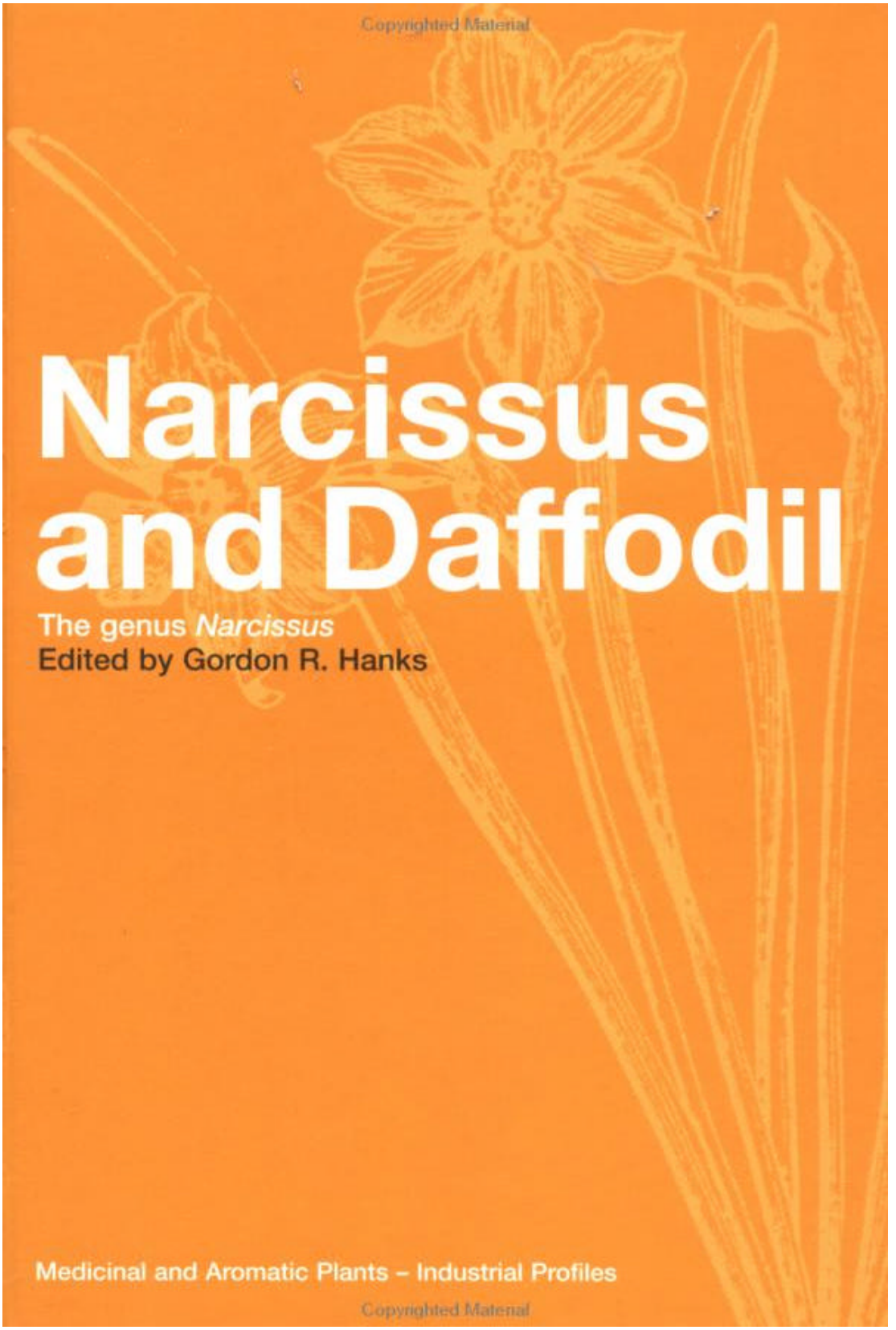

Narcissus and Daffodil

The genus *Narcissus*

Edited by Gordon R. Hanks

Medicinal and Aromatic Plants – Industrial Profiles

Narcissus and Daffodil

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Narcissus and Daffodil

The genus *Narcissus*

Edited by

Gordon R. Hanks

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Contents

Preface to the series

There is increasing interest in industry, academia and the health sciences in medicinal and aromatic plants. In passing from plant production to the eventual product used by the public, many sciences are involved. This series brings together information which is currently scattered through an ever increasing number of journals. Each volume gives an in-depth look at one plant genus, about which an area specialist has assembled information ranging from the production of the plant to market trends and quality control.

Many industries are involved such as forestry, agriculture, chemical, food, flavour, beverage, pharmaceutical, cosmetic and fragrance. The plant raw materials are roots, rhizomes, bulbs, leaves, stems, barks, wood, flowers, fruits and seeds. These yield gums, resins, essential (volatile) oils, fixed oils, waxes, juices, extracts and spices for medicinal and aromatic purposes. All these commodities are traded worldwide. A dealer's market report for an item may say 'Drought in the country of origin has forced up prices'.

Natural products do not mean safe products and account of this has to be taken by the above industries, which are subject to regulation. For example, a number of plants which are approved for use in medicine must not be used in cosmetic products.

The assessment of safe to use starts with the harvested plant material which has to comply with an official monograph. This may require absence of, or prescribed limits of, radioactive material, heavy metals, aflatoxin, pesticide residue, as well as the required level of active principle. This analytical control is costly and tends to exclude small batches of plant material. Large scale contracted mechanised cultivation with designated seed or plantlets is now preferable.

Today, plant selection is not only for the yield of active principle, but for the plant's ability to overcome disease, climatic stress and the hazards caused by mankind. Such methods as *in vitro* fertilisation, meristem cultures, and somatic embryogenesis are used. The transfer of sections of DNA is giving rise to controversy in the case of some end-uses of the plant material.

Some suppliers of plant raw material are now able to certify that they are supplying organically-farmed medicinal plants, herbs and spices. The European Union directive (CVO/EU No 2092/91) details the specifications for the obligatory quality controls to be carried out at all stages of production and processing of organic products.

Fascinating plant folklore and ethnopharmacology leads to medicinal potential. Examples are the muscle relaxants based on the arrow poison, curare, from

species of *Chondrodendron*, and the antimalarials derived from species of *Cinchona* and *Artemisia*. The methods of detection of pharmacological activity have become increasingly reliable and specific, frequently involving enzymes in bioassays and avoiding the use of laboratory animals. By using bioassay linked fractionation of crude plant juices or extracts, compounds can be specifically targeted which, for example, inhibit blood platelet aggregation, or have antitumour, or antiviral, or any other required activity. With the assistance of robotic devices, all the members of a genus may be readily screened. However, the plant material must be fully authenticated by a specialist.

The medicinal traditions of ancient civilisations such as those of China and India have a large armamentarium of plants in their pharmacopoeias which are used throughout South East Asia. A similar situation exists in Africa and South America. Thus, a very high percentage of the World's population relies on medicinal and aromatic plants for their medicine. Western medicine is also responding. Already in Germany all medical practitioners have to pass an examination in phytotherapy before being allowed to practise. It is noticeable that throughout Europe and the USA, medical, pharmacy and health related schools are increasingly offering training in phytotherapy.

Multinational pharmaceutical companies have become less enamoured of the single compound magic bullet cure. The high costs of such ventures and the endless competition from "me too" compounds from rival companies often discourage the attempt. Independent phytomedicine companies have been very strong in Germany. However, by the end of 1995, eleven (almost all) had been acquired by the multinational pharmaceutical firms, acknowledging the lay public's growing demand for phytomedicines in the Western World.

The business of dietary supplement in the Western World has expanded from the health store to the pharmacy. Alternative medicine includes plant based products. Appropriate measures to ensure the quality, safety and efficacy of these either already exist or are being answered by greater legislative control by such bodies as the Food and Drug Administration of the USA and the recently created European Agency for the Evaluation of Medicinal Products, based in London.

In the USA, the Dietary Supplement and Health Education Act of 1994 recognised the class of phytotherapeutic agents derived from medicinal and aromatic plants. Furthermore, under public pressure, the US Congress set up an Office of Alternative Medicine and this office in 1994 assisted the filing of several Investigational New Drug (IND) applications, required for clinical trials of some Chinese herbal preparations. The significance of these applications was that each Chinese preparation involved several plants and yet was handled as a single IND. A demonstration of the contribution to efficacy, of each ingredient of each plant, was not required. This was a major step forward towards more sensible regulations in regard to phytomedicines.

My thanks are due to the staff of the Publishers who have made this series possible and especially to the volume editors and their chapter contributors for the authoritative information.

Roland Hardman

Preface

My interest in flower-bulb crops started in 1973, when I began working with Dr Alun Rees at the Glasshouse Crops Research Institute, Littlehampton, a site consigned to horticultural history in 1995 as a result of the all-too-familiar 'cuts in government funding'. Alun had himself taken up a post at Littlehampton in 1962, when the (then) Agricultural Research Council started funding research on ornamental bulb crops. Alun succeeded in putting bulb growing and forcing on a sound scientific base, evidenced by the publication of his *The Growth of Bulbs* in 1972.[1] Fortunately for me, Alun's enthusiasm for these interesting crops was infectious.

Whilst my main research interests concerned the early forcing of bulbs, the use of plant growth regulators, narcissus propagation and other aspects relevant to the UK bulbs industry, uses of bulbs other than as ornamentals attracted my attention from time to time. For instance, one was aware of the eastern European literature, long ignored in the west, on pharmaceuticals such as galanthamine (galantamine) from *Galanthus* (snowdrops) and other genera. Only in 1995 did the growing of narcissus bulbs in the UK for processing for galanthamine extraction, and the clinical trials on the use of the compound in Alzheimer's disease, become public knowledge.[2] Whilst there is a wealth both of other alkaloids in narcissus, and of other potential uses of galanthamine, this case in particular led to the conception of the present volume. Reminyl, Shire Pharmaceuticals and Janssen-Cilag's Alzheimer's disease treatment derived from narcissus, received its first European approval in 2000.[3,4] Subsequently, Reminyl was recommended by the National Centre for Clinical Excellence.[5]

The impact of Alzheimer's disease was first brought home to me by examples of public figures. For example, there was ex-President Ronald Reagan's touching letter to the American people, relating the start of the 'journey that would lead him into the sunset of his life'.[6] Earlier, in the UK, Prime Minister Harold Wilson had unexpectedly retired from public life, and the cases of these two statesmen

1 Rees, A.R. (1972) *The Growth of Bulbs*. Academic Press, London.
2 Bonner, J. (1995) Flower bulbs slow brain disease. *New Scientist*, **145** (1964), 21.
3 Reminyl approval lifts Shire price, *The Times*, 4 March 2000.
4 New Alzheimer's drug approved. *The Pharmaceutical Journal*, **265** (22 July 2000), 122.
5 Press release 2001/002, NICE *issues guidance on drugs for Alzheimer's disease*, 19 January 2001, NICE.
6 Letter from Ronald Reagan revealing Alzheimer's disease, for example, see *http://www.law.umkc.edu/faculty/projects/ttrials/hinckley/ALZHEI~1.htm*

were poignantly contrasted in an article by Clair Woodward entitled 'Ending the taboo: Reagan was right to go public over Alzheimer's'.[7] Subsequently, I saw the suffering and death of my mother from Alzheimer's disease, in 1995. My hope, therefore, is that this volume will prove a useful reference for those researching the exciting field of Amaryllidaceae alkaloids.

I am pleased to be editor for the series of comprehensive reviews the *Narcissus* volume comprises, but am particularly gratified that much original material, and material accessible only with difficulty in the west, has been included.

Notes on nomenclature

1 Galanthamine is also referred to as galantamine; in this volume the former name has been used exclusively.
2 In popular UK usage, the term 'daffodil' is used for 'trumpet' or 'large cup' types of *Narcissus*, and 'narcissi' for smaller flowered types. In the present volume the term *Narcissus* (or narcissus) has been used to cover all types, using the Latin at first mention or where referring to a specific cultivar or other taxon, otherwise using the colloquial 'narcissus'. Where a reference is to a particular species or group of narcissus (e.g., Tazetta cultivars), this is stated.
3 The taxonomy of the genus *Narcissus* is subject to much debate (Brian Mathew discusses 'splitters' and 'lumpers' in chapter 3 of this volume). While the editor had hoped, optimistically, to achieve uniformity of narcissus names throughout this volume, this did not prove practical. Except in specialist chapters, however, names conform as far as feasible to those given in *The International Daffodil Register and Classified List 1998* of the Royal Horticultural Society.

Gordon R. Hanks

7 Woodward, C. (1995) Ending the taboo: Reagan was right to go public over Alzheimer's. *Signpost*, **1** (31), 18–19.

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I would like to thank our Series Editor, Dr Roland Hardman, for his helpfulness and kindly consideration during the preparation of the volume. I would also like to acknowledge the professionalism shown by the contributors to this book: it has been a pleasure working with you! As always, I appreciate the encouragement given by my wife Anita and daughter Esther.

Contributors

Dr. Jaume Bastida
Department of Natural Products
Faculty of Pharmacy
University of Barcelona
08028-Barcelona
Spain

Dr. Jairo K. Bastos
Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
de Ribeirão Preto
14040-903 Ribeirão Preto – SP
Brazil

Dr. Peter W. Bowers
Department of Dermatology
Treliske Hospital
Truro
Cornwall TR1 3LJ, UK

James B. Briggs (retired)
ADAS Consulting Ltd.
ADAS Park Farm
Ditton
Aylesford
Kent ME20 6PE, UK

Dr. David Brown
School of Pharmacy and
Biomedical Sciences
University of Portsmouth
St Michael's Building
White Swan Road
Portsmouth PO1 2DT
UK

Dr. V.N. Bulavka
Slavich Company
Mendeleev sq., 2
Pereslavl-Zalesskiy 152025
Russian Federation

Dr. O.A. Cherkasov
Department of Agriculture
All-Russian Research Institute of
Medicinal and Aromatic Plants (VILAR)
Grina Str. 7
Moscow 113628
Russian Federation

Dr. Carles Codina
Department of Natural Products
Faculty of Pharmacy
University of Barcelona
08028-Barcelona
Spain

Prof. Els J.M. Van Damme
Katholieke Universiteit Leuven
Laboratory for Phytopathology and
Plant Protection
Willem de Croylaan 42
3001 Leuven
Belgium

Anthony C. Dweck
Dweck Data
8 Merrifield Road
Ford
Salisbury
Wiltshire SP4 6DF
UK

Gordon R. Hanks
Crop and Weed Science Department
Horticulture Research International
Kirton, Boston
Lincolnshire PE20 1NN
UK

Dr. Kornkanok Ingkaninan
Faculty of Pharmaceutical Sciences
Naresuan University
Phitsanulok 65000
Thailand

Prof. Dr. Hubertus Irth
Screentec
Niels Bohrweg 11-13
2333 CA Leiden
The Netherlands

Dr. Celia G. Julian
Department of Dermatology
Treliske Hospital
Truro
Cornwall TR1 3LJ, UK

Dr. Mirko Kreh
Merck KGaA
Frankfurter Strasse 250
D-64271 Darmstadt
Germany

Brian Mathew
90 Foley Road
Claygate
Surrey
KT10 ONB, UK

Dr. Rita M. Moraes
National Center for Natural Products Research
Research Institute of Pharmaceutical Sciences
School of Pharmacy
University of Mississippi
University
MS 38677
USA

Dr. James R. Murray
Hunter-Fleming Ltd
Regus House
1 Friary
Temple Quay
Bristol, BS1 6EA
UK

Dr. N.P. Dhammika Nanayakkara
National Center for Natural Products Research
Research Institute of Pharmaceutical Sciences
School of Pharmacy
University of Mississippi
University
MS 38677
USA

Prof. Willy J. Peumans
Katholieke Universiteit Leuven
Laboratory for Phytopathology and Plant Protection
Willem de Croylaan 42
3001 Leuven
Belgium

Christian Remy
Laboratoire Monique Remy
Parc Industrial des Bois de Grasse
F-06130 Grasse
France

Prof. O.N. Tolkachev
Department of Chemistry and Technology of Natural Products
All-Russian Research Institute of Medicinal and Aromatic Plants (VILAR)
Grina Str. 7
Moscow 113628
Russian Federation

Prof. Rob Verpoorte
Division of Pharmacognosy
Leiden/Amsterdam Center for
Drug Research
Gorlaeus Laboratories, Leiden University
Einsteinweg 55, P.O. Box 9502
2300 RA Leiden, The Netherlands

Dr. Francesc Viladomat
Department of Natural
Products
Faculty of Pharmacy
University of Barcelona
08028-Barcelona,
Spain

Figures

Plates

Colour plates appear between pages 140 and 141

1 The biology of *Narcissus*

Gordon R. Hanks

INTRODUCTION

The genus *Narcissus* L. belongs to the Monocotyledon family Amaryllidaceae, to which it contributes some 80 species to its total of about 850 species in 60 genera (Meerow and Snijman, 1998). The taxonomy of *Narcissus* is difficult because of the ease with which hybridisation occurs naturally, accompanied by extensive cultivation, breeding, selection, escape and naturalisation (Webb, 1980; see Chapter 3, this volume). The genus is distinguished from other Amaryllids by the presence of a perigonal corona structure ('paraperigone') forming a ring ('cup') or tube ('trumpet') (Dahlgren *et al.*, 1985). Unlike other genera of the family, *Narcissus* has a mainly Mediterranean distribution, with a centre of diversity in the Iberian Peninsula, and the genus also occurs in south-western France, northern Africa and eastwards to Greece, while *Narcissus tazetta* is found not only in Spain and North Africa but in a narrow band to China and Japan (Grey-Wilson and Mathew, 1981). The eastwards distribution of *N. tazetta* may represent transfer along an ancient trade route, illustrating the long human interest in the genus as an ornamental plant, leading to its importance in commercial horticulture today (see Chapter 4, this volume).

The survival of a number of *Narcissus* species has been threatened by past overcollection and habitat destruction, not only in Spain and Portugal but also in Morocco, Turkey and Belgium (Oldfield, 1989; Koopowitz and Kaye, 1990). The 'Red List' currently gives three *Narcissus* as 'endangered', five as 'vulnerable' and six as 'rare' (WCMC, 1999). In the light of the environmentalist concerns in the 1990's, the collection of wild bulbs has been addressed by the industry. However, there is a need to maintain vigilance in the conservation of wild species and of their many variants, to establish genetic collections for future breeding programmes, and to develop sustainable production systems for their utilisation in commercial horticulture.

Hybridisation has resulted in commercial narcissus cultivars that are in most cases larger and more robust than their wild parents. Trumpet cultivars with coloured perianth and corona originated from *N. pseudonarcissus* and its varieties, and trumpet cultivars with white perianth and coloured corona from *N. pseudonarcissus* ssp. *bicolor*. Large-cupped cultivars were the result of crosses between *N. pseudonarcissus* and *N. poeticus*, back-crossed with *N. poeticus* to yield the small-cupped cultivars. Multiheaded cultivars (the 'Poetaz' group) comprise mainly hybrids of *N. poeticus* and *N. tazetta* (Doorenbos, 1954).

Information about the commercial horticulture of narcissus can be found in Rees (1972, 1985b, 1992) and Hanks (1993). Texts on the genus include Bowles (1934), Jefferson-Brown (1969, 1991), Blanchard (1990) and Wells (1989).

THE GROWTH CYCLE UNDER NATURAL CONDITIONS

The native habitats of *Narcissus* species are very varied, and include grassland, scrub, woods, river banks and rocky crevices, in both lowland and mountain sites (Webb, 1980), and the ecology of natural populations of wild daffodil (*N. pseudonarcissus*) has been intensively studied (Caldwell and Wallace, 1955; Barkham, 1980a,b, 1992; Barkham and Hance, 1982). The bulk of *Narcissus* species are synanthous and spring-flowering. Shortly after flowering rapid leaf senescence occurs, followed by a summer underground (or 'dormant') period that allows the bulb to conserve moisture and avoid predators (the alkaloids which are largely the subject of the present volume may give further disincentive to predation). Although the term 'dormancy' is used, this refers mainly to the lack of any obvious external growth, for there is little physiological dormancy because, once the leaves and roots have died down, there is intense activity of primordia within the bulb (Kamerbeek *et al.*, 1970; Rees, 1971, 1972). There is a requirement for a cold period before normal growth resumes in the spring, an arrangement that avoids most damage due to frosts in winter. The cold requirement, however, is not particularly long nor cold, so that in climates like the UK or the Netherlands it is easily satisfied by normal winters. The flowering date is then dependent on spring temperatures being sufficiently high for growth, the resultant variations in flowering date being described by Rees and Hanks (1996). The cold requirement is not an obligate one for stem extension, as stem growth will proceed even without a cold treatment, albeit slowly and with a gradual loss of flowers due to bud abortion or other causes (Rees and Hanks, 1984). The effect of the cold period is to produce rapid, synchronous stem extension and progress to anthesis (Figure 1.1). In nature, this occurs at a time when competition for pollinator insects is low and growth is relatively unhindered by shading or other competition from grasses or deciduous trees (Caldwell and Wallace, 1955; Shmida and Dafni, 1990).

The growth cycle just described has been utilised in commercial horticulture, being manipulated by controlled-temperature storage (Figure 1.2) to obtain 'forced' flowers in glasshouses over an extended season (ADAS, 1985; De Hertogh, 1989; Anon., 1998). Geophytic plants such as bulbs and corms lend themselves to horticultural usage, since their storage organs can be conveniently treated (whether by pesticides or environmental treatments), transported and traded, yet can be brought into flower in a relatively short time. The rapid growth and flowering of spring bulbs is dependent on the conversion of insoluble reserves, such as starch, into readily translocatable soluble sugars. In order to understand these processes and develop improved methods of flower forcing, carbohydrate metabolism and the related hormone-mediated processes have been investigated in flower-bulb crops, although less so in the case of narcissus than in species such as tulip and iris, perhaps because of the presence in narcissus of mucilaginous sap, which interferes with chemical extraction and separation. Carbohydrate metabolism in narcissus has been studied by Grainger (1941), Thomas *et al.* (1995) and Ruamrungsri *et al.*

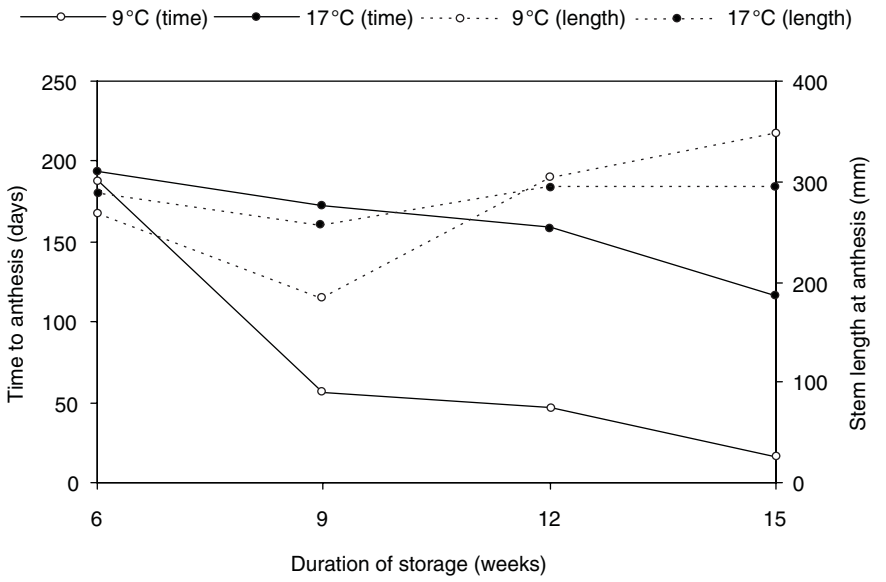

Figure 1.1 The effect of bulb storage at 9 or 17 °C for 6–15 weeks on (left axis) the time to anthesis (days in a glasshouse at 16 °C) and (right axis) stem length at anthesis for narcissus 'Fortune' (data from Rees and Hanks, 1984).

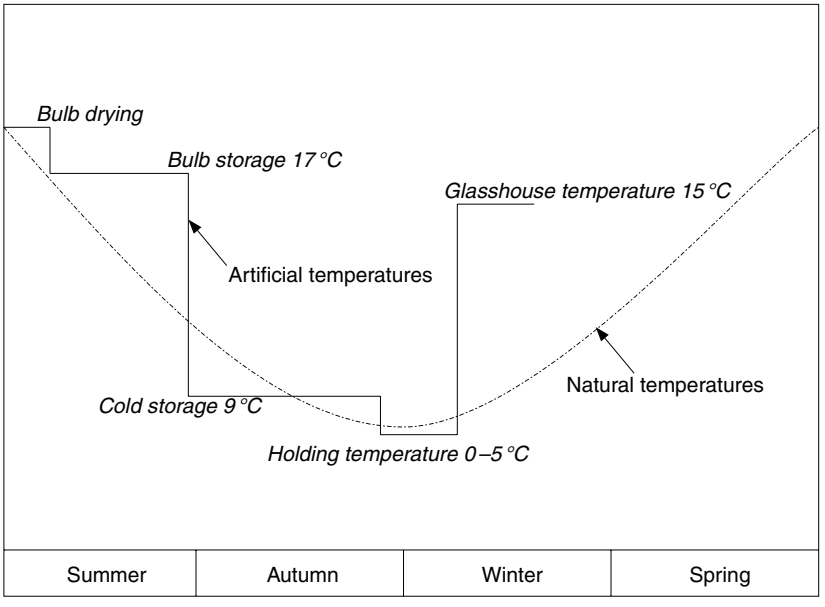

Figure 1.2 The temperature sequence during bulb forcing, compared with natural temperatures (after A.R. Rees, personal communication).

(1999), and the polysaccharides by Balbaa *et al.* (1980) and Rakhimov and Zhauynbaeva (1997). The auxins of narcissus have been studied by Edelbluth and Kaldewey (1976), gibberellins by Aung *et al.* (1969), cytokinins by van Staden (1978) and Belynskaya *et al.* (1990) and ethylene by Staby and De Hertogh (1970).

Cultivars derived from the *N. tazetta* group are unusual in that, while still 'summer dormant', they have no cold requirement and growth and anthesis can occur before winter if all other conditions are favourable (although cold treatments can increase stem length and speed anthesis; Roh and Lee, 1981; Rees and Hanks, unpublished data in Hanks, 1993). Tazetta narcissus, unlike most narcissus, respond to ethylene, which promotes flowering (Imanishi, 1997). These characteristics have enabled horticulturists to exploit Tazettas for flower production over a long season.

A few species – *N. elegans, N. serotinus, N. viridiflorus* and *N. humilis* – are autumn-flowering and generally hysteranthous. Studies on *N. tazetta* and other geophytes suggest that the syanthous-hysteranthous habit is facultative, with hysteranthy tending to be expressed in xeric habitats (Evenari and Gutterman, 1985; Halevy, 1990). The autumn-flowering species have not yet been exploited horticulturally, probably because their flowers are small or insignificant, although they clearly have potential for producing new types of commercial cultivars (Koopowitz and Kaye, 1990).

MORPHOLOGY AND DEVELOPMENT

The flowering plant

Detailed reports of the morphology and development of the narcissus plant have been given by Huisman and Hartsema (1933), Chan (1952), Okada and Miwa (1958) and Rees (1969, 1972), from which the following description has largely been compiled.

Bulb structure and bulb unit development

The 'dormant' narcissus bulb in autumn consists of a more-or-less disc-shaped stem plate (base or basal plate) bearing adventitious roots below and storage organs (bulb scales) surrounding a bud above. The bulb 'scales' consist of true scales, which are almost entirely within the bulb and serve a purely storage function, and the bases of foliage leaves; after anthesis the base of the flower stalk becomes flattened and is also scale-like in function. Leaf bases can be distinguished from bulb scales because the former have a thicker tip and a scar where the leaf lamina became detached. In the 'model' case of a narcissus bulb with a single, terminal growing point (a 'single nosed round' bulb), a transverse section of the bulb in autumn shows a series of concentric 'scales' surrounding the old flower stalk base and a terminal bud (Figure 1.3). The exception to the concentric nature of the 'scales' is that the inner leaf, which subtends the flower, has a semi-circular base with keeled margins only partly enclosing the flower stalk. The terminal bud consists of bulb scales surrounding leaves and a flower. In addition to the terminal bud, there is also a lateral bud.

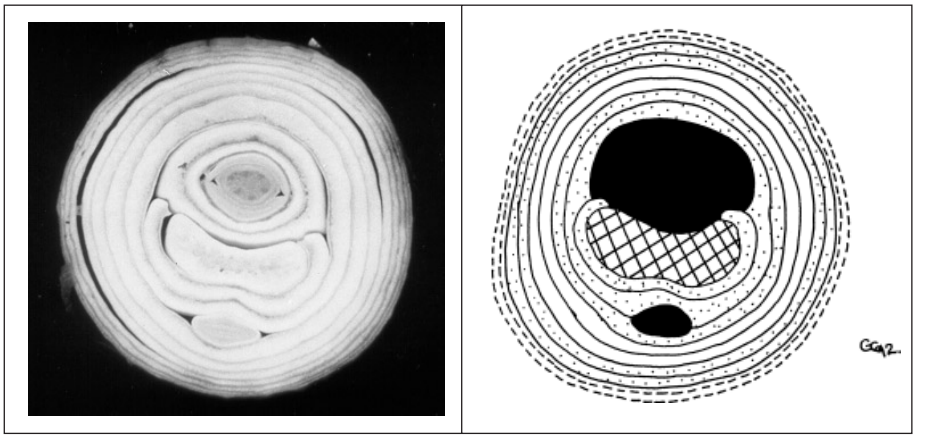

Figure 1.3 Left: transverse section of single-nosed, flowering-size narcissus 'Carlton' bulb in autumn. Right: diagram showing the generations of bulb units with their component parts. Shaded areas: the terminal (above) and lateral (below) units with next spring's leaves and flower (individual bulb scales, leaves and flower not shown). The unit that bore last spring's leaves and flower comprises the flattened remains of the stem (cross-hatched), three leaf bases (the innermost semi-sheathing) (stippled) and two bulb scales (unshaded). Beyond these scales are the remains of bulb scales and leaf bases of previous generations of bulb units, which are eventually shed as dry tunic (broken lines). (After Hanks (1993); reprinted from *The Physiology of Flower Bulbs*, ©1993, page 466, with the permission of Elsevier Science.)

