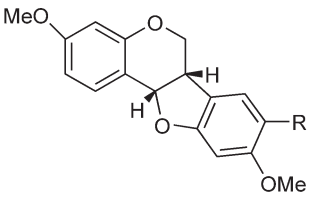
The polymethylated flavonoids 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**353**), 5-hydroxy-3,6,7,8,-tetramethoxyflavone (**354**), and 5,6-dihydroxy-3,7-dimethoxyflavone (**355**) and a chalcone, 4,4′,6′-trihydroxy-2′-methoxychalcone **356**, have all been isolated from cudweed, *Gnaphalium affine* (Compositae).[153] Although the flavonoids were present in small amount in the plant, they all exhibit high antifeedant activity against the common cutworm (*S. litura*) with ED_{50} values of 1.1×10^{-7} mol cm^{-2} for **353**, 2.0×10^{-8} mol cm^{-2} for **354**, and 2.5×10^{-8} mol cm^{-2} for **355**. The chalcone **356** was present in higher amounts, but had less activity (3.8×10^{-7} mol cm^{-2}).[153]



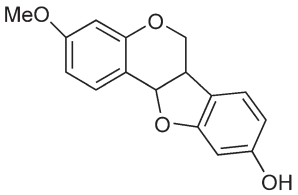
Homopterocarpin (**343**)



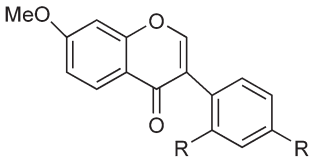
Pterocarpin (**344**)



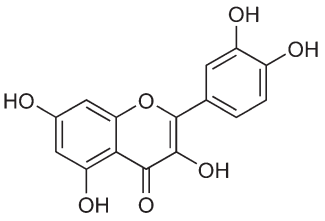
(–)-Hydroxyhomopterocarpin (**345**) R = OH
(–)-Methoxyhomopterocarpin (**346**) R = OMe



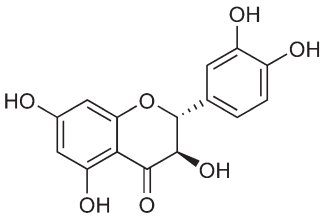
(±)-Isomedicarpin (**347**)



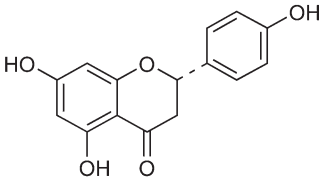
348 R = OH
349 R = OMe



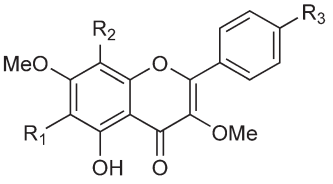
Quercetin (**350**)



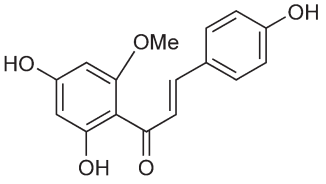
Taxifolin (**351**)



Naringenin (**352**)



353 $R_1 = R_2 = R_3 = OMe$
354 $R_1 = R_2 = OMe$, $R_3 = H$
355 $R_1 = OH$, $R_2 = R_3 = H$



356

4.11.6 Miscellaneous

4.11.6.1 Celastroidines

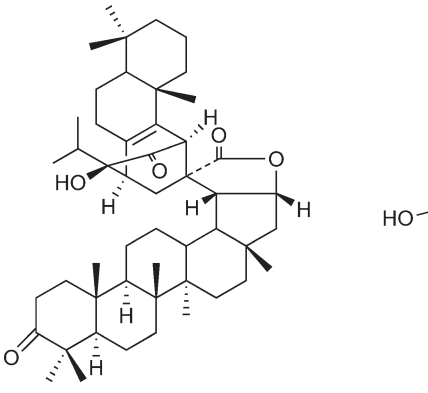
Although two novel celastroidines were isolated from the roots of *Hippocratea celastroides* (Celastraceae), only celastroidine A (**357**) strongly inhibited feeding by *Sitophillus zeamais*. Celastroidine A (**357**) is described as Diels–Alder adduct of a triterpene on a diterpene. It was isolated as a white powder.[154]

4.11.6.2 Cerebrosides

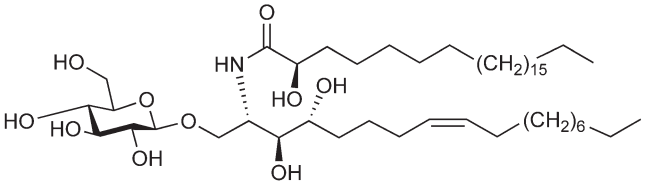
The cerebroside 1-*O*-β-D-glucopranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-*N*-(2′hydroxytetracosanoyl)-heptadecasphinga-8-ene (**358**) was isolated from the methanolic extract of the whole bodies of *Munronia henryi* (Meliaceae) and exhibited significant antifeedant activity against the larvae of *P. brassicae*, reducing feeding by 62 and 50% mortality.[155] Although two additional ceramides were isolated as well, neither of them exhibited antifeeding activity. A new weakly active antifeedant, the A,B-seco-tetranortriterpenoid lactam munroniamide (**359**), was also isolated.[155]

4.11.6.3 Cardenolides

Digitoxin (**360**) and cymarin (**361**) found in *Erysimum cheiranthoides* (Cruciferae) was previously found to be an active oviposition deterrent for two *Pieris* species.[156] Tested against the cabbage looper, *T. ni*, both were active antifeedants, reducing feeding by 50% at a concentration of 18.8 and 10.8 $\mu g\,cm^{-2}$, respectively.[146]



Celastroidine A (**357**)



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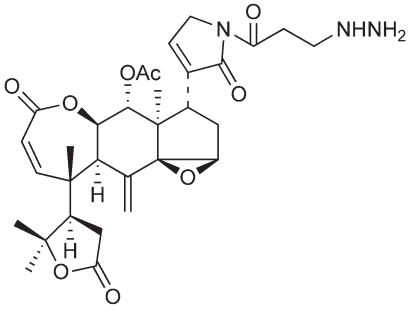
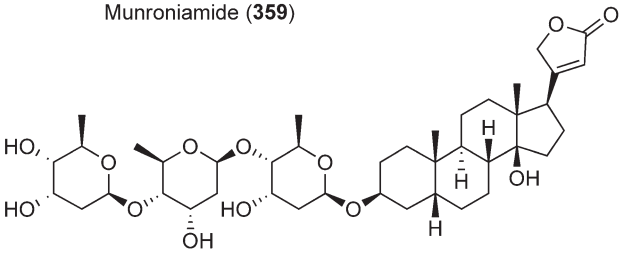
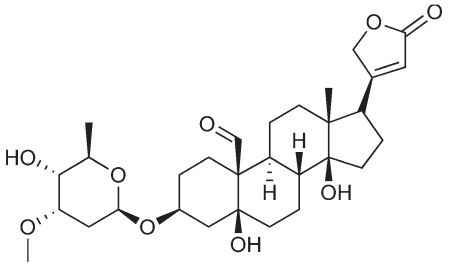


Munroniamide (**359**)



Digitoxin (**360**)



Cymarin (**361**)

4.11.6.4 Alkyl Derivatives

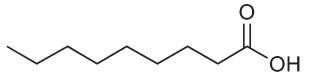
Nonanoic acid (**362**) isolated from the bark of linden *Tilia cordata* (Tiliaceae) possessed strong antifeedant activity against the pine weevil, *H. abietis*.[157] Several other long-chain acids were also tested for their antifeedant activity.[158] However, high activities were restricted to the smaller chain length, C-6–C-10, acids with nonanoic acid still the most active. Similarly, only the shorter 2-methyl branched alkanoic acids (up to C-10 chain length) were active as antifeedants. The C-9 alcohol, 1-nonanol (**363**), was also active.[158]

1-Hentriacontanol (**364**), a long-chain alcohol, was found in the leaf extracts of *V. negundo* (Verbenaceae) and deterred feeding by the larvae of the castor looper, *A. janata*,[91] with 78% feeding reduction after 24 h when tested at 10 $\mu g\,cm^{-2}$.

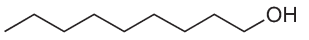
The gaur, *Bos frontalis*, a wild ox in India and other Asian countries, is known not to be troubled by biting arthropods, such as mosquitoes. Bovidic acids occur naturally only in the Bovidea family. 5-(1-Hydroxynonyl)-2-tetrahydrofuranpentanoic acid (an 18-carbon bovidic acid; **365**) was isolated from the gaur and tests showed that this compound acts as a feeding deterrent for the blood-sucking insect, *Aedes aegypti*.[159] Other bovidic acid analogues, 16-carbon and methyl esters, and enantiomers were all effective antifeedants, and as effective as the positive control *N*,*N*-diethyl-*meta*-toluamide (DEET), indicating that the absolute configuration of the furanoid ring is less important.

4.11.6.5 Cyclohexyl Derivatives

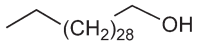
Kaempferia rotunda belongs to the Zingiberaceae family, perennial rhizomatous herbs important for their spices such as ginger and turmeric. Two polyoxygenated cyclohexane derivatives, (–)-zeylenol **366** and 2-acetylrotepoxide B **367**, were isolated among several others from *K. rotunda*. Both exhibited moderate antifeeding activity with a feeding inhibition of 50.8 and 42.8%, respectively, at 100 ppm against *S. littoralis*.[160]



Nonanoic acid (**362**)



1-Nonanol (**363**)



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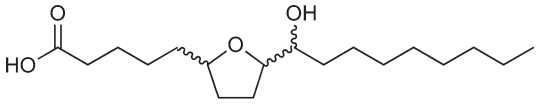

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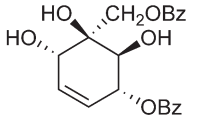
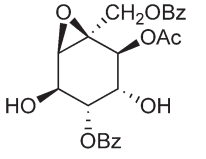


(–)-Zeylenol (**366**)



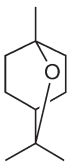
2-Acetylrotepoxide B (**367**)

4.11.7 Feeding Deterrence in Higher Animals

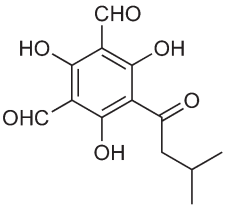
The search for effective feeding deterrents is not confined to arthropods and mollusks. There is also a search for deterrents against browsing animals and fruit-eating birds. Much damage to seedlings and saplings can be done by deer and elk. Deterring deer, moose, and caribou from grazing near roads to prevent accidents has been considered. The gray squirrel, *Sciurus caroliniensis*, can cause enormous damage to young trees of beech *Fagus sylvatica* (Fagaceae) and hornbeam *Carpinus betulus* (Corylaceae) in Europe. Many of these studies as yet have been made with crude products, such as feline urine.

Terpenes from *Eucalyptus* are thought to be involved in feeding deterrence of Australian possums, the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trochosurus vulpecula*). 1,8-Cineole (**368**) was identified as one of the active terpenes.[161] Diet with added jensenone (**369**), a diformylphloroglucinol compound (DFPC), was consumed less than the control diet.[161,162] Derivatives of jensenone (**369**)

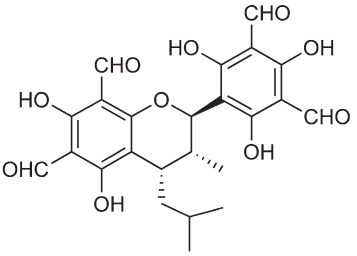
resulted in different feeding inhibition, leading to the conclusion that the aldehyde groups attached to the aromatic ring of jensenone were important for the activity, while the phenol groups (hydroxy groups attached to the ring) only play a minor role.[162] The dry matter intake by the common ringtail possum was also significantly reduced when sideroxylonal (**370**) was added.[163] Macrocarpal G (**371**) was the first compound isolated from *Eucalyptus* that exhibited feeding inhibition of any marsupial folivore. Macrocarpal G is an adduct of a DFPC and a bicyclogermacrene[164] and resulted in a 90% reduction of food intake at 2.1% of dry matter.



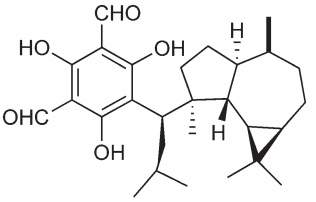
1,8-Cineole (**368**)



Jensenone (**369**)



Sideroxylonal A (**370**)



Macrocarpal G (**371**)

4.11.8 Conclusion

The many differently conceived bioassays, and the different ways of expressing results, makes comparison between compounds difficult. While some SAR have been established among groups of similar compounds, there is no general understanding of what affects palatability for phytophagous insects. No truly effective antifeedants for birds or higher animals have yet been found that might stimulate greater activity in this field of investigation.

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Biographical Sketches

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David Morgan is a member of the Chemical Ecology Group at Keele University. He was born in Newfoundland and had his university education there, at Dalhousie University and University of King's College in Halifax, Nova Scotia, and at Oxford. His doctorate thesis was on the lipids of *Mycobacterium tuberculosis*. He later worked at the National Institute for Medical Research in London, and for Shell Chemical Company and Shell Research under the direction of Sir Robert Robinson, O.M., Nobel Laureate. From 1966 he has been at Keele in Staffordshire as lecturer, senior lecturer, reader, and professor. He discovered the natural pesticide azadirachtin and collaborated with S. V. Ley for its final structure elucidation. He is the author of over 300 papers and reviews, mostly on insect chemistry, editor, and contributor to several volumes and author of the book 'Biosynthesis in Insects.'

4.12 Marine Natural Products as Antifeedants

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4.12.1 Introduction

The structural diversity and stereochemistry of secondary metabolites isolated from marine sources, together with issues of biosynthesis and ecology, has commanded the attention of the natural products community for close to 50 years. A good body of information has accumulated that marine organisms, particularly when soft-bodied and not well physically defended, may accumulate these metabolites to protect themselves from predation in the competitive environment underwater. The topic of antifeedants was summarized by Kobayashi and Ishibashi[1] in the earlier series of *Comprehensive Natural Products Chemistry* in a chapter on marine chemical ecology. This chapter updates the chemical strategies used by marine plants and animals to prevent predation by other species, and is based on published literature from 1999 onwards. The examples selected are representative of ecological interactions for which the chemical basis has been well defined; for this reason, reports that survey the feeding deterrency of crude extracts alone are not considered unless the data illustrate major ecological trends. Some well-understood examples of predator–prey interactions from the pre-1999 literature that were not described by Kobayashi and Ishibashi are also included.

The biosynthetic origin of marine natural products is a complex topic that has gained more clarity through application of molecular biological study; microbial symbionts may represent the true source in many sponges, ascidians, and bryozoans, even in cases in which localization studies have suggested an invertebrate source for

the compound. There are many documented examples where marine metabolites have accumulated in mollusks through predation or through complex food webs. In this review, individual topics that cross phylogenetic boundaries are cross-referenced to other sections.

Some of the metabolites described in this chapter have undergone structural or stereochemical revision since the ecological study was published. The chemical structures used have been derived from a recent compendium of marine natural product structures,[2] and so in some cases differ from the structures provided in the original article. The ongoing review series on 'Marine Natural Products' in *Natural Product Reports* should be consulted for definitive coverage of marine natural products; there were 18 reviews written between 1984 and 2002 by the late D. John Faulkner[3] and more recent coverage from 2003 onwards is provided by Blunt *et al.*[4] A number of major reviews on marine chemical ecology have appeared that discuss the topic of marine antifeedants.[5–9]

4.12.1.1 Experimental Methodology

Field observations underwater give rapid qualitative insights into predator–prey interactions. For quantitative work, crude extracts or purified metabolites are incorporated into suitable food at ecologically sound concentrations and provided to the test consumer in either field or laboratory (aquarium) assays. Measuring the concentrations of selected metabolites by HPLC is a convenient quantification method, and should be carried out prior to ecological study.[10] The choice of concentration is important since studies have revealed that deterrency can vary with concentration.[11] Where possible, assays should be conducted at volumetric concentrations rather than gravimetric concentrations since the assay food then more closely models natural tissue.[12–14] Deterrency outcomes can differ depending on whether the assays are conducted on a tissue mass or tissue volume basis.[14] Compounds should be freshly isolated since chemical changes during isolation or storage may affect deterrency properties.[15–19] Sample decomposition during artificial food preparation has also been reported.[20]

Metabolites may be incorporated into agar, alginate, or carrageenan blocks or strips that contain additives for nutritional and palatability requirements. The artificial food must have a similar nutritional value and color to the natural food; higher quality foods may be preferred even if an antifeedant is present, while some consumers, particularly fish, may use visual cues to determine food choices.[21]

Flavoring agents for algal work are often freeze-dried ground seaweeds. *Ulva* spp. are commonly used since this seaweed is known to lack chemical or structural defenses that might interfere with the assay. The seaweed is soaked in a diethyl ether solution containing the compound at the selected concentration, and solvents then removed *in vacuo*. Control foods contain seaweed soaked in solvent alone. Food strips are prepared by adding control or treated seaweed to hot agar, and pouring the mixture onto a plastic screen mesh. After cooling, the mesh can be cut into pieces. The amount of food eaten is assessed by counting the number of mesh squares that have been cleared of food.[11,22,23] An alternative protocol involves pouring food mixture into cube-shaped molds, then attaching the food cubes onto ropes for underwater studies.[24]

For assays that involve carnivorous consumers, freeze-dried brine shrimp, squid, tuna, urchin roe, and krill are typical flavoring and nutritional additives while agar, carrageenan, or calcium alginate have all been used as support matrix. Strips are prepared by heating, for example, carrageenan with deionized water and brine shrimp, adding the chemical of interest dissolved in an appropriate solvent, and then pouring the mixture into a plastic mold containing lengths of cotton string. After cooling, the strips are cut to size with a scalpel, and attached to ropes for deployment in the field or in large aquaria.[10,12] In assays involving consumers such as sea hares that require high levels of protein in their diet, catfish food can be incorporated into the cubes to provide sufficient protein.[11,25] The significance, if any, of a physical defense can be assessed by incorporating spicules or sclerites into the food cubes or strips; these are obtained prior to the ecological study by soaking sponge or coelenterate tissue in bleach followed by thorough rinsing.[12,26]

Assays may be of short-term exposure, usually a few hours duration, or involve extended or repeated exposure to the compound of interest that assesses learning/avoidance behavior in consumers. Assays conducted underwater should be monitored by observers or by video recording;[27] it can be valuable to record the fish species involved in feeding experiments with natural populations of reef fish.

In some palatability (choice) assays, fish are first trained to consume food particles; in the assay, pieces of dried brine shrimp or food pellets coated with extract are offered alternately to controls and fish behavior

(consumption, regurgitation, avoidance) is then monitored.[12,28,29] A convenient method of making pellets involves loading an alginic acid mixture containing the metabolite into a syringe, then extruding it into a 0.25 mol l^{-1} solution of $CaCl_2$ to harden into 'spaghetti' strands, which can be cut to the desired length.[13,29] A review article provides complete detail of feeding deterrency assay methods.[22]

4.12.2 Microorganisms

The antifeedant roles of marine microbial products have not been extensively explored. The best documented cases involve cyanobacterial metabolites.

Phytoplanktonic microalgae, which are important sources of food in both oceans and fresh water habitats, use an activated form of chemical defense to reduce grazing by predators. Damaged microalgal cells convert unsaturated fatty acids into unsaturated aldehydes which affect reproductive outcomes in herbivorous copepods and other planktonic grazers.[30] Representative products of these biotransformations include the C_{10} aldehydes **1** and **2** in the diatom *Thalassiosira rotula*, and C_8 diene hydrocarbons and the trienoic acid aldehyde **3** in *Asterionella formosa*.[31]

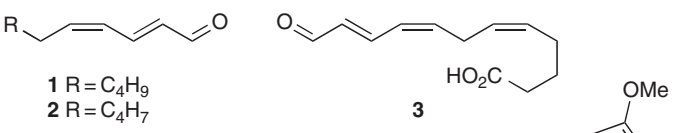
4.12.2.1 Cyanobacteria

Cyanobacterial blooms pose a public health risk and may have adverse effects on marine life (fish, turtles) that accidentally consumes them. The production of toxins or of antifeedant compounds, particularly in cyanobacterial strains that are slow growing can lead to rapid bloom production in areas of high herbivory, for example on tropical coral reefs.[8]

The cyanobacterial strain *Lyngbya majuscula* is a rich source of bioactive compounds that are often nitrogen functionalized. The presence of toxic compounds in *L. majuscula* combined with low palatability deters predation, however sea hares, notably *Stylocheilus* spp., are specialized feeders that sequester deterrent compounds from *Lyngbya* spp. although it is not clear whether this provides them an ecological advantage (see Section 4.12.8.1).

The cyanobacterial metabolites malyngamides A **4** and B **5**, majusculamides A **6** and B **7**, and malyngolide **8** all deter feeding by reef fishes.[32–34] Malyngamide A, malyngamide B, a mixture of majusculamides A and B all deter feeding by the pufferfish *Canthigaster solandri* and by the crab *Leptodius* spp. Although there were no significant differences in palatability of the cyanobacterial metabolites, malyngamide A was found to be more toxic than malyngamide B in assays using sea urchin embryos or brine shrimp nauplii, while the majusculamide mixture was less toxic than either malyngamide metabolite.[33] In Guam, the sea hare *Stylocheilus longicauda* is frequently found feeding on mats of *L. majuscula* that contain the majusculamides and malyngamides. When 13 different cyanobacterial metabolites were tested in feeding assays using an artificial diet, it was found that microcolin B **9** at 0.022%, ypaoamide **10** at 0.5%, and malyngolide **8** at 0.4% all deterred feeding by *S. longicauda*; these are close to natural concentrations. In contrast, the metabolites curacin A **11** and debromoaplysiatoxin **12** did not show significant feeding deterrency, while barbamide **13** stimulated sea hare feeding at the very low concentration of 0.007%. The malyngamides and majusculamides were attractants at low concentrations (0.5%) but deterred feeding at 4.0%, a concentration that may be above natural. The concentrations of the malyngamide and majusculamide metabolites in *L. majuscula* are highly variable, generally ranging up to 2% of the dry extracted mass. The feeding preferences of the sea hare *S. longicauda* may be concentration- and cue-dependent.[11] In aquarium assays, when tested at the natural concentration of 0.7%, ypaoamide **10** was deterrent to juvenile rabbitfish (*Siganus spinus* and *S. argenteus*), and to the parrotfish *Scarus schlegeli* and the sea urchin *Echinometra mathaei*.[35]

Crude extracts were prepared from an Australian strain of *L. majuscula* containing lyngbyatoxin A **14** and debromoaplysiatoxin **12** and used in laboratory assays in Guam to assess their palatability. The extracts were deterrent to a range of generalist predators (amphipods, sea urchins, crabs) and to natural populations of reef fish that would not have been previously exposed to these compounds since **12** and **14** are not detected in Guam samples. Extracts stimulated feeding by *Stylocheilus striatus*.[19] The cyclic peptide pitipeptolide A **15** isolated from a specific strain of *L. majuscula* deterred feeding in the sea urchin *E. mathaei*, in an algal-dwelling crab, and in two species of amphipods. However, the sea hare *S. striatus* consumed artificial food containing the peptide.[23]



1 R = C_4H_9
2 R = C_4H_7

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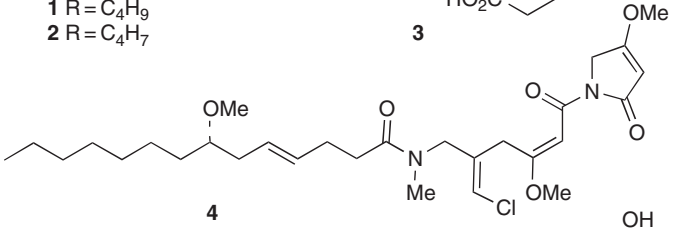

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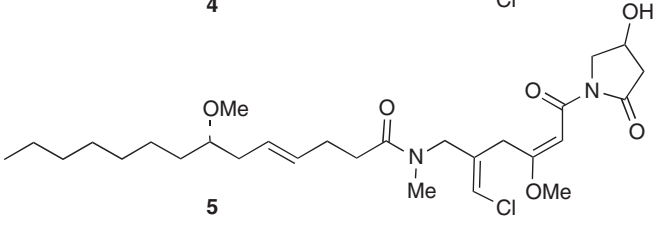

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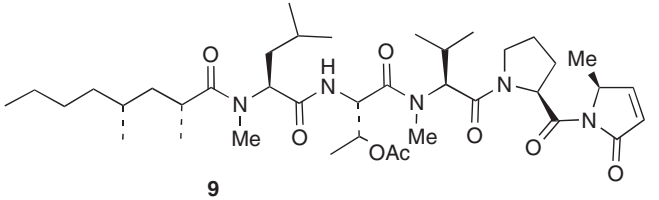

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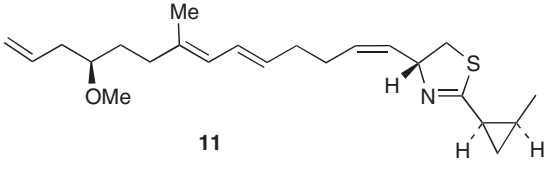

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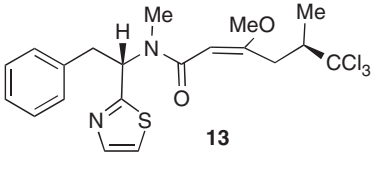

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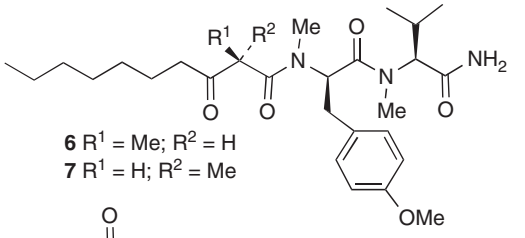
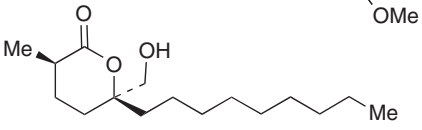


6 R^1 = Me; R^2 = H
7 R^1 = H; R^2 = Me



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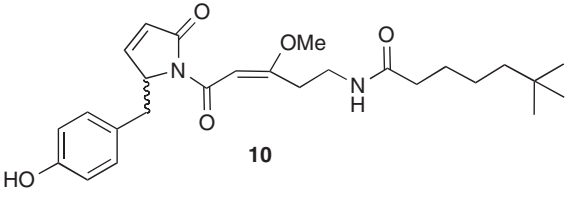

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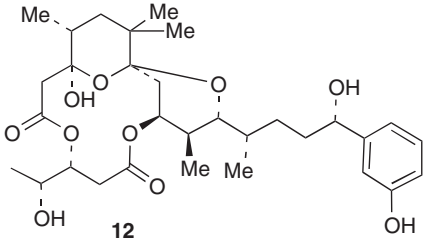

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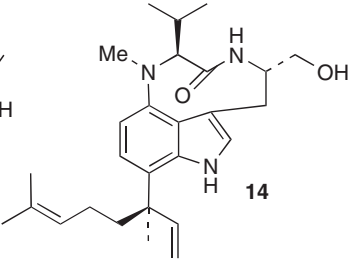

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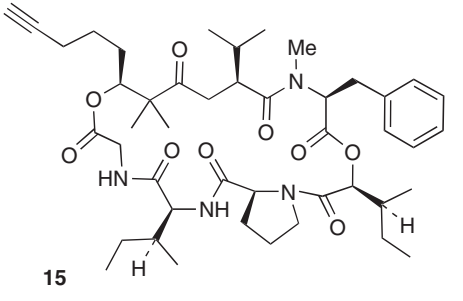

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4.12.3 Algae

Marine algae contain a structural diversity of metabolites and may play a variety of ecological roles, including as feeding deterrents against fish, sea urchins, mollusks, sea stars and crabs, and as antifouling agents. The high energetic cost of producing and maintaining novel biosynthetic capability, plus the need to compartmentalize the bioactive products, is compensated for by a defensive benefit to the host plant. Algal chemical defenses are often localized in key plant parts such as reproductive or actively growing tissue. The topic of algal chemical defenses has often been reviewed.[5,6,36]

In some seaweeds, levels of an existing deterrent molecule increase in response to an environmental cue or external stress; this is described as an 'induced' defense. A number of seaweeds use 'activated' chemical defenses, in which damage to the seaweed leads to rapid production of a more deterrent compound from a precursor molecule. A general introduction to activated chemical defense in seaweeds has been provided by Cetrulo and Hay.[18] They screened chemical extracts from algae collected from tropical and temperate waters for their deterrency against sea urchin and fish predators. Assay results were compared with data from algae whose tissues were ground prior to extraction. Of the 42 species tested, 7 species became less palatable on injury while 4 species became more palatable. Algae from both tropical and temperate regions showed activated chemical defenses; no taxonomic pattern was apparent since examples of both green algae (family Halimedaceae) and brown algae (Dictyotaceae) were deterrent, yet other species from these families were not deterrent. The rapid change in chemistry that can occur on cutting or crushing seaweeds has many implications, not least in quantitative studies of metabolite composition.

Tropical seaweeds are often well defended owing to high levels of predation by fishes on tropical reefs;[18,37] however, coldwater seaweeds also use chemical protection, mainly against sea urchins and crabs. A number of macroalgae from the Antarctic subcontinent possess chemical extracts that are deterrent to sea stars,[38,39] amphipods,[39,40] or fish,[39] while Arctic algae have been screened for deterrency toward sea urchins and amphipods.[41] Chemical defenses have also been detected in seagrasses, notably in the Mediterranean *Posidonia oceanica* whose extracts deter feeding by sea urchins and fish.[42]

As discussed in Section 4.12.8.1, sea hares are specialist feeders that have adapted to eat a range of macroalgae; their feeding behavior is often not influenced by the specific composition or concentration of metabolites.[43,44]

The following sections describe a selection of algal metabolites whose antifeedant effects have been investigated in some detail.

4.12.3.1 Terpenes

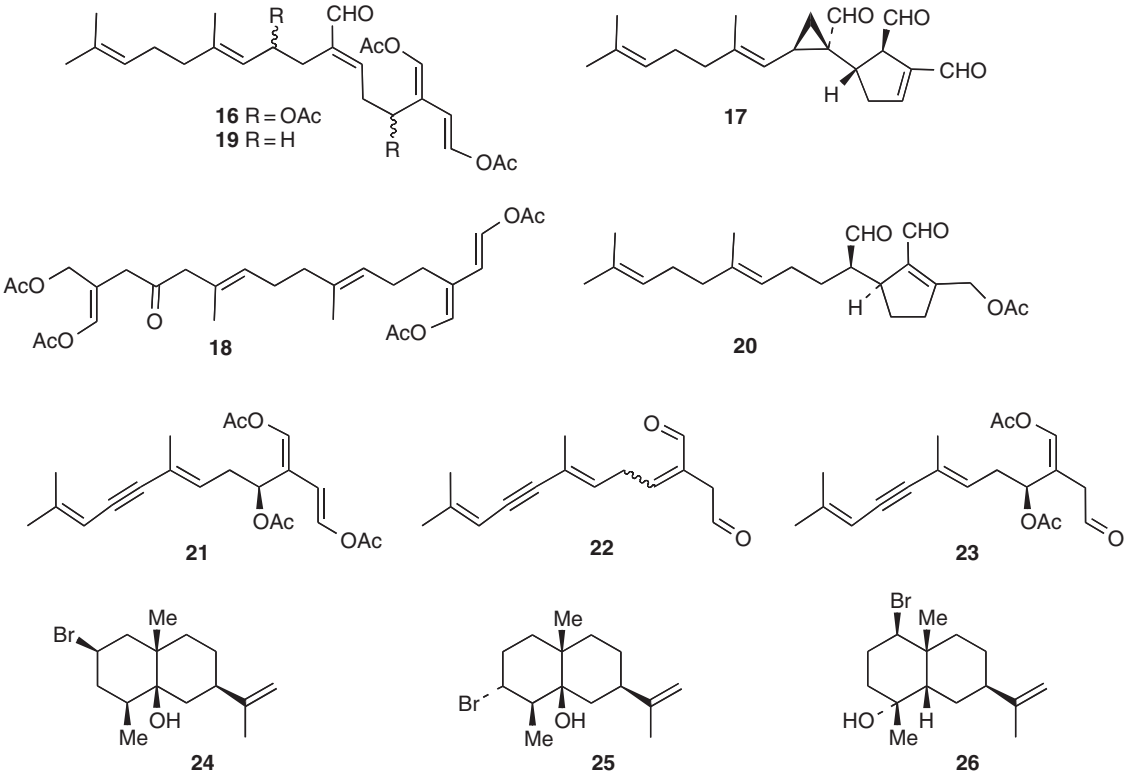
A survey of algae that are deterrent has identified chemical defenses based on terpenoid compounds.[37] *Halimeda* spp. are calcified green algae, commonly found on coral reefs, which produce diterpenoids that function as effective feeding deterrents against natural populations of reef fish.[36,45] Halimedatetraacetate **16**, whose enol acetate groups are masked aldehyde groups, is enzymatically converted into the trialdehyde halimedatrial **17** on plant damage. Halimedatrial is present in newly produced uncalcified tissue while the less deterrent halimedatetraacetate is present in older, more calcified tissue. The precursor **16** and the esterase enzymes involved may be separately compartmentalized in intact tissue in order to prevent self-toxicity.[46] There is evidence that the alga adjusts its metabolite composition in response to levels of herbivory in different habitats.[47] The 1,4-diacetoxybutadiene unit is found in the diterpene chlorodesmin **18**, which is an effective feeding deterrent in *Chlorodesmis* spp.,[37,48] and in udoteal **19** that may be transformed into petiodial **20** in *Udotea flabellum*.[45]

The sesquiterpene caulerpenyne **21** from *Caulerpa* spp., which also contains enol acetate functionality, is ineffective as a deterrent against fish despite being concentrated in plant parts prone to herbivory,[49,50] but is effective against the gastropod mollusk *Dolabella auricularia*[51] and the sea urchin *Echinometra lucunter*.[50] When *Caulerpa* tissue is damaged, rapid conversion of caulerpenyne into the unstable dialdehyde oxytoxin-2 **22** occurs through the action of esterases.[52,53] Both **22** and the related oxytoxin-1 **23** have also been isolated from mollusks, in which their chemical defense role has been further evaluated (see Section 4.12.8.2). In *Caulerpa*

spp., dialdehyde **22** crosslinks with the nucleophilic groups of proteins to form a polymeric plug that seals wounded tissue. Externally added nucleophiles compete with the algal proteins for **22** and suppress polymer formation.[54]

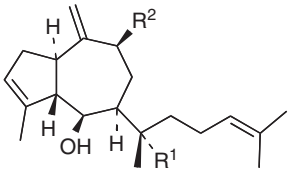
The calcified green alga *Neomeris annulata* manufactures a range of brominated sesquiterpenes **24**–**26**, which are concentrated in the fleshy tips of this algae,[55] and which are individually deterrent to parrotfishes and the sea urchin, although a mixture of the three metabolites does not increase deterrency.[56]

These various examples all demonstrate that green algae use both chemical and physical defenses against predators.[24,51,55]

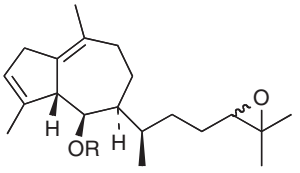


In brown algae, variable patterns of deterrency against herbivores are apparent even for closely related diterpenoid metabolites such as the metabolites pachydictyol-A **27**, and dictyol B acetate **28** from *Dictyota ciliolata*[57] and dictyol-E **29** from *D. menstrualis*.[58,59] The terpenes acutilol A **30**, acutilol A acetate **31**, and acutilol B **32** from *Dictyota acutiloba* showed contrasting effects in feeding studies using tropical and temperate fishes or sea urchins.[60] Small sedentary grazers such as amphipods often select chemically defended seaweeds as host plants to avoid predation by reef fishes. In the brown algae *D. menstrualis*, grazing by amphipods induces increased concentrations of dictyol metabolites and makes the seaweed less susceptible to attack by other predators.[59]

Recent examples of novel terpenes whose isolation was bioassay guided include the diterpenes dictyol H **33** and epoxypachydictyol A **34** from different collections of the Brazilian *Dictyota mertensii*, the meroterpenoid atomaric acid **35** from *Stypopodium zonale*. All these compounds deterred feeding by the crab *Pachygrapsus transversus*.[61,62] The major diterpene **36** from *Dictyota pfaffii* deters the sea urchin *Lytechinus variegatus* and reef fishes, but not *P. transversus*.[63] The two hydroxylated dictyodial metabolites **37** and **38** from *D. menstrualis* were identified following bioassay-guided fractionation using the amphipod *Amphithoe longimana*.[64] Menzoquinone **39** from the Antarctic brown alga *Desmarestia menziesii* deters feeding by sea stars.[38]



27 $R^1 = R^2 = H$
28 $R^1 = H$; $R^2 = OAc$
29 $R^1 = OH$; $R^2 = H$



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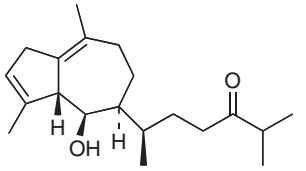
31 R = Ac



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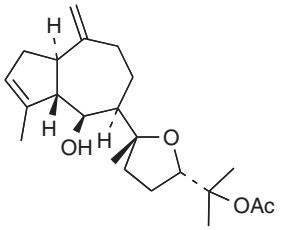

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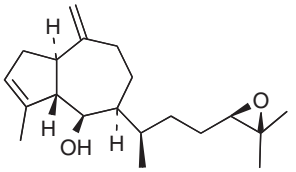

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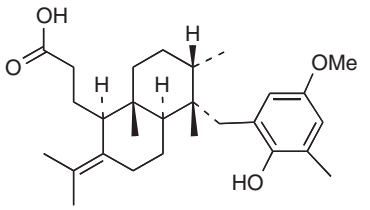

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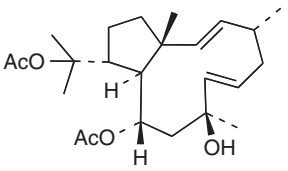

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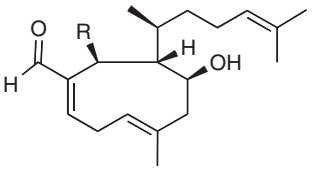

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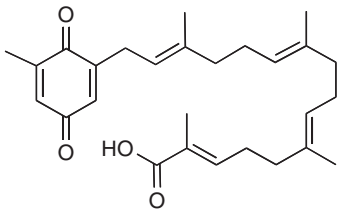
38 R = CH_2OAc



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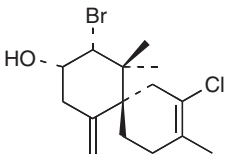

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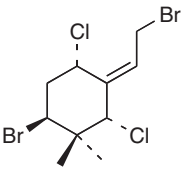

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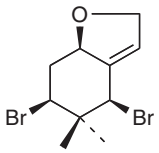

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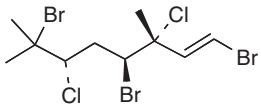

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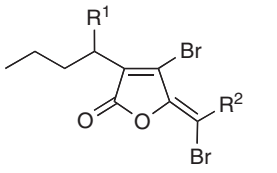
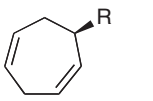
Elatol **40** isolated from several species of the red algal genus *Laurencia* deters feeding by reef fish, the crab *P. transversus*, and the sea urchin *L. variegatus*.[65] In red alga such as *Ochtodes secundiramea* and *Desmia hornemanni*, halogenated monoterpenes including ochtodene **41** act as effective feeding deterrents against various fishes, and against natural populations of herbivorous fish, however the structurally related chondrocole C **42** is not deterrent against reef fish. The individual terpenes **41** and **42** are not effective deterrents of amphipod feeding, whereas an unresolved monoterpene mixture from this alga is deterrent.[37,66] Halogenated terpenes, including anverene **43**, from *Plocamium cartilagineum* collected in Antarctica deter feeding by sea stars or amphipods.[38]

4.12.3.2 Other Acetate-Derived Metabolites

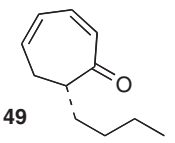
The temperate red alga *Delisea pulchra* produces a number of halogenated furanones known as the fimbrolides **44–47** that collectively defend it from a range of predators including sea urchins, gastropods, some amphipods,[67] and sea stars.[38] Metabolite **44** is a pronounced fish feeding deterrent at natural concentrations,[68] but does not deter feeding by the amphipod *Ampithoe ngana*,[67] while **45** has no deterrent effect when tested above natural concentration.[68] Although there is little variation in secondary metabolite levels in different plants of this algae, there can be large variations in individual metabolite levels; metabolites are also concentrated in the plant tips.[68]



44 R^1 = OAc; R^2 = H
45 R^1 = OH; R^2 = H
46 R^1 = H; R^2 = Br
47 R^1 = R^2 = H



48 R = $(CH_2)_3CH_3$



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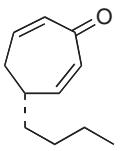

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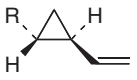

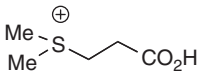

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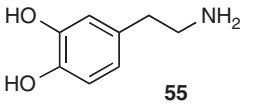

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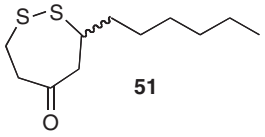
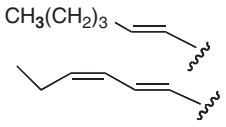
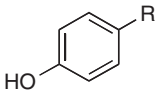


51

52 R =
53 R =





56 R = CHO
57 R = OMe

In many brown algae, long-chain fatty acids are converted into C_8 or C_{11} acyclic or cyclic hydrocarbons which act as pheromones in sexual reproduction; some of these hydrocarbons, and their decomposition products, also provide some protection from predation. Dictyotene **48** and its oxidative degradation products **49** and **50** deter feeding by the amphipod *A. longimana*, but not by the sea urchin *Arbacia punctulata*.[69] The sulfur-functionalized C_{11} compound **51** of *Dictyopteris membranacea* strongly deters feeding by the mesograzer *A. longimana*, but does not affect grazing by the sea urchin *A. punctulata*.[70] A 1:2 ratio of the hydrocarbons dictyopterene A **52** and -B **53** from *Dictyopteris delicatula* has been shown to deter grazing by reef fishes but has no effect on grazing by amphipods.[71]

4.12.3.3 Other Algal Metabolites

Activated chemical defenses based on dimethylsulfoniopropionate (DMSP) **54** are widely distributed among many species of green, brown, and red algae. However feeding deterrency trials have given inconsistent results; neither DMSP nor the two conversion products dimethyl sulfide and acrylic acid deter feeding by the sea urchin *E. lucunter*,[50] although artificial foods containing dimethyl sulfide or acrylic acid are avoided by the sea urchin *Strongylocentrotus droebachiensis*.[72] DMS, acrylic acid and triethylamine are not deterrent to the amphipod *A. longimana* when tested individually, yet in combination are deterrent at natural concentrations.[73]

The Northeastern Pacific green algae *Ulvaria obscura* uses dopamine **55** as a feeding deterrent.[74] Two simple aromatic compounds *p*-hydroxybenzaldehyde **56** and *p*-methoxyphenol **57** from the red algae *Myriogramme smithii* deter feeding by the sea stars *Perknaster fuscus* and *Odontaster validus*.[38]

Brown algae are rich in phlorotannin components that play an important role as a structural support and as photoprotective agents. A range of herbivore responses to phlorotannins has been described,[75] most recently in *Fucus vesiculosus*[76] in which a bioassay-guided fractionation revealed that a polar galactolipid contributed to feeding deterrency.[77] Increased phlorotannin levels may be induced by environmental cues and by injury or predation. Several reviews cover this specialized topic in considerable detail.[7,9,78,79] Some brown algae are chemically defended by the presence of sulfuric acid within cell vacuoles.[80]

4.12.4 Sponges

Marine sponges are conspicuous sessile members of the benthic fauna, and often brightly colored or soft bodied. Field observations suggest that they are avoided by fishes, crabs, and other generalist predators, although certain groups of mollusks, in particular the nudibranchs, are specialized sponge eaters. There is a considerable

body of experimental evidence that marine sponges are protected by the presence of deterrent chemicals, and this has been well summarized in recent literature.[6,9,81,82]

The most detailed field studies have been carried out on Caribbean sponges. Pawlik *et al.*[83] surveyed the deterrency of sponges collected from a range of habitats toward *Thalassoma bifasciatum*; the study found no correlation between sponge color and deterrency, indicating that the conspicuous color of many sponges is not a warning signal to predatory fish on Caribbean reefs. In this study, 69% of the 71 sponges screened were deterrent.[83] In later surveys, out of 30 Caribbean sponges screened against the hermit crab *Paguristes punticeps*, 26 samples (87%) were deterrent;[84] some extracts were also deterrent to sea stars.[85]

A number of studies highlight the critical relationship between predator density and deterrency, a topic that was first addressed by Bakus and Green,[86] who considered that tropical marine invertebrates should show a higher incidence of deterrency. When compared to Caribbean data, a survey of 16 Bermudan sponges found a lower incidence of deterrency against fish, ascribed to lower levels of predation in these waters.[87] A collection of 40 sponges from near San Diego yielded only 11 extracts that were strongly bioactive, although it should be noted that this study did not include deterrency data.[88] Numerous sponge extracts showing very high levels of bioactivity have been documented from the oceans around Southern Australian and New Zealand,[4] although only a few sound ecological studies have been conducted. For example, in South East Australia, habitats in which sea urchin density is high have been found to contain more deterrent sponges than habitats with lower numbers of sea urchin predators.[89] The most recent data on tropical versus temperate sponge defenses is from a survey of 20 sponges from 14 different genera that were collected from either Guam or from North East Spain. In assays against different types of reef fish, 35% of the sponges were deterrent in at least one assay, and defenses from tropical and temperate sponges were equally effective.[90] Perhaps the best evidence that cold water sponges have deterrent extracts is provided by studies on Antarctic sponges that involve sea stars and other predators.[91,92]

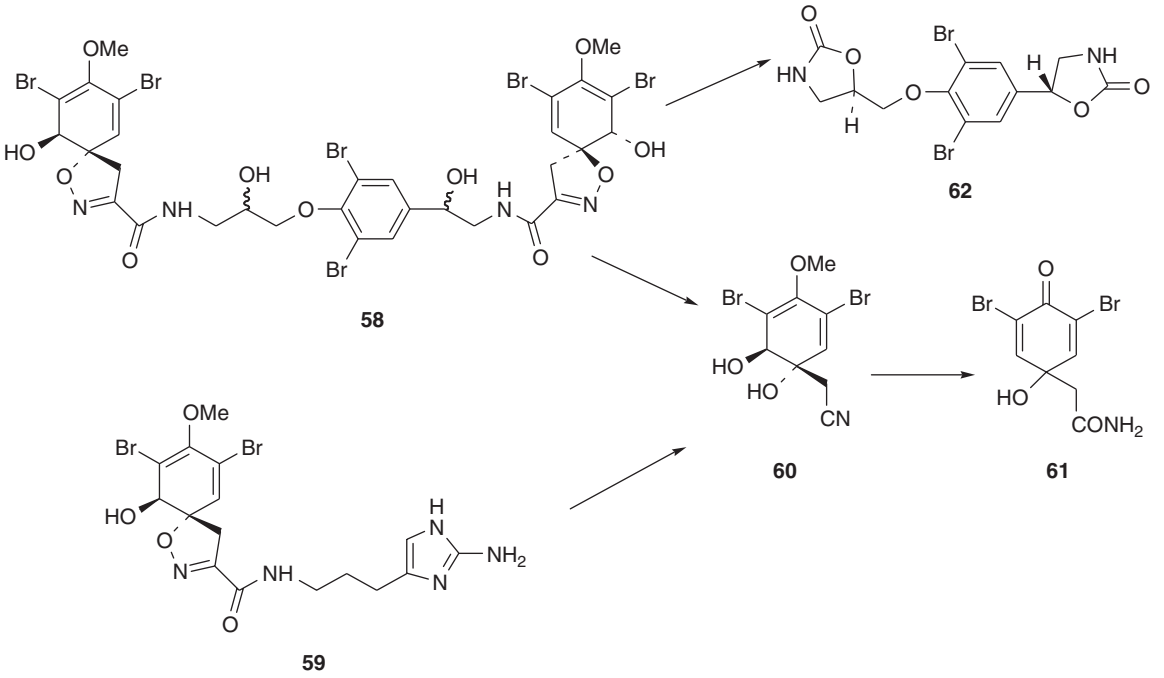
The deterrency of sponge extracts may be affected by their nutritional content and by whether the sponge has additional physical defenses such as spicules or a tough body. Extracts of high nutritional value may be eaten even when chemically defended.[93] There is limited evidence that spicules protect sponges from predation;[84,85,94–96] however, a combination of chemical and physical defenses may protect the North American sponge *Microciona prolifera* from predation by the hermit crab *Pagurus longicarpus*.[97]

4.12.4.1 Alkaloids

A number of studies illustrate that alkaloids isolated from sponges are potent feeding deterrents; two recent examples involve activated chemical defenses in which precursor compounds are converted into deterrent compounds when the sponge tissue is damaged.

The best documented example involves sponges of the order Verongida, which contain complex brominated metabolites, known to derive from tyrosine.[98] The isoxazoline alkaloids isofistularin-3 **58** and aerophobin-2 **59** are converted into aeroplysinin **60** and the dienone **61** following tissue damage in the Mediterranean sponge *Aplysina aerophoba* (**Scheme 1**). These conversions, which were demonstrated in cell-free extracts of several *Aplysina* sponges, are proposed to provide a chemical defense. A mixture of **60** and **61** was more deterrent to *T. bifasciatum* than a mixture of precursor alkaloids that included **58** and **59**,[99] but in testing against the fish species *Bennius sphinx*, the precursor alkaloids were more deterrent.[100] The ecological advantage of the biotransformation is however evident from additional experiments in which the gastropod *Littorina littorea* was repelled when exposed to seawater containing either of the conversion products; these compounds also inhibited the growth of marine bacteria and microalgae,[101] and so may provide protection against pathogens in wounded tissue.[102] Subsequent experiments on species of *Aplysina* from both the Mediterranean and from the Caribbean showed that the different chemical outcomes result from the wounding methods used. Mechanical damage involving grinding of sponge tissue is required; cutting or coring of tissue does not increase levels of **60**.[102,103] The biotransformation can be detected within 1 min of tissue damage.[102]

Evidence that supports an enzymatic basis to these biotransformations includes (1) cell free extracts from other sponges were unable to transform isoxazoline alkaloids; (2) when tissue from the sponge *Crambe crambe* or from the mollusk *Tylodina perversa* (see Section 4.12.8.4) that feeds on *Aplysina* spp. was spiked with **58**, no biotransformation was detected; (3) in contrast to the increased concentrations of **60** and **61**, the concentration

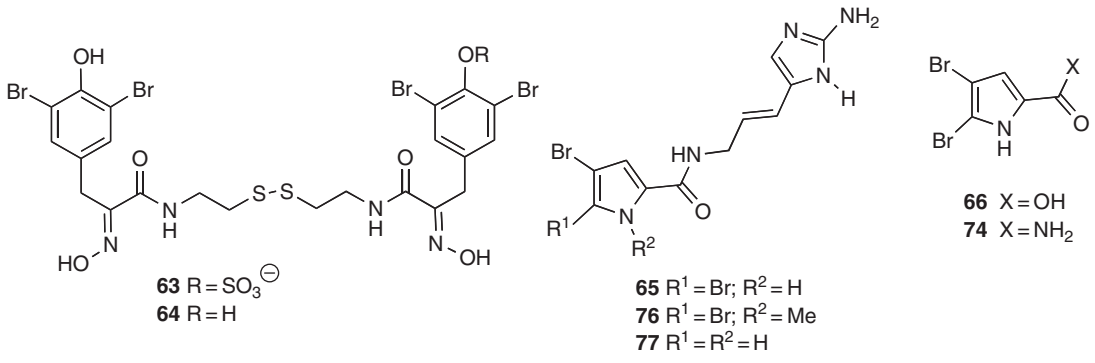


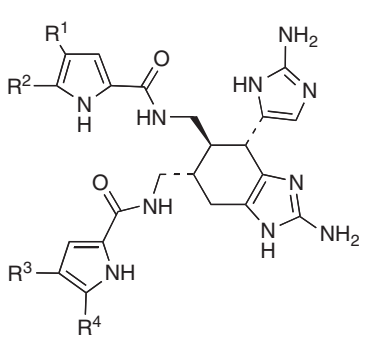
Scheme 1 Wound-induced conversion of brominated isoxazoline alkaloids in *Aplysina aerophoba*.[99,102]

of precursors **58** and **59** is decreased in tissue-damaged sponge;[102] (4) the bisoxazolidinone **62** could be detected during the biotransformation of **58**.[99]

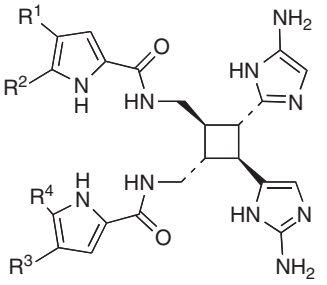
A second example of an activated chemical defense concerns the Indo Pacific sponge *Aplysinella rhax*, in which tissue damage results in the rapid enzymatic transformation of psammaplin A sulfate **63** into psammaplin A **64**; exposure of **63** to tissue from other sponges does not result in any conversion. Compound **63** deters feeding by reef fish, but when offered a choice between psammaplin A and its sulfate, both foods were avoided. In aquarium assays with *C. solandri*, extracts of damaged tissue were more deterrent than extracts from intact tissue, but both treatments were less palatable than control foods. In choice experiments, *C. solandri* preferred food treated with **63** over **64**.[104]

These results highlight the need to consider extraction methods in marine chemical ecology. Freeze-dried *Aplysina* spp. when extracted in methanol yields isoxazoline alkaloids, but when extracted in water or in aqueous methanol the major alkaloids recovered are **60** and **61**.[102] Freeze-dried *A. rex* provides **63** on extraction with methanol, but addition of water prior to extraction results in a high yield of **64**.[104]

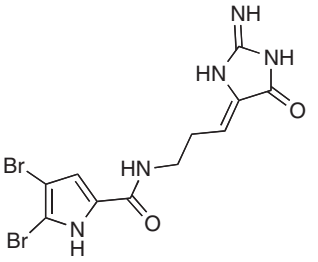




67 $R^1 = R^2 = R^3 = Br$; $R^4 = H$
70 $R^1 = R^2 = R^3 = R^4 = Br$
80 $R^1 = R^3 = Br$; $R^2 = R^4 = H$



68 $R^1 = R^3 = Br$; $R^2 = R^4 = H$
69 $R^1 = R^2 = R^3 = R^4 = Br$



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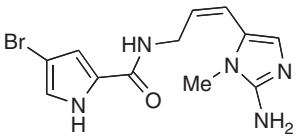

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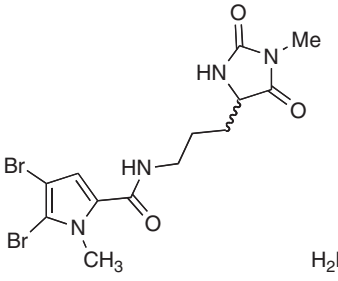

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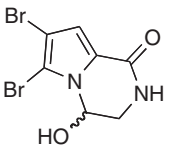

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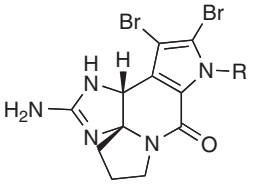
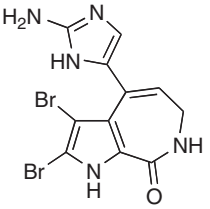


78 R = Me
79 R = H



81

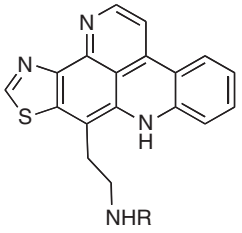
A number of surveys have shown that the sponge *Agelas clathrodes* is not consumed by spongivorous fishes on Caribbean reefs; its crude extracts are deterrent to reef fish in both field and laboratory assays.[83] Assay-guided fractionation provided oroidin **65** that was deterrent at natural volumetric concentrations (0.5–4.0 mg ml^{-1} of food) to *T. bifasciatum* in aquarium assays. Also isolated was 4,5-dibromopyrrole-2-carboxylic acid **66** which was deterrent at 1.0 and 2.0 mg ml^{-1} of food, but not at 0.5 mg ml^{-1}. A mixture of **65** and **66** was deterrent at 1.0 and 2.0 mg ml^{-1}.[105] Bromopyrrole chemicals have been detected in crude extracts of four other *Agelas* sponges (*A. conifera*, *A. dispar*, *A. sceptrum*, and *A. wiedenmayeri*).[105,106] *Agelas wiedenmayeri* contains the deterrent chemical bromoageliferin **67** in addition to **65** and **66**, while *A. conifera* is characterized by a series of dimeric bromopyrrole alkaloids including sceptrin **68**, dibromosceptrin **69**, dibromoageliferin **70** as well as bromoageliferin **67**. Sceptrin (the major metabolite with a mean natural concentration of 5.3 mg ml^{-1}) and bromoageliferin **67** were both deterrent to *T. bifasciatum* at concentrations of 1, 5, and 10 mg ml^{-1}, but bromoageliferin **67** was not deterrent at 0.24 mg ml^{-1}. Dibromosceptrin **69** and dibromoageliferin **70** were deterrent at 5 and 10 mg ml^{-1}, but not at 1.0 mg ml^{-1}. Sceptrin **68** was not significantly deterrent when tested at lower concentrations, while a recombined mixture of **67–70** was deterrent in the field at 1.5 mg ml^{-1}. A mixture of **65–67** had similar activity to **66** alone.[105] Extracts of *A. conifera* containing oroidin **65** and sceptrin **68** are reported deterrent to the reef fish *Stegastis partitus*. Cell separation on Ficoll confirmed a sponge cell origin, possibly in spherulous cells, for the bromopyrroles in *A. conifera*. The concentrations of sceptrin and of oroidin in *A. conifera* increase up to fourfold within 6 days of tissue wounding, suggestive of an induced chemical defense in the sponge.[106]

A series of 14 synthetic pyrroles, furans, thiophenes, and prolines were assayed using *T. bifasciatum*; the presence of bromine was important for activity, replacement of the pyrrolic N by either O or S did not affect activity, while the prolines tested were inactive.[107] A later study compared the feeding deterrency of **65** and **66**

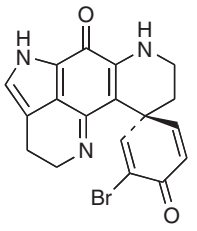
with a suite of other pyrrolic natural products including dispacamide A **71**, keramadine **72**, midpacamide **73**, 4,5-dibromopyrrole-2-carboxamide **74**, and racemic longamide A **75**, some of which were obtained by synthesis rather than from their *Agelas* sponges. The pyrrole nucleus was essential while addition of an imidazole group enhanced deterrency.[108]

A number of studies on Caribbean sponges have used deterrency-guided fractionation to locate antifeedant metabolites. The butanol extract of *Agelas sventres* contained sventrin **76** and hymenidin **77** each of which deterred feeding of *T. bifasciatum* at 3 mg ml^{-1} or above. The assays results agree with previous structure–activity data; hymenidin is less active than more highly brominated metabolites such as oroidin **65** while N-methylation, as in sventrin **76**, lowers activity.[109] The potent deterrent *N*-methyldibromoisophakellin **78**, active at 0.9 mg ml^{-1}, was isolated from *Stylissa caribica* along with the two known metabolites dibromoisophakellin **79** and ageliferin **80** that were isolated in trace quantities.[110] *Axinella corrugata* (= *Teichaxinella morchella*) provided the bromopyrrole stevensine **81**, which deterred feeding at concentrations above 2.2 mg ml^{-1} in aquarium assays with *T. bifasciatum*, and at ~12 mg ml^{-1} in field assays.[111]

Within a sponge, the location of sponge metabolites is often consistent with their proposed antifeedant role. The Micronesian sponge *Oceanapia* sp. is a conspicuous red-colored sponge that is only partially exposed to predation. Although fistules capped by a small fragile capitum protrude into the water column, the base of the sponge is buried in sandy substrate. A methanolic extract from sponge tissue was highly deterrent at base concentration (7.4% of dry mass) to reef fish in field assays and to the sponge-feeding angelfish *Pomacanthus imperator* in aquarium assays. The major secondary metabolites kuanoniamine C **82**, also known as dercitamide, and kuanoniamine D **83** were present in higher concentration in exposed sponge parts and deterred feeding by reef fish at fistule concentrations (cited as 0.4–1.0%). A synthetic sample of the minor sponge metabolite, *N*-deacetyl kuanoniamine-D **84** also deterred feeding in field assays.[112]



82 R = –COEt
83 R = –COMe
84 R = H



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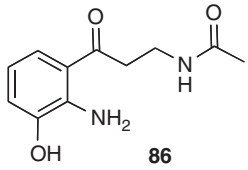

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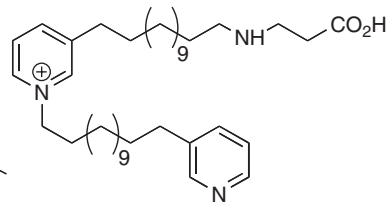

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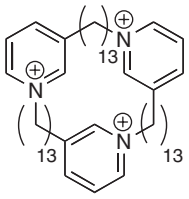

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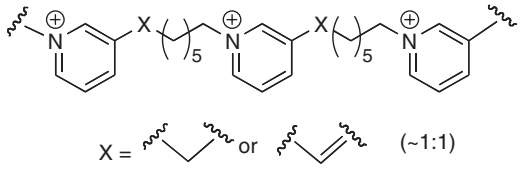

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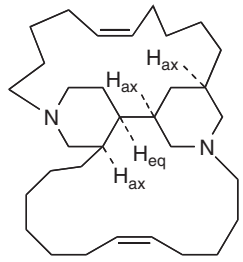


90

The Antarctic sponge *Latrunculia apicalis*, which has a distinctive spherical shape, concentrates discorhabdin G **85** in its surface tissues, optimally positioned as a chemical defense. Sponge extracts and purified **85** prevent predation by sea stars by eliciting a tubefoot retraction.[91] In the Antarctic sponge *Isodictya erinacea*, whose extract is rich in nitrogenous metabolites, the metabolite that triggered tubefoot retraction in sea star predators was identified as *p*-hydroxybenzaldehyde **56**,[113] also found in red algae.[38] The pigment erebusinone **86** reduces amphipod molting and leads to increased mortality when fed to the spongivorous amphipod *Orchomene plebs*.[114] Extracts from the Arctic sponge *Haliclona viscosa* that are unpalatable to amphipods contain the

3-alkylpyridinium alkaloids viscosaline **87**[115] and viscosamine **88**,[116] which both resemble amphitoxin **89**, a known fish antifeedant from the Caribbean *Amphimedon compressa*.[117] Other 3-alkylpyridine sponge metabolites reported to show antifeedant activity against reef fish include haliclonacyclamine A **90**, an antifungal metabolite from the soft-bodied sponge *Haliclona* sp. 628[118] that inhibits larval settlement.[119]

4.12.4.2 Terpenes

Erylus formosus contains formoside **91** together with other deterrent triterpene glycosides that are more deterrent when tested as mixtures rather than individually. Structural features that enhance activity include glycosylation, and/or side chain methylation or hydroxylation.[120–122] The active metabolites are more concentrated in the inner layers of *E. formosus*, and are not detected in seawater collected near the sponge.[122] In *Ectyoplasia ferox*, hydroxylated triterpene glycosides possessing two different carbon skeletons (the ectyoplasides A and B **92–93** and the feroxosides A and B **94–95**, and which are potent feeding deterrents in both field and laboratory assays, are instead concentrated in the top layer of the sponge. The triterpene metabolites also play a role in the antimicrobial and antifouling defenses of these two Caribbean sponges.[122]

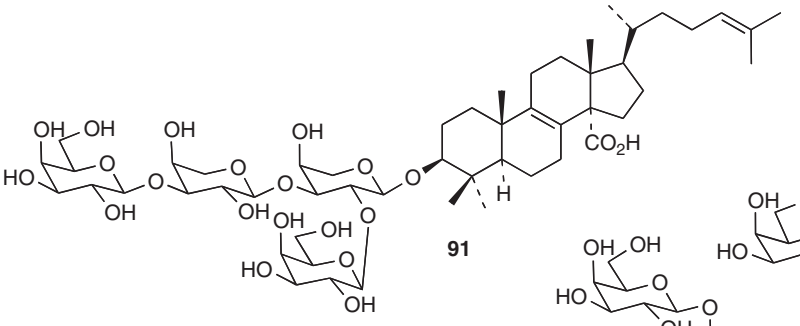
Pawlik *et al.*[123] have studied a group of Caribbean *Ircinia* species that have a characteristic garlic odor resulting from the presence of volatiles such as dimethyl sulfide and methyl isocyanide. Despite this, some fish and sea stars feed on these sponges. Crude extracts from *Ircinia campana*, *I. felix*, and *I. strobilina* all deter *T. bifasciatum* at natural volumetric concentration,[123] with linear furanosesterterpenoid metabolites such as variabilin **96** responsible for this activity. Volatile components such as dimethyl sulfide (refer Section 4.12.3.3) did not deter fish feeding. Extracts of Brazilian *I. strobilina* at natural concentrations deter feeding by tropical fish in field assays; variabilin **96** at 0.23% of dry mass was deterrent when incorporated into an alginate matrix, but was not deterrent when incorporated into a carrageenan matrix owing to decomposition during pellet preparation.[20] Mediterranean species of *Ircinia* gave the ircinins I **97** and II **98** that were deterrent to *T. bifasciatum*.[124] The lintenolides C–E **99–101** are a group of novel sesterterpenes isolated from *Cacospongia* cf. *linteiformis*; these and other terpenoid metabolites from the sponge show feeding deterrency to the freshwater *Carassius auratus*.[125] The Antarctic sponge *Suberites* sp. contains the sesterterpene suberitenone A **102**, and other similar metabolites, that deter feeding by the sea star *P. fuscus*.[126]

Sponge terpenes are not always effective as feeding deterrents. Sesterterpenes such as luffariolide **103** lack deterrency,[93] while manoalide **104** is reported active, but only when incorporated into a low-quality artificial food.[127] In *Aplysilla glacialis*, a dendroceratid (i.e., nonspiculated) sponge from the Caribbean that is rarely eaten by fish despite its fleshy nature, 7-dehydrocholesterol endoperoxide **105** and the diterpene manoöl **106** are both active against reef fish, but neither metabolite was active when tested against *Thalassoma lunare* in aquarium assays. Other diterpene metabolites were not deterrent.[128]

The sponge quinone avarol **107** from *Dysidea avara* deters feeding by a range of consumers including the sea urchin *Paracentrotus lividus*,[129] and the pufferfish *C. solandri*.[93] Reef fish and crabs consumed foods containing avarol; reef fish were only deterred when the metabolite was incorporated into artificial food of low quality.[93] The larvae of *D. avara* are known to be defended, but the chemical basis is not yet clear.[129] The deterrent effects of sesquiterpene metabolites from some other *Dysidea* spp. and of the scalaranes, a group of cyclic sesterpenoids from the sponge genera *Cacospongia*, and that show variable predator deterrency are covered in Section 4.12.8.3 since these sponges are the preferred foods of glossodorid nudibranchs.