The narcissus is a perennial branching system, and Rees (1969, 1972, 1987) used the term 'bulb unit' to describe each annual increment of growth, thereby distinguishing these structures from shorter lived entities such as the bulbs of tulip, where new bulbs (daughter bulbs) become separate entities each year. In narcissus, the growing point produces a new bud with bulb scales and leaves each year. This bud grows through its first year, and (if large enough) initiates a flower in its second year, its leaves and flower emerging in the next spring. Thereafter its 'scales' persist for perhaps two more years, so that the bulb unit has an overall lifespan of about 4 years. Since new buds are initiated in the centre of the bulb, the bulb 'scales' are gradually displaced outwards by the continued annual production of new bulb units within. In the year that a bulb unit reaches anthesis, its 'scales' (swollen with reserves) make up the bulk of the fresh weight of the 'bulb'; subsequently its 'scales' become depleted until they form the dry tunic (skin) of the bulb, and they are eventually lost through displacement from within or by abrasion (the latter accentuated by commercial bulb handling). Although bulb units may be called 'mother bulbs' in the year they reach anthesis and 'daughter' and 'granddaughter bulbs' previously, better terms might be pre-floral, floral and post-floral bulb units. What are termed 'bulbs' in common usage might be better called 'compound bulb units' or, more simply, 'bulb clusters'. A useful diagram showing narcissus development was presented by Alkema and van Leeuwen (1978) and is reproduced in Figure 1.4.

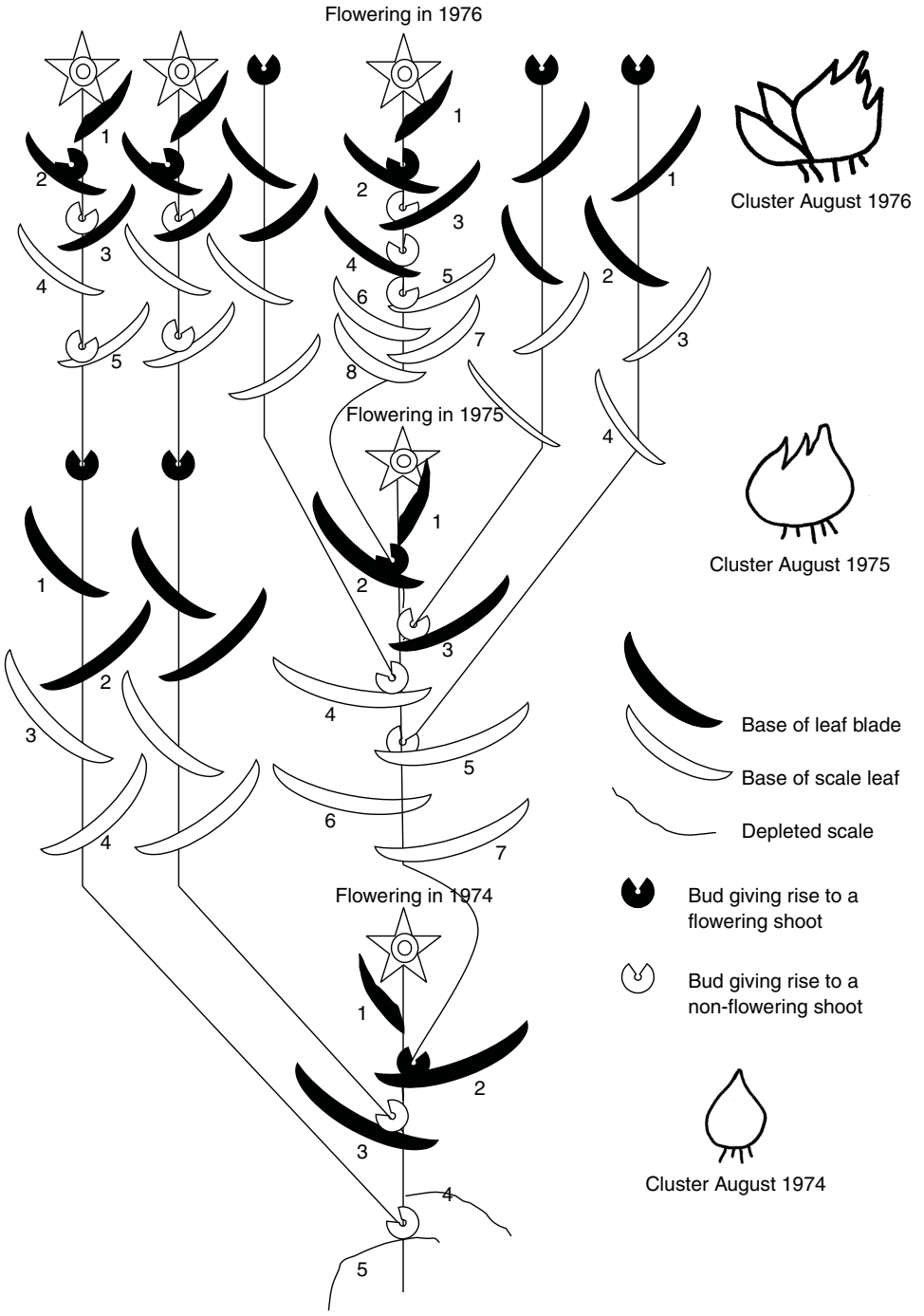

Figure 1.4 Schematic representation of a narcissus plant showing three years' development (redrawn after Alkema and van Leeuwen (1978), with permission from the Bulb Research Centre, Lisse, The Netherlands).

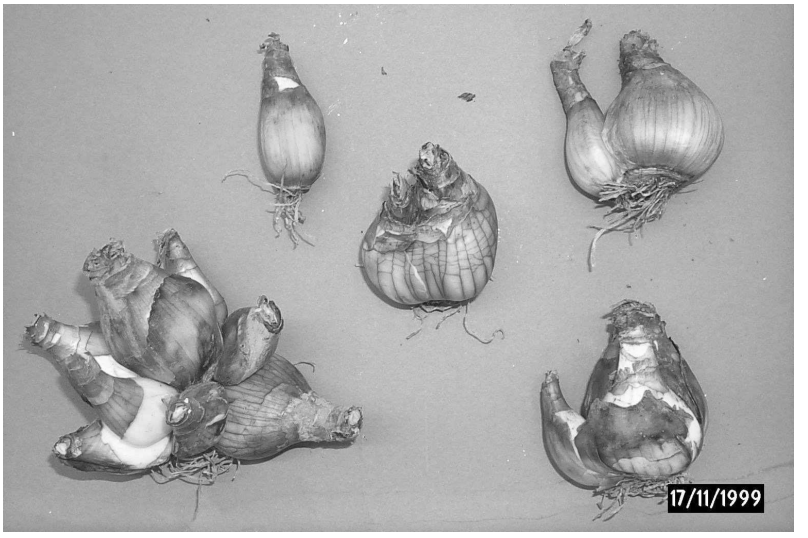

Figure 1.5 Centre: double-nosed bulb, narcissus 'Carlton'. Right: separation of offsets. Left: offset (top) and large 'mother bulb' (below). (Photograph: Horticulture Research International.)

As narcissus bulbs are branching systems, lateral bulb units are initiated as well as the terminal, replacement units. The presence of lateral as well as terminal bulb units, and the gradual separation from the cluster of bulb units derived from laterals ('offsets'), results in a variety of bulb shapes and sizes, from round single-nosed bulbs to double- and multi-nosed bulbs (the latter also termed 'mother bulbs'), together with smaller attached or detached, usually non-flowering, offsets (Figure 1.5). The life-span of individual branching systems is not known for narcissus cultivars, although Barkham (1980a) recorded half-lives of adult *N. pseudonarcissus* of 12–18 years, and Koopowitz (1986) suggested that, judging from other Amaryllids, they are likely to have a high longevity.

Terminal bulb units are initiated alongside the flower initial of the previous bulb unit, in the axil of the second leaf from its centre, at or shortly after floral initiation in May (Rees, 1969). Lateral bulb units are initiated, usually in the axil of the third leaf from the centre, in the following December. Supernumerary (lateral) bulb units may also be initiated in the same year as regular laterals or a year later, in the axil of a bulb scale (usually the innermost scale) or alongside the regular lateral. The classification of bulb units was elegantly described by Rees (1969) (Figure 1.6, Table 1.1). Each terminal bulb unit is replaced by two 'daughter' units, terminal and lateral, while lateral units rarely contain a lateral unit. This gives a slow increase in bulb unit numbers, increasing in a Fibonacci series 1, 1, 2, 3, 5, 8, 13 . . . , an increase tending to 1.6-fold per annum, producing a population made up of 38% lateral units. In commercial bulb growing, this progression is prevented by the grading-out of saleable bulbs, and, possibly, by the suppression of laterals under sub-optimal conditions. In the earlier study by Okada and Miwa (1958) on a

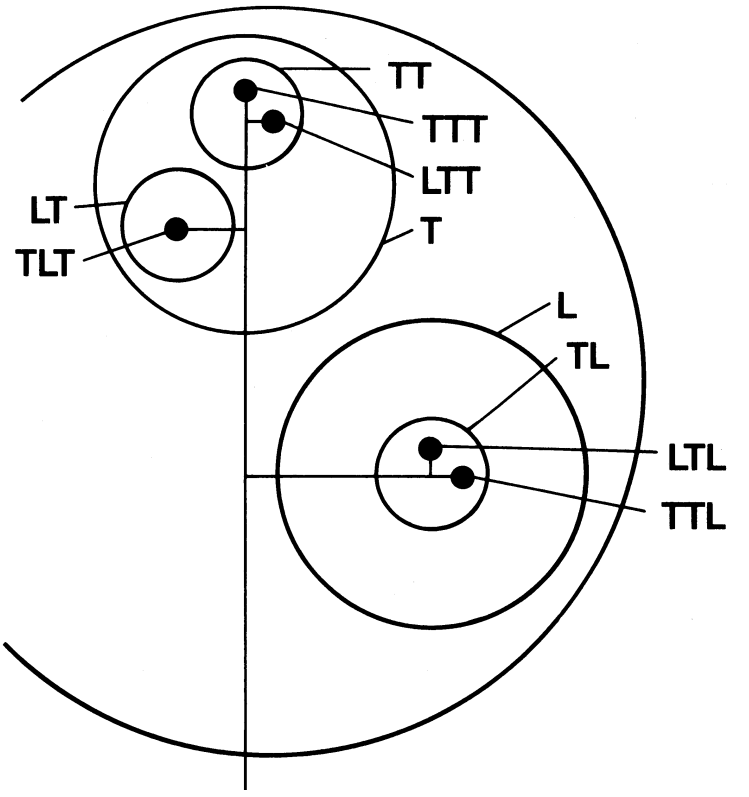

Figure 1.6 Diagrammatic representation of a large narcissus bulb showing the relationship between terminal (T) and lateral (L) bulb units. Each circle represents one bulb unit (or annual increment of growth in the branching system), the small black ones being those of the current season, labelled with a three-letter code. Successively older units are one size larger, and coded with one or two letters, respectively. (After Rees (1969), with permission of Academic Press Ltd.)

different cultivar, bulbs produced an average of 2.2 new bulb units annually, and in this case 19% were formed in the axils of bulb scales.

After terminal bulb units are initiated in May, they grow rapidly for three to four months and then more slowly through autumn and winter (Rees, 1969). Periods of alternating rapid spring-summer and slow autumn-winter growth continue, and the bulb unit reaches a peak dry weight in the May two years after its initiation, shortly after anthesis. There is some weight loss the following spring, corresponding to the rapid growth of the next generation of bulb units, after which weight is regained and maintained until August, following which the bulb unit becomes senescent and dies. Lateral bulb units show less distinct alternations of growth rates, as they are initiated later than terminals (Figure 1.7). Periods of rapid growth of bulb units begin in February, reserves being translocated from older bulb units. No new parts are being produced at this time, so this is a true bulbing

Table 1.1 The production of terminal (T) and lateral (L) bulb units in *Narcissus*[a]

Year	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Bulb unit types	T	T	T L	TT TL LT	TTT TTL TLT LTT LTL	TTTT TTTL TTLT TLTT TLTL LTTT LTTL LTLT	TTTTT TTTTL TTTLT TTLTT TTLTL TLTTT TLTTL TLTLT LTTTT LTTTL LTTLT LTLTT LTLTL
No. of bulb units	1	1	2	3	5	8	13
Increase over previous year	–	1	2	1.5	1.7	1.6	1.6

Note
[a]After Rees (1969).

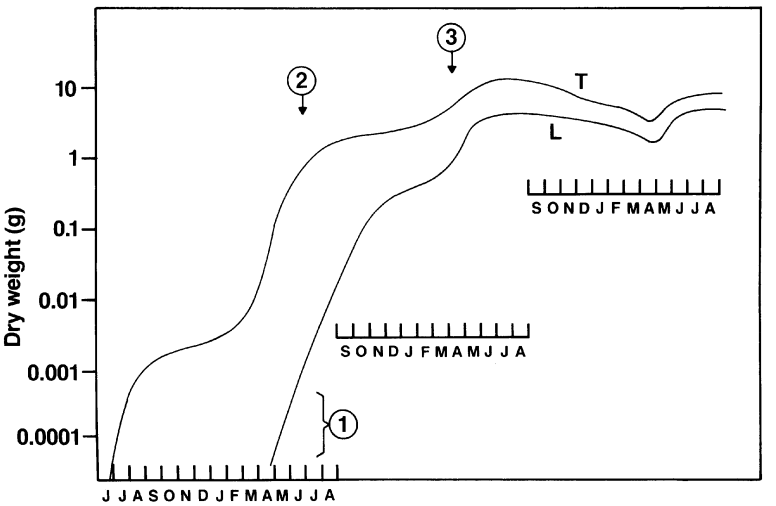

Figure 1.7 The growth pattern of terminal (T) and lateral (L) bulb units from initiation to senescence, narcissus 'Fortune'. The times of 'scale' initiation, floral initiation and anthesis are indicated by 1, 2 and 3. (Modified from Rees (1969), with permission of Academic Press Ltd.)

effect probably controlled by temperature; attempts to show a photoperiodic effect were not successful (Rees, 1972).

In both terminal and lateral bulb units the full complement of bulb scales and leaves is initiated during its first period of active growth, and the apices then become inactive until the initiation of the flower at the end of the second period of rapid growth of the terminal bulb unit, a year after the initiation of that unit

(Rees, 1969). When floral initiation does not occur, there may be renewed initiation of leaves, resulting in non-flowering bulb units with high numbers of leaves. If floral initiation occurs in lateral bulb units, it is after their period of rapid growth, and several months after floral initiation in the terminal bulb unit. In contrast to terminal bulb units, laterals have fewer parts, are lighter in weight, have less tendency to flower, and show almost complete suppression of further lateral units. Rees (1972) suggested that the differences between the two types of bulb unit were due to the later initiation of lateral bulb units, and because lateral units are subject to apical dominance by the terminal units and develop only once the terminal has become floral, losing its dominance. Large number of new bulb units are formed when the shoot apices are damaged by pests, disease or high temperature. This suggests that there is a capacity for regeneration at the base of the scales which is not normally expressed because of apical dominance (Rees, 1972), an observation confirmed by the effectiveness of propagation techniques such as twin-scaling (see Chapter 4, this volume).

The shoot apex and primordia initiation

The anatomy of the narcissus stem apex was described by Denne (1959). There is no conclusive evidence as to whether the flower of narcissus is terminal as a result of sympodial branching (in which case an axillary bud becomes the new growing point) or axillary as a result of monopodial branching (in which case the main axis continues vegetative growth) (Huisman and Hartsema, 1933). Rees (1972) summarised the arguments, favouring the sympodial view partly on the basis of the appearance of serial sections through the initiating apex. The floral primordium comes to dominate the apex in any case, with the vegetative apex remaining quiescent for some time.

Denne (1960) described the comparative development of bulb scales and leaves. The factors controlling the transition between scale and leaf production on the apex are not known, and there is a period of apical inactivity between the formation of the two types of primordia (Rees, 1972). While apex size was shown to be related to the width of leaves formed in terminal or lateral bulb units, it did not appear related to whether scales or leaves were formed first (Denne, 1960). Whereas in tulips, there are examples of laminae forming on bulb scales (after treatments that kill the foliage leaves), no such instances were noted in narcissus (Rees, 1972). The anatomy of bulb scales and leaves is similar up to a length of 1 mm: thereafter, the whole division is restricted to the base of bulb scales, whereas, in the leaf, cell division occurs in the basal sheath and in an intercalary region of the lamina, the latter continuing until the lamina is around half its final length (Okada and Miwa, 1958; Denne, 1960). The anatomy of scales and leaf bases is similar (Chan, 1952).

Observations have been made on the relative number of bulb scales and leaves in bulb units. Rees (1969) obtained data for cultivars ‘King Alfred’ and ‘Fortune’, but the commonest combination in the former (3 + 3) occurred only once in 594 units of ‘Fortune’ examined, suggesting there were varietal differences in the ratio of the two structures. Higher complements of scales and leaves occurred in terminal units than in laterals, and on terminal units in laterals than on its laterals, perhaps related to the earlier initiation of terminal units. Of terminal units, almost all

flowered, and those that did not produced four or more leaves; in contrast, few laterals flowered, usually if there were three or four leaves.

There are no experimental data on the factors which trigger floral initiation (Rees, 1972). Experimentation is difficult as initiation takes place before bulb lifting, and would be complicated by the perennial habit and, hence, possibly the effects of earlier years. However, bulb units appear to reach a critical weight before they are likely to contain flowers. In many bulbous ornamentals, flower initiation occurs at a fixed time in the normal annual cycle of development, and often, for example in tulip and iris, after a minimum number of leaves have been initiated, perhaps related to apex size (Rees, 1985a). A similar situation may exist in narcissus, as there appears to be some relationship between leaf numbers and flowering, albeit different in terminal and lateral bulb units (see above). Rees (1986) determined the critical weight for flowering, based on bulb unit weights: using bulbs dissected in August, the critical weight was about 1.15 g, although there was some overlap in the weight distribution of flowering and non-flowering units. The critical weight for flower initiation of bulb units, and hence critical weight for clusters, may vary from year to year (Dickey, 1940; Rees, 1986), with growing conditions (Roh *et al.*, 1978; Kim and Lee, 1982), and between bulbs propagated by chipping and 'ordinary' bulbs (ADAS, 1987).

The number of flowers per bulb or per weight of bulbs is important for commercial bulb producers. Rees (1986) examined the relationships between the number of bulbs and flowers per tonne of bulbs, the number of bulb units per bulb, and the number of flowers per bulb unit. Variations in flower yields might arise for a number of reasons, such as a large number of bulb units being below critical size, the occurrence of a few very large bulb units, or variations in the critical size itself. Over four years, the number of flowers obtained varied from 27.5 to 32.9 thousands/tonne in 'Carlton' and from 21.0 to 31.8 in 'Golden Harvest'. A major cause of variation in flower yield was the number of bulb units per unit weight in larger bulbs, which was only partly compensated by an increase in the number of bulb units per bulb, although the number of bulb units per tonne was a relatively consistent statistic. The number of flowers per bulb unit varied between cultivars and years, and the critical bulb unit weight for flowering varied from year to year. To obtain high numbers of flowers per tonne of bulbs, mean bulb unit weight should just exceed the critical weight, and cluster weights should be multiples of the critical weight, as illustrated by the analyses of Alkema and van Leeuwen (1978) and Kruyer (1981) (Figure 1.8). At present the factors controlling these responses are not known, and flower yields can be manipulated only crudely through changing the grade of bulbs planted, planting density and the duration of the crop (growing the crop for one or more years).

Experiments carried out by Gerritsen and van der Kloot (1936) and Hartsema (1961), involving lifting bulbs early (around or before floral initiation) and excising leaves, suggested that, under normal conditions, the presence of green leaves was essential for floral initiation. However, in bulbs cold-stored for 6 months, new flowers were sometimes initiated in the lateral bulb units in the absence of green leaves (Hartsema and Blaauw, 1935). This suggested that the effects of light are not essential for flower initiation to take place (Hartsema, 1961). Although the effects of temperature on floral initiation are largely unknown, there is much information on the effects of temperature on flower development and production

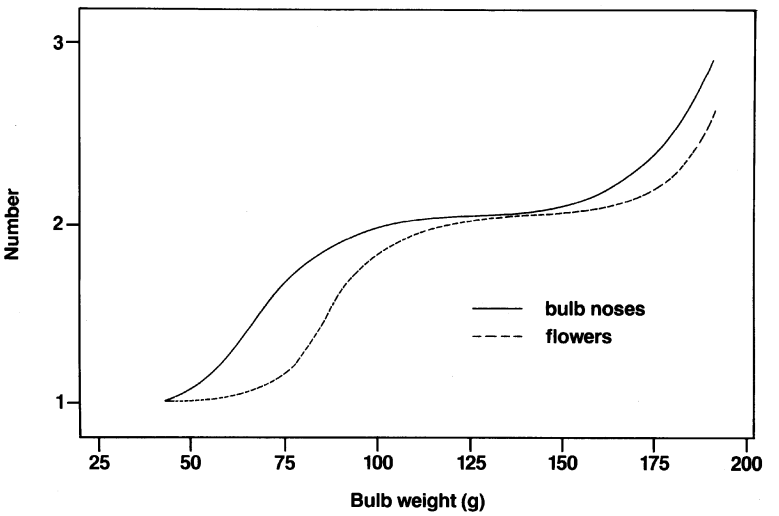

Figure 1.8 The relationship between bulb (cluster) weight and numbers of bulb noses (growing points) and first-quality flowers, narcissus 'Carlton'. (Modified after Kruyer (1981), with permission of the publishers of *Bloembollencultuur*.)

in relation to commercial flower forcing, where rapid and carefully timed stem extension and anthesis are necessary (e.g., see Rees, 1972; Hanks, 1993). Hartsema (1961) reported that the optimum temperature for flower formation was initially 20 °C, falling gradually to 13 °C as development progressed. Low temperatures (9–15 °C) immediately after lifting favoured early anthesis. For optimal growth subsequently, 11°C was required until there was 3 cm of growth, then 17 °C until 6 cm of growth, then 20 °C. In *N. tazetta* types, the optimum temperature for flower initiation was 25–30 °C (Yahel and Sandler, 1986; Koike *et al.*, 1994). Smoke and ethylene treatments induce flowering in bulbs of Tazetta narcissus below flowering size (Imanishi, 1997), but do not affect the flowering of standard daffodil cultivars (Tompsett, 1985).

Above-ground parts

The fully expanded foliage leaf consists of a basal sheath and a lamina, the prolongation of one side of the basal part. The lamina is ribbon-like, boat-shaped in transverse section (owing to an abaxial, mid-line ridge), with parallel venation and isobilateral symmetry, except in species such as *N. juncifolius* and *N. jonquilla* where the leaf is semi-cylindrical or rush-like (Chan, 1952). The mature bulb scale has a semi-transparent prolongation forming a short-lived sheath that extends above the soil, enclosing and supporting the foliage leaves early in their growth. Each side of the lamina has a similar anatomy, but at the base of the lamina elongated cells on the abaxial side contain raphides of calcium oxalate (Chan, 1952). Chan (1952) examined more than 100 *Narcissus* cultivars, of which 70% had two to four leaves, but some cultivars were consistently leafier, with seven to eight leaves.

The flower stalk is a leafless single internode (or scape), which contains abundant starch grains and raphides of calcium oxalate (Chan, 1952). The scape is topped by a single flower or a cyme, the receptacle of which bends to bring the flower into a horizontal position for anthesis and straightens after fertilisation to hold the capsule upright, a tropic response to gravity accentuated by light. Flowers also turn towards the light when growing in clumps (Rees, 1988). After flowers have been picked, the lower part of the stem that is left behind is capable of considerable growth from its basal meristem.

The flower bud is enclosed in a protective sheath or spathe, which is green during bud development and dries and splits before anthesis. Flower development in narcissus was described and reviewed by Huisman and Hartsema (1933) and Chan (1952). The flower consists of two whorls of three perianth segments (or tepals) which arise from a hypanthial tube, two whorls of three anthers, and a tricarpellate inferior ovary with two rows of anatropous ovules in each of three loculi. The corona (paracorolla, trumpet or cup), characteristic of the genus, is conspicuous between the perianth segments and the anthers (Webb, 1980); it is regarded as either a prolongation of the receptacle or as a distinct structure (Huisman and Hartsema, 1933; Guédès, 1966). The floral parts are formed in a spiral sequence from the perianth inwards to the gynoecium, although for horticultural purposes the completion of flower initiation (important as a key stage in beginning cold storage or hot-water treatment) is regarded as when the corona initial can be clearly seen on dissection, following the initiation of the gynoecium (Huisman and Hartsema, 1933; Cremer *et al.*, 1974). There are many varieties with double flowers, the structure of which varies in complexity (Reynolds and Tampion, 1983). Gross flower abnormalities (such as two flowers from the same apex) are rare (Chan, 1952), although physiological disorders may occur in flower development (Rees, 1972). The floral biology of *N. pseudonarcissus* has been described in detail by Caldwell and Wallace (1955). The corona and perianth tube lead to nectaries between the staminal filaments, the flowers being pollinated by bumble bees (*Bombus* spp.). Pollination is often poor due to a lack of insects in the relatively cool conditions, and little pollination occurs in the absence of insects. Pollination of *N. longispathus* in Spain was studied by Herrera (1995); the temperature inside the flower was up to 8 °C above ambient temperatures, raising the temperature of pollinating bees sufficient for flight. Di- and polymorphism with respect to style length has been reported for *N. tazetta* and *N. triandrus*, respectively (Arroyo and Dafni, 1995; Barrett *et al.*, 1997). The fruits are ovoid green pods which grow to full size in about two weeks and dehisce a few weeks later. The seeds are black and round and are distributed over a restricted area; seeds of commercial narcissus cultivars were described by Chan (1952).

Roots

The roots of narcissus are adventitious, unbranched, with a prominent root cap, and are generally considered to have no root hairs, although a few poorly developed root hairs can occur in some circumstances (Chan, 1952; Rees, 1972; Kawa and De Hertogh, 1992). However, Chilvers and Daft (1981) found root hairs in all cultivars examined, while Wilson and Peterson (1982) described the roots of *N. lobularis* as initially glabrous and later with hairs. Price (1977) examined the

root system of commercial narcissus, reporting a maximum of 112 functional roots for a bulb of 12–14 cm circumference, with 77% of root weight occurring in a 20 cm-deep layer beneath the bulb, indicating little depth or lateral spread. Some roots, shorter and more persistent than the rest, are contractile (Chen, 1969; Price, 1977). Contraction occurs near the base plate, shortening the root by 7–8 mm over a four week period in spring (Chen, 1969), and its relationship to cell wall structure was investigated by Wilson and Anderson (1979). Contraction in *N. tazetta* was stimulated by illumination of the lower part of the leaf laminae (Putz, 1996). The presence of endotrophic mycorrhizae in narcissus roots has been described (Kelley, 1950; Chan, 1952; Chilvers and Daft, 1981; Iqbal and Firdaus, 1986a,b). Roots rapidly became infected with mycorrhizae soon after planting, infected roots being less likely to bear root hairs (Chilvers and Daft, 1981). Iqbal and Firdaus (1986a,b) described mycorrhizae in foliage and dried sheathing leaves of bulbs of *N. poeticus*, as well as in the roots.

The seedling and development of the Juvenile plant

Germination and seedling growth were described by Chouard (1926, 1931) and Chan (1952). Germination is hypogeal. The cotyledonary sheath extends to 2–3 cm before the primary, grass-like leaf breaks through and appears above ground. After the growth of the radical, one or two adventitious roots appear, and the radical is later lost. The base of the cotyledonary sheath swells to form the outermost scale, as does the base of the foliage leaf within it, itself enclosing the apical meristem. The contractile roots pull the bulb down. The one-year-old bulb is about 1 cm long. There is no information on environmental factors controlling bulbing in the seedling, although this takes place under increasing daylength (Rees, 1972).

There is a juvenile phase of several years. In narcissus cultivars, the plant produces one or two scales and a single foliage leaf in its second and third years, a second leaf is produced in the fourth year, and three or more in the fifth (Chan, 1952; Rees, 1972). Anthesis has been reported to occur after three to eight years, at the shorter end of this range for some species and after a longer time for large-flowered cultivars (Chan, 1952; Jamiolkowska and Zawadzka, 1971; Rees, 1972; Koopowitz, 1986). When a bulb fails to flower, growth is then similar to that of a younger bulb, and at least one foliage leaf is produced in addition to the usual complement, and all foliage leaves then have completely sheathing bases (Chan, 1952). The growth and development of small bulbs propagated artificially (e.g., by chipping) is similar to that of seedlings of similar size.

CONCLUSIONS

Although ‘the processes which go on inside a bulb are very difficult to test’ (K. Goebel, quoted by Rees, 1972), the structure and development of the narcissus bulb have been well described, primarily by Chan (1952) and Rees (1969). On the other hand little is known of the endogenous or environmental factors which control processes such as bulb scale/foliage leaf differentiation or the initiation of bulb units or flowers. A few studies suggest there are varietal differences in floral initiation and bulb ‘scale’ numbers, while a few species have no cold requirement

or respond to ethylene. Further investigation of these features may facilitate the manipulation of narcissus growth and development, so that desirable characters – whether ornamental or industrial – could be exploited.

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2 The folklore of *Narcissus*

Anthony C. Dweck

INTRODUCTION

William Wordsworth probably did more for the daffodil than any other in his enchanting verse:

> I wandered lonely as a cloud
> That floats on high o'er vales and hills,
> When all at once I saw a crowd,
> A host of golden daffodils;
> Beside the lake, beneath the trees,
> Fluttering and dancing in the breeze.
>
> Continuous as the stars that shine
> And twinkle on the milky way,
> They stretched in never-ending line
> Along the margin of the bay:
> Ten thousand saw I at a glance,
> Tossing their heads in sprightly dance.

John Gerard sets the scene ideally for this chapter. The fair Lady Europa, entering with her Nymphs into the meadows, did gather the sweet smelling daffodils:

> But when the Girles were come into
> The medowes flouring all in sight,
> That Wench with these, this Wench with those
> Trim floures, themselves did all delight:
> She with the Narcisse good in sent,
> And she with Hyacinths content.

Gerard in his Herbal (Woodward, 1990) says 'It is not greatly to our purpose, particularly to seek out their places of growing wild, seeing we have them all and every one of them in our London gardens, in great abundance. The common wild Daffodil groweth wild in fields and sides of woods in the West parts of England.'

Today, the daffodil or narcissus is a very popular garden plant and an important commercial crop, with a large number of species, hybrids and varieties in cultivation (Figure 2.1). Gerard's Herbal lists 37 different types that were already in cultivation by the end of the l6th century, which demonstrates the popularity of

Figure 2.1 The narcissus, a plant with a rich folklore (from Bessette and Chapman, 1992).

the plant from the early days of horticulture. Many different daffodils are now found naturalised in grassland, hedge-banks, woodland margins, roadsides and waste ground throughout the British Isles, especially in the south.

THE LANGUAGE OF FLOWERS

In the Middle Ages, when the art of reading and writing was known only by a privileged few, there grew up a tradition of the language of flowers, whereby every flower had a meaning. It was a tradition that was revived by the early Victorians, who took great delight in this fanciful idea and collected together much of the information that survives to this day. The example that most of us would recognise

is the giving of red roses as a sign of love. In this tradition, the daffodil is for rebuttal in domestic situations: 'I do not share your feelings'. However, in battle emblems the daffodil is for regard and chivalry (Greenaway and Marsh, 1978; Pickles, 1990).

DERIVATION OF THE NAME DAFFODIL

There are a number of thoughts on how the name daffodil came into being. The popular English names daffodowndilly, daffodily and affodily may be corruptions of asphodel, since the daffodil was thought to be identical with the blossoms mentioned by the ancient Greeks. Another school of thought is that the name comes from the Mediaeval Latin *affodilus*, Latin *asphodilus* or Greek *asphodelus*, which was the name of that plant which grew across the meadows of the underworld and belonged to Persephone, the Queen of Hell (Grigson, 1996).

Pliny describes the narcissus as '*narce narcissum dictum, non a fabuloso puero*', which translated means 'named narcissus from *narce*, not from the fabulous boy'. The Greek *narkao*, meaning to be numb, originates in the narcotic properties of the plant (Genders, 1985).

The popularity of the daffodil in the British Isles is attested by the large number of common names used in various parts of the country (Dony *et al.*, 1986; Grigson, 1996; Grieve, 1998). These include:

Popular name	*Place*
Affodil, Affrodil	Cheshire
Bell-Flowers	Dorset and Somerset
Bell-Rose	Somerset
Butter and Eggs	Devon, Somerset and Northampton
Churn	Lancashire
Cowslip	Devon
Cuckoo-Rose	Devon and Somerset
Daffodil	England, Scotland, Ireland
Daffydowndilly	Somerset
Daffy-down-dilly	Somerset
Daffydilly	Northamptonshire
Dillydaffs	Somerset
Easter Lily	Devon and Somerset
Easter Rose	Somerset
Fairy Bells	Dorset
False Narcissus	Devon
Fleur de Coucou	Devon
Garden Narcissus	Devon
Giggary	Devon
Gylfinog	Wales
Gold Bells	Wiltshire
Golden Trumpets	Somerset
Gooseflop	Somerset
Goose-Leek	Isle of Man
Gracie Daisies	Devon and Somerset
Gracie Day	Devon
Hen and Chickens	Devon
Hoop Petticoats	Dorset
Jonquil	Hertfordshire

Julians	Hertfordshire
King's Spear	Somerset
Lady's Ruffles	Wiltshire
Lent-Cocks	Devon and Somerset
Lent-Lily	Cornwall, Devon, Dorset, Somerset, Isle of Wight, Gloucestershire, Sussex, Kent, Surrey, Suffolk, Warwickshire, Cheshire, Derbyshire, Lincolnshire, Yorkshire, Westmoreland
Lent Pitchers	Devon and Somerset
Lent-Rosen	Devon and Somerset
Lents	Cornwall, Devon, Lancashire
Lenty Cups	Somerset
Lent Lily	Cornwall
Lily	Scotland
Narcissus	Norfolk
Porillon	Norfolk
Queen Anne's Flowers	Norfolk
St Peter's Bell	Wales
Sun-Sonnets	Somerset
Whit Sunday	Devon
Wild Daffodil	Yorkshire
Wild Jonquil	Yorkshire
Yellow Maidens	Somerset
Fleur d'asphodèle	France
Pauvres filles de Sainte Claire	France

MYTHOLOGY AND LEGEND

According to Culpeper's Herbal (Potterton, 1983), yellow daffodils are under the dominion of Mars.

Daffodil flowers, though beautiful to the sight, leave a feeling of sadness when the history and folklore of the plant is examined. In classical mythology there was a handsome Greek shepherd boy named Narcissus. Though he was loved by all the wood nymphs, there was one called Echo who loved him more than the rest. Unfortunately, she could not tell him of her love, because she was only able to repeat his last words. It comes as no surprise to learn that Narcissus was totally unaware of Echo's love and adoration for him. He was equally unaware of the pain and suffering that his ignorance of her love was causing her. Echo became thinner and thinner as her love robbed her of her appetite, until she slowly pined away to nothing more than a spirit who took sanctuary in the mountains. Only her soft voice remained. Venus, the goddess of love, came to hear of Echo's hopeless devotion and immediately assigned the blame for her condition on Narcissus, who she decided should be punished. One day Narcissus was hunting in the forest. Little did he know that Venus had arranged with Cupid to set a magic spell on him so that he would fall in love with the first person that he saw. Coming to a crystal clear pool he stopped for a cooling drink to assuage his thirst and there in the water he saw another face rise up to meet his own as he leant over. Narcissus immediately succumbed to Cupid's spell and fell in love. Again and again he tried to catch the face of the spirit who appeared to live in the water. In vain he called

out to this vision, but all that could be heard was the faint and sad echo coming from the mountains. Narcissus had fallen in love with his own reflection. Every day he returned to the pool in the hope of capturing the face that he saw there, and every day his tears added to the water in the pool. Slowly, like Echo, he began to waste away with unrequited love. The Immortals were not totally heartless and turned him into a delicate white papery flower, which would grow forever by the pool in memory of the egotistical youth. Another story continues by saying that when the nymphs came to look for him, they only found 'A rising stalk with yellow blossoms crown'd', and that the cup in the flower's centre of all varieties contains the tears of Narcissus (Pickles, 1990).

This story has led to the name being used as the term 'narcissism' or 'narcissistic personality disorder', in which people described by this condition have a grandiose view of their own uniqueness and abilities; they are preoccupied with fantasies of great success. To say they are self-centred is an understatement (Davison and Neale, 1998). These characteristics have been validated in empirical studies (Ronnington and Gunderson, 1990) and are often a factor with borderline personality disorders (Morey, 1988). Such people are constantly seeking attention and adulation, and are, underneath, extremely sensitive to criticism and have a deep fear of failure. Many of the contemporary studies have been carried out by Heinz Kohut (Kohut, 1971, 1977; Kohut and Wolf, 1978).

The flower has another legend, which is even more gruesome than the former! Earth first put forth the flowers to lure the lovely Prosperine for Pluto, god of the underworld. The maid was so taken with the beauty of the daffodil that she stopped to admire it and as she stooped to pick it, the very worst happened. Pluto looking out from his hiding place took advantage of this momentary lack of attention and pounced out from his lair and seized her. It was, therefore, quite understandable why the ancients labelled the narcissus the flower of deceit. It was also the flower of imminent death, since it was the last bloom she plucked (MacFadyen, 1992).

Another version of this story is told by Perdita in William Shakespeare's *The Winter's Tale*, where it was Proserpina who was picking lilies and was subsequently captured by Pluto. However, in this story, as she dropped the lilies in her fear, they turned into daffodils as they touched the ground.

FOLKLORE AND RELIGIOUS CONNECTIONS

Narcissus tazetta, which grows on the Plain of Sharon, Israel, may be the plant referred to in the biblical reference '... The wilderness and the solitary place shall be glad for them; and the desert shall rejoice, and blossom as the rose ...' (Isaiah 35 v.1). The Hebrew word here translated 'rose' may indicate a bulbous plant, rather than a rose (Tenney, 1967).

Daffodils are considered by many to be unlucky, and they will not have the flowers in their house because they hang their heads, bringing tears and unhappiness. The sweet-scented old fashioned white narcissus, also called scented lily or white lily, is also known as grave flowers and unlucky to take indoors (Vickery, 1995).

In the Isle of Man it is unlucky to have the plant in the house till the goslings have hatched. The Manx name is *lus-ny-guiy* or goose herb. In common with primroses, daffodils were sometimes banned from the house by poultry-keepers, and, in

Herefordshire, if daffodils are brought in when the hens are sitting, they say there will be no chickens. However in Devon, the number of goslings hatched and reared is said to be governed by the number of wild daffodils in the first bunch of the season brought into the house (Vickery, 1995).

Robert Herrick alludes in his *Hesperides* to the daffodil as a portent of death, probably connecting the flower with the asphodel, which the ancient Greeks planted near tombs. Despite this he writes 'Fair daffodils, we weep to see/You haste away too soon . . . '

The occurrence of wild daffodils is sometimes said to indicate the former site of a religious foundation. At Frittlestoke, near Torrington, Devon, it was recorded in 1797 that the people of the village call daffodils by the name Gregories, a name that coincided with the order of a neighbouring monastery – the Canons of St Gregory (Britten and Holland, 1886). In both Hampshire and the Isle of Wight it was generally said that wild daffodils indicated the site of a monastery. St Urian's Copse is well known for its primroses and daffodils. There is a tradition that daffodils grow in profusion on one side of a track running through the copse because a religious building once stood there. The only sizeable population of wild daffodils in the London area is found at Abbey Wood, named after Lesney Abbey (Vickery, 1995).

HISTORICAL TALES

A crusader returned home to Churchill (in Avon, in the west country of England), having spent years fighting the Crusades in the Holy Land. A rich man before his departure, he had returned home poor. His wife was a lover of precious and rare flowers, and so he had carefully brought back with him two bulbs of the Primrose Peerless. The story is a sad one, since when he returned, it was to a wife who had been buried for four years. In despair he flung the cherished bulbs over the churchyard wall. He is said to have died of a broken heart. However, throughout the centuries the bulbs have grown and flourished and kept his memory alive (Vickery, 1995).

Both the daffodil and the leek are national symbols of Wales. The daffodil is associated with St David because it is traditionally said to bloom first on his day (1 March). It is an easier emblem to wear than the leek, and many a schoolchild in Wales sports one, real or artificial, on this date (Vickery, 1995).

Since 1990, National Daffodil Day has been promoted by Marie Curie Cancer Care. At about the same time, the Irish Cancer Society similarly adopted the daffodil as a symbol. In Australia, they also have a national fund raising day for cancer research (Anon., 1997).

On the Isles of Scilly, The Prince of Wales is paid one daffodil annually as rent for the untenanted lands of Scilly, paid by the local Environmental Trust.

HERBAL MEDICINES

Considering that narcissus are a rich source of alkaloids (see Chapter 6, this volume), it is not surprising that the genus has figured in herbal medicine. This

has been vindicated by recent developments. The Daily Mail (28 September 1996) carried a headline 'Shire says it with snowdrops'. 'Flower power could soon be helping sufferers of chronic fatigue syndrome. Shire Pharmaceuticals is testing galanthamine, a compound found in daffodils and snowdrops, on victims of "yuppie flu". The drug already has improved the mental performance of Alzheimer's patients.'

The Greek physician Hippocrates of Cos (460–377 BC) recommended a pessary prepared from *Narcissus* oil (probably *N. poeticus*) for the management of uterine tumours (Pettit *et al.*, 1986). Plants of the *Narcissus* genus have been used to treat a variety of human medical problems (Pettit *et al.*, 1995), and *N. poeticus* was described in the Bible as a well-established treatment for symptoms that would now be defined as cancer (Pettit *et al.*, 1990). Pliny the Elder (AD 23–77) also recorded the topical use of *N. poeticus* and another derived from *N. pseudonarcissus* for the treatment of uterine tumours. It is now known that *N. poeticus* contains 0.012% of the antineoplastic agent narciclasine in the fresh bulb (Piozzi *et al.*, 1969).

However, narcissus are not recommended for domestic use. A homoeopathic medicine is made from the bulbs and used for respiratory disease, particularly bronchitis and whooping cough, according to Culpeper's (1616–1654) Herbal (Potterton, 1983):

> The roots boiled and taken in posset drink cause vomiting and are used with good success at the appearance of approaching agues, especially the tertian ague, which is frequently caught in the springtime. A plaster made of the roots with parched barley meal dissolves hard swellings and imposthumes, being applied thereto; the juice mingled with honey, frankincense wine, and myrrh, and dropped into the ears is good against the corrupt and running matter of the ears, the roots made hollow and boiled in oil help raw ribed heels; the juice of the root is good for the morphew and the discolouring of the skin.
>
> Galen [AD 130–201] saith: That the roots of Narcissus have such wonderfull qualities in drying, that they consound and glew together very great wounds, yea and such gashes or cuts as happen about the veins, sinues, and tendons. They have also a certaine clensing facultie. The root of Narcissus stamped with hony and applied plaisterwise, helpeth them that are burned with fire, and joineth together sinues that are cut in sunder. Being used in manner aforesaid it helpeth the great wrenches of the ancles, the aches and pains of the joints. The same applied with hony and nettle seed helpeth Sun burning. Being stamped with the meale of Darnel and hony, it draweth forth thorns and stubs out of any part of the body.

Narcissus are also referred to in John K'Eogh's Irish Herbal (Scott, 1986). Narcissus was said to have a hot and dry nature. The roots, pounded with honey were good against burns, bruised sinews, dislocations and old aches. They take away freckles and heal abscesses and sores, and they draw out thorns and splinters. A decoction of the roots is a great emetic.

It has also been used as an application to wounds, for hard imposthumes, for strained sinews, stiff or painful joints, and other local ailments. The narcissus was

the basis of an ancient ointment called Narcissimum. The powdered flowers have been used as an emetic in place of the bulbs, and in the form of a syrup or infusions for pulmonary catarrh. A decoction of the dried flowers acts as an emetic, and has been considered useful for relieving the congestive bronchial catarrh of children, and also useful for epidemic dysentry. In France, narcissus flowers have been used as an antispasmodic. A spirit has been distilled from the bulb, used as an embrocation and also given as a medicine and a yellow volatile oil, of disagreeable odour and a brown colouring matter has been extracted from the flowers, the pigment being quercetin, also present in the outer scales of the onion. The Arabians commended the oil to be applied for curing baldness and as an aphrodisiac (Grieve, 1998). Conveniently, the bulbs of *N. tazetta* have also been used as a contraceptive (Matsui *et al.*, 1967). The influence of daffodil on the nervous system has led to giving its flowers and bulb for hysterical affections and even epilepsy, with benefit. It entered into the books as a purge and a vomitive and a cure for erysipelas and the palsy (Grigson, 1996).

Throughout the Middle Ages, the Arabian, North African, Central American and Chinese medical practitioners continued to use *Narcissus* oil in cancer treatment (Pettit *et al.*, 1993). For example, the bulbs of *N. tazetta* var. *chinensis*, cultivated in China as a decorative plant, were also used topically in folk medicine as a liniment for the treatment of tumours. In this case, pretazettine was proved to be one of the antitumour active compounds (Furusawa *et al.*, 1973; Ma *et al.*, 1986). The bulbs of *N. tazetta* continued to be used in Turkey as a home remedy for the treatment of abscesses, because of their antiphlogistic and analgesic property (Çakici *et al.*, 1997).

POISONOUS EFFECTS

Socrates called the narcissus the 'Chaplet of the infernal Gods', because of its narcotic effects. An extract of the bulbs, when applied to open wounds, has produced staggering, numbness of the whole nervous system and paralysis of the heart (Grieve, 1998)

There have been cases of poisoning when the bulbs have been eaten in mistake for onions (Culpeper's Herbal; Potterton, 1983). Lycorine or narcissine in warm-blooded animals acts as an emetic, causing eventual collapse and death by paralysis of the central nervous system: cattle, goats and pigs have been poisoned by the plant (Manning, 1965). With cats, narcissine causes nausea and purgation (Grieve, 1998). The poison acted speedily, high temperature did not destroy the toxicity of the poison and only a relatively small amount was needed (Grieve, 1998). Ingestion of narcissus bulbs produces severe gastroenteritis and nervous symptoms, apparently owing to the phenanthridine alkaloids contained therein (Tyler *et al.*, 1988).

When the bulbs have been mistaken for onions and eaten, either raw or cooked, symptoms including dizziness, stomach pains, nausea, vomiting and diarrhoea have developed shortly afterwards. In more severe poisoning there may be trembling, convulsions and paralysis. Vomiting has occurred in children who have eaten a few leaves, and there is also a report of a four-year-old child who died after sucking a narcissus stalk. Recovery, however, is usually complete in a few hours without any treatment being necessary. Those who pick and pack the flowers are liable to develop dermatitis, probably caused partly by the irritant effects of the sap

and partly by an allergic reaction. Animals rarely eat these plants, although, during the food shortage in the Netherlands in the Second World War, some cattle died after being given narcissus bulbs to eat. A tortoise which ate four daffodil leaves lost its appetite and became constipated and listless; it died 11 days later. In severe cases it may be necessary to induce vomiting or remove stomach contents (Cooper and Johnson, 1991). In South Africa, similar problems with toxicity are experienced. The bulbs of daffodil and narcissus are known to have caused death when eaten by mistake (Moll and Moll, 1989). A case of poisoning by daffodil bulbs, cooked by mistake in the place of leeks, was reported from Toulouse in 1923. The symptoms were acute abdominal pains and nausea, which yielded to an emetic (Grieve, 1998). The bulbs of *Narcissus poeticus*, the Poet's narcissus, are reported to be more dangerous than those of the garden daffodil, being powerfully emetic and irritant (Grieve, 1998).

ENDANGERED SPECIES

Large garden varieties of daffodils have recently been crossed with many of the small wild species to produce delightfully graceful blossoms. *Narcissus triandrus* and *N. cyclamineus* have been used in breeding for many years. The first species makes small clusters of blooms with very silky petals. *N. cyclamineus* usually gives genes for flowers with backswept petals, a long-waisted trumpet, and early flowering. These types of daffodils are constantly popular and the demand for miniature daffodils far exceeds the supply. Unfortunately, the ability to produce new kinds of miniature daffodils is hampered by the disappearance of many of the tiny wild species. *N. calcicola*, a tiny yellow jonquil from Spain and Portugal is considered endangered and *N. watieri*, possibly the most powerful tool for making miniature white daffodils, is already unobtainable (Koopowitz and Kaye, 1990). Occasionally *N. watieri* from North Africa's Atlas Mountains is advertised by unscrupulous bulb merchants who substitute another variety. The exact status of *N. watieri* is unknown. The only hope is that a few plants may still exist on some of the rocky ledges or hillsides where the species once thrived. If so, perhaps it can be introduced once again. More likely, the species has already been destroyed – either over-collected or eaten by goats (Koopowitz and Kaye, 1990).

FOOD USE

On the upper Nile, Grant found a narcissus about 20 cm high, with white flowers having a waxy, yellow corona and with leaves tasting of onions. The leaves, cooked with mashed groundnuts, he reported, make a delicious spinach (Hedrick, 1972).

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3 Classification of the genus *Narcissus*

Brian Mathew

CLASSIFICATION OF *NARCISSUS* SPECIES

It is generally acknowledged that the genus *Narcissus* presents great taxonomic problems, and there have been numerous attempts at its classification. Some authors have taken a very wide view of the concept of each species (e.g., Webb, 1980), resulting in as few as 26 recognised species, while some (e.g., Fernandes, 1969b) have taken a very narrow view which results in the recognition of a great many species (upwards of 60), often involving a complex hierarchy of infraspecific taxa. At the generic level, some researchers have taken a narrow view of the delimitation of genera; for example, in the system devised by Haworth (1831), many of the presently accepted subgenera, sections and subsections were recognised as separate genera (e.g., *Hermione* for the 'tazetta' group, *Corbularia* for the 'bulbocodium' group, *Ajax* for the 'pseudonarcissus' group, *Ganymedes* for the 'triandrus' group, etc.). On the other hand, Herbert (1837) took a rather wider view of the genera, and reduced the 16 genera recognised by Haworth to six, while Spach (1846) went a stage further and treated many of Haworth's genera as sections of the one genus, *Narcissus*. Many of the plants that had been described as species up to that time were of unknown (or garden) origin, and it was Baker (1875) who attempted to clarify the situation by considering only wild source material for his classification. All the segregate genera recognised by most of the previous authors were included by him in *Narcissus*, except for *Tapeinanthus*, which Baker regarded as sufficiently distinct to uphold at generic level; nowadays it, too, is often 'sunk' into *Narcissus* (e.g., Webb, 1980), although Cullen (1986) maintains it on the basis of the rudimentary corona and near-absence of a perianth tube.

Although the status (subgenus, section, etc.) bestowed upon the individual groups may vary somewhat from author to author, as does the status of the individual taxa within the groups, the actual content of each group is similar in the various classifications. Most of the groups – most frequently referred to as sections – are fairly obvious, for example the Trumpet daffodils, the Tazettas, the Pheasant's Eyes, the Hoop Petticoats, the Jonquils, and so on, and these are the basic divisions in the genus recognised here. It is, however, considered somewhat unsatisfactory to give all the infrageneric groupings similar status (in some recent classifications, e.g., Webb (1980), they are treated equally as sections). The classification used here is a combination and adaptation of the systems devised by previous researchers, notably those of Fernandes (1951, 1969a,b) and Webb (1980), in an attempt to

reflect the relationships more clearly. Some examples of species representing the main sections are illustrated in Figure 3.1.

An additional complication to the taxonomy is posed by hybridisation. Most species of *Narcissus* will hybridise but, significantly, there is great variation in the fertility of the offspring, depending upon the degree of relationship between the parents. The important cytological work by Fernandes (1951, 1969a,b), Brandham and Kirton (1987) and Brandham (1992) has done much to clarify the genetics of the genus. There has been a great deal of hybridisation in this very popular, garden-worthy genus, resulting in thousands of hybrid cultivars and selections (Kington, 1998), and doubtless this will continue. Although much of this work has been concerned with sophisticated selection for flower form and colour (e.g., pink and red coronas and apricot-coloured perianth segments), there are probably still some interesting lines of research that could be pursued using the many wild species. Taking just one possibility as an example, the autumn-flowering species (*Narcissus serotinus, N. elegans* and the green-flowered *N. viridiflorus*) could perhaps be utilised in the production of a race of larger-flowered autumnal narcissi, thus extending the overall flowering season of the garden forms by several months. With the great diversity of characters exhibited by the species and their numerous variants, there are great possibilities in this natural gene pool. However, some of the species are under threat in the wild, and many more will become so with increasing urban and tourist-based development. A good example is provided by *N. cyclamineus*, which in recent decades has been one of the most important species in the production of a wide range of 'Cyclamineus' daffodils of Division 6 in the Horticultural Classification: this species may already be extinct in the wild, or at best very scarce (Blanchard, 1990). Even in the case of widespread and well-known species, there are often local variants which could be of potential in breeding programmes. It is thus essential that steps are taken to ensure the survival of these wild progenitors of the garden daffodils, so that this valuable gene pool is not lost or severely depleted.

Although the family Amaryllidaceae as a whole may be seen as primarily tropical or subtropical in its distribution, the genus *Narcissus* is to be found largely in south-western Europe, notably Spain and Portugal, and in North Africa. A few species extend into France and Italy, and even fewer are found farther east in the Balkans (*N. poeticus*, *N. serotinus* and *N. tazetta*) and the eastern Mediterranean (*N. serotinus*). Records outside this area – for example, *N. tazetta* variants in western and central Asia, China and Japan – are almost certainly ancient introductions. The extent of the natural distribution northwards is also unknown: although there are apparently wild populations of *N. pseudonarcissus* or similar species in Britain, for example, these could well be the result of early introductions that have become naturalised.

OUTLINE OF CLASSIFICATION

1. **Subgenus *Narcissus***
   a. Section ***Narcissus***
   b. Section ***Pseudonarcissus***
   c. Section ***Ganymedes***

(1)

(2)

(3)

(4)

(5) (6)

Figure 3.1 Examples of *Narcissus* species of the main sections: (1) Subgenus *Narcissus*, Section *Narcissus*: *N. radiiflorus*; (2) Subgenus *Narcissus*, Section *Pseudonarcissus*: *N. cyclamineus*; (3) Subgenus *Hermione*, Section *Hermione*: *N. papyraceus*; (4) Subgenus *Corbularia*: *N. romieuxii*; (5) Subgenus *Narcissus*, Section *Ganymedes*: *N. triandrus*; (6) Subgenus *Narcissus*, Section *Jonquillae*: *N. gaditanus*. Photos: Brian Mathew. (*See Colour plate 1*)

d. Section ***Jonquillae***
   i. Subsection ***Jonquillae***
   ii. Subsection ***Apodanthi***
   iii. Subsection ***Chloranthi***
e. Section ***Tapeinanthus***

2. Subgenus *Hermione*
a. Section ***Hermione***
   i. Subsection ***Hermione***
      A. Series *Hermione*
      B. Series *Albiflorae*
   ii. Subsection ***Angustifolii***
   iii. Subsection ***Serotini***
b. Section ***Aurelia***

3. Subgenus *Corbularia*

CONSPECTUS OF THE GENUS *NARCISSUS*

1. Subgenus *Narcissus* [Subgenus *Eu-narcissus* Pax]

Description: Flowers usually vernal, rarely autumnal, umbels solitary- to several-flowered. Perianth segments well-developed; corona well-developed, trumpet-shaped, cup-shaped or rudimentary; perianth tube varying from ± absent to funnel-shaped or long and slender; stamens with ± straight filaments. Chromosome number x = 7 (usually 2n = 14).

Five sections of subgenus *Narcissus* are recognised, *Narcissus, Pseudonarcissus, Ganymedes, Jonquillae* and *Tapeinanthus*.

1a. Section Narcissus *[Section* Helena *(Haworth) Ascherson and Graebner]*

Description: Leaves linear or lorate, usually grey-green, flat or channelled. Flowers vernal, solitary, fragrant; perianth tube cylindrical; segments spreading or slightly reflexed; corona shallow, disk-like or very widely funnel-form, much wider than deep. Filaments much shorter than the anthers; anthers dorsifixed, the lower three usually included and the upper three at least partly exserted.
Type species: *N. poeticus* L.
Note: There have been great differences of opinion over the classification of the Poet's or Pheasant's Eye narcissus. At one extreme all the variations are considered to belong to one species, *N. poeticus*, while at the other extreme about ten separate species have been recognised. The current view, which appears to have gained most support, is that there are two species, *N. poeticus* and *N. radiiflorus*, with several named variants of each.

N. poeticus L. Petals broad and overlapping at the base, giving a substantial 'rounded' flower; stamens unequal.

var. ***poeticus***. Corona flattish and disk-like; petals at right angles to corona; flowers up to 7 cm diameter. Southern France, Italy.

var. ***hellenicus*** (Pugsley) Fernandes. Corona shallow cup-shaped; petals reflexed; flowers small, about 4.5 cm diameter. Greece.
var. ***majalis*** (Curtis) Fernades. Corona shallow cup-shaped, white-zoned below the red edge; flowers up to 7 cm diameter. France.
var. ***recurvus*** (Haworth) Fernandes. Corona shallow cup-shaped, green-yellow edged red; petals reflexed; flowers up to 7 cm diameter. Switzerland.
var. ***verbanensis*** Herbert. Corona shallow cup-shaped; petals very pointed at apex; flowers small, about 3.5–5 cm diameter. Italy.

N. radiiflorus Salisbury. Petals not overlapping at the base, the flower more 'starry' in appearance; stamens nearly equal.

var. ***radiiflorus***. Corona shallow cup-shaped, less than 1 cm across. Switzerland, Austria, northern Balkan Peninsula.
var. ***stellaris*** (Haworth) Fernandes. Corona shallow cup-shaped and about 1 cm across. Central and eastern Europe.
var. ***exertus*** (Haworth) Fernandes. Corona flattish and disk-like, yellow or yellowish-green with a red or orange edge. Switzerland, southern France.
var. ***poetarum*** Burbidge and Baker. Corona flattish, wholly red. Of unrecorded origin.