Certain groups of sponges such as the Indo-Pacific *Acanthella cavernosa* are avoided in the field by generalist predators and are the preferred food of phyllidid nudibranchs (Section 4.12.8.3). These sponges contain terpene isocyanides that have a characteristic odor,[130] however, the isocyanide metabolites are not confirmed feeding deterrents.[8,131] Sponge crude extracts are deterrent at half natural concentrations when tested against reef fish,[21] but individual terpene isocyanides lack deterrent effects in field trials. For example, axisonitrile-1 **108** from a Mediterranean sponge fails to deter feeding by marine and freshwater fishes, however, the compound is ichthyotoxic,[132] while axisonitrile-3 **109** is not active.[133]

In general, there is no clear trend on the antifeedant effects of sponge terpene metabolites, or on the contributions of individual functional groups to activity. Many terpenes contain functional groups that may be sensitive to oxidation or the effects of UV light, resulting in chemical changes or decomposition during bioassays.



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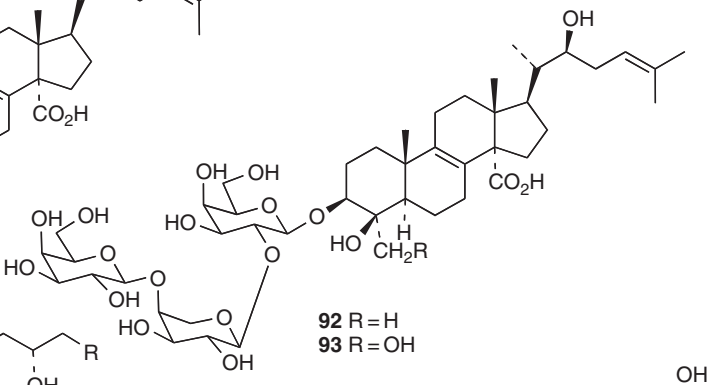
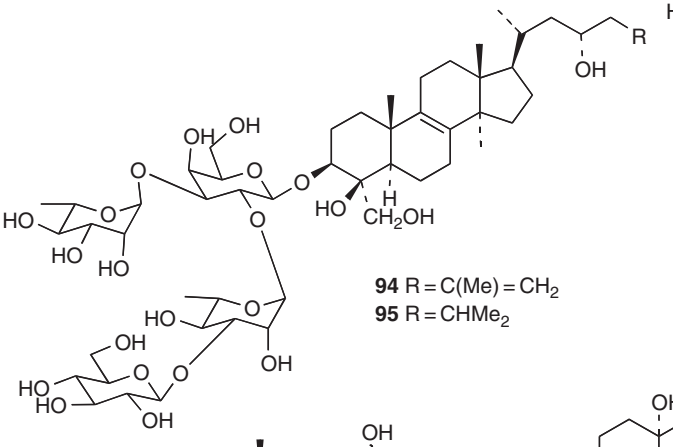
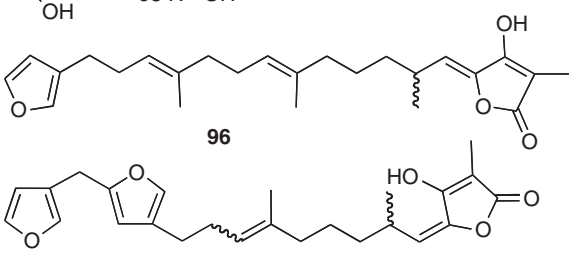


92 R = H
93 R = OH

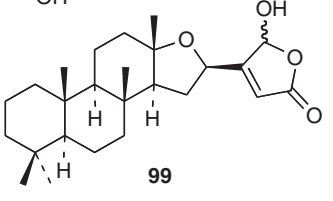


94 R = C(Me) = CH_2
95 R = $CHMe_2$



96

97 *E* isomer
98 *Z* isomer



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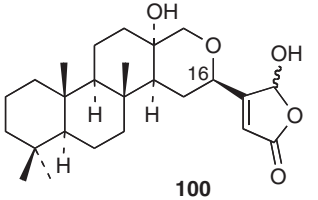
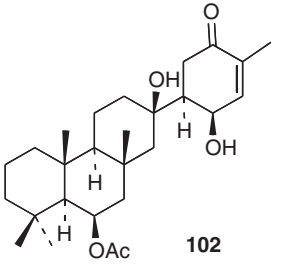


100
101 epimer at C-16



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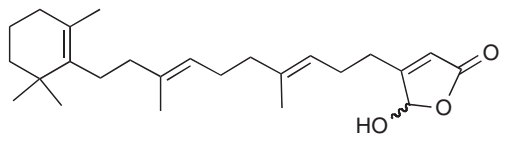

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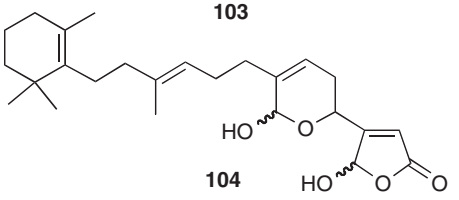

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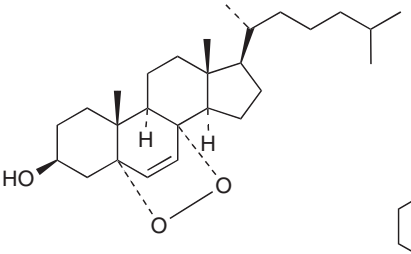

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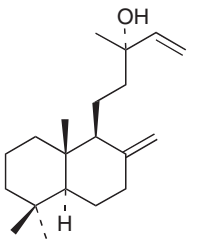

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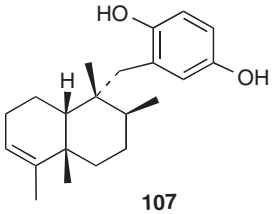

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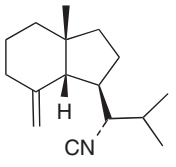

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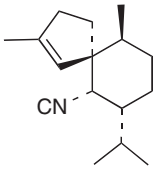

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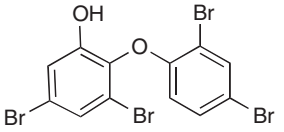

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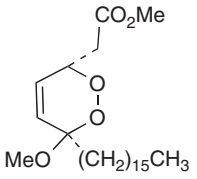

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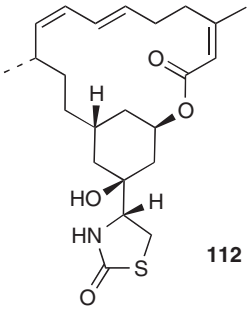

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4.12.4.3 Other Sponge Metabolites

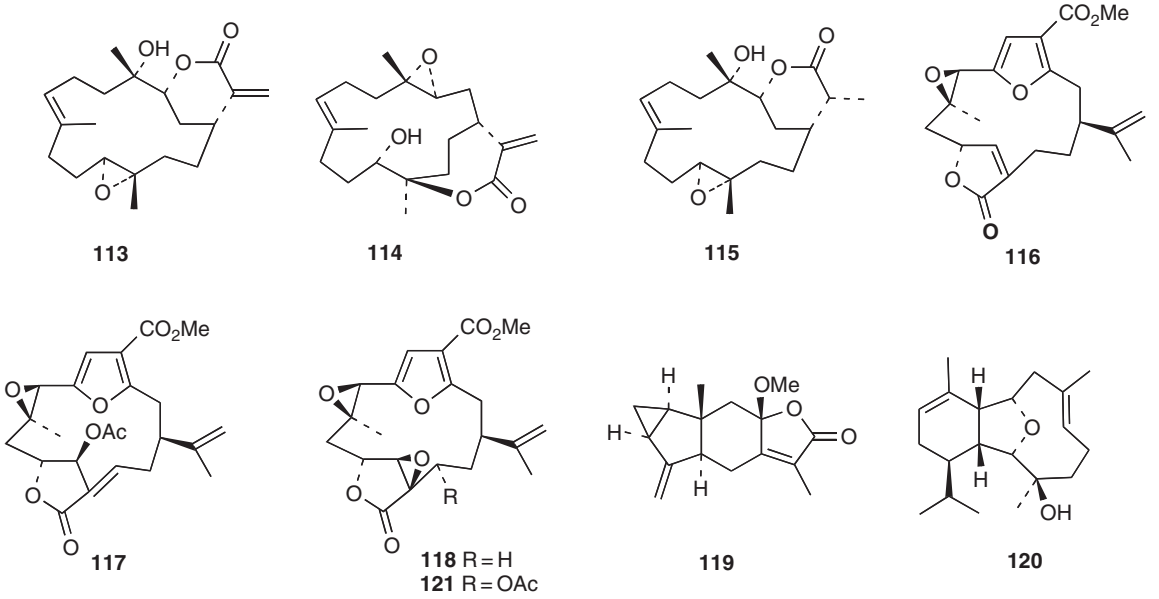
The brominated diphenyl ether (BDE) metabolite **110** first isolated from *Lamellodysidea (=Dysidea) herbacea* deters feeding by the crab *Leptodius* sp.[93] and by reef fish,[127] and is frequently used as a positive control in feeding studies. A flow cytometry study showed that BDEs were localized in the cyanobacterial symbiont *Oscillatoria spongeliae* present within sponge tissue, and consequently it was inferred that the BDE production took place in the symbiont cells, with BDEs then excreted into the sponge tissue and stored in crystalline form.[134] However in *Dysidea granulosa*, high quantities of BDEs are found in sponge internal (endosomal) tissue, along with bacteria, whereas cyanobacterial symbionts are localized in outer (ectosomal) tissue by electron microscopy. Exposure of the host sponge to light impacts on the production of these compounds in sponge tissue.[135] A microbial study identified BDEs in a culture of the bacterium *Vibrio* sp. isolated from *D. herbacea*.[136] Chondrillin **111** from the sponge genus *Plakortis* has variable effects on feeding by *C. solandri* and by the sea hare *S. longicauda*,[93] Latrunculin-A **112** from *Negombata magnifica* is described as an ineffective deterrent,[81] although the sponge is avoided by fish and its crude extracts are deterrent.[137]

4.12.5 Coelenterates

4.12.5.1 Soft Corals

The typical secondary metabolites of soft corals are diterpenoids, although some species also produce sesquiterpenoids. Despite the fleshy nature of soft corals relative to other invertebrates, predation is rare because the diterpene metabolites produced are believed to be potent feeding deterrents.[138]

A study of predator deterrency in Australian soft corals has compared the effects of flexibilide **113**, sinulariolide **114**, and dihydroflexibilide **115** from *Sinularia flexibilis*. In feeding trials with the mosquitofish *Gambusia affinis* food containing dihydroflexibilide at 1% of dry weight was rejected while foods containing sinulariolide and dihydroflexibilide were only rejected at 10% dry weight.[139] In Guam, *Sinularia* corals contain pukalide **116** and 11β-acetoxypukalide **117**,[26] each metabolite showing antifeedant activity.[140–142] A study of *Sinularia maxima* reveals that the content of the major deterrent metabolite **116** is reduced in bleached corals, and that bleached corals are subject to predation by *C. solandri*. Feeding assays conducted with **116** at concentrations found in bleached and unbleached corals confirm that *C. solandri* is not deterred by 'bleached' levels of **116**.[142] In *Sinularia flexibilis* from Australia, temporary bleaching results in an increase and decrease in flexibilide **113** and sinulariolide **114** levels respectively.[143]



Butterfly fish feed exclusively on chemically protected soft corals such as *Sinularia* spp., generally from the tips where the terpene concentration is maximal, and may have evolved mechanisms to detoxify the metabolites.[140,144] The structural defenses provided by sclerites, that are involved in defense against generalist reef fishes,[26] are ineffective against these specialized predators.[140] Although extracts from *Sinularia* soft corals are deterrent against reef fishes at 3–7%, much higher concentrations (19–20%) are required to deter feeding by *Chaetodon unimaculatus*, which selectively feeds on the corals. In this study, the major terpene isolated from *S. maxima* was 11β-acetoxypukalide **117** rather than pukalide **116**;[140] temporal differences in concentrations of **116** and **117** in *Sinularia* spp. have been noted.[145] The nudibranch *Phyllodesmium guamensis* also eats *Sinularia* spp. (see Section 4.12.8.3).

Soft corals eggs contain terpenes such as **116**, **117**, and epoxypukalide **118** that are believed to play a defensive role.[146,147] Extracts of *Sinularia polydactyla* larvae containing **116** and **117** are deterrent in assays with *C. solandri* which has been observed feeding on soft coral polyps in the field.[147]

Some species of algae, including the chemically protected *Halimeda* spp. (Section 4.12.3.1), and sea grasses grow in abundance at the base of *Sinularia* colonies, but rather surprisingly the soft coral chemical defense is not responsible for maintaining this association. Instead, the shape of the *Sinularia* colonies appears to influence fish grazing, thereby affording protection to the algal or sea grass colonies.[148]

Other soft coral metabolites reported to show antifeedant effects against fish include heterogorgiolide **119** and the eunicellane **120** from *Heterogorgia uatumani*,[149] and furanocembrenoid diterpenes, for example, 13-acetoxy-11β, 12β-epoxypukalide **121**, from *Lophogorgia violacea*.[150]

4.12.5.2 Gorgonians

Secondary chemicals are commonly used as a defense mechanism in sea fans and sea pens, but structural defenses are also important. Biogeographic studies have surveyed the chemical and structural defense mechanisms of 32 species of gorgonian corals from the Caribbean,[14] of 7 species from Pacific[151,152] regions, and of 8 species from Singaporean[17] waters. Crude extracts from all of the Indo-Pacific species studied, except *Viminella* sp. from Guam,[152] are deterrent to natural populations of reef fish, while all of the Caribbean extracts are deterrent to *T. bifasciatum* when tested at natural volumetric concentration. In most gorgonians, the deterrent chemicals are concentrated near the tips,[12,151] which are polyp-bearing regions and more prone to predation in coral reef environments than is basal tissue. Considered together, these studies emphasize the importance of gorgonian chemicals in deterring generalist predators.

A number of studies have investigated Caribbean *Pseudopterogorgia* spp. whose extracts are deterrent.[14] In *Pseudopterogorgia americana*, an aquarium assay with *T. bifasciatum* identified a fraction containing 9,11-secogorgosterol **122** and 9,11,-secodinosterol **123** that was deterrent, although the purified metabolites were only deterrent when tested as a mixture.[153] Biosynthetic data support the involvement of zooxanthellar symbionts in the production of secosterols.[154] The sesquiterpenoids curcuquinone **124** and curcuhydroquinone **125** from *Pseudopterogorgia rigida* are deterrent at below natural concentrations.[12]

The gorgonian *Pseudopterogorgia elisabethae*, whose crude extracts deter feeding by *T. bifasciatum* in aquarium assays,[14] contain pharmacologically important pseudopterosins A–D **126–129**. A fish feeding deterrency role has been suggested for these diterpene glycosides. Increased production of the pseudopterosins has been detected in response to feeding by the mollusk *Cyphoma gibbosum*, a specialist feeder on gorgonians, but not after grazing by the butterfly fish *Chaetodon capistratus*. Terpene formation in *P. elisabethae* is also induced by decreased levels of UV/Vis radiation.[155] Symbionts within the gorgonian are involved in the biosynthesis of these terpenes, a topic that has been studied in some detail.[156]

Chemical extracts of the Caribbean gorgonian *Briareum asbestinum* are strongly deterrent,[14] owing to the production of both asbestinane, for example **130**, and briarane, for example briathein Y **131** diterpenes for their chemical defense. There is considerable variation in the chemistry of individual colonies according to the collection site. At two sites in the Bahamas, briarane metabolites predominated while a collection from the Virgin islands contained more of the asbestinane metabolites. Although unexpectedly high levels of chemicals were found in deep water collections compared to shallow water collections, shallow water colonies transplanted to deeper waters retained their shallow water chemistry, suggesting that the chemistry shown by adult colonies is genetically determined rather than inducible.[157] The chlorinated briarane diterpenes erythrolide B **132** and -D **133** from *Erythropodium caribaeorum* are deterrent in field assays.[158]

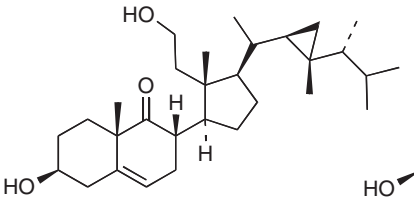
Extracts of *Pterogorgia anceps* contain a fraction enriched in ancepsenolide metabolites of the polyketide class, one of which (**134**) is individually deterrent in shipboard assays with *T. bifasciatum*.[10] This last study illustrates that chemical defense mechanisms in gorgonians do not solely rely on the terpenoid biosynthetic pathway.

Antarctic gorgonians use chemical defense against predatory seastars.[159] In the gorgonian *Ainigmaptilon antarcticus*, the antibacterial sesquiterpene ainigmaptilone A **135** deters predation by the seastar *O. validus*.[160]

The predator deterrent effects of *Gorgonia ventalina* and its nudibranch predator *Tritonia hamnerorum* are described in Section 4.12.8.3.

4.12.5.3 Hydroids

There have been few chemical studies on marine hydroids and consequently their use of chemical defense is poorly understood. Many hydroids have the physical protection of nematocysts that discharge proteins when in contact with predator tissue. The pinfish *Lagodon rhomboides* avoids eating hydroids compared to palatable control foods. When their nematocysts are discharged by KCl treatment, two species of hydroid become palatable while four other species including *Tridentata marginata* all remain unpalatable owing to the presence of deterrent extracts. *Tridentata marginata* contains tridentatol A **136** that deters feeding by pinfish and filefish, and other nondeterrent tridentatol metabolites while *Corydendrium parasiticum* contains 2-methyl-6-alkenyl-3-piperidinol alkaloids. The physical defense afforded by nematocysts is metabolically expensive and, since each nematocyst is a single weapon, deterrent chemicals may be more effective particularly in periods of intense predation. The



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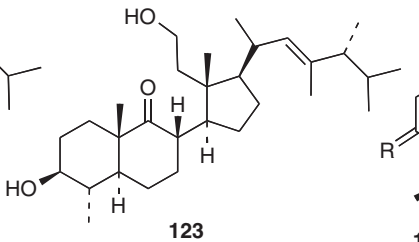

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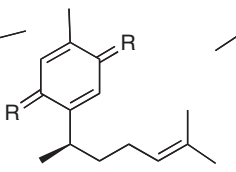
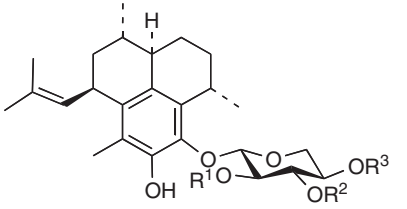
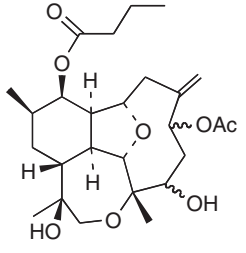


124 R = O
125 R = H, OH



126 $R^1 = R^2 = R^3 = H$
127 $R^2 = R^3 = H$; $R^1 = Ac$
128 $R^1 = R^3 = H$; $R^2 = Ac$
129 $R^1 = R^2 = H$; $R^3 = Ac$



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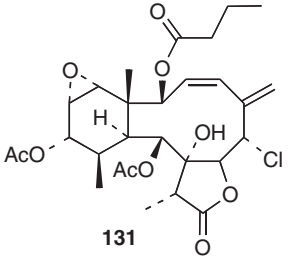

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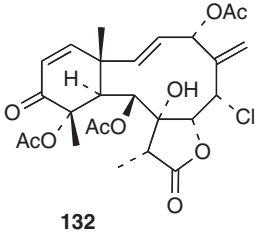

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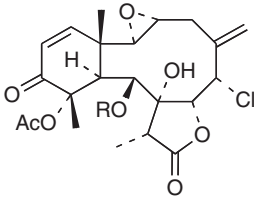
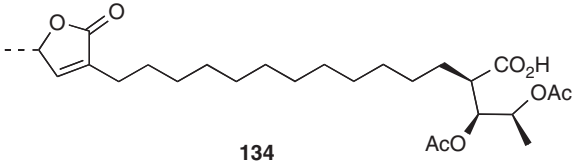
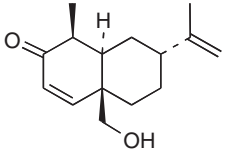


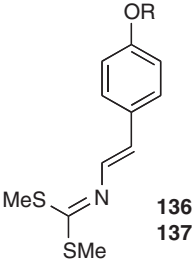
133 R = –COCH$_2$OAc



134



135



136 R = H
137 R = $SO_3^{\ominus}$ $Na^{\oplus}$

pattern of nematocyst morphology vs. bioactive chemicals has been contrasted with that found in soft and hard corals.[161] A comparison of the tridentatol composition of hydroids crushed just prior to extraction with that of uncrushed tissue has established that an activated chemical defense operates in *T. marginata*. In damaged tissue, the sulfate group of the nondeterrent tridentatol E **137** is cleaved enzymatically producing **136**. This experiment simulates the effect of a grazer and the resulting production of the more effective deterrent **137** is ecologically advantageous.[162]

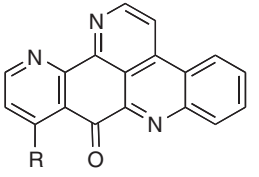
4.12.6 Ascidians

Ascidians are conspicuous marine animals that are generally soft-bodied and which lack obvious physical defenses against predation. Calcareous spicules found in some species have been proposed to play a role in chemical defense, as are tissue toughness and nutritional value. Ascidians show remarkable chemistry and accumulate both inorganic acids in bladder cells and high levels of vanadium salts, whose ecological functions may include deterrency and antifouling roles.[163,164] A study on vanadium chemistry and chemical defense in the Caribbean tunicate *Phallusia nigra* has shown vanadium to be concentrated in internal tissues and blood over the tunic surface. Food pellets into which acidic solutions of VCl_3 or $VOSO_4$ had been incorporated are unpalatable to *T. bifasciatum* in aquarium assays; however, the nonacidic vanadium complex $V(Acac)_3$ is ineffective in deterring predation.[164] In field and laboratory experiments involving *Cystodytes* spp., artificial foods containing spicules or sulfuric acid do not deter fish feeding.[165] A study of the palability of ascidian species from the Western Atlantic has revealed that 16 of 17 species tested showed deterrency to *T. bifasciatum*, while 9 species secrete inorganic acid in their tunics that leads to a lower incidence of predation.[13] Lipophilic crude extracts from the Antarctic ascidian *Distaplia cylindrica* deter feeding by seastars.[166] These studies are from diverse biogeographic regions and so support the general importance of secondary metabolite defense in ascidians.

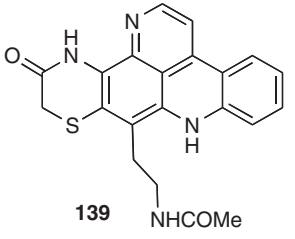
The Mediterranean ascidians *Cystodytes* spp., which generally lack epibionts and show few signs of predation, are physically defended by the presence of spicules and occur in a range of color morphs. A blue color morph of the ascidian *Cystodytes* sp. contains ascididemin **138** with antipredatory effects against pufferfish, damselfish, but not against sea urchins.[165] Studies using energy-dispersive X-ray microanalysis[167] or by MALDI-TOF[168] have shown that both shermilamine B **139** and kuanoniamine D **83** are present in pigmented granular cells in the tunic of a purple morph with their deacetylated forms present in both tunic and zooids. In blue and green morphs, ascididemin and 11-hydroxyascididemin **140** are present in both tunic and zooids, while in a brown morph ascididemnin alone is detected in these tissues. The seasonal variation in pyridoacridine alkaloid content in purple and blue morphs of this species, and its relationship to reproduction and energy budget, has also been investigated.[169,170] The co-occurrence of the same pyridoacridine alkaloids in both sponges (Section 4.12.4.2) and these ascidians may suggest a microbial-based biosynthesis, although the possibility of convergent evolution cannot be excluded. Studies using confocal microscopy have shown that in sponges these metabolites are localized in specialized cells containing inclusions rather than in intercellular microbial symbionts.[171]

The chemical basis of didemnid ascidian defense has been investigated with some compounds showing quite potent antifeedant effects. In *Didemnum conchyliatum*, the same cyclic peptides, didemnimides A–D **141**–**144**, have been noted for both adults and larvae.[28] In laboratory assays using *T. bifasciatum*, didemnimides C **143** and D **144** deter feeding, while didemnimide C also deters feeding in field-based assay, but didemnimides A **141** and B **142** are ineffective in both laboratory and field assays.[29] The ascidian *Didemnum granulatum*, which may also be chemically protected, stores granulatamide **145** and isogranulatamide **146** in tunic bladder cells.[172] All of these metabolites show some structural similarity to the bromopyrrole-imidazoles that are responsible for the lack of palatability of *Agelas* sponges (Section 4.12.4.1).

The Caribbean tunicate *Trididemnum solidum* and its larvae contain a complex mixture of didemnin/nordidemnin metabolites that act as feeding deterrents in field-based assays at below natural concentrations. The most potent metabolite is nordidemnin B **147**.[28] Crude extract mixtures enriched in didemnin B **148** induce vomiting in the spotted pinfish *L. rhomboides*, causing these fish to avoid feeding.[173] The larvae of other didemnid ascidians are also protected by the presence of deterrent chemicals.[28,174]



138 R = H
140 R = OH



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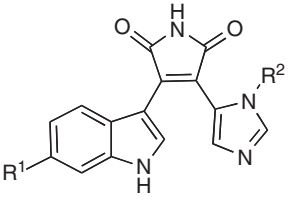
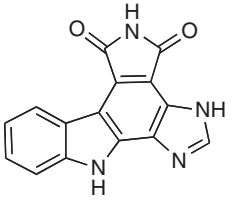


141 $R^1 = R^2 = H$
142 $R^1 = Br$; $R^2 = H$
143 $R^1 = H$; $R^2 = Me$
144 $R^1 = Br$; $R^2 = Me$



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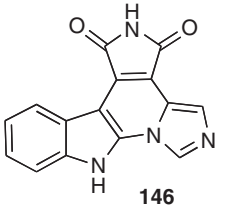

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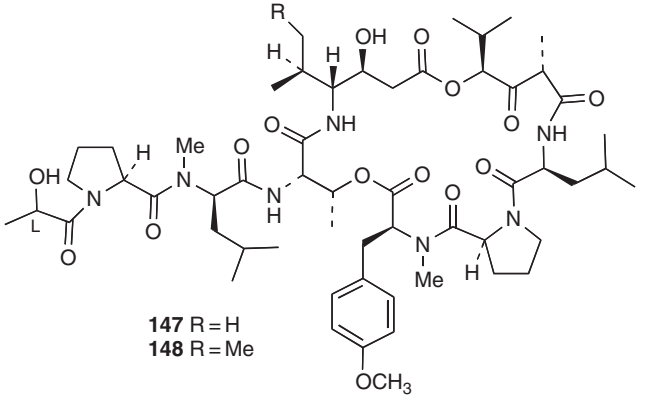
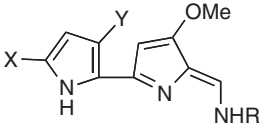
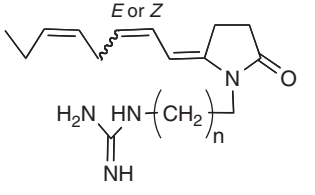


147 R = H
148 R = Me



149 X = Y = R = H
150 X = Y = H; R = CH_2CHMe_2
151 X = Y = H; R = Et
152 X = Y = H; R = $(CH_2)_2Ph$



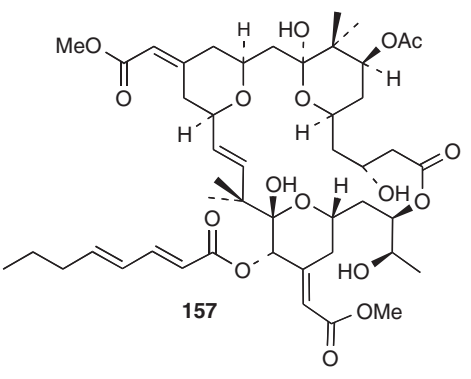
153 *Z*; *n* = 4
154 *E*; *n* = 4
155 *Z*; *n* = 3
156 *E*; *n* = 3

Other ascidian compounds whose antifeedant properties have been confirmed include tambjamine metabolites **149–152** from *Atapozoa* sp. and its nudibranch predator *Nembrotha* spp.[175] In field assays, the crude ascidian extract and tambjamines C **150** and F **152** show significant feeding deterrency at or below natural concentration. Although tambjamines A **149** and E **151** are not individually deterrent at natural concentration, a 1:1 mixture of tambjamines E and F is deterrent at below natural concentration.[175] Lindquist *et al.*[28] also report that the larvae of the Indo-Pacific ascidian *Sigillina* cf. *signifera* (= *Atapozoa* sp.) are protected by the presence of tamjamine C **150** (and refer to Section 4.12.7).

A mixture of polyandrocarpidine metabolites **153–156** from the ascidian *Polyandrocarpa* sp. deters feeding by hermit crabs and two species of snails.[28] Nitrogenous ascidian compounds that are reported to lack antifeedant effects include patellamide C (*Lissoclinum patella*),[28] and eudistomins G and H.[176]

4.12.7 Bryozoans

Two recent reviews emphasize the ecological significance of bryozoan metabolites.[8,177]



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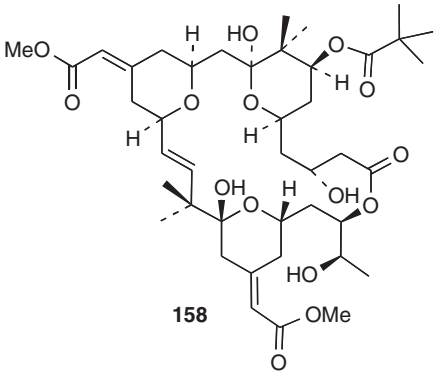
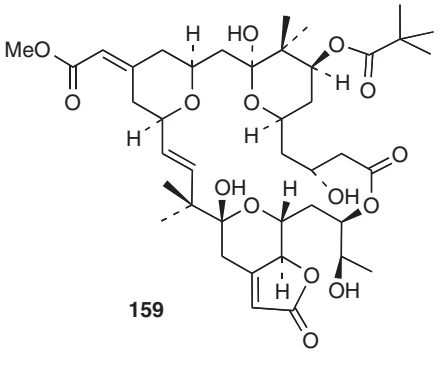


158



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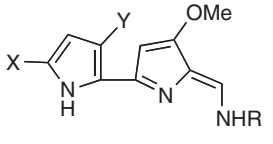


160 X = Br; Y = R = H
161 X = H; Y = Br; R = CH_2CHMe_2

The global fouling organism *Bugula neritina* produces the complex polyketide bryostatin group of metabolites that are of clinical significance owing to their potent antitumor activity. The compounds are in fact products of the symbiotic γ-proteobacterium '*Candidatus* Endobugula sertula' present in bryozoan tissues. In feeding assays crude extracts from larvae and juvenile stages of *B. neritina* are unpalatable, but crude extracts from adults and from larvae obtained without their bacterial symbionts following antibiotic treatment are palatable.[178,179] The chemical basis of the deterrency has been traced to bryostatins 1 (**157**), 10 (**158**), and 20 (**159**), which protect the larvae of *B. neritina* from predation.[180] The absence of bryostatins in the treated larvae confirms symbiont production of the metabolites.[178,181] This is the first reported example of a symbiont producing chemicals that function as the antipredator defense for the vulnerable larvae of their host.

Energy-dispersive X-ray microanalysis has shown that the brominated alkaloids present in the Australian bryozoan *Amathia wilsoni* may be associated with a surface bacterium,[182] consistent with a role in chemical defense. Tambjamines A–D **149**, **150**, **160**, and **161**, from the bryozoan *Sessibugula translucens* and nudibranch predators

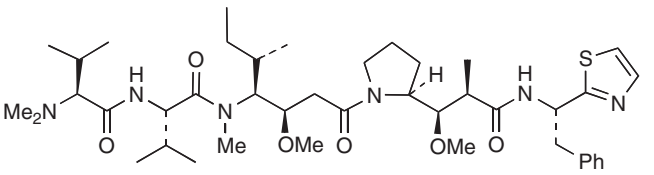
Tambja spp. and *Roboastra tigris* deter feeding by the spotted kelpfish *Gibbonsia elegans* in laboratory-based assays. This study also investigated the pheromonal role of tambjamine compounds in the *Tambja* slime trails, their role in deterring attack by *Roboastra tigris*, and reveals that *S. translucens* is a favored food of *Tambja* spp.[183]

4.12.8 Mollusks

A comprehensive review published in 1995 lists the chemicals isolated from opistobranch mollusks, and catalogues their anatomical locations and biological activities, providing useful background information on a wide range of antifeedant compounds.[184] Other review articles include descriptions of recent mollusk antifeedant studies.[7,9,185,186] Despite an extensive literature on mollusk chemistry, there is little experimental evidence that rigorously confirms the antifeedant properties of mollusk metabolites at ecologically relevant concentrations. The small amounts of compound that are typically isolated from individual sources limit broad scale ecological investigations.[21] The best understood examples involve mollusks that sequester antifeedant chemicals from their diet, since this enables a supply of compounds for ecological study to be obtained from the dietary source.

4.12.8.1 Sea Hares

Chemical studies have shown that sea hares sequester metabolites from dietary algae and cyanobacteria. An example of pharmaceutical significance concerns the anticancer compound dolastatin 10 (**162**) that, along with related metabolites, was first isolated from the sea hare *D. auricularia*. These compounds have now been shown to originate from the cyanobacterial species *L. majuscula* and *Symploca* spp.,[187–189] and are therefore of dietary origin.



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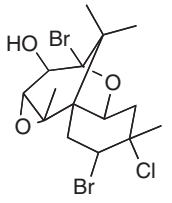

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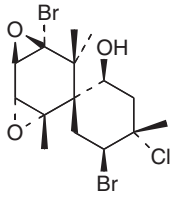

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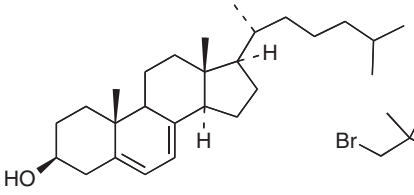

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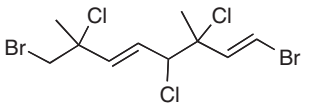

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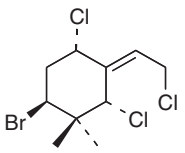

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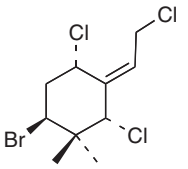

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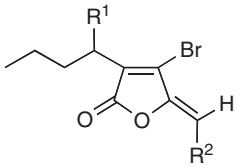
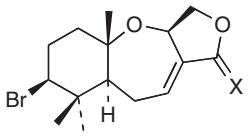
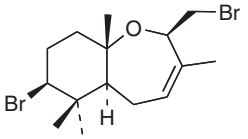


169 R^1 = OAc; R^2 = I



170 X = H_2
172 X = O



171

Sea hares of the genus *Stylocheilus* prefer the cyanobacteria *L. majuscula* over other foods, and often contain cyanobacterial metabolites. It was mentioned before (Section 4.12.2.1) that *S. longicauda* concentrates the deterrent metabolites malyngamides A **4** and B **5**; it further converts malyngamide B into an acetate derivative, which is a feeding stimulant rather than a deterrent.[32] Given the dietary selection of the chemically rich

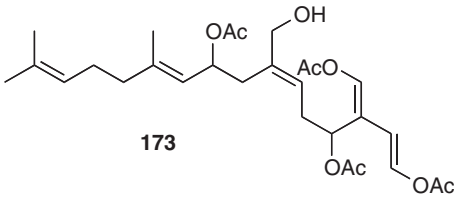
Lyngbya, it is perhaps unsurprising that this sea hare can accumulate a chemically diverse range of algal and sponge metabolites when these are provided in an artificial diet;[190] *S. striatus* also accumulates cyanobacterial compounds and may convert lyngbyatoxin A **14** into a less harmful acetate derivative.[191] The sea hare *D. auricularia* sequesters a range of algal compounds including the feeding deterrents johnstonol **163** and prepacifinol epoxide **164**,[192] caulerpenyne **21**, pachydictyol A **27**, and cyanobacterial metabolites such as malyngamide B **5** if these are provided as part of its diet.[190] However, anatomical studies have shown that the sequestered metabolites are stored in the digestive glands rather than in exposed body parts or ink.[190,192] In the ink of *D. auricularia*, the pigment aplysioviolin has been implicated in unpalatability, while the skin extracts appear to be rendered unpalatable by the presence of 7-dehydrocholesterol **165** rather than by algal compounds, which are only found in low concentrations in the skin. The unpalatability of mollusk egg extracts could not be traced to any dietary-derived compounds. Consequently, *D. auricularia* may not utilize dietary-derived algal or cyanobacterial compounds to protect itself from predation.[192] Sea hares may protect themselves from predation by choosing to inhabit host plants that are chemically defended, and may not have a dietary need for their preferred algal food.[15,43,193]

In Guam, *Aplysia californica* has been reported to sequester sesquiterpenes and halogenated monoterpenes (e.g., **166**) from its algal diet of *Laurencia pacifica* and *P. cartilagineum*, and concentrates metabolites in its digestive system.[190] *Aplysia parvula* from Guam grazes on *Portieria hornemannii* that contains the fish-feeding deterrents apakaochtodenes A **167** and B **168** and accumulates these metabolites in its digestive glands. The choice of diet confers some protection to the mollusk since body parts of *A. parvula* were unpalatable to reef fish; in contrast, body parts from animals that had been feeding on the chemically unprotected red alga *Acanthophora spicifera* were consumed. This study also provided some insight into the effect of dietary concentration on feeding behavior by sea hares. Low concentrations (<2.0% algal wet mass) of *P. hornemannii* crude extract did not deter feeding by *A. parvula*, however, testing at higher concentrations (4 or 6%) significantly deterred feeding. The purified metabolites **167** or **168** were deterrent at all concentrations tested, but this unexpected result may have been a consequence of the feeding protocol used.[43] In Southern Australia (NSW), sea hares of the genus *Aplysia* feed on red algae such as *Laurencia obtusa* and *D. pulchra*. In its natural habitat, *A. parvula* consumes larger quantities of *L. obtusa* than *D. pulchra*, yet this sea hare typically contains halogenated metabolites that are characteristic of *Delisea* plants rather than of *Laurencia* plants.[44] Halogenated furanones can be detected in high concentrations, on average 12–13% of the dry mass of an animal, and with the yield of metabolite **44** exceeding 30% of the dry mass in some animals. Furanone **44** is an effective fish antifeedant in contrast to the sequestered furanone **45**, which is present in lower concentrations to that present in the plant. Compound uptake is specific since some plant compounds, notably the acetylated **169**, are not detected in the sea hare. The sequestered metabolites are accumulated in the digestive glands of the mollusks.[44,68] *A. parvula* also ingests metabolites diagnostic of *L. obtusa* including palisadin A **170** and palisadin B **171** but these metabolites are not stored and can be detected in mucous and opaline secretions. *Aplysia dactylomela* also acquires **170** and may convert this into **171** and **172** prior to excretion.[44]

Recent studies have examined the chemical basis of sea hare response to attack and have identified amino acid constituents in the opaline glands and ink secretions of *A. californica* that stimulate a false feeding response ('phagomimicry') as well as confused behavior in the shiny lobster (*Panulirus interruptus*).[194,195] Both ink and opaline secretions are acidic, believed to enhance behavioral responses.[196] The importance of inking as a deterrent mechanism is revealed by feeding assays in which reef fish consumed frozen mollusks but did not eat live specimens.[43]

4.12.8.2 Sacoglossans

Sacoglossans are a group of mollusks with a specialized diet of green algae from which they sequester metabolites that may play a role in their chemical defense. Chloroplasts, also of algal origin, are functional symbionts in many species of sacoglossans and convert bicarbonate into sugar products that are ultimately used for the synthesis of 'polypropionate' metabolites. Experimental evidence in support of the proposed defensive role of either acquired or *de novo* metabolites in sacoglossans has been limited by metabolite instability or by the small amounts available for bioassays.



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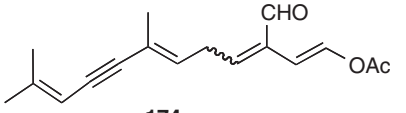

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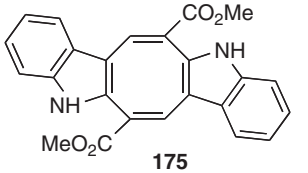

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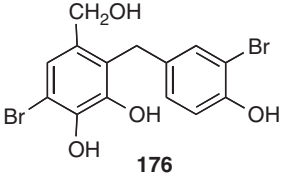

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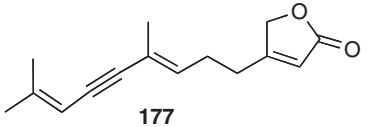

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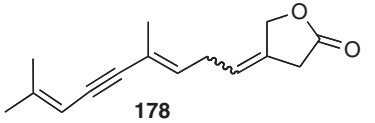

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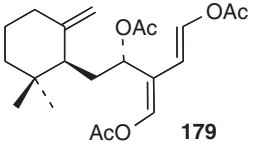

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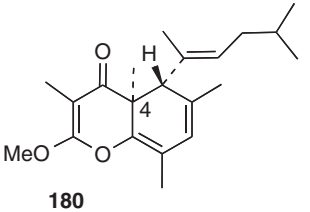

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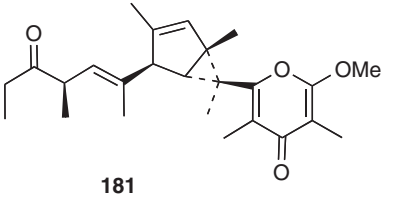

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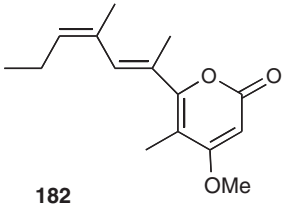

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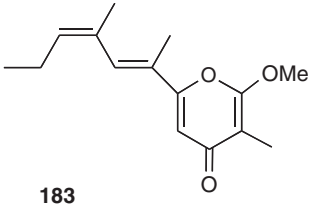

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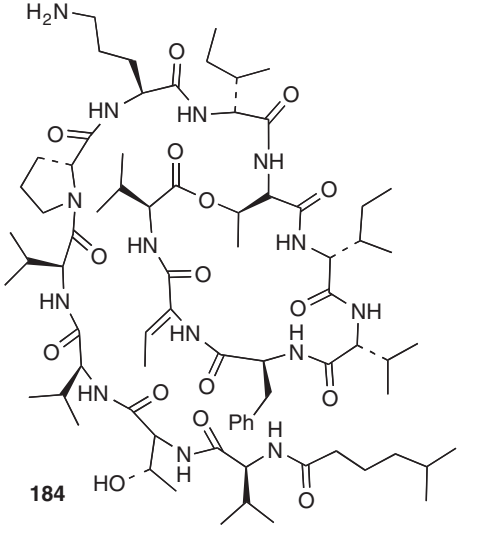


184

In a classic study, *Elysiella pusilla* (= *Elysia halimedae)* was shown to acquire the diterpenoid metabolite halimedatetraacetate **16** from its algal diet of *Halimeda macroloba* and to convert this metabolite into the alcohol **173** that is stored in high concentrations in its body, mucus, and egg masses. Both **16** and **173** deterred feeding by reef fish at natural concentrations, yet the alcohol is the single major metabolite in the mollusk, which does not sequester the more potent feeding deterrent halimedatrial **17**.[197] Oxynoid mollusks of the genera *Ascobulla* (= *Cylindrobulla*), *Oxynoe*, and *Lobiger* show an activated chemical defense strategy based on caulerpenyne **21** that is present in their *C. prolifera* diet (see Section 4.12.3.1). The Mediterranean species *Oxynoe olivacea*, *Ascobulla fragilis*, and *Lobiger serradifalci* contain the monoaldehyde oxytoxin-1 **23** in external parts. An anatomical study revealed that oxytoxin-1 is stored in the body parts of *O. olivacea* (tail) and *L. serradifalci* (parapodia) that detach when the mollusks are molested. In *O. olivacea* and *A. fragilis*, further modification to the dialdehyde oxytoxin-2 **22** also occurs.[198] The enzymatic processes involved in the biotransformation of the conjugated enol acetate to aldehyde functionality have been studied in a cell-free system from *O. olivacea*, from which the unstable metabolite pre-oxytoxin-2 **174** was characterized.[199] The mucus secretions of all three mollusk species, which are deterrent to marine fish, contain high concentration of oxytoxin-1, while *A. fragilis* alone retains the less deterrent caulerpenyne in its internal body parts. Oxytoxin-1 deterred feeding by the marine fish *Thalassoma pavo* when incorporated into an artificial diet at 4%; the chemical instability of oxytoxin-2 prevented its assay.[198] In a chemical study of Caribbean sacoglossans, six species contained halimedatetraacetate **16**, caulerpin **175**, or avrainvilleol **176**, all acquired from their algal diet, while five species were found to acquire and modify caulerpenyne, including *Ascobulla ulla*, which in contrast to *A. fragilis* contains ascobullin-A **177** and -B **178** in which the 1,4-dialdehyde has been further modified into a γ-lactone ring.[200] In contrast, *Elysia crispata* contained crispatenine **179**, likely derived from algal sources, in addition to the polypropionates tridachiahydropyrone **180** (for which revised stereochemistry is shown at C-4[201]) and crispatene **181**.[202,203] The Indo-Pacific sacoglossan *Cyerce nigricans* sequesters small amounts of the deterrent terpene chlorodesmin **18** from its dietary alga *Chlorodesmis fastigiata*. Although crude organic extracts of the mollusk strongly deter feeding in aquarium assays, neither chlorodesmin nor the two polypropionate metabolites **182** and **183** that were also isolated provide an effective chemical defense at the concentrations found in this mollusk species.[48,204] The chemical strategies of Mediterranean[205] and Caribbean sacoglossans have been compared and placed in an evolutionary perspective. The more primitive shelled species sequester or biotransform *Caulerpa* metabolites, while shell-less species of the Elysiodea frequently contain polypropionate metabolites.[200] Sacoglossan polypropionate compounds are biosynthesized *de novo*,[206] and various ecological roles have been proposed for them.[206,207]

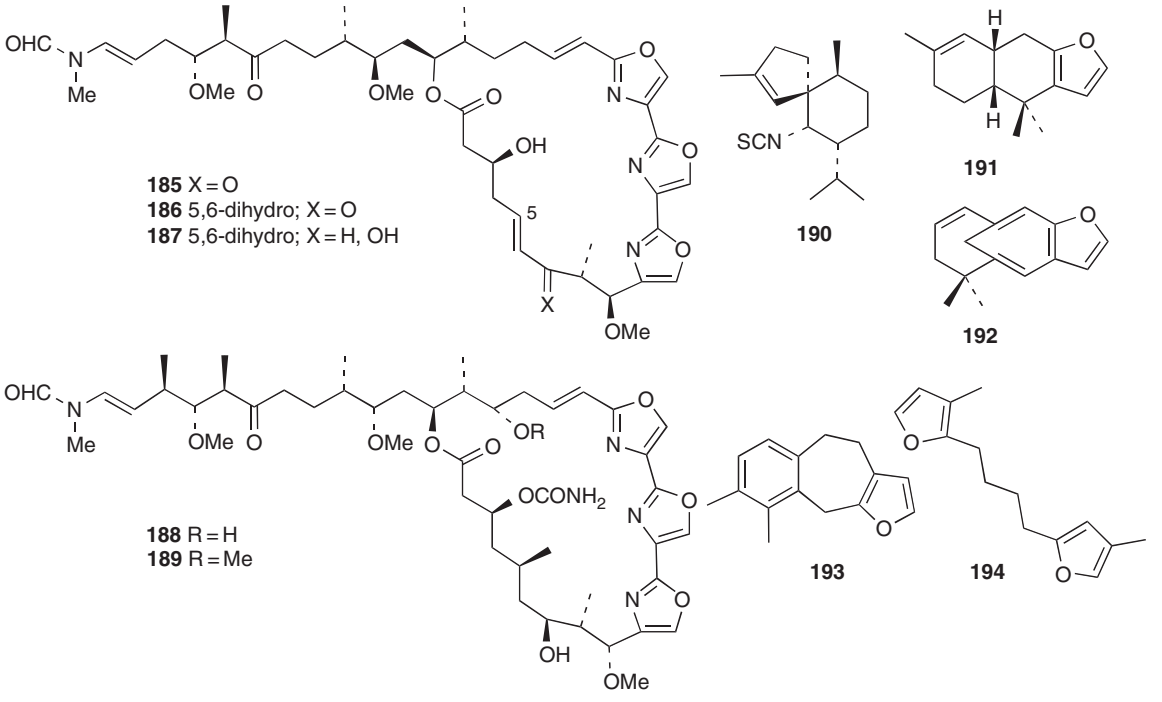
The Hawaiian sacoglossan *Elysia rufescens* sequesters the depsipeptide metabolite kahalalide F **184** from its algal diet *Bryopsis* sp. Both algal and mollusk extracts were deterrent when tested against natural populations of reef fish, as was the peptide metabolite.[208] Kahalalide F shows potent cytotoxicity against a range of tumor cell lines.[209]

4.12.8.3 Nudibranchs

Nudibranchs are soft-bodied mollusks that feed on sponges, ascidians, or soft corals, or on other mollusks. They often exhibit a range of chemical types and structures that reflect their choices of food. Many of the sequestered metabolites are toxic, and they are often localized in specialized spherical dorsal glands called mantle dermal formations (MDFs), or in the mantle border or in the gills, parts of the animals that may be expected to be prone to predation. Given the complex life cycle, communication, and reproductive needs of nudibranchs, the sequestered metabolites may play multiple ecological roles. Some nudibranchs manufacture their own metabolites, thereby reducing their dependence on an external food source; this strategy may have ecological benefits during reproduction.[210–212]

Chemically defended nudibranchs are often highly colored as a warning to predators. One of the first comprehensive studies on sponge–nudibranch chemistry involved the conspicuous Spanish dancer nudibranch *Hexabranchus sanguineus* and its dietary sponge *Halichondria* spp., which both contain macrolide metabolites. Halichondramide **185** was found only in sponge tissue, but its dihydro- and tetrahydro- analogues **186** and **187** were present in various body parts including the mantle, in the brightly colored egg masses, and in the mucus extract of the mollusk, suggesting that the mollusk had modified dietary **185**. Two other macrolides of sponge

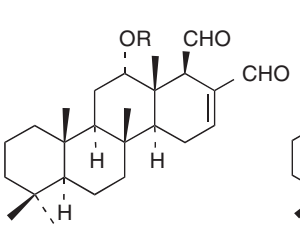
origin, the kabiramides B **188** and C **189**, were also found in *H. sanguineus* and its egg ribbons. Each macrolide deterred feeding by the reef fish *T. lunare*.[213]



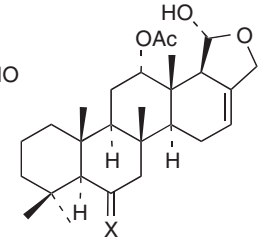
Although a link between deterrency and color was not confirmed in a study on sponge extracts,[83] it appears that color, taste, and olfactory cues together provide phyllidid nudibranchs with an effective antipredator defense against reef fish.[21] There are many reports of sponge-derived isocyano compounds in phyllidid nudibranchs, yet their ecological role in nudibranchs is unclear. Although some of these sponge and nudibranch crude extracts show deterrency in feeding assays, studies using purified sponge-derived isocyanide metabolites have given inconclusive results (Section 4.12.4.2). In a study that confirms dietary transfer, specimens of *Phyllidiella pustulosa* consumed ^{14}C-cyanide or thiocyanate-labeled specimens of the sponge *A. cavernosa*, from which they acquired radioactive axisonitrile-3 **109** and axisothiocyanate-3 **190**.[214]

In a second study on dietary transfer, the MDFs of *Hypselodoris picta* (= *webbi*) assimilate *ent*-furodysinin **191** or the spiniferins-1 and -2 **192** and **193** in addition to their 'regular' metabolite longifolin **194** when the mollusks feed on sponges (*Dysidea fragilis*; *Pleraplysilla spinifera*) known to produce these terpenes.[215] *ent*-Furodysinin, whose absolute configuration is inconsistently drawn in some literature,[216,217] deters feeding by the goldfish *C. auratus*[216] and by *T. pavo* at 4%[217] in aquarium assays. Longifolin was active at 300 μg cm^{-2} in feeding trials with *Carassius carassius* and with the marine fish *Chromis chromis*.[132] Two chromodorid nudibranchs, *Ceratosoma trilobatum* and *C. gracillimum*, also sequester **191** and concentrate this metabolite in their dorsal horn, where it may act as a lure to protect other parts of the mollusk.[218]

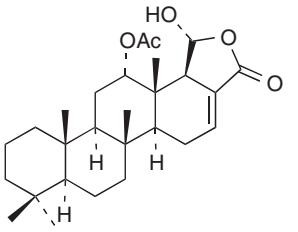
Nudibranchs may selectively concentrate certain allelochemicals alone from their sponge diet, or they may chemically modify the ingested compounds. The Mediterranean *Hypselodoris orsini* transforms the dietary dialdehyde metabolite scalaradial **195** into deoxoscalarin **196**, concentrated in the viscera, and into 6-keto-deoxoscalarin **197** found in MDFs.[219] In Guam, the nudibranch *Glossodoris pallida* converts scalaradial **195** from its sponge diet of *Cacospongia* sp. (a sponge previously described as *Hyrtios erecta*) into deoxoscalarin **196**. *G. pallida* also contains dietary desacetylscalaradial **198**, but does not sequester the major sponge metabolite scalarin **199**.[131,220] Other glossodorid nudibranchs feed on sponges that contain heteronemin **200**.[131] Collections of *G. pallida* from China, and of glossodorid mollusks from Australia contain a series of 12-oxo scalaranes that may also be formed by dietary modification.[221]



195 R = Ac
198 R = H



196 X = H_2
197 X = O



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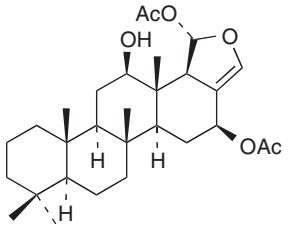


200

The ecological significance of the metabolite patterns in these mollusk species is not yet fully understood, although it is proposed that the oxidative transformations may provide detoxification mechanisms. Deoxoscalarin **196** is found in the reproductive system, eggs, and mantle border of *G. pallida*, while desacetylscalaradial **198** and scalaradial **195** are predominantly present in the mantle border. Although removal of the nudibranch mantle increases susceptibility to predation by reef fish, the specific location of the diet-derived terpenes in the mollusk was not considered significant.[220] Nor does the presence of deoxoscalarin **196** provide protection to the egg masses of *G. pallida* since these are eaten by a variety of fishes.[131] When tested for their deterrent effects against a range of predators, *Cacospongia* and *Glossodoris* extracts and metabolites have given variable results. Crude extracts from glossodorid nudibranchs do not deter feeding by the pufferfish *C. solandri* at ecologically relevant concentrations, but some host sponge extracts are deterrent.[131] Laboratory experiments first revealed that freshwater and marine fish rejected food after tasting pellets containing scalaradial **195** at 60 μg cm^{-2} or deoxoscalarin **196** at 250 μg cm^{-2}.[132] A later study established that scalaradial **195** and heteronemin **200** lack deterrency, while scalarin **199** was a feeding attractant when tested against *C. solandri*.[93,131] In laboratory assays using the crab *Leptodius* sp., scalaradial (**195**) was deterrent when incorporated into squid pieces at 2.5% dry mass.[220] In the field, scalaradial **195** and heteronemin **200** deterred feeding by reef fishes at one location (Haps Reef) when tested at 2.5 and 1.5% respectively, but scalaradial **195** was not deterrent when tested at 1.5% at a second site (Fingers Reef). A 1:1 mixture of scalaradial **195** and scalarin **199** was deterrent when tested at a combined concentration of 1% at Haps Reef.[131] Glossodorid nudibranchs may live on chemically defended sponges to avoid accidental predation rather than to acquire an antipredator chemical.[131,222] The glossodorid nudibranchs typically prefer to feed on basal pieces of sponge tissue where they are better protected from potential predators, even though these locations are less concentrated in defensive chemicals than are the tips. In contrast, *C. solandri* or reef fishes did not express any preference for foods containing low rather than high concentrations of the sponge extracts.[222] These studies highlight the importance of testing marine metabolites at varying concentrations against a range of potential predators.

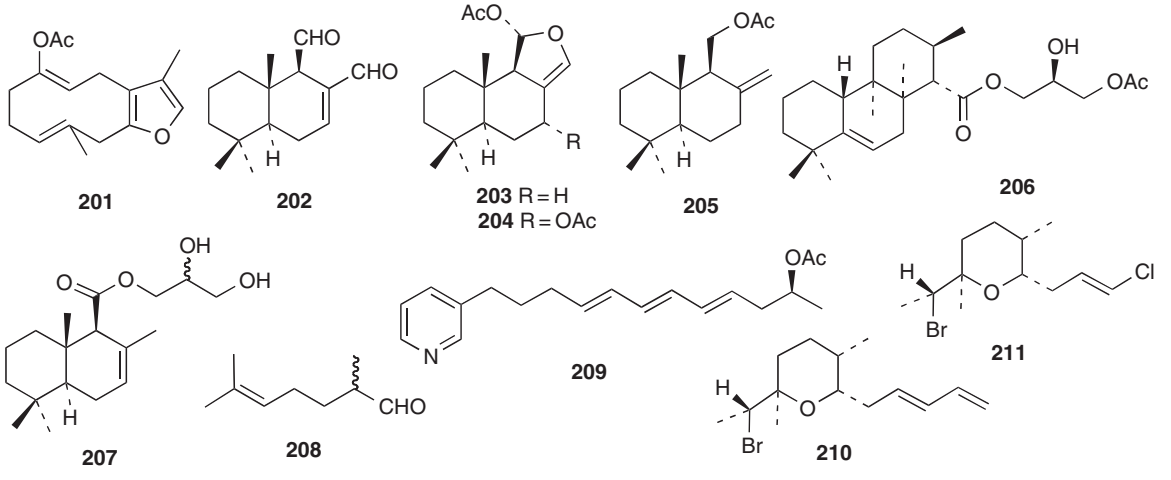
Some nudibranchs species feed on coelenterates, ascidians, or bryozoans rather than on sponges. In Guam the aeolid nudibranch *Phyllodesmium guamensis* is a cryptic, nocturnal species that lacks the nematocyst-based defense of other aeolid nudibranchs, and grazes on soft coral species including *S. maxima* and *S. polydactyla*. Intact nudibranchs or their cerata are rejected, in contrast to mantle or viscera, when provided to *C. solandri* in laboratory assays. Field assays give variable results; some reef fishes consume body parts while other fishes including the butterfly fish *Chaetodon auriga*, reject them. The mollusk bioaccumulates the diterpene 11β-acetoxypukalide **117** from *S. maxima* and stores this metabolite in its cerata. Mantle tissue contains lesser amounts of **117** while traces are also evident in mucus and egg masses of the nudibranchs. Metabolite uptake is selective since the related soft coral metabolite pukalide **116** was not sequestered. In the laboratory, 11β-acetoxypukalide deters feeding by *C. solandri* when mixed into an artificial food diet at 0.5% of dry mass; this concentration is an order of magnitude less than that found in the cerata, but similar to that found in the viscera. However, **117** was not found to be an effective defense when tested at 5 and 9% of dry mass in field assays that involved omnivorous reef fishes, a result which may explain the nocturnal behavioral pattern of this nudibranch.[223] In an earlier study, extracts from *Sinularia* soft corals at 3–7%, and terpene **117** at 2%, were deterrent against reef fishes, while much higher concentrations (19–20%) were required to deter feeding by *C. unimaculatus*, which selectively feeds on the corals.[140]

Dense colonies of the dendronotid nudibranch *T. hamnerorum* have been observed feeding on the sea fan *G. ventalina* from which the mollusks sequester defensive chemicals. In both field and laboratory assays, fishes rejected nudibranch tissue, while squid pellets containing natural volumetric concentrations of nudibranch extract deterred feeding by *T. bifasciatum* in laboratory assays. Feeding assay-guided fractionation of both nudibranch and gorgonian extracts led to the isolation of the sesquiterpene julieannafuran **201**, present in higher concentrations in nudibranch tissue compared to gorgonian tissue, and which was responsible for the deterrency. None of the other chemicals isolated from the nudibranch or the gorgonian showed any significant deterrency.[224] The shelled gastropod *Cyphoma gibbosum* that also inhabits *G. ventalina* did not contain any gorgonian metabolites.[224] In an earlier study, extracts of *G. ventalina* deterred feeding by *C. gibbosum* and by reef fishes when incorporated into an artificial diet at 12%. Sclerites afforded an additional defense; in contrast to the gorgonians described in Section 4.12.5.2, deterrent chemicals are uniformly distributed throughout the colony.[225]

Some groups of nudibranchs, typically those from temperate waters, manufacture their own defensive terpenoid chemicals; this may be a response to the lack of terpenes in the preferred sponge diet.[210,211] The ability to synthesize defensive metabolites confers an ecological advantage over sequestration, and is regarded as an evolutionary advance.[226]

De novo biosynthesis in nudibranchs was first demonstrated when Cimino *et al.*[227] incorporated radiolabeled mevalonate into polygodial **202**, a sesquiterpene metabolite of *Dendrodoris limbata* and of *Dendrodoris grandiflora*. Polygodial, first isolated from plants, is a well-established antifeedant against insects and worms. The hot taste of **202** may be linked to interaction of the aldehyde groups with the amino groups of taste receptors. In nudibranchs, **202** is concentrated in mantle tissue (up to 3.9 mg per animal), and shows potent antifeedant effects against the marine fish *C. chromis* and the freshwater fish *C. carassius*, consistent with a deterrent role *in situ*. Treated food particles were 'mouthed but immediately rejected by the fish, the minimum inhibitory concentration being 30 $\mu g\,cm^{-2}$', while a series of related sesquiterpene esters were inactive and may represent detoxification products.[132] A more recent study also highlights the repugnant effects of polygodial. Two species of fish offered food containing extracts from the nudibranch *Doriopsilla pharpa* learnt to reject the food; the antifeedant effect was then traced to the presence of **202**.[228]

7-Deacetoxyolepupuane **203** has been found in the hermaphrodite gland and in egg masses in several species of nudibranch. Even though **203** is also found in sponges of the genus *Dysidea*, its *de novo* origin from mevalonate was recently established in two nudibranch species, *D. limbata* and *Dendrodoris arborescens*. Time-course studies suggest that this metabolite may be a biosynthetic precursor for drimane metabolites such as **202** in these mollusks.[229] Although **203** has been described as less deterrent than **202**,[229] it significantly deterred feeding when incorporated into agar strips and provided to the spongivorous fish *Pomacanthus imperator* in laboratory assays. Further, the compound caused necrosis in adjoining sponges, including *Cacospongia* sp.[230] The related olepupuane **204** from *Doriopsilla* spp. represents a protected form of **202**[231] and inhibits feeding of the Pacific damsel fish *Dascyllus aruanus* at 15–20 $\mu g\,mg^{-1}$ of pellet, and is considered as effective as **202**.[232]



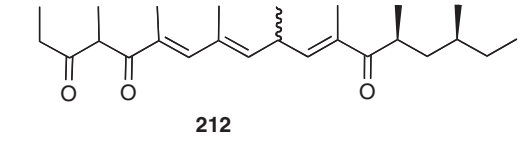
201 **202** **203** R = H, **204** R = OAc **205** **206** **207** **208** **209** **210** **211**

Other deterrent nudibranch metabolites that are shown to be biosynthesized *de novo* include the antifeedant[233] albicanyl acetate **205** in *Cadlina luteomarginata*,[234] the ichthyotoxic diterpenoic acid glyceride verrucosin A **206** from *Doris verrucosa*,[235] and the sesquiterpene-derived **207** from *Archidoris montereyensis*,[236,237] which was active in an assay using the tidepool sculpin *Ologocottus maculosus*.[236] 2,6-Dimethylheptenal **208**, whose mevalonate origin has been established, may be responsible for the repellent nature of gland extracts from *Melibe leonina*[238] although this could not be demonstrated by fish-feeding assay.[236]

4.12.8.4 Other Mollusks

The haminol metabolites are found in parapodia and other external parts of *Haminoea* spp. and their mollusk predators, and they act primarily as alarm pheromones rather than antifeedants.[239] Haminol-2 **209** has been shown to derive *de novo* in *Haminoea orbignyana* from nicotinic acid via the polyketide pathway.[240,241] Unusually, the related species *Haminoea cyanomarginata* contains the bromopyran metabolite **210**, a typical sponge metabolite, even though the mollusk is a herbivore. Compound **210** was unpalatable to the marine shrimp *Palaemon elegans* when provided in artificial food at a natural volumetric concentration.[242] In field assays, the related compound **211** of *H. cymbalum* from Guam deters feeding by carnivorous fishes.[243]

The opisthobranch mollusk *Bulla striata* contains aglajne 1–3 **212–214** for which a polypropionate origin has been established by radiochemical labeling;[244] these metabolites, which are deterrent in laboratory assays with the freshwater fish *C. auratus*, are localized in glands along the margin of the mantle and in mollusk secretions, consistent with a defensive role.[239] Carnivorous Aglajide mollusks feed on *Bulla* species from which they acquire the aglajne metabolites.[245] Vallartanone B **215** for which the C-8 configuration has been revised by total synthesis[246] is a metabolite of the pulmonate limpet *Siphonaria maura* that shows some fish-feeding deterrency in laboratory assays.[247] Experimental evidence supporting an antifeedant role for other siphonariid polypropionate metabolites is lacking, although the compounds are localized in the foot tissue and in the mucus trails of these mollusks.[206] Antifeedant activity has been suggested for membrenone A **216** from the notaspidean mollusk *Pleurobranchus membranaceus* on the basis of a preliminary bioassay against *C. auratus* and the localization of this polypropionate in the skin of this mollusk.[248] The Antarctic shell-less mollusk *Clione antarctica* contains the polyketide pteroenone **217** that is deterrent to fish. The amphipod *Hyperiella dilatata* attaches individuals of *C. antarctica* to its back, thereby taking advantage of this chemical defense to avoid predation.[249]



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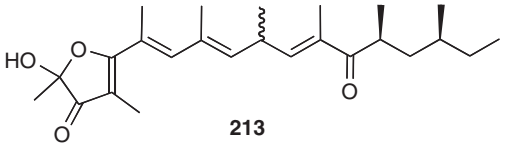
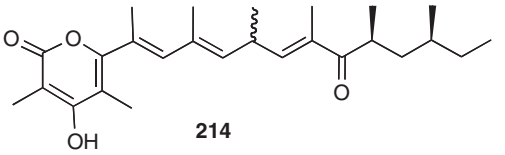
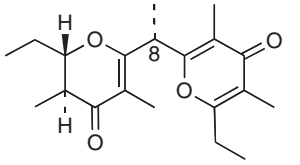


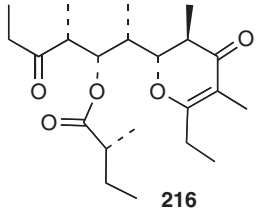
213



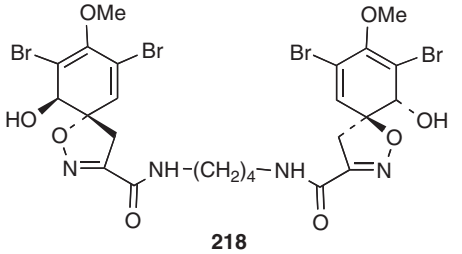
214



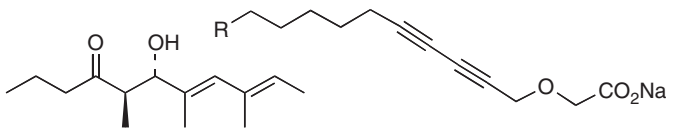
(215)



216



218



217

219 R = CH_2=CH
220 R = CH_3CH_2

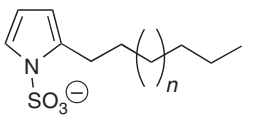
The cryptic gastropterid mollusk *Sagaminopteron nigropunctatum* and the brightly colored *Sagaminopteron psychedelicum* both feed on the sponge *D. granulosa* from which they sequester polybrominated diphenyl ethers.[250] In addition to cryptic coloration, the chemical deterrency of these mollusks provides a defense against predators. The major metabolite **110** which is concentrated in mantle tissue, mucus, and parapodia of both species, and in the egg masses of *S. nigropunctatum*,[250] deters feeding by pufferfish, crabs, and sea hares at concentrations below those found in these two mollusks.[93,127] In aquarium assays, pufferfish avoid eating *S. nigropunctatum*. This mollusk prefers to feed on sponge ectosomal tissue[250] where cyanobacterial symbionts responsible for bromoether synthesis are localized.[134] The same dietary strategy is shared by the gastropod mollusk *T. perversa*, which selects the ectosomal tissue of *A. aerophoba* from which it acquires bromotyrosine metabolites including aerophobin-2 **59**.[251] In the sponge, these compounds are localized in specialized surface (spherulous) cells that are known to have a defensive function. The mollusk stores the sequestered alkaloids in mantle, mucus, and egg masses.[252] At natural concentrations, crude extracts of *T. perversa* and from their egg masses were more deterrent to damselfish than were sponge extracts; individual alkaloids were not tested in these assays. The presence of aerothionin **218**, a metabolite of *Aplysina cavernicola* but not of *A. aerophoba*, in the mollusk may be due to biotransformation of sequestered alkaloids.[251]

The majority of mollusks search out food by as yet unknown mechanisms that may be related to the chemical composition of their diet. A recent report of interest is that the muricid gastropod *Drupella cornus* which is a voracious predator of coral tissue locates its food source *Montipora* sp. through the presence of water-soluble sodium salts of montiporic acids C and D, **219** and **220**, that are released in the mucus of this hard coral species.[253]

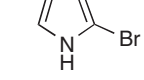
4.12.9 Other Marine Organisms

4.12.9.1 Worms

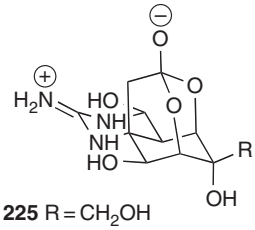
Bioassay-guided fractionation of the marine annelid worm *Cirriformia tentaculata* has led to the isolation of three 2-alkylpyrrole sulfamates **221–223**. Feeding deterrency studies using food pellets that nutritionally mimicked the worm revealed that mixtures of the three metabolites reduced feeding by *T. bifasciatum* by 80%.[254,255] The marine worm *Saccoglossus kowalevskii* is unpalatable to fishes, and this has been traced to the presence of 2,3,4-tribromopyrrole **224**. Other marine worms that are known to produce brominated metabolites failed to deter predation, as did a range of brominated aromatic compounds, previously reported as natural products, when tested at natural concentrations.[256,257] Like nudibranchs, marine worms are often brightly colored and this may be an important component of their survival strategy. A correlation between bright colors and unpalatability was found when testing marine worm extracts for antipredator effects;[258] an earlier study in sponges had found no such correlation.[83]



221 $n = 1$
222 $n = 2$
223 $n = 3$



224



225 R = CH_2OH
226 R = H

A polyclad flatworm (planocerid sp. 1) collected in Guam was found to contain the neurotoxin tetrodotoxin (TTX; **225** and 11-nortetrodotoxin-6(*S*)-ol **226** which it uses to kill mobile prey such as gastropod mollusks. Consistent with a role in prey capture, levels of TTX in the flatworm decreased after they were fed on cowries. Although an antipredation role has been implied for TTX in some terrestrial species, its presence does not prevent flatworms from being consumed by reef fish.[259]

4.12.10 Conclusions

The studies described above show that many factors, both acquired and environmental, determine the fitness of an organism in the marine environment. Many organisms have adapted to the competitive marine environment by production of chemical defenses that help ensure their survival; in some cases, a mixture of chemicals may act synergistically to provide an optimal defense. The complexity of marine food chains can result in the same molecule playing a deterrent role in a suite of organisms, and against a range of predators. Specialized chemical defenses may be required at certain stages of the organism's life cycle, notably during reproduction processes, or in egg masses. Many sponges and mollusks defend exposed tissues; even fast-growing algae protect their most vulnerable tissues. Most significant is that chemical defense does not reside in a single class of marine natural product; this survey has revealed the potency of all the main classes of marine natural product, including terpenes and other acetate or propionate-derived compounds, alkaloids, sterols, as well as aromatic products of shikimate metabolism or of mixed biosynthetic origin. It has also highlighted the need for careful chemical study to correctly analyze the deterrent molecules, and in particular to identify activated chemical defenses in which rapid chemical changes may occur on tissue treatment. In conclusion, ecological observations and studies on antifeedants provide detailed information about the intended biological effects of marine compounds (see Chapter 4.06), and increasingly also about their likely pharmacological profiles. This knowledge contributes to the development of novel therapeutic and agrochemical agents from marine sources.