1b. Section Pseudonarcissus *DC. [subgenus* Ajax *Spach]*

Description: Leaves usually grey-green, flat. Flowers vernal, usually solitary, rarely up to four, wholly yellow, wholly white or bicoloured; perianth tube broadly conical; segments usually spreading, rarely suberect or much reflexed; corona cylindrical, often widened at the mouth, much longer than wide. Filaments straight, subequal to or much longer than the anthers; anthers ± basifixed or rarely dorsifixed, exserted from the perianth tube, included within the corona.
Type species: *N. pseudonarcissus* L.
Note: These are the true daffodils in which the corona is in the form of a long more or less cylindrical trumpet rather than a cup, saucer or funnel. They tend to flower in mid spring, after the early Hoop Petticoats and Angel's Tears but before the *N. poeticus* forms. Numerous taxa have been described in this section and these have been accorded various taxonomic ranks, many of them as variants of *N. pseudonarcissus*. An added complication to the taxonomy is that they have been cultivated for centuries and many plants of unrecorded origin have also been described as species. A very thorough revision of the group is required. They are listed below in two groups, the very small ones such as *N. asturiensis* and *N. nanus* and the larger-flowered including *N. pseudonarcissus* and its relatives.

Group A. Plants small, usually less than 15 cm; flowers to 3.5 cm diameter

N. asturiensis (Jordan) Pugsley. Plant to 10 cm in height. Leaves grey-green. Flowers yellow; corona constricted in the middle. Northern Portugal, north-central and north-west Spain, on acidic soils. Var. *villarvildensis* Diaz and Prieto is a slight variant from central-northern Spain, as is var. *brevicoronatus* Pugsley which has a shorter corona.

N. cyclamineus DC. Flowers yellow, with sharply reflexed petals. Leaves bright green, not at all glaucous. North-west Portugal, north-west Spain.
N. jacetanus Casas.

> **var**. ***jacetanus***. Very similar to *N. asturiensis* but with a very incised corona. Northern Spain, on limestone formations.
> **var**. ***vasconicus*** (Casas) Casas. A very small-flowered variant, corona only 1.5 cm long. Northern Spain, on acid formations.

N. lagoi Merino. Up to 50 cm tall but with small flowers, similar to those of *N. asturiensis*. North-west Spain.
N. minor L. Similar to *N. asturiensis* but a larger plant to 15 cm with broader leaves. Flowers yellow, the corona not constricted in the middle. Long cultivated, and of unknown origin, possibly the result of hybridisation between *N. asturiensis* and *N. pseudonarcissus*.
N. nanus Spach. Early-flowering, bicoloured with pale yellow petals and a deeper yellow, straight-sided corona. A small trumpet daffodil of unknown origin.
N. provincialis Pugsley. Similar to *N. minor*, the flowers slightly larger to 4 cm long. Southern France.
N. parviflorus Jordan. Flowers ± pendent, with pale cream-yellow perianth segments and deep yellow corona. South-western France.
N. pumilus Salisbury. Similar to *N. nanus* but flowers wholly bright yellow, the corona very expanded and frilled at the margin. Origin unknown.
N. portensis Pugsley. Up to 20 cm in height with uniform bright yellow flowers; perianth segments noticeably shorter than the funnel-shaped trumpet. Northern Portugal, central and north-western Spain.

Group B. Plants often 15–60 cm or more and/or with large flowers, usually 5–12 cm diameter

N. abscissus (Haworth) Schultes and Schultes fil. Flowers bicoloured with cream perianth segments and yellow, parallel-sided corona not expanded at mouth. North-eastern Spain, south-western France.
N. albescens Pugsley. Flowers creamy-white with a pale creamy-yellow corona, held horizontally; corona much-expanded at the mouth. Origin unknown.
N. alpestris Pugsley. Flowers wholly white, pendent; perianth segments held alongside corona. North-eastern Spain.
N. bicolor L. Similar to *N. abscissus* but with pale yellow perianth segments and deeper yellow corona. Origin unknown.
N. bujei (Casas) Casas. Similar to *N. hispanicus* (below) but leaves less noticeably blue-green. Flowers wholly yellow, to 6.5 cm across; corona mouth not markedly frilled. Southern Spain.
N. calcicarpetanus Casas. Flowers suberect or horizontal, wholly yellow; corona frilled 2.5–3 cm long, longer than perianth segments. North-central Spain.
N. confusus Pugsley. Similar to *N. hispanicus* (see below) but with green leaves and corona less flared at mouth. Flowers wholly yellow, facing obliquely upwards. Central Spain.
N. fontqueri Casas and Rivas Ponce. Related and very similar to *N. nobilis* (below). Northern Spain.

N. gayi (Hénon) Pugsley. Probably a variation of *N. pseudonarcissus* (below). Origin unknown.

N. genesii-lopezii Casas. Described as a very glaucous-leafed plant with sulphur-coloured flowers. East-central Spain.

N. hispanicus Gouan. Tall, with flowers of 10 cm diameter, yellow; leaves bluish, twisted; corona very flared at mouth. Long-cultivated, origin obscure.

N. longispathus Pugsley. Tall with up to three usually wholly yellow flowers per umbel; spathes very conspicuous, to 10 cm long. South-eastern Spain.

N. macrolobus (Jordan) Pugsley. Up to 25 cm tall; pale yellow with corona slightly deeper yellow; probably a variation of *N. pallidiflorus*. North-eastern Spain, south-western France.

N. moleroi Casas. Similar to *N. alpestris* but flowers smaller, pale yellow. North-eastern Spain.

N. moschatus L. Up to 30 cm tall; flowers creamy-white, pendent with petals drooping alongside corona. Origin unknown, but probably from south-western France or north-eastern Spain.

N. nevadensis Pugsley. Flowers up to four in an umbel, bicoloured with pale yellow petals and corona slightly deeper. Southern Spain.

N. nobilis (Haworth) Schultes fil. Only 15–30 cm tall but flowers 8–12 cm diameter, bicoloured; petals white, corona deep yellow. Northern Spain, northern Portugal. Var. *leonensis* (Pugsley) Fernandes from north-central Spain is a larger-flowered variant, with flowers up to 12.5 cm diameter with a very widely-flared corona.

N. obvallaris Salisbury. To 30 cm but with relatively small flowers (4 cm diameter), wholly yellow. Central-southern Spain, naturalised in western Britain.

N. pallidiflorus Pugsley. Flowers very pale cream/yellow, to 7.5 cm diameter; perianth segments twisted, corona mouth much-toothed. Northern and north-eastern Spain, south-western France.

N. perez-chiscanoi Casas. Probably rather similar to *N. obvallaris* (above), with fairly small flowers. Central-south Spain.

N. primigenius (Suarez ex Lainz) Casas and Lainz. This appears to be very similar to *N. nobilis*, perhaps best treated as a variant of it. North-west Spain.

N. pseudonarcissus L.

> **ssp**. ***pseudonarcissus*** To 30 cm; bicoloured white/yellow flowers; corona not widely expanded and frilled. Spain, southern France, northern Italy; naturalised in several other countries in Europe.
>
> **ssp**. ***eugeniae*** (Casas) Casas. A smaller plant to 10 cm but flowers to 7.5 cm diameter; perianth segments not overlapping; leaves short and wide. Central-eastern Spain.

N. pseudonarcissus **ssp**. ***pugsleyanus*** Barra and Lopez. Appears to be closer to *N. nobilis*, but with smaller flowers and a perianth tube that is markedly shorter (only 1.2–2 cm). Central Spain.

N. radinganorum Casas. Probably close to *N. hispanicus* with wholly yellow flowers, but a smaller plant, 25–40 cm tall. South-eastern Spain.

N. tortuosus Haworth. Flowers sulphur-white with twisted petals, similar to *N. moschatus*. Northern Spain.

1c. Section Ganymedes *Salisbury ex Schultes and Schultes fil.*

Description: Leaves narrow, flat or ± cylindrical, usually dark green and slightly glaucous. Flowers vernal, one to about six in an umbel; perianth tube funnel-form; segments sharply reflexed; corona campanulate or obconical, about as wide as deep. Filaments of differing lengths, the lower three shorter than the anthers and with the anthers included, the upper three much longer than the anthers and the anthers exserted.
Type species: *N. triandrus* L.
Note: A very distinctive group, known as the Angel's Tears daffodils. They have pendent flowers with sharply reflexed perianth segments; it is the three prominent upper stamens that have given rise to the epithet *triandrus*. The taxa in this group have been treated as separate species by some authors, or at various infraspecific levels within *N. triandrus* by others. The latter view is accepted here, but it must be pointed out that even these taxa are not well defined.

N. triandrus L.

var. ***triandrus***. Flowers white or with a yellowish tinge on petals; leaves 4–5 mm wide. Northern Spain.
var. ***cernuus*** (Salisbury) Baker. Flowers creamy-white or very pale yellow; leaves very narrow, about 2 mm wide. Widespread in Spain and Portugal.
var. ***concolor*** (Haworth) Baker [syn. *N. triandrus* ssp. *pallidulus* (Graells) Webb]. Flowers bright yellow. Portugal, central, southern and eastern Spain.
var. ***loiseleurii*** (Rouy) Fernandes [syn. *N. triandrus* ssp. *capax* (Salisb.) Webb]. Similar to var. *triandrus* with white flowers; grows in sand by the sea on the Isles de Glenán, western France.

Notes: The most frequently cultivated variant of this popular *Narcissus* has flowers of a cream colour and is known as 'N. triandrus albus'; it is of unknown origin and should probably be regarded as belonging to var. *cernuus*.

N. lusitanicus Casas Dorda and Casas from central Portugal may belong here with *N. triandrus*, since it is described as having affinities with *N. pallidulus*, which is regarded by Webb (1980) as a synonym of var. *concolor*; it is said to have intensely yellow flowers.

1d. Section Jonquillae *De Candolle*

Description: Leaves narrow, dark green, cylindrical or subcylindrical. Flowers vernal or rarely autumnal, one to several in an umbel, very fragrant; perianth tube cylindrical, slightly widened towards the apex; segments spreading or slightly reflexed; corona a shallow cup, wider than deep. Filaments shorter than anthers; anthers dorsifixed, included within the corona or slightly exserted.
Type species: *N. jonquilla* L.

Three subsections of section *Jonquillae* are recognised, *Jonquillae, Apodanthi* and *Chloranthi*.

1d(i). Subsection Jonquillae

Description: Spring-flowering; flowers yellow throughout; corona cup-shaped, to 6 mm deep.
Type species: *N. jonquilla* L.

N. jonquilla L. Leaves dark green, ± terete, channelled on the adaxial surface, 1–4 mm wide. Flowers up to five in each umbel, fragrant, wholly yellow, 3–3.5 cm diameter; perianth tube straight, 2–3 cm long; segments spreading or slightly reflexed; corona 2–4 mm deep, 10–15 mm wide, shallowly crenate at the margin. Spain, Portugal. Note: The Jonquils are a confusing group with much natural variation. Botanists have for long disagreed as to how many species should be recognised. Listed below are the various taxa that have been described.
N. assoanus Dufour [syn. *N. juncifolius* auct. plur., *N. requienii* Roemer]. Flowers one or two per umbel, 1.5–2.2 cm diameter; corona 3–5 mm deep, 9–11 mm wide; tube straight. North-eastern and eastern Spain, southern France. Ssp. *praelongus* Barra and Lopez has a longer perianth tube (to 27 mm). Southern Spain.
N. baeticus Casas. Similar to *N. assoanus* but with a longer, narrower perianth tube. Southern Spain.
N. cerrolazae Ureña. Flowers one to four, 2.5–4 cm diameter; corona 5–7 mm deep, 11–16 mm wide; tube straight or curved. Southern Spain.
N. cordubensis Casas. Flowers up to three, about 3 cm diameter; corona 5 mm deep, 15 mm wide; tube slightly curved. Southern Spain.
N. fernandesii G. Pedro. Flowers up to four per umbel, 2.5–3.3 cm diameter; corona 6 mm deep, 8 mm wide; tube curved. South-central Portugal, south-central Spain. Var. *rivas-martinezii* (Casas) Casas from southern Spain is a variant with a straight perianth tube.
N. gaditanus Boiss. and Reuter. Flowers up to eight per umbel, 1–2 cm diameter; corona 2.5–7 mm deep, 4.5–8 mm wide; tube curved. Southern Spain, southern Portugal.
N. jonquilla* var. *henriquesii Sampaio. Flowers one or two per umbel, 3.5–4 cm diameter; corona 6 mm deep, 10 mm wide; tube straight. South-central Portugal.
N. marianicus Casas. Similar to *N. fernandesii* and probably best regarded as a variant of it. Central Spain.
N. minutiflorus Willkomm. Probably should be regarded as a small variant of *N. gaditanus* with flowers only 1 cm across. Southern Portugal, southern Spain.
N. palearensis Romo. This is described as being like *N. jonquilla* but usually with one to three flowers with corona 6–7 mm deep. North-eastern Spain
N. pallens Freyn and Willk. This is now regarded by Fernandez Casas as a pale version of *N. assoanus*, var. *pallens*.
N. willkommii (Sampaio) Fernandes. Flowers up to three per umbel, 1.5–2 cm diameter; corona 4–5 mm deep, 7–11 mm wide; tube straight. Southern Portugal, south-western Spain.

1d(ii). Subsection Apodanthi *(A. Fernandes) D.A. Webb*

Description: Leaves usually grey-green, with two or four keels on the abaxial surface, channelled on the adaxial surface. Flowers vernal, one to several in an

umbel, fragrant; perianth tube cylindrical or slightly widened towards the apex; segments spreading or slightly reflexed; corona cup-shaped, wider than deep. Filaments shorter than the anthers; anthers dorsifixed, included within the corona or the upper whorl of three exserted.
Type species: *N. rupicola* Dufour.
Note: Although very similar to the species of subsection *Jonquillae* in flower shape, having long-tubed fragrant flowers with small or shallow cup-like coronas, the leaves are very different, usually grey-green and angled in cross-section with conspicuous keels on the underside, not green and terete as in the *Jonquillae*. They are generally small plants, not more than 20 cm tall and the flowers are only 1.2–4 cm in diameter. There may be up to five flowers in an umbel but several taxa usually have solitary flowers.

N. albimarginatus Muller-Doblies. Flowers one or two, golden yellow with a white rim to the corona. This species exhibits heterostyly: at anthesis all six stamens of the short-styled plants are exserted from the corona, unlike other species in the group (but *N. gaditanus* does have the upper three exserted). Morocco.
N. atlanticus F.C. Stern. Flowers solitary, creamy-white, about 3.5 cm diameter; corona cup-shaped, 6 mm deep, 11 mm wide. Of unknown origin, presumed to be Morocco.
N. calcicola Mendonça. Flowers up to five, yellow, 1.7–2.5 cm diameter; corona cup-shaped, 4–8 mm deep, 6–9 mm wide. Central Portugal.
N. cuatrecasasii Casas, Laínz and Ruiz Rejón. Flowers one or two, yellow, 2.2–3 cm diameter; corona cup-shaped, 3–6 mm deep, 8–10 mm wide. Southern Spain. This was known previously as *N. rupicola* ssp. *pedunculatus*. The variety *segimonesis* (Casas) Casas is a variant from southern Spain which is said to have slightly smaller flowers, but this is not consistent.
N. rupicola Dufour.

ssp. ***rupicola***. Flowers solitary, yellow, 2–4 cm diameter; corona a wide funnel, 2–6 mm deep, 7–10 mm wide. Central and southern Spain, central Portugal.
ssp. ***marvieri*** (Jah. and Maire) Maire and Weiller. Flowers solitary, yellow, about 3.5 cm diameter; corona a wide funnel, *ca*. 10 mm deep, 15 mm wide. Morocco.
ssp. ***watieri*** (Maire) Maire and Weiller. Flowers solitary, white, about 3.5 cm diameter; corona a wide funnel, 2–6 mm deep, 7–10 mm wide. Morocco.

N. scaberulus Henriques. Flowers up to four, yellow, 1.2–1.7 cm diameter; corona cup-shaped, 2–5 mm deep, 5–7 mm wide. North-central Portugal.

1d(iii). Subsection Chloranthi *D.A. Webb [Sect.* Chloraster *(Haworth) E. Dorda and F.J. Fernández Casas]*

Description: Autumn-flowering; flowers green throughout; corona very small, *ca*. 1 mm deep.
Type species: *N. viridiflorus* Schousboe.

N. viridiflorus Schousboe. Leaves very narrow, dark green, not present at flowering time. Flowers very odourous/fragrant, one-five in an umbel, about 2–2.5 cm in

diameter, deep green throughout; perianth tube 1–1.5 cm long, segments narrowly oblong, reflexed; corona a shallow cup only *ca*. 1 mm deep, 6-lobed at the margin. Southern Spain, Morocco.

1e. Section Tapeinanthus *(Herbert) Traub [Sect.* Braxireon *(Rafinesque) B. Valdes]*

Description: Leaves filiform. Flowers autumnal, usually solitary; perianth tube very short; perianth segments spreading; corona rudimentary to ± absent.
Type species: *N. cavanillesii* A. Barra and G. López.

N. cavanillesii A. Barra and G. López [syn. *Tapeinanthus humilis* (Cavanilles) Herbert]. Leaves one or two per bulb, present at flowering time or appearing later, filiform, green. Flowers autumnal, usually solitary, erect or suberect, 2–2.5 cm in diameter, yellow; corona absent or rudimentary. South-west Spain, Morocco.
Note: Larger-flowered forms have been found in the Atlas Mountains of North Africa.

2. Subgenus *Hermione* (Salisbury) Spach [syn. Sect. *Tazettae* De Candolle]

Description: Flowers vernal or autumnal, usually several in an umbel; perianth tube usually long and slender; perianth segments well-developed, spreading or slightly reflexed; corona a shallow cup, sometimes ± absent. Filaments ± straight, shorter than anthers; anthers dorsifixed, included, or the upper three slightly exserted from the tube. Chromosome number x = 5 (2n = 10, 20, 30 or sometimes 22, 44).
Type species: *N. tazetta* L.

Two sections of subgenus *Hermione* are recognised, *Hermione* and *Aurelia*.

2a. Section Hermione

Description: Leaves flat. Flowers usually vernal, rarely autumnal, several in an umbel; perianth tube cylindrical/narrowly infundibuliform; perianth segments spreading; corona well developed, usually cup-shaped.
Type species: *N. tazetta* L.

Three subsections of section *Hermione* are recognised, *Hermione, Angustifolii* and *Serotini*.

2a(i). Subsection Hermione

Description: Leaves flat, to 2 cm wide. Flowers usually vernal, more rarely autumnal; corona cup-shaped, to 6 mm deep.
Type species: *N. tazetta* L.
Note: The taxa in this subsection, comprising the very fragrant, cluster-headed Tazetta and Paperwhite *Narcissus* species and their relatives, have been classified in very different ways. Some authors have regarded all those listed below as separate species, others have grouped them as subspecies or varieties of either N. *tazetta* (those with yellow or bicoloured flowers) or *N. papyraceus* (those with wholly white flowers), while others have 'sunk' some of the names altogether, without any

recognition whatsoever. Here, in order that interesting variations may not be overlooked, they have been retained with their specific names, and for convenience grouped in two series under the two 'umbrella' species, *N. tazetta* and *N. papyraceus*.

Series A. ***Hermione*** (including series *Luteiflorae* Rouy). *N. tazetta* and its relatives – perianth segments white or yellow, corona yellow or orange.
N. tazetta L. Leaves grey-green. Scape to 45 cm, flattened in section. Flowers up to 15 in an umbel, to 4 cm diameter, fragrant; perianth tube 1–2 cm long; segments white; corona deep yellow or orange. Widespread in the Mediterranean region.

Note: Some wild forms of *N. tazetta*, although predominantly winter/spring-flowering, sometimes flower in autumn in the wild but do not appear to do so consistently when introduced into cultivation. This species has a long history of cultivation and is naturalised widely from the Mediterranean to Japan; it is sometimes referred to as the Chinese Sacred Lily.

The following have been variously recognised as variants of *N. tazetta* or as separate species:

N. aureus Loiseleur. Leaves green. Perianth segments bright yellow; corona darker yellow-orange. A wholly yellow-flowered Tazetta, similar to the often-cultivated 'Soleil d'Or' which may be a selection of it. South-west France, north-west Italy.
N. bertolonii Parlatore. Leaves slightly greyish-green. Perianth segments pale to bright yellow; corona bright yellow to orange. Probably inseparable from *N. aureus*.
N. canaliculatus hort. (*N. tazetta* ssp. *lacticolor*). Leaves narrow (to 5 mm), grey-green. Scape usually less than 15 cm. Perianth segments white, corona yellow. Origin unknown.
N. corcyrensis (Herbert) Nyman. Leaves slightly grey-green. Perianth segments pale yellow, narrow and not overlapping at base, sometimes slightly reflexed; corona yellow or orange-yellow, conspicuously lobed at margin. Corfu, possibly also southern France, Italy and the Balkan Peninsula.
N. cupularis (Salisbury) Schultes. Leaves markedly blue-grey-green. Perianth segments and corona pale to bright yellow. Probably inseparable from *N. aureus*.
N. cypri Sweet. Leaves slightly grey-green. Flowers large (4–5 cm diameter) with white perianth segments and pale yellow corona, expanded at mouth. Cyprus.
N. italicus Ker-Gawler. Leaves green. Flowers large (4–5 cm diameter) with perianth segments pale creamy-yellow; corona yellow. Southern France, Italy, Sicily, Corsica, Sardinia.
N. ochroleucus Loiseleur. Leaves green. Scape subterete. Flowers 2.5–3.5 cm diameter; perianth segments white, corona pale lemon yellow. Similar to *N. italicus* and probably best regarded as a minor variant of it. Southern France.
N. patulus Loiseleur. Leaves grey-green. Scape up to 20 cm. Flowers 1.8–2.5 cm diameter; perianth segments slightly reflexed, white; corona deep yellow. Southern France, Italy, Sicily, Sardinia, Corsica, Balkan Peninsula.

Series B. Albiflorae Rouy. *N. papyraceus* and its relatives – perianth segments white, corona white.
N. papyraceus Ker-Gawler. Leaves markedly grey-green. Scape to 50 cm, rarely more, compressed. Flowers 2.5–4 cm diameter, in umbels of up to 20, very fragrant;

perianth tube 1.2–2.5 cm long; segments and corona wholly white; corona cup-shaped, usually 2–4 mm deep, slightly crenulate at the margin. South Europe and North Africa; widespread in the central and western Mediterranean region, westwards to Portugal.

The following have been treated as variants of *N. papyraceus* or as separate species:

N. barlae Parlatore. Leaves very grey-green. Flowers 2–2.5 cm diameter. Probably conspecific with *N. panizzianus* (see below).
N. canariensis Burbidge. Leaves greyish-green. Flowers small (at most 1.5 cm diameter) with pointed petals. Canary Islands.
N. pachybolbos Durieu. Bulb very large (5–7 cm diameter at maturity). Leaves pale grey-green. Flowers 1.5–2 cm diameter, corona less than a third as long as perianth segments. Algeria, Morocco.
N. panizzianus Parlatore. Leaves grey-green. Scape very strongly compressed, two-edged. Flowers 2–2.5 cm diameter; perianth segments pointed. South-east France, Italy, Spain, Portugal.
N. polyanthus Loiseleur. Leaves green. Scape terete. Flowers 2.5–4 cm diameter; corona entire. Southern France.

The following two, although wholly white-flowered, appear to be sufficiently distinct from *N. papyraceus* and its relatives to merit specific status:

N. dubius Gouan. Leaves dark green, slightly glaucous, 3–5 mm wide. Scape to 20 cm. Flowers up to six in each umbel, 1.5–2 cm diameter; perianth tube 1–1.4 cm long; corona half as long as petals, *ca*. 4 mm deep. Southern France, north-eastern Spain.
Note: Cytological investigations suggest that this has arisen by hybridisation between *N. assoanus* and *N. papyraceus*, but since it is now established over a wide area it is probably best regarded as a species in its own right. Although included here in subsection *Hermione*, there is a case for a separate subsection to house it.

N. tortifolius Casas. Leaves grey-green, twisted lengthwise, 5–8 mm wide. Scape terete-elliptical in section, to 28 cm. Flowers up to 16 in each umbel, about 1.5 cm diameter; perianth tube *ca*. 10 mm long; corona *ca*. 2 mm deep. South-eastern Spain.

2a(ii). Subsection Angustifolii *(A. Fernandes) F.J. Fernández Casas*

Description: Leaves narrow (usually under 0.5 cm wide), flat or subterete. Flowers autumnal; corona very shallow, 2 mm or less deep.
Type species: *N. elegans* (Haw.) Spach.

N. elegans (Haw.) Spach. Scape *ca*. 20 cm. Leaves usually 2–5 mm wide, flattish or subterete, grey-green. Flowers autumnal, fragrant, up to seven in an umbel, 2.5–3.5 cm in diameter; perianth segments white, cream or greenish-white; corona a shallow cup 1.5–2 mm deep with an incurved margin, green, greenish-brown or orange. West and south Italy, Sicily, Algeria, Libya, Morocco.

Note: *N. elegans* shows considerable infraspecific variation and some of the variants have been named: var. *elegans* forma *elegans* has narrow, pointed, white perianth segments and greenish corona; var. *elegans* forma *auranticoronatus* Maire has an orange corona; var. *fallax* Font-Quer has narrow, pointed, greenish-white perianth segments; var. *flavescens* Maire has narrow, pointed cream-coloured perianth segments; and var. *intermedius* J. Gay has perianth segments rather broader and more obtuse than in the other taxa.

2a(iii). Subsection Serotini *Parlatore*

Description: Leaves very narrow, ± filiform. Flowers autumnal, often solitary; perianth tube cylindrical; corona a very shallow cup. Filaments subequal to anthers, anthers dorsifixed.
Type species: *N. serotinus* L.

N. serotinus L. Scape 10–25 cm. Leaves absent at flowering time, filiform, green, to 25 cm long. Flowers autumnal, fragrant, one to three per umbel, up to 3.5 cm in diameter, white; corona saucer-shaped, to 2 mm deep, orange or yellow; perianth tube 1.2–2 cm long, slender. Widespread in the Mediterranean region.
Note: This is a variable species and some of the local variants have been given distinguishing names, but it is doubtful if they can be maintained.

2b. Section* Aurelia *(J. Gay) Baker

Description: Leaves flat. Flowers autumnal, several in an umbel; perianth tube cylindrical/narrowly infundibuliform; perianth segments spreading; corona rudimentary. Filaments longer than the anthers, anthers dorsifixed, exserted from the tube.
Type species: *N. broussonetii* Lagasca.

N. broussonetii Lagasca. Leaves present at flowering time, flat, grey-green, 1–1.5 cm wide. Flowers autumnal, fragrant, up to ten (rarely to 12) in an umbel, about 3 cm in diameter, white; corona represented by only a tiny rim, white. Morocco.

Note: A large-flowered (about 3.5 cm diameter) tetraploid variant has been distinguished as forma *grandiflorus*.

3. Subgenus *Corbularia* (Salisb.) Pax [Section *Bulbocodium* De Candolle]

Description: Leaves narrow, semi-terete, usually dark or bright green, sometimes slightly glaucous. Flowers vernal (sometimes in late autumn or winter at low altitudes and in cultivation), solitary, usually held just below to just above the horizontal; perianth tube widely obconical; segments narrow, suberect or occasionally nearly patent; corona narrowly conical to widely funnel-shaped and constituting the main part of the flower. Filaments very much longer than the anthers, deflexed in the lower part, curved-ascending in the upper part, anthers dorsifixed, exserted from the tube, usually included within the corona but sometimes exserted. Chromosome number x = 7 (2n = 14 to 56).

Type species: *N. bulbocodium* L.
Note: The 'Hoop Petticoat' daffodils cannot be confused with any other group of species, with their very narrow perianth segments and prominent, broadly funnel-shaped corona. However, within the group they present great problems of classification. Many taxa have been described, at varying levels, but the account of Fernandes (1967) is that usually followed, recognising five species within the section – *N. bulbocodium, N. romieuxii, N. cantabricus, N. obesus* and *N. hedraeanthus*, with several named variants of each of the first three of these; this classification is largely followed here. Progress will probably only be made via a thorough field study of the plants comprising this section using modern statistical methods, coupled with molecular investigations. Although many of the taxa, as listed below, have characters which overlap those of others, thus rendering the treatment unsatisfactory, it would be even more unsatisfactory, especially for the purposes of communication, to lump all of them together under one species, *N. bulbocodium*.

N. blancoi Barra and Lopez. This is the plant known previously as *N. cantabricus* ssp. *luteolentus* Barra and Lopez (see below).
N. bulbocodium L. Flowers in varying shades of yellow. Anthers usually included or equalling the corona.

ssp. ***bulbocodium*** Usually spring-flowering. Flowers pale to bright yellow, usually 2.5–4.5 cm long; corona obconical, not narrowed at the mouth.

var. ***bulbocodium***. Pedicel usually up to 2 cm long; perianth usually up to 3 cm long. Spain, Portugal, south-western and western France, Morocco.
var. ***nivalis*** (Graells) Baker. Similar to above; abaxial surface of leaves deeply striate, scape ridged. Mountain plants from Spain, Portugal, Morocco. Plants described from Spain as *N. jeanmonodii* Casas, *N. juressianus* Casas and *N. subnivalis* Casas all appear to be very similar to this.
var. ***quintanilhae*** Fernandes. This is described as large, 15–40 cm tall with flowers up to 3.5 cm long. Central-eastern Portugal.
var. ***conspicuus*** (Haworth) Baker. Robust plants with leaves to 2.5 mm wide. Pedicels usually more than 2 cm long. Flowers usually 3–3.5 cm long; corona *ca*. 2 cm diameter at the mouth. Spain, Portugal, western France.
var. ***serotinus*** (Haworth) A. Fernandes. Similar to above but leaves to 4 mm wide. Flower usually 3.5–5 cm long; corona *ca*. 3 cm diameter at mouth. Western Portugal.
var. ***citrinus*** Baker [syn. *N. lainzii* Barra and Lopez]. Flowers lemon yellow, 3.5–5 cm long; corona *ca*. 2.5 cm diameter at the mouth, crenulate. Northern Spain.
var. ***graellsii*** (Webb) Baker. Dwarf plants with primrose coloured flowers with exserted stamens. Central Spain.
var. ***ectandrum*** Casas. Dwarf plants with prostrate leaves; flowers yellow with a widely flaring corona and spreading perianth segments. Central Spain.
var. ***pallidus*** (Gatt. and Weiller) Maire and Weiller. Robust, with primrose yellow flowers; corona a wide, wavy-margined funnel *ca*. 3.5 cm across. Morocco.

ssp. ***praecox*** Gatt. and Weiller. Winter-flowering; flowers primrose yellow, 4.5–5 cm long; corona widely funnel-shaped, to 3.7 cm diameter. Morocco.

var. ***praecox***. Perianth segments with six veins. Morocco.
var. ***paucinervius*** Maire. Perianth segments with three veins. Morocco.

N. cantabricus DC. Flower pure white or greenish-white, usually produced in winter; anthers usually included or equalling the corona.

ssp. ***cantabricus***. Leaves usually more than one per bulb.

var. ***cantabricus***. Leaves usually two per bulb, spreading or prostrate. Flowers pure white. Spain.
var. ***foliosus***. Leaves three to eight per bulb, erect. Flowers milky-white, born on a distinct pedicel; corona 2–3 cm diameter. Morocco.
var. ***petunioides***. Leaves one to three per bulb. Flowers white; corona up to 4 cm diameter, very widely flared and markedly crenulate. Algeria.
var. ***kesticus***. Leaves usually two to four per bulb. Flowers milky- or greenish-white; corona 2.5–3 cm diameter. Morocco. Plants described as *N. perocci-dentalis* Casas from western Morocco are probably the same as this, with flowers often not pure white.

ssp. ***luteolentus*** Barra and Lopez. This was described from south-east Spain (Albacete) and is said to have noticeably yellowish flowers and dark brown spathes.
ssp. ***monophyllus*** (Dur.) A. Fernandes. Leaf one per bulb, prostrate. Flowers pure white; corona 1–2 cm long, 2–3.5 cm diameter. South-eastern Spain, Morocco, Algeria.
ssp. ***tananicus*** (Maire) A. Fernandes. Leaves erect, three to five per bulb. Flowers *ca*. 5 cm long with off-white corona and pale yellow perianth segments; corona narrowly conical, 2–2.5 cm diameter. Morocco.

N. hedraeanthus (Webb and Heldr.) Colmeiro. Dwarf plant (scape 5–8 cm) with small straw yellow flowers facing obliquely upwards or suberect; perianth segments wide (*ca*. 5 mm); stamens exserted from corona. South-eastern and south-central Spain.
N. jacquemoudii. Casas is a little-known species from Morocco, said to resemble *N*. [*bulbocodium* var.] *graellsii*.
N. obesus Salisbury. Leaves prostrate. Flowers large, usually deep yellow; corona with incurved margin. Western and southern Portugal, Morocco.
N. romieuxii Braun Blanquet and Maire. Flowers rather pale, sulphur yellow to greenish-white; anthers usually exserted from corona.

ssp. ***romieuxii***. Flowers pale sulphur yellow.

var. ***romieuxii***. Pedicels absent or very short, to *ca*. 5 mm; flowers large, up to 4 cm long; perianth segments almost as long as corona. Morocco.

Note: *N. romieuxii* var. *mesatlanticus* Maire is probably just one of the variants of var. *romieuxii*.

var. ***rifanus*** (Emb. and Maire) A. Fernandes. Pedicels to 1 cm long; flowers small, usually 2–3.5 cm long, petals longer than corona. Morocco.

ssp. ***albidus*** (Emb. and Maire) A. Fernandes. Flowers white with a greenish or yellowish tint.

var. ***albidus***. Flowers suberect to erect, perianth segments distinctly longer than corona. Morocco.
var. ***zaianicus*** (Maire, Weiller and Wilczek) A. Fernandes. Flowers near horizontal, perianth segments about as long as corona. Morocco.

N. tingitanus. Casas, from Tangier, is described as having large pale yellow flowers becoming cream-coloured, then white with age.

THE HORTICULTURAL CLASSIFICATION OF *NARCISSUS* CULTIVARS

Historical developments

The development of the horticultural classification of *Narcissus* cultivars was described by Kington (1998). Prior to 1884, garden varieties of daffodils were known by 'pseudo-botanic names', resulting in elaborate examples such as 'Chalcedonicus Fimbriatus Multiplex Polyanthos' and 'Gallicus Major Flore Pleno'. In 1884, the Royal Horticultural Society (RHS) Daffodil Conference reviewed the classification of garden varieties for the first time. Varieties were put into 33 groups split between three divisions, the Magnicoronati, Mediicoronati and Parvicoronati. These groups and divisions were based partly on Baker's (1869) classification of *Narcissus* species and partly on other criteria devised by the Conference Committee. While the group names were largely abandoned, elements of the 1884 classification – such as the use of both 'arbitrary' features (e.g., relative corona size) and 'natural' features (the characteristics of the species) – remain in the system of classification used today. Variety names were now in the vernacular, rather than in Latin, and some 400 were listed (Barr and Moore, 1884). With a great increase both in the numbers of varieties and in hybridisation between the three divisions, later RHS classification saw the three divisions amended to seven in 1908 and to 11 in 1910. The 1910 scheme introduced sub-divisions indicating colour for trumpet, large-cupped and small-cupped cultivars. The 1910 scheme survived, with only minor amendments, until 1950 (Kington, 1998).

The revised classifications introduced by the RHS in 1950 and 1998 were designed to be more logical, easier to use, and adaptable to further developments in daffodil breeding (Kington, 1998). In 1950, the trumpet, large-cupped and small-cupped divisions were sub-divided according to a one-letter colour code: (a) perianth and corona coloured, (b) perianth white, corona coloured, (c) perianth and corona white, and (d) other combinations. A more comprehensive colour coding was introduced in 1975. Main changes in 1998 included the sub-division of split-corona cultivars into Collar and Papillon types, and the establishment of a division for Bulbocodium cultivars. With the huge number of hybrids and intense interest among enthusiasts, it is likely that the classification of daffodil cultivars will continue to evolve.

(1)

(2)

(3)

(4)

(5)

(6)

Figure 3.2 Examples of *Narcissus* cultivars of the main cultivar groups, with their classification: (1) Small-cup: 'Barrett Browning', 3 WWY-O; (2) Large-cup: 'Hollywood', 2 Y-O; (3) Trumpet: 'Dutch Master', 1 Y-Y; (4) Double: 'Papua', 4 Y-Y; (5) Tazetta: 'Grand Soleil d'Or', 8 Y-O; (6) Split-corona: 'Canasta', 11a W-Y. Photos: Horticulture Research International. (*See Colour plate 2*)

The present classification

The RHS 1998 scheme of classification into 13 divisions is shown in Table 3.1 (Kington, 1998). Once a selection has been distinguished by a cultivar name, whether of cultivated or wild origin, it is placed in one of twelve divisions, division 13 being reserved for those *Narcissus* (including hybrids) distinguished solely by a botanical name. The majority of large-flowered cultivars fall into divisions 1 to 3, the trumpet, large-cupped and small-cupped varieties, respectively, the separation depending on the ratio of the lengths of perianth segments and corona. Other distinct flower types are the double cultivars (division 4) and the split corona cultivars (division 11), the latter being classified as Collar Daffodils (11a) or Papillon Daffodils (11b) on the basis of the corona and perianth segments being opposite or alternate, respectively. Cultivars with clearly evident characteristics of *Narcissus triandrus, N. cyclamineus*, section Jonquilla or Apodanthi, section Tazettae, the *N. poeticus* group and section Bulbocodium fall into divisions 5 to 10, respectively. Division 12 is reserved for those cultivars which do not fit the description of any other division. Since 1975, a more comprehensive system of defining flower colour has been used, the colour code being appended to the division number. The present

Table 3.1 Horticultural classification of *Narcissus* cultivars[a]

Division		
1	Trumpet daffodils	Corona ('trumpet') as long or longer than perianth segments
2	Large-cupped daffodils	Corona ('cup') more than one-third the length of the perianth segments, but not as long
3	Small-cupped daffodils	Corona ('cup') not more than one-third the length of the perianth segments
4	Double daffodils	Corona and (or) perianth segments with doubling
5	Triandrus daffodils	Characteristics of *N. triandrus* clearly evident
6	Cyclamineus daffodils	Characteristics of *N. cyclamineus* clearly evident
7	Jonquilla and Apodanthus daffodils	Characteristics of Sections Jonquillae or Apodanthi clearly evident
8	Tazetta daffodils	Characteristics of Section Tazettae clearly evident
9	Poeticus daffodils	Characteristics of *N. poeticus* group
10	Bulbocodium daffodils	Characteristics of Section Bulbocodium clearly evident
11	Split corona daffodils:	Corona split:
	(a) Collar daffodils	with corona segments opposite the perianth segments
	(b) Papillon daffodils	with corona segments alternate to the perianth segments
12	Other daffodils	Those that do not fit descriptions of any other division
13	Daffodils distinguished solely by botanical name	

Note
[a]after Kington (1998).

scheme describes the colour (White, Green, Yellow, Pink, Orange or Red) of the perianth segments followed by that of the corona. Either component may have a single-letter colour code if substantially of one colour, or a three-letter code describing the colour of the distal, mid and proximal regions of the perianth segments or of the proximal, mid and distal regions of the corona, respectively. Full details of these criteria are given by Kington (1998). Some cultivars representing these divisions and classifications are shown in Figure 3.2.

The classified lists

The first RHS list of daffodil cultivars, including about 1500 names, appeared in 1907 (RHS, 1907), followed by a series of Classified Lists beginning in 1908 (RHS, 1908) and, following the appointment of the RHS as the International Registration Authority for *Narcissus* varieties in 1955, a series entitled Classified List and International Register starting in 1958 (RHS, 1958). The International Daffodil Checklist of 1989 (Kington, 1989) preceded publication in 1998 of the 23rd cumulative list

of names as The International Daffodil Register and Classified List (Kington, 1998), containing about 25 000 distinct names after accounting for synonymy (S. Kington, personal communication). In order to avoid confusion due to the re-use of earlier names, from 1998 the Register re-instated a quantity of names that had previously been deleted because they had been deemed to be extinct or of no historical interest. The Register is updated annually by supplements of newly registered names (RHS, 1999 and earlier years). A further advantage of the current series (1998 Register onwards) is that parentages and descriptions are being included where available.

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4 Commercial production of *Narcissus* bulbs

Gordon R. Hanks

INTRODUCTION

Narcissus (daffodil) bulbs have been an important floricultural crop in western Europe since the late nineteenth century, although the bulbs have been grown in the Netherlands since the sixteenth century and *Narcissus hispanicus* has been cultivated in the UK for over 300 years as '*N. maximus*' or '*N. maximum superbus*' (Doorenbos, 1954). In a 1998 survey of consumers in the UK, daffodils were rated eighth in popularity amongst cut-flowers and achieved sixth position in value of sales, despite being relatively inexpensive and not available throughout the year (FPA, 1999). At the start of the twenty-first century, the narcissus or daffodil remains one of the major ornamental bulb crops grown in temperate regions, with large areas of field-grown crops providing both bulbs and flowers, while bulb 'forcing' in glasshouses provides flowers and pot-plants over an extended season. Rees (1993) estimated that the area of narcissus grown in gardens, parks, cemeteries, etc., is five-times the area grown commercially. The histories of commercial bulb growing in the UK, Netherlands and US, the major producing countries, have been described by Dobbs (1983), Krelage (1946) and Gould (1993), respectively.

After a brief review of the statistics of narcissus bulb production, this chapter will describe the methods used in growing the crop. The objective is to provide clear guidance on how narcissus are grown, commenting on how the requirements of producing bulbs for processing might differ from the production of bulbs as ornamentals. Research findings will also be considered, for these may give insight into how the methods of production of narcissus as industrial crops might be varied or improved.

PRODUCTION STATISTICS

World production of *Narcissus* bulbs

The areas of field-grown narcissus in the major producing countries are given in Table 4.1. Production is dominated by the UK and Netherlands, with some 4200 and 1800ha, respectively, although it should be noted that in the UK the crop is grown on a two-year-down basis so that only half the area is lifted each year. It should also be noted that, since 1996–1997, the practice in the UK has been to include statistics on narcissus grown for ornamental use only, and examination of recent figures (MAFF, 1999a) suggests that a few hundred hectares have been

grown for galanthamine production. Over 400ha are grown in the USA, and other significant areas of bulb production include Australia and Canada (British Columbia). Significant areas are also grown in Jersey (Channel Islands), although this is mainly for flower, not 'dry bulb', production. Tazetta narcissus bulbs are produced mainly in Israel. In general, comparisons of areas of the field-grown crops over the past 10 years show that production areas are generally stable, with a small increase (Table 4.1). However, recent trends include a significant increase in the area grown in the Republic of Ireland, while in Poland the area has decreased markedly, largely due to virus infection (Mynett, 1990).

The annual output of narcissus bulbs can be estimated from the area lifted annually, the average planting density (say about 17.5 t/ha), and the average percentage

Table 4.1 World production areas of field-grown *Narcissus*

	1980s[a]		*1990s*		
	Year	*Area (ha)*	*Year*	*Area (ha)*	*Reference*
Australia	–	na	1999	200	G. Guy (personal communication)
Canada (British Columbia)	–	na	1991	149	Gould (1993)
Denmark	1982	36	–	na	
England and Wales	1990	3972	1998[b]	3808	MAFF (1999a)
France	1981	21	1999	22	M. le Nard (personal communication)
Germany[c]	1984	29	1996	14	Heinrichs (1999)
Republic of Ireland	1985	26	1991	73	Heinrichs (1999)
Israel	1987	144	1999	150	G. Lurie and H. Lilien-Kipnis (personal communication)
Italy[c]	1982	80	1994	27	Heinrichs (1999)
Japan	1987	28	1997	44	Japanese Ministry of Agriculture and Fisheries (K. Ohkawa, personal communication)
Jersey[c]	1990	298	1998	175	Jersey Department of Agriculture and Fisheries (I.K. Norris, personal communication)
Netherlands	1990	1639	1998	1756	PT/BKD (1999)
New Zealand	–	na	1999	70	J. Catley (personal communication)
Northern Ireland	–	na	1996	4	Department of Agriculture Northern Ireland (personal communication)
Poland	1982	200	1999	50	D. Sochacki and K. Mynett (personal communication)
Scotland	1987	255	1997	390	M.W. Sutton (personal communication)
South Africa	–	na	1992	12	De Hertogh *et al.* (1992)
USA	1989	467	1998	410	G.A. Chastagner (personal communication)
Total of above		7195		7354	

Notes
[a]1980s figures from Hanks (1993).
[b]Excludes area grown for processing.
[c]Mainly for flower production.
na: not available.

Table 4.2 Areas (ha) of field-grown *Narcissus* in the UK and the Netherlands

	1988[a]	*1989*	*1990*	*1991*	*1992*	*1993*	*1994*	*1995*	*1996*	*1997*	*1998*
UK[b]	3826	3961	3972	3702	3943	4048	3830	4334	3993	3936	3808
Netherlands[c]	1763	1799	1652	1533	1343	1350	1406	1496	1517	1574	1756

Notes
[a]1988 means 1988–1989, etc.
[b]MAFF (1999a), 1998 figure provisional.
[c]PVS/BKD (1993), PT/BKD (1999), 1998 figure provisional.

weight increase from planting to lifting (say 150%); disposable yield is the yield after taking out the weight required as re-planting stock. This gives an annual disposable bulb output of about 30 000 tonnes for both the UK and the Netherlands. A tonne of narcissus bulbs typically contains about 20 000 'medium sized' bulbs.

Narcissus bulbs are sold as commercial planting stocks, for commercial bulb forcing, for amenity or landscape use, and for retail sales either loose or in 'prepacks', the last involving garden centres, multiple retailers, mail order and other outlets. In England and Wales, commercial bulb forcing currently accounts for over 4000 tonnes of narcissus bulbs (about 80 million bulbs) annually (MAFF, 1999b). The prices of narcissus bulbs may change markedly over periods of a few years, and there is an interplay between the price of bulbs and flowers, the areas of field-grown crops from which flower crops are taken, and the number of bulbs used for forcing. In the UK, recent trends have increased the significance of cut-flowers taken from field-grown crops and have resulted in fewer bulbs being forced. When bulb prices are favourable, bulbs used for forcing may be re-claimed and planted back in the field. Bouwman (1993) described a simulation model for the supply and demand of bulbs: on the demand side there was a relationship between price asked and quantity demanded, and, on the supply side, between price asked and acreage grown. The economic aspects of narcissus bulb production are considered in Chapter 5 of this volume.

Bulb growing areas in the main producing countries

In England and Wales, some two-thirds of the field-grown narcissus are grown in eastern England (Lincolnshire, Norfolk and Cambridgeshire) and much of the remainder in the south-west, in Cornwall and the Isles of Scilly. Census figures for 1998 give the following areas for bulb and outdoor flower crops (predominantly narcissus) of 2016 ha (south Lincolnshire), 463 ha (Norfolk), 364 ha (Cambridgeshire) and 1472 ha (Cornwall and Isles of Scilly), out of a total of 5325 ha (MAFF Statistics Branch, personal communication). For narcissus alone, it is now (1999) considered that some 50% of the production in England and Wales is in the eastern counties, and 40% in the south-west, with a gradual shift towards the latter (J.B. Briggs, personal communication). South Lincolnshire is an area with strong historical links to the Netherlands, evidenced, for example, by the district name 'South Holland'. The area is characterised by large fields, extensively mechanised agriculture, and the efficient production of cereals, potatoes and vegetables as well as narcissus bulbs, a logical development as there is scope for sharing equipment

such as drying stores with potato, onion or cereal growing (e.g., see Williams, 1996). Until recent years, flowers cropped from the field were considered of secondary importance to the bulbs. This area has traditionally supplied the relatively close markets of London and the industrial Midlands, and is now a major distribution centre for fresh produce for the UK, the EU and world-wide. Cornwall has been traditionally important for the production of early fresh produce, including flowers and potatoes, from its mild, relatively frost-free climate. With improved road links eastwards, and EU support, the area has become increasingly important for narcissus bulb production. Bulb growing has a very high labor requirement for flower picking and bulb handling, and so is important in the local economy. Bulb quality is enhanced by soils free of potato cyst nematode, a requirement for bulbs exported to the USA, Canada and Norway. Unlike eastern England, fields are sometimes relatively small, of irregular topography and have stony soils. The extreme of this type of agriculture is found in the Isles of Scilly, where Tazetta narcissus are cropped over a long season in the frost-free climate: fields are small and often surrounded by hedges or artificial shelter for protection from the strong, salt-laden winds. The high transport costs here limit growing to high-value produce, and there is an interdependence between agriculture and tourism. Other areas of narcissus growing include Scotland and Jersey, the latter mainly for flower production. Growing narcissus over the broad geographic range of the British Isles ensures flower production over a long season and confers a variety of other climatic advantages, such as frost-freedom in the extreme south-west, and relative freedom from insect pests in the colder north.

In the Netherlands, bulbs have been traditionally grown around Hillegom, in the *Bloembollenstreek*. The advantages to bulb production here are considerable: ideal sandy soils with a controlled water table, abundant traditional expertise, a strong R&D base, the availability of family labour for labour-intensive bulb handling, and a superb infrastructure for marketing, selling and logistics. With the pressure on agricultural land in this region in recent years, however, much narcissus production has moved to the heavier soils of the polders of North Holland, more akin to the situation in eastern England. About 55% of the production area is in *Noord-Holland* and 33% in the *Bloembollenstreek* (de Vroomen and de Groot, 1991).

In USA, the most commercial bulb production takes place in the ideal climatic conditions of the coastal area of the Pacific North-West, with almost all narcissus production in Washington State. Most growers now produce field-grown flowers as well as bulbs, although formerly the production of bulbs for glasshouse forcing in the east and mid-west was important. The tourism associated with bulbs has a major impact in the communities where bulbs are grown, with a number of bulb festivals: it is estimated that the economic input of bulb growing on tourism is five-times the value of the crop itself (G.A. Chastagner, personal communication).

Exports and imports

Dutch exports and imports of narcissus bulbs are shown in Table 4.3 (although the words ‘exports’ and ‘imports’ are used here in a general sense, since the advent in the EU of the Single Market, the terms strictly apply only to trade with non-EU (‘third’) countries). The predominant Dutch export destinations are Germany, North America, the UK and France. Dutch imports of narcissus bulbs are dominated

Table 4.3 Dutch exports and imports of *Narcissus* bulbs (1990 figures)[a]

	Exports		*Imports*	
	Number (million)	*Weight (1000 t)*	*Number (million)*	*Weight (1000 t)*
Austria	9	0.7	0	0
Belgium and Luxembourg	6	0.4	0	0
Canada	11	0.6	0	0
Denmark	8	0.4	0	0
Finland	7	0.6	0	0
France	29	1.6	8	0.3
Germany	88	5.6	4	0.2
Israel	0	0	11	0.5
Italy	4	0.2	0	0
Norway	4	0.3	0	0
Sweden	13	1.0	0	0
Switzerland	8	0.5	0	0
UK	37	1.3	50	2.8
USA	70	4.0	0	0
Total	294	17.2	73	3.8

Note
[a]From PVS (1990a,b).

by supplies from the UK, along with Tazetta bulbs from Israel (PVS, 1990a,b). The trade in narcissus bulbs between the UK and the Netherlands consists largely of bulbs of 'mainstream' cultivars (sold by weight) exported from the UK to the Netherlands, and bulbs of 'choicer' cultivars (sold by number) traded in the opposite direction. UK also exports bulbs to Germany, North America, Scandinavia and France. UK exports of bulbs (predominantly narcissus) were valued at £9.451 m in 1998, while imports of narcissus bulbs amounted to £3.462 m (MAFF, 1999a). Israel exports some 30 million Tazetta bulbs annually (I. Gerstein and H. Lilien-Kipnis, personal communication), to the Netherlands and the USA, while China and Japan export small quantities to the USA (Oldfield, 1989). US imports about 120 million narcissus bulbs annually (Gould, 1993), including some 90 million from the Netherlands (1994–1995 figures, W.B. Miller, personal communication). The Netherlands and UK also export large volumes of narcissus cut-flowers. On the assumption that narcissus make up the bulk of the cut-flower exports from the UK, these were valued at over £10m in 1998 (MAFF, 1999a). Pot-grown dwarf narcissus are an important export from the Netherlands: sales of pot narcissus in Germany reached 1.79 million in 1998 (Heinricks, 1999).

Cultivars grown

Full statistics for the cultivars grown are available only for the Netherlands, and they are summarised in Table 4.4 by cultivar groups and in Table 4.5 by top cultivars. In recent years, the main change has been the expansion in growing Cyclamineus types (now 30% of the total narcissus area), predominantly involving

Table 4.4 Areas of *Narcissus* cultivar groups grown in the Netherlands in 1990–1991 and 1998–1999[a]

Cultivar group	*1990–1991*		*1998–1999*		*Change in area over 8 year period*
	Area (ha)	*(No. of cultivars)*	*Area* (ha)	*(No. of cultivars)*	
Cyclamineus	185	(25)	535	(32)	+189%
Large-cup	666	(107)	433	(164)	–35%
Trumpet (yellow)	375	(40)	294	(61)	–22%
Double	189	(40)	202	(58)	+7%
Tazetta	58	(10)	73	(17)	+26%
Jonquilla	20	(18)	53	(26)	+165%
Triandrus	15	(9)	34	(12)	+127%
Split Corona	30	(33)	25	(43)	–17%
Trumpet (bicolor)	27	(15)	19	(16)	–30%
Small-cup	34	(14)	18	(27)	–47%
Species	6	(10)	17	(10)	+183%
Trumpet (white)	16	(7)	13	(12)	–19%
Poeticus	6	(3)	6	(8)	0%
Unknown	9	(–)	33	(–)	–
Totals	1639	(331)	1756	(488)[b]	+7%

Notes
[a]Data from PVS/BKD (1991) and PT/BKD (1999); data or cultivars grown by only one grower are excluded.
[b]Numbers do not total due to some un-classified cultivars.

Table 4.5 Areas of the 'Top 20' *Narcissus* cultivars grown in the Netherlands in 1998–1999[a]

Cultivar	*Division and colour code*[b]	*Area* (ha)
Tête-à-Tête	12Y-Y	449
Carlton	2Y-Y	153
Dutch Master	1Y-Y	135
Ice Follies	2W-W	101
Golden Harvest	1Y-Y	55
Minnow	8Y-Y	46
Dick Wilden	4Y-Y	45
February Gold	6Y-Y	33
Standard Value	1Y-Y	32
Jetfire	6Y-O	30
Salome	2W-PPY	28
Tahiti	4Y-O	24
Bridal Crown	4W-Y	22
Gigantic Star	2Y-Y	19
Quail	7Y-Y	18
Van Sion	4Y-Y	16
Hawera	5Y-Y	15
Cheerfulness	4W-Y	15
Geranium	8W-O	15
Yellow Sun	2Y-Y	13

Notes
[a]From PT/BKD (1999).
[b]Kington (1998).

'Tête-à-Tête', a dwarf cultivar important as a scented pot-grown bulb and for garden use. There have also been significant increases in the area of other more specialist types (Tazetta, Jonquilla, Triandrus and species), while the area of trumpet, large-cup and small-cup cultivars has declined, including once popular cultivars such as 'Golden Harvest'. Examination of the figures in Table 4.4 shows a great increase in the number of different cultivars grown in the Netherlands, even in cultivar groups of which the overall area is declining. This may indicate the development of more discerning consumer tastes. In 1998, 488 cultivars were listed (those cultivars grown by a single grower were excluded from these statistics), of which the top five cultivars ('Tête-à-Tête ', 'Carlton', 'Dutch Master', 'Ice Follies' and 'Golden Harvest') made up 51% of the total area (PT/BKD, 1999). In the UK, one publication listed 35 cultivars as 'important' (ADAS, 1985a); the main ones included 'Golden Harvest', 'Dutch Master', 'Carlton', 'Fortune', 'Ice Follies' and 'Cheerfulness'. However, there is now a trend away from 'Golden Harvest' and 'Carlton', which are susceptible to base rot disease.

BULB PRODUCTION

As the UK and the Netherlands account for some 76% of the world area of field-grown *Narcissus* bulbs, the information on bulb production methods in the present chapter is largely derived from British and Dutch advisory material for bulb growers. UK advisory material formerly included a range of booklets produced by the Ministry of Agriculture, Fisheries and Food (MAFF) and MAFF's former Agricultural Development and Advisory Service (ADAS), in particular ADAS (1985a). Dutch material includes the handbook produced by the *Ministerie van Landbouw, Natuurbeheer en Visserij* (Ministry of Agriculture, Nature Management and Fisheries) with the *Consulentschap in Algemene Dienst voor de Bloembollenteelt* (General Service Consultancy Unit for Flower-bulb Cultivation) (Langeslag, 1990). Neither of these publications has been updated. Information quoted in this account without specific reference citations is taken from these two publications. Other useful accounts of commercial narcissus growing include: from the USA, NBGA (1957, 1961); from the Netherlands, Krabbendam and Baardse (1964); and, from the UK, ADAS (1970) and MAFF (1984). In growing bulbs as ornamental crops, either the bulbs or the flowers, or both, may be important, depending on local circumstances and the economic situation. In growing bulbs for pharmaceutical or other industrial uses, quite different considerations may apply, and it may be appropriate to modify agricultural practices accordingly. More specialised protocols may be needed, at least in the initial stages of bulking suitable stocks or screening cultivars for potential use. On the other hand, where the aim is industrial processing, flower production and the visual aspects of bulb quality may be unimportant.

Rees (1972) described the annual cycle of narcissus growth as characterised by alternating periods of growth and quiescence, which enable the genus in its natural, typically Mediterranean, habitat to condense its above-ground growth into the relatively short period between cool winters and hot, dry summers. This provides horticulturists with an annual opportunity to treat, grade and market the bulbs during the 'dormant' summer period (during which there is, in fact, active formation and growth of the young bulbs, shoots and root initials within the bulb).

Following planting in late-summer or autumn, root outgrowth is rapid, and shoot growth continues inside the bulb until slowed by falling temperatures. Except for Tazetta narcissus, the plants have a cold requirement for rapid, synchronous shoot growth and anthesis, and, once this requirement has been met by normal winter temperatures, shoots grow at a rate determined by ambient temperatures. Bulb growth is rapid from around the time of anthesis, but is soon curtailed by the prompt onset of foliar senescence in summer, perhaps as a means of conserving water. In the UK, initiation of the flower begins in May and its differentiation is completed in July or August. Further details of the annual pattern of narcissus growth are given in Chapter 1 of this volume.