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Biographical Sketch

Mary Garson obtained a Ph.D. in organic chemistry under Professor Jim Staunton at the University of Cambridge, UK in 1977. After postdoctoral work at the Universita Cattolica del Sacro Cuore, Rome, Italy, followed by 2 years in the pharmaceutical industry, she migrated to Australia in 1983 as a Queen Elizabeth II Research Fellow at James Cook University of North Queensland where she began to study isocyanide biosynthesis in marine sponges. She has held academic positions at The University of Wollongong and The University of Queensland, where she is now a Professor of Chemistry and Deputy Head of the School of Chemistry and Molecular Biosciences. Her research interests are in determining how the structures of marine natural products relate to their biological functions, and therefore encompass biosynthesis and ecology. Additionally she runs a research program exploring the chemistry of medicinal plants from South East Asia. She is a keen scuba diver, especially in the warm waters of Queensland. From 2003 to 2005, she was chair of the Australian Science Olympiads, and she is currently secretary of Division III (Organic and Biomolecular) of IUPAC.

4.13 Allelochemicals for Plant–Plant and Plant–Microbe Interactions

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Masahiro Natsume, Tokyo University of Agriculture and Technology, Tokyo, Japan

4.13.1 Allelochemicals in Plant–Plant Interactions

4.13.1.1 Introduction

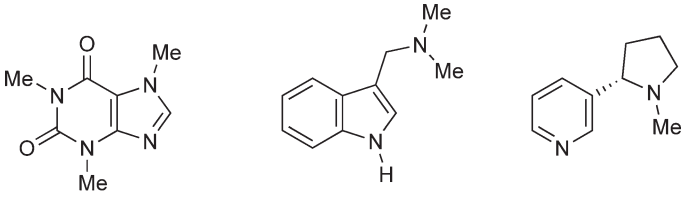
Allelochemicals are chemicals that are released from donor organisms into the environment and affect the growth and development of receiver organisms. In this section, the chemistries and functions of allelochemicals involved in plant–plant interactions are reviewed. However, it is likely that some of the compounds active in plant–plant interactions also participate in the interactions between plants and microorganisms, insects, and other animals. Although simple inorganic compounds and primary metabolites released from plants and their decaying residues often have pronounced effects on the growth and development of plants in the vicinity, these will not be considered as allelochemicals here.

Plants release allelochemicals as volatiles, leaf leachates, and root exudates. In addition, all the constituents of plant residues are eventually released into the environment through microbial decomposition. During this process, most allelochemicals lose their activity but some compounds, for example, benzoxazinoids (cyclic hydroxamic acids), can be activated after hydrolysis.[1–3]

4.13.1.2 Allelochemicals

4.13.1.2.1 Alkaloids

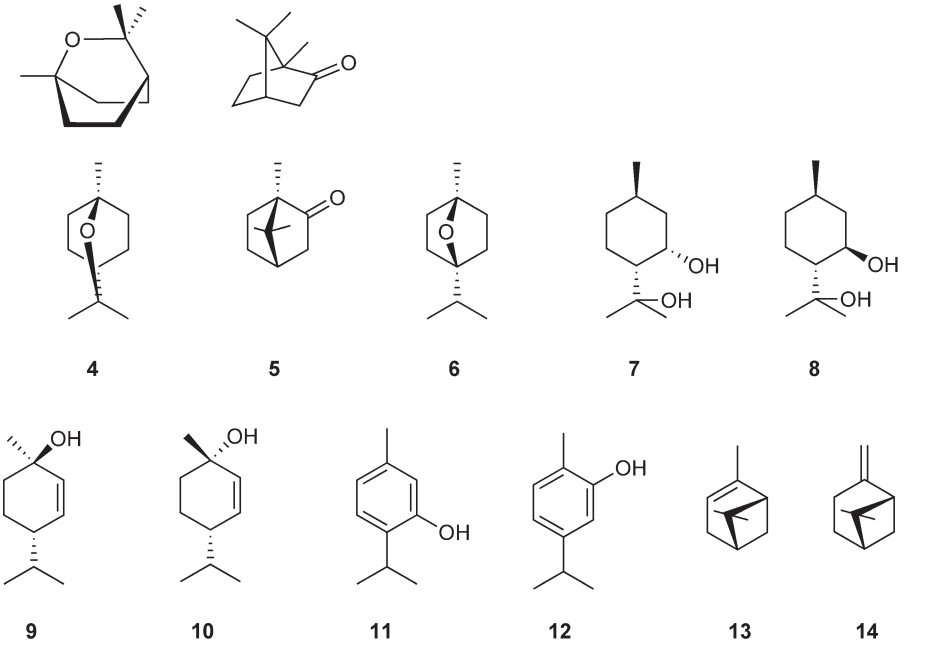
Although diverse plant alkaloids are active as defensive compounds against invertebrate and vertebrate herbivores, only a few appear to be involved in plant–plant interactions. For example, it is well known that the purine alkaloid caffeine (**1**) causes autotoxicity in coffee and tea plantations.[4] Most plant alkaloids including gramine (**2**) and nicotine (**3**) affect seed germination and shoot growth, but at relatively high concentrations (>0.1%). This is higher than the active concentrations of other allelochemicals, for example, phenolic compounds, that are active at 10–200 ppm.



1 **2** **3**

4.13.1.2.2 Terpenoids

There are a number of allelochemicals among the mono-, sesqui-, and diterpenoids. In particular, plants in arid and semiarid regions produce diverse volatile terpenoids with allelopathic activity.[5] Among the volatile monoterpenes, 1,8-cineole (**4**) and camphor (**5**) exhibit strong growth inhibitory effects on plants and are considered to be involved in plant competition. 1,4-Cineole (**6**), a minor isomer of 1,8-cineole, is a potent inhibitor of asparagine synthetase.[6] *p*-Menthane-3,8-diols (*cis* **7** and *trans* **8**), *p*-menth-2-en-1-ols (*cis* **9** and *trans* **10**), thymol (**11**), carvacrol (**12**), 1,8-cineole, α-pinene (**13**), and β-pinene (**14**) were isolated as allelopathic monoterpenes from *Eucalyptus* species.[7] *Eucalyptus* trees also produce allelopathic sesquiterpenes including spathulenol (**15**), and α-, β-, and γ-eudesmols (**16–18**).[7]

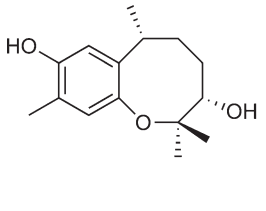


4 **5** **6** **7** **8**

9 **10** **11** **12** **13** **14**

15 **16** **17** **18**

Plants belonging to the family Asteraceae, including asters, daisies, and sunflowers, are rich sources of allelopathic sesquiterpenes and sesquiterpene lactones. Heliannuol A (**19**) and its related compounds heliannanes, found in sunflower leaves, are thought to be involved in the allelopathic effects displayed by the plants.[8] Some sesquiterpene lactones such as dehydrocostuslactone (**20**), santamarine (**21**), and reynosin (**22**) have been shown to induce seed germination of root parasitic plants,[9] especially of sunflower broomrape *Orobanche cumana*.[10]



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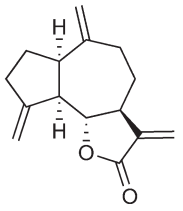

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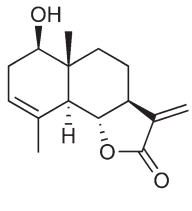

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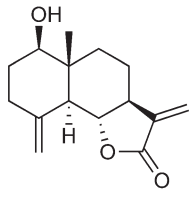

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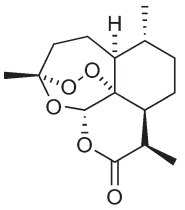
Artemisinin (**23**), isolated from a Chinese medicinal plant, annual wormwood (*Artemisia annua* L.), is a unique sesquiterpene lactone bearing an endoperoxide moiety. This compound displays a strong antimalarial activity and inhibits seed germination and plant growth.[11]

Rice plants produce various diterpene phytotoxins when elicited by biotic and abiotic stresses. Among these phytotoxins, momilactones[12] (momilactone B, **24**) and oryzalexins[13] (oryzalexin C, **25**) exhibit strong growth inhibitory effects on cress and other plant species. In particular, momilactone B (**24**) is a highly potent growth inhibitor; its concentration in rice tissues and the amounts released from rice plants into the environment are high enough to suppress the growth of susceptible plant species in the vicinity.[14]



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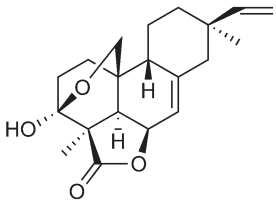

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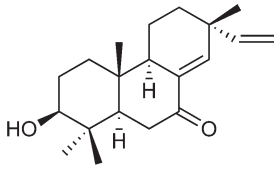

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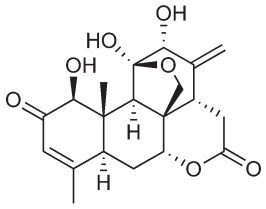
Quassinoids are bitter components produced by members of the Simaroubaceae. They exhibit diverse biological activities including anticancer, antimalarial, insecticidal, and phytotoxic activities. Ailanthone (**26**), chaparrinone (**27**), and other quassinoids, along with indole alkaloids, contribute to the invasiveness of the Chinese tree tree-of-heaven (*Alianthus altissima* Swingle), in Europe.[15]



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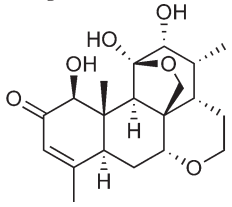

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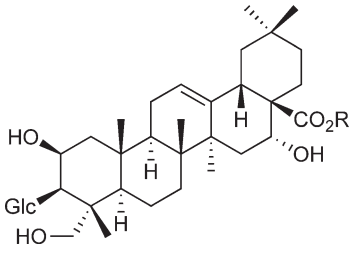
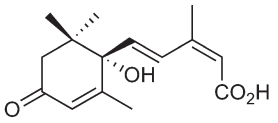
Durantanins I (**28**), II (**29**), and III (**30**), the triterpenoid-type saponins, are allelochemicals from *Duranta repens* L.[16]



R	
Rha-Api-Rha-Ara	**28**
Rha-Xyl-[Api]-Rha-Ara	**29**
Rha-Xyl-Rha-Ara	**30**

There are two important classes of allelochemicals synthesized by oxidative cleavages of tetraterpene carotenoids. One is the plant hormone abscisic acid (ABA, **31**) that plays important roles in growth and development of plants, especially in seed development and dormancy.[17] Dry dormant seeds contain relatively large amounts of ABA, particularly in the seed coats. ABA and phenolic allelochemicals in the seed coats are easily released into the environment when the seeds are imbibed, resulting in inhibition of seed germination and seedling growth of plants in the vicinity. Both ABA and the phenolic compounds are rapidly broken down in the soil, and therefore the inhibition is short-lived.

The other important apo-carotenoids are strigolactones, which induce seed germination of root parasitic plants[18] and hyphal branching in symbiotic arbuscular mycorrhizal (AM) fungi.[19] Strigol (**32**) and strigyl acetate (**33**), the first strigolactones, were isolated from root exudates of a nonhost plant, cotton, as germination stimulants for *Striga lutea* (*Striga asiatica*).[20] Later, strigol was identified in root exudates of *Striga* hosts, including sorghum, maize, and proso millet.[21] Sorgolactone (**34**) was isolated from sorghum.[22] Orobanchol (**35**) was isolated as the first *Orobanche* germination stimulant from red clover root exudates.[23] Although alectrol was first purified from cowpea root exudates as a germination stimulant for *Alectra* and *Striga gesnerioides*,[24] it was recently identified as orobanchyl acetate (**36**).[25] Orobanchol and orobanchyl acetate were found in root exudates of various plant species including red clover, soybean, and cowpea. 5-Deoxystrigol (**37**), isolated from root exudates of *Lotus japonicus* as the first branching factor for AM fungi[19] and later as a germination stimulant,[26] is one of the major strigolactones in both monocotyledonous[27] and dicotyledonous plants.[28] 5-Deoxystrigol is a key precursor of strigolactones; the allylic hydroxylation yields strigol or orobanchol. The oxidation of one of the gem-dimethyl groups affords sorgomol (**38**) and the subsequent decarboxylation leads to sorgolactone.[29,30] Tobacco plants were found to produce at least five different germination stimulants, three of which were identified as solanacol (**39**), 2′-epi-orobanchol (**40**), and orobanchol.[31] Solanacol and 2′-epi-orobanchol are the first strigolactones to be identified having a benzene ring and a 2′-epi stereochemistry, respectively. In addition to these strigolactones, there are several novel strigolactones with structures that are yet to be determined. Since plants exude a mixture of strigolactones, qualitative and quantitative differences may contribute to the host recognition by both root parasitic plants and AM fungi. Strigolactones on their metabolites are novel class of plant hormones inhibiting shoot branching.[32,33]



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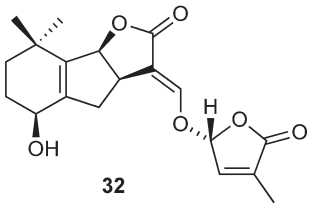

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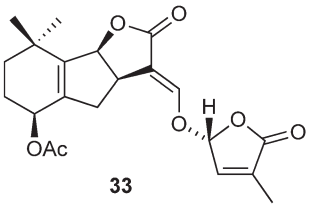

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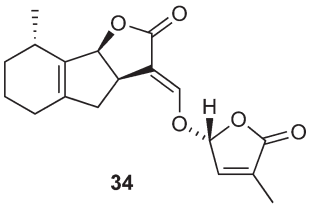

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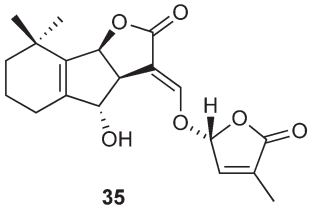

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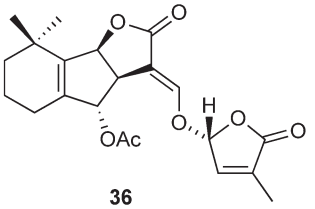

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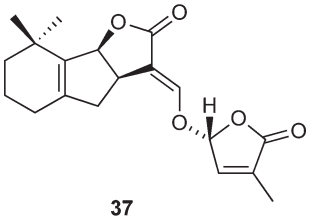

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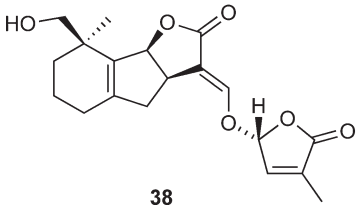

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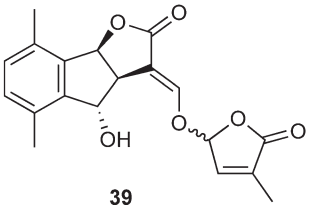

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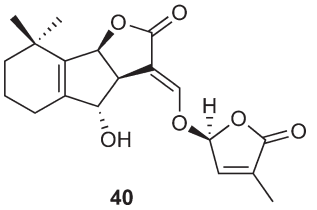

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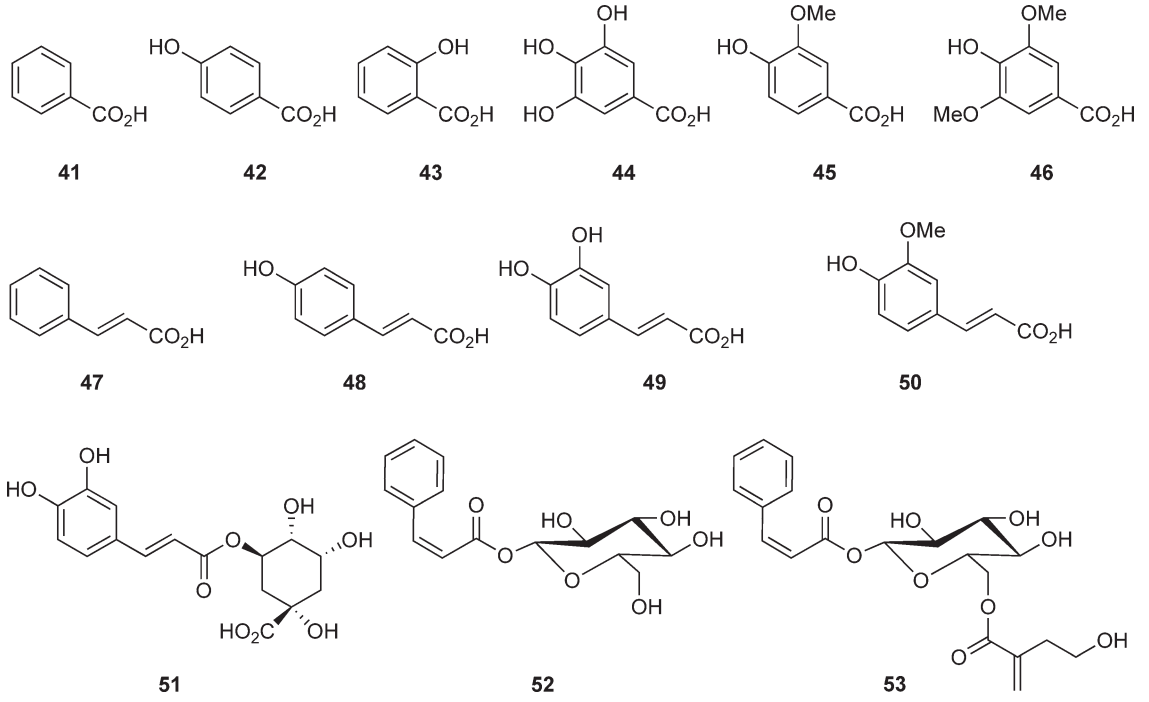
4.13.1.2.3 Phenolic compounds

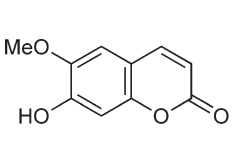
Phenolic compounds constitute a major group of allelochemicals including simple benzoic and cinnamic acid derivatives, quinones, and flavonoids. Benzoic and cinnamic acid derivatives are the most common plant-originated allelochemicals. Some examples of these substances are benzoic acid (**41**), *p*-hydroxybenzoic acid (**42**), salicylic acid (**43**), gallic acid (**44**), vanillic acid (**45**), syringic acid (**46**), *trans*-cinnamic acid (**47**), *p*-coumaric acid (**48**), caffeic acid (**49**), ferulic acid (**50**), and chlorogenic acid (**51**). These simple phenolics are thought to influence forest ecosystems, successions, and autotoxicity phenomena.[2] Glucosides of these phenolic compounds also exhibit plant growth inhibitory effects; for example, two glucosides of *cis*-cinnamic acid, 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG) (**52**) and 6-*O*-(4′-hydroxy-2′-methylenebutyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BG) (**53**), isolated from the leaves of *Spiraea thunbergii* Sieb.[34] Although these compounds are abundant in plant tissues, leaf leachates, root exudates, and plant litters, their inhibitory activities on seed germination and seedling growth are rather weak. In addition, their concentrations in the soil may not be high enough to exhibit inhibitory effects due to the losses by rapid degradation, strong binding to humic acids, and adsorption to soil particles. These phenolics have diverse biological activities; salicylic acid, for example, is an inducer of systemic acquired resistance (SAR) against pathogen attack.[35]



Coumarins and their glucosides are ubiquitous and widely distributed secondary metabolites in the plant kingdom. Among them, scopoletin (**54**), umbelliferone (**55**), and esculetin (**56**) are representative allelochemicals. They exhibit various biological activities. In general, these compounds inhibit growth of plants but some display growth promotion at lower concentrations.[36]

5-Hydroxy-1,4-naphthoquinone, juglone (**57**), is a potent allelochemical produced by black walnut (*Juglans nigra* L.). Juglone is a strong inhibitor of hydroxyphenylpyruvate dioxygenase (HPPD), the key enzyme in plastoquinone biosynthesis.[37] Since plastoquinone is a cofactor for phytoene desaturase in the carotenoid biosynthetic pathway, the inhibition of HPPD results in the depletion of carotenoids that protects chlorophylls from photooxidation. Therefore, rapid photobleaching occurs in plants treated with HPPD

inhibitors such as juglone. Juglone also inhibits photosynthetic and respiratory electron transport systems, and the latter appears to contribute to the strong allelopathic effect of juglone.[38]



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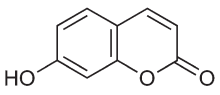

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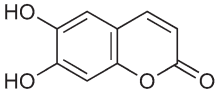

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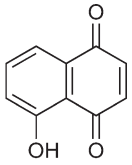

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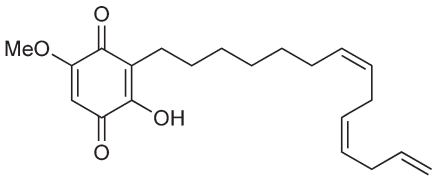
Sorgoleone (**58**) and related compounds are responsible for the allelopathic effect displayed by sorghum. Sorgoleone, as expected from its structural resemblance to plastoquinones and ubiquinones, is a potent inhibitor of both photosynthetic electron transport in chloroplasts and oxidative electron transport in mitochondria.[39] In addition, sorgoleone strongly inhibits HPPD.[37] Dihydrosorgoleone or SXSg (**59**), the reduced form of sorgoleone, was identified as the first *Striga* germination stimulant from a natural host, sorghum.[40] This hydroquinone is exuded as oily droplets from sorghum root hairs and is rapidly oxidized to the quinone sorgoleone. Therefore, the hydroquinone is a nonphytotoxic precursor and its contribution as a germination stimulant of root parasites would be limited as compared to other germination stimulants such as strigolactones.

The haustorium is the attachment organ of parasitic plants, through which the parasites gain water and nutrients from their hosts. In the case of the root parasitic plants *Striga* spp., host root–derived chemicals have been shown to induce haustoria formation in the radicles of germinating seeds. Some quinones including 2,6-dimethoxy-2,4-benzoquinone (DMBQ, **60**) and 5,7-dihydroxynaphthoquinone (**61**) have been identified as haustorium inducers.[41] Xenognosins A (**62**) and B (**63**),[42,43] and the flavonoid peonidin (**64**) also induce haustoria formation.[44]



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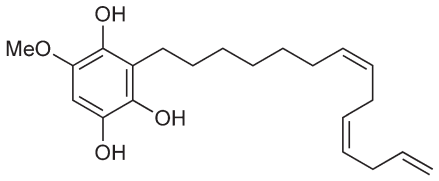

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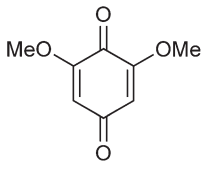

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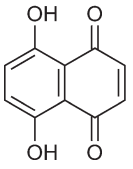

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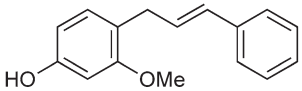

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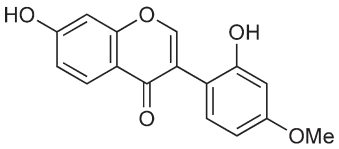

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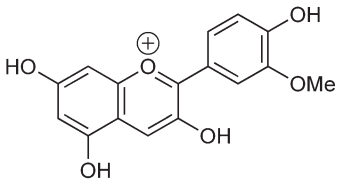
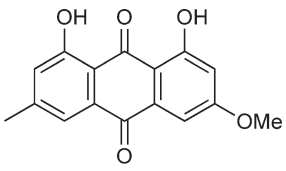


64

Two naphthoquinones, emodin (**65**) and physcion (**66**), and their glucosides were identified as allelochemicals from the rhizomes, aerial parts, and fallen leaves of giant knotweed (*Polygonum sachalinense* Fr. Schm.).[7] In addition, the concentrations of emodin and physcion in the soil samples collected from the weed community were high enough to suppress seedling growth of susceptible plant species.



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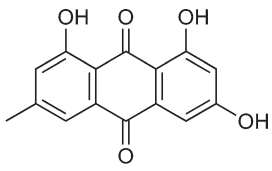
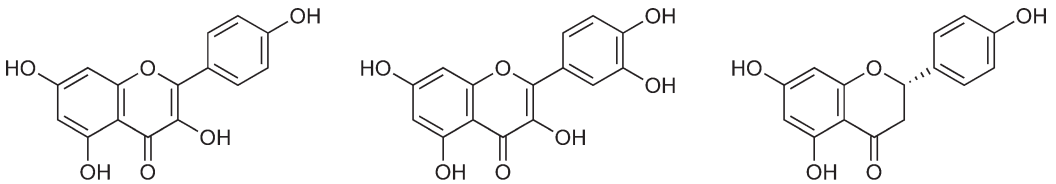
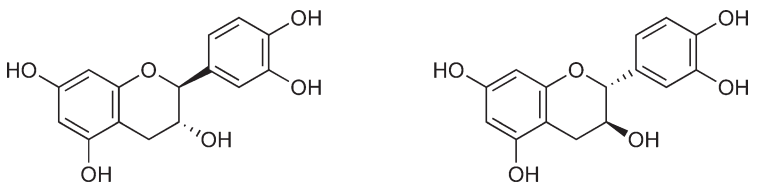


66

Flavonoids are important secondary metabolites involved in pigmentation of flowers, disease resistance, and so on. Among the flavonoids, kaempferol (**67**), quercetin (**68**), and naringenin (**69**) are most often cited as allelochemicals. (±)-Catechin was identified in the root exudates from spotted knapweed (*Centaurea maculosa*) as the compound responsible for its invasive behavior.[45] The plant uses (−)-catechin (**70**) as an allelochemical facilitating its invasion and (+)-catechin (**71**) as a defense compound against pathogens.



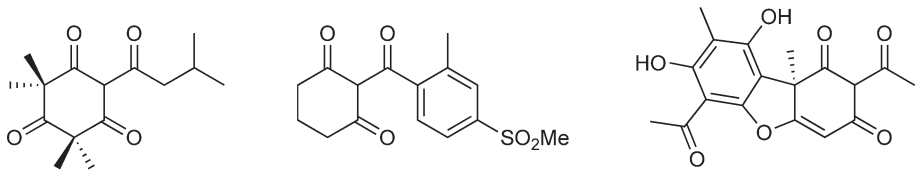
67 **68** **69**



70 **71**

Leptospermone (**72**) is an allelochemical produced by the bottlebrush plant (*Calispermon* spp.).[46] This compound is herbicidal, causing bleaching symptoms, and is a potent inhibitor of HPPD. The triketone-type HPPD-inhibiting herbicides such as sulcotrione (**73**) were developed based on the structure of leptospermone.[47]

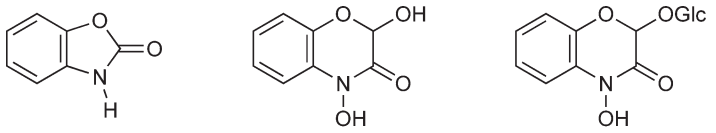
Among phytotoxic compounds produced by lichens, usnic acid (**74**) is one that is unique and relatively abundant.[48] Usnic acid also effectively inhibits HPPD.[37]



72 **73** **74**

4.13.1.2.4 Benzoxazinoids

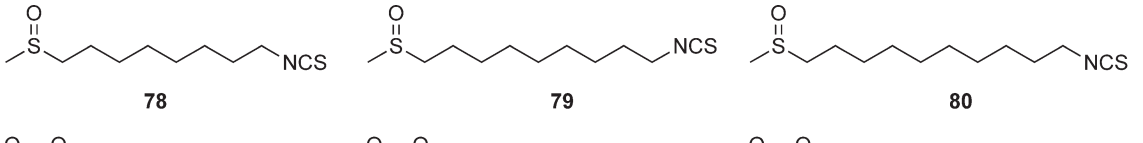
Benzoxazinoids or cyclic hydroxamic acids are typical examples of allelochemicals that have been recognized as natural herbicides, insecticides, and fungicides. They are produced by and released from plants belonging to the family Poaceae. These compounds are also found in plants belonging to the Acanthaceae, Ranunculaceae, and Scrophulariaceae. They are produced by enzymatic degradation of their inactive forms (glucosides) and subsequent chemical degradation.[49] For example, benzoxazoline-2(3*H*)-one (BOA, **75**) is formed by the chemical degradation of 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA, **76**), which is produced by the enzymatic cleavage of the corresponding 2-β-D-glucoside (DIBOA-Glc, **77**). Glucosides stored in the vacuole are released into the environment by root exudation or by mechanical or insect wounding, where enzymatic and/or microbial degradation to benzoxazinoids occurs. Decomposition of plant residues also releases these glucosides and benzoxazinoids.



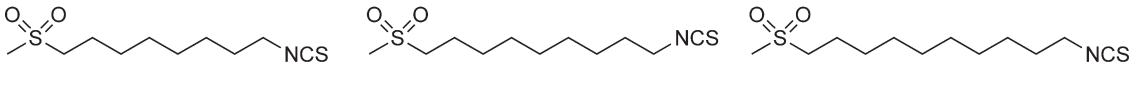
75 **76** **77**

4.13.1.2.5 Glucosinolates and isothiocyanates

Glucosinolates are precursors of biologically active isothiocyanates that play defensive roles against attacks by insects and microorganisms. They are found in plants belonging to the Brassicaceae, Resedaceae, and Capparidaceae.[50] These isothiocyanates and related compounds also inhibit seed germination and seedling growth. Hirsutin (**78**), arabin (**79**), camelinin (**80**), and related ω-methylsulfonylalkyl isothiocyanates **81**–**83** are allelopathic chemicals produced by *Rorippa indica* (L.) Hiern.[51]



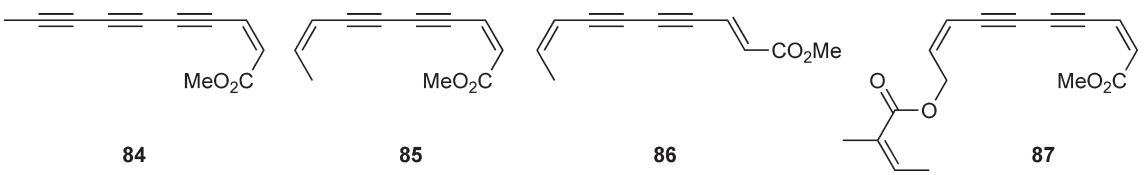
78 **79** **80**



81 **82** **83**

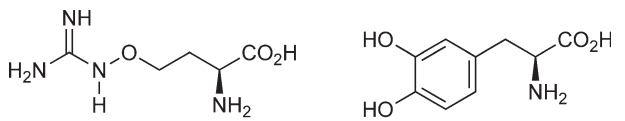
4.13.1.2.6 Other allelochemicals

cis-Dehydromatricaria ester (*cis*-DME, **84**), *cis*-matricaria ester (*cis*-ME, **85**), *trans*-matricaria ester (*trans*-ME, **86**), and *cis*-lachnophyllum ester (*cis*-LE, **87**) are C_{10}-polyacetylenes with strong growth inhibitory activities.[52] *cis*-DME is a major allelochemical produced by *Solidago altissima* L. It was found with its *trans*-isomer, being formed by *cis*–*trans* isomerization, in the soil at the border of *S. altissima* communities at concentrations high enough to suppress the growth of other plant species. *cis*- and *trans*-MEs and *cis*-LE are allelochemicals produced by *Erigeron* species. These polyacetylenes are thought to contribute to the competitiveness of these plant species in early stages of secondary succession in urban wastelands or abandoned fields. In addition, accumulation of phytotoxic polyacetylenes in soil results in autotoxicity, and therefore the occupation by these plant species is relatively short.



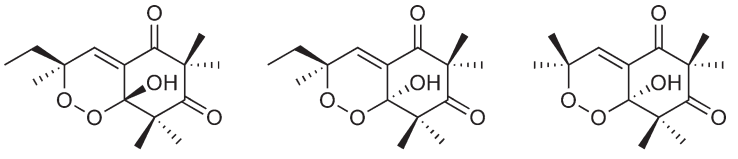
84 **85** **86** **87**

L-Canavanine (**88**), a nonprotein amino acid in legumes such as jack bean (*Canavalia ensiformis* (L.) DC.), has been shown to inhibit growth of susceptible plant species.[53] L-3,4-Dihydroxyphenylalanine (L-DOPA, **89**) has been isolated as an allelochemical from a cover crop, velvet bean (*Mucuna pruriens* (L.) DC. var. *utilis*).[54]

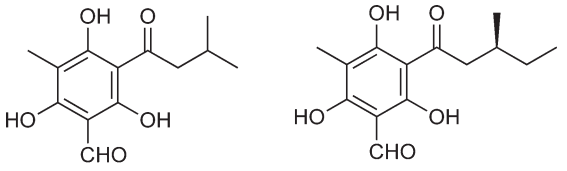


88 **89**

Three rooting inhibitors, G1–G3 (**90–92**), were isolated from *Eucalyptus grandis*.[55] In addition, grandinol (**93**) and homograndinol (**94**), were isolated as inhibitors of seed germination and photosynthesis.[56,57]

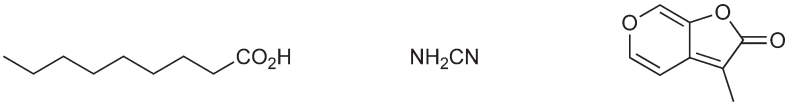


90 **91** **92**



93 **94**

Simple fatty acids (C6–C22) commonly found in plant tissues exhibit moderate to strong growth inhibitory activity against plants.[58] In particular, nonanoic acid (**95**) is a commercial herbicide. Cyanamide (**96**), which has been produced industrially and used as a fertilizer and herbicide, was recently identified as a major plant growth inhibitor in the leaves and stems of a winter cover crop, hairy vetch (*Vicia villosa* Roth).[59] Smoke from the combustion of plant material has been found to stimulate germination of a wide range of plant species from Australia, North America, and South Africa.[60] A butenolide (karrikinolide, **97**) was isolated as one of the potent germination stimulants from the less complex cellulose-derived smoke, and its presence in plant-derived smoke was confirmed by gas chromatography–mass spectrometry (GC–MS).[61] This compound also promotes seedling growth in some plant species. Although karrikinolide and related compounds, karrikins, are not plant secondary metabolites, they may be considered as allelochemicals in a broad sense as they are derived from plant material.



95 **96** **97**

4.13.1.2.7 Allelochemicals and allelopathy

Allelochemicals involved in plant–plant interactions are diverse in their structures. Some are unstable and their activities are short-lived. In addition, some are highly hydrophobic and thus are barely soluble in water. Therefore, there are arguments that only stable and water-soluble compounds contribute to plant–plant interactions in the field. However, in the case of strigolactones, for example, seeds of root parasitic plants and also AM fungi use these unstable short-lived rather lipophilic compounds as host recognition signals. Therefore, unstable short-lived allelochemicals should be further explored to identify unknown signaling chemicals involved in plant–plant interactions.

4.13.2 Allelochemicals in Plant–Microbe Interactions

4.13.2.1 Introduction

Allelochemicals involved in plant–microbe interactions are reviewed in this section. Many microbial metabolites that exhibit biological activity toward plants are known, and vice versa. However, allelochemicals, that is, bioactive metabolites that were proven to be secreted from plants or microbes and to show activity in the ecosystem, are not many.

Allelochemicals that operate between plants and microbes exhibit a variety of different activities. In some cases, chemically different types of compounds affect the same enzyme or pathway, and in other cases, a compound attacks various enzymes. In this section, allelochemicals are summarized based on their relevant biological phenomena.

4.13.2.2 Symbiosis between Plants and Microbes

4.13.2.2.1 Root nodules

The root nodule is the selective symbiont between legumes and leguminous bacteria. Nodule formation is regulated by chemical signals between plants and microbes, and is one of the most well-studied chemical communications. The first step of nodule formation starts with the leguminous bacterium receiving a signal from the host plant. The signal molecules are mostly flavonoids and their glycosides **67–69** and **98–116**, and their biosynthetic precursors, chalcones **117**, **118**. The betains stachydrine (**120**) and trigonelline (**121**), and aldonic acids, erythronic acid (**122**) and tetronic acid (**123**), were also identified as signal molecules exuded from alfalfa (*Medicago sativa*) and lupin (*Lupinus albus*), respectively. These signal molecules trigger the expression of the bacterial genes required for nodulation. Nodulation gene inducers classified by skeletal structure and their plant of origin are summarized in **Table 1**.[62]

Legumes also secrete antagonists that inhibit the flavonoid-mediated activation of nodulation genes (**Table 2**).[63,64] Some act as inducers in another symbiotic system. Antagonists generally have structures similar to those of inducers and inhibition can be overcome by increasing the concentration of inducers, hence they are considered as competitive inhibitors.[63]

Table 1 Nodulation gene inducers secreted by leguminous plants[61]

Compound		*Plant of origin*
Flavone		
4′,7-Dihydroxyflavone	**98**	Clover (*Trifolium repens*) Alfalfa (*Medicago sativa*)
4′-Hydroxy-7-methoxyflavone	**99**	Clover (*T. repens*)
4′,7-Dihydroxy-3′-methoxyflavone (geraldone)	**100**	Clover (*T. repens*)
4′,5,7-Trihydroxyflavone (apigenin)	**101**	Pea (*Pisum sativum*)
4′,5,7-Trihydroxy-3′-methoxyflavone (chrysoeriol)	**102**	Alfalfa (*M. sativa*)
3,4′,5,7-Tetrahydroxyflavone (kaempferol)	**67**	Common bean (*Phaseolus vulgaris*)
3′,4′,5,7-Tetrahydroxyflavone (luteolin)	**103**	Alfalfa (*M. sativa*)
3′,4′,5,7-Tetrahydroxyflavone (eriodictyol)	**104**	Common bean (*P. vulgaris*) Pea (*P. sativum*)
3,3′,4′,5,7-Pentahydroxyflavone (quercetin)	**68**	Common bean (*P. vulgaris*)
3,3′,4′,5,5′,7-Hexahydroxyflavone (myricetin)	**105**	Common bean (*P. vulgaris*)
Flavanone		
4′,7-Dihydroxyflavanone (liquiritigenin)	**106**	Alfalfa (*M. sativa*) and vetch (*Vicia sativa* subsp. *Nigra*)
3′,7-Dihydroxy-4′-methoxyflavanone	**107**	Vetch (*V. sativa* subsp. *Nigra*)
4′,7-Dihydroxy-3′-methoxyflavanone	**108**	Vetch (*V. sativa* subsp. *Nigra*)
4′,5,7-Trihydroxyflavanone (naringenin)	**69**	Common bean (*P. vulgaris*) and vetch (*V. sativa* subsp. *Nigra*)
3′,5,7-Trihydroxy-4′-methoxyflavanone	**109**	Vetch (*V. sativa* subsp. *Nigra*)
4′,5,7-Trihydroxy-3′-methoxyflavanone	**110**	Vetch (*V. sativa* subsp. *Nigra*)
3,3′,5,7-Tetrahydroxy-4′-methoxyflavanone	**111**	Vetch (*V. sativa* subsp. *Nigra*)
Isoflavone		
4′,7-Dihydroxyisoflavone (daidzein)	**112**	Soy bean (*Glycine max*)
4′,5,7-Trihydroxyisoflavone (genistein)	**113**	Common bean (*P. vulgaris*) and soy bean (*G. max*)
Flavylium		
3,4′,5,7-Tetrahydroxy-3′,5′-dimethoxyflavylium (malvidin)	**114**	Common bean (*P. vulgaris*)
3,4′,5,5′,7-Pentahydroxy-3′-methoxyflavylium (petunidin)	**115**	Common bean (*P. vulgaris*)
3,3′,4′,5,5′,7-Hexahydroxyflavylium (delphinidin)	**116**	Common bean (*P. vulgaris*)

(*Continued*)

Table 1 (Continued)

Compound		*Plant of origin*
Chalcone		
4,4′-Dihydroxy-2′-methoxychalcone	**117**	Alfalfa (*M. sativa*), Vetch (*V. sativa* subsp. *nigra*)
2′,4,4′-Trihydroxychalcone (isoliquiritigenin)	**118**	Vetch (*V. sativa* subsp. *nigra*) and soy bean (*G. max*)
Others		
Coumestrol	**119**	Soy bean (*G. max*)
Stachydrine	**120**	Alfalfa (*M. sativa*)
Trigonelline	**121**	Alfalfa (*M. sativa*)
Erythronic acid	**122**	Lupin (*Lupinus luteus*)
Tetronic acid	**123**	Lupin (*L. luteus*)

Skeletal structures and numbering of flavone (**98**–**105**), flavanone (**106**–**111**), isoflavone (**112**, **113**), flavylium (**114**–**116**), and chalcone (**117**, **118**) are as follows. Other structures are mentioned before.

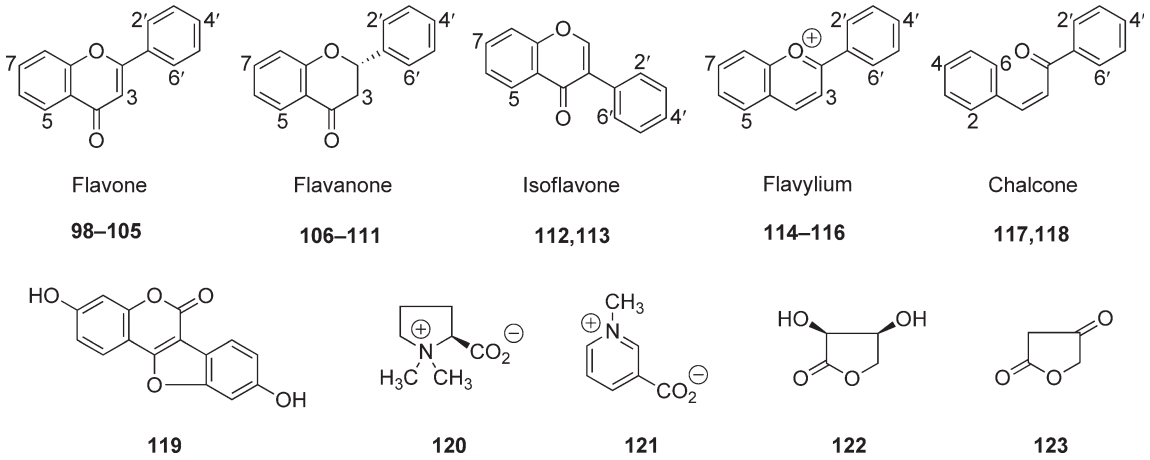


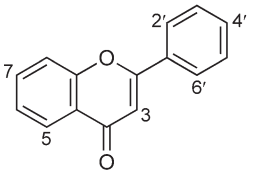
Table 2 Antagonists of Nod gene inducers[62]

Compound		*Plant of origin*
Flavone		
Flavone	**124**	Soy bean (*Glycine max*)
7-Hydroxy-5-methylflavone	**125**	Soy bean (*G. max*)
5,7-Dihydroxyflavone (chrysin)	**126**	Soy bean (*G. max*)
3,4′,5,7-Tetrahydroxyflavone (kaempferol)	**67**	Pea (*Pisum sativum*) and soy bean (*G. max*)
3,3′,4′,5,7-Pentahydroxyflavone (quercetin)	**68**	Alfalfa (*Medicago sativa*)
2′,3,4′,5,7-Pentahydroxyflavone (morin)	**127**	Alfalfa (*M. sativa*)
Isoflavone		
4′,7-Dihydroxyisoflavone (daidzein)	**112**	Pea (*P. sativum*)
4′,5,7-Trihydroxyisoflavone (genistein)	**113**	Pea (*P. sativum*)
7-Hydroxy-4′-methoxyisoflavone (formonetin)	**128**	Clover (*T. repens*)
Miscellaneous		
7-Hydroxycoumarin (umbelliferone)	**55**	Alfalfa (*M. sativa*), clover (*T. repens*), and common bean (*Phaseolus vulgaris*)

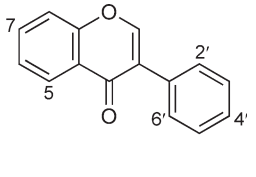
(*Continued*)

Table 2 (Continued)

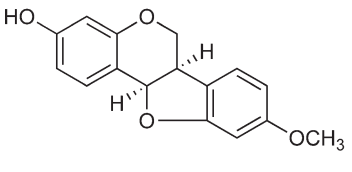
Compound		*Plant of origin*
Coumestrol[63]	**119**	Alfalfa (*M. sativa*)
Medicarpin[63]	**129**	Alfalfa (*M. sativa*)
4-Acetyl-2-methoxyphenol (acetovanillone)	**130**	Pea (*P. sativum*)
4-Hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)	**131**	Pea (*P. sativum*)
4-Acetyl-2,6-dimethoxyphenol (acetosyringone)	**132**	Common bean (*P. vulgaris*)



Flavone
124–127



Isoflavone
128



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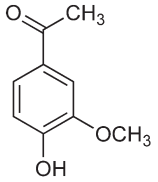

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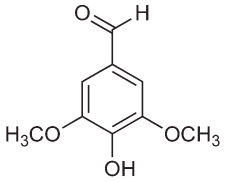

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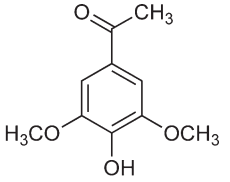

132

When induced, nodulation genes cause leguminous bacteria to produce the Nod factor (**133**), which is an *N*-acetylglucosamine oligomer that is highly modified by fatty acid, sugar, and methyl, acetyl, carbamoyl, and sulfonyl groups.[65–67] Representative structures of Nod factors, their producing bacteria, and target plants are summarized in **Table 3**. Most species produce several Nod factors with distinctive substituent(s) and diversified part(s). The range and quantity of the Nod factor varies with inducing materials and among strains in the same species. The skeletal structure of the Nod factor is biosynthesized by common enzymes in leguminous bacteria and then modified by specific enzymes for each individual modification.[65] Nod factors secreted from leguminous bacteria induce expression of *nodulin* genes and morphological differentiation of the host plant to accept bacterial cells. Specificity between host plant and bacterial species depends on the specific recognition of the *nod* gene inducer by bacteria, and recognition of the nod factor by host plants.

The symbiosis between the host plant and leguminous bacteria is tightly controlled; the host plant uses the phytohormone ethylene to control the nodule number and the nodulation zone (autoregulation), because excessive nodule formation causes exploitation of photosynthetic products by bacteria. The leguminous bacterium *Bradyrhizobium elkanii* produces rhizobitoxine (**134**), which was isolated as a chlorosis-inducing phytotoxin, and is now known to be an inhibitor of 1-aminocyclopropane-1-carboxylate synthase, a key enzyme in ethylene biosynthesis. *Bradyrhizobium elkanii* is thought to gain a competitive advantage over species that do not produce rhizobitoxine by suppressing the ethylene level of the host plant and reducing the plant's regulation of nodulation.[68]

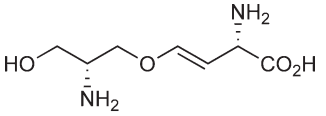

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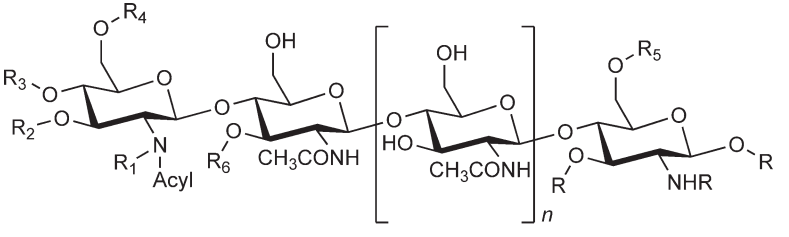
Table 3 Representative Nod factors

n	R_1	R_2	R_3	R_4	R_5	R_6	*Acyl*	*Producer*	*Target plant*
0, 1, 2	H	H	H	H, Ac	SO_3H	H	C16:2(Δ*E*2,9), C16:3(Δ*E*2,*E*4,*Z*9),	*Sinorhizobium meliloti*	Alfalfa (*Medicago sativa*)
1, 2	H	H	H	Ac	H	H	C18:1(Δ*Z*11), C18:4(Δ*E*2,*E*4,*E*6,*Z*11)	*Rhizobium leguminosarum* bv. *viciae*	Pea (*Pisum sativum*) and Vetch (*Vicia sativa*)
0, 1, 2	H	H	H	Ac	H	H	C16:0, C16:1, C18:0, C18:1, C18:3, C20:2, C20:3, C20:4	*R. leguminosarum* bv. *trifolii*	Clover (*Trifolium repens*)
2	Me, H	$CONH_2$, H	$CONH_2$, H	H	4-*O*-Ac-Fuc, Fuc	Fuc, H	C18:0, C18:1, C20:1	*Mesorhizobium loti*	*Lotus japonicus*
2	H	H	H	H, Ac	2-*O*-Me-Fuc	H	C16:0, C18:1(Δ*Z*9)	*Bradyrhizobium japonicum*	Soy bean (*Glycine max*)
2	Me	$CONH_2$ (R_3 or R_3)		H	4-*O*-Ac-Fuc, SO_3H, H	H	C18:1(Δ*Z*11)	*Rhizobium etli*	Common bean (*Phaseolus vulgaris*)

Nod factors having substituent(s) at R in the structure are produced by other leguminous bacteria (see the works of Zuanazzi *et al*.,[64] Downie *et al*.,[65] and Spaink[66] and the original papers cited therein).



4.13.2.2.2 Mycorrhizae

Mycorrhizae are more widely distributed than root nodules throughout plant–microbe symbiotic systems; more than 80% of terrestrial plants are able to establish mutualistic symbiotic associations with AM fungi. Hyphal branching of germinated spores of AM fungi is the crucial step for contact with plant roots.

Effects of flavonoids on spore germination, hyphal growth, and branching in AM fungi were extensively studied,[69] since flavonoids were known to be plants' signaling compounds in root nodule formation. Flavonoids, although they certainly exhibited some activities, were eventually found not to be indispensable plant signal compounds in AM symbiosis; maize plants deficient in chalcone synthase activity, which is necessary for flavonoid biosynthesis, were equally colonized with AM fungi as wild-type maize.[70]

A sesquiterpene that triggers hyphal branching in an AM fungus *Gigaspora margarita* was isolated from the root exudates of *L. japonicus*, and was identified as 5-deoxystrigol (**37**).[19,71] Strigol (**32**), sorgolactone (**34**), and orobanchol (**35**) were previously isolated as germination stimulants of root parasitic plants (see Section 4.13.1.2.2), and also displayed hyphal branching activity.

There is evidence that hyphae from germinating spores of AM fungi secrete an 'Myc factor' that enables plants to accept AM fungi. The factor can diffuse across a dialysis membrane and its molecular weight is estimated to be less than 3.5 kDa, but its chemical nature has not yet been revealed.[72]

4.13.2.3 Utilization of Plant Products by Microbes

Flavonoids exuded from plants play an important role in the symbiosis between legumes and leguminous bacteria. At the same time, they attract zoospores and induce spore germination of phytopathogenic fungi.

4.13.2.3.1 Host-specific zoospore attractants

Zoospores of the phytopathogenic fungi *Pythium* and *Phytophthora* are attracted to plant roots and root exudates. The first description of attractants other than nutrients such as amino acids and sugars was an extensive investigation on chemical products including alcohols, aldehydes, and organic acids with 1–9 carbon atoms. In that study, isovaleraldehyde was shown to attract zoospores of *Phytophthora palmivora*.[73] Two plant-derived structurally irrelevant attractants, 3-indolecarbaldehyde (**135**) and isoflavone prunetin (**136**), were isolated from cabbage and pea, and attracted *Aphanomyces raphani* and *Aphanomyces euteiches* zoospores, respectively. Host specificity was shown by these substances (**Table 4**).[74,75] Since then, the isoflavones daidzein (**112**) and genistein (**113**), cochliophilin A (**137**), and *N-trans*-feruloyl-4-*O*-methyldopamine (**138**) have been isolated from the host plants.[76–78] The relationships between the structures of compounds and their biological activities have been reported: isoflavones and their attraction for *A. euteiches* zoospores;[79,80] flavonoids and their related compounds and the chemotactic behavior of *Phytophthora sojae* zoospores;[81] and effects of **137** and **138** and their analogues on *Aphanomyces cochlioides* zoospores.[82] Substances resulting in repellent **140** and attractant **139** zoospore chemotaxis were isolated from the root extract of the nonhost plant *Portulaca oleracea*.[83] The signaling and communication between phytopathogenic fungal zoospores and host and nonhost plants have been reviewed with special reference to *A. cochlioides*.[84]

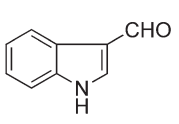
4.13.2.3.2 Germination inducers of phytopathogens

Flavonoids stimulate spore germination in the soilborne phytopathogenic fungus *Fusarium solani*. Spore germination of *F. solani* f. sp. *pisi*, which causes disease in pea, is stimulated by flavanone hesperetin (**141**), flavone apigenin (**101**), the pterocarpan phytoalexin pisatin (**142**), and so on. In contrast, germination of the bean pathogen *F. solani* f. sp. *phaseoli* is stimulated by the pterocarpans maackiain (**143**) and medicarpin (**129**), and isoflavones biochanin A (**144**), and so on but not by pisatin.[85]

It was reported that there is a factor stimulating the germination of the resting spores of the clubroot pathogen *Plasmodiophora brassicae* in the root exudates of turnip (*Brassica campestris* subsp. *rapa* rapifera group) and lettuce (*Lactuca sativa*).[86] The leaf extract of a seagrass, *Posidonia australis*, was found to stimulate germination of resting spores, and it was known to contain phenolic compounds such as chicoric acid (**145**). The stimulatory activities of caffeic acid (**49**), one of the final metabolites of chicoric acid, coumalic acid (**146**) and corilagin (**147**) were examined, and they were shown to stimulate germination of resting spores of *P. brassicae*.[87]

Table 4 Zoospore attractants and repellent

Compound	*Affected microorganism*	*Secreting plant*
Host-specific attractant		
3-Indolecarbaldehyde (**135**)	*Aphanomyces raphani*	Cabbage (*Brassica oleracea*)
4′,5-Dihydroxy-7-methoxyisoflavone (prunetin, **136**)	*Aphanomyces euteiches*	Pea (*Pisum sativum*)
4′,7-Dihydroxyisoflavone (daidzein, **112**)	*Phytophthora sojae*	Soy bean (*Glycine max*)
4′,5,7-Trihydroxyisoflavone (genistein, **113**)	*P. sojae*	Soy bean (*G. max*)
5-Hydroxy-6,7-methylenedioxyflavone (cochliophilin A, **137**)	*Aphanomyces cochlioides*	Spinach (*Spinacia oleracea*)
N-trans-feruloyl-4-*O*-methyldopamine (**138**)	*A. cochlioides*	Pigweed (*Chenopodium album*)
N-trans-feruloyltyramine (**139**)	*A. cochlioides*	*Protulaca oleracea*
Repellent		
1-Linoleoyl-2-lysophosphatidic acid monomethyl ester (**140**)	*A. cochlioides*	*P. oleracea*



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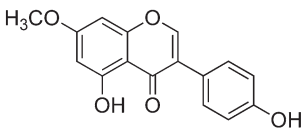

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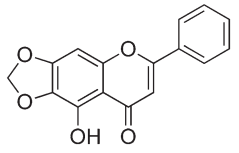

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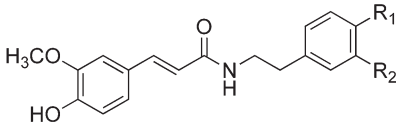
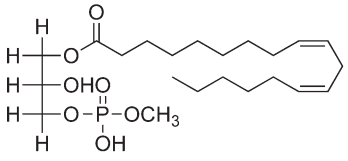


138 : $R_1 = OCH_3$, $R_2 = OH$
139 : $R_1 = OH$, $R_2 = H$



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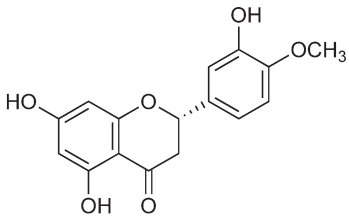
Volatile thiols and sulfides, such as di-*n*-propyl disulfide (**148**) and diallyl disulfide (**149**), trigger germination of sclerotia of *Sclerotium cepivorum*, the causal pathogen of white rot in *Allium*. These compounds are metabolites of thiosulfinates such as allicin and their precursors alk(en)yl cysteine sulfoxides, which are exuded from alliaceous plants and metabolized by the soil microflora. Allicin exhibits strong antimicrobial activity as described later. The sclerotium is a dormant organ of fungi that can survive for more than 10 years, and thus germination stimulants are expected to be a new agent to reduce disease.[88,89]



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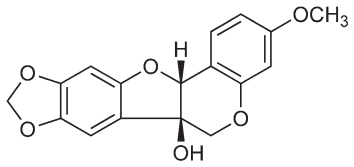

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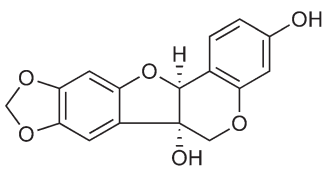

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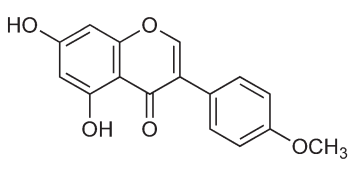

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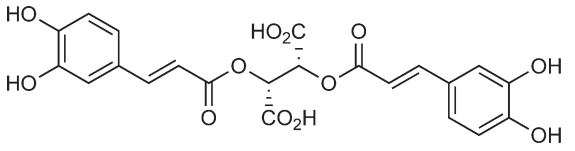

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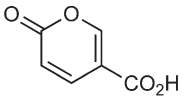

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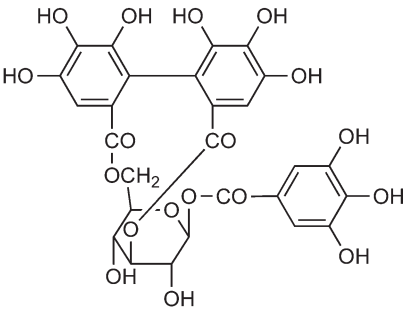

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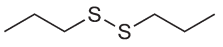

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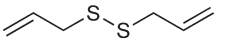

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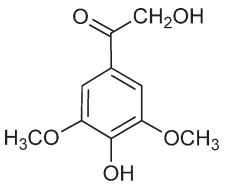
4.13.2.3.3 Other plant chemicals affecting microbial activity

Agrobacterium tumefaciens, a phytopathogenic bacterium in soil, transforms dicotyledonous plant cells to cause the neoplastic disease crown gall, and is used as a tool for genetic transformation of plant cells. This bacterium initiates the expression of virulence genes in response to phenolic signals acetosyringone (**132**) and α-hydroxyacetosyringone (**150**), which are released from wounded and metabolically active plant cells.[90]



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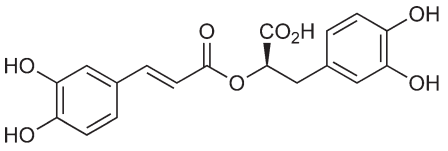
4.13.2.4 Self-Defensive Allelochemicals in Plants

Phytoalexins are antimicrobial substances produced by plants in response to infection by the pathogen or elicitation by abiotic agents. Since isolation of the pterocarpan pisatins (**142**),[91] chemically diverse compounds have been isolated from the plant families Leguminosae, Gramineae, Rosaceae, Cruciferae, Compositae, and so on, structures of these phytoalexins are summarized in the earlier edition of this book[92] and also in this volume (Section 4.08.2.3). There are, however, some cases where an elicitor induces secretion of antimicrobial substances from the plant body. Rosmarinic acid (**151**) was secreted in the root exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) in response to a fungal cell wall elicitor, and it exhibited antimicrobial activity against a range of soilborne microorganisms.[93] Secondary metabolites in the root exudates of *Arabidopsis thaliana* were profiled after the roots had been treated with fungal cell wall elicitor, and vanillic acid (**45**), *trans*-cinnamic acid (**47**), ferulic acid (**50**), butanoic acid (**152**), *o*-coumaric acid (**153**), *p*-hydroxybenzamide (**154**), methyl *p*-hydroxybenzoate (**155**), 3-indolepropanoic acid (**156**), and syringic acid (**46**) were shown to have antimicrobial activity against soilborne fungi and/or bacteria at the concentration detected.[94]



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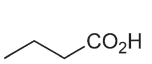

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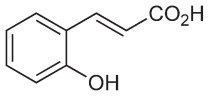

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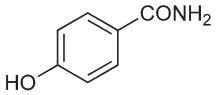

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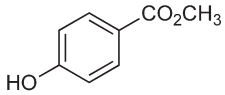

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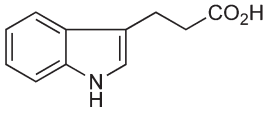

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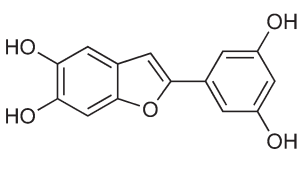
Rotation of crops is a long-established practice that reduces disease damage; a certain crop reduces disease damage to the succeeding crop, which indicates that the former excretes some antimicrobial substances. 2-(3′,5′-Dihydroxyphenyl)-5,6-dihydroxybenzofuran (**157**) was isolated from the root exudates of alfalfa (*M. sativa*) as an antifungal substance against *Fusarium oxysporum* f. sp. *phaseoli*, after observing that soilborne disease of kidney bean (*Phaseolus vulgaris*) was less in fields where alfalfa was cultivated as the preceding crop.[95] This compound also increased the solubility of ferric phosphate, which is only slightly soluble. Screening for antimicrobial substances has also been conducted with the root exudates of species used as mating sources in breeding resistant cultivars, and as rootstock; the sesquiterpenoids solavetivone (**158**), lubimin (**159**), lubiminoic acid (**160**), and aethione (**161**) were isolated from the root exudates recovered from *Solanum aethiopicum*.[96] They exhibited antifungal activity against *F. oxysporum* and/or *Verticillium dahliae*. Maize (*Zea mays*) is commonly used in the control of soilborne disease caused by tomato *Fusarium* wilt by *F. oxysporum*, and in the control of brown stem rot of adzuki bean (*Vigna angularis*) by *Cepharosporium gregatum*. (6*R*)-7,8-dihydro-3-oxo-α-ionone (**162**) and (6*R*,9*R*)-7,8-dihydro-3-oxo-α-ionol (**163**) were isolated from the root exudates of maize.[97] They exhibited antifungal activity against *F. oxysporum* and inhibited germination of *C. gregatum*. It is also worth noting that 6-methoxybenzoxazolinone (MBOA, **164**) and 6,7-dimethoxybenzoxazolinone (DMBOA) (**165**) were isolated from the root extract. These compounds were identified as phytoalexins in several gramineous plants.