PESTS, DISEASES AND DISORDERS

In the commercial production of narcissus bulbs, considerable effort is needed to control pests and diseases. As for other crops, pesticide applications in the field are important, but bulbs also present a convenient opportunity for pesticide applications during the 'dormant' stage between bulb lifting and re-planting. Many pests and diseases are exacerbated by the techniques of modern husbandry, such as high planting densities, reduced sorting of bulbs by hand, bulk handling and two-year-down growing (Price, 1977a,b), but, since it would be uneconomic to change these practices, control measures have to be highly effective to work under these exacting conditions.

Methods of control (or management) of narcissus pests and diseases, and for preventing physiological disorders, are incorporated into the following description of narcissus bulb production. Further descriptions and methods of control of pests, diseases and disorders are available in a number of texts. For the UK, information on pests is given in Lane (1984), on diseases and disorders in Moore *et al*. (1979) and ADAS (1986a), and generally in Rees (1972, 1992), Linfield and Cole (1989), Hanks (1993) and Linfield (1994). Dutch information includes Bergman *et al*. (1978) and Langeslag (1990), and US information includes Gould and Byther (1979) and Chastagner and Byther (1985).

Narcissus bulbs are unusual in that the major pest, stem nematode ('eelworm', *Ditylenchus dipsaci*), is controlled by immersing bulbs in a hot-water treatment (HWT). Other pests are also controlled by HWT, including the larvae of large narcissus fly (*Merodon equestris*), small narcissus flies (*Eumerus strigatus* and *E. tuberculatus*), other nematodes, narcissus leaf miner (*Norellia spinipes*), bulb scale mite (*Steneotarsonemus laticeps*) and bulb mites (*Rhizoglyphus* and *Histiostoma* species). Of these, the large narcissus fly and bulb scale mite are the most significant pests, while small narcissus flies and bulb mites attack only damaged bulbs. Besides *D. dipsaci*, other nematode pests include the narcissus bulb and leaf nematode (*Aphelenchoides subtenuis*) and the root-lesion nematode *Pratylenchus penetrans* that can attack bulbs causing a root rot in conjunction with the fungus *Nectria radicicola*. Nematodes transmitting narcissus viruses are *Trichodorus* and *Paratrichodorus* spp. (transmitting tobacco rattle virus), *Longidorus* spp. (tomato black ring and raspberry ringspot viruses) and *Xiphinema diversicaudatum* (arabis mosaic and strawberry latent ringspot viruses). Potato cyst nematode (PCN, *Globodera* spp.) does not attack narcissus bulbs, but its presence may cause the rejection of bulbs for export

to some countries. Slugs attack bulbs and flowers, and the garden swift moth (*Hepialus lupulinus*) is an occasional pest. Aphids do not usually colonise narcissus, but several common species spread viruses through exploratory probings. Vertebrate pests are not usually troublesome in narcissus growing.

Narcissus fungal diseases include bulb rots and foliar diseases, of which the most significant is base rot (basal rot) caused by *Fusarium oxysporum* f.sp. *narcissi*. Many of the procedures in narcissus growing are designed to control base rot, although incidentally helping to control other fungal pathogens. The control of base rot involves a range of measures, of which the use of fungicides is only one (Melville, 1980; Tompsett, 1980a; ADAS, 1989b). Some of the most widely grown narcissus cultivars, such as 'Golden Harvest' and 'Carlton', are susceptible to base rot. Apart from base rot (Tompsett, 1986), relatively little is known of varietal differences in susceptibility to other diseases and pests, although Beaumont (1950) gave some useful information. Neck rot is related to base rot, and is also generally caused by *F. oxysporum* f.sp. *narcissi*, although *Penicillium hirsutum* and *Botrytis narcissicola* are also implicated (Davies *et al.*, 1998; Carder, 1999). Although reported by Hawker in 1935, neck rot has caused concern recently as it is seen in pre-export inspections, and it attacks a wider range of cultivars than base rot (Davies *et al.*, 1998). It is important to distinguish pathological neck rot, which is a rot clearly spreading down from the bulb neck, from 'physiological neck rot', which is simply the natural presence of the dead bases of leaves in the bulb neck. Less usual, or less serious, fungal rots include grey bulb rot (*Rhizoctonia tuliparum*), soft rot (*Rhizopus* spp.), *Penicillium* rots, black slime (*Sclerotinia bulborum*) and white root rot (*Rosellinia necatrix*). The major fungal foliar diseases are smoulder (*Botrytis narcissicola*), fire (*Sclerotinia polyblastis*), leaf scorch (*Stagonospora curtisii*) and white mould (*Ramularia vallisumbrosae*). A number of fungi attacks narcissus bulbs resulting in skin diseases that give a downgraded appearance, with darker, greasy, multi-layered or irregular skins (Bergman *et al.*, 1978).

Bacterial diseases are not generally associated with narcissus. Although mycoplasma-like organisms have been reported in unhealthy narcissus plants (Bellardi *et al.*, 1990), there is no information as to the wider significance of this discovery. On the other hand, some 21 viruses are known to infect narcissus, of which about 13 are considered to be of economic importance, especially the aphid-borne narcissus yellow stripe, narcissus late seasons yellows and narcissus white (or silver) streak viruses (Brunt, 1995). Most have aphid or nematode vectors, although for some the vector is not known or mechanical transmission has been demonstrated. Aphid- and nematode-borne viruses are unlikely to be spread by handling healthy and infected plants alternately, and Mowat (1980a) found no evidence for the mechanical transmission of narcissus tip necrosis virus by handling and flower cropping. However, narcissus mosaic virus is easily spread by mechanical inoculation (Brunt, 1966) and can be spread by flailing (Mowat, 1987). Only the ringspot viruses are seed-borne. Most narcissus stocks are heavily infested with viruses, and while their effects may be generally less dramatic than those of stem nematode or base rot, they are important and their significance should not be under-estimated by growers.

Narcissus crops may be affected by a number of physiological disorders, including damage from frost, waterlogging and sun scorch. Damage due to chemical or other treatment can be due to herbicides, HWT, formaldehyde or mechanical damage. 'Grassiness' ('horses teeth') can result when the main shoot of a bulb is

lost, resulting in the production of many offsets. The disorders 'chocolate spot' and 'root rot' may have the appearance of fungal diseases, but no pathogen has been associated with them. Finally, there are a number of disorders in flower development, resulting in the death or deformity of the flower bud or floral organs.

STANDARD PRODUCTION OF *NARCISSUS* BULBS IN THE FIELD

Planting material

In obtaining bulbs as planting material, the potential grower needs to consider cultivar, source and quality, and bulb grade.

Cultivars

Although many narcissus cultivars are grown, few are cultivated in significant amounts, so stocks of relatively few cultivars can be obtained in quantity. The choice of planting stock will depend on the characteristics required (e.g., flower quality or galanthamine concentration) and the amount of biomass produced, linked with the price of different cultivars; these aspects are considered in Chapter 10 of this volume. General sources of information on the main commercial cultivars grown include ADAS (1982b, 1985a) and IFC (undated). The classified list of narcissus names is published by the Royal Horticultural Society (RHS), the International Registration Authority for the genus (Kington, 1998). The American Daffodil Society (ADS) publishes an abridged list (Throckmorton, 1989) and an illustrated database on CD (ADS, in press). Data from cultivar trials have been reported from the UK (Fry and Shepherd, 1961; ADAS, 1963, 1967, 1971; Hanks, 1994a; Hanks and Withers, 1998), Czechoslovakia (Petrová, 1983), Germany (Loeser, 1979), Poland (Szlachetka, 1989) and the USA (Nelson, 1988). Dutch information on narcissus cultivars appears in the annual guide *Narcissengids*, published by the *Coöperatieve Nederlandse Bloembollencentraal*, Lisse, and in the descriptive lists of ornamental plant cultivars issued by *RIVRO* (the National Institute of Crop Variety Research). Further information appears in articles in the periodicals *Bloembollencultuur, HOBAHO* and *Vakblad voor de Bloemisterij*. There are several public collections of narcissus cultivars in the Netherlands, including the CNB-Showtuin 'Hein Schrama' (Heemstede), Narcissen Showtuin K.J. van der Veek (Burgervlotbrug), HOBAHO Keurtuin 'De Buitenhof' (Lisse) and 'Hortus Bulborum', a collection of old cultivars (Limmen) (P.J.M. Vreeburg, personal communication). In the UK, bulb cultivars are displayed at Springfields Garden (Spalding) and are trialled at the RHS garden at Wisley, while there are a number of private collections under the aegis of the National Council for the Conservation of Plants and Gardens (NCCPG, 1999).

Bulb source and quality

Bulbs may be obtained by private sales or from auctions, or may be grown under contract arrangements. Obviously, a reputable source should be used to ensure trueness-to-type and quality, and, if possible, the stock should be inspected during the previous growing season. Stocks should be checked for the main pests and

diseases – stem nematode, bulb rots, foliar diseases and viruses. Bulb stocks should be obtained in good time to carry out HWT prior to prompt planting, and bulbs should be properly stored until planting.

In the EU, a 'Plant Passport' scheme is in operation for bulb sales within the 'Single Market'. In the UK, for example, bulb stocks are entered by the grower with the Plant Health and Seeds Inspectorate (PHSI) of MAFF. Stocks are then inspected for stem nematode, the only EU quarantine pest of narcissus, in a growing season inspection (GSI), and a dry bulb inspection (DBI) is carried out after cleaning and grading bulbs to ensure freedom from soil. Bulbs are eligible for the grower to issue a plant passport if both inspections are passed; if they are not, it may be permitted for the bulbs to be sold for retail sales or landscape use only. Similar arrangements exist in other EU countries. For exporting bulbs to 'third countries' (i.e., outside the EU), plant health inspections and their criteria depend on the requirements of the importing country: for example, for export to the USA and Canada, the requirements also include strict freedom from soil on the bulbs, that bulbs are grown on certified PCN-free land, and that they undergo field inspections for freedom from other pests and diseases as well as pre-export inspections. When narcissus bulbs are bought in from a different climate, they may take some time to become synchronised with local stocks. Abbiss and Craze (1948) showed that Dutch stocks took at least three years to synchronise with local stocks in south-west England.

Most ordinary narcissus stocks have widespread virus infection (Stone, 1973; Brunt, 1980), and VT propagation schemes offer the best opportunity for improving stocks (Hollings and Stone, 1979). Limited quantities of 'virus-free' narcissus stocks, more properly called virus-tested (VT) or virus-indexed stocks, are available from nuclear stocks produced from meristem-tip culture or from indexed plants (Brunt, 1985; Asjes, 1990; Lawson, 1990). VT stocks of narcissus should be grown in 50 m isolation from ordinary stocks (Broadbent *et al.*, 1962) or in sterilised substrate in vector-proof fine mesh tunnels (Moore *et al.*, 1979). VT stocks offer the general advantages of improved vigour. VT bulbs may have lower critical weights for flowering, and more, larger and brighter flowers (Stone, 1973). VT stocks may show increased bulb yields of 10–20%, compared with ordinary stocks, as well as earlier growth (Sutton *et al.*, 1986, 1988). In some trials there was a consistent yield advantage for VT stocks over ordinary stocks in Cornwall, but not in Lincolnshire, suggesting that VT stocks were able to take advantage of the different conditions in Cornwall (Hanks, 1992a). Much greater advantages of VT stocks were reported in New Zealand by Allen and McIntosh (1994). When growing VT stocks, cultural practices may have to be adjusted to take account of their greater vigour, e.g., using lower planting densities. VT stocks of some cultivars are re-infected only slowly by viruses if grown in isolation (Hanks, 1997).

In the UK, there are three grades of VT certification for narcissus under the voluntary Plant Health Propagation Scheme: (1) Virus-Tested Mother Stocks are the highest grade, strictly maintained in vector-proof conditions with zero tolerance for pests, diseases and off-types; (2) Foundation, field-grown stocks grown in isolation with low tolerance for pests and diseases (e.g., 0.05% for severe virus symptoms and 0.5% for mild virus symptoms); and (3) Élite, for which isolation and tolerances are less exacting (e.g., 2% for severe virus symptoms) (MAFF Plant Health Division, 1999). Methods for selecting, producing and certifying

pathogen-tested nuclear stock of narcissus have been described (Anon., 1993). Commercial ELISA testing services for narcissus are becoming available (e.g., Monro and Johnstone, 1992). However, 'virus-freedom' may be difficult to define, given the advances in virus identification and detection (e.g., Mowat *et al.*, 1988a,b, 1989; Boonekamp *et al.*, 1990; Langeveld *et al.*, 1997; Sochacki *et al.*, 1997).

Narcissus bulbs from glasshouse forcing are commonly reclaimed and can be 'plant passported' for sale, but such 'ex-forced' bulbs must be labelled as such. Although they may be somewhat small and desiccated, if properly hot-water treated and grown-on for two or three years, good growth should be obtained for an economical starting price.

Bulb size and shape

Yields are mainly controlled by the grade of bulbs and their planting density. Narcissus bulbs are graded by circumference, with bulbs of the middle sizes (12–16 cm) being in demand for dry bulb sales and bulb forcing. Small bulbs (10–12 cm grade) are considered good planting material, since their percentage weight increase can be high. Large bulbs (>16 cm) are often re-planted in a stock because they are said to 'increase vigour', and they have been shown to emerge earlier and have larger flowers than small bulbs (Strojny, 1975). The presence of many small, round bulbs in a stock may indicate declining vigour, and it has been suggested that bulbs <8 cm in circumference should be culled (Dickey, 1940). Planting bulbs as a mixture of sizes (or even ungraded) will maintain a variety of shapes and sizes in the harvest.

Bulb shape is also important. Stocks with a good proportion of 'flat offsets' are excellent planting material, for as narcissus bulbs are graded on a long (slotted) riddle, flat bulbs have a high weight in proportion to their grade. Large offsets grow into large round or double-nosed (DN) bulbs in one year, while small offsets take two or three years. In the Netherlands, DN bulbs are graded in increasing sizes as DN-III, DN-II and DN-I, with up to 375, 275 and 175 bulbs per 50 litre, respectively. Bulbs derived from 'chipped' stocks may be more uniform, and therefore more suited to planned production (Vreeburg, 1984c, 1986).

Site considerations

Narcissus bulb growers should consider a number of factors in relation to site selection: climate, topography, soil, crop rotation and site history, and economic factors.

Climate

Climate affects crop growth, crop management, and pests and diseases. For example, mild maritime climates, such as south-west England or the bulb-growing area of Washington State, favour crop growth with an earlier and longer growing season, compared with more continental climates like eastern England or the Netherlands, but their higher rainfall and temperatures favour pests, diseases and weeds and make bulb lifting and drying more difficult. Cooler climates, such as Scotland, may have fewer pests (such as aphids and large narcissus fly), and,

although the growing season is delayed, growth then takes place under higher light levels and longer days. Rees (1972) compared the growth of identical stocks of narcissus 'Fortune' grown in south-west and eastern England. In the south-west, emergence, anthesis and senescence occurred 2–4 weeks earlier than in the east. After planting, the rate of dry weight loss was faster in the south-west and minimum plant weight was reached sooner, presumably because of the higher soil temperatures. Although bulb weight also increased sooner in the south-west, the eventual bulb yields were higher in the east. Annual temperatures and rainfall for important bulb-growing areas are given in Figure 4.1.

There are large differences between years in narcissus yield, quality and timing, much of which may be attributable to weather conditions (Hanks, 1996a). Szlachetka and Romanowska (1990) carried out long-term trials, and reported that weather conditions, especially in December, had an effect on bulb yield greater than the effect of the grade of planting material used. Where the mean monthly air temperature was above 0 °C or slightly below (–0.8 °C) and the soil was not frozen, bulb yield was high; if the temperature was below –3.6 °C and the soil was frozen, yield was decreased by 14–48%.

Narcissus bulbs are generally regarded as suitable for naturalising in US climatic zones 4 (annual minimum temperature about –30 °C) to 8 (about –10 °C) (De Hertogh, 1989; De Hertogh *et al.*, 1993). Information on the hardiness and performance of a range of cultivars is available from trials such as those described by Klingaman and Eaton (1983) and Nelson (1988). In the Netherlands and similar climates, narcissus crops are covered (e.g., with straw) to protect them from frost or other injury. In the UK, crop covers are not used, as soils seldom freeze to sufficient depths to damage bulbs of most cultivars. Tazetta narcissus cultivars are hardy in the Isles of Scilly, whereas cultivars with Tazetta parentage (e.g., 'Tête-à-Tête') may be damaged by low temperatures in eastern England. Data are available on the cold tolerance of narcissus cultivars, although there are some discrepancies between studies, perhaps because of the different experimental techniques used (van der Valk, 1971; Sakai and Yoshie, 1984).

An annual rainfall of about 100cm is considered ideal for narcissus growing. Evenly distributed rainfall favours crop growth, especially in April to June when the bulb is growing rapidly, and also facilitates bulb planting and lifting. In appropriate climates, the availability of irrigation water should be considered. Waterlogging reduces crop growth, although narcissus are not specially prone to damage and can tolerate waterlogging if the water is well aerated (Gibson, 1935). However, wet soils assist the spread of stem nematode in the soil water and in waterlogged furrows. Growing in light sandy soil with a controlled water table is the ideal bulb-growing environment, except that the spread of nematodes is favoured.

In windy situations crops may be flattened, reducing yields. MacKerron and Waister (1975) investigated the effects of shelter on narcissus performance, and concluded that reducing the wind run from 164 to 118 km/day was probably not economic for increasing yields.

Topography

Large flat fields are the most economic to farm, while small, sloping fields present a number of practical problems (Figure 4.2). Warm sites, such as south-facing

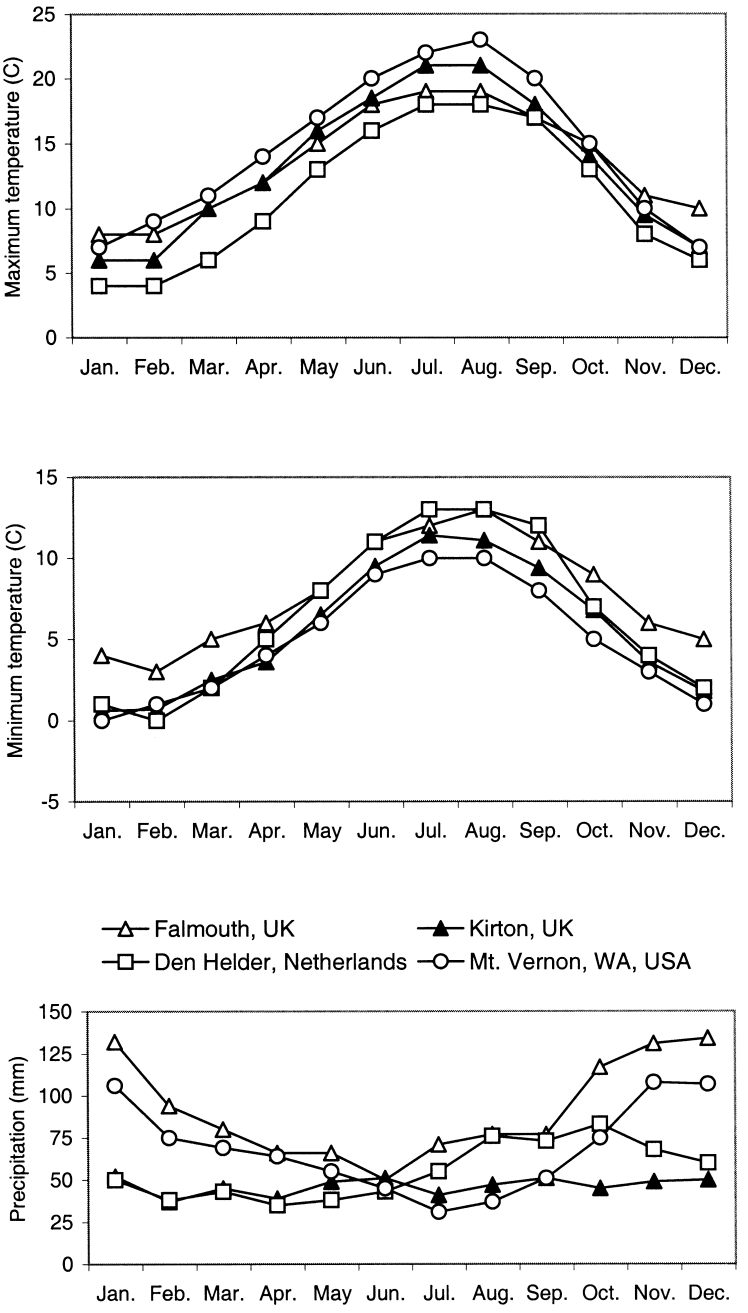

Figure 4.1 Mean monthly weather data for Falmouth (south-west England), Kirton (eastern England), Den Helder (the Netherlands) and Mt Vernon (Washington State). Data are long-term means, for at least 30 years to 1999, from Washington Post historical weather data and Horticulture Research International.

slopes, may favour the development of bulb rots, while nearby shelter (hedges, etc.) may favour the survival of large narcissus fly. Nearby bulbs in gardens, field margins, etc., may also act as a source of infection of large narcissus fly. Sloping sites can potentially give rise to problems with surface run-off of pesticides.

At the present time, pesticide applications are an essential part of narcissus husbandry, and topography and soil type may have an effect on pesticide run-off. Field operations associated with growing narcissus, potatoes, etc., often result in the movement of turbid water, and soil-rich run-off from ill timed operations

Figure 4.2 Typical narcissus fields of south Lincolnshire (top) and old daffodil beds on St. Marys, Isles of Scilly (bottom) (Photographs: Horticulture Research International and Andrew Tompsett, respectively).

(as well as natural erosion) on sloping sites can move pesticide residues into watercourses. Concentration of pesticides has been recorded in colluvium and in the sediment of surface pools where the watertable was high (Harrod *et al.*, 1991; Harrod and Rickson, 1994).

Soil

Silts or very fine sandy loams are ideal for narcissus growing. In the UK, bulbs are grown successfully on a variety of silt, silt loam, brick-earth and peat soils. In the Netherlands, narcissus were traditionally grown in sandy soil and it was once considered that bulb quality could not be maintained on heavy silt clay soils (de Vroomen and de Groot, 1991). However, they are now grown on the heavier polders, factors involved including the pressure on prime agricultural land and the need, on sandy soils with a high water content, to practice regular soil sterilisation to control nematodes. There have been few comparisons of growth in different soil types, although Szlachetka (1976) reported better narcissus growth in light black-earth soils than in heavy alluvial soils. It has been reported that narcissus bulbs were larger and more susceptible to base rot when grown on peat, rather than mineral, soils (ADAS, 1982a).

According to ADAS (1985a), bulb soils should be deep, fertile, well drained and moisture retentive (water holding capacity >40 mm per 300 mm depth), while the level of organic matter should be >3% and the pH value should generally be between 6.0 and 7.5. In the bulb growing areas of eastern England, typical mineral soils have 2.5–3.0% organic matter, while peat soils may have up to 25% (J.B. Briggs, personal communication). Weed control is difficult in highly organic soils. Narcissus bulbs are susceptible to poor growth due to compaction (De Haan and van der Valk, 1971). Although the observed root spread of narcissus was similar in different soil types, it was low in comparison with tulips, so a good soil structure is needed (Rees, 1972). Heavy soils, stones and clods can damage bulbs and make lifting difficult, requiring the use of stone and clod removers. In 'sticky' soils it may be difficult to produce soil-free bulbs unless bulbs are washed, a process which spreads diseases and creates disposal problems (ADAS, 1987).

Crop rotation and site history

Crop rotation should aim to maintain a good soil structure and reduce pest, disease and weed problems. Leys, peas, barley or early potatoes often form part of the rotation with narcissus. Preceding crops leaving a high residue (e.g., cauliflowers) should be avoided, as high levels of soil nitrogen may encourage basal rot (Hanks *et al.*, 1998a). Rotations should allow adequate time for soil preparation before bulb planting. Narcissus should not follow crops treated with sulphonylurea herbicides, which can cause morphological damage (Greenfield, 1987). With 'two-year-down' growing, foliar fungal diseases generally become a problem in the second year, so first- and second-year narcissus crops should not be planted adjacent to each other (Melville, 1980).

At least four years should elapse between growing narcissus in a rotation, or six years where stem nematode has been found. In sandy soils, deep ploughing

can be used to bring up fresh soil and allow a shorter rotation. Where tulips are grown in the same rotation, they should follow narcissus, since the tulip race of stem nematode attacks narcissus bulbs but the narcissus race does not attack tulips. Other hosts of the stem nematode, which should be avoided, include (1) crops such as onion, maize, peas, beans, sugar beet, mangold, carrot, turnip, red clover and strawberries, (2) bulbs such as bluebells and hyacinth, and (3) weeds such as speedwell, scarlet pimpernel, chickweed, cleavers and black bindweed (Jones and Jones, 1984). For the export of bulbs to certain countries, they must be grown on soil certified free of potato cyst nematode (PCN): although PCN does not attack narcissus, the cysts can be carried on soil attached to dry bulbs. Spores of the base rot fungus are widespread, and have been found even in soils not previously known to have grown narcissus (Price, 1975a,b). Base rot spores may remain viable for up to 10 years, at least under artificial conditions (C.A. Linfield, personal communication). Diagnostic methods for the base rot fungus have been investigated (Linfield, 1993). Field populations of *Fusarium oxysporum* f.sp. *narcissi* are being characterised using molecular tools with the ultimate goal of developing sensitive and specific detection systems for this pathogen in bulbs and soils. These in turn may lead to diagnostics capable of predicting the disease potential of fields selected for narcissus production (J.H. Carder, personal communication).

Bulbs left behind in the ground after harvesting ('volunteers' or 'groundkeepers') are difficult to eliminate, but the removal of these bulbs is important in controlling pests and diseases. Contact herbicides (such as paraquat or glyphosate), cultivation and picking by hand should be used (Tompsett, 1974).

Economic factors

The site chosen for narcissus bulb production should take account of the local availability of experienced labour, if required for labour-intensive operations such as flower cropping or bulb cleaning. The location of markets, local infrastructure and availability of logistics services should also be considered.

Pre-planting operations in the field

Cultivation

Whenever soil is compacted, or suspected of being so, it should be sub-soiled (deep-ploughed) prior to growing narcissus. Good agricultural practices must be used, for example, carrying out sub-soiling when the soil is dry enough to burst the compacted layer but not wet enough to cause smearing. Perennial weeds should be removed by cultivation or by using a translocated herbicide. The land should be ploughed well in advance of planting, and when the soil is dry enough for structure to be maintained. Either the whole field should be ploughed and roadways taken out later, or permanent headlands should be established. After ploughing, the land may need further cultivation, to 25 cm depth in clay or loam, or 35 cm in sand. If the bulbs are not ready to plant and conditions are dry, the prepared land may be rolled to conserve moisture. In appropriate soils, the use of stone and clod separators before planting is becoming more usual. To avoid soil

compaction and its effects on clod formation and reduced crop growth, bed formers are used to produce an even soil particle density prior to planting. All cultivations should take account of the need to reduce soil compaction by reducing machinery movements and the area affected by wheelings.

Soil disinfection

In the UK, where bulbs are grown on heavier soil, it is not usual to use soil disinfection on a field scale. However, nematodes move more freely in sandy soils, and in the Netherlands soil disinfection is routinely used in bulb growing. Prior to assigning a field for narcissus production, soil samples should be sent to a diagnostics laboratory for identification and counts of nematodes present, including stem nematode, virus vector nematodes and PCN, as appropriate. Sterilants used in the Netherlands include metam-sodium and dichloropropene, which should be used at sufficient depth to control the target organisms (up to 40 cm for *Pratylenchus*). Soil disinfection may also be used to control soil-borne fungi such as white root rot (Mantell and Wheeler, 1973).

Fertilisers

Fertilisation for narcissus should aim to maintain adequate levels of phosphate (P_2O_5) and potash (K_2O) in the soil and to supply adequate (but not excessive) nitrogen (N). Nutritional experiments with narcissus have proved difficult because of the reserves within the bulbs (Bould, 1939; Hewitt and Miles, 1954). Long-term experiments showed that there is little short-term response to N or P_2O_5, that adequate K_2O is needed, and that fertilisers do affect growth in subsequent years (NAAS, 1961; Wallis 1966, 1967b, 1968). K is beneficial in the second year of growth (Fodor and Sólymos, 1975).

Fertiliser recommendations for general bulb growing under UK conditions are given in MAFF (1994). Soil samples should be taken well in advance of planting for the determination of P_2O_5, K_2O and magnesium (Mg) levels, while N requirements are usually based on the previous cropping of the land, the highest rates being applied following demanding crops such as cereals. The maximum rates suggested, where the index has fallen to zero for a particular nutrient, were (in kg/ha) 100 N, 125 P_2O_5, 250 K_2O and 150 Mg, after deducting the amount of nutrients applied as organic manures. If necessary to apply magnesium, kieserite or calcined magnesite is used, unless liming is also necessary, in which case magnesian limestone is used. P_2O_5, K_2O and Mg are usually applied after ploughing and worked in before planting, but it is recommended that N is applied later as a top-dressing, shortly before crop emergence (to avoid scorching the foliage); this reduces leaching by winter rainfall. In practice, growers may prefer to apply a compound fertiliser before planting. Manganese deficiency may occur in narcissus grown on soil where the pH is high, and manganese sprays should be applied in advance of deficiency becoming evident.

There may be advantages to applying fertilisers as split-dose applications (pre-planting and as a spring top-dressing) (Parker, 1935) and to counter the effect of rain (Lyakh, 1988), but there was no advantage of using repeated high rates of N (Lees, 1960). No advantage was demonstrated in trials of applying fertiliser into

the ridges at planting, instead of broadcast (ADAS, 1970). No fertilisers are applied in the second year of the crop, unless growth is obviously poor in the first, in which case a low rate of N (50 kg/ha) is top-dressed before crop emergence.