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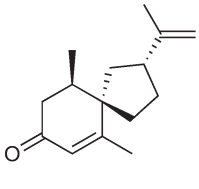

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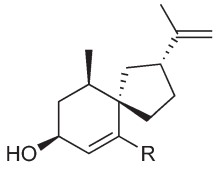
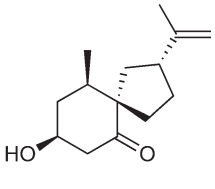


159 : R = CHO
160 : R = CO_2H



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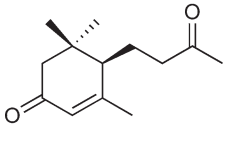

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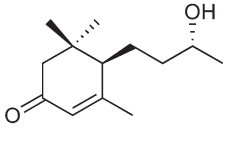

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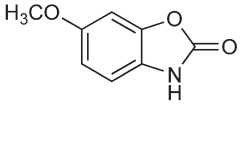

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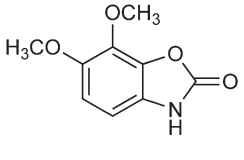
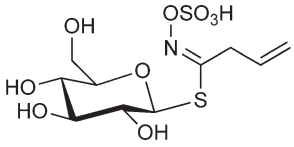


165

Disease control by green manure is an artificial application of allelopathy. Isothiocyanates released from cruciferous plants are an example of this; allyl isothiocyanate (**166**), which is derived from the glucosinolate sinigrin (**167**), is the most popular example.[98] Application of *Geranium carolinianum* for the control of *Ralstonia solanacearum*, the cause of bacterial wilt of potato, and potato scab, which is induced by some *Streptomyces* spp., was reported.[99,100] One of the antibacterial constituents was shown to be ethyl gallate (**168**).[101]

166



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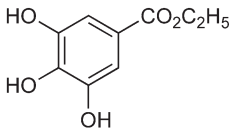
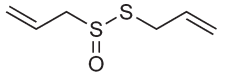


168

Alliaceous plants release characteristic volatile compounds. The most well-known compound is the allylthiosulfinate allicin (**169**), which is produced from alliin (**170**) by alliinase and exhibits strong antibacterial and antifungal activities. For this reason, alliaceous plants are used as companion plants of tomato, cucumber, strawberry, and so on.

Plant volatiles have been surveyed to find safe and environmentally friendly postharvest fumigants. Hexanal (**171**), 1-hexanol (**172**), (*E*)-2-hexen-1-ol (**173**), (*Z*)-6-nonenal (**174**), (*E*)-3-nonen-2-one (**175**), methyl salicylate (**176**), and methyl benzoate (**177**) exhibit potential to control the gray mold, *Botrytis cinerea*.[102] (*E*)-2-hexenal (**178**), carvacrol (**179**), (*E*)-cinnamaldehyde (**180**), and citral (**181**) exhibited consistent fungicidal activities against *Penicillium expansum*, the cause of blue mould of pear.[103] (*E*)-2-hexenal (**178**), carvacrol (**179**), and citral (**181**) were also effective against *Monilinia laxa*, the cause of brown rot in stone fruit.[104]



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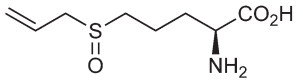

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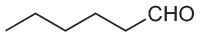

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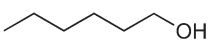

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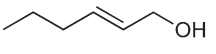

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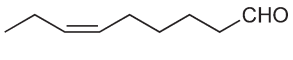

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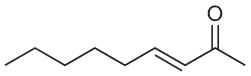

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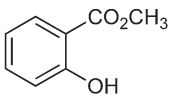

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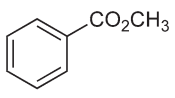

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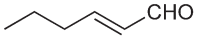

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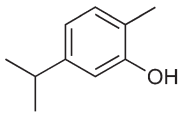

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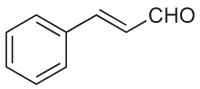

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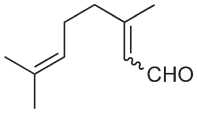
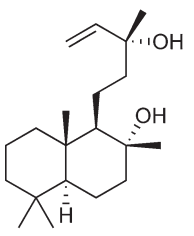


181

Some antimicrobial metabolites leached from the surface of leaves are known, namely, sclareol (**182**) and isosclareol (**183**) from *Nicotiana glutinosa*,[105] parthenolide (**184**) from *Chrysanthemum parthenium*,[106] and rugosal A (**185**) from *Rosa rugosa*,[107] and so on. The possible roles of these compounds in host–pathogen interactions are discussed in the original papers.



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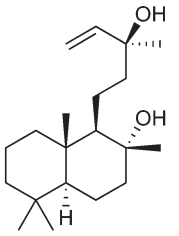

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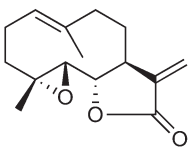

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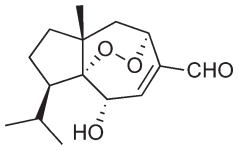
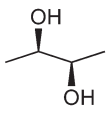


185

4.13.2.5 Phytoactive Substances Produced by Microbes

Phytohormones and phytotoxins are two major classes of compounds produced and secreted by microorganisms that act on plants. These will be discussed in another chapter (4.10). Recently, production of 2,3-butanediol (**186**) and acetoin (**187**) by two strains of plant growth-promoting rhizobacteria (PGPR) was reported. These compounds promote growth of *A. thaliana* and trigger induced systemic resistance (ISR) in the plant.[108,109]



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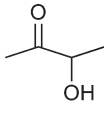


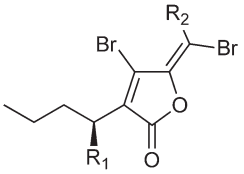
187

4.13.2.6 Allelochemicals in Aquatic Ecosystems

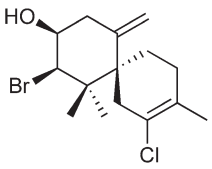
Many antimicrobial substances have been isolated from algae and were thought to be potential chemical defense substances against pathogenic bacteria. However, it is difficult to examine whether they are secreted from the alga, or whether they are present at concentrations high enough to exhibit antimicrobial activity. Only a few have been proven to be allelochemicals in the aquatic ecosystem. Halogenated furanones **188–191**, which were isolated from the red alga *Delisea pulchra*, are rare compounds that have been shown to act as allelochemicals.[110] Halogenated sesquiterpenes elatol (**192**), iso-obtusol (**193**), and lembyne-A (**194**) isolated from the red alga *Laurencia* sp. exhibit antibacterial activity against marine bacteria isolated from the algal habitats.[111]

Halogenated furanones **188–191** deterred the settlement and growth of a range of ecologically relevant fouling organisms.[112] These compounds also exhibited inhibitory activity toward the quorum sensing system mediated by acylated homoserine lactones (AHLs) in Gram-negative bacteria.[113] The quorum sensing system, a mechanism to induce gene expression when increasing bacterial population density, results in increased concentrations of signal compounds, for example, AHLs. This system provides a collective response of bacteria in bioluminescence, biofilm formation, virulence factor expression, antibiotic production, etc. Zoospores of a green alga *Ulva intestinalis* respond to AHLs such as *N*-(3-oxododecanoyl)-homoserine lactone (**195**) and identify bacterial biofilms for preferential settlement.[114] Higher plants, including pea, rice, soybean, tomato, and crown vetch, secrete compounds that affect the quorum sensing system in bacteria, although their principles have not been clarified.[115]

Many green algae lose their normal foliose morphology when they are grown under aseptic conditions. Many bacteria were isolated from marine algae, and screened for their ability to induce normal growth of unicellular *Monostroma oxyspermum*. A morphogenetic factor was isolated from an epiphytic marine bacterium on a green alga and named thallusin (**196**).[116] Absolute stereochemistry was revised later by total synthesis.[117]



	R_1	R_2
188 :	H	Br
189 :	H	H
190 :	OAc	H
191 :	OH	H



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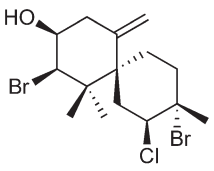

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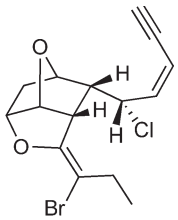

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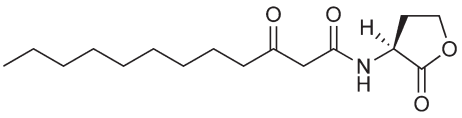

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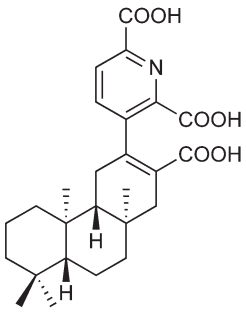

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Biographical Sketches

Koichi Yoneyama was born in a rural mountainous city, Matsumoto, Japan, and graduated from the University of Tokyo with an M.S. degree in Agricultural Chemistry in 1978. He immediately joined as an assistant professor at Utsunomiya University and is currently a professor in the Weed Science Center. He received a Ph.D. degree in Agriculture from the University of Tokyo in 1984. His research interests are mainly directed toward biologically active natural compounds of plant origin and their synthetic modifications. His current research focuses on the chemistry of strigolactones, host-recognition signals for both symbiotic AM fungi and root parasitic plants.

Masahiro Natsume was born in Tokyo, Japan, in 1956. He graduated from Nagoya University with an M.S. degree in Agricultural Chemistry in 1981. In 1987, he was appointed assistant professor in Agriculture at Nagoya University. He was transferred to Tokyo University of Agriculture and Technology in 1991 and currently works as a professor. He obtained a Ph.D. degree in Agriculture from Nagoya University in 1991 entitled "Study on Bio-organic Chemistry of Aerial Mycelium-differentiation in Actinomycetes." His research interests focus on search for the regulatory substances of morphological differentiation and secondary metabolism in microorganisms and of plant–microbe interactions, and elucidation of their mode of action.

4.14 Allelochemicals in Plant–Insect Interactions

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4.14.1 Introduction

The majority of insects are herbivores that depend on plants for their nutrients. The chemicals produced by plants and insects can have significant effects on their lives, and insect–plant interactions have therefore been of long-standing interest in chemical ecology. Biological and chemical analyses of the interplay between insect pests and crops are of great importance from the perspective of future agriculture as the need to develop environmentally benign pest control agents for practical use continues to grow.[1] Phytochemicals, particularly secondary metabolites, play important and often crucial roles in many types of insect behaviors, and can even regulate their growth and reproduction in various ways, thus ultimately exerting considerable influence on their fitness. The abilities of insects to counteract the plants' chemical barriers, which might have developed through coevolutionary interactions between insects and plants, allow them to make use of certain phytochemicals as cues to locate and recognize suitable food or host plants. Various attractants, stimulants, repellents, and deterrents are usually involved in this process. Some insects even sequester and store noxious phytochemicals to use for their own chemical defenses. Owing to the complexity of insect–plant relationships, the allelochemicals mediating these interactions include a wide array of chemical compounds with a variety of ecological functions.

In this chapter, we have focused our attention on selected topics in which significant progress has been made during the last decade, and have not discussed those chemicals of little or no ecological relevance. Since antifeedants are covered in another chapter, plant chemicals that deter food ingestion have also been omitted. This chapter initially deals with plant constituents involved in host selection: those affecting oviposition by females and those stimulating feeding by both larvae and adults. Later, pollination strategies of plants involving

manipulation of their floral scents to lure or repel insects are reviewed, along with the gustatory responses of foragers to a few groups of nutrients and other chemicals in the nectar. Finally, we have addressed the multitrophic interactions among plants, insects, and their parasitoids, which have aroused increasing interest in recent years, and further commented on induced plant volatiles that mediate the attraction of predators to prey-infested plants.

4.14.2 Host Selection

Host selection by phytophagous insects usually consists of two phases: food choice through preimaginal feeding (also feeding by adults in certain insect taxa) and host-plant seeking and assessment by ovipositing females. Among the most important factors influencing host selection by insects are the chemical constituents present in the plants, although other factors such as visual and/or mechanical stimuli also act concurrently on deciding whether to accept potential host plants or not.

4.14.2.1 Phytochemicals Involved in Oviposition

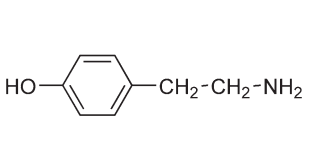
Behavioral, sensory, and phytochemical bases for egg-laying by moths and butterflies have previously been fully reviewed.[2,3] Subsequently a number of newly identified plant chemicals serving as oviposition stimulants or deterrents have been reported for many lepidopterans and other insects.

The majority of swallowtail butterflies of the genus *Papilio* (family Papilionidae) exclusively utilize plants of the family Rutaceae as hosts, with a few species exploiting limited plant species of the families Apiaceae or Lauraceae. The North American black swallowtail butterfly, *Papilio polyxenes*, a specialist on members of carrot family (Apiaceae), has already been shown to lay eggs in response to a mixture of two chemotactile stimulants, luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucoside and *trans*-chlorogenic acid, identified from one of its major host plants, *Daucus carota* (wild carrot). Further study revealed that the oviposition response by the butterfly to another host plant, *Pastinaca sativa* (wild parsnip), was evoked by a combination of tyramine (**1**), *trans*-chlorogenic acid, and a neutral fraction from the plant.[4]

The zebra swallowtail butterfly, *Eurytides marcellus*, is an Annonaceae-feeding specialist. One of three hydroxycinnamoyl derivatives of 1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid, that is, 3-caffeoyl-*muco*-quinic acid (**2**), isolated from the foliage of its primary host plant, *Asimina triloba*, was active alone in stimulating oviposition.[5] The action of compound **2** is unique because other papilionid butterflies for which oviposition stimulants have been reported are induced to oviposit by the synergistic action of multiple components.[3] However, *E. marcellus* females were deterred from egg-laying by flavonoids (rutin and nicotiflorine) co-occurring in the host plant, thus using both qualitative and quantitative information about these compounds to assess host quality.[6] Interestingly, the same compound (**2**), also identified from *Sassafras albidum* (Lauraceae), was found to act as an oviposition stimulant for the spicebush swallowtail, *Papilio troilus*, which feeds exclusively on plants of the family Lauraceae. This compound was inactive alone, but increased the oviposition activity of the females when combined with other as yet uncharacterized stimulant(s).[7]

Two oviposition stimulants, (−)-4-(*E*)-caffeoyl-L-threonic acid (**3**) and (−)-2-*C*-methyl-D-erythrono-1,4-lactone (**4**), have been reported[8,9] for a Rutaceae-feeding swallowtail butterfly, *Papilio bianor*, which preferentially utilizes *Orixa japonica* as its principal host in the western districts of Japan. The former compound singly stimulated egg-laying by the females to a certain extent, while the latter on its own evoked no oviposition response, but turned out to be active when assayed in combination with other fractions derived from a methanolic extract of the plant. However, no synergistic action of the two compounds in stimulation of oviposition was shown. *Papilio macilentus* also feeds specifically on *O. japonica*, which ovipositing females chemically recognize as a host, partly by foretarsal detection of a benzofuran derivative, cnidioside A (**5**). Although a few species of Japanese swallowtail butterflies exploit *O. japonica* as larval food, the plant is rejected by many other swallowtails. *Papilio xuthus*, a typical *Citrus* feeder, never accepts *O. japonica*. The inhibition of colonization of this plant by the butterfly has been attributed to the presence of at least two compounds, 3,4-*O*-disyringoylaldaric acid (**6**) and 5-{[2-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosyl]oxy}-2-hydroxybenzoic acid (**7**), which potently deter both larval feeding and female oviposition.[10]

Papilio polytes is a tropical swallowtail butterfly specializing on only a few rutaceous plants in Japan, such as *Toddalia asiatica* and *Citrus depressa*, while another sympatric potential host, *Murraya paniculata* (Rutaceae), remains entirely unexploited by the butterfly in its natural habitat. Strong oviposition responses to *T. asiatica* were found to be elicited by synergism between *trans*-4-hydroxy-*N*-methyl-L-proline (weakly active alone) (**8**) and 2-*C*-methyl-D-erythronic acid (**9**).[11] On the contrary, trigonelline (**10**) present in *M. paniculata* was partly responsible for the avoidance of the plant by ovipositing females.[12] *Glycosmis citrifolia*, a relatively rare rutaceous plant occurring in sympatry with the above plants, is occasionally infested by *P. polytes* in nature. Oviposition on this plant also proved to be due to the presence of compounds **8** and **9**.[12]



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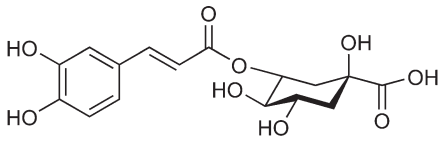

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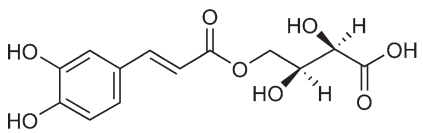

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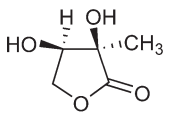

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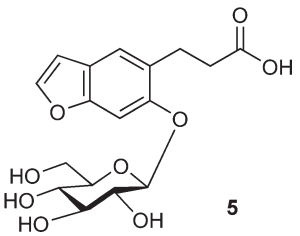

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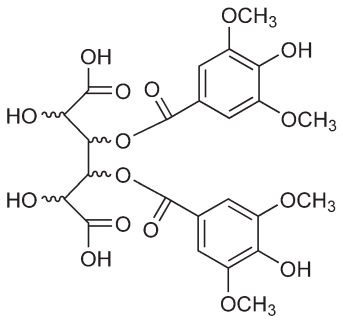

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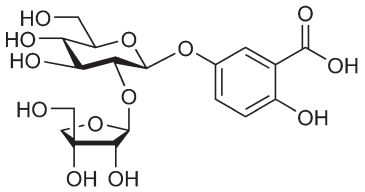

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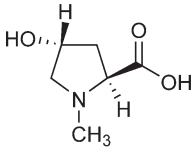

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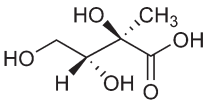

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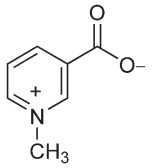


10

Pyrrolizidine alkaloids (PAs) are a typical class of plant secondary metabolites, which certain butterflies and moths in particular groups, that is, Danainae, Ithomiinae (Nymphalidae), and Arctiidae, sequester as larvae or adults and utilize as chemical defensive substances against predatory enemies, probably due to their bitter taste and hepatotoxicity.[13] PAs also serve as precursors of male pheromones of PA-storing lepidopterans.

The primitive danaine butterfly, *Idea leuconoe* (occurring in the Old World tropics), is a specialist on plants of the genus *Parsonsia* (Apocynaceae), which contain PAs, and uses the alkaloids as crucial cues in host recognition. The females were induced to oviposit by host-plant-specific macrocyclic PAs, including parsonsianine, parsonsianidine, and 17-methylparsonsianidine (**Figure 1**), each of which individually showed significant

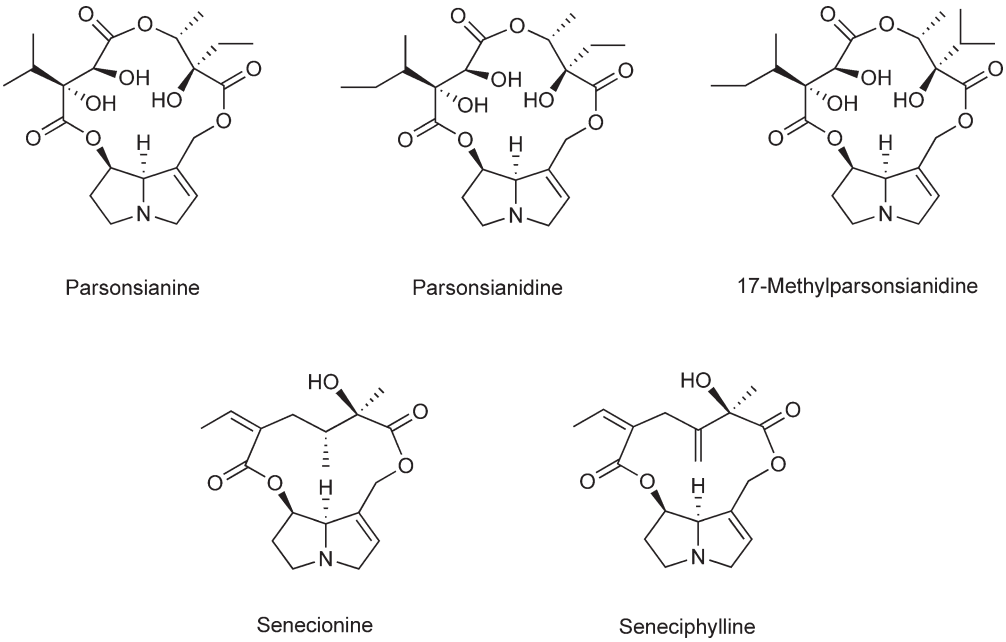


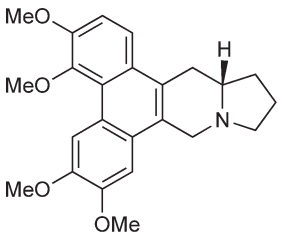
Figure 1 Oviposition stimulants for *Idea leuconoe* (danaine butterfly; upper) and *Tyria jacobaeae* (arctiid moth; lower) feeding on pyrrolizidine alkaloid-containing plants.

stimulatory activity.[14] The cinnabar moth, *Tyria jacobaeae* (Arctiidae), also depends exclusively on the PA-containing plant, *Senecio jacobaea* (Asteraceae), for larval growth. Two PAs of host-plant origin, senecionine and seneciphylline (**Figure 1**), and monocrotaline of nonhost origin stimulated oviposition by the females,[15] although they strongly preferred to oviposit on substrates treated with a PA mixture derived from *S. jacobaea* over single PA, suggesting the involvement of multiple PAs in oviposition preference.

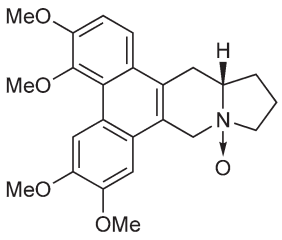
A different group of alkaloids has been shown to mediate egg-laying by another danaine butterfly, *Ideopsis similis*, which exclusively exploits *Tylophora* plants as hosts (Asclepiadaceae), known to contain various phenanthroindolizidine alkaloids. Of 12 tested alkaloids isolated from one of its host plants, *Tylophora tanakae*, at least five components, that is, isotylocrebrine, its *N*-oxide, 3-demethyisotylocrebrine, 6-demethyltylocrebrine, and 7-demethyltylophorine (**Figure 2**), individually stimulated oviposition,[16] although an equivalent blend of these five compounds was much preferred by ovipositing females to any one of them alone. This clearly indicates that synergism in stimulation of oviposition occurs not only among structurally unrelated compounds (papilionid butterflies) but also among those with structural similarities.

The chestnut tiger, *Parantica sita*, is also an Asclepiadaceae-feeding danaine butterfly, which, along with the monarch butterfly, is known to migrate northwards in spring and southwards in late autumn in Japan. From one of its major host plants, *Marsdenia tomentosa*, three unsaturated cyclitols have been identified as oviposition stimulants:[17] conduritol A, conduritol F, and conduritol F 2-*O*-β-D-glucoside (**Figure 3**). Of these, conduritol F 2-*O*-β-D-glucoside (trace component) was most active by itself, conduritol A (predominant cyclitol) showed moderate activity, while conduritol F was inactive alone. However, a combination of these cyclitols evoked the highest oviposition responses from females.

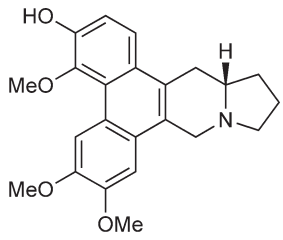
Plants of the Crucifer family (Brassicaceae) are characterized by the presence of peculiar secondary metabolites called glucosinolates, which are thought to be defensive chemicals against herbivores.[18] The diamondback moth, *Plutella xylostella* (Plutellidae), a notorious worldwide pest of cruciferous crops, has previously been shown to lay eggs in response to diverse glucosinolates (sinigrin, etc.) present in crucifers, synergized by leaf epicuticular waxes. A recent study has reported new oviposition stimulants for the moth from cabbage (*Brassica oleracea*), which fall into the chemical group of isothiocyanates. Among others, those with a sulfur atom in the side chain, such as iberin, iberverin, and sulforaphane (**Figure 4**), elicited significant behavioral and electroantennographic responses.[19] Females of other pests on crucifers, *Hellula undalis*



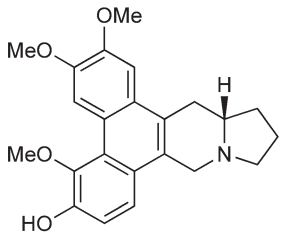
Isotylocrebrine



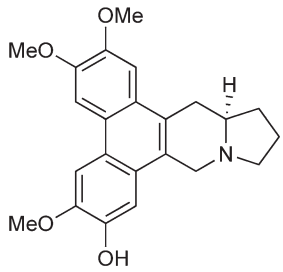
Isotylocrebrine *N*-oxide



3-Demethylisotylocrebrine

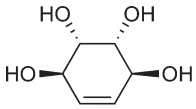


6-Demethyltylocrebrine

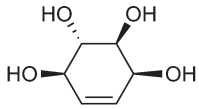


7-Demethyltylophorine

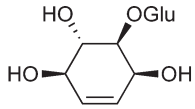
Figure 2 Oviposition stimulants for *Ideopsis similis* from *Tylophora tanakae*.



Conduritol A

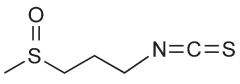


Conduritol F

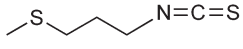


Conduritol F 2-*O*-glucoside

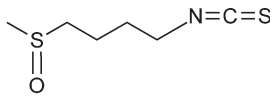
Figure 3 Oviposition stimulants for *Parantica sita* from *Marsdenia tomentosa*.



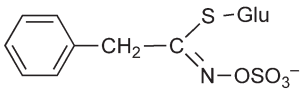
Iberin



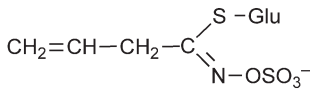
Iberverin



Sulforaphane



Benzylglucosinolate



Allylglucosinolate

Figure 4 Oviposition stimulants for crucifer pests, *Plutella xylostella* (upper) and *Hellula undalis* (lower).

(Pyralidae) and *Mamestra brassicae* (Noctuidae, polyphagous moth), were attracted to substrates treated with allyl isothiocyanate (breakdown product of allylglucosinolate) through upwind orientation flight, while benzyl- and allyl-glucosinolates (**Figure 4**) exerted potent stimulatory activity on egg-laying by *H. undalis*.[20]

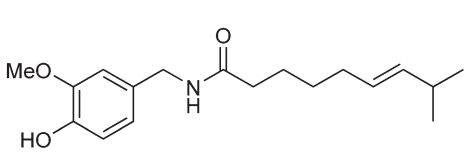
(*E*)-Capsaicin (**11**) has been identified from the fruits of red pepper, *Capsicum annuum* (Solanaceae), as a principal oviposition stimulant for the oriental tobacco budworm, *Helicoverpa assulta* (Noctuidae), an oligophagous moth infesting primarily solanaceous plants.[21] Another related moth, *Heliothis armigera*, is a polyphagous pest utilizing a wide variety of plants as hosts, such as pigeon pea, cotton, tobacco, maize, and sunflower. A sesquiterpene hydrocarbon, α-bulnesene (**12**),[22] green leaf volatiles (GLVs) ((*E*)-2-hexenal and esters of (*Z*)-3-hexen-1-ol), and monoterpenes including α-pinene, β-myrcene, limonene, and linalool, were found to be electrophysiologically active to gravid females of *H. armigera*.

The European corn borer, *Ostrinia nubilalis* (Crambidae), is also a highly polyphagous moth, which attacks several major crops including maize, tomato, and cotton. Each of the *n*-alkanes (C_{26}–C_{29}) and tritriacontane (C_{33}) present in the leaf epicuticular wax of corn, *Zea mays*, was shown to be responsible for eliciting oviposition on the plant.[23] In the oligophagous *Ostrinia* moth, *Ostrinia latipennis*, feeding on knotweeds (*Fallopia* spp.) in Japan, females were stimulated to oviposit by a mixture of leaf epicuticular wax chemicals present in *Fallopia japonica* (Polygonaceae) consisting of *n*-alkanes of C_{16}–C_{33} (nonacosane (C_{29}) being dominant) and free fatty acids of C_9–C_{22} (hexadecanoic acid (C_{16}) being dominant).[24]

The spotted stem borer, *Chilo partellus* (Pyralidae), and *Sesamia nonagrioides* (Noctuidae) are serious pests of maize and sorghum. Their oviposition responses to maize, however, have been reported to differ greatly in different cultivars; some compounds that deter female oviposition have been suggested to occur in resistant cultivars. Oviposition deterrence evoked by resistant cultivars of *Z. mays* was attributed partly to larger quantities of specific components: 1-nonadecanol and 1-heptadecanol for *C. partellus,* and aldehydes of C_9–C_{14}, for *S. nonagrioides*.[25,26]

The host orientation of the codling moth, *Cydia pomonella* (Tortricidae), a major pest of apple (*Malus domestica*; Rosaceae) and stone fruit, is thought to be guided by volatiles emitted from larval host fruit. Although some fruit odors (α-farnesenes, (*E*)-β-farnesene, acetic acid, ethyl (2*E*,4*Z*)-deca-2,4-dienoate), which may act as larval feeding attractants, have been demonstrated to attract adult males and/or females, little information is available on the phytochemicals involved in host finding and oviposition by females. Recently, a female-specific attractant from apple fruit was identified as butyl hexanoate (**13**).[27] In addition, certain sugars and sugar alcohols detected from the surfaces of apple fruits and foliage, particularly fructose, sorbitol, and *myo*-inositol, were shown to stimulate egg-laying by the moth.[28] A more recent investigation has revealed that gravid females of the oriental fruit moth, *Cydia molesta*, which also causes severe damage to stone fruit and pome fruit, were attracted to a mixture of three GLVs ((*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate), benzaldehyde, and benzonitrile[29] identified from peach shoot extract. The synthetic mixture was as attractive as the natural peach shoot volatiles. The antennae of mated females of the European grapevine moth, *Lobesia botrana* (Tortricidae), sensitively responded to many components of host-plant (grape; *Vitis vinifera*) odor, including (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT), β-caryophyllene, (*E,E*)-α-farnesene, (*E*)-β-farnesene, 1-octen-3-ol, 2-ethyl-1-hexanol, linalool, (*E*)- and (*Z*)-linalool oxide furanoside, methyl salicylate, and they were actually attracted to a synthetic 10-component blend in flight tunnel experiments. However, ternary, but not binary, mixtures of these compounds were sufficient to release upwind flight of females, suggesting synergism and redundancy in host-plant volatiles that lure females.[30] *L. botrana* females make use of sugars as cues for oviposition site choice, that is, fructose and glucose (much less active), which are perceived not by tarsal sensilla but by ovipositor sensilla.

The banded sunflower moth, *Cochylis hospes* (Cochylidae), is an important pest of cultivated sunflower (*Helianthus annuus*, Asteraceae) and the larvae are restricted to feeding on several sunflower species. Two diterpenoid alcohols, *ent*-kauran-16α-ol (**14**) and *ent*-atisan-16α-ol (**15**), isolated from prebloom sunflower heads, were identified as oviposition stimulants for the moth.[31] These compounds individually induced egg-laying, with linear dose responses. The former was more stimulative than the latter and they had no synergistic effect on oviposition. Females of the pyralid moth, *Ephestia kuehniella* (Mediterranean flour moth), which infests cereal products, were stimulated to lay eggs by each of three electrophysiologically active (antennae used) volatile components from chocolate products, namely, benzyl alcohol, nonanal, and phenylacetaldehyde.



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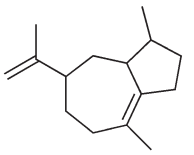

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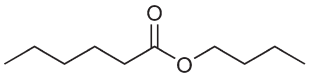

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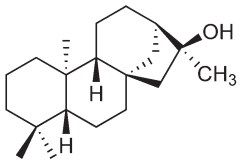

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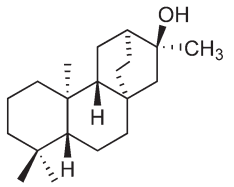

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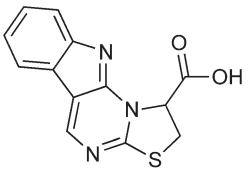
Certain species of Diptera have been shown to be of considerable importance in terms of agricultural damage and their indirect impacts on other herbivores. The cabbage root fly, *Delia radicum* (Anthomyiidae), attacks crucifers, with the larvae feeding on the roots and stems of the plant and often causing serious damage. To locate potential hosts, gravid females of the fly use visual and olfactory (isothiocyanates, etc.) cues, while the final decision on host acceptance is mediated by chemotactile stimuli, evoked by specific phytochemicals detected by taste receptors located on the tarsi during walking on the leaf surface and stem after alighting on the plant. Apart from glucosinolates, which have long been known to be important cues allowing females to recognize suitable host plants, two new and more powerful oviposition stimulants have been identified from cabbage leaves, *B. oleracea* (Brassicaceae): 1,2-dihydro-3-thia-4,10,10b-triazacyclopenta[.a.]fluorene-1-carboxylic acid (major) and its glycine amide[32] (**Figure 5**). The major component occurs at a very low concentration (1 ng per leaf) on the leaf surface. Subsequently, another structurally related and very active stimulant, 1,2-dihydro-6-methoxy-3-thia-4,10,10b-triazacyclopenta[.a.]fluorene-1-carboxylic acid (**Figure 5**), was found from the roots of *Brassica napus*, together with the above two compounds.[33] The turnip root fly, *Delia floralis*, a close relative of *D. radicum*, has also been reported to positively respond to glucosinolates and thia-triaza-fluorenes in host assessment.[34]

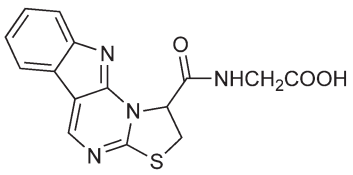
The Hessian fly, *Mayetiola destructor* (Cecidomyiidae), is a major pest of wheat, *Triticum aestivum*, but occasionally infests rye, barley, and a number of wild grasses in the genera *Elymus*, *Aegilops* (Poaceae), and others. Two oviposition stimulants, octacosanal and 6-methoxy-2-benzoxazolinone (**Figure 5**), have been identified from wheat leaves upon which females deposit eggs. These compounds exert a synergistic effect on elicitation of oviposition.[35]

The American serpentine leafminer, *Liriomyza trifolii* (Agromyzidae), is a worldwide generalist pest attacking plants of more than 21 families, including Solanaceae and Cucurbitaceae. The sweet pepper, *C. annuum* (Solanaceae), is fiercely infested in its young stages by *L. trifolii*, but becomes resistant to the insect at more mature stages. One apolar component, (*E*)-phytol, three water-soluble components, namely, 4-aminobutanoic acid, *trans*-4-hydroxy-*N*-methyl-L-proline, and cytidine, and a flavonoid, luteolin 7-*O*-*β*-D-apiofuranosyl-(1→2)-*β*-D-glucopyranoside (**Figure 6**), have been identified as oviposition deterrents from the foliage of mature *C. annuum*, accounting for the chemical defense of the plant from *L. trifolii*.[36,37] Interestingly, 4-hydroxy-*N*-methyl-L-proline serves as an oviposition stimulant for the Rutaceae-feeding swallowtail butterfly, *P. polytes* (see Section 4.14.2.1), whereas it acts as an oviposition deterrent to this polyphagous fly. Bitter gourd, *Momordica charantia*, belonging to the Cucurbitaceae family, is widely cultivated in tropical Africa and Asia, but rarely infested by *L. trifolii*. Several key substances that deter oviposition by *L. trifolii* on this plant were identified as cucurbitane triterpenoids and their glucosides, including 7,23-dihydroxy-3-*O*-malonylcucurbita-5,24-dien-19-al, momordicine I, momordicine II, momordicine IV, and momordicine V[38,39] (**Figure 6**).

The cerambycid beetle, *Monochamus alternates*, is a vector of the pine wood nematode, *Bursaphelenchus xylophilus*. Mortality of pines is caused by the combination of beetles and nematodes. The following 10



1,2-Dihydro-3-thia-4,10,10b-triazacyclopenta-[.a.]fluorene-1-carboxylic acid



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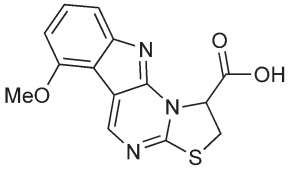

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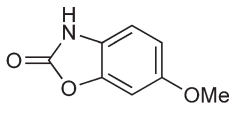
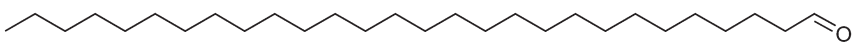


6-Methoxy-2-benzoxazolinone



Octacosanal

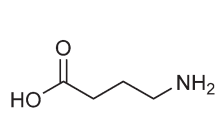
Figure 5 Oviposition stimulants for the cabbage root fly (*Delia radicum*) and the Hessian fly (*Mayetiola destructor*).

compounds have been isolated from *Pinus densiflora* and identified as oviposition stimulants for *M. alternates* (**Figure 7**): (+)-catechin,[40] (−)-2,3-(*E*)-dihydroquercetin-3′-*O*-*β*-D-glucopyranoside, polymeric proanthocyanidins, procyanidin B-1, procyanidin B-3,[41] dihydroconiferyl alcohol-9-*O*-*β*-D-glucopyranoside, cedrusin-4′-O-*β*-D-glucopyranoside, cedrusin-4′-*α*-L-rhamnopyranoside, 7-*O*-methylcedrusin-4′-*O*-*α*-L-rhamnopyranoside, and 1-(4′-hydroxy-3′-methoxyphenyl)-2-[4″-(3-hydroxypropyl)-2″-hydroxyphenoxy]-1,3-propanediol-4′-*O*-*β*-D-xylopyranoside.[42] These individual compounds do not stimulate oviposition, but some combinations of them are active.

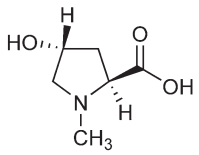
4.14.2.2 Feeding Attractants and Stimulants

Many coleopteran beetles use host-plant volatiles for host location. Strawberry leaf beetle, *Galerucella vittaticollis*, feeds on polygonaceous plant leaves and also on strawberry leaves. The main component of both polygonaceous plant and strawberry leaf volatiles is the compound, (*Z*)-3-hexenyl acetate.[43] (*Z*)-3-Hexenyl acetate attracts *G. vittaticollis* at concentrations of 0.01–0.05%. Quercetin glycosides are also characteristic components of polygonaceous plants, as well as organic acids, and are also found in strawberry leaves. The quercetin glycosides, quercitrin, rutin, avicularin, hyperoside, and isoquercetin, stimulate *G. vittaticollis* feeding (**Figure 8**).[44] Thus, both (*Z*)-3-hexenyl acetate as the feeding attractant and quercetin glycosides as feeding stimulants play important roles in the host selection of *G. vittaticollis* for polygonaceous plants and strawberry.

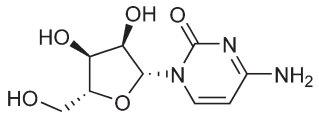
The Colorado potato beetle, *Leptinotarsa decemlineata*, is one of the most serious pests of potato. Two-component blends of three chemicals, (*Z*)-3-hexenyl acetate, (±)-linalool, and methyl salicylate, are attractive to *L. decemlineata* when (*Z*)-3-hexenyl acetate is one of the components of the blend,[45] but individual compounds are inactive. Low levels of (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol can substitute for (*Z*)-3-hexenyl



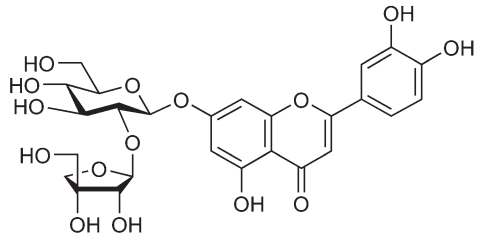
4-Aminobutanoic acid



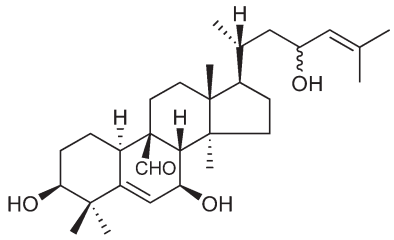
trans-4-Hydroxy-*N*-methyl-L-proline



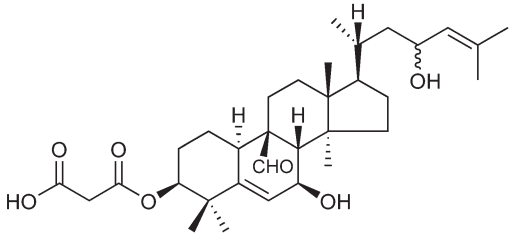
Cytidine



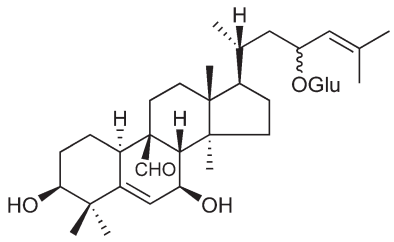
Luteolin 7-*O*-β-D-apiofuranosyl-(1 $\rightarrow$ 2)-β-D-glucopyranoside



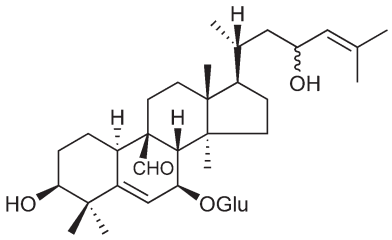
Momordicine I



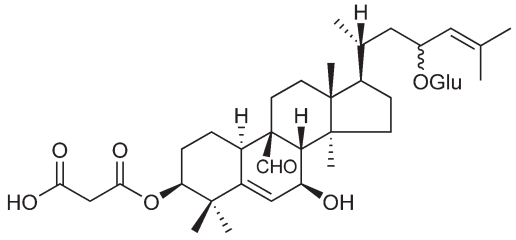
7,23-Dihydroxy-3-*O*-malonylcucurbita-5,24-dien-19-al



Momordicine II



Momordicine IV

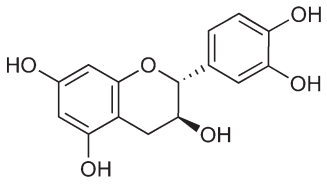


Momordicine V

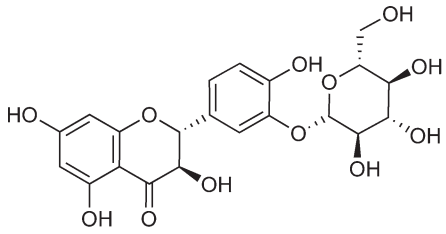
Figure 6 Oviposition deterrents to the American serpentine leafminer (*Liriomyza trifolii*).

acetate when combined with (±)-linalool. However, blends containing relatively high amounts of (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol are unattractive. Emission of these compounds is greatly increased by the feeding *L. decemlineata* larvae, which may alert predators or parasitoids to the presence of potential prey.

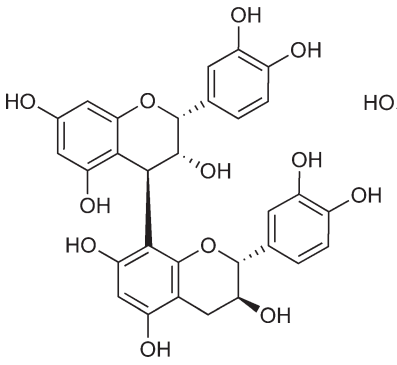
Many specific plant odors are achieved by combinations of widely distributed constituent components in specific ratios characteristic to particular plant species.[46] Therefore, many insects that use general compounds as feeding attractants, such as *L. decemlineata*, are attracted to blends of several components, but not to the individual compounds. Fifteen compounds, (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol,



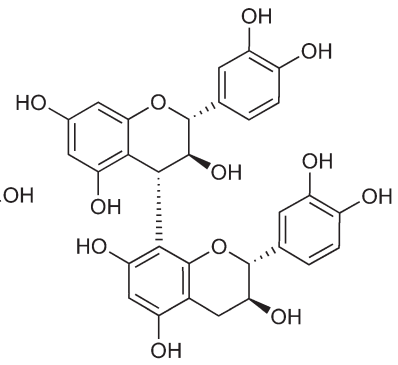
(+)-Catechin



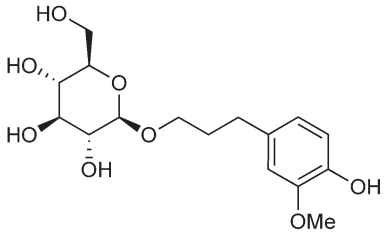
(–)-2,3-(*E*)-Dihydroquercetin-3′-*O*-β-D-glucopyranoside



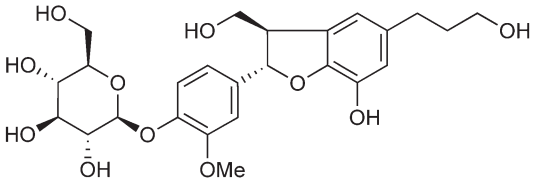
Procyanidin B-1



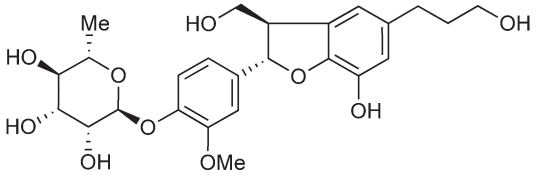
Procyanidin B-3



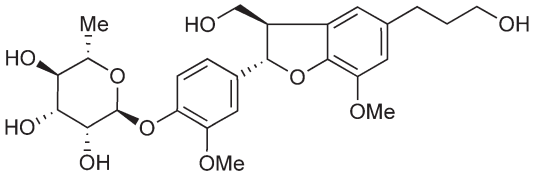
Dihydroconiferyl alcohol-
9-*O*-β-D-glucopyranoside



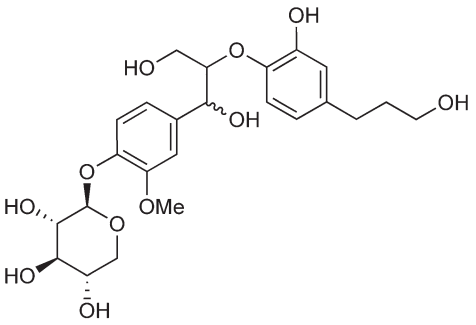
Cedrusin-4′-*O*-β-D-glucopyranoside



Cedrusin-4′-*O*-α-L-rhamnopyranoside



7-O-Methyl cedrusin-4′-*O*-α-L-rhamnopyranoside



1-(4′-Hydroxy-3′-methoxyphenyl)-2-[4″-(3-hydroxypropyl)-2″-
hydroxyphenoxy]-1,3-propanediol-4'-*O*-β-D-xylopyranoside

Figure 7 Oviposition stimulants for *Monochamus alternatus* contained in *Pinus densiflora*.

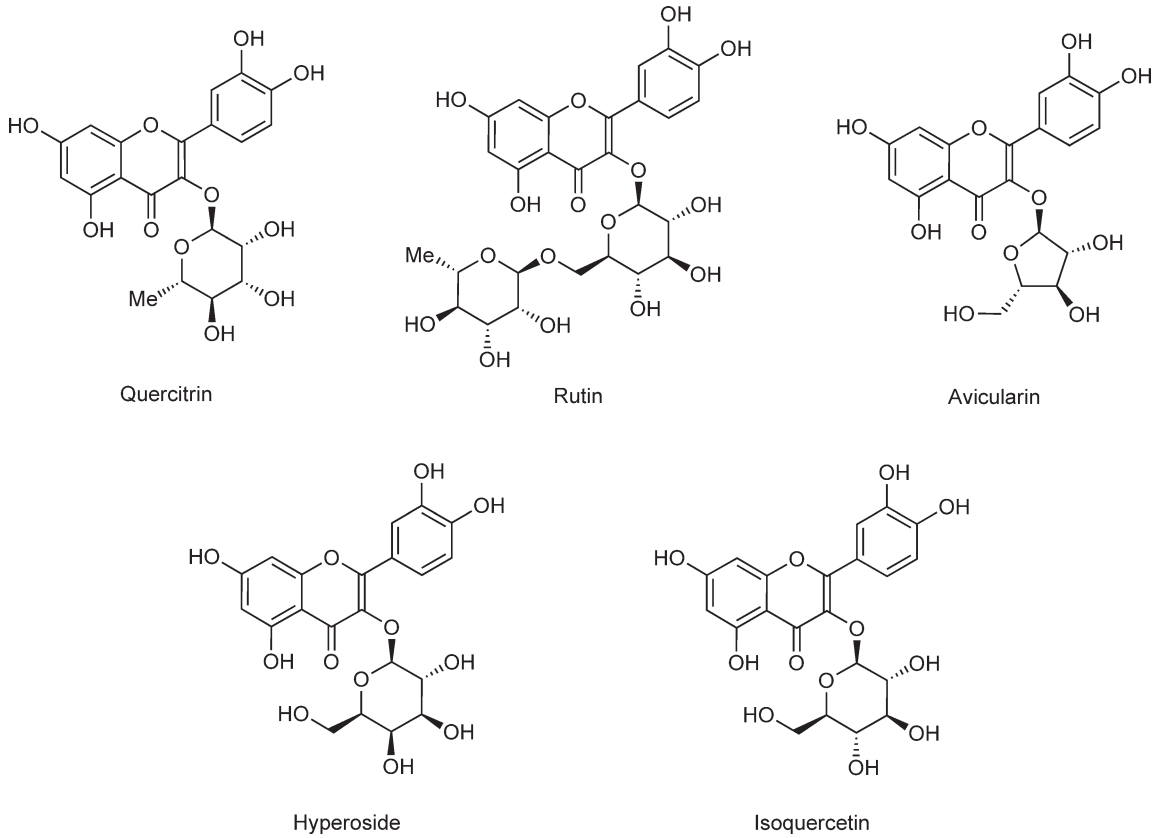


Figure 8 Quercetin glycosides as feeding stimulants for *Galerucella vittaticollis*.

1-hexanol, heptanal, (*Z*)-3-hexenyl acetate, octanal, (*E*,*E*)-2,4-heptadienal, benzyl alcohol, nonanal, decanal, (2*E*,6*Z*)-2,6-nonadienal, indole, and (*E*,*E*)-α-farnesene, from saltcedar, *Tamarix* spp., which is the host of the leaf beetle, *Diorhabda elongate*, induce antennal response in this beetle.[47] A natural ratio blend of four GLVs, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenal, and (*Z*)-3-hexenyl acetate, strongly attracts the beetles. The Fuller's rose weevil, *Pantomorus cervinus*, which is a polyphagous weevil, is also attracted to the blend of general compounds.[48] The headspace of clover, which is one of the weevil's hosts, contains two GLVs, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate. The weevils are attracted to a synthetic blend of these GLVs over a range of concentrations (0.01, 0.1, and 1 mg μl^{-1}), as strongly as to clover leaves. The Japanese beetle, *Popillia japonica*, is a polyphagous insect feeding on the fruit, flowers, or foliage of about 300 species of plants. It is attracted to many naturally occurring plant volatiles, including phenylacetonitrile, (*Z*)-3-hexenyl benzoate, nerolidol, (*Z*)-3-hexenyl hexanoate, (*Z*)-3-hexenyl 2-methylbutyrate, (+)-limonene, and (+)-α-pinene, with the attraction increasing as the number of components in a volatile blend increases[49] (see Chapter 2.02).

Some plant volatiles, however, are highly specific and composed of compounds not found in unrelated plant species.[46] Allyl isothiocyanate, a specific odor component, is a breakdown product of glucosinolates in oilseed rape, *B. napus*. This compound attracts the crucifer flea beetle, *Phyllotreta cruciferae*, which is consistently found feeding in oilseed rape or canola fields.[50]

The blossoms of Cucurbitaceae are well known to be highly attractive to many species of *Diabrotica* beetles. However, the volatile compounds that act as attractants differ in different *Diabrotica* species. Cinnamaldehyde strongly attracts the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*, whereas 4-methoxycinnamaldehyde is a specific attractant for the western corn rootworm, *Diabrotica virgifera virgifera*.[51] The northern corn rootworm, *Diabrotica barberi*, and *Diabrotica cristata* are attracted to eugenol, cinnamyl alcohol, and 2-(4-methoxyphenyl)ethanol. Volatiles of maize, one of the host plants, also attract *D. v. virgifera*

and *D. barberi*. *Diabrotica barberi* is strongly attracted to the maize volatiles geranylacetone[52] and *syn*-benzaldoxime,[53] while *D. v. virgifera* is attracted to (+)-α-terpineol, linalool,[52] and β-caryophyllene.[53] Attraction of *D. v. virgifera* to (±)-linalool, (+)-α-terpineol, or β-ionone is enhanced by methyl salicylate.[53]

Dendroctonus beetles are one of the most serious pests of coniferous trees. *Dendroctonus valens* is attracted to (−)-β-pinene, (+)-α-pinene, and (+)-3-carene from the resin volatiles of its hosts, *Pinus ponderosa* and *Pinus lambertiana*.[54] Three sympatric coniferous nonhost species have the same attractive monoterpenes, but produce less resin. *Dendroctonus pseudotsugae* is attracted to the synthetic bole volatile of its coniferous host, *Pseudotsuga menziesii* (a blend of (−)-α-pinene, (+)-α-pinene, and (−)-β-pinene).[55] On the other hand, *Dendroctonus rufipennis* is attracted to the synthetic bole and/or foliage volatiles of its coniferous host, *Picea glauca* (bole: a blend of (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, and (+)-3-carene; foliage: a blend of (+)-α-pinene, (+)-camphene, myrcene, (−)-limonene, and (−)-bornyl acetate). *Dendroctonus pseudotsugae* can discriminate among volatiles of sympatric host and nonhost conifers. The attraction of this beetle to aggregation pheromone traps is increased by coniferous host volatiles, but decreased by nonhost volatiles. These results indicate that pioneers of some *Dendroctonus* beetles can locate their coniferous hosts by attraction to the host volatiles, even without the aggregation pheromone, and followers can quickly locate the hosts by using pheromones and by discriminating between sympatric host and nonhost conifers.

Constituents of plant volatiles change according to plant age and developmental stage, sometimes causing changes in the behavioral responses of the insects to the host plants. The concentration of (*E*)-2-hexenal in the volatile component of 1.5-year-old red clover roots is higher than that in 2.5-year-old roots.[56] Conversely, the limonene content of 1.5-year-old plants is lower than that of 2.5-year-old plants. (*E*)-2-Hexenal attracts the root borer, *Hylastinus obscurus*, which feeds on the clover roots, whereas limonene repels it and *H. obscurus* therefore shows different behavioral responses to red clover, depending on plant age:[57] an increase in limonene content and a decrease in (*E*)-2-hexenal content correlate with reduced attractiveness of 2.5-year-old clover roots.

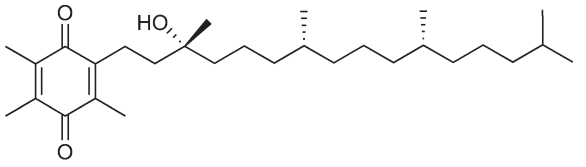
The balance of attractants and repellents is also important in the orientation of insects to the plants. Volatile extracts from storage roots and aerial plant parts of sweet potato are attractive to female sweet potato weevils, *Cylas formicarius*.[58] Three oxygenated monoterpenes from storage roots, nerol, (*Z*)-citral, and methyl geranate, are responsible for attracting the female weevils, while sesquiterpenes, such as α-gurjunene, α-humulene, and ylangene, from storage roots and aerial plant parts repel them. Differences in the relative attractiveness of sweet potato cultivars are inversely correlated with the composite concentration of headspace sesquiterpenes. Host-finding behavior in the granary weevil, *Sitophilus granarius*, also depends on the balance of positive and negative volatile stimuli from grain.[59] *Sitophilus granarius* adults have the ability to respond behaviorally to a wide range of cereal volatiles. However, 1-hexanol, butanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 2-pentanone, 2-hexanone, 2-heptanone, 2,3-butanedione, and furfural repel the weevils, while 1-butanol, 1-pentanol, 3-methyl-1-butanol, pentanal, (*E*,*E*)-2,4-heptadienal, maltol, phenylacetaldehyde, and vanillin attract them. Among the latter components, 1-pentanol, (*E*,*E*)-2,4-heptadienal, and phenylacetaldehyde also repel them at relatively high concentrations. The response of weevils to the cereal odor may change according to storage period because relative concentrations of individual cereal volatiles change during storage.

Some plant volatiles act synergistically with aggregation pheromones. Aggregation of the American palm weevil, *Rhynchophorus palmarum*, on host plants is mediated by host-plant volatiles and a male pheromone (rhynchophorol).[60] Acetoin, one of the major volatile components of the host plants *Cocos nucifera*, *Saccharum officinarum*, *Jacaratia* spp., and *Elaeis* spp., plays an important role in the aggregation of weevils on these plants. Although the pheromone alone is weakly attractive, its attraction is enhanced by acetoin.

Several reports have revealed that host-plant volatiles show synergistic attraction with host visual cues:[61] cinnamyl alcohol and (*E*)-anethole, common flower scent components, attract *Epicometis hirta* (which damages the reproductive parts of flowers of several orchard trees and many ornamental bushes[62]) and enhance the attractiveness of light blue color.[63]

Leaf surface compounds provide important information about host-plant acceptability to coleopteran insects. Although the tortoise beetle, *Cassida canaliculata*, is only weakly attracted to odors from host plants, it shows strong preferences for host plants when additional contact cues are provided.[64] The cottonwood leaf beetle, *Chrysomela scripta*, which is a pest of cottonwood, poplar, and willow, is stimulated to feed by leaf surface chemicals produced by a beetle-preferred poplar clone.[65] The feeding stimulants have been isolated and identified as 1-docosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-triacontanol, and

α-tocopherylquinone (α-TQ) (**16**). Beetle feeding is not stimulated by fatty alcohols and α-TQ alone, but is synergistically stimulated by a mixture of alcohols and α-TQ. The beetles prefer to feed on *Populus* clones with α-TQ, rather than clones without α-TQ.[66] As the amount of α-TQ increases, the feeding preference increases, but it then decreases again as the amount of α-TQ increases further. The potato leaf surface contains kunzeaol (**17**),[67] which acts as a feeding stimulant for *L. decemlineata*. Large aggregations of *L. decemlineata* have been observed on potato varieties that produce relatively high amounts of kunzeaol.



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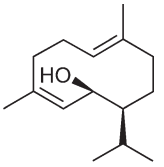
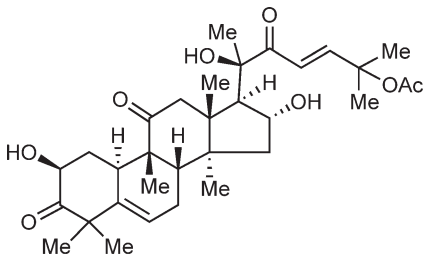
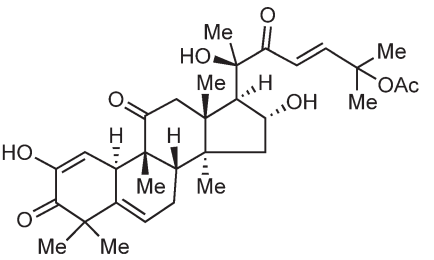


17

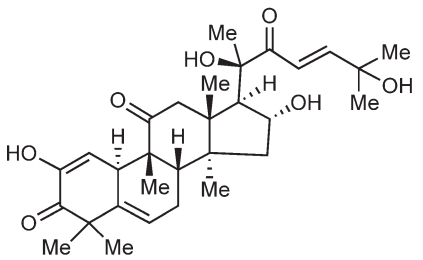
It is well known that cucurbitacins act as feeding stimulants for cucurbitaceous-feeding beetles (**Figure 9**). *Aulacophora indica* and *Aulacophora lewisii* are strongly stimulated to feed by cucurbitacin B, E, I, and E-glucoside, at concentrations ranging from 0.001 to 0.5 mg ml^{-1}, while *Aulacophora nigripennis* is weakly stimulated to feed only by 0.01 mg ml^{-1} of cucurbitacin B.[68] *Diabrotica undecimpunctata howardi*, whose adults feed on leaves of many crops such as cucumber, soybean, cotton, and bean, is stimulated to feed by cucurbitacin E-glucoside.[69] *Epilachna admirabilis* and *Henosepilachna boisduvali* are cucurbitaceous-feeding lady beetles, and cucurbitacin B stimulates feeding of both adults and larvae of both species. Cucurbitacin E, I, and E-glucoside do not stimulate feeding of adult *H. boisduvali* although these three cucurbitacins stimulate larvae of both species and adults of *E. admirabilis*.[70] *Henosepilachna vigintioctomaculata* and *Henosepilachna vigintioctopunctata*, which attack both solanaceous and cucurbitaceous plants, also are stimulated to feed by cucurbitacin B, E, I, and E-glucoside. Although cucurbitacins play an important role in the host selection of many cucurbitaceous-feeding beetles, their activities differ among species, or between adults and larvae within species. The differences in host ranges among the beetle species may be related to the differences in responses to cucurbitacins. The striped cucumber



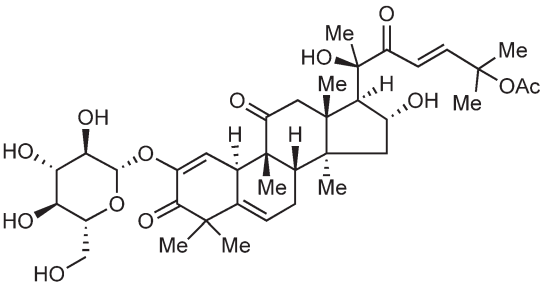
Cucurbitacin B



Cucurbitacin E



Cucurbitacin I



Cucurbitacin E-glucoside

Figure 9 Structures of cucurbitacins that act as feeding stimulants.

beetle, *Acalymma vittatum*, sequesters cucurbitacins from cucurbitaceous plants. However, the response of *A. vittatum* to cucurbitacin diminishes with continued sequestration.[71] The beetles ingest cucurbitacins until they have sequestered enough to make them sufficiently bitter to repel enemies, but sequestration of additional cucurbitacins entails a fitness cost.

Lignans and neolignans are a widely distributed and structurally diverse class of phytochemicals. Most of them and their intermediate products exhibit various biological activities such as fungal-growth and insect-growth inhibitory activities, as well as insecticidal and antifeeding activities. Lignan biosynthesis plays important roles in the host-plant defense system. However, two minor lignans, 1-acetoxypinoresinol and (−)-olivil (**Figure 10**) from olive trees, act as feeding stimulants for the olive weevil, *Dyscerus perforates*, which feeds on the leaf and bark of the olive tree.[72] This weevil uses the compounds, which inhibit many herbivores from attacking the plants, as key stimulants for feeding. A secoiridoid glucoside, oleuropein (**Figure 10**), has also been known to stimulate feeding of this weevil on olive trees.[73]

Mixtures of dominant amino acids, such as proline, alanine, asparagine, and γ-aminobutyric acid, contained in pollens from plants such as sweet corn, winter squash, sunflower, and Canada goldenrod, stimulate feeding in the pollen-feeding beetle, *D. v. virgifera*.[74] The beetle's feeding is stimulated by three major sugars, fructose, glucose, and sucrose.[75] In pollen, the flavonoids, isorhamnetin 3-*O*-neohesperidoside from maize pollen[75] and isoquercetin from sunflower pollen,[76] have been identified as feeding stimulants for *D. v. virgifera* (**Figure 11**). Isorhamnetin 3-*O*-neohesperidoside interacts additively with the mixture of three sugars and 21 amino acids. From sunflower pollen, N^1,N^5,N^{10}-tri[(*E*)-*p*-coumaroyl]spermidine, α-linolenic acid, phosphatidylethanolamine, phosphatidic acid, phosphatidylcholine, and 1,2-dilinolenoyl-3-palmitoylglycerol also have been identified as feeding stimulants (**Figure 11**).[76] α-Linolenic acids are essential nutrients for almost all insects, including *Diabrotica*. Linolenoyl-rich lipids could serve as appropriate pollen-selective taste cues for pollen-feeding specialists.

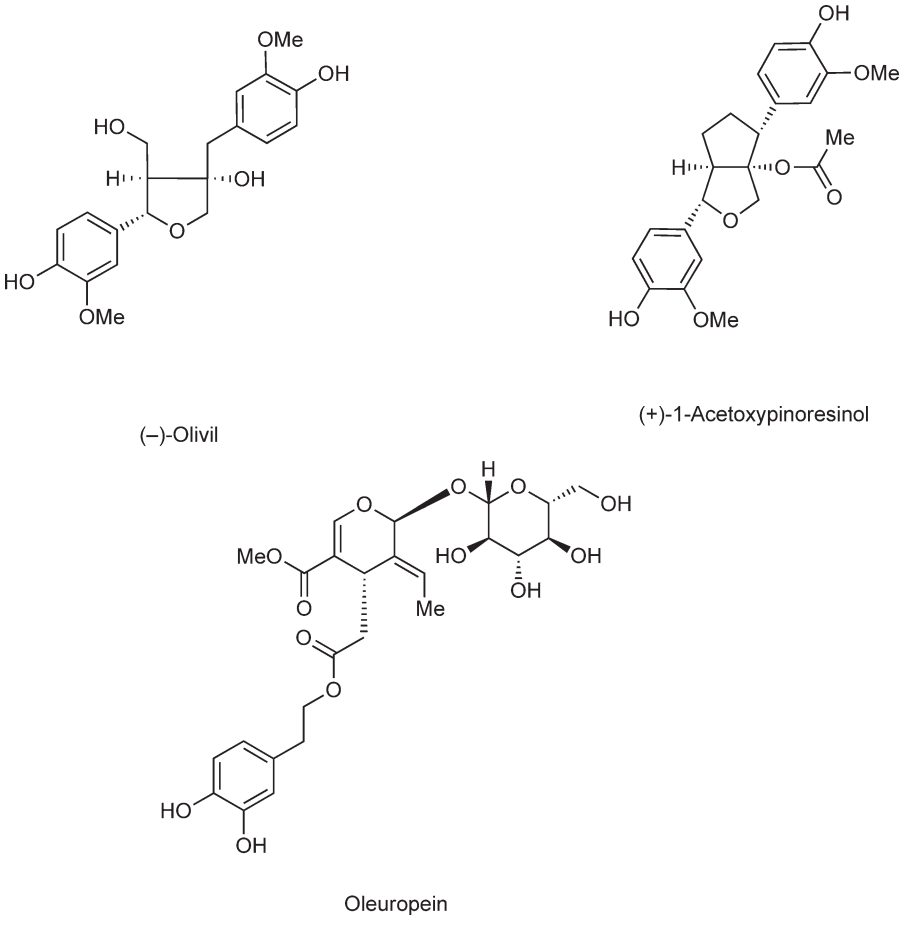
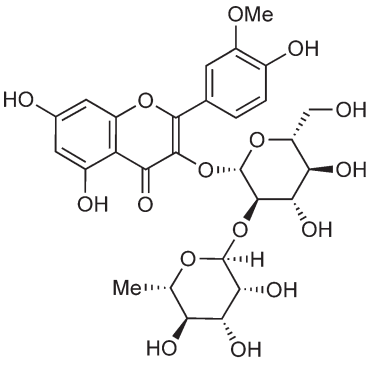
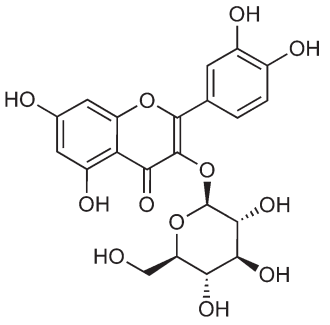


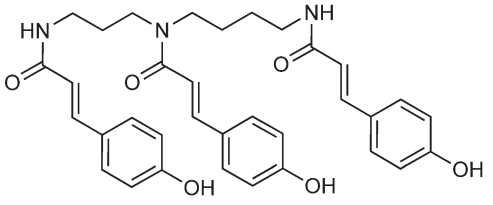
Figure 10 Structure of feeding stimulants identified from olive tree.