Chilvers and Daft (1980, 1981) reported on the widespread occurrence of endomycorrhizal fungi (*Glomus* spp.) in narcissus in Scotland. Mycorrhizal infections assist in the uptake of phosphates and possibly other nutrients, and attempts had been made to introduce more active mycorrhizal species into field situations. Iqbal and Firdaus (1986a,b) described mycorrhizal infections in *N. poeticus*. In pot experiments with *N. tazetta*, inoculation with *Azospirillum* spp. improved productivity in sandy soils even when nitrogen fertilisers were applied (Naggar and Mahmoud, 1994).

From hydroponics experiments, Ruamrungsri *et al.* (1996a,b, 1997) reported decreased bulb dry weight only where nitrogen (N), calcium (Ca) or magnesium (Mg) were omitted from the nutrient solution, although flower quality was not affected. Omitting N resulted in stunted shoot growth, with small, thin yellow leaves, while omitting Ca depressed root and shoot growth. Omitting Mg gave severe interveinal chlorosis near the leaf tips, omitting iron (Fe) gave chlorosis near the base of leaves, and omitting boron (B) resulted in water-soaked areas in the basal part of leaves. The only other report concerning trace element effects was from a study that found few differences in yields between different fertiliser treatments, but that flower yield was increased when B was applied (Emsweller *et al.*, 1938). The importance of Ca for yields was also shown by Hewitt and Miles (1954). Sun *et al.* (1991) reported that bulb treatment with paclobutrazol increased Fe uptake and reduced zinc (Zn) uptake in hydroponically grown plants, but the effects on growth were not reported.

Dutch investigations indicated that excessive amounts of fertiliser are often used on bulb crops, and that narcissus have lower N requirements than other bulb crops (van Berkum, 1987; van Berkum and Braam, 1991). A level of 125 kg N/ha was recommended, and it was suggested that soil mineral nitrogen should be analysed and the amount of N applied should be reduced by this amount. In the UK, a scale of N applications has been recommended, depending on soil type, from a maximum of 120 kg/ha in sandy or shallow soils, decreasing to zero in peaty soils (C.R. Rahn, personal communication). Excessive N levels are reported to lead to more bulb rots, splitting and bruising in narcissus (Biekart, 1930; McClellan and Stuart, 1947; Rikhter, 1976; Hanks *et al.*, 1998a); for example, Rikhter (1976) found highest yields with N applied at 120 kg/ha, with more fungal disease at 180 kg/ha. The lowest rates of base rot occurred when potassium was added (McClellan and Stuart, 1947). The excessive use of phosphate fertiliser remains an important consideration in Dutch bulb growing (Pasterkamp *et al.*, 1999).

Organic fertilisers may be used, but high rates should not be applied shortly before planting. Allen (1938) reported that ammonium sulphate (0.5 t/ha), 5:10:5 (N:P:K) fertiliser (1.5 t/ha) and rotted manure (4 t/ha) each about doubled bulb yield. Well rotted farm-yard manure applied to the previous crop at up to 75 t/ha may be useful in soils where organic matter is low, but trials showed no advantages of using particular types of bulky organic fertilisers (Lees, 1961). The use of sewage sludge on bulbs is not well known, but its use is not recommended where bulbs are being grown for overseas sales because of the possibility of contamination with PCN. Properly composted waste from bulb growing (including waste containing

bulbs with base rot) can be safely spread on bulb fields, according to Dutch trials (van Dijk, 1990). Narcissus growing produces 3–5 m^3/ha of waste from the field or 1 t/ha from bulb forcing (Bouma, 1990). Care should be exercised in applying plant wastes from processing to cropping land, because of the possible effects of residual solvents present.

Pre-planting bulb treatments

Following the receipt of bulbs, the main treatments to be considered before planting are storage and hot-water treatment (HWT). In all bulb handling operations, a reasonable standard of hygiene should be maintained, particularly of containers, bulb stores and equipment, as soil and other debris can be a major source of stem nematode, bulb scale mite, and fungal propagules. Nematode 'wool' (the dehydrated fourth stage juvenile stage of the stem nematode) can survive for 25 years in its dry state. For use at room temperature, phenols (including cresylic acid) and iodine/phosphoric acid disinfectants were most effective in killing stem nematode (Lole, 1990). Alternatively, bins, etc., can be treated in HWT tanks for 10 minutes at 50 °C. Stored bulbs can be fumigated by specialist contractors with methyl bromide to control bulb scale mite, without phytotoxicity (Gurney and Gandy, 1974; Murdoch, 1975; Powell, 1977).

Bulb storage – general

Ideally, bulbs should be stored after receipt at 17–18 °C in a controlled-temperature store with good air movement, some exchange of fresh air, and a relative humidity below 75%. Temperatures below 17 °C slow shoot development, and high temperatures will encourage base rot. In practice, bulbs are often stored outdoors (in which case they should be protected from sun and rain) or in sheds at about ambient temperatures. If stored in bulb trays, air circulation may be adequate; if stored in loose bulk or in bulk bins, it will be necessary to maintain some air movement through the bulbs, through ducts or fans, to prevent dampness which could lead to premature rooting and fungal growth. Roots of narcissus bulbs form over a short time and emerge simultaneously, so if they grow in storage and are broken at planting, this damage can be serious (Rees, 1972). When narcissus roots were repeatedly excised (up to four times), new roots developed from the base plate, but their number was progressively reduced and, increasingly, plants failed to flower (Yasuda and Fuji, 1963).

Hot-water treatment (HWT)

HWT is the key aspect of growing narcissus bulbs, and its practice is described in Gratwick and Southey (1986) and ADAS (1985b). It is essential to apply HWT (colloquially called 'sterilising' in the UK) to all narcissus planting stocks to control stem nematode, and other pests are also killed by HWT. It is important to use HWT even if bulb stocks have no history of stem nematode, as very few nematodes are needed in a bulb to cause its destruction (Hesling, 1971), while the treatment of stem nematodes at sub-lethal temperatures can result in only temporary inactivation (Hastings *et al.*, 1952). Early experiments on the HWT of narcissus were

carried out by Hewitt (1914), but the treatments used (1–6 hours at 48.9 °C) resulted in the death of the bulbs. The technique was further developed by Ramsbottom (1918, 1919), Staniland (1933) and Staniland and Barber (1937), resulting in a standard recommendation of 3 hours at 43.5 °C. The early studies have been fully described by several authors (Slootweg, 1962; Turquand, 1966; Tompsett, 1982; Lane, 1984; Gratwick and Southey, 1986). Treatments were later increased to 3 hours at 44.5 °C or 4 hours at 43.5 °C, to improve control (e.g., van Slogteren, 1931; Chitwood and Blanton, 1941; Woodville and Morgan, 1961). Lees (1963) gave the critical treatments for crop damage. Growth was not impaired by extending a 3 hour treatment to 46.1 °C, nor by extending a 43.5 °C treatment to 6½ hours, but a 3 hour treatment at 47.2 °C impaired vigor and at 54.4 °C a 45 minute treatment was lethal. For *N. tazetta* var. *chinensis*, Lin *et al.* (1987) reported that bulbs grew normally after HWT for 45 minutes at 50 °C or 25 minutes at 55 °C, although complete control of the target nematodes (in this case *Aphelenchoides* spp.) was not achieved. The chemicals applied do not penetrate beneath the bulb scales at ambient temperatures unless a vacuum treatment is applied (Newton *et al.*, 1933). 'Vapour heat treatment' was also investigated, but the regime necessary to control stem nematodes – 8 hours at 47.7 °C – had adverse effects on the crop (Chitwood and Blanton, 1941). Other methods of treating stem nematode (such as nematicidal dips or field applications) are either ineffective, or suitable chemicals are not now available (Hesling, 1971; Damadzadeh and Hague, 1979; Windrich, 1986).

With two-year-down growing, effective HWT becomes even more important, and this is reflected in the UK recommendation to treat all planting stocks for a full 3-hour period at a temperature of 44.4 °C. The treatment period is usually taken as starting when the tank temperature regains 44.4 °C after the cooling effect of loading the bulbs. In the Netherlands, using one-year-down growing and particularly where soil sterilisation is used to control nematodes, it is usual to apply a milder treatment (2 hours at 43 °C), adequate to control mites and narcissus fly larvae. Where a nematode problem is suspected, or when using two-year-down growing, a longer, hotter regime (4 hours at 47 °C) is recommended, and a 4 hour treatment at 48 °C is used for ex-forced bulbs (Vreeburg *et al.*, 1999). At the time of writing, anecdotal evidence is that growers in the UK and the Netherlands are treating bulbs at up to 49 °C. Warm-storage and pre-soaking are necessary prior to HWT in these cases (see below).

A variety of designs of HWT tanks is used, the usual type being a front-loaded design where bulk bins of bulbs are loaded and unloaded using a fork-lift truck whilst the dip solution is temporarily pumped to a holding (or 'slave') tank (Figure 4.3). Tank capacity varies depending on the scale of the operation, but may be from 0.5 to 10 t of bulbs. Since the temperature of HWT is critical for killing nematodes without causing unnecessary damage to the bulbs, good temperature control and tank circulation are key factors in HWT tank design. Many other factors are also important, such as rapid heating, good insulation, a suitable water:bulb ratio, temperature monitoring and recording, operator safety and convenience of use. Aspects of the design and use of HWT tanks have been described by Gratwick and Southey (1986). Although HWT may be used to control pests and diseases in a variety of other plant material, flower bulbs (in particular narcissus) are the main subjects for HWT, and an HWT facility is likely to be the major dedicated outlay

Figure 4.3 Modern front-loading hot-water treatment tanks. Two four-tonne units with overhead slave tank for holding dip when loading and unloading bulbs (Photograph: Lyn Secker, Secker Welding, Holbeach).

for bulb producers. HWT tanks can also be utilised for sterilising bulb containers and other equipment, and other produce requiring dipping in pesticide may make use of them at ambient temperatures. HWT of narcissus bulbs may be carried out under contract, but in UK it is usual for bulb growers to treat their own bulbs.

The correct timing of HWT involves the overall logistics of the farming operation. To minimize crop damage, narcissus bulbs are treated after all flower initials have been formed and are visible under a hand-lens on dissection of the bud, but before the root initials have developed too far (shown by the roots erupting from the base plate). The internal stage of development (ISD) of 'complete flower differentiation', called 'Stage Pc' by bulb growers from the paracorolla (trumpet or cup), the last formed part of the flower bud, is illustrated in Preece and Morrison (1963), Cremer *et al.* (1974) and ADAS (1990b). In practice, this means that in the UK, for example, bulbs should be treated from late-July onwards, aiming to complete HWT before the end of August, a window of some four weeks. HWT tank capacity should be calculated to suit this treatment window and the cultivars grown. Narcissus cultivars which produce fine or early roots (Poeticus, Cyclamineus and Jonquilla cultivars) should be given HWT first (Benczur, 1976), while for mainstream cultivars the accepted order is small-cup, large-cup, then trumpet cultivars, although earlier HWT will give better control of base rot (ADAS, 1974; Millar, 1976; Price and Briggs, 1976). There should be a gap of at least a week between removal of offsets and HWT, as recent offset removal can increase infection, especially in certain cultivars (Kruyer, 1978). Bulbs re-claimed from forcing may be given HWT early, in May or June (Vreeburg and Korsuize, 1991). Where flower quality is unimportant, an earlier start to HWT may be acceptable, although the critical development stages or earliest dates for avoiding damage to leaf initials has

not been reported; late HWT is probably more damaging, because of the potential loss of leaf area and roots. In cases where a problem with nematodes is suspected or known in a stock, bulbs should be lifted early (June), cleaned, graded and given HWT promptly. Earlier recommendations (e.g., Hastings and Newton, 1934) and some Dutch recommendations state that HWT in this case should be done within three to four weeks of lifting, but current recommendations in the UK are to treat sooner than this (J.B. Briggs, personal communication). The increase in stem nematode numbers in bulbs during storage was shown by Winfield and Hesling (1966).

Even when applied correctly, HWT can reduce crop vigor, but this disadvantage is outweighed by the control of stem nematode and, in any case, the loss of vigour is insignificant in two-year growing. If HWT is carried out too early or too late, for too long a time or at too high a temperature, or following the storage of bulbs at low temperatures, a variety of damage occurs. This varies from mild damage to the flowers (unimportant where crops are being grown for processing), through damage to the leaves (from mottling of the leaf tips to severe distortion or stunting resulting in bulb yield loss) or roots (resulting in severe yield loss), to the death of the bulbs. Excessive HWT temperatures may result in the abnormal production of additional bulblets (Edwards, 1965; H.Y. Alkema, personal communication).

Hot-water treatment – the use of chemicals

While stem nematodes in the bulbs are killed by the high temperatures of HWT alone, chemicals are added to HWT tanks to control pests and diseases better. The basic material added is the disinfectant formaldehyde, used as 'commercial formalin' (containing 38–40% formaldehyde) (Hawker, 1944). For California, Qiu *et al.* (1993) reported that the standard HWT regime was 4 hours at 44 °C, but showed that such a treatment was sufficient to control stem nematode without added formaldehyde; using formaldehyde, 150 minutes was sufficient and caused no crop damage. However, the time for the centres of bulbs to attain the target temperature after placing in hot water should be added to these basic times: this was calculated as equal (in minutes) to $-15 + 3.4x$, where x is bulb circumference in cm (Qiu *et al.*, 1993). Higher temperatures could be used for shorter times: without formaldehyde, stem nematode was controlled by 60 or 15 minute treatments at 46 or 48 °C, respectively, without crop damage; with formaldehyde, control was achieved by 90, 45 and 30 minute treatments at 46, 48 and 50 °C, respectively, but this caused crop damage. HWT for 4 hours at 44 °C reduced the number of fungal colonies (*Penicillium* sp., *Fusarium oxysporum* f.sp. *narcissi* and *Mucor plumbeus*) recovered from bulbs, but the effects of formaldehyde, glutaraldehyde and sodium hypochlorite in controlling these fungi were variable.

Despite these possibilities for dispensing with a disinfectant, it is generally considered essential to add formaldehyde, which is effective at HWT temperatures in killing free-swimming stem nematodes (nematodes that escape from the bulbs into the tank dip and which are more resilient to high temperatures), as well as the spores of the base rot fungus. Formaldehyde is usually added as 5 litres commercial formalin per 1000 litres (0.2% a.i.). Increasing the concentration of formaldehyde gives little extra benefit in fungicidal activity, but can result in crop toxicity, with fewer and deformed flowers (Price and Briggs, 1976; Linfield, 1991), so any recommendations to use higher rates (e.g., Higgins, 1999) should be treated with

caution. Higher rates of formalin can also damage the base plates of bulbs, producing corky areas, especially when HWT is being carried out early or soon after lifting (Briggs, 1988), and in dwarf cultivars and others which form early, fine roots and which are susceptible to formaldehyde damage (Vreeburg, 1984b; van der Weijden, 1989). However, formaldehyde continues to be the material of choice for most growers because of its effectiveness, cheapness and availability. The use of formaldehyde is now coming under scrutiny due to health issues (Zell, 1984), and it may be unavailable in some localities. Linfield (1991) evaluated alternative disinfectants for killing the chlamydospores of the base rot fungus under HWT conditions: commercial preparations of glutaraldehyde, hydrogen peroxide–peracetic acid and thiabendazole were highly effective and non-phytotoxic. A disinfectant based on peroxyacetic acid (peracetic acid) was effective in killing stem nematodes (both free-swimming and in the 'wool' stage) and chlamydospores of the base rot pathogen at HWT temperatures (Hanks and Linfield, 1997, 1999). In this study, peroxyacetic acid killed the chlamydospores of the base rot fungus within 1 hour, whereas total kill was not achieved with formaldehyde even after 4 hours. Used in HWT, the peroxyacetic acid-based disinfectant was not phytotoxic to bulbs. Glutaraldehyde was shown to be effective against stem nematodes at HWT temperatures (M.J. Lole, personal communication). Alternatives such as bleach and chlorine dioxide are being evaluated by Chastagner (1999).

Fungicides and insecticides may be added to HWT tanks along with formaldehyde. In the UK, thiabendazole is the material of choice for controlling the base rot fungus and other pathogens such as *Penicillium*. Thiabendazole, although used in agriculture and horticulture as a fungicide, was originally introduced to farming as an anthelminthic, and it appears to have useful activity against stem nematode in HWT (Hanks and Linfield, 1999). Because thiabendazole is more soluble at very acidic pH values, and, like many pesticides, is broken down by alkaline hydrolysis, for bulb dips it is used in an acidic formulation ('Storite Clear Liquid' in the UK). To further reduce the pH value of the dip an acidifier, sodium hydrogen sulphate (sodium bisulphate), is sometimes added to HWT tanks by growers on the basis of anecdotal evidence, and this is now being investigated experimentally (Hanks, 1999). For stocks severely affected by base rot, thiabendazole is used in HWT even when it has been applied post-lifting, this double treatment providing the greatest initial reduction in the number of diseased bulbs (Hanks, 1996b). Prochloraz-based fungicides are also used, while a number of other fungicides have been found to be effective, including thiram, benomyl and carbendazim (ADAS, 1974, 1976; de Rooy, 1975). Fungicides used in the Netherlands include zineb/maneb, captan, benomyl, carbendazim and Topsin M, sometimes used in a mixture, especially for dwarf cultivars (Anon., 1987). Recent recommendations are for a combination of formaldehyde, captan, prochloraz and carbendazim (van der Weijden, 2000). Lower concentrations are used for ex-forced bulbs, because of the extra uptake by the drier bulbs.

The insecticide chlorpyrifos may be added to HWT tanks to prevent subsequent infection of bulbs by the larvae of the large narcissus fly, although this gives protection only in the first year (Tones and Tompsett, 1990; Hanks and Linfield, 1997). Cold dips or application at planting were less effective.

It is usual to add a non-ionic wetter to HWT tanks to enhance the effects of pesticides, and other types of wetters should be avoided because of the possibility

of phytotoxicity (Wallis, 1966, 1967a). In trials with carbendazim, the addition of compounds believed to increase fungicide uptake (dimethyl sulphoxide, indolylacetic acid and hydrochloric acid) showed no benefit to disease control (ADAS, 1976). The generation of foam in HWT tanks, which could reduce pesticidal effectiveness, may be reduced by the addition of an anti-foam preparation, which has no adverse effect on the crop (Tompsett, 1977). Where problems in handling bulbs are likely due to the applied pesticides, an anti-dust 'sticker' may be added, as is practised in the Netherlands (Anon., 1987).

Some pesticide labels state that bulb dip solutions should be freshly made up each time, but this is impractical. It is usual to top up bulb dip tanks and to re-use them for as long as practical, providing gross contamination with debris (bulb skins, soil, etc.) can be avoided. After dipping, tanks are topped up with water and the appropriate amounts of disinfectant, pesticide, etc., are added so that the top-up is given at the original strength. Dutch recommendations to top-up formaldehyde at twice the original strength have now been modified, and 0.75% commercial formalin is now recommended (van der Weijden, 2000). For pesticides, manufacturers' recommendations should be consulted, although these are often not specific. The thiabendazole fungicide 'Storite Clear Liquid' is an exception, as definite top-up procedures are given. As active ingredients can be lost in use, it is advisable to have the concentration of formaldehyde and pesticides determined by specialist laboratories for sets of samples, at least until experience of their behaviour is obtained, and particularly as little published guidance exists on the stability of pesticides in bulb dips. Recent Dutch recommendations suggest using slightly higher top-up rates for prochloraz, but using other pesticides at the original strength (van der Weijden, 2000).

Treatments to reduce damage due to HWT

HWT damage can be reduced by warm-storing bulbs for 1 week at 30 °C before HWT, a procedure used in south-west England to reduce damage to flowers in the year after HWT (Wood, 1944; Slootweg, 1962; Tompsett, 1975). Warm-storage is always recommended when treating sensitive narcissus such as Poeticus cultivars, and is beneficial in cultivar 'Tête-à-Tête' (ADAS, 1988b). The exact treatment is not critical, 3 to 8 days at 30 to 35 °C having been used (Rees and Turquand, 1967; Turquand and Rees, 1968). Such warm-storage, however, by partly desiccating the bulbs, induces stem nematode to migrate to the outside of the bulbs, often near the base plate, where they form 'nematode wool' which can escape into the bulb dip during HWT. Warm-storage is, therefore, used in conjunction with pre-soaking bulbs (for 3 or 4 hours or, preferably overnight, at ambient temperatures with formaldehyde) immediately before HWT, in order to hydrate the 'wool'. With this regime a higher HWT temperature, 46 or 47 °C, is necessary to kill stem nematodes. It is important not to omit pre-soaking when pre-warming is used. Warm-storage in itself may decrease crop vigor (Wallis, 1965, 1967a). Warm storage appears to induce dormancy in the shoot initials, making them less sensitive to damage by high temperatures, and thus extends the period over which HWT can be safely used (Wallis, 1967c). The lower limit of the warm-storage effect is about 18 °C. If bulbs are stored at 18 °C for 2 weeks before HWT (where storage may otherwise have been at slightly lower ambient temperatures), HWT damage

can also be prevented, but without the need to pre-soak bulbs or to use a treatment temperature higher than 44.4 °C. By this means (sometimes called 'partial pre-warming'), the window for the safe HWT of narcissus bulbs in eastern England can be extended to late-September (Hanks, 1995a). An equivalent treatment of 1 week at 20 °C is recommended in some Dutch advisory material.

There is a difference in Dutch and UK advice on the warm-storage of early-lifted bulbs known to be infected with stem nematode. The former suggests a warm-storage of at least one week at 30 °C after lifting, while in the UK it is stated that pre-warming should be avoided because of the danger of inducing the formation of 'wool' if the bulbs are allowed to dry out.

Bulb treatment after HWT

The increasing practice in UK is to plant bulbs soon after HWT, which means they can be planted while still damp. This avoids the need for re-drying and storage and reduces handling, but it means that the bulbs are planted into relatively warm soil in August, possibly encouraging base rot. When removed from the HWT tank, the bulbs should be allowed to drain and then should be cooled, ventilated and surface dried using fans in a drying wall or similar arrangement. Although there is no evidence from trials to show a distinct benefit from rapid cooling to ambient temperatures after HWT (Tompsett, 1973), this seems sensible. From the viewpoint of operator safety, the ventilation of bulb handling areas is critical, especially when bulbs are being removed from the HWT tanks and moved to the drying facility. As an alternative to immediate planting, bulbs can be dried after HWT, followed by appropriate storage and replanting in September, when soil temperatures have fallen sufficiently to slow the development of base rot. In this case, rapid drying is important in controlling base rot (Hawker, 1935, 1940).

Other pre-planting pesticide applications

The application of insecticides, other than in HWT, has been evaluated for the control of large narcissus fly. Separate pre-planting dips of chlorpyrifos were less effective than using the material in HWT (Tones and Tompsett, 1990). One-hour dips, either immediately after lifting or before planting, were evaluated by Bogatko (1988) and Bogatko and Mynett (1990): several insecticides were found to be effective in controlling larvae (including isofenphos and carbofuran).

Bulb storage treatments to manipulate growth

In addition to the considerations relating to bulb storage generally, storage temperatures may be altered, in the period before planting, to manipulate the subsequent growth of the crop. This was investigated by Rasmussen (1976b), who compared the growth of narcissus bulbs after storage at ambient outdoor temperatures and planting bulbs in September, with storage at 20 or 23 °C and planting in October. Emergence, but not anthesis or senescence, was delayed by controlled-temperature storage and later planting. Better yields of bulbs or flowers resulted from using the higher temperatures, such that best results were obtained from the ambient regime in warm winters and from 23 °C storage in cool winters. More

usually, however, interest has centred on the cool storage of bulbs before planting as a means of advancing (or 'forwarding') field-grown cut-flowers. In south-west England, 'pre-cooling' narcissus bulbs for 2–6 weeks at 9 °C before planting gives flowers earlier and over a longer period, although the flowers may be of poorer quality and subsequent bulb yield is reduced (Rees and Wallis, 1970; Rees, 1972; ADAS, 1982d). The optimum treatment for advancing flowering was 6 weeks at 9 °C, with no advantage of using longer or colder treatments or of augmenting the treatment by covering the growing crops with polythene film (ADAS, 1982c; Flint, 1983). Flowering dates 8–28 days earlier than untreated controls were reported in these studies. Cocozza (1972) investigated pre-cooling before planting bulbs outside in Italy, successfully using a treatment at 3 °C for up to 6 weeks. In France, Le Nard (1975) reported that flowers were obtained 30–45 days earlier than from untreated bulbs, by warm storage (7 days at 34 °C) followed by cooling (8 weeks at 9 °C) and covering rows with a narrow polythene tunnel. To produce late outdoor flowers, bulb storage at 20–26 °C from August to October has been investigated, but this delayed flowering by less than a week (ADAS, 1972). Tazetta narcissus can be warm-stored over winter for planting in spring (see under Production of Tazetta Narcissus).

While most interest in these procedures relates to optimising crop growth and producing earlier flowers, they also offer the possibility of shifting the growth pattern to one more suited to particular needs, for example to fit a more convenient or programed production schedule. The effect of temperature on narcissus growth and development is being studied with a view to developing predictive models (Hanks *et al.*, 1998b,c). As narcissus growth and yield show marked year-to-year differences (Rees, 1972; Hanks, 1996a), such crop models would be useful management tools.

Bulb planting

The main factor to consider is the type of growing system – whether to grow the bulbs in ridges or beds, and whether to lift bulbs annually (one-year-down growing) or every two years. Because it is considered more economic, a 'two-year-down' growing system in ridges has generally been adopted. Other factors to be considered include planting date, rate, depth and arrangement; the use of pesticides at planting, crop covers, cover crops and growing in nets also need to be considered.

Planting and growing in beds or ridges

Bulbs (including narcissus) were traditionally grown in flat beds and were planted and lifted annually by hand. Bulb planting and lifting are now mechanised. In the UK, bulb crops are now grown in ridges (as potatoes), and ridges are also used in the Netherlands where narcissus are grown on heavier soils. Growing bulbs in flat beds allows better utilisation of the available area, but large volumes of soil have to be moved in planting and lifting bulbs. Growing in ridges has been adopted largely on practical grounds, and is useful as it allows sharing of equipment with potato growing.

Bulbs are planted in ridges using specialised planting machines that typically feed bulbs from a hopper, planting two rows at a time (Figure 4.4). The planting

Figure 4.4 A typical bulb planting machine feeding bulbs from a hopper into two ridges (Photograph: Horticulture Research International).

rate should be calibrated by adjusting the hopper aperture or the speed of the delivery belt. High planting rates may be difficult to achieve with standard machinery, and blockages often occur, so that constant attention from the operator will be needed. When planting in flat beds, small or uniform bulbs are planted using a row planter, planting four rows at a time. Larger bulbs are planted using bed digging and filling machines that lift the soil and transfer it to the adjacent planting bed.

'One-' or 'two-year-down' growing

Rees *et al.* (1973) compared one-, two- and three-year-down narcissus growing in south-west England, and concluded that costs were too high for a one-year-down system to be economic. UK narcissus growers now use this two-year-down growing system, half of the stock being lifted and re-planted in alternate years. This 'biennial' growing involves some loss of bulb yield, because an ideal planting density obviously cannot be achieved in both years. Too low a planting density is inefficient in the first year, while too high a density results in subsequent overcrowding. At a planting density of 10 t/ha, half the bulb weight increase occurred in each year in two-year growing, but at high densities all the increase was in the first year (Rees *et al.*, 1973). Some data (ADAS, 1993) are illustrated in Figure 4.5. In ornamentals production, however, when most bulb growth takes place in the first year, this has the advantage that higher flower yields are obtained in year two, and there are high yields of relatively small bulbs which are advantageous for retail bulb sales because they produce more flowers per tonne. The flowers produced in the second year are unaffected by any adverse effects of HWT two years earlier, so quality is improved. There is also an element of 'compensation' in yield,

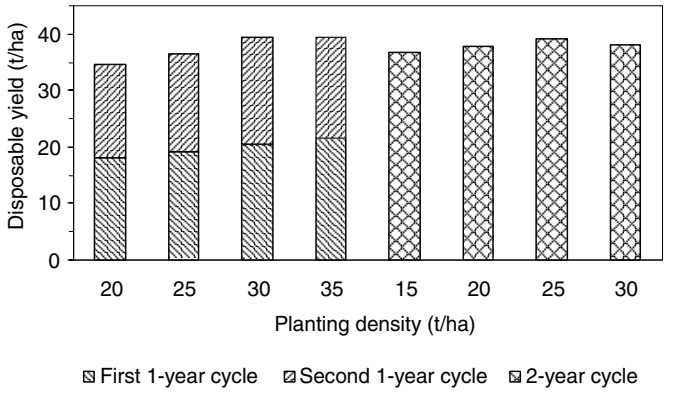

Figure 4.5 Narcissus bulb yields for one- and two-year-down growing (data from ADAS, 1993).

if poor growth occurs in one of the two years of the growing cycle, for example due to early senescence or pesticide damage. Two-year-down growing has enabled UK narcissus growers to become very efficient by reducing the annual demand for labor, land, storage and other resources (ADAS, 1986b). In some cases, growers may leave narcissus crops down for a third year for economic reasons, perhaps because bulb prices are low or the flower crop is more important, but this is not a practice that is recommended as good husbandry. Other reports of trials comparing one- and two-year-down growing have been reported from Denmark (Rasmussen, 1976a) and Poland (Sochacki and Mynett, 1996), confirming the advantages of the two-year-down system.