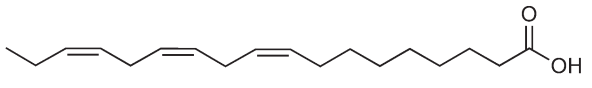
Isorhamnetin 3-*O*-neohesperidoside



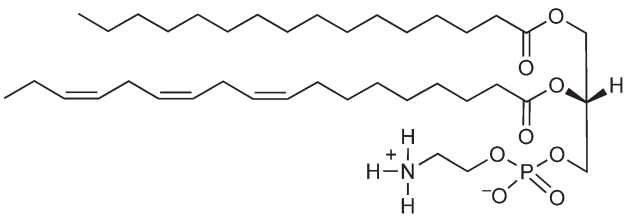
Isoquercetin



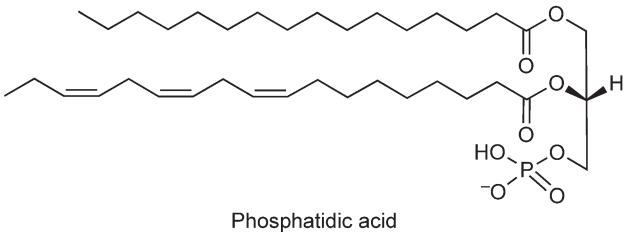
N^1,N^5,N^{10}-tri[(*E*)-*p*-coumaroyl]spermidine



α-Linolenic acid

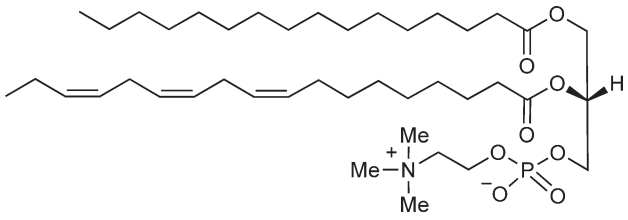


Phosphatidylethanolamine

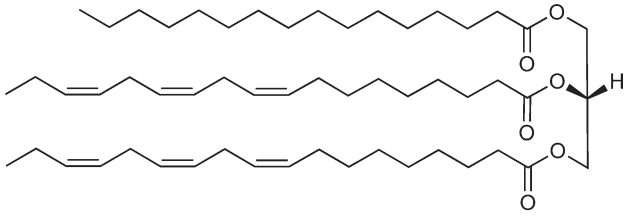


Phosphatidic acid

Figure 11 (Continued)



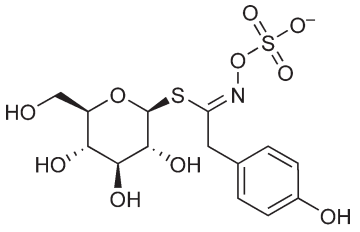
Phosphatidylcholine



1,2-Dilinolenoyl-3-palmitoylglycerol

Figure 11 Feeding stimulants for *Diabrotica virgifera virgifera* identified from maize and sunflower pollen.

Some compounds show feeding stimulatory activity when mixed, even though they show no, or weak, activity alone. Methanol extract of one of the asteraceous plants, ragweed, *Ambrosia artemisiifolia*, stimulates feeding in the asteraceous-feeding leaf beetle, *Ophraella communa*. α-Amyrin acetate, β-amyrin acetate, chlorogenic acid, and 3,5-dicaffeoylquinic acid have been isolated as feeding stimulants from this extract (**Figure 12**). Both triterpenoid derivatives, α-amyrin acetate and β-amyrin acetate, show feeding stimulatory activities when combined with chlorogenic acid or 3,5-dicaffeoylquinic acid, even though the activities of individual triterpenoid derivatives are weak.[77] Methyl linolenate from potato leaves has been identified as the feeding stimulant for *H. vigintioctomaculata*.[78] Although methyl linolenate alone is inactive, it acts synergistically with sugars. The feeding activity stimulated by methyl linolenate combined with sugars is maximal at the concentration found naturally in potato leaves. Feeding stimulation of the mustard leaf beetle, *Phaedon cochleariae*, is also based on a combination of biosynthetically distinct metabolites.[79] The glucosinolate and flavonoid fractions from *Sinapis alba*, which is a host of this leaf beetle, stimulate it to feed, while the combination of both fractions evokes higher feeding stimulatory activity than the individual fractions. The flavonoid fraction has only weak stimulatory activity, while sinalbin (**18**), the most abundant glucosinolate in *S. alba*, induces approximately 50% of the beetles to feed. Hence, sinalbin might be the active stimulant in the glucosinolate fraction, and flavonoids might act synergistically with sinalbin.



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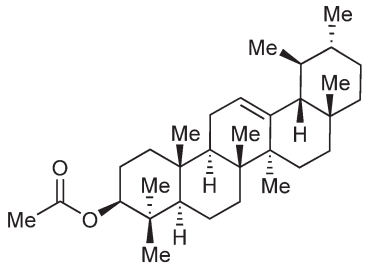
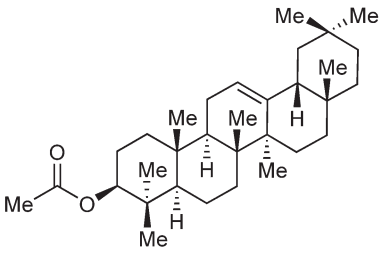
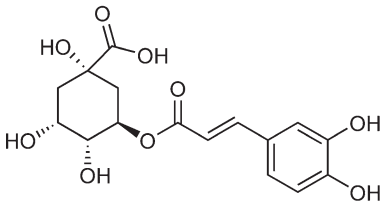
The balance of feeding stimulants and deterrents is important for host selection by insects. *Salix integra* leaves contain both feeding stimulants and deterrents for the willow beetle, *Plagiodera versicolora*, which feeds on several species of willow, including *S. integra*. 1,2-Di[(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoyl]-3-β-D-galactopyranosyl-*sn*-glycerol (MGDG) (**19**) acts as a feeding stimulant and 3,5-dicaffeoylquinic acid acts as a



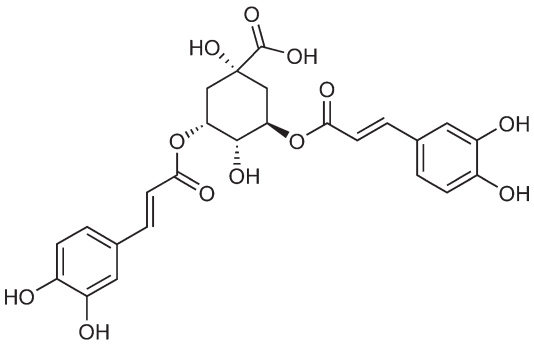
α-Amyrin acetate



β-Amyrin acetate



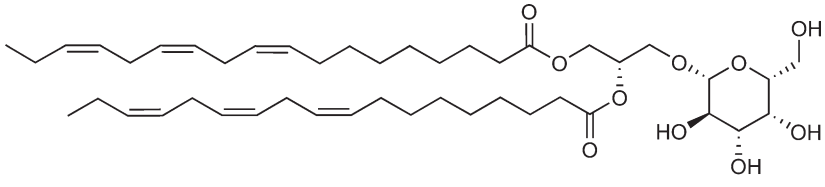
Chlorogenic acid



3,5-Dicaffeoylquinic acid

Figure 12 Feeding stimulants for *Ophraella communa* identified from *Ambrosia artemisiifolia*.

deterrent.[80] In Japan, chlorogenic acid from *S. integra* stimulates feeding in *P. versicolora* on the island of Honshu, but deters them on the island of Hokkaido. The balance between feeding deterrence and stimulation by these compounds plays an important role in the acceptance of *S. integra* by *P. versicolora*. Luteolin 7-*O*-glucoside (**20**) contained in the leaves of *Physalis alkekengi* acts as a feeding stimulant for the two related lady beetle species, *H. vigintioctopunctata* and *H. vigintioctomaculata*.[81] However, *H. vigintioctopunctata* feeds on *P. alkekengi,* while *H. vigintioctomaculata* cannot feed on this plant at all. Feeding deterrents contained in *P. alkekengi* cause its rejection by *H. vigintioctomaculata*; for *H. vigintioctomaculata*, the activities of feeding deterrents in *P. alkekengi* overcome the activities of feeding stimulants, such as luteolin 7-*O*-glucoside (see Chapter 1.25 for feeding deterrents).



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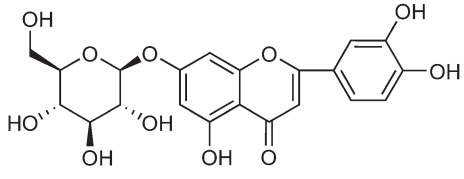

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4.14.3 Flower-Visiting and Foraging

4.14.3.1 Floral Volatiles

Many species of insects visit angiosperm flowers to acquire nutrition or reproductive reward and coincidentally contribute to pollination. The scents, as well as the colors, of angiosperm flowers serve as primary cues for mediating the long-range attraction of insects.[82] In general, floral scents are composed of a number of compounds belonging to different chemical classes, and the chemical composition of floral scents varies greatly among plant species. To understand the ecological significance of these variations, characterization of floral scents and their relationships with different types of insect pollinators has been attempted for many years (**Table 1**).[83]

Thus far, more than 1700 compounds from around 1000 plant species have been identified as floral volatiles.[84] Twelve compounds in particular, namely, limonene, (*E*)-β-ocimene, β-myrcene, α-pinene, β-pinene, linalool, 6-methyl-5-hepten-2-one, β-caryophyllene, benzyl alcohol, 2-phenylethanol, benzaldehyde, and methyl salicylate, have been found to occur in more than 50% of the plant families investigated, and these are regarded as the most common floral volatiles. Various insects use these ubiquitous volatiles for flower selection, for example, honeybee, butterfly, and moth commonly show preferential responses to linalool, benzaldehyde, and/or phenylacetaldehyde contained in the floral scents of various plants.[85–87] These volatiles not only serve as important floral cues by themselves but also contribute to the floral scents unique to each plant species, which are distinguishable for flower visitors. On the other hand, it has recently been revealed that several plant species are pollinated exclusively by particular insect species, and that the floral scents play important roles in such pollination.

4.14.3.1.1 Attractants in nursery pollination

Nursery pollination systems are examples of reciprocal mutualism between pollinators and plants, in which the female pollinators oviposit on the ovaries of plants and the hatched larvae feed on pollinated fruits and seeds. At least 13 nursery pollination systems have been identified.[88] Floral volatiles significantly contribute to attracting specific pollinators in several systems.

Fig–fig wasp interactions are an example of highly specialized and diversified mutualism. Approximately 900 species of *Ficus* (Moraceae), when receptive, release floral scents that attract specific wasps (Agaonidae) for pollination. The chemical compositions of the floral scents differ considerably among different *Ficus* species, and several volatiles such as (*E*)-β-ocimene, α-pinene, linalool, *trans*-linalool oxide furanoside (**21**), 1,8-cineol, β-caryophyllene, germacrene D, α-copaene, and benzyl alcohol have been identified as the dominant components of these scents.[89] Such differences in composition of the floral scents of figs induce species specificity in the attraction of fig wasps.[90] In addition, in fig flowers, the scent composition differs between the receptive and

Table 1 Characteristics of floral volatiles of general-, coleopteran-, dipteran-, hymenopteran-, and lepidopteran-pollinated plants

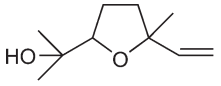
Types of insect pollinators	*Traits of floral volatiles*
Generalist (diverse insects)	Fatty acid derivatives, terpenoids, and benzenoids
Coleoptera	
Tropical scarab beetles	Methoxylated benzenoids
Other tropical beetles	Fatty acid esters, benzenoid esters, and terpenoids
Temperate beetles	Variable, with N-compounds frequently
Diptera	
Typical flies and hoverflies	Fatty acid derivatives (acids and alcohols) and N-compounds
Hymenoptera	
Bees	Variable, with terpenoids commonly abundant
Wasps	Variable
Lepidoptera	
Butterflies	Benzenoids and terpenoids, with N-compounds occasionally
Settling moths	Benzenoids and terpenoids, with fatty acid esters and N-compounds occasionally
Hovering moths	Benzenoids, terpenoids, and N-compounds

the postpollinated states, for example, in *Ficus hispida*, the amounts of several dominant components, such as linalool and linalool oxide, decrease and disappear after pollination by wasps, while that of 1-hydroxylinalool increases.[91]

Yucca plants (Agavaceae) are distributed in the arid areas of North and Central America and rely exclusively on *Tegeticula* and *Parategeticula* yucca moths for pollination. The floral scent of *Yucca filamentosa* consists mainly of homoterpenes and long-chain aliphatic hydrocarbons, among which DMNT (**22**) has been identified as the dominant component, and two dioxygenated compounds with unknown structures have also been found. Since no compositional differences in floral scents have been observed among various yucca populations pollinated by different *Tegeticula* moths, a stable combination of unique compounds may contribute to the highly selective attraction of yucca moths.[92]

Hadena moths use *Silene* flowers (Caryophyllaceae) as both nectar sources and as host plants for depositing larvae. The floral scent of *Silene latifolia* is characterized by the presence of several fatty acid derivatives, benzenoids, and monoterpenoids, while the most abundant compounds are the lilac aldehyde isomers ((2*S*, 2′*S*, 5′*S*), (2*R*, 2′*S*, 5′*S*), (2*S*, 2′*R*, 5′*S*), and (2*R*, 2′*R*, 5′*S*)) (**23**), veratrole, and benzyl acetate.[93] However, several chemotypes of these dominant components are found in *S. latifolia*, suggesting variability in attracting pollinators. Wind tunnel experiments have revealed that *Hadena bicruris* is strongly attracted by lilac aldehyde isomers; although the moths respond electrophysiologically to all eight stereoisomers of lilac aldehyde, only four are present in the floral scent.[94]

More than 300 species of *Glochidion* trees (Phyllanthaceae) are pollinated at night exclusively by local species-specific moths of the genus *Epicephala*. The floral scents of the five *Glochidion* species contain mainly (*R*)-(−)- and (*S*)-(+)-linalools, (*E*)- and (*Z*)-β-ocimenes, as well as another 6–20 minor components. The compositions of the scents are species specific, especially in terms of their minor components, and two-choice bioassays indicated that *Epicephala* moths selected their host on the basis of floral scent.[95]



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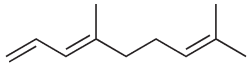

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4.14.3.1.2 Attractants in sexual mimicry

Several orchids have remarkably characteristic pollination systems employing sexual deception; in this system, the flowers mimic the visual, tactile, and olfactory traits of the females of particular hymenopteran species and so deceive conspecific males into attempting to mate with the flowers. In particular, the floral scent, which has a composition identical to that of female sex pheromones, is a key mediator of this exclusive and species-specific mode of pollination.

The flowers of *Ophrys sphegodes* produce the same compounds as those found in the female sex pheromone of the solitary bee *Andrena nigroaenea* (**Table 2**).[96] The floral scent triggers copulation behavior and significant electrophysiological responses in male bees.[97] *Ophrys fusca* and *Ophrys bilunulata* are specifically pollinated by *A. nigroaenea* and *Andrena flavipes*, respectively. The two sympatric and closely related orchids differ in the relative amounts of alkenes present in their floral scents, which are responsible for the selective attraction of pollinators.[98] After pollination, *O. sphegodes* shows a significant change in the chemical composition of the floral scent and an increase in the amount of (*E*,*E*)-farnesyl hexanoate (**24**), which serves as a repellent volatile for the bee pollinators.[99]

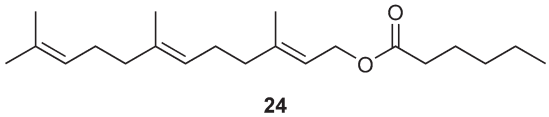
Male adults of the scoliid wasp *Campsoscolia ciliate* are strongly attracted to the flowers of *Ophrys speculum*, and attempt to copulate with the flower labellum. The floral scent contains many volatiles, including trace amounts of (ω-1)-hydroxy and (ω-1)-oxo acids, especially 9-hydroxydecanoic acid (*R*:*S* = 6:4) (**25**). These characteristic substances are the major components of the female sex pheromone in the scoliid wasp and induce copulatory behavior by male wasps.[100]

The Australian orchid *Chiloglottis trapeziformis* relies on exclusive pollination by the thynnine wasp *Neozeleboria cryptoides*. The flowers have been found to release 2-ethyl-5-propylcyclohexane-1,3-dione, called chiloglottone (**26**), which is responsible for attracting male wasps and is also produced by female wasps as a sex

Table 2 Chemical compositions of extracts of virgin *Andrena nigroaenea* females and *Ophrys sphegodes* flower labellum

	Abundance (%)	
Compounds	*Andrena bee*	*Ophrys flower*
Heneicosane	1.6	1.8
Docosane	0.6	0.5
Tricosane	28.7	30.6
Tetracosane	2.0	3.1
(*Z*)-9-Pentacosene	3.4	0.6
Pentacosane	34.9	20.2
Hexacosane	1.6	2.1
(*Z*)-12+(*Z*)-11-Heptacosenes	0.7	6.0
(*Z*)-9-Heptacosene	5.1	7.6
Heptacosane	11.2	11.5
(*Z*)-12+(*Z*)-11-Nonacosenes	3.7	6.7
(*Z*)-9-Nonacosene	6.6	9.4

pheromone.[101] Interestingly, the closely related orchid *Chiloglottis valida* also uses the same compound for attracting the specific wasp *Neozeleboria monticola*. Although both wasps are attracted to dummies scented with chiloglottone, *N. cryptoides* preferentially attempted copulation with dummies a few centimeters above the ground, while *N. monticola* preferred those closer to the ground. Since the two orchids differ with regard to flower height, that is, *C. trapeziformis* is nearly twice the height of *C. valida*, these behavioral differences between two thynnine wasps are responsible for the reproductive isolation of the orchids.[102]



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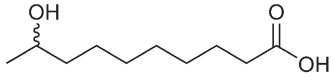

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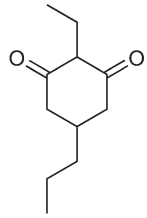

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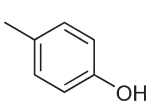
4.14.3.1.3 Attractants in abiotic mimicry

Not all floral scents consist of fragrant volatiles. Unpleasant odors associated with dung, carrion, urine, rotting fungi, and decaying cabbage and onion have been found in abiotic mimicry in many plant families such as Araceae, Aristolochiaceae, Orchidaceae, and Apocynaceae.[103] Several saprophagous insects are attracted to these flowers and act as selective pollinators.

The inflorescence of the dead horse arum *Helicodiceros muscivorus* (Araceae), distributed in the western Mediterranean regions, mimics a dead mammal and emits an oligosulfide odor, which includes dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide.[104] The flower odor is crucial for attracting blowflies, which serve as important pollinators for this flower. In addition, the thermogenesis and respiration of the inflorescence reinforce the stimulation for fly pollination.[105]

The stapeliad flowers (Apocynaceae) that inhabit the regions from the African continent to India are known as 'carrion flowers' due to their strong fetid scents and their exclusive pollination by flies. However, the floral scents of 15 stapeliad species are highly diverse in their compositions, and on this basis, the species can be divided into four groups that appear to reflect different types of mimicry: species with *p*-cresol (**27**) as the dominant component of their scent (herbivore feces mimicry); species with dimethyl oligosulfides as the major scent components (carnivore/omnivore feces or carcass mimicry); species with large amounts of heptanal and octanal (carnivore/omnivore feces or carcass mimicry); species with scents in which hexanoic acid is the major component (urine mimicry).[106]

The genus *Amorphophallus* (Araceae) is well known for large-sized flowers in some of its species and for very strong and obnoxious floral scents. The floral scents of 12 *Amorphophallus* species have gaseous or carrion-like odors with a simple chemical composition consisting mainly of dimethyl oligosulfides. Floral scents containing dimethyl oligosulfides are also found in the closely related genus *Pseudodracontium* (Araceae). Several *Amorphophallus* species produce different floral scents: the strong fried fish odor of *Amorphophallus brachyphyllus* in which trimethylamine is the dominant component; the strong cheesy odor of *Amorphophallus elatus* in which 4-methylpentanoic acid (**28**) is the main component; an unusual banana-like odor of *Amorphophallus haematospadix*, which contains large amounts of isoamyl acetate and ethyl acetate; and the anise-like odor of *Amorphophallus albispathus* containing 2-(4-methoxyphenyl)ethanol (**29**).[107]



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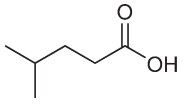

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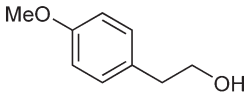

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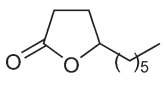
4.14.3.1.4 Repellent volatiles in floral scents

Several insects exert negative effects on plant fitness by behaving as nectar robbers, opportunistic flower visitors, and flower-tissue feeders. Recently, it has been revealed that floral scents can act not only as attractants but also as repellents to specific flower visitors. The flowers of the sweet olive *Osmanthus fragrans* (Oleaceae) attract few species of lepidopteran adults despite their strong scents. The major floral volatiles, namely, γ-decalactone (**30**) and linalool oxide isomers (furanoside (**21**) and pyranoside), discourage flower visiting by the cabbage butterfly, *Pieris rapae*.[108] Wild tobacco, *Nicotiana attenuate* (Solanaceae), produces flower nectar that is rich in nicotine (**31**), which comprises 1% of the floral headspace and acts as a deterrent for the hawkmoth, *Manduca sexta*, so reducing nectar removal.[109]



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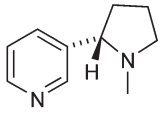

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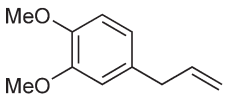
4.14.3.1.5 Transfer of volatiles from flowers to insects

Several insects collect floral oils (scent components) as rewards for pollination and utilize them for their intraspecific communications.

For pollination, the flowers of several *Bulbophyllum* orchids (Orchidaceae) attract males of *Bactrocera* fruit flies with characteristic fragrances rich in several phenylpropanoids. Although these orchid species never produce nectar, the fruit flies are rewarded by the uptake of floral phenylpropanoids from the orchids, and these compounds are the raw materials of their pheromones. The fruit flies can be classified into two groups based on their sensitivities to the two phenylpropanoids, methyl eugenol (ME) (**32**) and raspberry ketone (4-(4-hydroxyphenyl)-2-butanone; RK) (**33**). The ME-sensitive fruit fly species convert the ingested ME into male sex pheromonal components, such as *trans*-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol, while the RK-sensitive species directly sequester and use the ingested RK as a component of male pheromones.[110] Both the ME- and RK-sensitive species are attracted by zingerone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) (**34**), which is responsible for the pungent odor produced by the ginger orchid *Bulbophyllum patens*. Further, *Bactrocera papayae* takes up zingerone, a precursor of sex pheromones.[111] In addition, other phenylpropanoids analogous to sex pheromones, for example, rhododendrol (4-(4-hydroxyphenyl)-2-butanol), *trans*-3,4-dimethoxycinnamyl alcohol, and euasarone (5-allyl-1,2,4-trimethoxybenzene), have been detected in floral scents.[112] The differential compositions of the floral scents of orchids mediate species-specific pollination by fruit flies.



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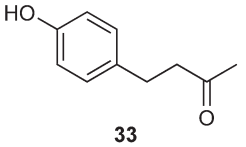

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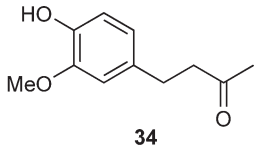

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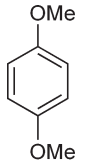
Males of neotropical euglossine bees (Apidae), called orchid bees, collect odoriferous substances from flowers of orchids and other plants. The floral scents of these species display relatively simple chemical compositions dominated by one or two major components, mostly terpenoids and aromatic compounds such as α-pinene, 1,8-cineol, eugenol, *p*-dimethoxybenzene (**35**), 2,3-epoxygeranyl acetate (**36**), nerolidol, 4-methoxycinnamaldehyde (**37**), and benzyl benzoate.[113] Since the orchid bees have odor preferences, their collection of fragrances leads to specialized pollination of particular plant species. Male bees absorb the floral volatiles with their tarsal hairs, form species-specific bouquets, and finally accumulate them in their hind tibial pouches. These bouquets have potential roles in courtship displays and marking territories.[114,115]



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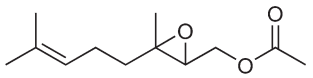

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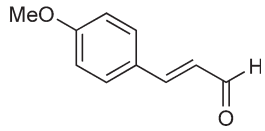

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4.14.3.2 Food Constituents Affecting Foraging

Floral nectar and pollen have ecological significance as food resources for various insects. There is quantitative and qualitative variability in the nutrients (including sugars, amino acids, sterols, lipids, and vitamins) found in the floral nectar and pollen of different plant species. In addition, other minor components, including organic acids, terpenes, alkaloids, flavonoids, and glycosides, are also present. Most flower-visiting insects assess the nutritional values of foods and demonstrate food selection by gustatory perception of these constituents.

4.14.3.2.1 Sugars

Sugars are the main floral nectar solutes and represent the major energy source for flower-visiting insects. Sucrose, glucose, and fructose are the most frequent nectar sugars. However, the sugar composition of floral nectars varies greatly among plant species and the concentrations of each sugar and the sucrose/hexose ratio are strongly related to pollinator type.[116,117] Recently, xylose has been identified as the fourth major nectar sugar, but honeybees and beetles show weak preference for this pentose sugar.[118] In sweet corn (*Z. mays*) pollen, sucrose is the predominant sugar, comprising 98% of the total sugars.

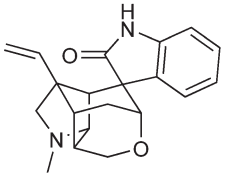
4.14.3.2.2 Amino acids

Amino acids, as well as sugars, have received considerable attention in terms of their role in flower–insect interactions. All 20 of the normal amino acids found in proteins have been identified in various floral nectars, where alanine, arginine, serine, proline, glycine, isoleucine, threonine, and valine are the most prevalent.[119,120] Despite their low levels in floral nectar, several amino acids significantly affect flower selection by insects. Honeybees prefer proline-rich nectar because they are thought to utilize this amino acid as fuel for the initial stages of flight.[121] Butterflies also display a foraging preference for floral nectar containing large amounts of amino acids, and the uptake of amino acids enhances fecundity in several butterfly species.[122] In sweet corn pollen, proline has been identified as the most abundant free amino acid, followed by serine and alanine.[123]

4.14.3.2.3 Toxic components

Many plants produce floral nectar-containing components that are toxic or repellent to some visitors. Particular minor components, including alkaloids, phenolics, glycosides, and sometimes sugars and amino acids, have been

described as being responsible for this toxicity and repellency. Several hypotheses have been proposed for the possible roles of these components, including encouraging specialist pollinators, deterring nectar robbers, preventing microbial degradation of nectar, and altering pollinator behavior.[124] Alkaloids in nectar are typically toxic or repellent to several flower-visiting insects. Nicotine (**31**) is contained at variable concentrations in the floral nectar of *Nicotiana* spp. and the floral nectar with a high concentration of nicotine discourages visiting or nectaring by moths and ants.[109] In the toxic nectar of Carolina jessamine, *Gelsemium sempervirens* (Loganiaceae), gelsemine (**38**) has been identified as the major repellent, not only for nectar robbers but also for most potential pollinators.[125] Triptolide (**39**) found in the nectar of the perennial vine, *Tripterygium hypoglaucum* (Celastraceae), negatively affects visiting frequency and nectaring time of honeybees[126] (see Chapter 4.08).



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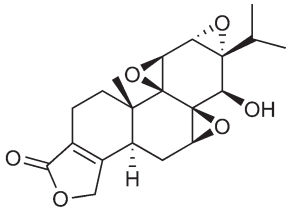

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4.14.4 Insect–Plant Interface in Multitrophic Interactions

As a response to feeding damage or egg-laying by herbivores such as insects and mites, plants emit various volatiles called herbivore-induced plant volatiles (HIPVs).[127–129] The composition changes in HIPVs can be exploited by the natural enemies of these herbivores, that is, parasitoids and predators, to locate the plant on which the hosts or prey are feeding. Therefore, plants indirectly attract natural enemies to defend themselves.[127,129] Release of HIPVs occurs a few hours after the initial damage. Emission of HIPVs is not limited to the site of feeding but can systemically occur from the whole plant,[130,131] so even undamaged leaves of a damaged plant release volatiles.[130,132–134]

The timing and location of the emission of HIPVs from corn plants have been studied by chemical analysis.[135–137] Immediately after leaf damage, the corn plant releases several typical octadecanoid-derived GLVs from the damaged leaf. Additionally, elicitors in the herbivore's oral secretions induce a systemic release of volatiles that are mainly composed of terpenoids but also include some phenolics, such as indole and methyl salicylate.

4.14.4.1 Parasitic Wasps

A number of hymenopteran parasitoids are attracted to herbivore-infested plants that emit HIPVs.[127]

In several species of *Cotesia* spp. (Hymenoptera: Braconidae), the damaged plant volatiles attractive for these wasps are emitted when the plant is damaged by the host herbivores. Among these species, it was first demonstrated that *Cotesia marginiventris* is responsive to HIPVs from corn seedlings damaged by *Spodoptera exigua*.[138] Artificially damaged seedlings did not release these volatiles in significant amounts, but HIPVs were released in significant enough amounts to become attractive to the wasps if oral secretions from the caterpillars were applied to the damaged site.

Cotesia kariyai is the dominant braconid parasitoid of the common armyworm *Mythimna separata* (Lepidoptera: Noctuidae). The female wasp uses chemical cues from the host and host–plant complex to find their hosts.[139,140] A wind tunnel was used with conditioning method to evaluate the learning of different synthetic blends such as host-infested and unspecific blends.[141] A blend of four chemicals, geranyl acetate, β-caryophyllene, (*E*)-β-farnesene, and indole (**Figure 13**), known to be specifically released from corn plants infested with host larvae (host-infested blend) (**Figure 14**) elicited a flight response in naïve *C. kariyai*, but did not enhance the response after conditioning. Another blend of five chemicals, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, β-myrcene, and linalool (**Figure 15**), known to be released not only from plants infested by host larvae but also from artificially damaged or undamaged plants (unspecific blend) (**Figure 16**), elicited

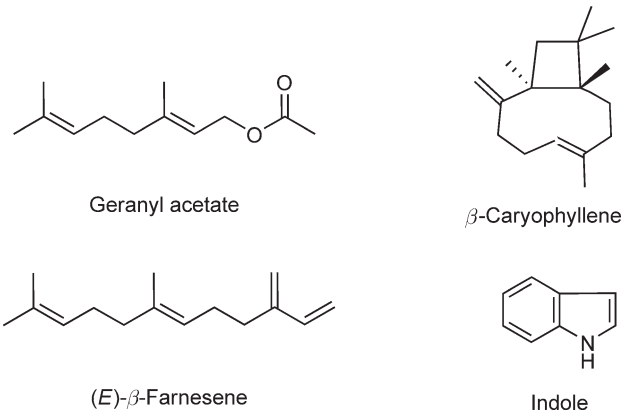


Figure 13 Volatiles selected as 'host-infested blends' specifically released from corn plants infested with host larvae.

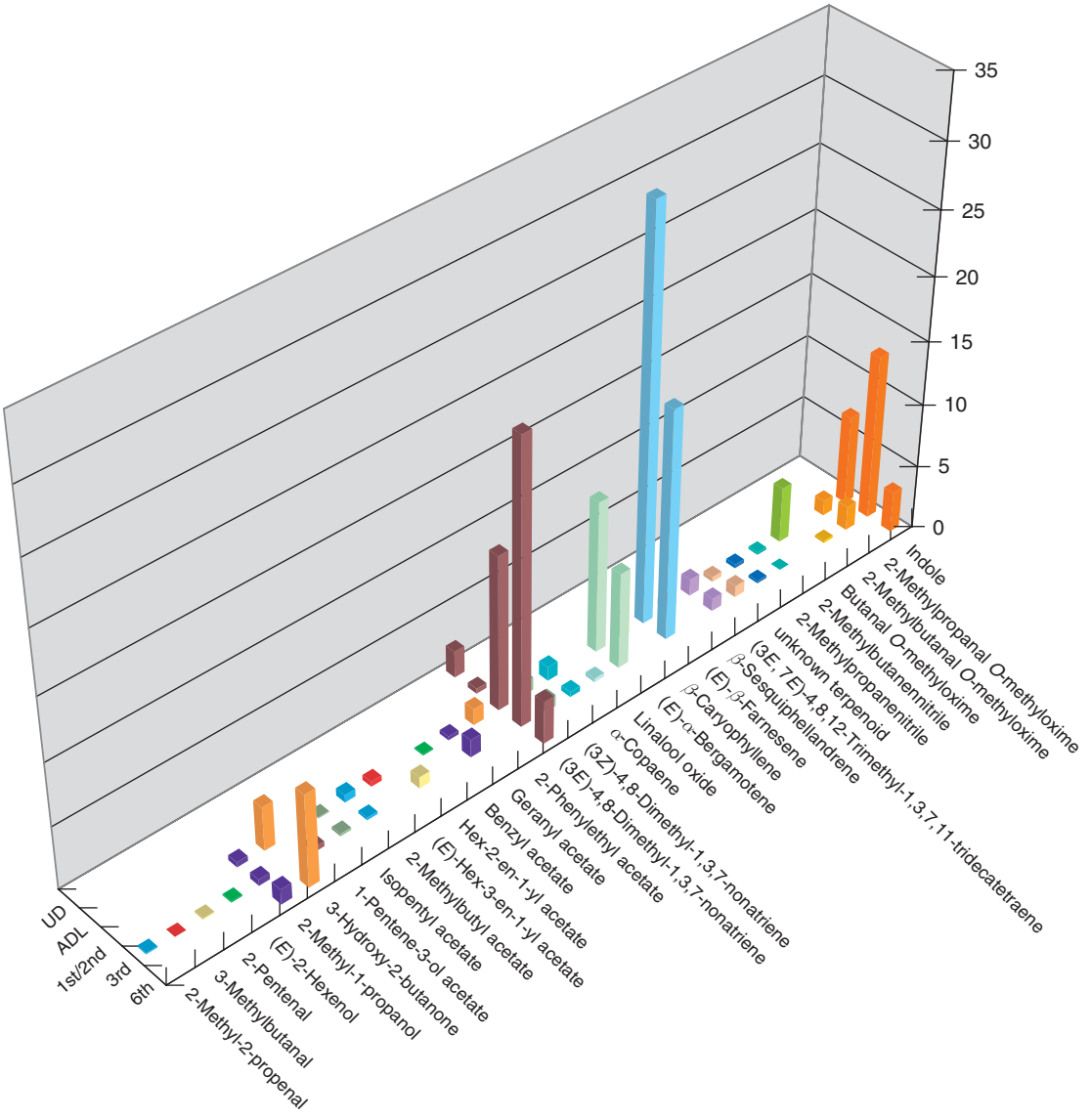


Figure 14 Volatile compounds identified from headspace of infested corn leaves but not (or in trace amount) in uninfested leaves. UD: undamaged leaves; ADL: artificially damaged leaves; 1st/2nd, 3rd, 6th: host instars infesting corn leaves. Reproduced from J. Takabayashi; S. Takahashi; M. Dicke; M. A. Posthumus, *J. Chem. Ecol.* **1995**, *21*, 273–287.

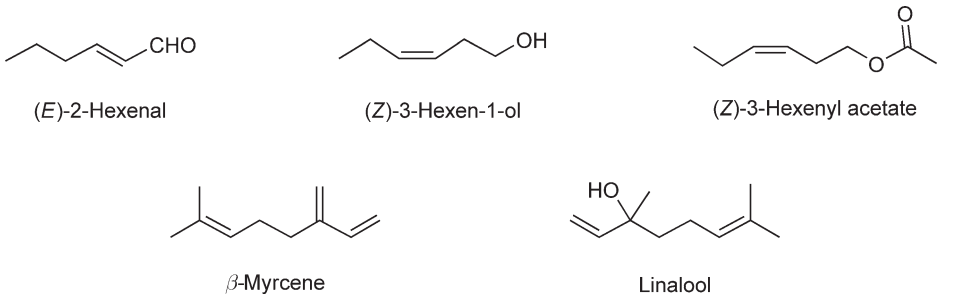


Figure 15 Volatiles selected as 'unspecific blend' released from artificially damaged or undamaged corn plants.

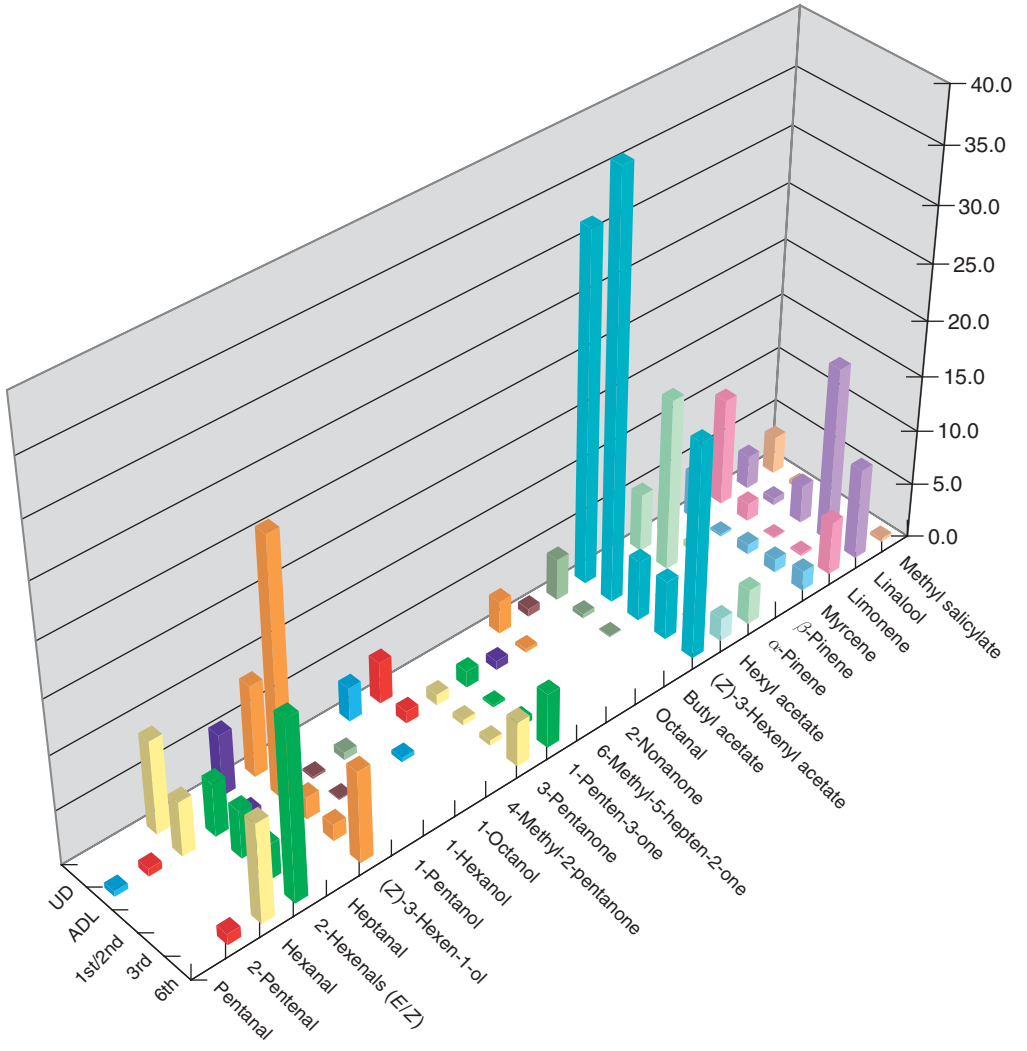
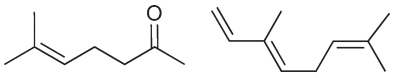


Figure 16 Volatile compounds identified from headspace of uninfested, artificially damaged, and infested corn leaves. Reproduced from J. Takabayashi; S. Takahashi; M. Dicke; M. A. Posthumus, *J. Chem. Ecol.* **1995**, *21*, 273–287.

little response in naïve wasps, but significantly enhanced the responses of wasps after conditioning. Wasps could learn a blend of the above nine chemicals at lower concentrations than they did the nonspecific blend.[141] These results indicate that both the host-induced and unspecific volatile compounds are important for the female *C. kariyai* to learn the necessary chemical cues for host location.

Aphidius ervi (Hymenoptera: Aphidiidae), a parasitoid of the pea aphid, responded more to aphid-infested plants than to an uninfested plant. The gas chromatography–electroantennographic detection (GC-EAD) active compounds were identified and candidate compounds were further tested by electroantennography (EAG) and wind tunnel assays. For *A. ervi* females, 6-methyl-5-hepten-2-one (**40**) was most attractive with linalool, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene (**41**), (*Z*)-3-hexen-1-ol, and (*E*)-β-farnesene, all eliciting oriented flight behavior.[142]



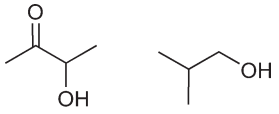
40 **41**

Deposition of insect eggs induces plant volatiles that attract egg parasitoids in elm,[143,144] pine,[145] and beans.[146] In *Brassica*, *Pieris*, and *Trichogramma,* egg deposition appears to induce changes in plant surface chemicals and arrest the egg parasitoids by contact chemical cues near the host eggs.[147] An egg parasitoid *Oomyzus gallerucae* (Hymenoptera: Eulophidae) attacks the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae) that feeds on the field elm (*Ulmus minor*). Before laying eggs, the female elm leaf beetles gnaw a groove into the undersurface of the leaf, and then glue an egg mass into this groove with oviduct secretions. The leaf begins to emit volatiles that attract *O. gallerucae*, caused by egg deposition not by feeding.[143]

4.14.4.2 Parasitic Flies

Compared to parasitoid wasps, only a few species of dipteran parasitoids are reported to respond to HIPVs.[148–153] Host-habitat location remains largely unknown in parasitoid flies.

The parasitic fly, *Exorista japonica* (Diptera: Tachinidae), is a gregarious and polyphagous parasitoid that attacks a number of lepidopteran pests, particularly noctuid larvae. Females of *E. japonica* lay heavy-shelled macrotype eggs on last instar host larvae. The first instar larvae emerge and penetrate the host integument after incubating for approximately 4 days. A previous study showed that *E. japonica* females were attracted to odors from corn plants infested with final instar larvae of *M. separata*.[151,153] When corn plants are infested with final instar larvae of *M. separata*, the plants release a nonspecific blend ((*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, hexanal, (*Z*)-3-hexen-1-ol, and linalool) (**Figure 16**) and a host-induced blend that includes the homoterpene DMNT (**22**), indole, 3-hydroxy-2-butanone (**42**), and 2-methyl-1-propanol (**43**) (**Figure 14**). *E. japonica* females show a high response to a synthetic mixture of the nonspecific and host-induced blends, but not to the nonspecific blend or the synthetic host-induced blend separately.[153] This suggests that the fly uses a mixture of nonspecific and host-induced blends as an olfactory cue for locating host-infested plants and may well explain the situation in nature.

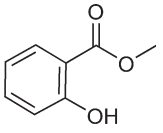


42 **43**

4.14.4.3 Predators

Compared to parasitoid wasps, relatively fewer studies have been reported on the attraction of predators to prey-infested plants. Among those predator species, the predatory mite *Phytoseiulus persimilis* was first reported to be attracted to volatiles from lima bean leaves infested with the two-spotted spider mite *Tetranychus urticae*.[154] Those prey-induced volatiles were identified as linalool, methyl salicylate (**44**), (*E*)-β-ocimene (**41**), and DMNT, all attractive to *P. persimilis*.[155] Similar attraction was demonstrated in the predatory mite *Amblyseius womersleyi*[156] and insect predators *Scolothrips takahasii* (Thysanoptera: Thripidae)[157] and *Oligota kashmirika benefica* (Coleoptera: Staphylinidae).[158] In a generalist predator mite, *Neoseiulus californicus*, among

the chemicals identified from *T. urticae*-infested and artificially damaged lima bean leaves, linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate were active in Y-tube olfactometer bioassays.[159]



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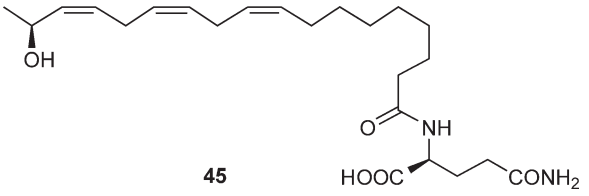
4.14.4.4 Elicitors

Feeding by herbivorous insects results in mechanical damage and adhesion of regurgitant to the wounded leaves, which cause the emission of HIPVs from the plant. The regurgitant is called an elicitor.

First, β-glucosidase was isolated and identified as an elicitor from *Pieris brassicae* larvae that causes the release of volatiles from *Brassica* plants.[160] Volicitin (*N*-[(*S*)-17-hydroxylinolenoyl]-L-glutamine) (**45**) was identified as the active substance from the larval regurgitant of *S. exigua*.[161] Application of this elicitor to mechanically damaged maize plants activated the same biochemical response in the plant as crude larval regurgitant, causing the plant to release the attractant volatiles.[162]



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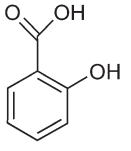
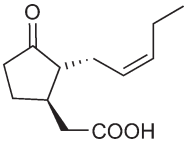
Another elicitor called inceptin was identified from *Spodoptera frugiperda* larval secretions.[163] Inceptin promotes cowpea ethylene production and triggers increases in the defense-related phytohormones, salicylic acid (SA), and jasmonic acid (JA) (**Figure 17**), then elicits the release of HIPVs. Inceptin is a peptide derived from the proteolytic cleavage of chloroplastic ATP synthase γ-subunits (cATPC) that elicits a plant defense response in cowpea leaves during herbivory by sixth-instar fall armyworms.[163] If the larvae contact damaged cowpea leaves, inceptin elicits increased production of JA, ethylene, SA, and HIPVs commonly associated with attracting predators and parasitoids. Those HIPVs are methyl salicylate (**44**) and DMNT.[163,164] Interestingly, the neonate fall armyworm exploits inceptin-treated plant volatiles as host-plant location and recognition cues.[165]

Studies on induced resistance against insects and pathogens have clarified the roles of SA and JA, revealing that SA regulates resistance to pathogens and JA resistance to herbivory. Treatment of plants with SA, JA, and synthetic mimics elicits the same metabolic changes that lead to resistance induced by pathogens and insects.[127] Under field conditions, treatment of tomato plants with JA increased parasitism of caterpillars (*S. exigua*) by *Hyposoter exiguae* (Hymenoptera: Ichneumonidae). This may be due to JA-induced increase in odor emissions[166] (see Chapter 1.20).



Salicylic acid



Jasmonic acid

Figure 17 Phytohormones closely related to the production of HIPVs.

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Biographical Sketches

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4.15 Human–Environment Interactions (1): Flavor and Fragrance

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4.15.1 Introduction

Odor perception, along with that of taste, has been one of the fundamental interactions between life and its environment ever since the early stages of evolution. It has a critical role in spotting and evaluating food, reproduction, and avoiding life-threatening hazards. In the case of humans, odorous compounds are restricted to a molecular weight of less than 300; despite this regulation, an estimated 400 000 odorous compounds are giving our lives a tremendous perspective. The fascinating nature, therefore, has been a target of sparking curiosity. A low threshold of 1-octen-3-one is a good example.[1] As a key compound of the metallic musty smell when human skin contacts metal objects, it at the same time exhibits the smell of blood and is actually its odor component. This presumably reflects the situation that seeking or avoiding blood smell had a close relationship with survival in the evolutionary struggle, leaving 1-octen-3-one a potent odorant for humans even after such ability has remained dormant for a long time.

In this chapter, chemistry related to odor in terms of aroma use, in other words, flavors and fragrances (F&F), is focused on as human–environment interactions. The following subsections deal with analytical methods, extraction of various aroma products, perfumery use, and flavor chemistry. In conclusion, the olfactory system is explained from the aspect of human interaction.

Considering the vast history of perfuming and the inevitable destiny to feed ourselves, written history of aroma naturally dates back to the beginning of human civilization (**Table 1**).[2,3] Along with the industrial revolution, the F&F use had also gained a greater leap powered by the emerging knowledge of chemistry, establishing the F&F industry.

4.15.2 Outline of the Flavor and Fragrance Industry

Taking advantage of the major role of odor perception in our daily lives, the F&F industry is now fully involved in this phenomenon. In other words, the use of F&F industry as an axis and a viewpoint of this science will facilitate the process of grasping the outline. In the following parts, the history and function of a typical company and the application of its products are described.

Table 1 Aroma usage before the industrial revolution period

Period/year	*Topics*
c. 35 C BC	Apparatus presumably used for distillation in Mesopotamia
25 C BC	Myrrh obtained by Pharaoh Sahure from Punt (oldest written record of aroma material trade)
12–10 C BC	*Ayurveda* compiled in India
1 C AD	*The divine farmer's herb-root classic* compiled in China
c. 12 C AD	Enrichment of ethyl alcohol content by distillation
c. 12 C AD	Essential oil production by steam distillation
1370	Hungary water (first alcoholic perfume)
15 C AD	Koh-doh established in Japan
1533	Marriage of Henri II with Catherine de' Medici (introduction of fragrance production in Grasse, France)

4.15.2.1 Brief History of Flavor and Fragrance Industry

The dawn of this field recognizable as industry can be traced back to the sixteenth century when commercial production of essential oil was initiated in Italy.[2,3] This attainment was then brought into France as a result of the marriage of Catherine de Médicis and Henri II, leading to the establishment of many fragrance companies in Grasse. The rise of organic chemistry in the nineteenth century led the industry to integrate the scientific aspects. As a result of the progress in natural product chemistry, isolation, characterization, structure elucidation, and synthesis of aroma-active substances were made possible. The strategy established was effectively practiced by engagement with synthetic organic chemistry, making production more affordable than ever before (**Table 2**). Nowadays, many important aroma materials are economically and stably supplied with the help of chemistry, making synthetic materials a reliable and indispensable player of the industry.

4.15.2.2 Outline of a Typical Flavor and Fragrance Company Operation

As is clear from the name, F&F company operation[4,5] can be categorized into two fields. Flavor is used toward any product that has interaction with the oral system and, besides conventional foodstuffs, includes applications like chewing gum, toothpaste, tobacco, and so on.[6,7] On the other hand, anything employed toward products that are appreciated through direct nasal interaction is considered as fragrance, and its field includes products like perfume, shampoo, detergent, deodorizers, and so forth.[8–10] Products of both fields consist of numerous materials from natural and/or synthetic sources combined together.

Flavorists and perfumers, who are experts in the respective art, design this combination. Generally, at least 5–10 years of training is a prerequisite to attain sufficient ability. Analytical data and supply of synthetic compounds as well as processed natural material will aid these specialists. Thus, a modern F&F product is an interesting collaborative work of creative artists supported by technology pursued by scientists and engineers. These products are in many cases further processed to accommodate the customer's preferences. Such processing may include dilution in various solvents, replacement of solvent media, balancing specific gravity, emulsion/powder preparation,[6,11,12] microencapsulation,[13] precursor synthesis,[14] controlled-release designing, and so forth.

4.15.2.2.1 Materials used in flavor and fragrance production

Ingredients used in the industry typically fall into three categories: natural aroma material, synthetic compounds, and isolated aroma chemicals.

Table 2 Major events influencing the flavor and fragrance industry

Year	*Event/topics*	*Discoverer/inventor*
1709	Launch of the first eau de cologne	J. M. Farina
1818–19	Isolation of benzaldehyde from bitter almond	Vogel, Martres
c. 1830	Major advancement in elemental analysis	J. von Liebig
1851	Appearance of artificial flavoring in World Expo (London)	W. J. Bush & Co.
1868	First synthesis of coumarin	W. Perkin
1887	Proposal of isoprene rule in terpene chemistry	O. Wallach
1888	Nitro musk (first artificial fragrance compound)	A. Baur
1900	Presentation of solvent extracts in World Expo (Paris)	Antoine de Chiris, Roure Bertrand Fils
1926	Structure of muscone and civetone determined	L. Ruzicka
1928	Concept of aromatherapy proposed	R. M. Gattefosse
1934	X-ray structure analysis by Patterson function	A. L. Patterson
1941	Theory of gas chromatography (GC)	A. J. P. Martin, R. L. M. Synge
1946	Observation of nuclear magnetic resonance (NMR) of paraffin	E. M. Purcell
1959	First demonstration of GC–MS	R. S. Gohlke
1964	GC-olfactometry (GC–O) brought into practice	G. H. Fuller *et al.*
1983	Asymmetrical synthesis of (−)-menthol and development of industrial production thereof	R. Noyori *et al.*, Takasago International Corp.
1991	Molecular genetic research of the olfactory receptor	R. Axel, L. Buck

In the dawn of F&F industry, ingredients were of natural origin. Natural extracts are prepared mainly by steam distillation, cold press method, and organic solvent extraction.[15–17] Supercritical CO_2 extraction (**Figure 1**) is widening its application in recent years.[18] A free-flowing oil obtained by steam distillation or cold press method is referred to as an essential oil while others are generally called extracts. Residual waxy mass left after evaporation of extraction solvents is called concrete and resinoid (oleoresin), the former from flowers and the latter from other sources.[15] Concrete and resinoid can be reextracted with ethanol to remove this waxy mass and concentrated to furnish absolute.[15] Historically, before industrial production of petrochemical solvents, deodorized animal fat and alcohol were used to extract raw materials, mainly flowers. The alcohol extract thus acquired is referred to as tincture.[15] Although vanilla tincture is still popular, massive production of tinctures has almost ceased nowadays. Animal fat extraction is accomplished by immersing raw materials into hot melted fat (maceration).[15] Alternatively, the scent of raw material is transferred simply by laying it upon fat at room temperature (enfleurage; **Figure 1**).[15] Aromatic fat thus obtained is called pomade and can be further processed in the same manner as concrete to furnish absolute. Maceration and enfleurage are labor-intensive and therefore nearly obsolete. These essential oils and extracts are discussed in Section 4.15.4. In flavorings, seasoning oil is another basic material used especially in savory flavorings. Seasoning oil is usually prepared by heating raw materials (e.g., onion, leek, sugar, amino acids, etc.) in edible oil, thereby scenting the oil with the odor of the cooked material.[19] Maillard reaction and degradation of sugars and amino acids are commonly the vital parts in the aroma formation of this sort.[20,21] Concentrated fruit juice production gives rise to another material called recovered aroma, which is stripped off during raw juice concentration.[22] Smoke flavor (liquid smoke) obtained by pyrolysis of botanical mass is additionally important.[23] Various other processed food materials are incorporated and increasingly recognized as flavoring ingredients (for instance, extracts from meat, seafood, fermented food – especially dairy products, enzymatically processed foodstuff, etc.). Spinning cone column[24] (SCC) and membrane filter concentration[25,26] find application because of their useful concentration characteristics. Seasoning oil, recovered juice aroma, smoke flavor, and other food-oriented flavoring materials are essential ingredients nowadays, but are usually not regarded as conventional aroma materials. Products are quality controlled by various analytical methods and finally certified by flavorists and perfumers through sensory evaluation.

Aroma-active molecules of natural origin are mainly formed via well-known biosynthetic pathways.[17,27–29] The major class is the terpenoids followed by phenylpropanoid compounds (see Chapters 1.15, 1.16, and 1.24). Enzymatic and biosynthetic transformation and cleavage of fatty acid is another important source of aroma-active compounds (see Chapter 8.07). Transformation of amino acids and carbohydrates by fermentation is also

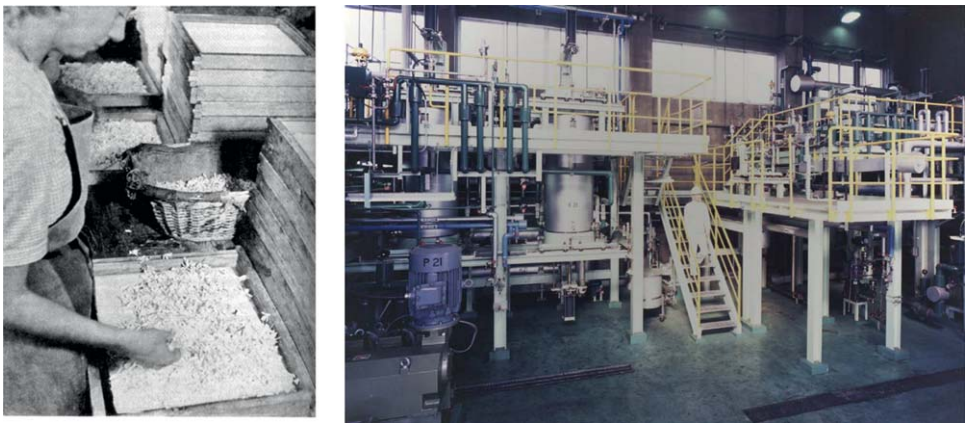


Figure 1 Coexistence of the past and present techniques in F&F industry: classical enfleurage process (photo on the left) and a supercritical carbon dioxide extraction facility as modern factory equipment (on the right). The photo on the left shows a stock of jasmine flowers in the basket (center) that are spread upon a wooden frame (chassis) that secures a glass plate coated with fat. The chassis is then piled to allow diffusion of fragrant components (note that the fat is applied on both sides of the glass plate to gain access to the headspace volume made by the chassis underneath). Enfleurage process photo reproduced from E. Guenther, *The Essential Oils*; with permission from Krieger Publishing Company: Melbourne, FL, USA, 1948 (reprinted 2006); Vol. 1, p 192.

well known[21,30] (see Chapters 3.22 and 3.26). Some of these molecules are stored as glucoside precursors and are released through various cleavage pathways (see Chapter 8.11). There are still many other molecules the formation pathway of which is not understood fully.

Synthetic compounds are advantageous in terms of price range, production capacity, and stable quality. They are further classified into two categories: nature identical and artificial.[31] Nature-identical compounds are substances found in nature that are prepared by means of synthetic chemistry. Artificial compounds are substances not found in nature but offer interesting aromatic profiles. They are not necessarily confined to the expectation that they should resemble a certain aroma of natural source. They may or may not have a similar chemical structure when compared with aroma compounds in nature. Artificial compounds are used extensively in fragrances, while nature-identical compounds are generally preferred in flavorings, mainly due to the consumer's demand for food additives of more of a so-called natural character.[32] Both nature-identical and artificial compounds are marketed as chemicals and are called aroma chemicals when emphasis is put on their odor properties. These synthetic chemicals are prepared by standard organic chemistry, and this will not be discussed in this chapter.

Isolated aroma chemicals are aroma-active substances isolated from natural sources mainly by means of crystallization, distillation, and adduct formation/decomposition. Although synthetic materials are in many cases convenient to use, isolated aroma chemicals continue to be advantageous, especially when chirality is the issue. Even if chirality is not a problem, in some cases (e.g., 1,8-cineole (**1**), eugenol (**2**), and limonene (**3**)), isolated natural chemicals serve better than their synthetic counterpart (**Table 3**).[33] Isolated aroma chemicals can be useful as such for the industry, and they are also utilized as starting materials for further synthetic manipulations.[34,35]

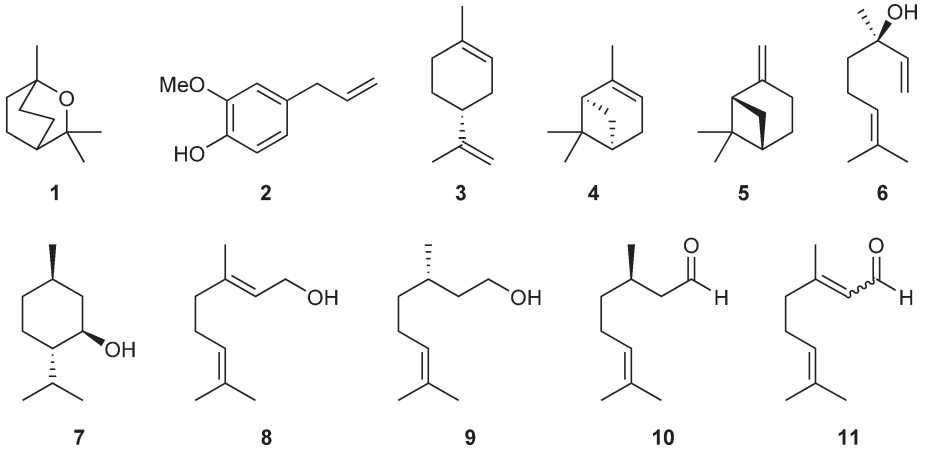
4.15.2.2.2 Aroma creation and odor perception characteristics

In F&F creation,[36–39] balance between ingredients of different odor characteristics – taking into account volatility, tenacity, and intensity – is of great significance. Especially in fragrance design, a smooth and harmonious transfer of scent as ingredients eventually evaporate is deemed a fundamental requirement (see Section 4.15.4). Synergic effect – either positive or negative – also has to be taken into consideration.

Table 3 Example of isolated aroma chemicals

Substance	*Source (Enantiomer)*	*Substance*	*Source (Enantiomer)*
Limonene (**3**)	Sweet orange (*R*)	Citronellol (**9**)	Geranium (*S*)
α/β-Pinene (**4**, **5**)	Pine wood ((+)-α[a], (−)-β[a])	Citronellal (**10**)	*Eucalyptus* (*E. citriodora*, ±), citronella (*R*)
Linalool (**6**)	Ho (*R*), rosewood (±)	1,8-Cineole (**1**)	*Eucalyptus* (*E. globulus*)
Menthol (**7**)	Japanese mint (−)	Citral (**11**)	Lemongrass, *Litsea cubeba*
Geraniol (**8**)	Palmarosa	Eugenol (**2**)	Clove

[a] The major enantiomer depends on the species.



The relationship between concentration and odor quality is yet another interesting argument. A good example is the odor quality of indole.[40] Indole at high concentration and in certain combinations with other malodorous compounds has an unpleasant fecal smell and is actually one of the compounds found in excrement. At the same time, indole is present in many flower extracts (in relatively small amounts) and when blended at lower concentration with other flowery aroma, it exhibits a dramatic lifting effect in odor quality, making it an invaluable ingredient in fragrance creation. Sotolone (**12**) is another example of such compounds.[41] It smells rather offensive with fenugreek-like smell accompanying a character of hydrolyzed vegetable protein (HVP) in high concentration, whereas in low concentration it offers a typical pleasant smell of burnt sugar. Depending on concentration and circumstances, aroma-active compounds can be either off-flavor or key aroma compounds: thiols like 3-methyl-2-butene-1-thiol (an important ingredient of coffee flavorings but an off-flavor component in beer[42]) and 1-alken-3-one like 1-nonen-3-one (found in yogurt[43]) are such examples. Many F&F ingredients have these characteristics, which deserves attention in product design.

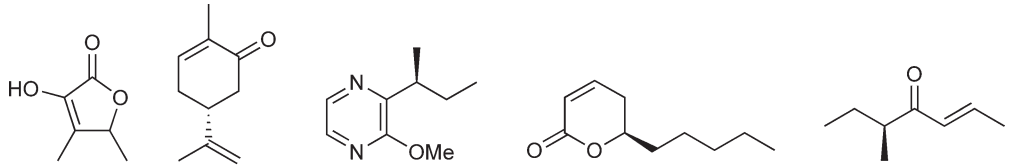
Enantiomeric property is another important issue, since some enantiomer sets differ in intensity and odor quality (**Figure 2**).[44,45] For example, (*R*)-carvone (**13**) (a predominant component of spearmint oil) has a spearmint-like smell with an odor threshold being 1/14 of its antipode while (*S*)-carvone (found in dill and caraway oil) is known to have a caraway-like scent.[44] In the case of ethyl 2-methylbutanoate, the (*S*)-isomer found in nature shows a threshold of 1/150 against its antipode, a stranger in natural product chemistry.[44] It is worth noting, however, that the presence in nature is not necessarily a prerequisite to be superior in terms of threshold level. Considering 2-(1-methylpropyl)-3-methoxypyrazine (**14**) as an example, the (*S*)-isomer found abundant in nature shows 10 times higher threshold compared with its counterpart, which is not yet found in nature as a dominant enantiomer.[44] There are other examples like 2-decen-5-olide[46] (**15**) and sotolone[47] (**12**), in which both odor quality and threshold show little difference between the enantiomers. Furthermore, many cases are known in which the enantiomeric purity of a certain compound in natural source is moderate in the first place. As an example, filbertone (**16**) in hazelnut differs between enantiomers in terms of both odor quality and threshold (dominant (*S*)-isomer showing 1/10 threshold of its antipode), but the enantiomeric excess (e.e.) found in hazelnut is only around 65% e.e.[48]

4.15.2.2.3 *Financial aspects of flavor and fragrance industry*

From the financial point of view, the size of the F&F market is relatively small with global size being around 18 billion US dollars (year 2006).[49] The proportion thereof is as follows: fragrance 36%, flavor 34%, cosmetic ingredients 16%, and aroma chemicals 14%. The top five players are Givaudan, International Flavors & Fragrances (IFF), Firmenich, Symrise, and Takasago, which together claim around 60% of the market. Thus, the market is generally considered fragmented.

4.15.2.3 Application of Flavor and Fragrance Products

F&F products find extremely wide diversity in terms of their application. Flavorings are used is beverages, confectionary (candy, cookie, cake, etc.), chocolate, ice cream, sherbet, snacks, soup, frozen food, chewing gum, toothpaste, and tobacco to name just a few.[7] Other food materials such as sauces, dressings, seasoning powder, and oil used in fast-food production, restaurants, and bars may also be flavored. The primary objective of flavorings is to supplement, add, or strengthen the aroma of foodstuff, but this can be extended to keeping the



12 **13** **14** **15** **16**

Figure 2 Representative examples of chiral odor-active molecules exhibiting interesting odor profiles influenced by enantiomeric purity.

aroma quality consistent in industrial manufacturing. Recent trends in appreciation of diets low in calories, fat, and salt demand flavorings to compensate the less appealing nature of these foods. Masking (disguising) an unpleasant flavor (for instance, in barium meals in X-ray diagnosis and syrup-type medication for children) is another function. Off-flavor[50–53] formed as a result of food processing may also be improved in the same manner. Animal feed can be yet another example of flavoring application.[54,55] Turning to fragrances, they find application in perfume, cosmetics, shampoo, soap, detergent, fabric softener, deodorant, and many other household chemicals.[8–10] In peculiar applications, they find use in scratch and sniff stickers, toys, scented erasers, and so on. Unlike flavorings, masking of unpleasant odors in, for instance, shampoo compounds, body odor, bleach, and rubber products (latex gloves, dental dam, condom, etc.) is the primary function of fragrances. Actually, the use of fragrance in tannery is one of the most classical applications. Industrial fragrance use includes stench gas odorizers (e.g., 2-methylpropane-2-thiol, tetrahydrothiophene) added to fuel gas for swift detection of leakage.[56]

Functions further sophisticating F&F products are the controlled release of aroma compounds by employing encapsulation technology[13] and precursors.[14] In recent applications, these technologies are combined together with the function of sensual stimulating agents[57] conveying hot,[58] cold,[59,60] tingling, stimulating, and various other sensations along with flavor or fragrance acceptance. Extension of additional functionality and safety is expected to continue as consumer demands become more and more intense, diverse, and complicated.

4.15.2.4 Safety and Regulations

Safety is an important issue in F&F products.[3,61–63] Considering their immense presence in daily life and diverse biological functionality, F&F products are subject to potential health hazards. Therefore, appropriate measures are implemented in many countries, although differing in details. Typically, flavorings are classified as natural, nature identical (molecules found in nature that are prepared synthetically), and artificial, and evaluated and regulated accordingly. The most well-known regulation of this sort is the generally recognized as safe (GRAS) designation by the Food and Drug Administration (FDA). Organizations such as International Organization of the Flavor Industry (IOFI) and UN committees like Codex Alimentarius (CODEX) and Joint Expert Committee on Food Additives (JECFA) cooperatively maintain, update, and harmonize internationally these regulations. In the fragrance field, similar organizations like International Fragrance Association (IFRA) and the Research Institute for Fragrance Materials (RIFM) are in operation to secure safety. Flavoring regulation may also arise from religious aspects. Certified standards such as Halal in Islam and Kashrut in Judaism are effective toward certain classes of F&F products.

4.15.3 Analytical Techniques

The content of aroma compounds is, in general, low, and compositions of these compounds are often complex. Therefore, at the dawn of analytical chemistry, aroma compounds were extracted from a huge mass of raw material. Fractionation was carried out by means of distillation, and various other classical procedures (e.g., crystallization, pH control in extraction, derivatization) were employed. Quite obviously, compounds revealed using these procedures were inevitably restricted to a set of major constituents, if any. Occasionally, before the 1950s, additional techniques like UV–IR spectroscopy and open-column chromatography were employed and were helpful to some extent.

4.15.3.1 Common Analytical Methods

In general, any analytical equipment or procedure used in the field of natural products chemistry and environmental engineering is also helpful in aroma analysis.[64,65] The history and principles of such art are described in detail elsewhere and will not be featured here. Gas chromatography (GC), GC–mass spectrometry (MS), and nuclear magnetic resonance (NMR) are the most frequently used techniques along with rather specialized setups such as proton transfer reaction–mass spectrometry[66] (PTR–MS) used in retronasal aroma analysis (see Chapters 9.02, 9.06, 9.10–9.11).

In modern art, analytical techniques are of increased importance in flavor design. Sense of flavor is in general conservative and the use of artificial substances in this field is far less encouraged compared with fragrances. Consequently, reproduction of natural aroma is favored, and therefore analysis of foodstuff is the dominating force in innovation. However, naturally, techniques used in flavor analysis are applicable to fragrance analysis as well.

4.15.3.2 Analyzing Methods in Flavor and Fragrance Art

Since the F&F industry incorporates a sensation of odor, some analytical methods used in this field are more or less specialized to handle this field of subjective and enigmatic sense. Representative analytical techniques considered important in this field are described in the following sections.

4.15.3.2.1 Gas chromatography–olfactometry

In aroma analysis, odor profile of an unknown compound is also helpful in its determination. Therefore, a human nose as an organoleptic sensor is employed in the detection of GC effluents. This technique is referred to as GC–olfactometry (GC–O) or GC-sniffing.[67–69] The procedure can be applied to discover key aroma components that actually reflect and constitute the aroma of a target material. It is by nature a qualitative analysis, but may also be quasi-quantitative by incorporating supplemental measures (see Section 4.15.3.2.2). An interesting extension of GC–O called OASIS method (original aroma simultaneous input to the sniffing port method) is known in which GC effluents are combined with a flow of other odorous compounds.[70] This can realize detection of trace compounds (not necessarily an impact aromatic substance by itself) that exhibit remarkable synergic/interactive effects with other components.

4.15.3.2.2 AEDA, CHARM, and odor unit determination

These methods were developed to quantify and visualize the intensity of aroma as a chromatogram. A specific system named combined hedonic and response measurement (CHARM) was initially developed. Later on, aroma extract dilution analysis (AEDA) (**Figure 3**), a new method using a conventional GC–O system, was proposed. They share the same strategy; aroma extract is diluted to a certain extent and then GC–O methodology is applied. In an AEDA procedure, if such a maximum extent of a dilution that allows the detection of a certain component is n times diluted from the original sample, this component is referred to have a flavor dilution (FD) factor of n. CHARM value corresponds to FD factor in a CHARM procedure. These values represent the contribution of the volatile; the larger these values are, the more important they are considered as key components.

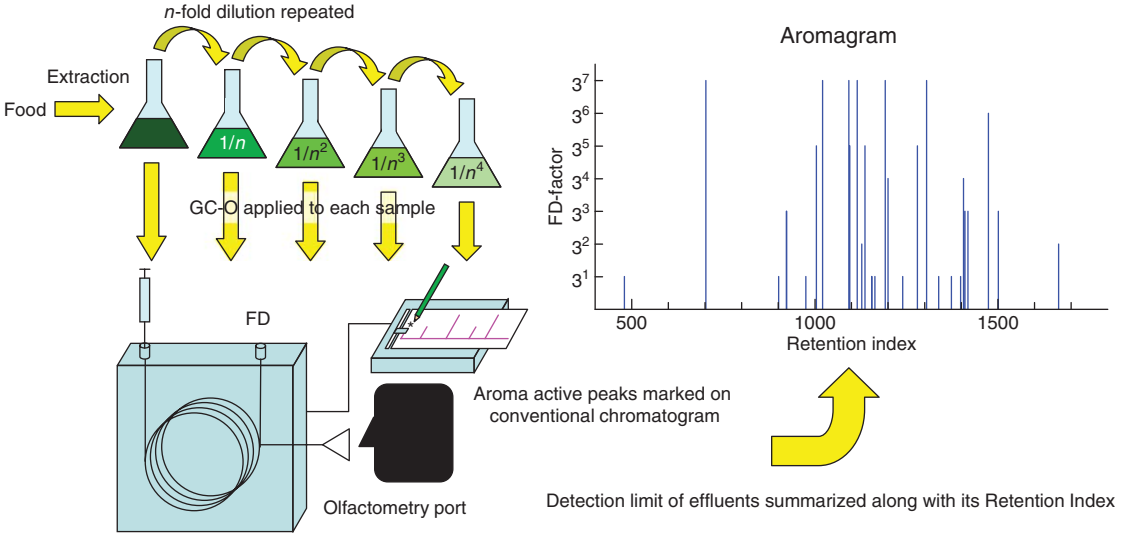


Figure 3 Aroma extract dilution analysis: concept scheme.

The intensity of aroma compounds found by AEDA and CHARM may be determined with further accuracy by subjecting them to odor unit (also called odor activity value) measurement.[71,72] This is done by first measuring the odor threshold of a compound while the concentration of this compound in the specimen is determined using internal standards (for instance, isotopic samples in contemporary approach). Dividing the latter concentration with the odor threshold will give the odor unit value, naturally being higher when a compound better contributes to the total aroma.

4.15.3.2.3 Multidimensional GC (MDGC, GC × GC)

Aroma composition is usually very complex and thus on many occasions coelution of components may take place. Utilizing GC columns with different separation characteristics in a tandem manner may allow resolution of such peaks. In a typical setup, a certain portion of an effluent from the first column is concentrated using a cold trap (named cryofocus) and then sent into the second column for further separation. This analyzing technique combining multiple GC columns is referred to as multidimensional GC (MDGC).[73,74] Cryofocus repeated in short intervals in combination with a short second column will furnish a whole two-dimensional chromatogram useful in complex aroma analysis (comprehensive GC × GC).

4.15.3.2.4 Chiral stationary phase application

Aroma compounds originate from biosynthetic pathways inside an animal, a botanical body, and other life-forms as well as enzymes and thus frequently carry chiral components within the molecule. Determination of such enantiomeric properties can, in many cases, be accomplished using a GC column with a chiral stationary phase (CSP) application.[75–79] These columns, usually called chiral GC column, will provide diastereometric interaction that could lead to resolution of enantiomers. Commercially available chiral GC columns predominantly utilize cyclodextrin derivatives as CSPs. Chiral columns consisting of multiple cyclodextrin derivatives intending synergic effect in resolution property[80] are also successful in the market. In practice, these columns are mainly operated as secondary columns in MDGC technique.

4.15.3.2.5 Quantification and stable isotope dilution assay

Quantification of aroma compounds using GC and internal reference has long been a problematic issue.[81] Detection response factor, peak shape, discrimination phenomenon at the injector port, and, of course, disproportion during sample preparation were more or less unavoidable. Stable isotopes used as internal standards combined with an MS detector have realized reproducible and far more accurate quantification. The major drawback of this method is the tedious process of preparing the isotope-labeled standards.

4.15.3.3 Sample Preparation Method

Aroma analysis is most often performed utilizing GC–MS. This demands separation of volatile constituents from nonvolatile matrices. Additionally, higher concentrations of analytes are favorable to allow detection of trace key compounds.[16,64,65,82] Therefore, various preparation methods derived from aroma extract production were developed. The composition of the concentrate may differ depending on the method used and thus selected to accommodate the aim.

Direct extraction and subsequent concentration of the extract (extraction method) along with direct distillation and steam distillation are the most classical procedures. Techniques like solvent distillation extraction (SDE),[83] solvent-assisted flavor extraction (SAFE),[82] vacuum headspace sampling (VHS),[84,85] direct thermal desorption (DTD),[86] and lyophilization[16] fall into this distillation category. Adsorption method can be tracked back to the enfleurage procedure. Nowadays, polydimethylsiloxane (PDMS) and poly-2,6-diphenyl-*p*-phenylene oxide (Tenax) are used extensively as adsorbents. Adsorption method is applied mostly toward analysis of the headspace gas, and two modifications exist.[84] In a static headspace procedure, the specimen is left standing still in a sealed chamber and the headspace is sampled under equilibrium. On the other hand, in a dynamic procedure, headspace gas is constantly purged to enhance vaporization and then put through the adsorbent. Solid-phase microextraction (SPME)[87] and its advanced form, solid-phase aroma concentrate extraction (SPACE),[88] are classified as static methods. Aquaspace is a modified form of dynamic headspace procedure, which makes use of water-saturated air as dynamic gas media.[89] Adsorption performed in aqueous

media is in practice as well. Stirrer bar sorptive extraction (SBSE) is one such procedure, which employs a magnetic stirring bar coated with adsorbents allowing concentration of aroma compounds as it stirs the aqueous media.[82] In any case, aroma-active compounds concentrated using the above adsorptive methods are released using solvents or by subjecting them to thermal desorption. Cryofocus apparatus also finds application by concentration of trace materials that are otherwise difficult to be characterized by a conventional GC–MS setup.

4.15.3.4 An Example of Aroma Analysis – Spotted Shrimp

Determination of aroma-active substances is carried out by a combination of aroma concentrate preparation and analyzing methods described above.[90] As a specific example, analysis of roasted spotted shrimp (*Sergia lucens*) – consumed as an ingredient of Kakiage (a sort of Tempura), Sushi, and rice snacks – will be shown here (**Figure 4**). Sun-dried spotted shrimp was heated to 160 °C in an electric cooking plate and the resulting aroma compounds were drawn into a Tenax TA adsorbent using an aspiration pump. The adsorbed aroma constituents were stripped off by repeated extraction using diethyl ether. Careful evaporation of diethyl ether furnished the aroma concentrate, which was further analyzed by GC, GC–MS, and AEDA procedures. AEDA method has revealed 31 aroma constituents with an FD factor of 3 or more. Among these compounds, six potent aroma constituents exhibiting an FD factor of 3^7 (2187), namely methanethiol (**17**), 1-pyrroline (**18**), *N*-(2-methylbutyl)pyrrolidine (**19**), *N*-(3-methylbutyl)pyrrolidine (**20**), isopropyl methyl disulfide (**21**), and 3-methylpyrazine (**22**), were found.

4.15.3.5 Quality Control

Materials of natural origin are prone to adulteration, especially when production costs are high and when quality and supply are unstable. In natural extracts, detection of adulteration can be accomplished by profiling

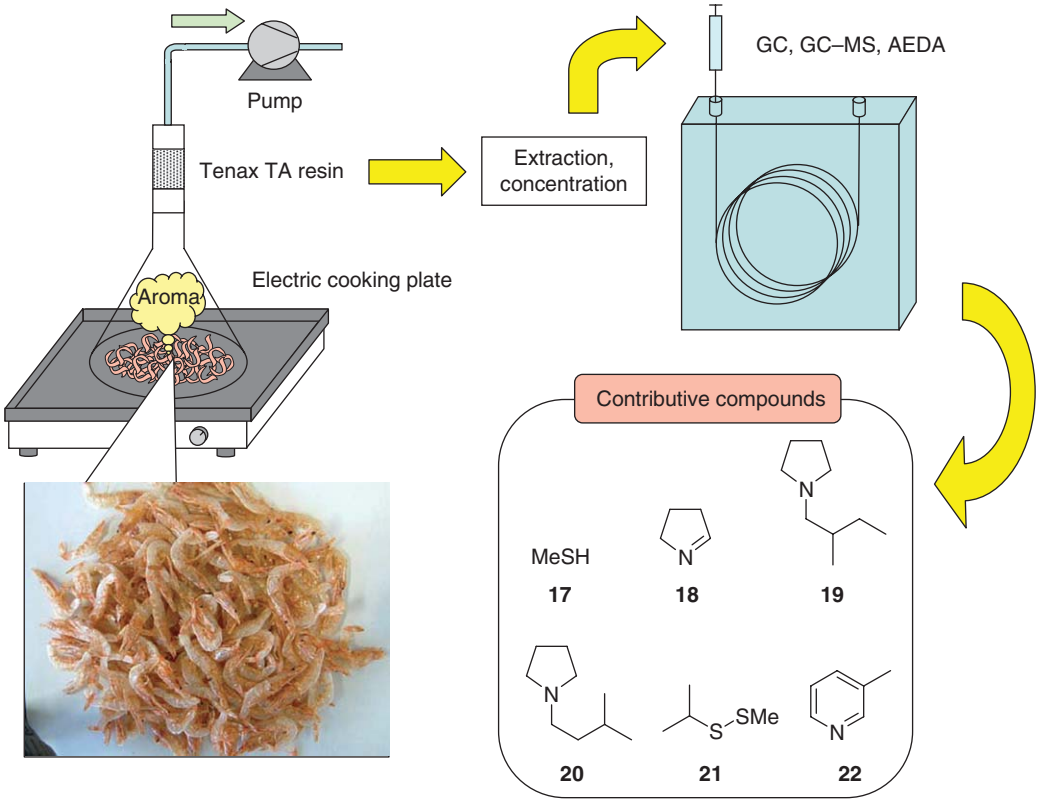


Figure 4 Aroma analysis of roasted spotted shrimp.

the proportion of complex substances by principal component analysis combined with GC, GC × GC, and LC–GC. Since many substances of natural origin are chiral and in many cases are enriched with a specific enantiomer, enantiomeric excess determination on chiral columns may also provide indication.[75] However, effectiveness of chiral analysis in authentication is under discussion in some cases.[91,92] A complementary way is isotopic ratio analysis taking the advantage of these ratios being different among location, species, elevation, and source material (either natural or petrochemical origin).[75] Genetically modified organism (GMO) in the food sector is also an issue.[93,94] The above-mentioned methods as well as biological measures can be applied in this case.

4.15.4 Flavor and Fragrance of Natural Origin

Although limited in quantity, natural extracts still enjoy an irreplaceable status in smoothing and integrating the aroma of products.[33,95,96] Raw materials used for F&F can be categorized depending on the part of the plant used. In the following subsections, several representative raw materials from flower, leaves, root, grass, seed, wood, resin, the miscellaneous botanical mass as well as those from animal sources are described.

4.15.4.1 Aroma Products from Flowers

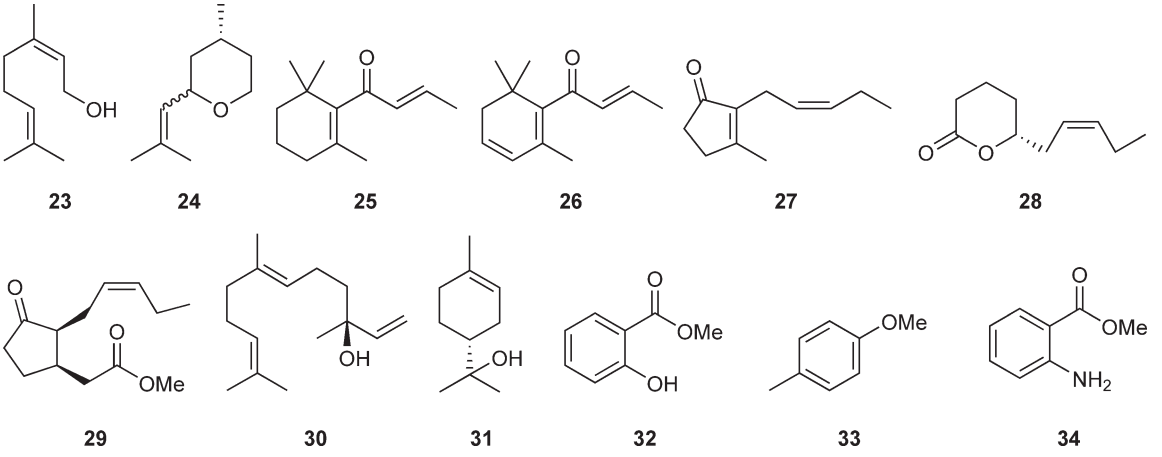
Flower scent is considered as the most fundamental and classical component in fragrance design and still deserves a first place in description (**Table 4**).[97] The rose has many cultivars of which two species are important, offering its invaluable function even in minute amounts. Jasmine[98] is cultivated in Egypt, Turkey, and India. Jasmine used to flavor Chinese tea is a different type of jasmine (*Jasminum sambac*).[99] Neroli oil is a steam distillate of freshly picked bitter orange flowers, and it offers a peculiar sweet-terpeny note. Ylang-ylang, meaning 'the flower of flowers,' is produced in Madagascar and Reunion. Narcissus absolute is known as one of the truly rare extracts with annual production being less than 200 lb (91 kg).

4.15.4.2 Aroma Products from Leaves and Allied Plant Materials

Essential oils from plant leaves are main ingredients in many products.[100] Geranium oil serves as a source of (*S*)-citronellol (**9**), obtained by saponification and subsequent fractional distillation. Eucalyptus is classified into two groups – the globulus type and citriodora type – which differ considerably in terms of their composition. Peppermint is cultivated mainly in the United States and also in China and India. Another species usually referred to as Japanese mint is grown in China, Brazil, India, and Japan and is used for *l*-menthol (**7**) isolation. Spearmint oil is a good modifier of peppermint and two species (Native and Scotch) are recognized as important. The main producer is the United States while India and China have also emerged in recent years. Citronella also has two separate species in cultivation (Sri Lanka and Java type), of which Java type is particularly important as an industrial source of (*R*)-citronellal (**10**). Likewise, lemongrass continues to be important as a citral (**11**) source, but to a less degree due to the advent of synthetic citral. Patchouli is cultivated in Sumatra, Seychelles, Madagascar, etc., and it is used in many men's fragrances. Lavender is grown in southern France while Lavandin, a hybrid developed by crossing *Lavandula officinalis* with spike or aspic lavender (*Lavandula latifolia*), is cultivated in France and northern Africa. Lavandin was once regarded as a less expensive substitute of lavender oil but now has attained a firm position in its own right. It is inexpensive and reliable with consistent quality. Basil is one of the representative cooking herbs (especially in Italy and Thailand) and also an important F&F product source. Thyme is well known for its antibacterial and antifungal properties. Three species (two from Europe and one from Mexico) are known as oregano even though each of them belongs to a different genus, naturally exhibiting discrete odor profiles. Rosemary is cultivated in Spain, France, and Croatia, and besides being a cooking herb it also has a long history as a deodorant and pesticide, and nowadays its extract is used as an antioxidant agent in processed food (**Table 5**).

Table 4 Aroma extract from flowers

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Rose (Morocco)	*Rosa centiofolia*	(*S*)-Citronellol, geraniol, nerol (**23**), 2-phenylethanol, (4*R*)-rose oxide (**24**), β-damascone (**25**), β-damascenone (**26**)	95,96
Rose (Bulgaria, Turkey)	*Rosa damascena*		
Jasmine	*Jasminum grandiflorum*	Benzyl acetate, (*S*)-linalool, indole, (*Z*)-jasmone (**27**), (*R,Z*)-7-decen-5-olide (**28**), methyl (+)-*epi*-jasmonate (**29**)	95,96,98
Neroli	*Citrus aurantium*	(*R*)-Linalool, (*R*)-linalyl acetate, (*S,E*)-nerolidol (**30**), (*R*)-α-terpineol (**31**)	95,96
Tuberose	*Polyanthes tuberosa*	Methyl benzoate, 1,8-cineole, methyl salicylate (**32**)	95
Ylang–ylang	*Cananga odorata*	Benzyl acetate, (*R*)-linalool, *p*-cresyl methyl ether (**33**), methyl benzoate	95
Narcissus	*Narcissus tazetta*	Linalool, methyl anthranilate (**34**), benzyl acetate, α-terpineol	95
	Narcissus poeticus		



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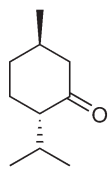
29 30 31 32 33 34

4.15.4.3 Aroma Products from Seeds

Many kinds of seeds are known to have fragrance properties.[100,101] Some are simply crushed and steam distilled, and others are dried, fermented, or cured prior to such processes. Among them, pepper was well recognized as one of the most precious trading goods. Black pepper[102] is prepared from whole unripe berries, while white pepper solely consists of pepper seeds. Piperine (**44**) is known to contribute to its hot taste. Vanilla[103,104] has two major sources (Madagascar/Reunion type and Tahiti type). In the case of vanilla, the pods that house the tiny seeds are important as an aroma source. Furthermore, raw pods are virtually odorless and need a process (called curing) that includes enzymatic cleavage of glucosides to give the key component vanillin along with other fairly complex aroma constituents. Coriander seed, when unripe, gives off an unpleasant smell mainly due to decanal. Therefore, ripe fruit is harvested and processed while the full part of the plant is occasionally used for herb oil production. Cumin is famous as a fundamental spice ingredient of curry. Anise is used for candy and baked confectionery. (*E*)-Anethole (**45**) is the dominant substance in its oil with content reaching 95%. Seeds of fennel and star anise are also known to have high (*E*)-anethole content. Fennel is used for fish dishes while star anise is used extensively in Chinese cuisine (**Table 6**).

Table 5 Aroma extract from leaves

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Geranium	*Pelargonium graveolens*	(*S*)-Citronellol, geraniol, (4*S*)-rose oxide	95,96
Eucalyptus	*Eucalyptus globulus*	1,8-Cineole	95
	Eucalyptus citriodora	(±)-Citronellal, (±)-citronellol	95
Peppermint	*Mentha piperita*	(−)-Menthol, (−)-menthone (**35**), (−)-menthyl acetate (**36**)	95,100
Japanese mint	*Mentha arvensis*	(−)-Menthol	95
Spearmint	*Mentha spicata*	(*R*)-Carvone	95,100
Citronella	*Cymbopogon winterianus*	(*R*)-Citronellal, geraniol, (*R*)-citronellol	95
Lemongrass	*Cymbopogon flexuosis* *Cymbopogon citratus*	Citral	95
Patchouli	*Pogostemon patchouli*	(−)-Patchoulol (**37**)	95,96
Lavender	*Lavandula officinalis*	(*R*)-Linalool, (*R*)-linalyl acetate, (*R*)-lavandulyl acetate (**38**)	95,96
Basil	*Ocimum basilicum*	(*R*)-Linalool, estragole (**39**)	95,100
Thyme	*Thymus vulgaris*	Thymol (**40**), carvacrol (**41**)	95,100
Oregano	*Origanum vulgare*	Carvacrol, thymol	95,100
	Coridothymus capitatus	Carvacrol, thymol	95,100
	Lippa graveolens	Thymol, carvacrol, 1,8-cineole	95,100
Rosemary	*Rosmarinus officinalis*	1,8-Cineole, (+)-camphor (**42**), (−)-borneol (**43**)	95,100



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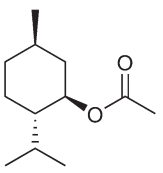

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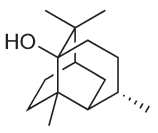

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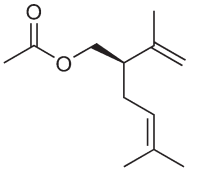

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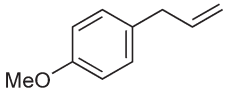

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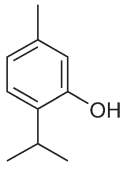

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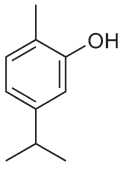

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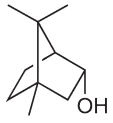

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4.15.4.4 Aroma Products from Roots

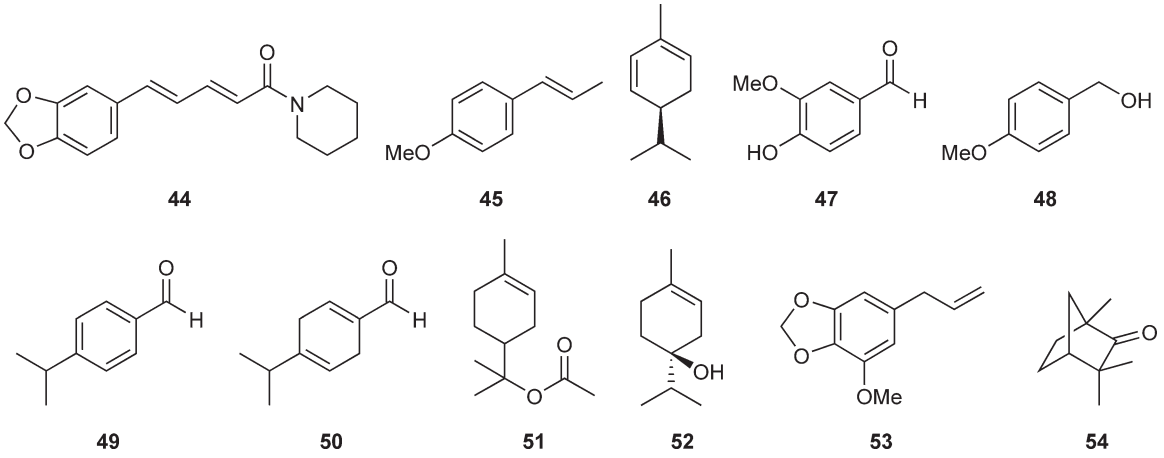
Vetiver is cultivated in southern India, Indonesia, and the Philippines.[101] While functioning as a fixative, at the same time it is a contributor in various perfumery applications. It is also recognized as a source of a synthetic material named vetiver acetate used in perfumes. Iris extract is obtained from rhizomes. The rhizome is peeled and dried under sunlight. Freshly harvested rhizomes bear a green note like peeled potato, but when further aged for 2–3 years they give rise to a characteristic scent of iris. Valerian[105] is used for personal care and as insect repellent (**Table 7**).

4.15.4.5 Essential Oils from Peel (Citrus Oil)

Citrus oil dominates this class of essential oil. It is obtained by the cold press method with the exception of lime oil, which is also prepared by steam distillation of essential oil separated during the production of juice.[106,107] Aside from bergamot, these oils are primarily monoterpene hydrocarbon mixtures of which (*R*)-limonene (**3**) is usually the dominant compound. Since odor contribution of this monoterpene compound is low, it is often removed by distillation or repeated solvent extraction. The resulting oil rich in odor-active compounds is called terpeneless oil and is used extensively. In the case of bergamot and lemon oils, psoralen derivates like bergaptene (**64**) causing photosensitivity are problematic, and those for fragrance use are rectified to remove it (**Table 8**).

Table 6 Aroma extract from seeds and surrounding sections

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Pepper	*Piper nigrum*	(−)-β-Pinene, (±)-limonene, (*S*)-α-phellandrene (**46**), (±)-linalool	95,101,102
Vanilla	*Vanillia planifolia*	Vanillin (**47**)	103,104
	Vanillia tahitensis	Vanillin, *p*-anisalcohol (**48**)	
Coriander	*Coriandrum sativum*	(*S*)-Linalool, (−)-camphor, geraniol	95,100
Cumin	*Cuminum cyminum*	Cuminaldehyde (**49**), *p*-mentha-1,4-dien-7-al (**50**)	95,100
Cardamom	*Elettaria cardamomum*	α-Terpinyl acetate (**51**), 1,8-cineole	95,101
Nutmeg	*Myristica fragrance*	(*S*)-Terpinen-4-ol (**52**), myristicin (**53**)	95,101
Anise	*Pimpinella anisum*	(*E*)-Anethole (**45**)	95,100
Fennel	*Foeniculum vulgare*	(*E*)-Anethole, (+)-fenchone (**54**)	95,100
Star anise	*Ilicium verum*	(*E*)-Anethole	95



44 **45** **46** **47** **48**

49 **50** **51** **52** **53** **54**

4.15.4.6 Aroma Materials from Wood

Sandalwood has been highly appreciated since prehistoric ages. The oil serves as base note, offers fixative properties, and harmonizes with various other materials, making it one of the most fundamental ingredients. The essential oil of cedarwood is steam distilled from the sawdust and other waste wood from the lumber mills while essential oil from its leaves is also produced. Camphor, native to Japan and Taiwan, was considered as a precious wood having high concentration of (1*R*)-camphor (**42**), which is used in medicine, although synthetic camphor introduced in the 1920s rapidly replaced the natural oil (**Table 9**).

4.15.4.7 Aroma Products from Resin

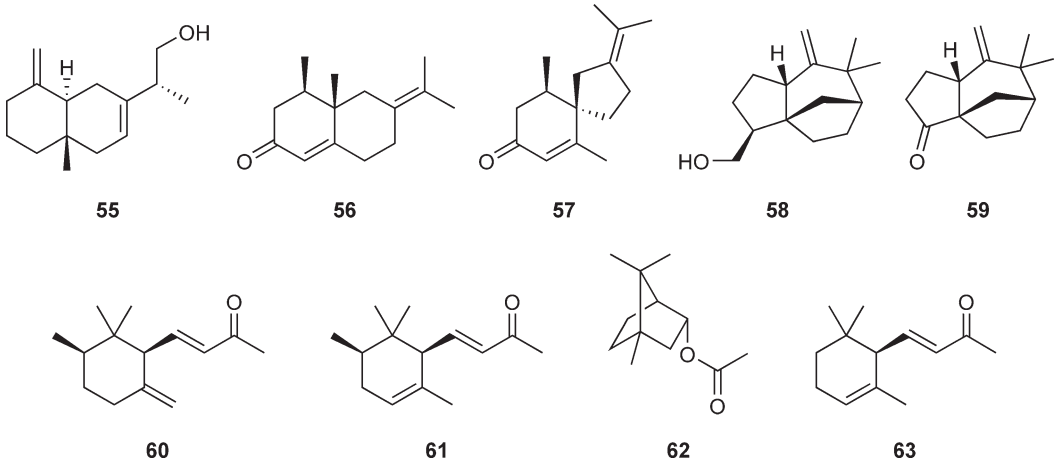
Peru balsam[108] has a typical balsamic odor. Galbanum, like labdanum and myrrh, is used primarily in oriental blends. Myrrh resinoid[109,110] refers to heerabol myrrh, while that with different amounts of α-bisabolene (**76**) is called opopanax[109] nowadays. The two raw materials have distinctly different odor characteristics. In perfumery, it serves as a fixative and an ingredient of oriental type. Olibanum[111] is the frankincense in the Bible and is used heavily in oriental-type perfumes (**Table 10**). In perfumery, these aroma materials from resin serve as a fixative and, in many cases, as ingredient of oriental type products.

4.15.4.8 Aroma Material from Other Botanical Sources

Oakmoss is a sort of lichen that grows on oak trees, and its extract has an important role in fragrance design.[101] Cinnamon oil is obtained from the bark of the cinnamon tree, although small-scale production of leaf oil is also known. Cassia oil is obtained from the bark, small branches, and leaves of the Cassia tree. Clove oil is prepared by steam distillation of clove buds, while clove leaf oil is made from leaves (**Table 11**).

Table 7 Aroma extract from roots

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Vetiver	*Vetiveria zizanoides*	(−)-Vetiselinenol (**55**), (+)-α-vetivone (**56**), (−)-β-vetivone (**57**), (+)-khusimol (**58**), (−)-khusimone (**59**)	96
Iris (orris)	*Iris pallida*	(+)-*cis*-γ-Irone (**60**), (+)-*cis*-α-irone (**61**)	96
Angelica root	*Angelica archangelica*	(−)-α-Pinene, 15-pentadecanolide	95
Valerian	*Valeriana officinalis*	(−)-Bornyl acetate (**62**), 3-methylbutanoic acid	95,105
Ginger	*Zingiber officinale*	Citral, geranyl acetate, geraniol	95,101
Costus	*Saussurea lappa*	Linalool, (*R*)-α-ionone (**63**)	95



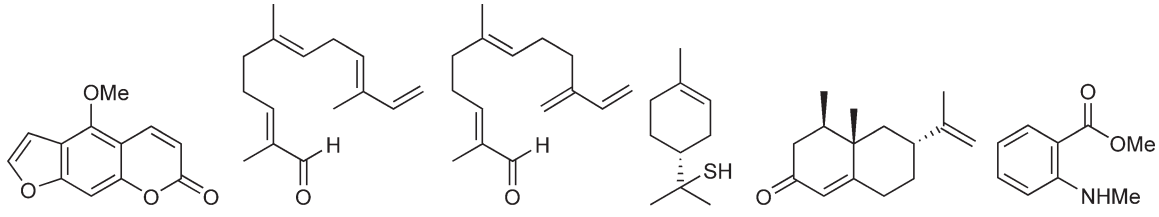
55 **56** **57** **58** **59**

60 **61** **62** **63**

Table 8 Aroma extract from peel (citrus oil)

Oil	*Source plant*	*Aroma-active constituents*	*References*
Sweet orange	*Citrus sinensis*	(*S*)-Linalool, octanal, decanal, α/β-sinensal (**65**, **66**)	95,96
Grapefruit	*Citrus paradisi*	(*R*)-1-*p*-Menthen-8-thiol (**67**), (+)-nootkatone (**68**)	95,96
Mandarin	*Citrus reticulata*	Methyl *N*-methylanthranilate (**69**)	95,96
Lemon	*Citrus limone*	Citral, (*R*)-linalool	95,96
Lime	*Citrus aurantifolia*	Citral, (±)-α-terpineol[a]	95,96
Bergamot	*Citrus bergamia*	(*R*)-Linalool, (*R*)-linalyl acetate	95,96

[a] In distilled oil.



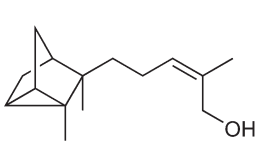
64 **65** **66** **67** **68** **69**

4.15.4.9 Aroma-Active Material from Animals

There are mainly four aroma products from animal sources.[112] In terms of extensive commercial use, all of them are substituted by synthetic materials. Musk[113] is a material obtained from an abdominal gland (called musk pods) of the male musk deer. Civet[113] extract is prepared from the perianal secretion of civet cat and exhibits a

Table 9 Aroma extract from wood

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Sandalwood	*Santalum album*	(+)-α-Santalol (**70**), (−)-β-Santalol (**71**)	95,96,111
Cedarwood	*Juniperus virginiana*	(+)-Cedrol (**72**), (+)-α-cedrene (**73**), (−)-thujopsene (**74**)	95,96
Camphor	*Cinnamomum camphora*	(+)-Camphor, safrole (**75**), 1,8-cineole	95
Rosewood (Bois de Rose)	*Aniba rosaeodora*	(±)-Linalool	95



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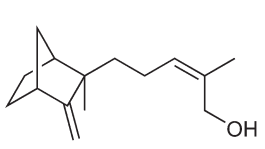

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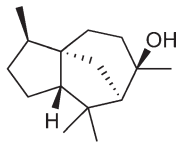

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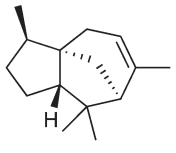

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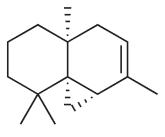

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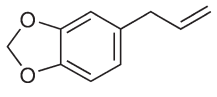

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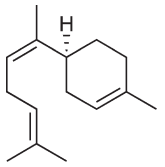
Table 10 Aroma extract from resin

Oil	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Styrax	*Liquidambar orientalis*	Cinnamyl alcohol, 3-phenyl-1-propanol, styrene	95
Peru balsam	*Myroxylon pereirae*	(*S*,*E*)-Nerolidol, methyl cinnamate	108
Galbanum	*Ferula gummosa*	(−)-β-Pinene, (3*E*,5*Z*)-1,3,5-undecatriene (**77**), 2-methoxy-3-isopropyl-5-methylpyrazine(**78**)	95,96
Myrrh	*Commiphora myrrha*	(+)-Furanoeudesma-1,3-diene (**79**), curzerene (**80**), lindestrene (**81**)	96,109,110
Opopanax	*Commiphora guidottii*	β-Ocimene (**82**), (*S*,*Z*)-α-bisabolene (**76**), α-santalene (**83**)	109
Olibanum	*Boswellia carterii*	1-Octyl acetate, 1-octanol	95,96,109,111



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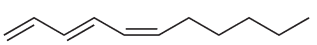

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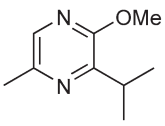

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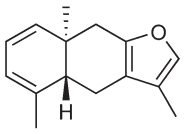

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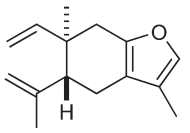

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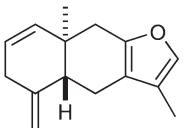

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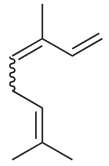

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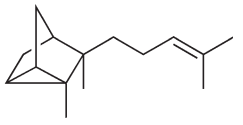

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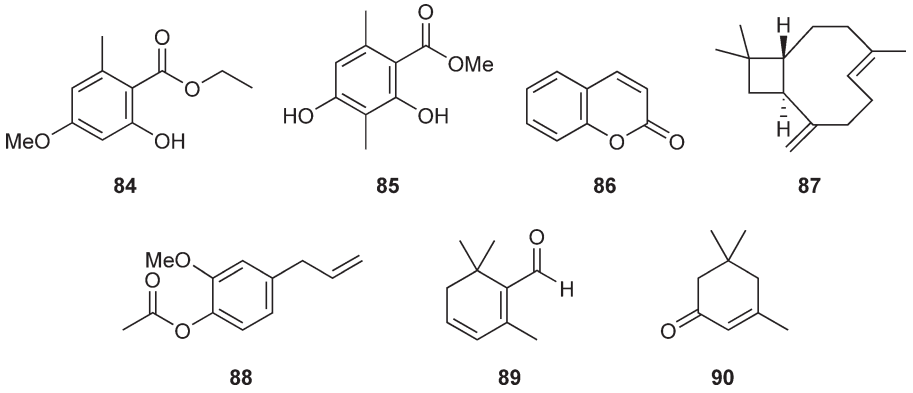
Table 11 Aroma extract from other botanical sources

Oil	*Source plant*	*Aroma-active constituents*	*References*
Oakmoss	*Evernia prunastri*	Ethyl everninate (**84**), methyl β-orcinolcarboxylate (**85**)	95,97
Cinnamon	*Cinnamomum zeylanicum*	Cinnamaldehyde, cinnamyl acetate, (*R*)-linalool, eugenol[a]	95,101
Cassia	*Cinnamomum cassia*	Cinnamaldehyde, coumarin (**86**), cinnamyl acetate	95,101
Clove	*Eugenia caryophyllata*	Eugenol, (−)-caryophyllene (**87**), eugenyl acetate (**88**)	95,101
Saffron	*Crocus sativus*	Safranal (**89**), 3,5,5-trimethyl-2-cyclohexen-1-one (**90**)	95,101

[a] Major constituents in leaf oil.



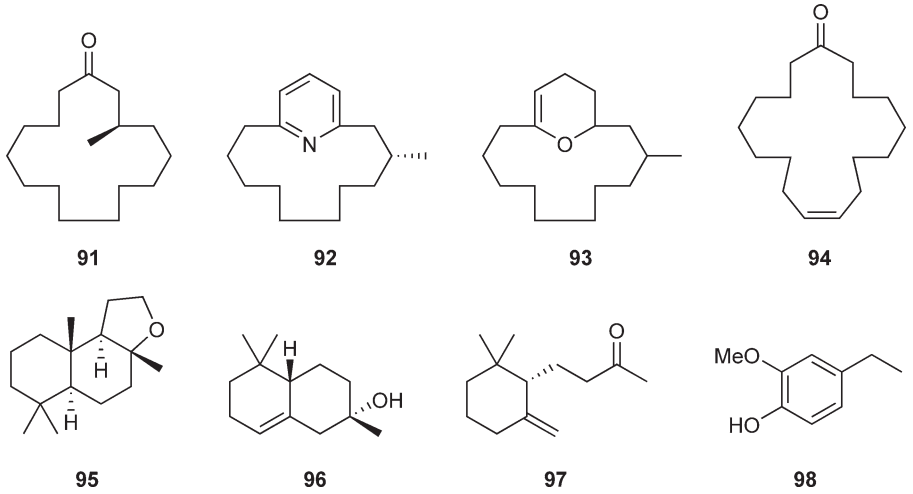
84 **85** **86** **87**

88 **89** **90**

musky animalic note. Sperm whale is the source of ambergris[114,115] prepared from the biliary secretion of its intestines. Castoreum[116,117] is produced from the glandular secretion of beaver, and its unique leather note differentiates it from other aroma materials of animal source (**Table 12**).

Table 12 Aroma-active material from animals

Oil	*Animal of origin*	*Aroma-active constituents*	*References*
Musk	*Moschidae moschus*	(*R*)-Muscone (**91**), (*R*)-muscopyridine (**92**), 7-methyl-1,5-epoxy-1-cyclopentadecene (**93**)	112,113
Civet	*Viverridae civetta*	Civetone (**94**), cycloheptadecanone	112,113
Ambergris	*Physeter macrocephalus*	(−)-Ambrox (**95**), (−)-α-ambrinol (**96**), (*S*)-dihydro-γ-ionone (**97**)	112,114,115
Castoreum	*Castoridae castor*	Borneol, 4-ethylguaiacol (**98**), trimethylpyrazine	112,116,117



91 **92** **93** **94**

95 **96** **97** **98**

4.15.5 Perfumery (Fragrances)

4.15.5.1 Fragrance as Natural Products Chemistry

Until the rise of modern chemical industries, the art of extraction and essential oil preparation was mainly targeting fragrance use, and that for flavorings (e.g., vanilla essence) was much more limited. Flavorings continue to put emphasis on the use of natural or nature-identical synthetic materials. On the other hand, fragrance nowadays is heavily dependent on artificial substances. In modern practice, the use of artificial materials is so extensive that even the basic odor description in the training course of perfumers is in many cases referenced by such substances. Thus, fragrance production from the point of view of natural products chemistry has faded away, although some exceptions apply in the field of citrus oil use.

4.15.5.2 Fragrance Creation

The fundamental idea in the design of fragrances is the classification and combination of materials according to volatility: top note, middle note, and base note.[118,119] A harmonized alternation of odor intensity and quality upon time is considered the heart of creation. Thus, highly volatile materials provide top note (first impression), which is gradually succeeded by middle note (modifier) of moderate intensity and tenacity, which makes up the main body. In the very last stage, the base note (last note) will offer the concluding theme in the design. Base note materials also work as fixatives, but fixatives solely for its function are also employed. Altogether, a complete fragrance creation can be compared to a work of a composer or a novelist, incorporating a consistent theme into a flow of dynamic changes.

Fragrance creation is not necessarily confined to natural aroma substances and many rely heavily on artificial compounds. Therefore, classical perfumes categorized into fundamental types along with the representative natural products are covered here (**Table 13**).

4.15.5.3 Artificial Fragrance Materials

Artificial substances are employed in flavorings as well but to a relatively less extent and most of them are analogues of natural products.[120] On the other hand, artificial compound use in fragrance is popular, and these compounds are not necessarily similar to substances found in nature. This is partially due to the scarcity of natural raw material used in fragrances. At the same time, the fact that fragrance design is less confined compared with flavorings – a conservative sense – has to be taken into account. This allows more freedom enjoyed by perfumers, leading to considerable use of artificial chemicals.

The most classical artificial substances used in perfumery are as follows (**Figure 5**). Hydroxycitronellal (**99**) was found to exhibit the odor of lily of the valley, the essential oil of which is not possible to prepare in the first place. Other classics include α-amylcinnamaldehyde (**100**) and musk ketone (**101**), which were discovered to be good substitutes for jasmine and musk, respectively. Furthermore, perfume No. 5 (Chanel, launched 1921) contained 2-methylundecanal,[121] with so unique an aroma character that it established No. 5 as the pioneer of a new fragrance class – floral aldehydic.

Further development in synthetic chemistry in the second half of the twentieth century brought bloom to another tide of artificial fragrance materials. In terms of musk substitutes,[113,122] mainly three new categories

Table 13 Classic perfumes and their contributing natural products

Type	*Representative materials*	*Representative perfume (manufacturer)*	*Year*
Floral	Jasmine, rose	Joy (Jean Patou)	1930
Oriental	Myrrh, opopanax, vanilla	Shalimar (Guerlain)	1925
Chypre	Bergamot, oakmoss, patchouli	Chypre de Coty (Coty)	1917
Citrus	Bergamot, neroli, lemon, lime	4711 (Mulhens)	1792
		Jean Marie Farina (Roger & Gallet)	1806
Fougere	Lavender, geranium, oakmoss	Fougere Royale (Houbigant)	1882

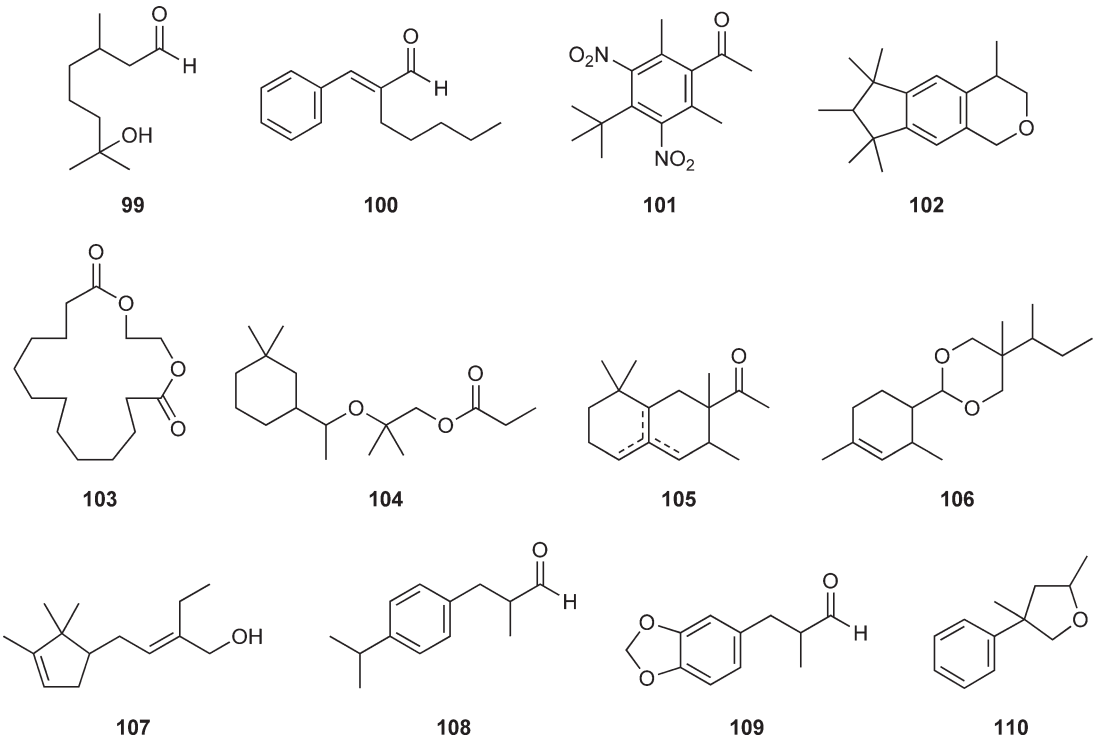


Figure 5 Typical molecules regarded as important fragrance materials.

were developed: the polycyclic aromatic musk[123] compounds led by Galaxolide (**102**), macrocyclic compounds[124] resembling muscone such as ethylene brassylate (**103**), and alicyclic musk compounds represented by Helvetolide (**104**).[120] Diversity in structure offering amber note is even more pronounced than musk.[114,120] Thus, amber note is now realized by many substitutes on the market and Iso E Super (**105**) and Karanal (**106**) can be given as typical examples. Bacdanol (**107**) and allied compounds that bear 2,2,3-trimethylcyclopent-3-en-1-yl group are known to have a sandalwood note.[125] Phenylpropanal derivatives such as cyclamen aldehyde (**108**) exhibit lily of the valley odor[126] and have given rise to compounds like Helional (**109**), a typical marine-ozone note material. The merge of Helional and the related material proved successful, making it a milestone in fragrance design – the ozone note. Rhubafuran (**110**), a substance with a rhubarb-like odor, is a similar example of peculiar modifiers established as important fragrance materials.[120]

4.15.6 Flavor of Foodstuffs

Along with taste, texture, color, and temperature, the flavor that accompanies food has great influence toward acceptance and appreciation of food.[127] Therefore, flavor analysis has attracted the interest of many scientists, now also known as food chemists. In this section, a brief description of flavor compounds of various foodstuffs is given.

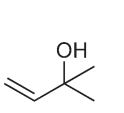
Biosynthetic pathways of aroma material in food are naturally identical with those found in nature. On the other hand, Maillard reaction that takes place especially when food is thermally processed is a formation pathway of aroma-active compounds that are characteristic to cooking art.

4.15.6.1 Fruits

Aroma components of citrus fruit juice[128–132] are different from that of essential oils (see Section 4.15.4.5 for composition of the latter).[133–135] Other familiar fruits like apples,[136] grapes,[137] strawberries,[129,138] bananas,[139] and pineapples[140] were analyzed as well. Place of origin (e.g., grapes) and ripeness of the obtained fruit (e.g., pineapples), in many cases, bring substantial difference in its composition and enantiomeric properties (**Table 14**).[141]

Table 14 Flavor compounds from fruits

Sample	*Source plant*	*Aroma-active constituents*	*References*
Valencia orange (juice)	*Citrus sinensis*	Ethyl butanoate, 2-methyl-3-buten-2-ol (**111**), ethyl acetate, ethyl 3-hydroxyhexanoate (**112**), 1-propanol, ethyl butanoate, wine lactone (**113**), (*Z*)-3-hexenal	106,107,113
Apple	*Malus domestica*	Hexyl acetate, ethyl butanoate, butyl acetate, 2-methylbutyl acetate, hexanal	128,131,136
Grape (European)	*Vitis vinifera*	Ethyl acetate, nerol, (*S*)-linalool, neryl acetate	128,131,137
Grape (American)	*Vitis labrusca*	Methyl anthranilate, (*E*)-2-hexenal, ethyl 3-hydroxybutanoate	128,131,137
Strawberry	*Fragaria ananassa*	(±)-Furaneol (**114**), (±)-mesifuran (**115**), ethyl hexanoate, (*E*)-2-hexenal	129,131,138
Banana (Philippines)	*Musa sapienturn*	2-Pentanone, 2-methylbutyl butanoate, 2-pentyl acetate, 3-methylbutyl acetate	128,132,139
Banana (Cuba)	*Musa sapientum*	2-Heptyl acetate, 2-heptyl hexanoate, 3-methylbutyl acetate, 2-methylbutyl acetate, elemicin (**116**)	128,132,139
Pineapple	*Ananas comosus*	Furaneol, ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, methyl 2-methylbutanoate, (3*E*,5*Z*)-1,3,5-undecatriene, β-damascenone, (*R*)-δ-decalactone (**117**), ethyl 3-(methylsulfanyl)propionate (**118**)	128,132,140



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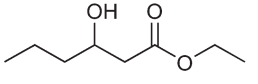

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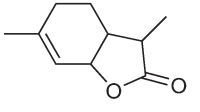

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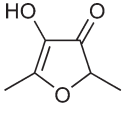

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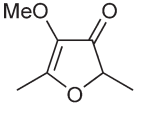

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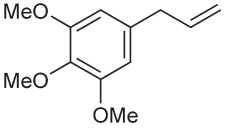

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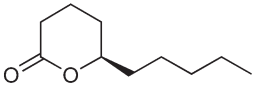

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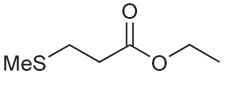

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4.15.6.2 Cereals

Rice is an important calorie source mainly in Asia and also throughout the world. Some rice cultivars are especially rich in 2-acetyl-1-pyrroline (**119**) content and are referred to as Kaori-mai (fragrant rice).[142] Bread is also recognized to play a major role in the human diet. The aroma of bread differs substantially between the crust and the crumb, and both of them have been analyzed by AEDA method.[143,144] Maize[145] is respected as the staple food in Mexico and southern Africa and is also consumed in various forms throughout the world (for instance, popcorn) (**Table 15**).

4.15.6.3 Nonalcoholic Beverages

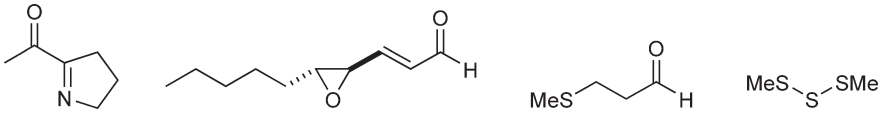
The composition of the aroma of coffee is extremely complex with more than 900 compounds determined[146,147] (see Chapter 3.25). The aroma of tea is greatly influenced by the degree of fermentation (green tea[148] and black tea[149] to mention a representative example) (see Chapter 3.23). Research of cacao mass volatiles using AEDA was also conducted (**Table 16**).[150]

4.15.6.4 Alcoholic Beverages

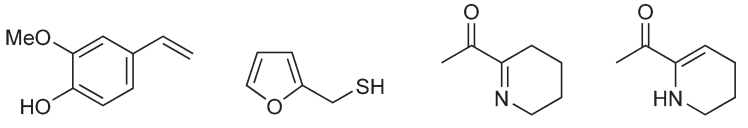
Nihonshu (commonly known as Sake) is an alcoholic beverage drunk in Japan.[151,152] Notable characteristics are the simultaneous fermentation allowing high alcohol concentration and the use of Koji-kabi (*Aspergillus oryzae*) for saccharification of starch. The fruity scent that accompanies high-quality sake is a contribution of esters like 3-methylbutyl butanoate and ethyl hexanoate[153] In terms of wine, varieties of cultivars of grape as well as differing production areas influence the character[154–159] (see Chapter 3.26). Some types of wine (e.g., Sauvignon Blanc) are characterized by trace amounts of sulfur-containing compounds. Beer aroma is quite complex in aroma composition (**Table 17**) [42,160] (see Chapter 3.22).

Table 15 Important flavor compounds in cereals[161]

Sample	*Aroma-active constituents*	*Reference(s)*
Rice (cooked)	2-Acetyl-1-pyrroline (**119**), (*E,E*)-2,4-decadienal, nonanal, hexanal, octanal, (*E*)-2-nonenal	142
Bread (crust)	2-Acetyl-1-pyrroline, (*E*)-2-nonenal, 3-methylbutanal	143,144
Bread (crumb)	(*E*)-2-Nonenal, (*E,E*)-2,4-nonadienal, (2*E*,4*R**,5*R**)-4,5-epoxy-2-decenal (**120**)	143,144
Rye bread (crust)	Methional (**121**), 3-methylbutanal, (*E*)-2-nonenal, (*E,E*)-2,4-nonadienal	143,144
Rye bread (crumb)	Phenylacetaldehyde, (*E*)-2-nonenal, (*E,E*)-2,4-nonadienal	143,144
Maize (cooked)	Dimethyl sulfide, dimethyl trisulfide (**122**), acetaldehyde, 4-vinylguaiacol (**123**), 2-acetyl-1-pyrroline	145
Maize (as popcorn)	2-Acetyl-1-pyrroline, (*E,E*)-2,4-decadienal, 2-furylmethanethiol (**124**), 4-vinylguaiacol, 2-acetyltetrahydropyridine (**125a**, **125b**)	161



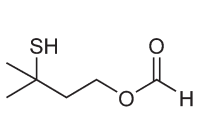
119 **120** **121** **122**



123 **124** **125a** **125b**

Table 16 Flavor compounds in nonalcoholic beverages

Sample	*Aroma-active constituents*	*Reference*
Coffee (Arabica)	β-Damascenone, 2-furylmethanethiol, 3-sulfanyl-3-methylbutyl formate (**126**), homofuronol (**127**), furaneol, guaiacol, 4-vinylguaiacol, methional, sotolone, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, vanillin, 4-ethylguaiacol, homosotolone (**128**)	147
Green tea (Sencha)	(*Z*)-1,5-Octadien-3-one (**129**), 4-methyl-4-sulfanyl-2-pentanone (**130**), methional, (2*E*,6*Z*)-2,6-nonadienal, 3-methyl-2,4-nonanedione (**131**), methyl *epi*-jasmonate, indole	148
Black tea (Darjeeling)	(*R*)-Linalool, geraniol, (2*E*,4*E*,6*Z*)-2,4,6-nonatrienal, β-damascenone, 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, 3-methyl-2,4-nonanedione, (2*E*,6*Z*)-2,6-nonadienal	149
Cacao mass	2-/3-Methylbutanoic acid, 3-methylbutanal, ethyl 2-methylbutanoate, hexanal, 2-methoxy-3-isopropylpyrazine (**132**), (*E*)-2-octenal, 2-methyl-3-(methyldisulfanyl)furan (**133**)	150



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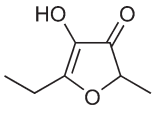

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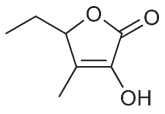

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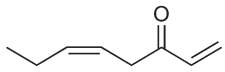

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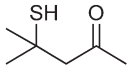

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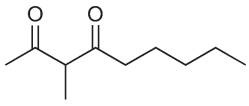

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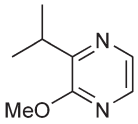

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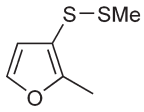

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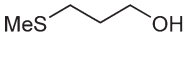
Table 17 Flavor compounds in alcoholic beverages

Sample	*Aroma-active constituents*	*Reference(s)*
Nihonshu (Sake)	Ethyl hexanoate, 3-methylbutyl acetate	153
White wine (Riesling)	Ethyl 2-butenoate, ethyl hexanoate, butanoic acid, 3-methylbutanoic acid, methionol (**134**), γ-undecalactone (**135**), 4-vinylguaiacol	156
Red wine (Cabernet Sauvignon)	Ethyl 2-methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate, 3-methyl-1-butanol, γ-nonalactone (**136**), eugenol	157
Rose wine (Grenache)	3-Sulfanyl-1-hexanol (**137**), β-damascenone, 3-methylbutyl acetate, ethyl octanoate, ethyl hexanoate	158
Beer (Pale lager)	β-Damascenone, ethyl butanoate, 3-methylbutanol, ethyl hexanoate, 2-phenylethanol, 4-vinylguaiacol, furaneol	42,160



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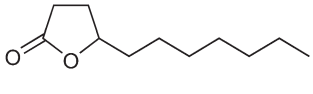

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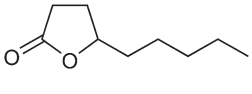

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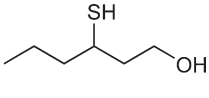

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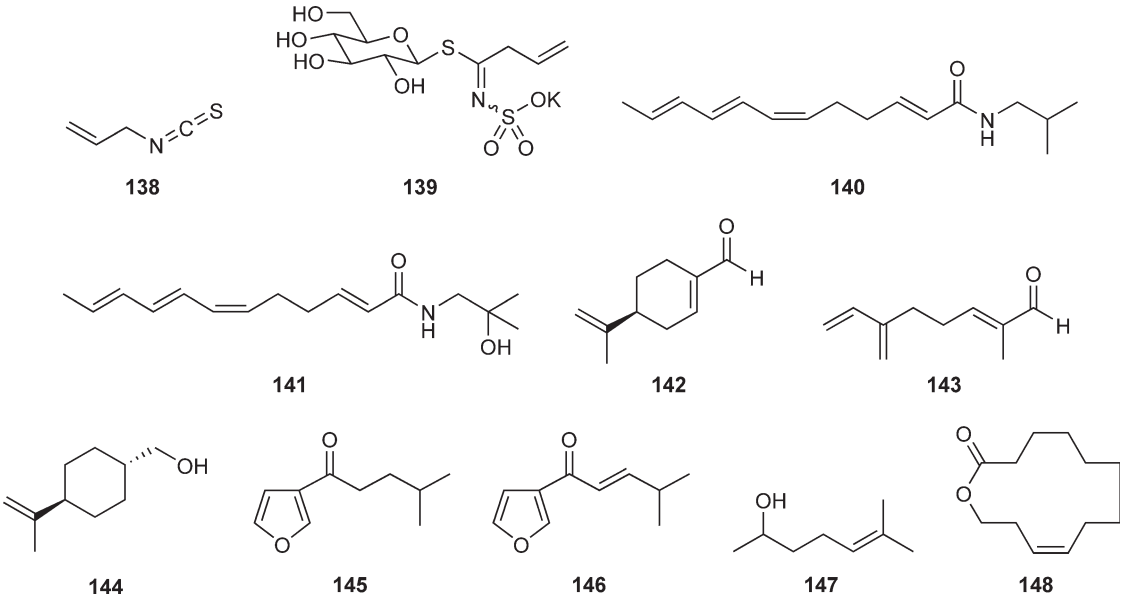
4.15.6.5 Condiments

The pungent aroma of wasabi is a result of allyl isothiocyanate[162] (**138**), the enzymatically cleaved component of its precursor sinigrin (**139**).[163] Sansho (*Zanthoxylum piperitum*) is used in Japan, and its Chinese counterpart – Sichuan pepper (*Zanthoxylum simulans*) – is consumed in some parts of China.[164] Shiso (*Perilla frutescens*) is a herb

Table 18 Flavor compounds in condiments

Sample	*Source plant*	*Aroma-active constituents*	*Reference*
Wasabi	*Wasabia japonia*	Allyl isothiocyanate (**138**)	162
Sansho	*Zanthoxylum piperitum*	Citronellol, α-sanshool (**140**), α-hydroxysanshool (**141**)	164
Sichuan pepper	*Zanthoxylum simulans*	Linalool, α-hydroxysanshool	164
Shiso	*Perilla frutescens var. crispa*	(*S*)-Perillaldehyde (**142**), 2-methyl-6-methylene-2,7-octadienal (**143**), trans-shisool (**144**)	165
Egoma	*Perilla frutescens var. frutescens*	Perillaketone (**145**), isoegomaketone (**146**)	166
Yuzu	*Citrus junos*	Dimethyl trisulfide, 6-methyl-5-hepten-2-ol(**147**), (*Z*)-9-dodecen-12-olide (**148**), 8-methylnonanal, 8-methyldecanal	167



of the Lamiaceae family of Chinese origin and many variants and chemotypes coexist. In Japan, *P. frutescens* var. *crispa* is known,[165] while another major variant of this plant, *P. frutescens* var. *frutescens* (known as beefsteak plant), is used extensively in Korea.[166] Yuzu (*Citrus junos*), a citrus fruit of Chinese origin, has a unique freshening flavor and is used as a condiment in Japanese cuisine (**Table 18**).[167]

4.15.6.6 Vegetables

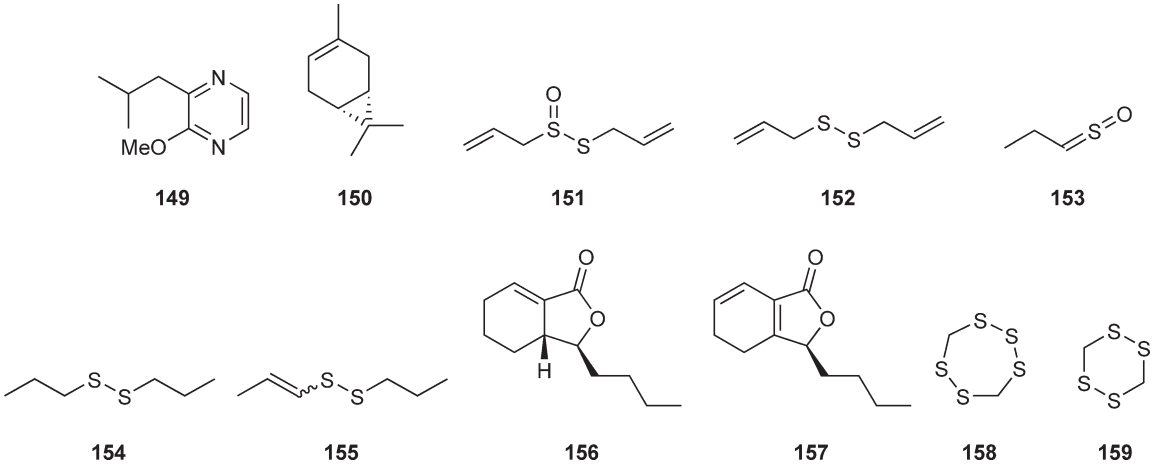
Representative examples of vegetables along with their characteristic aromatic compounds are listed in **Table 19**.[131,168] Usually, the aromatic composition of vegetable aroma is influenced by food processing (see for instance, tomato[169] and onions[170–172]). Naturally, the cultivated varieties of vegetables will also result in different aroma profiles.[173–179]

4.15.6.7 Dairy Products

The scent of raw milk[180,181] is generally considered faint and delicate. This aroma composition may be greatly altered by transformation of milk to various dairy products such as butter,[182] cheese,[183] and yogurt[43] (**Table 20**).

Table 19 Flavor compounds in vegetables

Sample	*Source plant*	*Aroma-active constituents*	*Reference(s)*
Potato	*Solanum tuberosum*	(2*E*,4*R**,5*R**)-4,5-Epoxy-2-decenal, methional, 2-acetyl-1-pyrroline, dimethyl trisulfide, 2,3-diethyl-5-methylpyrazine	131,173
Tomato (fresh)	*Solanum lycopersicum*	(*Z*)-3-Hexenal, hexenal, 1-octen-3-one, methional	131,169
Bell pepper (green)	*Capsicum annuum* var. *grossum*	2-Isobutyl-3-methoxypyrazine (**149**), 2,3-butanedione, octanal, dimethyl trisulfide	174
Bell pepper (red)		2-Isobutyl-3-methoxypyrazine, (+)-3-carene (**150**), dimethyl trisulfide	174
Garlic	*Allium sativum*	Allicin (**151**), di(2-propenyl) disulfide (**152**), dimethyl trisulfide	131,171
Onion (raw)	*Allium cepa*	Propanethial *S*-oxide (**153**), dipropyl disulfide (**154**), 1-propenyl propyl disulfide (**155**)	131,170,171,172
Celery	*Apium graveolens*	(3*S*,3a*R*)-Sedanolide (**156**), (3*S*)-sedanenolide (**157**)	131,175
White truffle	*Tuber magnatum*	Dimethyl sulfide, bis(methylsulfanyl)methane, 1-octen-3-ol	176
Pfifferling	*Cantharellus cibarius*	1-Octen-3-ol, 3-octanol, 2-heptanol, benzaldehyde	177
Matsutake mushroom	*Tricholoma matsutake*	(*R*)-1-Octen-3-ol, methyl cinnamate	178
Shiitake mushroom	*Lentinula edodes*	Lenthionine (**158**), 1,2,4,5-tetrathiane (**159**)	179



149 **150** **151** **152** **153**

154 **155** **156** **157** **158** **159**

4.15.6.8 Meat and Seafood

The aroma of meat products[184–186] and seafood[90,187,188] is mainly a result of Maillard reaction in cooking. See **Table 21** for representative aroma compounds of this class.

4.15.7 Human Interactions

As shown in the case of 1-octen-3-one in the beginning of this chapter, aroma-active compounds show an interesting indication as media in human–environment interactions; the role of this often disliked compound has some similarity to alarm pheromones. Flavor or smell of food can be categorized into food attractant molecules from this point of view. Infants are able to recognize the body odor of their mother and are attracted to it.[189] An opposite example is represented by the notorious ability of skunks to spray, in which thiol compounds such as (*E*)-2-butene-1-thiol and 3-methyl-1-butanethiol are used as potent repellents[190](see Chapter 4.09).

Table 20 Flavor compounds in dairy products

Sample	*Aroma-active constituents*	*Reference(s)*
Cow's milk (raw)	Ethyl hexanoate, ethyl butanoate, dimethyl sulfone, nonanal	180,181
Butter (Irish sour cream)	2,3-Butanedione, (*R*)-δ-decalactone, butanoic acid, (*Z*)-γ-6-dodecenolactone (**160**)	182
Cheddar cheese	Butanoic acid, 2,3-butanedione, methional, homofuronol, (*R*)-δ-decalactone, furaneol, (*Z*)-γ-6-dodecenolactone	183
Yogurt	2,3-Butanedione, acetaldehyde, hexanoic acid, dimethyl sulfide, 2,3-pentanedione	43

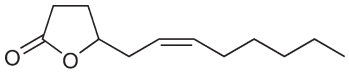


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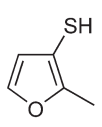
Table 21 Flavor compounds in meat and seafood

Sample	*Aroma-active constituents*	*Reference*
Beef	12-Methyltridecanal, 2-methylfuran-3-thiol (**161**), 2-furylmethanethiol	185
Pork	Methanethiol, 2-methylfuran-3-thiol, 2-furylmethanethiol	185
Chicken	Methanethiol, nonanal, acetaldehyde	185
Salmon (boiled)	(*Z*)-1,5-Octadien-3-one, (*2E,6Z*)-2,6-nonadienal, propanal, acetaldehyde, methional, (*E,E*)-2,4-decadienal	187
Lobster	Trimethylamine, 2,3-butanedione, (*Z*)-4-heptenal, 1-octen-3-one, 2-acetyl-1-pyrroline, 2-acetyl-3-methylpyrazine (**162**)	188
Spotted shrimp (roasted)	Methanethiol, 1-pyrroline, *N*-(2-methylbutyl)pyrrolidine, *N*-(3-methylbutyl)pyrrolidine, isopropyl methyl disulfide, 3-methylpyrazine	90



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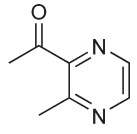

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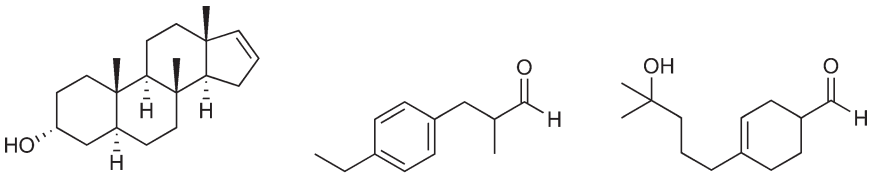
The bad odor of rotten food is also an example of chemicals recognized as repellents. The extremely unpleasant feature of thiol smell, on the other hand, can contribute to modern life as a safety measure due to the odorizing fuel gas used to detect gas leaks at an early stage.[56] An application of sex attractant in human culinary culture is observed in the classical method to seek out truffles. The constituent of truffles, an androstenol(**163**)-related molecule (**Figure 6**), is known to be coincidentally identical to the sex pheromone of truffle hogs, making them keenly attracted to them.[191] On the other hand, an unintended ecological interaction of human culture toward hornets is suggested. In this case found in Japan, a combination of 2-pentanol, 3-methyl-1-butanol, and 1-methylbutyl 3-methylbutanoate, a formula often present among F&F materials, in a certain ratio could provoke attacks by the world's largest hornet (*Vespa mandarinia*), which claims dozens of fatalities annually in the island state.[192]

One has to take into account that in human society, the function of the above-mentioned volatiles is substantially influenced by the cultural background of the person who perceives it, for instance, when the scent of fermented food is under discussion. Just to mention a few, cheese, fish sauce, stinky tofu, surströmming, takuan[193] (pickled daikon raddish), kusaya,[194] and natto[195] (fermented soybeans) are notable examples. Many other fermented foodstuffs around the world are famous for their characteristic smell, which renders them repellent or attractant depending on the person who perceives it. Similarly, the amine odor of fish markets and the smell of roasting fish are generally more tolerated (or even appreciated) by consumers who are used to



163 **164** **165**

Figure 6 Ecological aspects of aroma-active molecules in relation with our daily lives.

fish-eating tradition whereas for those not familiar with it, they are considered as an unpleasant smell of marine animals deteriorating by bacterial activity (however, this can also occur regardless of hygienic environment, due to endogenous enzymatic activity).[196] Herbs and condiments like coriander and celery are sometimes disliked since they carry a distinctive characteristic odor. Those who avoid coriander leaves frequently argue that its scent resembles that of shield bugs, also known as stink bugs of the Hemiptera family, which indeed reflects the fact that coriander leaves contain (*E*)-2-decenal, a constituent of stink bug defensive spray.[197] Thus, the evaluation of odor is substantially influenced by the individual experience and memory that accumulate as a result of it.

So far, human pheromones are not clearly identified[198] (see Chapter 4.05). However, menstrual synchrony observed among women with a higher ability to sense 3α-androstenol (**163**) can be presented as an indication.[199] Body odor, which is major histocompatibility complex (MHC) dependent, is known to modulate mating behavior in mice while reports show a comparable result in humans in terms of preference of body odor accumulated on worn T-shirts.[200] The emotional effect of fragrant materials is considered as taken-for-granted knowledge in human history; the art and culture of perfumery, aromatherapy, use of incense in religious rituals worldwide, and Koh-doh (incense ceremony), for example, are based upon it. Subsequently, there are several types of odor that are recognized as sexy, of which musk, civet, and jasmine (methyl *epi*-jasmonate (**29**)) are included but without common objective effectiveness. Olfactory receptors are present in human sperm cells and an interesting effect of a fragrance chemical like bourgeonal (**164**) and lyral (**165**) (**Figure 6**) toward olfactory receptors of sperm cells is known – an analogous microscopic counterpart of the attractant/repellent role of chemical mediators in the reproduction scene.[201]

In this section, aroma science in terms of human interaction will be highlighted. In the first part, the human olfactory system from the biological aspect is summarized. Genetic background in the perception of odor molecules is briefly summarized. Human interaction in terms of aromatherapy is briefly described to end the section.

4.15.7.1 Olfactory System

Odor perception is perhaps one of the most enigmatic senses, still not well understood. Compared to other senses, the physical aspect of olfaction is complex. Typical stimuli perceived by humans and animals, namely, light, sound, pressure, balance, temperature, electric and magnetic fields, and so forth, can be recorded, reproduced, or analyzed relatively easily, owing to the fact that they are combinations of comparatively simple physical characteristics. On the other hand, odor perception is an integrated result of a large number of interactions caused by various molecules. Furthermore, the sense of odor is more experience- and memory oriented. Probably many people share the experience of recalling very old or irrelevant memories, triggered by a certain scent, which would otherwise never come to mind. Odor perception is subjective and is reliant upon the function of the brain, which processes the signals provided by the receptors. Odor fatigue is a good example of such characteristics. Although adaptation is found in other senses, a substantial decrease in the sensitivity of a specific adapted odor while allowing detection of newly incoming odorants is an interesting phenomenon.

4.15.7.2 Biological Background of Olfactory Perception

A biogenetic approach toward the mechanism of the olfactory system led to a major breakthrough in 1991, when Buck and Axel discovered that the G-protein-coupled receptors (GPCRs) were the essential components of odor receptors.[202] In mammals, there are as many as 1000 genes that represent the receptors and they amount

to 2–4% of the genome, making them the largest family of genes. Specifically, in the human olfactory system, about 350 of them are functional receptor genes.[203] Having gained knowledge concerning other types of GPCRs, the methodology employed in the receptor function research of these systems may also be applied to odor receptors. The odorant receptors are not necessarily strictly specific: a certain odorant receptor can bind different kinds of odorants and, conversely, one odor-active molecule may interact with several odor receptors.[204] Odorant receptor genes in mammals are categorized into class I (fish-type) and class II (terrestrial animal-type), suggesting possible gene duplication during evolution from fish to terrestrial animals.

What happens at the instant when an odor-active molecule comes in touch with our nasal cavity?[205] The first interaction of odorant molecules takes place in the olfactory receptor neurons, which are embedded in the pseudostratified columnar epithelium (or simply, olfactory epithelium), which is located in the posterior nasal cavity in the case of mammals. Olfactory sensory neurons express receptor proteins on the surface membrane of the cilia, which gain access to the extracellular region covered with mucus. The airborne odorants are dissolved into the mucus, bind with the receptors, and then the receptor protein triggers a signal transduction cascade. This results in the opening of the cation channel that would depolarize the sensory neuron and eventually elicit a train of action potentials in the axon. The olfactory axon leads to the olfactory bulb through basal lamina and lamina propria.

To properly recognize the smell of a particular molecule, each sensory neuron should express a single odorant receptor and respond to an appropriate range of odorants with similar molecular characteristics. This presumption (one receptor–one olfactory neuron rule) was deemed acceptable for quite some time but did not have a firm experimental basis until recently when it was demonstrated in the olfactory system of mice.[206]

Following the detection of odorant molecules, the sensory neurons send signals through their axons and these stimulation signals are gathered at the olfactory bulb, which is a primary processing site of olfactory information in the brain. There are thousands of glomeruli, which are the convergent site of axons distributed on the surface of the mouse olfactory bulb, and the connection of the axon, which represents 1000 different types of receptors, made so that a certain kind of olfactory neurons project their signals to a small number of corresponding fixed glomeruli. This relation between the axons and the glomeruli, combined with the one receptor–one olfactory neuron rule, elucidates the one glomerulus–one receptor rule (**Figure 7**).[207] In other words, the brain only needs to know which glomeruli are activated to know which receptor was activated by the inhaled odorants. Therefore, the glomerular sheet at the surface of the olfactory bulb by itself is a map of odorant receptors.

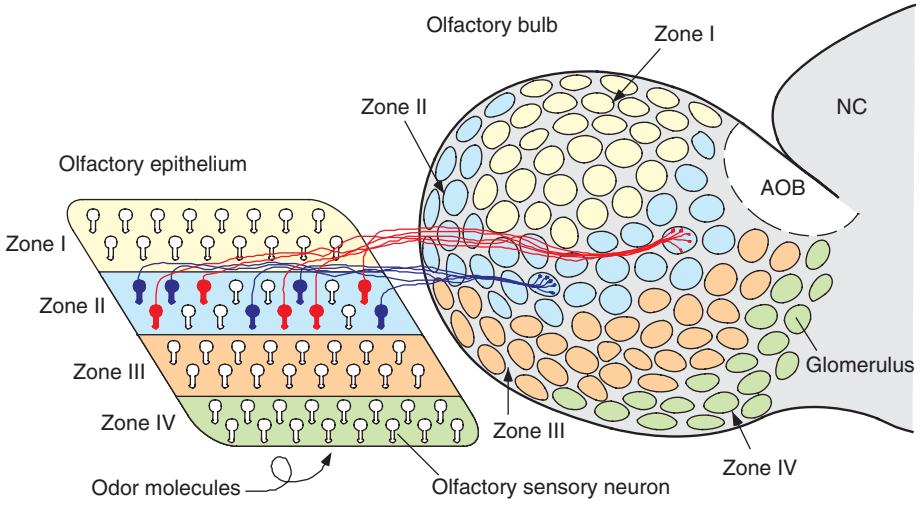


Figure 7 Schematic diagram demonstrating the connection system between the nasal odor receptors and the (main) olfactory bulb. Sensory neurons expressing identical odorant receptors converge their axons to a limited number of defined glomeruli. AOB, accessory olfactory bulb; NC, neocortex. Reproduced from K. Mori; H. Nagao; Y. Yoshihara, *Science* **1999**, *286*, 711–715, with permission from AAAS.

Mapping the odorant-induced glomerular activity revealed the following three characters of its spatial organization: (a) individual glomeruli typically respond to a range of odorants that share a specific combination of molecular features; (b) each glomerulus appears to be unique in its odorant selectivity; and (c) glomeruli with similar odorant selectivity situate themselves in proximity and form molecular-feature clusters. These molecular-feature clusters might be a structural unit in the spatial organization of a glomerular sensory map.[208] A number of studies have demonstrated a close relationship between molecular features of odorants and their perceived odor; functional groups like carboxyl and hydroxy groups remarkably influence the quality of odor. Accordingly, if clusters reflecting molecular features are identified along with their specific subsets of odorant receptors, they may lead to an interesting indication of such clusters being related to submodality of subjectively perceived odor.

Mitral and tufted cells in the olfactory bulb project their axons to the olfactory cortex, the site thought to integrate the signals from distinct glomeruli. The olfactory signals processed in the olfactory cortex are sent to a variety of higher centers of the brain, which include insular cortex, orbitofrontal cortex, amygdale, hippocampus, and the nucleus accumbens.[205]

Considering the fact that humans are able to distinguish far more varieties of odor than the types of receptors present, these higher centers of the brain must be responsible for recognizing and processing the complex patterns of reactions given by the receptor–olfactory bulb system. Although preprocessing of signals including lateral suppression mechanism in the olfactory bulb is a known function, an upstream research from the nasal receptors toward the brain has indicated that the remaining part – the higher center of the brain – is the essential part of the olfactory mechanism. Interaction (either enhancing or suppressive) between multiple aroma compounds including pattern recognition, as well as stimulation of memorized matter, adaptation, and habituation of odor can be deemed to be a function of the higher centers of the brain, although details are yet to be uncovered. In other words, excluding a small number of basic chemicals that possess instinctive and seemingly absolute characters, evaluation of odorous compounds is in nature an acquired sense. Mind-only perception is pronounced when compared to other senses, making olfaction a good example of impersonality in the context of annata (nonself). Hence, research of the olfactory system is likely to become even more challenging as this aspect becomes more notable – the fundamental and eemotionally influential senses being dependent upon the relational environment with the consciousness or essence of personality vaguely blurred therein. This is in agreement with recent studies concerning the olfactory perception and cultural background differences together with the evaluation of odor in relationship with familiarity.[209]

4.15.7.3 Aromatherapy as Human Interaction

Aromatherapy, a concept established in the first half of the twentieth century, originates from herbalism practiced since prehistoric ages, and therefore is one of the oldest human–environment interactions (see Chapter 3.14). The word aromatherapy was proposed by the French chemist R. M. Gattefossé in the 1920s, who coincidentally noticed the fact that lavender oil effects rapid healing of burns.[210] The term can be defined as therapeutic use of essential oils by inhalation, topical application, and ingestion to improve a person's health or to balance the psychological condition. Although there are criticisms that scientific evidence with regard to the merit of aromatherapy is not sufficient, it is widely practiced and appreciated as a complementary or alternative medicine nowadays.[211,212] As an example, chemical substances that belong to a class of aldehydes (in particular, α,β-unsaturated aldehydes) and phenols are often known for their antibacterial functions.[213] Representative essential oils and extracts employed in aromatherapy as well as their typical use are listed in **Table 22**.[210] Owing to the lack of full scientific insight into aromatherapy, bioactivity and the synergic effects of these materials toward human health are not fully understood. Adverse effect and toxicity as potential hazards have to be always taken into account in their use, especially when they are taken into the body by ingestion.

4.15.8 Outlook

Development history of F&F industry represents the eagerness of mankind toward discovering the enigma shrouding the science of odor. This was, and will continue to be, one of the major driving forces in this field of chemistry and biology. Nowadays, aroma science, like the mechanism of odor perception (discussed in Section 4.15.7) and fine aroma chemistry taking into account the concept of chirality, for instance, continues

Table 22 Typical essential oils used in aromatherapy

Oil	*General usage/target symptoms*
Basil	Nerve corroborant, expectorant, febrifuge, insect bite
Bergamot	Disinfectant, eczema, psoriasis, bronchitis, antidepressant
Chamomile	Anti-inflammatory, analgesic, gynopathy, sedation
Eucalyptus	Febrifuge, expectorant, disinfectant
Geranium	Neuralgia, perimenopausal symptom, insecticide, gallstone, urinary stone, skin problems
Lavender	Sedation, anti-inflammatory, skin infection, sunburn prevention, insect bite
Marjoram	Sedation, warming, contusion, sprain, purgative
Peppermint	Analgesic, sedation, coolant, expectorant, dermatitis, insect repellent
Rosemary	Neuropathy, bronchitis, rheumatism, myalgia

to be at the leading front of chemistry as it expands further to interdisciplinary areas. Genetics, molecular biology, neuroscience, and integration of taste and smell will most probably be the next phase of challenge in the F&F research (see Chapter 4.16).

Abbreviations

CODEX	Codex Alimentarius
F&F	Flavor(s) and Fragrances
FDA	Food and Drug Administration
GC	Gas Chromatography
GRAS	Generally Recognized as Safe
IOFI	International Organization of the Flavor Industry
IR	Infrared
JECFA	Joint Expert Committee on Food Additives
MHC	major histocompatibility complex
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PTR-MS	Proton Transfer Reaction Mass Spectrometry
RIFM	Research Institute for Fragrance Materials
UN	United Nations
UV	Ultraviolet

Nomenclature

% e.e.	% enantiomeric excess
lb(s)	pound(s) = 0.454 kg

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4.16 Human–Environment Interactions – Taste

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4.16.1 Introduction

Humans have evolved to consume plants and animals in their environment and have acquired a sense of taste. It has been suggested that our ability to taste bitter and sour evolved to identify potentially dangerous food. On the contrary, our ability to recognize a sweet taste developed to identify an energy source, while salty and umami tastes are a signal of minerals and proteins, respectively. As time progressed, humans began to use bitter, pungent, and

even astringent tastes that were initially considered to be unpleasant. This may have been because we became aware that such substances were effective at improving health or even treating disease. Human food culture progressed further with the development of cooking methods that use spices and more dramatically through the enjoyment of fermentation products such as cheese, beer, wine, soy sauce, and miso (fermented soybean paste).

Henning[1] proposed, along the lines of a concept that was first introduced by the ancient Greeks, that the four tastes, that is, sweet, sour, salty, and bitter, constitute a psychological continuum, and mixed tastes can be placed on the surface of a psychological continuum in which each of the four tastes is placed at one of the apexes of a regular tetrahedron. Prior to Henning's tetrahedron theory, Ikeda[2] had discovered that monosodium glutamate (MSG) was a savory-tasting substance and named it umami in 1909. Since the umami taste sensation could not be explained by the four basic tastes theory, the umami taste was recognized as a fifth taste modality in Japan. However, until recent molecular-biological findings, only sweet, sour, salty, and bitter have been regarded as the basic taste qualities in the rest of the world. Although umami has previously been referred to as flavor and not taste, recently the term 'umami' has become accepted worldwide as one of the five basic tastes.[3–5]

Recently, a series of major discoveries on taste receptors have been reported. Specifically, the receptors that corresponded to sweet,[6] bitter,[7] and umami[8] were identified and characterized. These receptors are distributed in taste buds, which are flower-bud-shaped organs containing taste receptor cells in taste papillae on the tongue. On the apical surface of taste receptor cells, taste receptor proteins provide molecular specificity to taste receptor cells, which are innervated by afferent nerve endings that transmit information to the taste centers of the cortex through synapses in the brain stem and thalamus. More recently, it was revealed independently by two groups that the receptor for sour taste consisted of polycystic-kidney-disease-like ion channel (PKD2L1) molecule.[9,10] The receptor for salty taste is also a subject of intense scrutiny, with particular focus on epithelial Na^+ channels (ENaCs).[11]

Taste intensity changes in proportion to the level of the stimulus. The recognition threshold is defined as the minimum concentration of a substance at which a particular taste can be recognized by a significant number of taste panels. The threshold value cannot be used to determine the relative taste intensity, since the relative taste intensity and spectrum of a substance change with concentration. Generally, the bitter taste is the most sensitive to concentration, followed by salty, sour, and sweet. Bitter and salty sensations have a wide range of responses to the concentration of the substance. However, the perception of sweet and sour occurs within a much narrower concentration range and can become saturated, for example, by a high concentration of sugar. In addition, quinine and caffeine have different thresholds even though they offer the same kind of taste sensation. On the contrary, a taste may actually change with the concentration. For example, saccharin tastes sweet at low concentration, but is bitter at high concentration. It is also well known that a salty-tasting compound can sometimes enhance sweet sensation. Taste-modifying substances are also known, and both gymnemic acid and miraculin are typical compounds that exhibit such activity. It has been reported that gymnemic acid suppresses sweet sensation probably by competitive binding with the receptor, while miraculin can modify the sour taste of citrate to a sweet taste.[12]

On the contrary, somatosensory stimuli such as pungency, astringency, tingling, and cooling sensations are believed to be transmitted directly to the brain through trigeminal nerve endings in the mouth, although this notion is still controversial.

Several review articles have described the chemistry of taste and structure–activity relationships.[13,14] Two comprehensive reviews have been published in Japan (in Japanese), with particular focus on taste compounds.[15,16] However, there has been no recent comprehensive review written from the perspective of natural products chemistry. In this chapter, several taste sensations found in natural products are described along with their structures. Unfortunately, however, it is still very difficult to anticipate the taste quality and intensity from the structure of an organic compound, even for the thoroughly studied sweet and bitter sensations, although some regularity has been observed. It is expected that recent progress in the study of receptors will contribute to a full understanding of the relationship between taste sensation and chemical structure.

4.16.2 Natural Products Associated with Sweetness

Sweet substances are the most desirable taste for humans, who have enjoyed them in fruits and honey since ancient times. It is believed that the derivation of sugar from sugarcane and sugar beet is a fairly recent practice and was started only 500–600 years ago. Initially, purified sugar was very expensive and could be enjoyed only

by the rich. However, the volume of sugar produced is currently enormous due to an increase in the cultivated area of sugarcane and sugar beet, and therefore the price is not so high. In addition, the discovery of new low-calorie artificial sweeteners such as aspartame, acesulfame K, and sucralose has expanded the sweetener market.

Meanwhile, intensive studies have been performed on the structure–sweetness relationship and many hypotheses have been proposed.[17,18] Based on these hypotheses, new, highly intense sweeteners are currently being designed. As a result, several compounds that are hundreds of thousands of times sweeter than sugar have been synthesized.[19]

Very recently, intensive studies on the receptor for sweet molecules using gene technology and knockout mice have been performed to clarify the mechanism of the perception of a sweet taste. For instance, the recognition of sugars is the function of specialized G protein-coupled receptors (GPCRs) in the gustatory system. Recently, three members of a novel subfamily of GPCRs (T1R1, T1R2, T1R3) have been proposed to function as taste receptors based on their expression in taste cells. The subfamily found in human and mouse contains a long extracellular region composed of a highly conserved amino acid sequence with about 570 residues and is called the T1R family. Notably, while each receptor expressed by cultured cells does not react at all with sweet substances by itself, cells that coexpress T1R2 and T1R3 show this reactivity. Based on these results, it is believed that a heterodimer of T1R2 and T1R3 acts as a sweet receptor.[20]

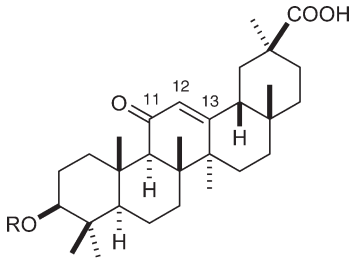
This section describes sweet-tasting natural products that have been found to date. Taste-modifying compounds and antisweet substances are also discussed.

4.16.2.1 Low-Molecular-Weight Sweet Substances

4.16.2.1.1 Glycyrrhizin

Liquorice (licorice) is the root of *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*, from which a sweet substance can be extracted. The licorice plant is a legume (related to beans and peas) and is native to southern Europe and parts of Asia. Licorice extract (derived from the ancient Greek words for 'sweet root') is traded in both solid and syrup forms. Its major active component is an oleanane-type triterpene glycoside, glycyrrhizin, which is used as a sweetener and is more than 50 times sweeter than sugar (sucrose).[21] Glycyrrhizin and its ammonium salt also have a variety of pharmaceutical effects and are used particularly for the treatment of peptic ulcers and as expectorants. Although glycyrrhizin is sweet, its taste is different from that of sugar. The sweetness of glycyrrhizin has a slower onset than sugar and lingers in the mouth for sometime. In the United States, glycyrrhizin is 'generally recognized as safe' (GRAS) as a flavoring agent, but not as a sweetener. Glycyrrhizin is used as a flavoring in some candies, pharmaceuticals, and tobacco products.

Monoglucuronide of glycyrrhetinic acid (MGGR) was found to be 941 times sweeter than sucrose.[22] MGGR is produced from glycyrrhizin by selective removal of the terminal glucuronide unit of glycyrrhizin by an enzyme from *Cryptococcus magnus* MG-27 (yeast).



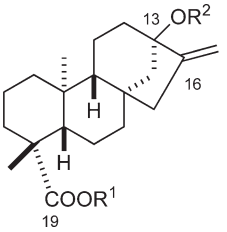
Glycyrrhizin: β-glcA^{2}–β-glcA
MGGR: β-glcA
GlcA = D-glucuronopyranosyl

4.16.2.1.2 Stevioside

Several *ent*-kaurenoid diterpene glycosides with steviol as a common aglycon have been isolated from *Stevia rebaudiana*, which is native to subtropical and tropical South America and Central America.[23–27] Among the

glycosides, stevioside is the most abundant followed by rebaudioside A. Stevioside is 140 times sweeter than sucrose, while rebaudioside is 240 times sweeter. Rebaudioside A has a better quality of sweetness. In Japan, stevia sweeteners have been produced commercially and are widely used in food products such as soy sauce, pickles, and boiled fish paste. Steviol glycosides are stable enough to remain sweet in processed foods.

The leaves of *Rubus suavissimus* S. Lee (Rosaceae), which is found wild in Guang Xi province in China, show potent sweetness and are used as a drink (sweet tea; *tian-cha*). Rubusoside has been isolated as a major sweet component from the leaves.[28] This compound has the same aglycon structure as stevioside but with one glucose less and can be obtained from stevioside by enzymatic transformation. Rubusoside is 130 times sweeter than sucrose. A comprehensive review of stevioside has been published.[29]



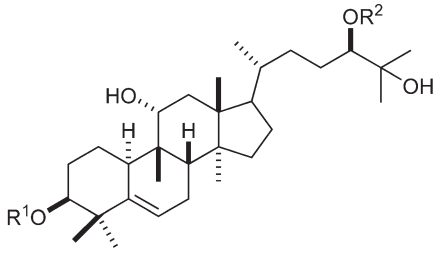
Stevioside: R^1 = β-glc, R^2 = β-glc^2-β-glc
Rebaudioside A: R^1 = β-glc, R^2 = β-glc^2-β-glc
|
β-glc^3
Rebaudioside B: R^1 = H, R^2 = β-glc^2-β-glc
|
β-glc^3
Rebaudioside C: R^1 = β-glc, R^2 = β-glc^2-α-rha
|
β-glc^3

Rebaudioside D: R^1 = β-glc^2-β-glc, R^2 = β-glc^2-β-glc
|
β-glc^3
Rebaudioside E: R^1 = β-glc^2-β-glc, R^2 = β-glc^2-β-glc
Dulcoside A: R^1 = β-glc, R^2 = β-glc^2-α-rha
Steviolbioside: R^1 = H, R^2 = β-glc^2-β-glc
Rubusoside: R^1 = β-glc, R^2 = β-glc

glc = D-glucopyranosyl; rha = L-rhamnopyranosyl

4.16.2.1.3 Mogroside

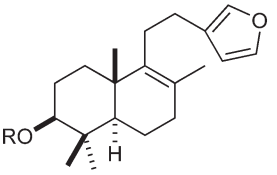
Siraitia grosvenorii is an herbaceous perennial vine that is native to southern China and is best known for its fruit, the lo han kuo (luo han guo). The fruit extract is nearly 300 times sweeter than sucrose and has been used as a natural sweetener in China for nearly a millennium due to its flavor. It has also been used in traditional Chinese medicine for the treatment of cold and sore throat. It is also used as an additive for drinks and candies in Japan and the United States. Two cucurbitane-type triterpene glycosides, mogrosides IV and V, were isolated as major sweet components of this fruit, and have been found to be 233–392 and 250–425 times sweeter than sucrose, respectively.[30,31] Mogrosides are classified by the US Food Drug Administration (FDA) as a GRAS product. There are no restrictions on consuming the fruit or its extracts. Mogroside V has been reported to be nonmutagenic.



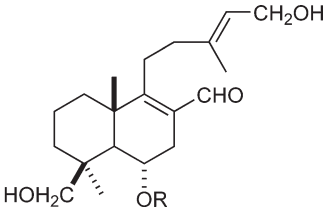
Mogroside IV: R^1 = β-glc^6–β-glc, R^2 = β-glc^2–β-glc
Mogroside V: R^1 = β-glc^6–β-glc, R^2 = β-glc^2–β-glc
|
β-glc^6

4.16.2.1.4 Baiyunoside and gaudichaudioside A

Baiyunoside, a labdane-type diterpene glycoside, was isolated from the root of *Phlomis betonicoides*, which is native to southern China and Tibet and which has been used as a traditional Chinese medicine.[32] Baiyunoside is 500 times sweeter than sucrose and has a long-lasting taste. Nishizawa and Yamada.[33] reported the synthesis of the aglycon, (+)-baiyunol, as well as its isomer, *ent*-baiyunol, and the structure–activity relationship in a series of glycosides. In addition, gaudichaudiosides A–F were isolated as other labdane-type diterpene glycosides from the stem of *Baccharis gaudichaudiana* DC. (Compositae), which is native to Paraguay. Among them, gaudichaudioside A was found to be 55 times sweeter than sucrose.[34,35]



Baiyunoside: R = β-glc^2-β-xyl
glc = D-glucopyranosyl
xyl = D-xylopyranosyl



Gaudichaudioside A: R = α-ara
ara = L-arabinose

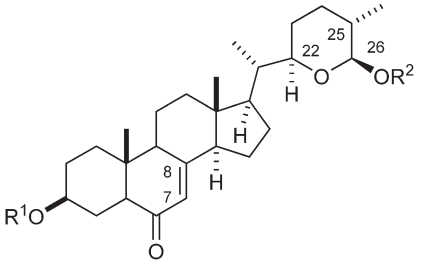
4.16.2.1.5 Steroidal saponins

Osladin, a steroidal saponin, was isolated as a sweet principle of the fern *Polypodium vulgare* L. (Polypodiaceae). Later, Nishizawa and Yamada.[36] reinvestigated the structure of osladin and revised the stereochemistry at C-22, C-25, and C-26. Although osladin has been reported to be 3000 times sweeter than sucrose, this value was also revised to 500 times.

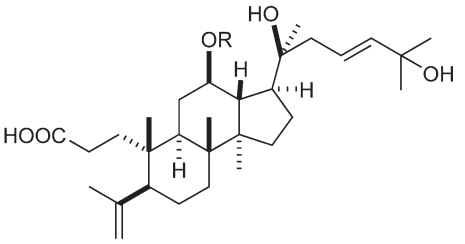
Three steroidal saponins, polypodosides, were isolated from the rhizomes of *Polypodium glycyrrhiza* DC. Eaton (Polypodiaceae).[37,38] According to the corrected structure of osladin, the structure of polypodoside was also revised.[39] Polypodoside A was shown to be highly sweet (600 times sweeter than sucrose).

4.16.2.1.6 Pterocaryosides A and B

Secodammarane saponins, pterocaryosides A and B, were isolated from *Pterocarya paliurus* Batal. (Juglandaceae), which is native to China.[40] Pterocaryosides A and B differ only in the structure of the sugar moiety bound to aglycon. Pterocaryosides A and B have been reported to be nontoxic in terms of mutagenicity and an acute toxicity test. Pterocaryoside A is 50 times sweeter than sucrose (2% solution) while pterocaryoside B is 100 times sweeter. In addition, dammarane glycoside, cyclocaryoside A, which is 200 times sweeter than sucrose, was isolated from the leaves of *Pterocarpa* (*Cyclocarya*) *paliurus*, which is native to China.[41]



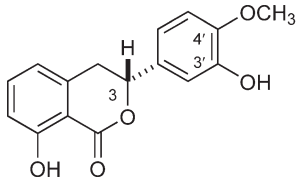
Osladin: R^1 = β-glc^2-α-rha, R^2 = α-rha, 7,8-dihydro
Polypodoside A: R^1 = β-glc^2-α-rha, R^2 = α-rha
glc = D-glucopyranosyl
rha = L-rhamnopyranosyl



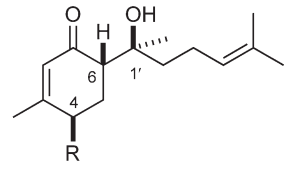
Pterocaryoside A: R = β-qui
Pterocaryoside B: R = α-ara
qui = D-quinovopyranosyl
ara = L-arabinopyranosyl

4.16.2.1.7 Phyllodulcin

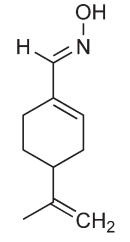
Phyllodulcin-8-*O*-β-D-glucoside, which is found in the leaves of *Hydrangea macrophylla* Seringe var. *thunbergii* Makino (Saxifragaceae), does not show any sweet taste. However, its aglycon, D-(+)-phyllodulcin, produced by enzymatic hydrolysis, is intensely sweet.[42] This compound is called 'amacha (sweet tea)' and is used for the sweet flavor of ceremonial tea. Phyllodulcin is structurally dihydroisocoumarin and was found to be a 3*R*-stereoisomer in 1959. In subsequent studies, it was revealed that unprocessed leaves contained a mixture of *R* and *S* isomers in a ratio of 5:1.[43] Phyllodulcin is reported to be 600–800 times sweeter than sucrose. Purified phyllodulcin has no mutagenicity and its acute oral toxicity in mouse is greater than 2 g per kg body weight. One drawback for its use as a sweetener is its very low solubility in water.



Phyllodulcin



Hernandulcin: R = H
4β-Hydroxyhernandulcin: R = OH



Perillartine

4.16.2.1.8 Hernandulcin

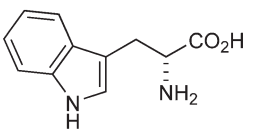
Hernandulcin is a bisabolane sesquiterpene isolated from the herb *Lippia dulcis* Trev. (Verbenaceae), which is native to Mexico, and has been reported to be 1500 times sweeter than sucrose.[44,45] The natural product has a 6*S*, 1′*S* configuration, and of the four possible stereoisomers, only this one has intense sweetness.[46,47] Another sweet substance, 4β-hydroxyhernandulcin, was isolated from a sample native to Panama.[46] The sweetness and bitterness of hernandulcin have been reported to linger in the mouth for sometime. This compound is rather thermolabile.

4.16.2.1.9 Perillartine

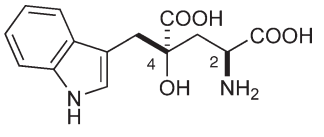
Perillartine, α-*syn*-oxime of perillaldehyde, is reported to be 2000 times sweeter than sucrose.[48,49] Perillaldehyde is a principal volatile oil of *Perilla frutescens* (L.) Britton (Labiatae) and is reported to be only slightly sweet. Perillartine is used as a replacement for maple syrup and licorice for the sweetening of tobacco in Japan. However, due to its low solubility in water as well as a menthol-licorice off-taste, there is some limitation to its use.

4.16.2.1.10 Amino acids

4.16.2.1.10(i) Proteinogenic amino acids The taste of proteinogenic amino acids involving a D-isomer was reviewed by Birch and Kemp,[50] Haefeli and Glaser,[51] and Wieser *et al.*[52] Among L-amino acids, alanine, serine, and glycine are sweet, while most of the D-amino acids are sweet. D-Tryptophan is the most intensely sweet amino acid and is 35 times sweeter than sucrose. Studies on the structure and sweetness–activity relationship have been reported by Shallenberger *et al.*[17] and Kier.[18]



D-Tryptophan



(2*S*,4*S*)-Monatin

4.16.2.1.10(ii) Monatin Monatin is an amino acid-type sweetener isolated from the bark of the roots of a spiny-leafed hardwood shrub, *Schlerochiton ilicifolius*, which is native to the northwestern Transvaal in South Africa.[53] From 160 kg of roots, 1.73 g of a crude mixture of monatin salts was obtained. Recrystallization of the salts from water–acetic acid–ethanol gave free amino acid. After this amino acid was transformed into the lactone derivative (methyl-(2*S*,4*S*)-2-(indol-3-ylmethyl)-4-(2,4-dinitroanilino)-5-oxo-2,3,4,5-tetrahydrofuran-2-carboxylate) and examined by nuclear magnetic resonance (NMR), the relative stereochemistry of the 2- and 4-positions was determined. Its absolute stereochemistry was further determined to be (2*S*,4*S*) by applying the observed optical rotation value to the Clough–Lutz–Jirgenson rule. Monatin is reported to be 800 times sweeter than sucrose at its threshold concentration and 1200–1400 times sweeter in a sucrose solution of 5–10%. It has a slight licorice aftertaste. In the patent literature, it was reported that all four stereoisomers of monatin have a sweet taste, and among them the (2*R*,4*R*) isomer is the most intense, while (2*S*,4*R*) is the weakest.[54] It has also been reported that monatin has no toxicity in the Ames test and no mutagenicity.[55]

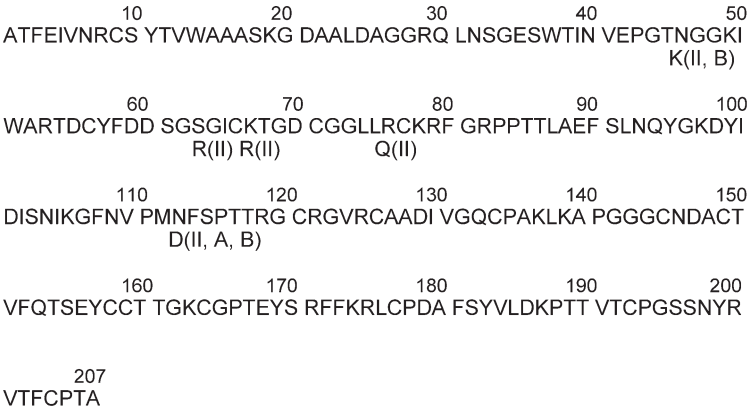
4.16.2.1.11 Sugars

Sugars are the simplest form of carbohydrates, and sugars such as monosaccharides, oligosaccharides, acyclic polyhydroxy alcohols, and cyclic sugar alcohols are well-known sweeteners. Shallenberger[56] has written a good review on the structure–sweetness relationship of sugars.

4.16.2.2 Sweet Proteins

4.16.2.2.1 Thaumatin

Thaumatin is a 22 kDa sweet protein that was isolated from the arils of the katemfe fruit of *Thaumatococcus daniellii* Benth, which is native to West Africa, by van der Wel and Loeve. It is a basic protein with an isoelectric point of approximately 12 and is 1600 times sweeter than sucrose. It also gives a cooling sensation and a slight licorice aftertaste. A water–sweet aftertaste was also reported.[57] Thaumatin is cultivated on a commercial scale and used as a sweetener, flavor enhancer, and flavor modifier. An aqueous solution of commercially available thaumatin is stable under the conditions of pH 2–10. There may be several related proteins in the plant, but there are two main forms: thaumatins I and II. Thaumatins I and II are each composed of 207 amino acids with eight intramolecular disulfide bonds shown in the figure. Thaumatins I and II differ in the amino acid sequence at 46, 63, 67, 76, and 113, which suggests that the two proteins are 98% identical.[58,59] Since the results of amino acid sequencing of the proteins were inconsistent with those of cDNA,[59] Lee *et al.* reinvestigated and isolated two proteins named thaumatins A and B, and found that they differ in only one amino acid at position 46, that is, Asn for A and Lys for B. The residue at 113 in thaumatin I is Asn, whereas it is Asp in thaumatins II, A, and B. Furthermore, thaumatin I did not make a refolding product, while thaumatins A and B expressed in yeast showed intense sweetness after refolding. Therefore, it has been suggested that there might have been an error in the determination of the residue at 113.[60] The tertiary structure of thaumatin I was analyzed by X-ray at resolutions of 3.1[61] and 1.65 Å.[62] It has been reported that thaumatin elicits a sweet taste in humans, and caused a significant electrophysiological response in the chorda tympani and glossopharyngeal nerves in the Old World monkey, but not the guinea pig or rat.[63] However, it was revealed that in Slc:ICR mice, chorda tympani and taste receptor cell response profiles and the behavioral results for monellin and thaumatin are similar to the response profiles for sucrose.[64] Thaumatin has been approved as a sweetener in Israel and Japan. In the United Nations, it is listed in Table III of the Codex General Standard for Food Additives (GSFA), which means that it is permitted for use in food in general.

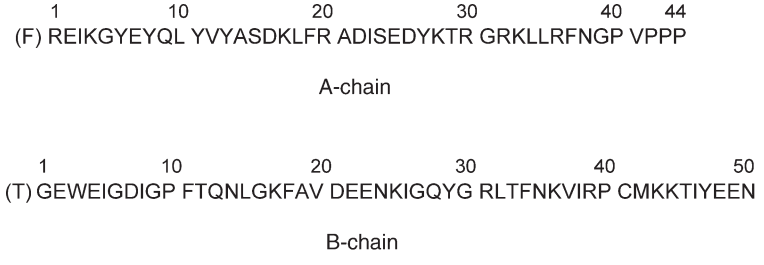


Thaumatin. II, A and B in parentheses correspond with thaumatin II, A and B, respectively. There are eight disulfide bonds between C9–C204, C56–C66, C71–C77, C121–C193, C126–C177, C134–C145, C149–C158, and C159–C164.

4.16.2.2.2 Monellin

Monellin is a sweet protein that was isolated from the fruit of *Dioscoreophyllum cumminsii* (*Stapf*) *Diels*, which is known as the serendipity berry and is native to West Africa. It is a basic protein with an isoelectric point of approximately 9.3 and is 3000 times sweeter than sucrose.[65,66] Perception lasts for more than 1 h and leaves an aftertaste. Heat denatures monellin proteins; they lose their sweetness when heated over 50 °C at low pH. Monellin has a molecular mass of 10.7 kDa. Monellin has two noncovalently associated polypeptide chains: chain A contains 44 amino acid residues and chain B has 50 residues. In 1976, the primary structure of monellin was proposed independently by three groups but their results all differed somewhat.[67–69]

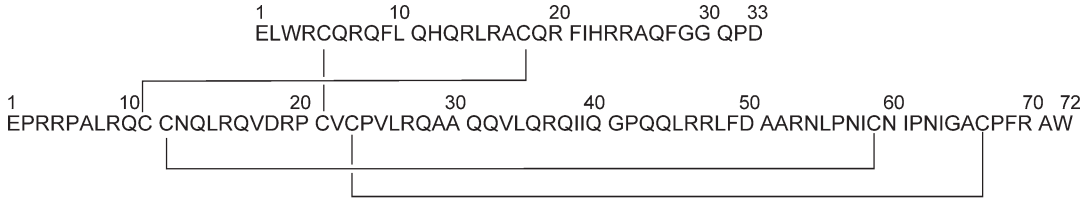
Recently, the amino acid sequence was reinvestigated[70] and it was revealed that chain A was consistent with that of Frank and Zuber,[68] while chain B coincided with the results of Bohak and Li (see figure).[67] The enzymatic hydrolysis product of monellin does not exhibit sweetness.[71] Since chains A and B are not sweet individually,[67,72] it is considered that expression of the sweet taste requires a natural three-dimensional structure. X-ray structural analysis of monellin was carried out at resolutions of 3[73] and 2.75 Å.[74] A structure–sweetness relationship study of monellin analogues strongly suggested that the Asp residue at the 7-position of chain B (AspB7) plays an important role in eliciting a sweet taste.[75–77] Meanwhile, a single-chain monellin was expressed by combining the C-terminus of chain B and the N-terminus of chain A. This compound was reported to be stable at higher temperature and over a wide range of pH,[78] and its tertiary structure was analyzed by X-ray at a resolution of 1.7 Å.[74] The main issue regarding its use as a sweetener is that monellin has no legal status in the European Union or the United States.



Monellin. 10% of A-chain has F in the N-terminus and 19% of B-chain has T in the N-terminus G of N-terminus is deleted in 24% of B-chain.

4.16.2.2.3 Mabinlin

Mabinlins are sweet-tasting proteins extracted from the seed of Mabinlang (*Capparis masaikai* Levl.), a Chinese plant that grows in Yunnan province. They have long-lasting but weak sweetness of 0.1% threshold.[79] There are at least five homologues. Mabinlin-I, Mabinlin-III, and Mabinlin-IV have molecular masses of 12.3, 12.3, and 11.9 kDa, respectively. Mabinlin-II is a 10.4 kDa protein and the most abundant homologue in nature.[80] It is also a basic protein with an isoelectric point of approximately 11.3 and is a heterodimer consisting of two different chains, A and B, like monellin. Chain A is composed of 33 amino acid residues and chain B is composed of 72 amino acid residues. Chain B contains two intramolecular disulfide bonds and is connected to chain A through two intermolecular disulfide bridges shown in the figure.[81] Mabinlin-II was estimated to be about 400 times sweeter than sucrose on a weight basis, which makes it less sweet than thaumatin (3000 times), although it elicits a similar sweetness profile. (Mabinlin-II is 375 times sweeter than sucrose on a molar basis and 10 times sweeter on a weight basis, and therefore mabinlin is not as sweet as other sweet proteins.) It has also been suggested that the difference in the heat stability of the different mabinlin homologues is due to the presence of an arginine residue (heat-stable homologue) or glutamine (heat-unstable homologue) at position 47 in chain B.[82] It has been reported that the precursor of mabinlin-II is a single-chain protein composed of 155 amino acid residues.[83]

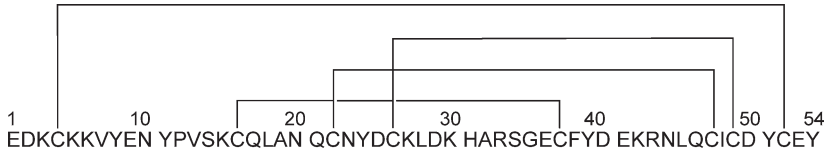


Mabiblin II

4.16.2.2.4 Brazzein

Brazzein is a sweet protein that was isolated from the fruit of the West African climbing plant Oubli (*Pentadiplandra brazzeana* Baillon). Along with pentadin, which was discovered in 1989, brazzein is the second sweet protein that was discovered in this fruit. Like other natural sweet proteins such as monellin and thaumatin, it is highly sweet. On a weight basis, brazzein is 500 times sweeter than sucrose when compared to 10% sucrose solution and 2000 times sweeter when compared to 2% sucrose solution. Its sweet perception is more similar to that of sucrose than that of thaumatin, and it presents a clean sweet taste with a lingering aftertaste. Brazzein is stable over a broad pH range from 2.5 to 8 and is heat stable at 80 °C for 4 h.[84]

The monomer protein, consisting of 54 amino acid residues with eight disulfide bonds shown in the figure,[84,85] is the smallest among the sweet proteins, with a molecular mass of 6.4 kDa. Chemical synthesis[86] of brazzein was performed and recombinant proteins were successfully produced by *Escherichia coli*.[87] Based on X-ray analysis[88] and NMR studies,[89] residues 29–33 and 39–43, plus residue 36, as well as the C-terminus were found to be involved in the sweet taste of the protein. The charge of Arg-43 in the protein also plays an important role in its interaction with the sweet taste receptor.[90]



Brazzein

4.16.2.2.5 Pentadin

Pentadin is a sweet-tasting protein that was isolated from the fruit of Oubli (*P. brazzeana* Baillon), a climbing shrub that is native to West African countries. Pentadin's molecular mass is estimated to be 12 kDa. It is reported to be 500 times sweeter than sucrose on a weight basis. The primary structure has not yet been determined, although amino acid analyses were carried out.[91]

4.16.2.3 Sweetness-Inducing Proteins and Substances

4.16.2.3.1 Miraculin

Miraculin is a basic glycoprotein that was extracted from the miracle fruit plant, a shrub that is native to West Africa (*Synsepalum dulcificum* or *Richadella dulcifica*). Miraculin itself is not sweet, but the human tongue, once exposed to miraculin, perceives ordinarily sour foods, such as citrus, as sweet for up to 2 h afterward. This small red berry has been used in West Africa to improve the taste of acidic foods. Since the miracle fruit itself has no distinct taste, this taste-modifying function of the fruit had been regarded as a miracle. The active substance, isolated by Kurihara, was named miraculin after the miracle fruit.[92] Miraculin was first sequenced in 1989 and was found to be a glycoprotein consisting of 191 amino acids and some carbohydrate chains.[93] The molecular mass of the glycoprotein is 24.6 kDa, including 3.4 kDa (13.9% of the weight) of sugar consisting (on a molar basis) of glucosamine (31%), mannose (30%), fucose (22%), xylose (10%), and galactose (7%). The sugar is linked with Asp-42 and Asp-186.

Miraculin occurs as a tetramer (98.4 kDa), a combination of four monomers grouped into dimers. Within each dimer, two miraculin glycoproteins are linked by an intramolecular disulfide bridge.[92–94] The formation of three intrachain disulfide bridges at Cys-47–Cys-92, Cys-148–Cys-159, and Cys-152–Cys-155, and one interchain disulfide bridge at Cys-138 was determined by amino acid sequencing and a composition analysis of cystine-containing peptides isolated by high-performance liquid chromatography (HPLC). It was concluded that native miraculin in pure form is a tetramer of a 25 kDa peptide and native miraculin in a crude state or denatured, nonreduced miraculin in pure form is a dimer of the peptide. Both tetramer miraculin and native dimer miraculin in a crude state have taste-modifying activity.

A cDNA clone encoding miraculin was isolated and sequenced. The encoded precursor of miraculin was composed of 220 amino acid residues, including a possible signal sequence of 29 amino acids.[95,96] Attempts have been made to express these proteins in *E. coli*, yeast, and tobacco. While it was confirmed that these proteins were expressed in these organisms, miraculin expressed in *E. coli* and yeast showed no activity.[97] The perception of 0.1 mol l^{-1} of citrate after taking 1 μmol l^{-1} of miraculin corresponds to the sweetness of 0.4 mol l^{-1} of sucrose, which means that it is 400 000 times sweeter than sucrose on a molar basis. Interestingly, a mixture of miraculin with citrate did not elicit sweetness.[97] Miraculin was denied approval for this purpose by the FDA. Miraculin also has no legal status in the European Union.

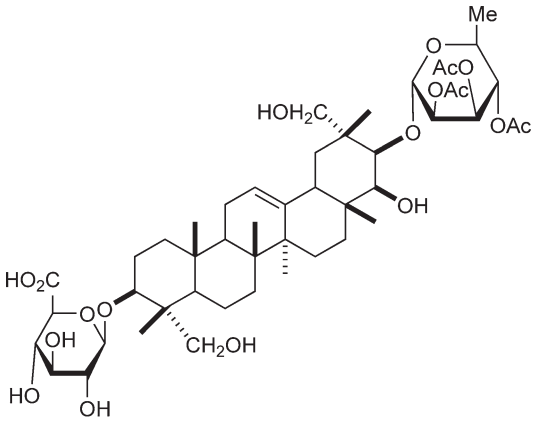
4.16.2.3.2 Curculin and neoculin

Curculin is a sweet protein that was isolated in 1990 from the fruit of *Curculigo latifolia* (Hypoxidaceae), which grows in Malaysia. Like miraculin, curculin exhibits taste-modifying activity. However, unlike miraculin, it also elicits a sweet taste by itself. After the consumption of curculin, water and sour solutions taste sweet. Curculin was reported to be a homodimer of two proteins connected through two disulfide bridges.[98] The molecular mass of curculin monomer is reported to be 12 kDa. It contains a sequence of 114 amino acids with an isoelectric point of 7.1. Curculin was crystallized and an X-ray structural analysis was performed.[99] Although both curculin and miraculin elicit a sweet taste, the two proteins are not homologous. However, based on an enzyme immunoassay and immunoblot analysis of curculin, curculin and miraculin showed cross-reactivity to both antibodies, and therefore it is considered that a common structure elicits sweet activity for both proteins.[100] Curculin is rather stable in acidic solution, and heating at 55 °C for 1 h does not reduce its potency. A 10 μmol l^{-1} curculin solution is as sweet as a 0.35 mol l^{-1} solution of sucrose. Thus, curculin is 35 000 times sweeter than sucrose. After curculin is held in the mouth for a short time, the sweet taste diminishes. However, the sweet taste is elicited again upon exposure to clear water. This can be explained as follows.[101] The sweet taste is suppressed by the reaction of curculin with divalent cations (Ca^{2+} and Mg^{2+}) in saliva. Upon exposure to water, the sweet taste is induced again since water washes the cations away. It is believed that the taste-modifying

protein strongly binds to the membrane surfaces of the taste cells in the presence of an acid such as citric acid.[102] In 1997, curculin was expressed in *E. coli* and yeast, but recombinant curculin did not exhibit 'sweet-tasting' or 'taste-modifying' activity.[103] Recently, neoculin, which is composed of an acidic glycoprotein subunit with 113 amino acid residues and a basic curculin subunit, was isolated from the same fruit as a heterodimer.[104] Another group successfully cloned curculin-2, which is highly homologous to curculin, and demonstrated that the heterodimer of curculin and curculin-2 elicits intense sweetness.[105] These results suggest that curculin itself may also be a heterodimer.

4.16.2.3.3 Strogin

Five new oleanane-type triterpene glycosides, strogins I–V, were isolated by Kurihara and coworkers[106] from the leaves of *Staurogyne merguensis* Kuntze, which is native to Malaysia. Strogin itself has a sweet taste. In addition, after strogins I, II, and IV are held in the mouth, the sweet taste can be recovered with exposure to water, as with curculin. In contrast, strogins III and V had no such activity. A 1.0 mmol l^{-1} strogin solution is as sweet as a 0.15 mol l^{-1} solution of sucrose. Furthermore, sweetness corresponding to 300 mmol l^{-1} sucrose is induced by holding 1.0 mmol l^{-1} strogin followed by water. Strogin's sweetness induction was temperature-dependent[107] while curculin's sweetness induction was independent of temperature. While Ca^{2+} and Mg^{2+} suppressed curculin's induction, strogin's induction was not suppressed by divalent cation. Thus, the mechanism of sweetness induction by strogin is different from that of curculin.

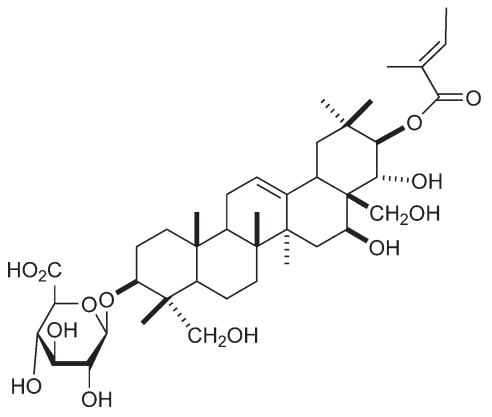


Strogin II

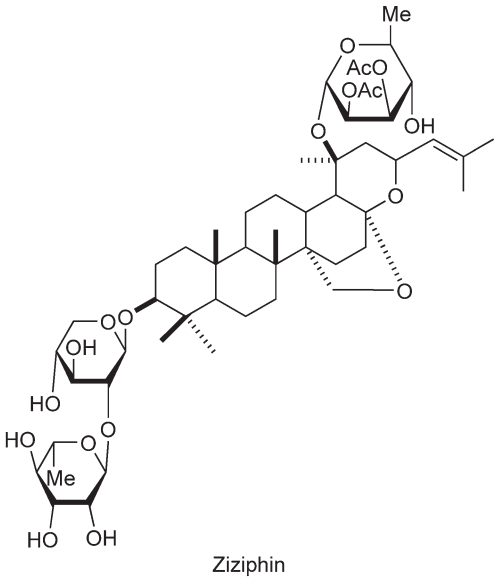
4.16.2.4 Antisweet Substances

4.16.2.4.1 Gymnemic acid

Gymnemic acids were isolated from the leaves of *Gymnema sylvestre* (Asclepiadaceae), which is native to India and southern China.[108,109] Gymnemic acids are glycosides of triterpene that suppress sweetness in humans. After the leaves are chewed, solutions that have been sweetened with sucrose taste like water. It is thought that gymnemic acid inhibits the binding of a sweet substance to the sweet receptor. Several gymnemic acid homologues with different acyl groups were purified from the leaves of *G. sylvestre* and their structures were determined. Interestingly, deletion of the acyl group diminishes the antisweet activity.[110] It suppresses the sweetness of most of sweeteners, including intense artificial sweeteners such as aspartame and natural sweeteners such as thaumatin, a sweet protein. The herb is traditionally used for the treatment of diabetes in India and Gymnema extracts are sold in Japan for the control of obesity.



Gymnemic acid I



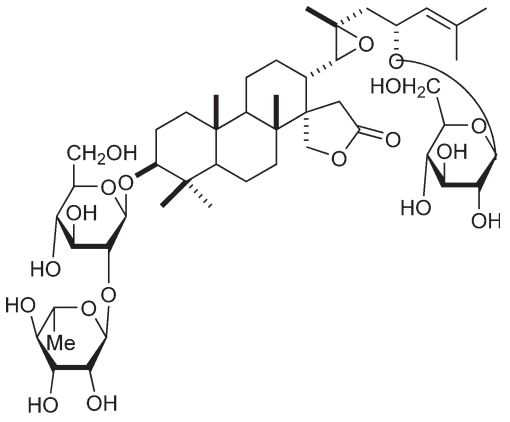
Ziziphin

4.16.2.4.2 Ziziphin

The sweetness-inhibiting substance ziziphin was isolated from the leaves of the plant *Zizyphus jujuba* (Rhamnaceae), which is native to China, by Kurihara *et al.*[111,112] Like gymnemic acids, ziziphin is a glycoside of triterpene that suppresses sweetness in humans. Removal of the acyl group under mild hydrolytic conditions led to complete abolishment of its antisweet activity.

4.16.2.4.3 Hodulcin

Hodulcin was extracted from the leaves of *Hovenia dulcis* (Rhamnaceae), which is native to China and Japan, and has been shown to selectively reduce sweetness perception in humans.[113] Hodulcin appears to be a triterpene glycoside, as are the gymnemic acids and ziziphins. NMR spectra indicated that the aglycon structure of hodulcin is different from that of gymnemic acid and similar to but not the same as that of ziziphin. Later, Arihara and coworkers[114] isolated five new dammarane glycosides named hodulosides I–V (e.g., hoduloside I) from the fresh leaves of *H. dulcis*. Their structures were determined on the basis of chemical and spectral evidence. All the compounds showed antisweet activity.



Hoduloside I

4.16.3 Bitter-Tasting Natural Products

There are many bitter-tasting compounds in nature.[115,116] Many of them are alkaloids, terpenoids and their glycosides (saponins) as well as amino acids and peptides. The threshold values of bitter compounds, as represented by alkaloids such as quinine, are extremely low (as low as ppm concentration) compared with compounds that elicit other basic tastes. It is considered that many bitter compounds possess some toxicity and therefore animals have become highly sensitive at tasting such bitter compounds. In many cases, an appropriate amount of a bitter-tasting compound can have a useful pharmacological effect. For example, it has long been known that the bitter-tasting component extracted from *Gentiana lutea* (Gentianaceae) can directly stimulate acid production by the gastric mucosa.[117]

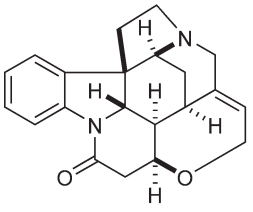
The bitter taste in foods is not always disliked by people. There are unexpectedly many cases in which a bitter taste has a positive effect of adding richness to food such as beer, coffee, and green tea. In these cases, if the bitter taste is eliminated or replaced by other compounds, the intrinsic value of the food might be completely lost. Therefore, the bitter taste is essential for these foods. On the contrary, however, methods to eliminate the unpleasant bitterness of cheese and grapefruit have also been investigated. These efforts also contribute to the progress in research on the bitter taste.[118]

Intensive studies on the bitter taste receptor are also in progress, as with the sweet taste receptor.[119] In fact, it has been reported that T2R receptors are necessary and sufficient for the detection and perception of bitter compounds.

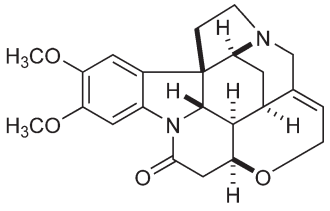
4.16.3.1 Bitter-Tasting Alkaloids

4.16.3.1.1 Strychnine

Strychnine is a highly toxic (LD_{50} i.v. in rats: 0.96 mg kg^{-1}) alkaloid that was isolated from the seeds of *Strychnos nux-vomica*, which is native to India and East Asia, and named after the tree. The structure of strychnine was determined by Woodward in 1948.[120] The total synthesis was also achieved by many chemists including Woodward. While it is barely soluble in water, its hydrochloride and nitric acid salts are water soluble. Strychnine elicits an intense bitter taste with a threshold of 0.000 001 6.[115] In India, China, and Japan, the seeds have long been used as a bitter-tasting gastric medicine, Vomica. Strychnine has also been used as a pesticide, particularly for killing small vertebrates such as rodents. Strychnine causes muscular convulsions and eventually death through asphyxia or sheer exhaustion.[121] At present, it is not used for clinical treatment, but is used in a pharmacological test as an analeptic. In nature, strychnine is produced by biosynthesis from tryptophan.



Strychnine



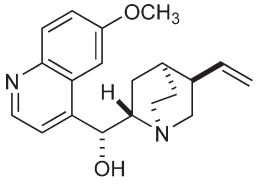
Brucine

4.16.3.1.2 Brucine

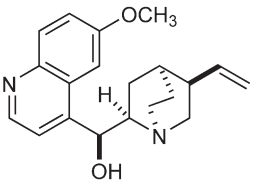
Brucine was also extracted as a principle, along with strychnine,[122] from the seeds of *S. nux-vomica*. Brucine is thought to be the most bitter-tasting alkaloid with a threshold of 0.000 000 7.[115] It is used for the chiral resolution of optically active carboxylic acids by diastereomeric salt formation. Brucine is isostructural to strychnine with methoxy groups, rather than hydrogen, at the 9- and 10-positions of the aromatic ring. Brucine is reported to be less toxic than strychnine. Nevertheless, a human consuming more than 2 mg of pure brucine will almost certainly suffer symptoms similar to strychnine poisoning.[123]

4.16.3.1.3 Quinine

Quinine was first extracted from the bark of the South American cinchona tree and isolated. In 1944, the total synthesis of quinine was achieved by Woodward and Doering.[124] Quinine exhibits specific toxicity against Plasmodium and has antipyretic (fever-reducing) activity. Therefore, it has long been used as an antimalarial drug. Although many other antimalarial drugs such as chloroquine have been developed based on the structure of quinine, it is still widely used since it is the sole compound to which Plasmodium has no resistance. Before 1820, the bark was first dried, ground to a fine powder, and then mixed into a liquid (commonly wine), which was then drunk. Quinine is a flavor component of tonic water, bitter lemon, vermouth, and cocktails. In the United States, the FDA limits quinine in tonic water to 83 ppm. Quinine is used as a standard substance for a bitter taste (threshold of sulfate salt: 0.000 008) in gustatory physiology.[125] It is also useful as an optical resolution agent and as an asymmetric catalyst.[126,127] Quinidine, a diastereomer of quinine, is also extracted from the bark and elicits a bitter taste. It is reported to have an antiarrhythmic effect.[128]



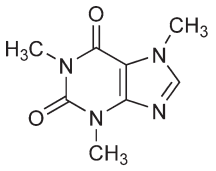
Quinine



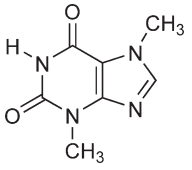
Quinidine

4.16.3.1.4 Caffeine

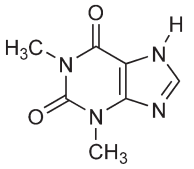
Caffeine is a bitter-tasting purine alkaloid.[129] Its threshold (0.000 7) indicates that the bitter intensity of caffeine is weaker than quinine and brucine. Caffeine was extracted from coffee and named after it. Caffeine is also contained in cola, black tea, green tea, cocoa, chocolate, and so on. It is well known that caffeine has antihypnotic, antipyretic, and diuretic effects.[130] Caffeine is a central nervous system and metabolic stimulant and is also used medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs. Sometimes, caffeine is incompatible with other drugs. For example, it has been reported that cimetidine decreased the systemic clearance of caffeine. Overdosing of caffeine may lead to a condition known as caffeinism.[131] Caffeinism usually combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, tremulousness, muscle twitching (hyperreflexia), insomnia, headaches, respiratory alkalosis, and heart palpitations. Today, the global annual consumption of caffeine has been estimated at 120 000 tons. The US FDA lists caffeine as a Multiple Purpose GRAS Food Substance.



Caffeine (coffee)



Theobromine (cocoa)



Theophylline (green tea)

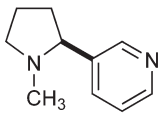
4.16.3.1.5 Theobromine and theophyllin

Theobromine was isolated from the seeds of the cacao tree and then shortly afterward was synthesized from xanthine by Fischer.[132] Theobromine is the primary bitter-tasting alkaloid found in cocoa and chocolate; chocolate contains 0.5–2.7% theobromine. Theobromine is water insoluble and is an isomer of theophylline as well as paraxanthine. Theobromine is categorized as 3,7-dimethylxanthine while theophylline is 1,3-dimethyl-7*H*-purine-2,6-dione and paraxanthine is 1,7-dimethylxanthine. Theophylline is known to be a bitter-tasting principle of green tea. Theobromine is used as a vasodilator (a blood vessel widener), as an aid in urination, and

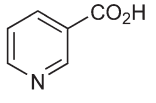
as a heart stimulant. Although the theobromine content in chocolate is small enough to be safely consumed by humans, it is reported that animals such as dogs metabolize theobromine more slowly and may succumb to theobromine poisoning.[133]

4.16.3.1.6 Nicotine

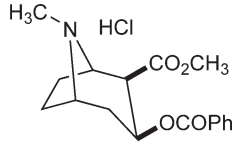
Nicotine is a bitter-tasting alkaloid found in the nightshade family of plants (Solanaceae), predominantly in tobacco. At low doses (an average cigarette yields about 1 mg of absorbed nicotine), nicotine acts as a stimulant in mammals and is one of the main factors responsible for the dependence-forming properties of tobacco smoking.[134] The biosynthesis is carried out from tryptophan via nicotinic acid. Nicotinic acid reacts with a piperidine compound derived from lysine to give anabasine as a homologue of nicotine. Nicotine exists in tobacco leaves as salts with malic acid or citric acid. There are more than 30 analogues of nicotine. Nicotine is essential for the synthesis of a water-soluble vitamin, nicotinic acid (niacin). Nicotine has an addictive nature and has been found to activate reward pathways – the circuitry within the brain that regulates feelings of pleasure and euphoria. The LD_{50} of nicotine is 3 mg kg^{-1} for mice, and 40–60 mg (0.5–1.0 mg kg^{-1}) for adult humans can be a lethal dosage.[135] This means it is deadly poisonous. It is more toxic than many other alkaloids such as cocaine, which has an LD_{50} of 95.1 mg kg^{-1} when administered to mice.



Nicotine



Nicotinic acid



Cocaine

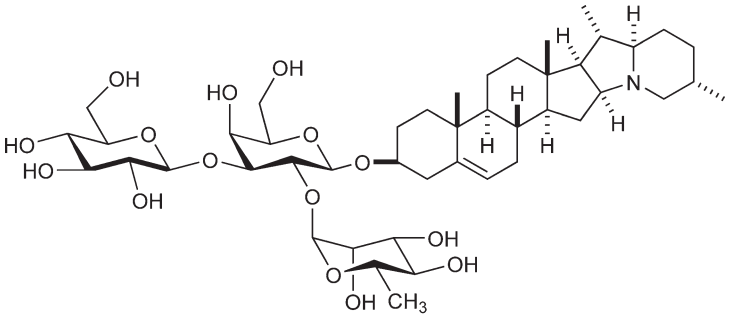
4.16.3.1.7 Cocaine

Cocaine is a bitter-tasting alkaloid extracted from the leaves of the coca plant (*Erythroxylon coca* Lam.).[136] It has a tropane skeleton and is synthesized from ornithine in nature. In medicine, cocaine is used in a limited manner as a topical anesthetic for nasal and lacrimal duct surgery. Cocaine is a potent central nervous system stimulant. Its effects can last from 20 min to several hours, depending upon the dosage. The initial signs of stimulation are hyperactivity, restlessness, increased blood pressure, increased heart rate, and euphoria. The euphoria is sometimes followed by feelings of discomfort and depression and a craving to experience the drug again. Side effects include twitching, paranoia, and impotence. Cocaine addiction is reported to be physical and psychological dependence may develop with regular use. It may result in physiological damage, lethargy, depression, or a potentially fatal overdose.[137] Therefore, its possession, cultivation, and distribution are illegal for nonmedicinal and non-government-sanctioned purposes in virtually all parts of the world. On the contrary, coca herbal infusion (referred to as Coca tea) is used in coca-leaf-producing countries much as any herbal medicinal infusion would be elsewhere in the world. The free and legal commercialization of dried coca leaves in the form of tea bags to be used as 'coca tea', as a drink with medicinal powers,[138] has been actively promoted in Peru and Bolivia. It is also used to help visitors overcome the malaise of high-altitude sickness.

4.16.3.1.8 Solanine

Solanine is a bitter-tasting steroidal alkaloid saponin that has been isolated from all nightshades, including tomatoes, capsicum, tobacco, and eggplant.[139] However, the most widely ingested solanine is from the consumption of potatoes. Potato leaves, stems, and shoots are naturally high in this saponin. When potato tubers are exposed to light, they turn green and increase saponin production. This is a natural defense mechanism to prevent the uncovered tuber from being eaten. It is very toxic even in small quantities. The poisoning is primarily manifested by gastrointestinal and neurological disorders.[140] Symptoms include nausea, diarrhea, vomiting, stomach cramps, burning of the throat, heart arrhythmia, headache, and dizziness. Hallucinations, loss of sensation, paralysis, fever, jaundice, dilated pupils, and hypothermia have been reported in more severe cases. It is suggested that doses of 200–400 mg for adult humans can cause toxic

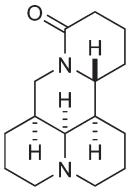
symptoms (20–40 mg for children). Most commercial potatoes have a solanine content of less than 0.2 mg g^{-1}.[141] However, potatoes that have been exposed to light and have started to turn green can show higher concentrations.



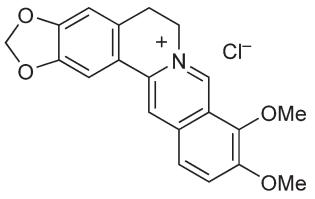
Solanine

4.16.3.1.9 Matrine and berberine

An herbal medicine, kushen, is obtained from the roots of *Sophora japonica* (*Sophora flavescens*) and is used as an anti-inflammatory and bitter-tasting stomachic. The principal component of kushen is the piperidine alkaloid matrine.[142] Berberine, which is isolated from the bark of *Phellodendron amurense* (Rutaceae) as well as *Coptis japonica* Makino (Ranunculaceae), is also an intensely bitter-tasting alkaloid that is named after Berberis.[143] There are a variety of known derivatives such as berberine hydrochloride, hydrosulfate, and tannic acid salt according to the kind of counteranion, and all are used as stegnotic agents. There is some controversy regarding the antibiotic activity of berberine. In Japan, berberine hydrochloride is available commercially. It is contraindicated for use in hemorrhagic colitis and bacteriogeneous diarrhea, since it might make the symptoms worse and prolong the duration of treatment.



Matrine



Berberine chloride

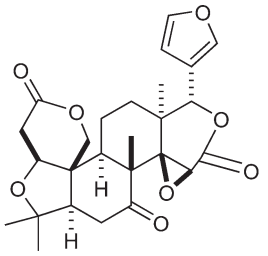
4.16.3.2 Bitter-Tasting Terpenoids

There are many bitter-tasting terpenoids in the plant kingdom. Among them, limonoids in citrus fruits, the cucurbitacin in members of the family Cucurbitaceae, and humulon analogues in hop have been intensively investigated. The structure–activity relationships of bitter-tasting terpenes have been studied for many years, and Kubota[144] and Beets[145] have proposed hypotheses.

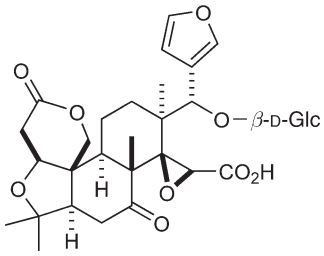
4.16.3.2.1 Limonoids

Several limonoids are known to be bitter principles of citrus (Rutaceae). A typical example is limonin. Although fresh juice does not elicit a bitter taste, sometimes it becomes bitter after heating or storage. This is explained by the formation of bitter-tasting limonin by deglycosylation and further cyclization from limonin glucoside, which is present in citrus fruit tissue and seeds and does not exhibit bitterness.[146] Recently, it was reported that limonin had antitumor activity.[147] Besides limonin, nomilin and obakunone, which are considered to be

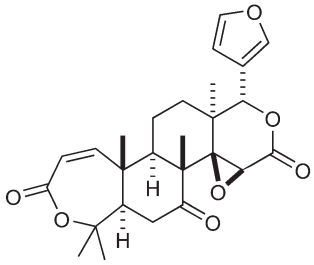
biosynthetic intermediates of limonin, have also been isolated as bitter-tasting components.[148] These three limonoids are formed from squalene via cyclization and oxidation.



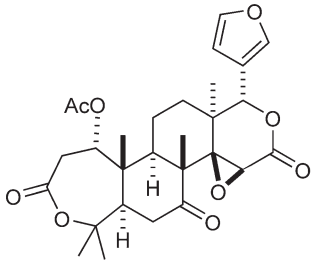
Limonin



Limonin glucoside



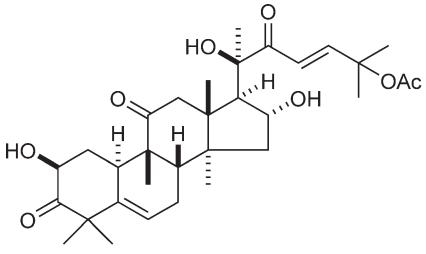
Obacunone



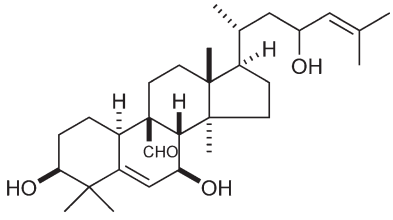
Nomilin

4.16.3.2.2 Cucurbitacin and momordicine

Cucurbitacin is a bitter-tasting principle that can be isolated from members of the family Cucurbitaceae, such as cucumber (*Cucumis sativus*) and melon (*Cucumis melo* L.). In particular, cucurbitacin[149] and momordicine,[150] which have an intensely bitter taste, are contained abundantly in *Momordica charantia* (bitter melon in English, go-yaa in Okinawa, Japan), which people enjoy due to its bitterness. There are more than 18 kinds of cucurbitacin, and among them cucurbitacin B is a typical component. It has been reported that cucurbitacin exhibits anticancer activity.[151] In addition, it is used for the treatment of hepatic disease in traditional Chinese medicine. It is also found in some herbal teas.



Cucurbitacin B

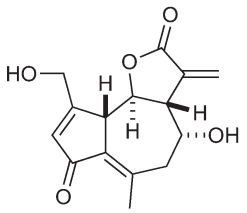


Momordicine I

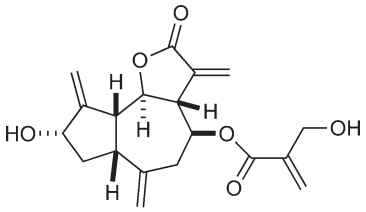
4.16.3.2.3 Lactucin and cynaropicrin

Lactucin is a bitter principle of the leaf vegetable chicory (*Cichorium endivia*), which is cooked or used for salads in western Europe.[152] It is also contained in the form of *p*-hydroxyphenyl acetate as lactucopicrin, which is known to have a sedative effect on the central nervous system.[153] On the contrary, a bitter-tasting sesquiterpene

lactone, cynaropicrin, can be isolated from artichoke (*Cynara scolymus*). Cynaropicrin has been reported to exhibit immunomodulatory effects.[154]



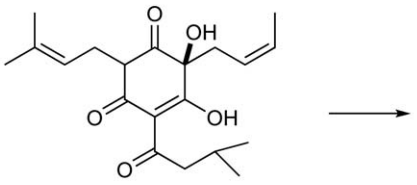
Lactucin (chicory)



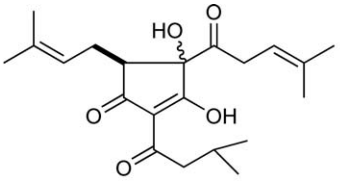
Cynaropicrin (artichoke)

4.16.3.2.4 Humulone

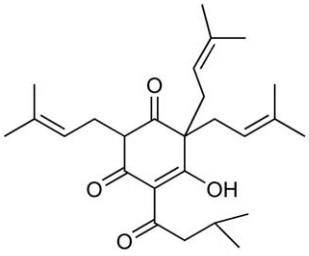
Humulone is a well-known bitter principle of beer. At least 32 derivatives of humulone such as luplone, cohumulone, and adhumulone have been isolated, many of which are not contained in hop (*Humulus lupulus*) but rather are formed during fermentation and preservation.[155] For example, humulone is transformed into isohumulone to elicit a bitter taste in beer. Hop (*Humulus*) is a small genus of flowering plants, native to the temperate Northern Hemisphere. The female flowers, commonly called hops, are used as flavoring and stabilizers during beer brewing. Hop is part of the family Cannabaceae, which also includes the genus *Cannabis* (also known as hemp). Beer has been used as folk medicine in Europe for a long time due to its diuretic and stomachic effect. Recently, new medicinal uses and properties of humulone derivatives are being explored.



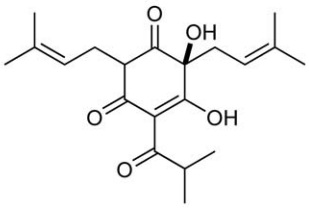
Humulone



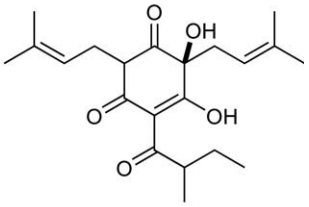
Isohumulone



Lupulone



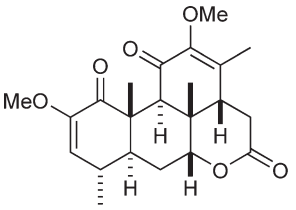
Cohumulone



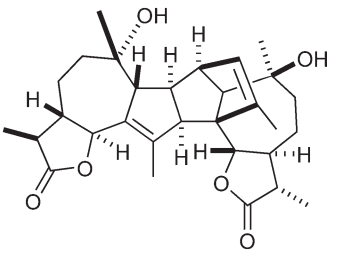
Adhumulone

4.16.3.2.5 Quassin

Quassin is a bitter-tasting substance that can be extracted from the quassia tree (bitter tree, *Picrasma quassioides* Benn).[156] It is said to be the most bitter substance found in nature. Quassin is used in traditional Chinese medicine. Besides quassin, modified triterpenes, the so-called quassinoid, are the principal component of the bitter taste.



Quassin



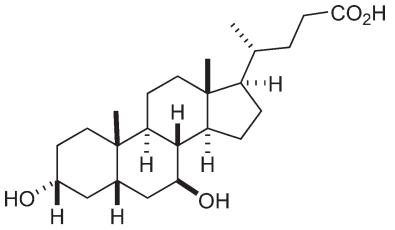
Absinthin

4.16.3.2.6 Absinthin

Absinthin is one of the most bitter substances known and is extracted from various plants of the genus *Artemisia*, but most commonly absinth wormwood.[157] The simple maceration of wormwood in alcohol without distillation produces an extremely bitter drink because of the presence of water-soluble absinthin. Wormwood extract can cause renal failure and death due to excessive amounts of thujone, which in large quantities acts as a convulsive neurotoxin. Absinthe was once portrayed as a dangerously addictive, psychoactive drug; thujone was blamed for most of its deleterious effects. Therefore, it was prohibited in several European countries and the United States. However, it has since been verified that no evidence shows it to be anymore dangerous or psychoactive than ordinary alcohol. An absinthe revival began in the 1990s, as countries in the European Union began to reauthorize its manufacture and sale.

4.16.3.2.7 Ursodiol

Ursodeoxycholic acid (ursodiol), which is found in large quantities in bear bile, and which elicits an intense bitter taste, has long been used in traditional Chinese medicine not only as a stomachic but also as a universal drug for the alimentary system.[158] Currently, the drug is generally derived by chemical synthesis rather than from animals.



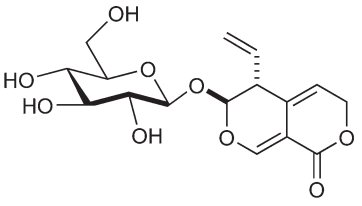
Ursodeoxycholic acid

4.16.3.3 Bitter-Tasting Saponins

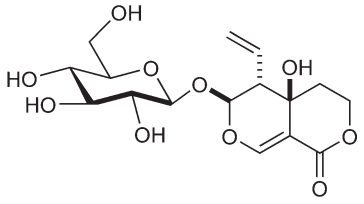
4.16.3.3.1 Secoiridoid saponins

Senburi is a biennial herb, *Swertia japonica* (*Ophelia japonica*), Gentianaceae, that is native to Japan.[159] Senburi is considered as one of the most popular medicinal herbs in Japan and is the most bitter Japanese herb. Senburi, also called 'touyaku' in traditional medicine, literally means 'still bitter after one thousand times infusion'. It is used for the treatment of gastrointestinal disease, diarrhea, and bellyache, and also as a digestive stimulant. The principal components of Senburi are bitter-tasting saponins such as swertiamarin, sweroside, amarogentin, amaroswerin, and gentiopicroside. Among them, amaroswerin is one of the most bitter-tasting natural products.[160]

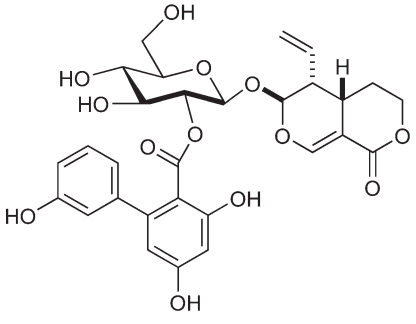
Gentiopicroside is also contained in the herbal medicine Gentiana (Ryutan), which is the extract of the root of *Gentiana lutea* and has gastroprotective effects.[117] It has a secoiridoid structure, which is a common constituent in members of the family Gentianaceae.



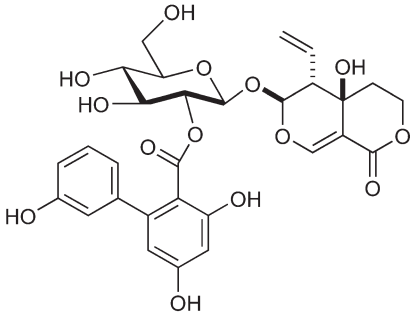
Gentiopicroside



Swertiamarin



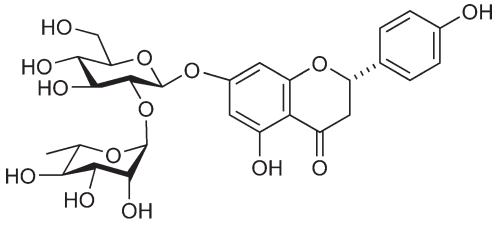
Amarogentin



Amaroswerin

4.16.3.3.2 *Flavonoid glycoside*

Naringin is a flavonoid glycoside that is abundantly contained in the skin of grapefruit and orange and is the origin of their bitterness.[161,162] Its aglycon is naringenin, which is synthesized by a shikimic acid pathway and occurs naturally in citrus fruits. To remove the bitterness of naringin in the production of canned citrus juice, an enzymatic hydrolysis process using naringinase is sometimes employed.[163] It has been reported that naringin exerts a variety of pharmacological effects such as antioxidant activity, anticarcinogenic activity, and inhibition of selected cytochrome P450 enzymes, which may result in several drug interactions *in vitro*. However, this notion is still controversial.[164]



Naringin

4.16.3.4 Bitter-Tasting Amino Acids and Peptides

4.16.3.4.1 *Amino acids*

Ever since Fischer, many chemists have focused their attention on the taste of amino acids. Generally, natural L-amino acids exert either no taste or a bitter taste while unnatural D-amino acids elicit a sweet taste almost without exception. Proteinogenic L-amino acids that exhibit a bitter taste include Trp (0.133%), Phe (0.069%), Tyr (0.017%), Leu (0.011%), Arg, Val, Ile, and Pro, and the remaining amino acids exert either no taste or a sour taste. The values in parentheses show the caffeine concentration that provides the same bitterness as a 0.3% amino acid solution.[165] However, different authors have reported different values for the strength of their

bitterness. A basic amino acid, arginine, exhibits bitterness along with a sweet taste. Glycine and alanine elicit a sweet taste. The bitter taste of an amino acid will be enhanced with an increase in the bulkiness of any substituent.

4.16.3.4.2 Peptides

Studies of bitter-tasting peptides arose from interest in the bitter constituent of cheese. Murray reported that the bitter taste accumulated after casein was hydrolyzed by various proteases and the principal component of its bitterness was peptides.[166] Afterward, several groups successfully isolated the bitter peptides from the hydrolyzate of casein. For example, formation of the bitter peptide QNKIHPFAQTQSLVYPFPGPIP was identified during the maturation of cheddar cheese.[167] Furthermore, hydrolysis of the constituent with peptidase makes the bitter taste worse. On the contrary, Fujimaki and coworkers[168] successfully eliminated the bitter taste of soybean peptides using a plastein reaction. These peptides characteristically contain mainly hydrophobic amino acids as constituents. Interestingly, a diketopiperazine, cyclo (Trp-Leu),[169] exhibits a bitter taste regardless of the optical isomer and cyclo (Val-Phe) and cyclo (Pro-Phe) have been reported to be bitter substances formed from cacao beans during roasting.[170]

4.16.3.5 Masking the Bitter Taste

As described above, many natural products elicit a bitter taste. Thus, it is an important issue in the formulation of pharmaceuticals to determine how to mask this bitterness. Although this issue has been somewhat overcome by coating technology in the manufacture of tablets, pills, and granules, the method used in liquid medicines is still unsatisfactory, though some approaches that involve the addition of high concentrations of sugars and acids have been developed. Kurihara and coworkers[171] reported that the bitter taste in response to quinine could be selectively suppressed with phosphatidyl acid in phospholipids of soybean.

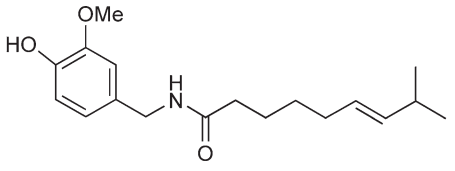
4.16.4 Pungent Natural Products

Pungency is generally associated with fiery stimuli and is sometimes an intolerable sensation for humans. However, it is effective at increasing appetite in many cases and is used in the cuisine of several cultures. Pungency is not one of the five basic tastes, but rather is characterized by trigeminal sensation in the mouth through the sensory modalities of touch, thermal sensation, and pain. There are several subtypes of pungency that basically depend on the chemical properties of the pungent constituents. In addition to pungency (hot sensation), a tingling effect and a cooling sensation on the tongue are important for cuisine. As a measure of the 'hotness', or more correctly, piquancy, of a chili pepper, the Scoville scale has been proposed.[172,173] Fruits of the *Capsicum* genus contain capsaicin, which stimulates chemoreceptor nerve endings in the skin, especially the mucus membranes. The number of Scoville heat units (SHU) indicates the amount of capsaicin present; however, it cannot be used as a measure of piquancy in foods without capsaicin.

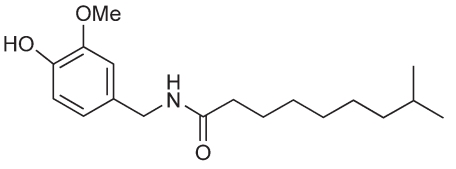
4.16.4.1 Capsaicin

Normally, spices used for cooking provide a pungent sensation. A typical pungent spice is red pepper (hot chili pepper), which contains amide derivatives, including capsaicin, as pungent constituents.[174] It elicits intense pungency and is an interesting compound from the viewpoint of its pharmacological action. Thus, it has been reported that capsaicin exhibits hypermetabolism as well as sweating by promoting the secretion of adrenaline.[175] In addition, it also has strong antibacterial activity along with an antisepsis function.[176] Capsinoids are amides of vanillylamine with a variety of aliphatic acids. Capsaicin is the main capsinoid in chili peppers, followed by dihydrocapsaicin. These two compounds have almost the same potency and other compounds such as nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin have been isolated as minor capsinoids. Dilute solutions of pure capsinoids produce different types of pungency; however, these differences were not noted using more concentrated solutions. Nakatani and Masuda.[177] isolated capsaicinol with only one hydroxy group in capsaicin. Intriguingly, they reported that capsaicinol did not exhibit pungency. Very recently, Yazawa

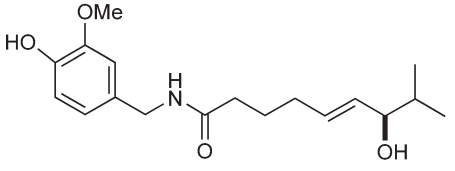
et al.[178] found that a nonpungent red pepper, CH-19 Sweet, contained only a trace amount of capsaicin. Instead, capsiate and dihydrocapsiate, which have an ester bond with vanillyl alcohol, were the major constituents of CH-19 Sweet.[179] Interestingly, the pungency disappeared when the amide was changed to an ester and this finding has attracted considerable attention to solve the mechanism of pungency perception, and intensive studies on the vanilloid receptor subtype 1 (VR1) are in progress.[180] Recently, it has also been shown that the glucosylation of capsaicin diminished its pungency.[181] Therefore, studies on glucosylation are ongoing. Studies on the prevention of obesity by capsinoid and capsaicin glucoside are also in progress.[182]



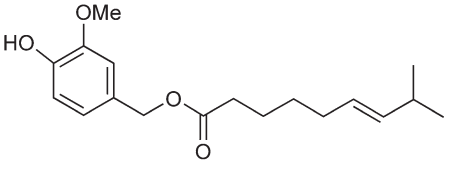
Capsaicin



Dihydrocapsaicin



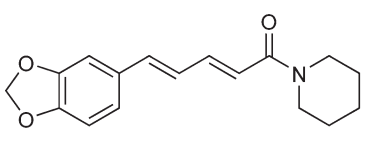
Capsaicinol



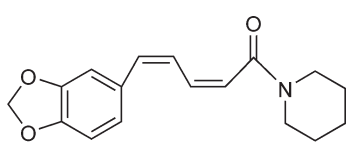
Capsiate

4.16.4.2 Piperine

Piperine is the alkaloid responsible for the pungency of black pepper, *Piper nigrum* (Piperaceae), and *Piper longum* L., commonly known as long pepper.[183] Three geometrical isomers of piperine (chavicine, isochavicine, and isopiperine) and piperanine (dihydro- form of piperine) are other constituents of these plants. Previously, chavicine was believed to cause the particular taste of pepper. However, it was reported later that only piperine has a strong pungent taste.[184] Although an increment in the three isomers in ground pepper after exposure to sunlight was observed by monitoring the MH^+ ion on liquid chromatography/atmospheric pressure chemical ionization mass spectrometer (LC/APCIMS), the degree of increase varied very little. Therefore, it is questionable whether the disappearance of the pungency in older ground pepper is derived from the formation of tasteless isomers by photochemical changes in piperine.[185] Piperine is a solid substance that is essentially insoluble in water. It is initially tasteless, but leaves a burning aftertaste. Piperine belongs to the vanilloid family of compounds, which also includes capsaicin, the pungent substance in hot chili peppers. Piperine may have bioavailability-enhancing activity for some nutritional substances and for some drugs.[186] It has putative anti-inflammatory activity and may be active at promoting digestive processes. Recently, with advances in analytical methods, many piperine derivatives have been isolated and their structures have been determined by Tsuda and coworkers.[187]



Piperine

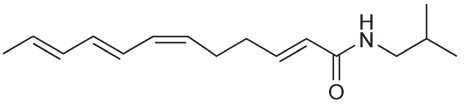


Chavicine

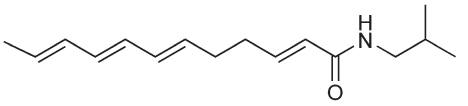
4.16.4.3 Sanshool and Gingerol

Sanshool is the pungent constituent in Sichuan pepper (Sansho in Japan).[188] It is contained in the outer pod of the tiny fruit of several species of the genus *Zanthoxylum* (most commonly *Zanthoxylum piperitum*, *Zanthoxylum simulans*, *Z. piperitum sansho*, and *Zanthoxylum schinifolium*), which are widely grown and consumed in Asia as a spice. Despite the name, it is not related to black pepper or chili peppers. It is widely used in the cuisine of Japan. Several sanshools have been isolated, such as α- and β-sanshool, both of which have an amide structure.[189] Sanshools exhibit several biological activities such as an insecticidal effect. A similar amide, spilanthol, is also found in the aerial part of *Spilanthes acmella* and *S. acmella* var. *oleracea*.[190]

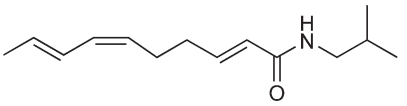
Gingerols are the pungent constituents of the rhizome of ginger, which is often used as a folk medicine and food.[191] Each gingerol has a phenol group as a substituent. The major constituent is 6-gingerol, which has vanillyl ketone (gingerone or zingerone) as a framework, similar to vanillylamine of capsaicin, but without an amide bond. Gingerone itself is also found in ginger root.[192] (6)-Shogaol is also found as a pungent component of ginger and is contained in semidried ginger but is rarely found in fresh ginger.[193]



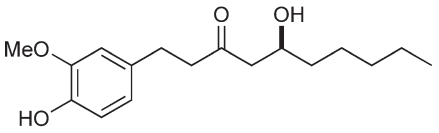
α-Sanshool



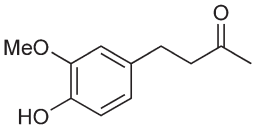
β-Sanshool



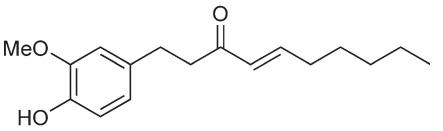
Spilanthol



(S)-(+)-[6]Gingerol



Gingerone (Zingerone)

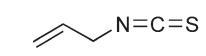


(6)-Shogaol

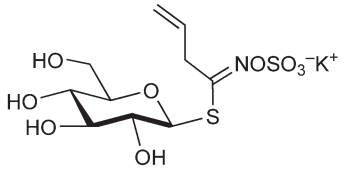
4.16.4.4 Isothiocyanates

Wasabi (*Wasabia japonica*, *Cochlearia wasabi*, or *Eutrema japonica*) is a member of the Brassicaceae family and is also known as Japanese horseradish. The rhizomes are used as a spice. Wasabi is enjoyed with sushi and sashimi, usually accompanied by soy sauce. It has an extremely strong and stimulating flavor with burning sensations. Its hotness is more akin to that of hot mustard than capsaicin in chili pepper, in that it produces vapors that irritate the nasal passages more than the tongue. Allyl isothiocyanate (AITC) and several other isothiocyanates are known to be the pungent constituents, and among them AITC is the main constituent (reported to be $\sim$2.0 g kg^{-1}).[194] It is thought to be beneficial to eat raw fish with wasabi because it has a sterilizing effect. AITC itself does not exist in wasabi, but it is formed immediately upon grating the root very finely. Thus, a glucosinolate (known as sinigrin) present in wasabi reacts with the enzyme myrosinase upon grating, and this leads to the production of AITC.[195] This suggests that sinigrin and myrosinase are present in different parts of the plant tissue. There are almost 30 known glucosinolates other than sinigrin, the biosynthetic pathway of which has also been clarified.[196] Based on its sterilizing effect, AITC is used in Japan to maintain freshness in

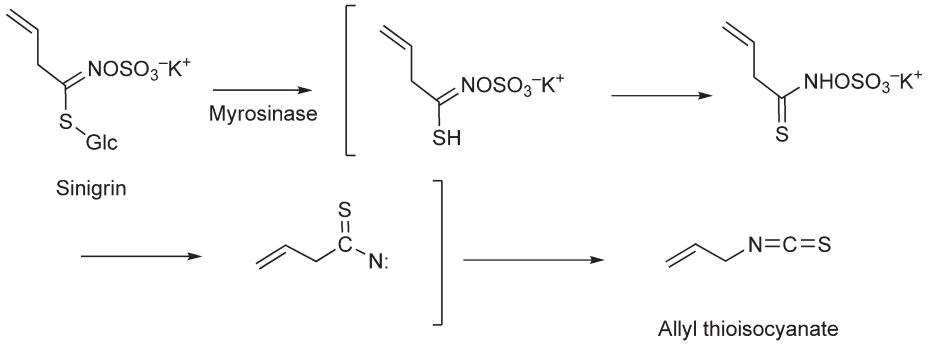
refrigerators. The pungent constituent of the horseradish root is also AITC, and methyl-3-butenyl isothiocyanate is the constituent in the Japanese radish.[197]



Allyl thioisocyanate
pungent taste of wasabi



Sinigrin

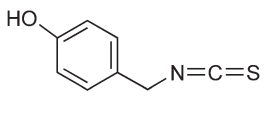


Sinigrin

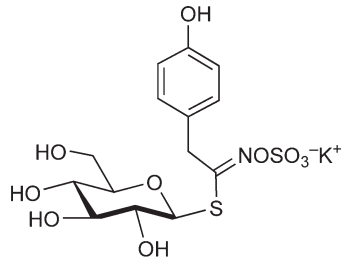
Allyl thioisocyanate

Formation mechanism of allyl thioisocyanate

Mustard is prepared from the ground seeds of mustard plants (white or yellow mustard, *Sinapis hirta*; brown or Indian mustard, *Brassica juncea*; or black mustard, *Brassica nigra*) by mixing them with water and adding ingredients such as flour. Mustard sensation can cause the eyes to water, burn the palate, and inflame the nasal passages. The pungent constituent of black mustard is sinigrin, as in wasabi, whereas that of white mustard is sinalbin.[198] In this case, *p*-hydroxybenzyl isothiocyanate is formed in the reaction with the enzyme myrosinase. The pungency of these spices is represented as hot in red (chili) and black pepper, and as sharp for the isothiocyanate family.



p-Hydroxybenzyl thioisocyanate
(pungent taste of white mustard)



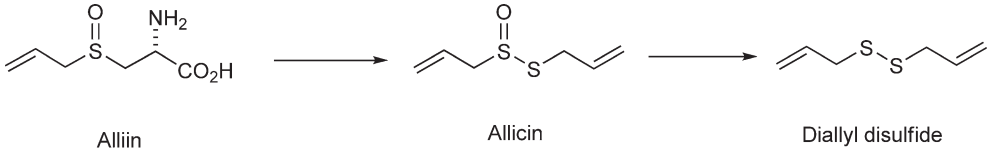
Sinalbin

4.16.4.5 Disulfides

Garlic, *Allium sativum* L., is a species in the onion family, Alliaceae. Onion, shallot, and leek are close relatives. Garlic has been used throughout history for both culinary and medicinal purposes. It has a pungent 'hot' sensation that mellows and sweetens considerably with cooking. A large number of sulfur compounds contribute to the smell and taste of members of the onion family. Diallyl disulfide and diallyl sulfide are

believed to be important odor and flavor components. Allicin has been found to be the constituent that is most responsible for the spiciness of raw garlic.[199] However, alliin, rather than allicin, is present in the plant before the cells are damaged. When a cell is broken, allicinase triggers the breakdown of alliin to give allicin, which has a strong smell and is converted to diallyl disulfide by reduction.[200]

The pungent constituents of onion are somewhat different from those of garlic and this accounts for their different odors and pungency. It has been reported that the major pungent constituents of onion are di-*n*-propyl disulfide and methyl-*n*-propyl disulfide.[201]



Formation mechanism of diallyl disulfide

4.16.5 Astringent Natural Products

4.16.5.1 Tannin

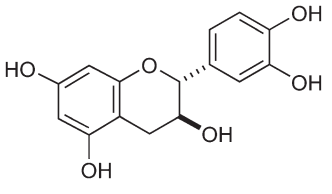
Tannins are astringent-tasting polyphenols found in plants that can bind and precipitate proteins. While the term tannin was originally derived from the use of tannins in tanning animal skins to make leather, the term is widely applied to any polyphenolic compound that forms strong complexes with proteins. The molecular weight of tannins ranges from 500 to more than 3000. There are two types of tannins, hydrolyzable tannins and condensed tannins.[202] At the center of a hydrolyzable tannin, there is a carbohydrate such as D-glucose. The hydroxyl groups of the carbohydrate are partially or totally esterified with phenolic acids such as gallic acid (in gallotannins). Hydrolyzable tannins can be hydrolyzed by weak acids or weak bases to produce carbohydrate and phenolic acids. Condensed tannins, known as proanthocyanidins, are widely distributed in plants and strongly affect the quality of foods. They are polymers of 2–50 (or more) flavonoid units, which are not susceptible to hydrolysis. Some very large condensed tannins are insoluble, while hydrolyzable tannins and most condensed tannins are soluble in water. All phenolic compounds are highly unstable and are rapidly transformed into various reaction products when the plant cells are damaged (for instance, during food processing), thus adding to the complexity of dietary polyphenol compounds. This accounts for the complexity of polyphenols in food.

Holding tannins in the mouth produces a strong sensation of astringency. It is believed that tannins bind to proteins in the tongue and mucous membrane in the oral cavity and then induce their denaturation.[203] This type of denaturation of proteins in the oral mucosa by tannins is called a shrinking effect. Strictly, it is considered that astringency is closely related to the pain and tactile sensation generated by protein denaturation. To feel the astringent effect of tannins, they must be dissolved in saliva. Accordingly, an increase in the molecular weight by polymerization causes a lack of astringency due to the insolubility of tannins. Some persimmons are highly astringent and therefore inedible due to the presence of soluble tannins. However, when they are dried or ripened, the astringency disappears due to the insolubility of tannins.[204] These condensed tannins consist of a high-molecular-weight- (∼15 000) polymer formed from catechins. They exhibit strong protein coagulation activity and therefore are used to clarify Japanese sake as well as a preservative.

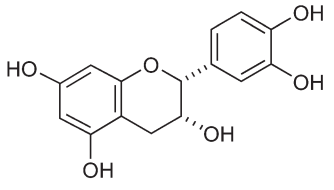
4.16.5.2 Catechins in Green Tea

Astringency in green tea (Japanese tea) is due to catechins, which are a kind of condensed tannins. The largest source is various teas derived from the tea plant *Camellia sinensis*. Catechins have various biological activities,

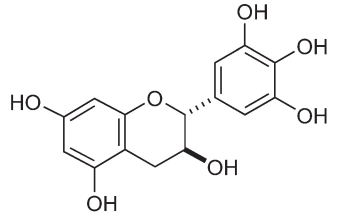
such as antibacterial and antiviral activities. The health benefits of catechins have been studied extensively in humans and in animal models. A reduction of carcinogenesis has been observed *in vitro*. The catechin content in the tea plant, and therefore its astringency, increases with an increase in exposure to sunlight. Although green tea contains several constituents, such as proteins, amino acids, vitamins, and caffeine, catechins are the major constituents and have attracted attention due to their health benefits. The catechins in green tea include epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), and catechin.[205] The most abundant (50%) of these is EGCG, which exhibits strong antioxidant activity.[206] Recently, an air filtration system against virus was developed in Japan using tea catechins.



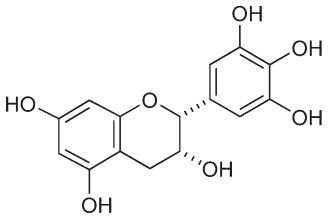
(+)-Catechin



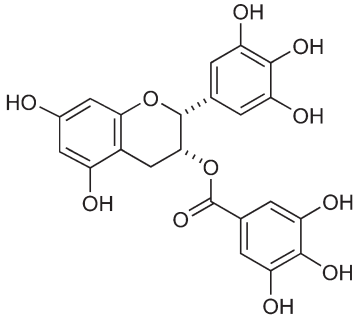
(–)-Epicatechin



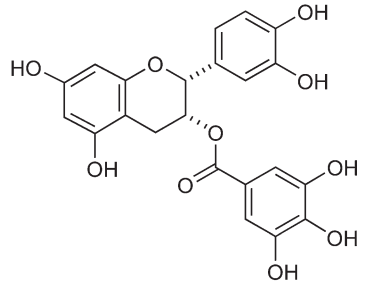
(+)-Gallocatechin



(–)-Epigallocatechin



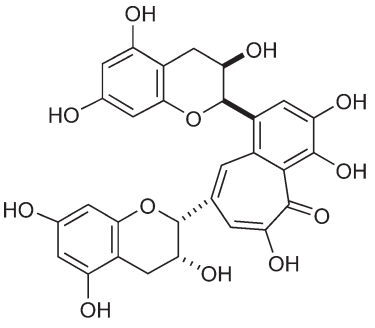
(–)-Epigallocatechin gallate



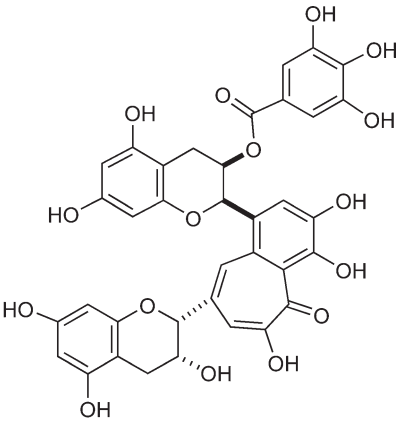
(–)-Epicatechin 3-gallate

4.16.5.3 Theaflavins and Thearubigins in Black Tea

Tannin constituents such as thearubigins and theaflavins present in black tea are formed by the enzymatic oxidation of EC and EGC followed by condensation, and this causes the characteristic astringency.[207] Theaflavins have benzotropolone structures and therefore give a red color in a black tea fusion. It has been reported that the relative proportions of theaflavins in black tea are theaflavin (18%), theaflavin-3-gallate (18%), theaflavin-3′-gallate (20%), theaflavin-3,3′-digallate (40%), and minor derivatives such as theaflavic acids.[208] These compounds are contained in oolong tea (a traditional Chinese tea), but are not as abundant as in green tea. It has been reported that theaflavins have various biological activities, such as antioxidant and anticancer activities. Thearubigins comprise 10–20 % of the dry weight of black tea. However, due to their high solubility in water, they account for 30–60% of the solids in black tea infusion. They are polymeric catechins that are formed during the enzymatic oxidation (called fermentation in the tea trade) of tea leaves. In contrast to theaflavins, thearubigins contain polysaccharides and proteins in the polymer. Gallic acid, cyanidins, and delphinidin are formed by acid hydrolysis and catechins are produced by reductive hydrolysis. These results indicate that thearubigins are a mixture of proanthocynidins containing flavonoid residues.[209]



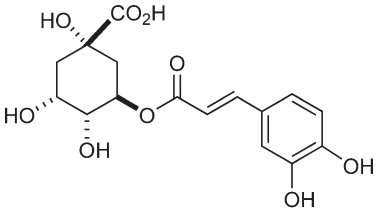
(–)-Theaflavin



Theaflavin 3′-gallate

4.16.5.4 Chlorogenic Acid in Coffee

Chlorogenic acid was isolated from green coffee beans.[210] It has also been found in the seeds and leaves of many dicotyledonous plants. It is thermally unstable and is readily decomposed to quinic acid and caffeic acid. Chlorogenic acid accounts for 5–10% of coffee beans, which is a much larger amount than caffeine (1–2%). Chlorogenic acid strongly influences the taste of coffee, such as astringent, sweet, and sour tastes, which change with the concentration. It is also considered to be the origin of the unpleasant complex taste found after prolonged brewing. It forms greenish-black compounds in the presence of Fe(III) ion. Due to its radical-capturing ability, an antioxidant activity is expected.[211]

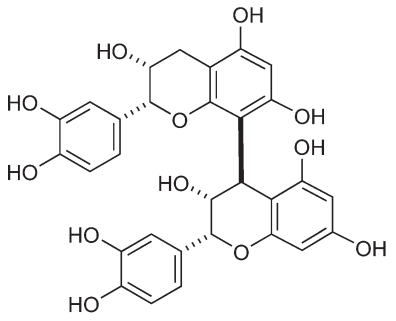


Chlorogenic acid

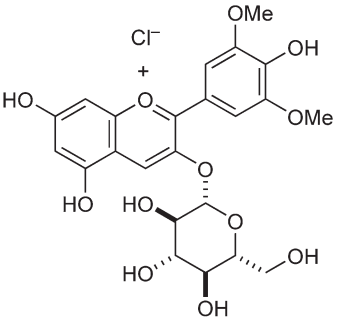
4.16.5.5 Anthocyanins in Red Wine

Anthocyanins (red pigments) and tannins are particularly important components of red wine. The changes in color and taste observed during the aging of red wine have been ascribed to anthocyanin–tannin reactions. The structures and properties of the tannins and pigmented tannins from these reactions are often misunderstood. Current research on phenolic compounds in wine has revealed that (1) reactions of tannins yield both larger polymers and smaller species, (2) anthocyanin reactions can generate colorless species as well as polymeric and various small pigments, (3) some polymeric pigments undergo sulfite bleaching while some low-molecular-weight pigments do not, (4) polymers are both soluble and astringent, so the loss of astringency during aging may involve cleavage rather than polymerization, and (5) sensory properties of anthocyanins and tannins are modulated by interactions with other wine components.[212] However, while great advances have been made in the field of red wine chemistry in recent years, a better understanding of the effect of wine polyphenol–salivary protein interaction is needed to gain a comprehensive understanding of red wine astringency.

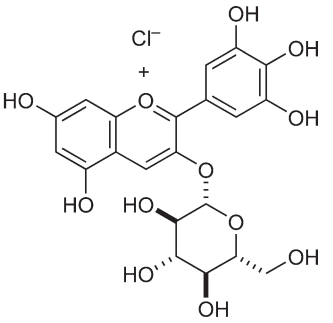
Anthocyanins are known to have antioxidant activity. Recently, they were also reported to have potential effects against cancer, aging and neurological diseases, inflammation, diabetes, and bacterial infections.[213]



Procyanidine B-2



Malvidin 3-glucoside



Delphinidin 3-glucoside

Structures of tannin derivatives in red wine

4.16.6 Umami and Kokumi

4.16.6.1 Umami-Tasting Natural Products

The postulate that umami is a basic taste quality was not generally accepted for a very long time. As a result of mounting research evidence obtained not only by Japanese but also by Western researchers from North America and Europe, the umami taste was finally recognized as the fifth basic gustatory quality at the first International Symposium on Umami held in Hawaii in 1985. Today, the Japanese word 'umami' has been established as the worldwide technical term for this savory taste quality.[4] The analysis of sensory evaluation data carried out by the multidimensional scaling method has provided numerical validation to substantiate the notion that the umami taste of substances such as sodium glutamate lies outside the gustatory space formed by the traditional four basic gustatory qualities. It has been established that (1) taste cells have receptors that bind with umami substances and produce an electrophysiological response to umami substances, (2) there are taste nerves that transmit umami stimuli, and (3) there are sites in the brain that respond to umami stimuli in the same manner as they respond to the other four basic tastes. Recent research has progressed to the point of cloning potential umami taste receptors. Currently, two types of receptors for the umami taste have been proposed: mGluR4[214] and T1R1/T1R3 heterodimer.[215,216]

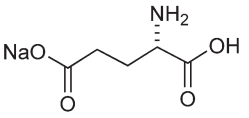
4.16.6.1.1 Monosodium glutamate

In 1908, Ikeda isolated MSG as the principal component of the savory taste contained in soup stock of kelp and named the sensation umami (delicious taste in Japanese).[2] MSG is now widely used as a flavor enhancer and seasoning, and current annual consumption worldwide is estimated to be 1.8 million tons. The present production method relies on fermentation, mainly of glucose. Arai *et al.*[217] reported that the intensity of the umami taste of free glutamic acid and its disodium salt is weak, and the taste was further diminished by esterification or amide formation. Homocysteinic acid with γ-sulfonate in place of γ-carboxylate of glutamic acid also exhibits a strong umami taste. While it has been suggested that the sensitivity to MSG shows racial differences, the threshold for Japanese is 0.015%, which is lower than that for sucrose (0.16%) and closer to that for table salt (0.0086%).[218] In general, the threshold value is lower for bitter- and sour-tasting compounds because of their warning nature, and higher for sweet compounds like sugar, which are taken in large quantities. Glutamic acid is the most abundant among the amino acids that constitute proteins in natural food. It is also contained in a free form in natural food and mother's milk and therefore can be considered a sign of proteins. The sensitivity of glutamate is reinforced tremendously by its 1:1 combination with 5′-mononucleotides such as inosine 5′-monophosphate (IMP) and guanosine 5′-monophosphate (GMP).[219,220] The synergistic action between MSG and nucleotides has been explained in terms of an allosteric effect by Kurihara *et al.*[221]

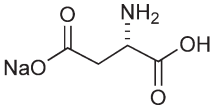
Monosodium aspartate also elicits an umami taste, although its intensity is not as high. It has also been reported to have a synergistic effect with IMP.[222]

4.16.6.1.2 Other proteinogenic amino acids

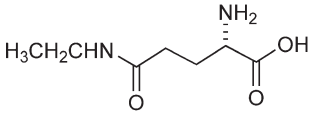
As described in Section 4.16.6.1.1, L-α-amino acids with an acidic side chain, glutamate and aspartate among proteinogenic amino acids, elicit umami taste. Several other proteinogenic amino acids without an acidic side chain, such as glycine, alanine, serine, threonine, asparagine, and glutamine, have umami taste as a side taste though their dominant taste is sweet (see Section 4.16.2.1.10(i)).[223] Their umami intensities are enhanced synergistically by adding 5′-mononucleotide similar to acidic amino acids.[8,224] It has been reported that amino acids such as glutamic acid, glycine, alanine, and arginine as well as guanylic acid, sodium cation, calcium cation, and chlorine anion are essential constituents that form the taste of scallops.[225]



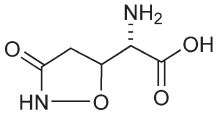
Monosodium glutamate



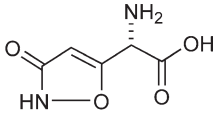
Monosodium aspartate



Theanine



Tricholomic acid



Ibotenic acid

4.16.6.1.3 L-Theanine

L-Theanine is abundantly contained in green tea (*C. sinensis* and *Thea sinensis*) and is an umami-tasting constituent along with glutamic acid.[226] Theanine was named after *T. sinensis*. The theanine content in dry leaves is 1–2% and is much higher in high-grade tea. It has been approved as a food additive in Japan, and theanine produced by fermentation is now commercially available. Besides theanine, green tea also contains many amino acids such as glutamic acid, aspartic acid, arginine, and serine.

4.16.6.1.4 Tricholomic acid and ibotenic acid

L-Tricholomic acid and L-ibotenic acid were isolated as umami taste principles from Japanese mushrooms, *Tricholoma muscarium* and *Amanita strobiliformis*, respectively.[227,228] Their umami intensities are much stronger than that of L-glutamic acid. These nonproteinogenic amino acids were found during the screening of insecticides. Although they have a very strong umami intensity, they are not used as seasonings.

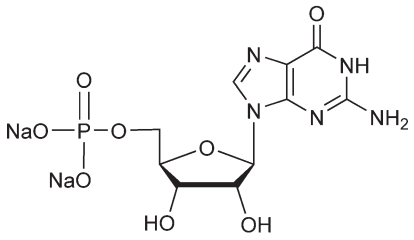
4.16.6.1.5 Organic acids

In the sixteenth century, succinic acid was isolated as colorless crystals by the dry distillation of amber. Takahashi found that a large amount of succinic acid was accumulated during the cultivation of a microorganism, and it had a delicious flavor.[229] Later, Aoki[230] reported that the umami-tasting constituent of shellfish such as *Corbicula japonica* (Asian clams) was succinic acid. Succinic acid is widely distributed in plants and animals and is used as a seasoning as well as a pharmaceutical ingredient. The threshold value is 0.02% and there is no synergistic effect with MSG or IMP.

4.16.6.1.6 Nucleotides

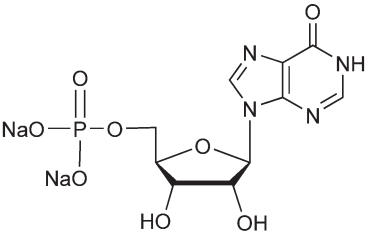
Five years after the discovery of MSG as an umami taste, Kodama, who was a senior pupil of Ikeda, found that inosine 5′-monophosphate (IMP) was an umami-tasting constituent of dried bonito, which has also been used for soup stock in Japan and East Asia.[231] Kuninaka.[232] further studied umami-tasting substances and found that guanosine 5′-monophosphate (GMP) obtained by the enzymatic hydrolysis of yeast RNA had an intense

umami taste. GMP was also found as a constituent of dried mushrooms (*Cortinellus shiitake*) cultivated in Japan.[233] Among natural nucleotides, umami-tasting compounds are 5′-nucleotides with purine as a nucleic acid base, such as IMP, GMP, and AMP. Mononucleotides with phosphate at the 2′- or 3′-position do not elicit an umami taste. Nucleosides and purine bases also do not give a savory taste. The relationship between the nucleotide structure and the intensity of umami was extensively studied.[234,235] As described in Section 4.16.6.1.1, it was confirmed that IMP and GMP have synergistic action with MSG and therefore these nucleotides are used with MSG as seasonings. Both IMP and GMP are industrially produced by enzymatic transformation and fermentation technology. Interestingly, the umami taste increases in stored meat and fish compared to very fresh food. It is thought that ATP present in the meat is transformed to IMP through AMP.



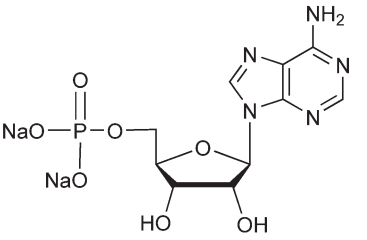
Disodium guanosine monophosphate

(GMP)



Disodium inosine monophosphate

(IMP)



Disodium adenosine monophosphate

(AMP)

4.16.6.1.7 Peptides

It was reported that α-glutamyl peptides, particularly peptides with hydrophilic amino acids, such as Glu–Asp, Glu–Thr, Glu–Ser, and Glu–Glu, elicited an umami flavor.[236] These peptides were isolated from the umami constituents in the enzymatically hydrolyzed products of soybean proteins. The same author also reported that tripeptides such as Glu–Gly–Ser also elicited the umami taste. The threshold value (0.15%) of these peptides is greater than that of MSG. The flavor of meat extract can be reproduced using these peptides with MSG and IMP. Recently, *N*-lactoyl glutamic acid, which is a condensation product of lactic acid with glutamic acid, was shown to elicit a weak umami taste, similar to MSG.[237]

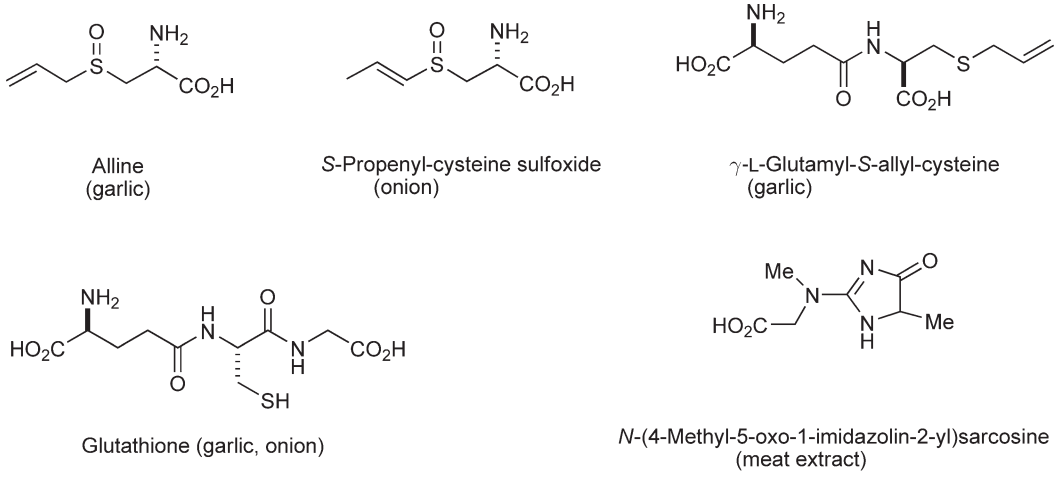
4.16.6.2 Kokumi

In Japan, 'koku' or 'kokumi' refers to the delicious taste of food. In particular, it is used when the flavor cannot be represented by any of the five basic taste qualities. It has been reported that 'kokumi' can be classified more concretely into thickness, continuity, mouthfullness of flavor, and harmony of taste.[238] Previously, there have been many reports on the constituents or fractions that enhance the flavor relevant to kokumi. In this section, examples of the constituents that provide kokumi are described by illustrating their structures.

4.16.6.2.1 Kokumi-inducing natural products

Ueda *et al.*[239] studied the kokumi-inducing effect of garlic and attempted to isolate the kokumi-inducing compound. Through repeated ion exchange chromatographic purification of garlic extracts, they obtained kokumi-inducing fractions as determined by sensory tests. As a result, sulfur-containing amino acids and peptides were characterized as kokumi-inducing constituents of garlic. These compounds are alliin (*S*-allyl-L-cysteine sulfoxide), *S*-methyl-L-cysteine sulfoxide, glutathione (γ-glutamyl-cysteinylglycine), and γ-glutamyl-*S*-allyl-L-cysteine. Ueda *et al.*[240] further investigated the kokumi-inducing constituents of onion using the same methodology and identified them as sulfur-containing amino acids, *S*-(1-propenyl)-L-cysteine sulfoxide and *S*-methyl-L-cysteine sulfoxide, and peptides such as glutathione (γ-glutamyl-cysteinylglycine) and γ-glutamyl-*S*-(1-propenyl)-L-cysteine sulfoxide. By themselves, these compounds exhibit only slight flavor in water solution. However, when they are added to an umami solution or various kind of foods, they substantially enhance the thickness, continuity, and mouthfullness of the taste.

Shima *et al.* noted that the taste of beef bouillon could not be reproduced with combinations of the compounds known to be contained and sought to identify the unknown compound that gives bouillon its brothy taste.[241,242] Broth prepared from beef was analyzed successively by dialysis, electrodialysis, gel filtration chromatography, chelate affinity chromatography, and carbon partition chromatography, and finally three fractions that gave the 'brothy taste' were obtained. One fraction contained the component responsible for this taste in the highest purity. A structural analysis was carried out using positive fast atom bombardment tandem mass spectrometry (FAB-MS) and various NMR methods and the main compound of the fraction was elucidated to be the novel compound *N*-(4-methyl-5-oxo-1-imidazolin-2-yl)sarcosine. The structure was also confirmed by X-ray structural analysis.[243] This compound is estimated to be synthesized by the reaction of creatine in meat extract with methylglyoxal generated from sugar. This compound does not exhibit a brothy taste by itself in water solution. However, when added to soup stock, it substantially enhances kokumi, such as the thickness, continuity, and mouthfullness of the taste as well as a thick sour taste. It was also reported that glutathione, which is a kokumi-inducing constituent, enhanced the umami flavor response, particularly for IMP, by a neurophysiological approach in which the response of tympani chord in mouse was observed.[244] Very recently, it was found that the addition of a nearly tasteless aqueous extract isolated from edible beans (*Phaseolus vulgaris* L.) to a model chicken broth enhanced the savory taste sensation.[245] The key molecules inducing the kokumi were identified as γ-L-glutamyl-L-valine, γ-L-glutamyl-L-leucine, and γ-L-glutamyl-L-cysteinyl-β--alanine (homoglutathione). It is expected that further progress in understanding the neurophysiology and molecular biology of the kokumi receptor may clarify this phenomenon at a molecular level.



Kokumi-inducing natural products

4.16.7 Sour and Salty Tastes

4.16.7.1 Sour Taste Receptors

The sour taste is one of the five basic tastes and is elicited by acids. However, it is unclear how taste cells transduce a sour taste because acids (specifically protons) have diverse effects on cell membranes. It has been shown that acids in a single receptor cell may block ion channels, permeate ion channels, change intracellular pH, and alter transporter function. Although the variety of effects and potential targets are well recognized, until recently there has been little success in characterizing the molecular species involved in the transduction machinery. Very recently, two groups independently revealed that the acid receptor consisted of the molecule PKD2L1. Zuker *et al.*[9] investigated the acid receptor using bioinformatics based on the genome data of mice and found that PKD2L1 was expressed in the taste bud. This molecule belongs to the TRP family (transient receptor potential). It was revealed that a transmembrane ion channel protein encoded by the gene PKD2L1 is a

taste receptor protein of the sour taste receptor system. At almost the same time, Matsunami *et al.*[10] showed that two TRP channel members, PKD1L3 and PKD2L1, are coexpressed in a subset of taste receptor cells in specific taste areas, and the PKD1L3 and PKD2L1 heterodimer may function as a sour taste receptor.

4.16.7.2 Sour-Tasting Natural Products

Sour-tasting compounds are called as acidulants giving a sharp taste to foods. They also act as preservatives. Many natural foods are acidic. For example, oranges, lemons, apples, and yogurt contain natural acids, such as citric acid, that give them their characteristic taste. Acids have been used for centuries as important contributors to flavor and the acid environment they produce prevents the growth of many microorganisms. Organic acids employed as food additives are listed below.[13]

4.16.7.2.1 Citric acid

Citric acid is a sour principle of citrus fruits such as orange and lemon and exhibits a mild and refreshing sour taste. It is widely used to add an acidic (sour) taste to soft drinks, jams, candies, and so on. It is also used as a natural preservative. By taking advantage of the buffer action of citric acid, sodium citrate is used in seasonings, and as a pH controller and emulsifier for processed cheese. In biochemistry, it is important as an intermediate in the citric acid cycle and therefore occurs in the metabolism of almost all living things. It also serves as an environmentally benign cleaning agent and acts as an antioxidant. Citric acid is produced by the fermentation of glucose. Approximately, 35 000 tons are consumed annually in Japan.

4.16.7.2.2 Malic acid

In nature, malic acid is found in the L-form in many fruits such as apple, and indeed it is sometimes called apple acid, and contributes to the sour taste of green apples. The chemically synthesized product is racemic, but there appears to be no difference in the quality of taste or sour intensity. Racemic malic acid has been approved as a food additive in Japan. While it is almost as sour as citric acid, it gives a slightly stimulating and continuous sour taste quality. It is used as a single dose, normally along with other organic acids in soft drinks, lactobacillus beverages, sherbet, jams, and pickles. It is produced industrially from maleic acid by hydration.

4.16.7.2.3 Tartaric acid

L-Tartaric acid is an abundant constituent of many fruits such as grapes and bananas and exhibits a slightly astringent and refreshing sour taste. It is one of the main acids found in wine. It is added to other foods to give a sour taste and is normally used with other acids such as citric acid and malic acid as an additive in soft drinks, candies, and so on. It is produced by acid hydrolysis of calcium tartrate, which is prepared from potassium tartrate obtained as a by-product during wine production. Optically active tartaric acid is used for the chiral resolution of amines and also as an asymmetric catalyst.

4.16.7.2.4 Lactic acid

Lactic acid is a sour principle of yogurt and lactobacillus beverages. It exhibits a soft and thick sour taste quality with slight astringency. It is a syrupy liquid produced by fermentation with a lactobacillus and is formed in the body by the metabolism of sugars. Due to its pH-controlling effect, it is used in soft drinks, pickles, Japanese sakes, sherbets, and so on. It is industrially produced by the fermentation of glucose and chemical synthesis. Approximately 12 000 tons are consumed annually as a food additive in Japan.

4.16.7.2.5 Succinic acid

Succinic acid is an umami-tasting constituent of shellfish, as well as a kokumi-tasting substance in Japanese sake. It is sometimes added to Japanese sake and soy sauce to improve the taste quality. It is industrially produced from maleic acid by hydrogenation and subsequent purification. It is also approved as a food additive in Japan. Recently, an efficient fermentation method has also been studied.[246]

4.16.7.2.6 Fumaric acid

Fumaric acid is a naturally occurring sour-tasting compound found in many plants such as *Fumaria officinalis* L. (Fumariaceae), *Boletus scaber* Bull. (Boletaceae), and *Fomes igniaries* (Fries) Kickx. (Pluporaceae). It is an essential component for respiration in plant and animal tissues. It is produced by fermentation with mold, such as *Rhizopus nigricans*, or by chemical synthesis. It is also used in soft drinks and ice cream and as an acidulant along with citric acid.

4.16.7.3 Salty Taste

It is believed that saltiness is induced by compounds passing directly through ion channels in the tongue, which leads to an action potential. Recently, it was reported[247] that both amiloride-sensitive and amiloride-insensitive mechanisms contribute to NaCl taste transduction. The amiloride-sensitive mechanism relies on the ENaC, which is widely expressed on the apical membrane of fungiform taste cells. The amiloride-insensitive mechanism, which predominates in circumvallated and foliate taste buds, was also reported to involve a variant of the nonselective cation channel transient receptor potential vanilloid receptor subtype 1 (TRPV1).[248] It has been suggested that additional mechanisms must contribute to the amiloride-insensitive NaCl response. Unfortunately, no naturally occurring product is known to exhibit a salty taste, despite several attempts to identify or synthesize one.

4.16.8 Conclusion

As reflected in this chapter, nature creates great diversity in plants and animals, even in terms of taste quality. However, we still do not have a thorough understanding of taste sensation. Recently, there are accumulated evidences for fatty substance receptors using rodents.[249] It is speculated that humans may also have the same receptors. Fat has occasionally been proposed as a possible basic taste. It is highly expected that recent progress in our understanding of neurophysiology with molecular-biological tools will help to clarify the nature of all of the taste sensations and the results should be useful for addressing several issues that bear on human health, such as low-calorie sweeteners for obesity, salt substitutes for patients with hypertension, and novel taste-modifying compounds for drug delivery systems.

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Biographical Sketches

Kunisuke Izawa was born in Hyogo, Japan, in 1945, and received his B.A. in 1968 and Ph.D. in 1973 from Osaka University under the direction of Professor Takayuki Fueno. He then joined the Central Research Laboratories of Ajinomoto Co., Inc., where he studied the cobalt-catalyzed amidocarbonylation (Wakamatsu) reaction. After studying a natural product synthesis as a postdoctoral fellow at MIT (with Professor George H. Buchi) for 2 years, he returned in 1981 to Basic Research Laboratories in the same company aiming at the discovery of new methodology for pharmaceuticals. In 1990, he moved to the Process Research Laboratories as a general manager. Since then, he has been engaged in the process development of pharmaceutical fine chemicals in Ajinomoto. In 2006, he became an advisor at AminoScience Laboratories in the same company, after serving as a corporate executive fellow for 7 years. He is also serving as regional president in the Society of Synthetic Organic Chemistry, Japan, from 2007. His research interest is in the field of organic synthesis utilizing amino acids, nucleosides, and carbohydrates.

Yusuke Amino was born in Japan in 1958. He received his master degree in 1983 and Ph.D. in 1991 from Kyoto University under the direction of Professor Takeo Saegusa and Professor Yoshihiko Ito. In 1983, he joined the Central Research Laboratories of Ajinomoto Co., Inc. He studied a natural product synthesis at Colorado State University (with Professor R. M. Williams) from 1991 to 1993. After studying the chemistry of sweet peptides at UCSD (with Professor M. Goodman) in 1994, he returned to Ajinomoto Co., Inc. Since then, he has been working on the structure–activity relationships of taste compounds.

Masanori Kohmura was born in Tokyo, Japan, in 1962. He received Bachelor of Agriculture in 1985 from the University of Tsukuba, Japan. Then he joined the Central Research Laboratories of Ajinomoto Co., Inc., where he studied peptide synthesis and structure–taste relationships of sweet protein. He received Ph.D. from the University of Tsukuba in 1994. In 1996, he moved to the Food Research and Development Laboratories in the same company and studied processed flavor and its precursor compound analysis. In 2001, he moved to the Life Science Institute of the same company. In 2004, he moved to the Quality Assurance & External Scientific Affairs Department of the Corporate Headquarters as a Manager. In 2007, he was promoted as Associate General Manager, and then moved to the ASEAN Regional Headquarters in Bangkok as a director. From 2002 to 2007, he served as the editorial board member of an academic journal *Food Science and Technology Research* published by the Japanese Society for Food Science and Technology.

Yoichi Ueda was born in Hokkaido, Japan, in 1956, and received his B.A. in 1979 and M.D. in 1981 from the University of Tokyo under the direction of Professor Kanehisa Hashimoto. He joined the Central Research Laboratories of Ajinomoto Co., Inc., where he engaged in the investigation of novel flavor-active natural compounds in foodstuffs such as garlic and meat. He received Ph.D. in 1998 from the University of Tokyo under the direction of Professor Shugo Watabe. After the research on enrichment of glutathione in yeast extract, he worked for the Seasoning Products Development Center of the company. In 2003, he moved to the Quality Assurance & External Scientific Affairs Department for working to improve the quality assurance system of Ajinomoto group companies. His research interest includes flavor interaction among constituents in delicious food materials and their application to new food products.

Motonaka Kuroda was born in Tokyo, Japan, in 1964, and received his B.A. in 1986 and Ph.D. in 2003 from Tsukuba University under the direction of Professor Hiroshi Imagawa and Professor Tetsuo Ozawa. In 1988, he joined the Central Research Laboratories of Ajinomoto Co., Inc., where he studied the flavor components of various soup stock materials such as dried-bonito broth (katsuo-bushi dashi), beef soup stock, and chicken broth. In 2002, he moved to the Food Research Institute as a general manager. In 2007, he moved to the Research Institute for Health Fundamentals. His research interest is in the field of flavor components of food and the health function of traditional food.