The main disadvantage of two-year-down growing is that HWT and other useful procedures (such as post-lifting fungicide treatment or high-temperature drying) can be carried out only in alternate years, so there is no opportunity every year to control stem nematode and other harmful organisms. Fungal pathogens in the soil and stem nematodes in bulbs can build to serious levels over a two-year period, while foliar fungal diseases (such as smoulder) are serious problems only in the second-year of narcissus crops. In the summer between the two growth periods, bulbs remain in the soil when temperatures are high and liable to encourage bulb rots, and the benefits of early bulb lifting (the avoidance of narcissus fly and aphid-borne virus infection late in the growing season) are lost. When narcissus are left in the ground, they begin growing earlier, and earlier rooting may allow more effective infection by soil-borne pathogens while soil temperatures are still high. Second-year narcissus shoot earlier, and may therefore be more damaged by frosts. Where base rot is a major problem, a temporary return to one-year-down growing should be considered (Tompsett, 1984).

When growing narcissus for processing, rather than as ornamentals, different considerations may apply. If high yields and the avoidance of pests and diseases are important, one-year-down growing may be more appropriate. Where the most economical growing system is required, two-year-down growing will be appropriate, but there is unlikely to be any advantage of growing cycles longer than two years.

Planting date

The practice of planting narcissus bulbs soon after HWT has been mentioned above, and, since the date of HWT is dependent on the stage of the crop, this also governs the date of planting. Planting immediately after HWT, in August or early-September, means that the bulbs are planted into relatively warm soil, increasing the likelihood of infection by the base rot fungus when the roots are erupting from the base of the bulb (Gregory, 1932; Hawker, 1935). Dutch recommendations, and earlier recommendations for the UK, are to plant narcissus bulbs in (late-)September. It is usual to plant cultivars in the same order in which they received HWT. In one-year-down growing, there is a steady decline in bulb yield when bulbs are planted later than September. One study showed yields of 120% from September planting and only 63% from December planting (Wallace and Horton, 1935), although in another trial (Allen, 1938) significant yield loss did not occur until planting was even later. In two-year-down growing, however, poor growth in the first year would be compensated by better growth in the second.

Planting density

The rate of planting will depend on the vigor of the cultivar, bulb price, the percentage bulb weight increase and size of bulbs required, and the growing system adopted. In the UK, narcissus bulbs for two-year-down growing are often planted at densities between 12.5 and 17.5 t/ha. A planting density in the lower range would be used where vigorous bulbs were being planted, where a high rate of increase was required (for example, where bulking an expensive cultivar), or where large bulbs were required; a high density could be used, for example, when growing a cheap cultivar where the requirements were a reasonable rate of increase coupled with reducing land and labor requirements, or where a good yield of smaller grade bulbs was needed. Higher planting rates are more economic of land and labor: the preferred planting rate is the one which gives the best financial returns, rather than the greatest yield (Rees, 1972, 1975; ADAS, 1976). The effects of planting density and planting grade in ridges were investigated by Rees (1972) in eastern England. For smaller planting grades, the optimum densities were above the highest used in the trial. For larger grades, there were large differences in optimum densities and financial returns between years, and over three years the mean optimum densities were 42 and 27 t/ha for 10/12 and 12/14 cm grade bulbs, respectively, considerably higher than the planting rates used commercially. Figure 4.6 shows the profitability for the one-, two- and three-year growing of small offsets and large DN bulbs grown in ridges in south-west England at a range of planting densities. Experimental work in Lincolnshire and Cornwall indicated that planting rates up to 22.5 t/ha could be profitable where a high production of saleable (middle grade) bulbs was required. Higher planting densities lead to longer stems and increase crop support on windy sites, so are useful where the flower crop is important. For growing narcissus on a one-year-down system, Dutch planting rates vary from 14 t/ha for vigorous, small offset planting material, to 35 t/ha for large 'mother bulbs' where splitting to more saleable grades of bulbs is desired. Dwarf narcissus such as Cyclamineus cultivars are usually planted at about half the density of standard cultivars.

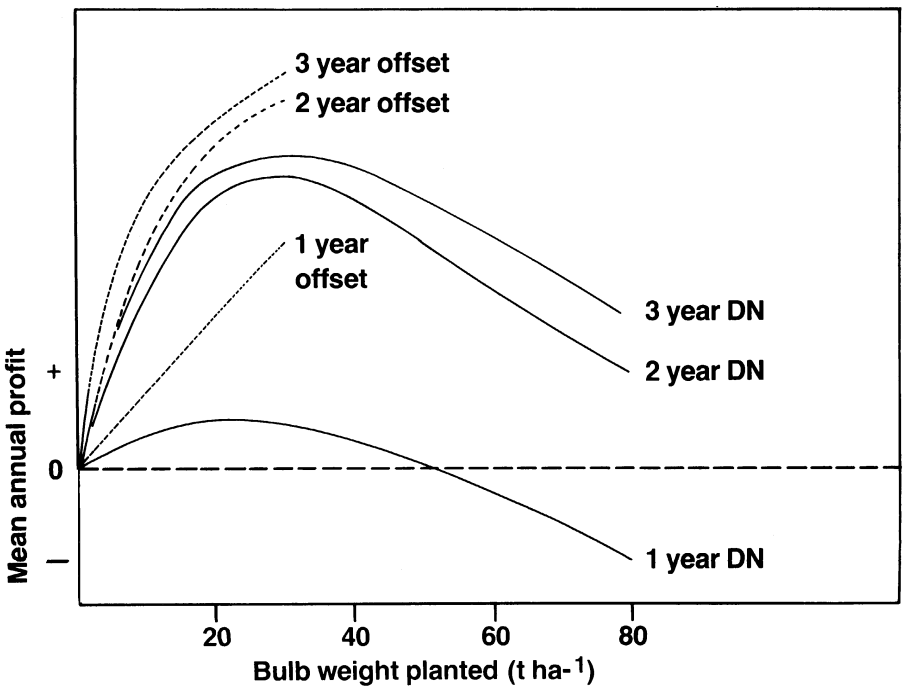

Figure 4.6 Effect of planting density, bulb grade (offset or double-nosed (DN) bulb) and crop duration (1, 2 or 3 years) on profitability. Data for narcissus 'Fortune' grown in ridges, from Rees *et al.* (1973), with permission from *The Journal of Horticultural Science and Biotechnology*.

High planting densities may accentuate problems with pests and diseases, create handling problems at planting and lifting, and require changes in irrigation or fertiliser practices. Planting density has a large effect on the spread of base rot. Linfield (1987) placed healthy bulbs 0, 10 or 20 cm laterally from 'inoculator' bulbs, and after one growing season 60, 27 and 6% of the healthy bulbs were affected, respectively. The base rot fungus can infect bulbs 30 cm away (Price, 1975c). For all bulbs to be at risk of infection requires 10% affected bulbs at a density of 10 t/ha, but only 5% at 20 t/ha, on ridges 76 cm apart; lower percentages would be required using 90 cm ridges (Tompsett, 1980a).

Planting depth

Narcissus bulbs are usually planted about 13 cm deep (from the base of the bulb to the top of the ridge). Taking care to adjust machinery to ensure an even planting depth, especially on undulating sites, produces a uniform environment and aids bulb lifting. Deeper planting may produce better growth, but bulbs are harder to lift, while shallower planting may lead to damage from cultivation and herbicides, and the bulbs are in warmer soil. Narcissus bulbs have contractile roots, and planting bulbs 20 cm deep results in the typical bulb shape, whereas when planted at 5 or 10 cm deep bulbs become elongated as a result of root action (Tompsett, 1977;

Hanks and Jones, 1986; cf. Allen, 1938). With increasing planting depth (8–23 cm), Wallis (1964) reported that emergence and flowering were progressively later, while bulb yields were greatest at intermediate depths. Narcissus bulbs can probably be planted much deeper than this, although this would be suitable only for garden and landscape use, where it would avoid damage from surface cultivation. Hagiladi *et al.* (1992) planted Tazetta bulbs at depths up to 90 cm. Deeper planting delayed emergence, and planting deeper than 60 cm resulted in fewer leaves and a net loss of bulb yield, although some shoots emerged even from bulbs planted 90 cm deep.

Planting arrangement

When bulbs were planted by hand in beds, they were placed evenly, 1½–2 bulb diameters apart and upright in the rows (Rees, 1972). Machine planting tumbles bulbs into the planting furrow, so they are more-or-less randomly orientated within the ridges, presumably resulting in less uniform growth and shape. The effect of bulb orientation was investigated when planting machines were introduced (NAAS, 1961; Wallis, 1964). Although random planting produced bulbs with bent necks, orientation (vertical, inverted, diagonal or horizontal) did not affect bulb yields, although vertical planting gave earlier crops. In the UK, ridges are often arranged at distances of 90 cm, centre-to-centre, but 76 cm ridges are also used. Planting machinery is often set to plant bulbs in a 20–25 cm wide band within the ridge, so the actual planting density within the planted area (the planting band) may be 3–4 times the overall (field) area. In trials, the width of the planting band (20–35 cm) had little effect on percentage bulb weight increase or bulb grade-out with planting densities from 20 to 30 t/ha (Millar, 1978; ADAS, 1983). In trials with bed-grown narcissus in the Netherlands, changes in planting arrangements, from 95 cm planting bands in 140 cm-wide beds, to 105 cm planting bands in 150 cm-wide beds, had practical advantages as well as allowing 2% more bulbs to be planted in the same area with little impact on labor requirements (van Dam and Schaap, 1987).

Rees *et al.* (1968) investigated the effects of planting density, rectangularity and row orientation in bed-grown narcissus in south-west England. Density did not affect anthesis date, but high densities increased stem length by over 20%. Flower numbers increased with density, but the number of flowers per bulb fell at the highest density. Lifted bulb weight increased with density, but did not peak within the range of densities used in this experiment (up to 150 bulbs/m^2), although yields of larger bulbs reached a maximum above 100 bulbs/m^2. Bulb yields were considered more than adequate, even at the highest planting density. Bulb yield declined with increasing rectangularity in east-west rows, but was unaffected by rectangularity in north-south rows, probably an effect of wind via shelter or light interception. Plants in north-south rows had longer stems.

In trials comparing ridge- and bed-growing of offsets and double-nosed bulbs at 54–216 bulbs/m^2 for 1–3 years, also in south-west England, planting at 20–30 t/ha gave the highest combined financial returns for bulbs and flowers (Wallis, 1968; Rees, 1972; Rees *et al.*, 1973). Flower yield in the first year was directly related to density, thereafter declining at the higher densities and with larger bulbs. Ridges were out-yielded by beds by 26–29%, except at the lowest density where the difference

was 15%. Two- and three-year-down growing was more efficient than one-year growing, especially at medium densities. Similar studies in eastern England showed that the response to density varied considerably between years, so it was difficult to draw general conclusions.

Pesticide application at planting

Bulb planting provides an opportunity to apply pesticides directly around the bulbs. The main use is for insecticides that prevent the larvae of the large narcissus fly entering bulbs, but this method is not currently used because no suitable pesticides are available. Until its withdrawal, the persistant insecticide aldrin was used successfully in this way in south-west England (Tompsett, 1973). Chastagner (1997) reported that most narcissus growers in the Pacific North-West applied a nematicide, usually fenamiphos, in-furrow at planting.

Crop covers and cover crops

In the UK, covers or cover crops are not used with narcissus, although they have been tested on an experimental scale. Despite the possibility of damage by frost, sensitive cultivars like 'Tête-à-Tête' are now being grown successfully, without covers, in England. In the Netherlands, however, it is common to protect narcissus and other bulbs by covering the land with straw or reeds, or by sowing a cereal crop, after bulb planting. For example, bulbs planted in beds may be covered with straw (10 t/ha, or 15–20 t/ha for frost-sensitive cultivars). The straw is removed in February, either mechanically or by burning, although lower rates (up to 10 t/ha) may be left in place. Alternatively, a lower rate of straw may be used, in combination with sowing rye at 250 kg/ha, and the cereal is killed by spraying a contact herbicide before the bulb shoots emerge. Where narcissus are grown in ridges, rye or barley (250 kg/ha) is sown before planting (if sown after the ridges have been formed much of the seed falls into the furrows). Rust from plant material used as a crop cover may sometimes infect narcissus foliage (Boerema, 1962).

Trials have been conducted in several countries to evaluate different crop covers and cover crops. In the Netherlands, plastic film and a covering of reeds were found to be much more effective than straw or rye in insulating bulbs from frost penetration (Meijers, 1979). In Danish trials, a number of covering materials was shown to increase soil temperatures by 1–2 °C, slightly hastening shoot emergence and significantly increasing bulb yields: the materials tested were chopped straw (10 t/ha), converted household refuse (100 t/ha), sphagnum peat (130 m^3/ha), bark (70 t/ha) and sawdust (70 t/ha) (Rasmussen, 1976c). In the colder areas of Poland, peat, straw, chaff and cow manure have been reported as mulches on bulb crops (Dargiewicz, 1971).

As well as increasing soil temperatures in winter, crop covers reduce soil temperatures in summer. Leaving a straw cover in place may reduce base rot by decreasing soil temperature in susceptible cultivars like 'Golden Harvest' and 'Carlton' (Tompsett, 1986). McClellan (1952) used late-season mulches (straw, foil, etc.) in an unsuccessful attempt to reduce soil temperature and losses due to base rot, probably because a calculated temperature reduction of 5 °C was needed but only a 2 °C drop was attained.

On sloping sites, ground cover crops (cereals, grass or oilseed rape) successfully prevented soil erosion if sown immediately after bulb planting and killed before crop emergence, provided the growing season was long enough (ADAS, 1987).

Planting in nets

The technique of planting bulbs in netting was developed as an aid to bulb recovery in heavier soils (Bijl, 1990). In small-scale trials in the UK, the use of netting did not reduce bulb yields in 'Tête-à-Tête', compared with growing bulbs loose (ADAS, 1988b).

Operations in the field

This section covers the control of weeds, diseases and pests as well as flower cropping or de-heading, irrigation, roguing and inspection, and (for two-year-down growing) maintenance of the crop between the two growing seasons.

Weed control

Bulb growers generally aim for good weed control in order to prevent competition, clean up crops between the two growing seasons, and to assist harvesting (weeds can clog lifting machinery). The use of crop covers (such as straw) may be a way of reducing reliance on herbicides.

The effects of weed competition on narcissus yield were examined by Lawson (1971, 1976) and Lawson and Wiseman (1972, 1976, 1978). Even when weed cover was substantial, weeds had little effect on early spring growth and first-year flowering. However, when they resulted in shading during the period of rapid bulb growth, leaves and stems grew longer at the expense of bulb yield; shading from late-June had no such effect. Narcissus foliage senesced quicker on weedy plots, reducing bulb yields, but if weeds were killed late in the season the narcissus foliage lodged. Over-wintering weeds that grew up with the narcissus foliage were most damaging, producing smaller, less vigorous bulbs. Under weedy conditions bulb yield losses approached 20%, or 35% in very dry conditions. Poor weed control often relates to failure to control a relatively few resistant species (Wood and Howick, 1958; Lawson and Wiseman, 1972). It is possible that over-wintering weeds may help the crop by giving winter protection and conserving moisture (Lawson, 1971), although this aspect has not been researched. Weeds can also lower soil temperature at bulb depth by 4 °C, compared with a weed-free plot, which may affect the development of base rot (Tompsett, 1980a).

Herbicides are used at four stages: (1) contact herbicides are used in autumn/winter before crop emergence; (2) pre-crop-emergence residual herbicides are used as late as possible before crop emergence; (3) early-post-emergence residual herbicides are used, usually before shoots are about 10 cm tall; (4) a late-season herbicide may be used after flowering, although the materials available are restricted and application at this stage is difficult because the crop foliage has often flopped to shield the soil surface by this time. The post-flowering period is difficult for weed control, because the previous herbicide 'seal' on the soil surface may be broken by the feet of flower pickers, and also because the new flower initials are

being formed at this time and may be sensitive to damage by herbicides; trials have taken place on herbicides suitable for application immediately after the flower cropping stage, before the crop foliage has spread to cover the furrows completely (Briggs and Hanks, 1997).

Suitable herbicides are given in the standard texts and elsewhere (Mével, 1979; ADAS, 1990a), and herbicide trials have been published in several countries (e.g., Turquand, 1968; BBLF, 1972; Briggs, 1972a,b; Lawson and Wiseman, 1976; Ryan and MacNaeidhe, 1978; Rupasava *et al.*, 1981; Rusalenko *et al.*, 1981; Smith and Treaster, 1982, 1984, 1989, 1990; Koster and Kruyer, 1983; Bing, 1985; Skroch *et al.*, 1988, 1994; Howard *et al.*, 1990; al Khatib, 1996). Several types of herbicide damage can occur, including leaf scorch and chlorosis, abnormalities such as distorted flowers, damage to the basal leaf meristem resulting in flaccidity, and reduced growth (Ivens, 1966). Cereal seed may be a problem where crops have been covered with straw (Koster and de Rooy, 1981; Koster, 1983; Koster and van der Meer, 1986). The control of cereal, potato and other 'volunteers' (plants left from previous crops) in narcissus crops may also prove difficult.

Fungicide sprays

It is usual to apply fungicide sprays to narcissus crops to control foliar diseases such as smoulder, leaf scorch, fire and white mould, and this is more important where bulbs are being grown on a two-year-down basis because of the build-up of disease in bulbs, debris or soil. Spray programs are not always successful in controlling these diseases (Melville, 1980), although they may help in the control of pathogens involved in bulb rots (Davies *et al.*, 1998). A general effect of fungicides, especially if a programme of sprays is used, is to delay foliar senescence, probably by controlling fungi that degrade the leaf cuticle (Rees, 1972; Jones, 1978). Since bulbs are usually lifted before the leaves have died down, this has the disadvantage that crops are even greener at lifting.

There is little specific information on the most effective fungicides or fungicide programs for controlling particular diseases, and in the UK it is usual to apply several fungicides with different modes of action, which also reduces the likelihood of the development of resistance to fungicides. Typical fungicides used in the UK and the Netherlands include chlorothalonil, iprodione, vinclozolin, mancozeb, zineb/maneb, benomyl, carbendazim, thiophanate-methyl and procymidone, sometimes involving tank-mixes. Spraying often begins soon after shoot emergence, continuing at 7- to 10-day intervals until flowering, with one or two further sprays after flowering to control infections resulting from the damage of flower cropping (O'Neill and Mansfield, 1982; O'Neill *et al.*, 1982). In practice, it may be difficult to apply fungicides at target dates because of unsuitable weather, a particular problem in wet and windy areas where suitable 'spraying days' may be relatively few. In the year of lifting, the spray programme is often curtailed to encourage the foliage to die down. At present, no detailed recommendations are available about the critical times for spraying to take place (Hanks and Briggs, 1999), but key times are thought to be when frost-damage occurs, after flowering or cropping (because of the damage caused by cropping or the presence of decaying flowers if not cropped), as well as in periods of damp weather.

Insecticide and nematicide applications

In climates where the large narcissus fly is a problem, it is advisable to apply appropriate insecticides if available. Regular sprays against adult flies should be applied, preferably making use of pest forecasting models to target applications accurately (Finch *et al.*, 1990; Collier and Finch, 1992). Suitable insecticides include omethoate (Conijn, 1990; Conijn and Koster, 1990), sprayed just before or during the oviposition period. The application of granular and liquid insecticides to the ridges during the growing season, to target the newly hatched larvae, has also been used. Many insecticides have been tested, but their effects are not always consistent each year (Bogatko, 1988; Bogatko and Mynett, 1990; Tones *et al.*, 1990; Ben-Yarkir *et al.*, 1997).

Foliar applications of oxamyl were evaluated for the control of stem nematode by Westerdahl *et al.* (1991) as an alternative to HWT with formaldehyde, pre-planting soil sterilisation with 1,3-dichloropropene, or applying phorate at planting. Several rates and timings of oxamyl application reduced nematode levels in bulbs and leaves without any phytotoxicity. Earlier, Bergeson (1955) had applied three, weekly applications of Systox or other systemic phosphates to narcissus in pot trials, and reported that nematode numbers in the leaves and bulb were reduced by these treatments, without toxicity at lower rates.

In the case of high-health status stocks or VT stocks, the regular application of aphicides should be considered in warmer weather when populations are high. While aphids only rarely colonize narcissus, several common species spread viruses during exploratory probings. Frequent applications of anti-feedant insecticides (pyrethroids) can be used, although Broadbent *et al.* (1957) showed that systemic insecticides can increase virus spread, possibly by increasing the irritability and probing of aphids before death. Alternatively, mineral oil sprays, which disrupt normal transmission of virus particles, may be applied, although they are not always effective (Mowat *et al.*, 1984). Mineral oil sprays can, however, reduce narcissus yield, in one trial by 50% when sprays were applied weekly (ADAS, 1982c). Some dwarf cultivars, such as 'Tête-à-Tête' and 'Hawera', appear to be more sensitive to mineral oil sprays (Vreeburg and Korsuize, 1987).

Other chemical treatments

The application of ammonium nitrate sprays can delay leaf senescence and increase bulb yields, perhaps by replacing the failing uptake from senescent roots (Rees, 1972). No beneficial effects on bulb yield have been reported from UK trials in which a range of plant growth regulators (PGR) were applied in the field (ADAS, 1984). However, for *N. tazetta*, El Sallami (1997) reported a range of effects when PGR were used as bulb soaks or foliar sprays, including increased bulb production with ethephon.

Flower cropping and de-heading

Depending on local practices, flowers from narcissus crops are either cropped routinely, only when market prices make this worthwhile, or not at all if bulb production is paramount. When grown for processing, it is unlikely that flowers

will be cropped because of the physical damage this causes, the likely spread of disease, the loss of photosynthetic area and the high labor requirements, and perhaps also because of contractual arrangements. Flower cropping may increase the incidence of smoulder because of the opportunity for the fungus to invade damaged surfaces (Gray and Shiel, 1975, 1987; Dixon, 1985, 1986).

On the other hand, de-heading crops may increase bulb production by removing a sink to nutrients, and by eliminating decaying flowers that may encourage fungal diseases. However, the results of trials have been variable. Thus, Wallace and Horton (1935) reported instances of 40 and 103% greater yields of bulbs when the flowers were not cut, whereas Allen (1938) reported only minor effects of flower or flower stem removal on bulb yield. Grainger (1941) found that the stem contributed little to bulb growth, and suggested it should be removed. Kalin (1954, 1956) reported highest bulb yields after de-heading: yields were reduced by 3% when flowers were left intact or cropped half-way up the stem, by 5% when the flowers were cropped at bud stage, and by 7% when cropped at full bloom. Removing the flower bud as soon as the stem had grown enough to allow it gave yields similar to those of de-heading. When these practices were repeated annually, the effects were cumulative. De Vlugt and Kruijer (1975) confirmed similar yield losses as a result of not de-heading (1%) or not picking flowers (5%). The differences between different trials may be due to cultural practices, cultivars, location or how carefully the cropping and de-heading treatments were carried out, although, in the case of de Vlugt and Kruijer's (1975) study, the results were similar whether 'careful' or 'commercial' standards of removal were used. Some cultivars have brittle stems, and large amounts of damage would be expected from de-heading (Tompsett, 1976). Removing leaves at cropping, compared with cropping flowers alone, further reduced yields by 66 and 36% when only one or two leaves were left attached, respectively (Allen, 1938). Overall, de-heading is probably not economically worthwhile. The removal of non-cropped flower heads is recommended in the Netherlands to control fire, but, although de-heading machines have been tested, this is not usually practised (van Aartrijk, 1990). Whether flowers are cropped, de-headed or left intact, a fungicide spray programme is important for different reasons.

Irrigation

Trials have shown that bulb yields are best in soils near field capacity (Strojny, 1975; Goniewicz *et al.*, 1976). A rise in soil moisture over the range 40–95% of available water capacity had no effect on N, P or K levels in the bulbs, but levels of P and K in the roots rose with increasing moisture levels (Dabrowska, 1975). Water availability also altered root anatomy and stomatal numbers in narcissus (Goniewicz *et al.*, 1976).

In the Netherlands, irrigation of bulb crops in sandy soils is normal, often through the control of the water table. It is recommended that narcissus should be irrigated when it becomes difficult to squeeze the soil round the roots into a ball, and water should be applied in applications of 15–20 mm, as higher applications damage soil structure. Narcissus crops are not usually irrigated in the UK, although some trials have shown that irrigation increases the yield of larger bulbs, especially at higher planting rates (ADAS, 1985d). Moderate irrigation may improve

bulb growth, especially in April-May when rapid growth is taking place, and it also improves soil conditions for bulb lifting. Excessive irrigation late in the growing season (late-May onwards) may increase bulb weight but may delay the 'ripening' of the bulb, as well as causing split scales due to uneven growth (ADAS, 1970). Benefits of irrigation were demonstrated in trials in New Zealand (McIntosh and Allen, 1992).

Roguing and selection

When grown as ornamentals it is important to inspect crops and physically remove rogue cultivars and other off-types, but whatever narcissus are grown for they should be inspected for signs of stem nematode lesions ('spickels'), disease 'primaries' (such as smoulder) and virus symptoms. Affected plants should be removed and destroyed. Roguing is a skilled and labor-intensive operation: traditionally, affected bulbs were dug out with a 'roguing iron' inserted into the ridge under the bulb. Various methods of roguing using herbicides (e.g., paraquat or glyphosate guns, gloves, sticks or aerosol sprays) have been tried (Millar, 1977, 1979; Ryan *et al.*, 1979; Bijl, 1981). To reduce the spread of viruses, crops should be inspected regularly: although severe infestations may be seen early in the season, some symptoms become evident only later. Virus spread is proportional to the number of infected plants, so this must be kept at a low level by roguing. In a three-year period, 16, 46 and 90% of healthy plants became infected in plots with initial infector levels of 10, 20 and 50%, respectively (Haasis, 1939; Broadbent *et al.*, 1962). Improved stocks (called 'greenstocks') can be built-up through vigorous roguing and by selecting the largest bulbs (ADAS, 1978) or plants with the desired characteristics (Chen *et al.*, 1988).

Crop inspection

Where narcissus bulbs are to be sold for growing-on commercially, they may need to be inspected and certified by the appropriate plant health authority. In the UK, growing season inspections are carried out by PHSI to ensure freedom from stem nematode. Checking for freedom from other pests and diseases is the responsibility of the grower.

Operations between growing seasons

During the summer between the two growing years, crops may be re-ridged to maintain good conditions around the bulbs, to remove dried bulb foliage and to seal the soil surface. It is important that all crop foliage is removed and the soil surface is cultivated to close cracks, so that contact or translocated herbicides subsequently applied do not reach and damage the bulbs (de Rooy and Koster, 1978; ADAS, 1987). It is not clear whether there are implications of re-ridging for disease control: Millar (1978, 1979) reported that the effects of re-ridging on neck rot and smoulder were variable, and Melville (1980) reported that, although leaf debris is a prime source of infection by smoulder, burning debris did not control the disease. Waterlogged furrows could assist the spread of stem nematode, which can move up to 1 m in a year, so, where the furrows have been, or are liable to

become, waterlogged, it is useful to improve drainage by breaking up the soil with a single tine in early-August, when roots will not be damaged.

Bulb lifting

Lifting date

Traditionally, bulb crops were lifted, once, most (95%) of the foliage had died down, in July in the UK. This maximizes yield and ensures that bulbs are 'mature', with well developed outer skins, when lifted. One disadvantage is that bulbs then remain in the ground when soil temperatures are increasing, encouraging diseases such as base rot: infection occurs late in the growing season, when moribund roots are present (Hawker, 1935, 1943). McClellan (1952) reported that infection with the base rot pathogen was related to soil temperature, occurring only above 13 °C and reaching a maximum of 29 °C. Further, when bulbs are lifted at this time it may not be possible to dry, clean and grade bulbs and meet sales deadlines or produce bulbs for early forcing. Early lifting, overcomes these problems, and may be useful in pest avoidance: for example, the larvae of the large narcissus fly hatch and invade bulbs late in the growing season (June), while if foliage is allowed to senesce naturally, a three-fold increase in virus levels occurs (Mowat, 1980a). For these reasons, but mainly to have bulbs ready for the export market, it is usual in the UK to lift bulbs from early-June onwards, before foliage senescence is well advanced, which requires the foliage to be removed prior to lifting. When growing bulbs for processing, other considerations may apply, but bulb lifting is possible over a window of at least two months. If lifting is delayed when the soil is moist, there is a danger of bulbs re-rooting before lifting. Very late bulb lifting may also mean that HWT, if necessary, is given well after the ideal date.

When bulbs are lifted early, yields are reduced because photosynthesis and assimilation have been limited, and the choice of lifting date is a balance between an acceptable loss of yield and the advantages of early lifting described. After planting, total plant dry weight falls until March, and this is followed by a period of rapid weight gain. The curve of growth is sigmoidal, with the linear phase extending from late-April to early-June (in southern England) (Rees, 1972) (Figure 4.7). Yield losses are therefore severe if bulbs are harvested before June, as shown, for example, by the data of Allen (1938) and van der Weijden (1987). Very early lifting (in May) leads to reduced flower numbers (Allen, 1938; Rees and Hanks, 1984). Reporting a trial on the date of defoliation carried out with a number of cultivars, Kingdom (1981) stated that removing leaves two weeks after flowering was very detrimental to yield, after four weeks was adverse but not destructive in all cultivars, and after six weeks gave results comparable with those of intact controls. In any case, bulbs should be lifted early if they are known to be infested with stem nematode, so that they can receive HWT early, and such bulbs should not be allowed to dry out before HWT because of the likely formation of nematode 'wool'. Early lifting and HWT also improves the control of base rot.

Where it is desired to maximize yields, and pest and disease considerations are not significant, there may be advantages of delaying foliar senescence through the continued use of a fungicide sprays programme. The critical factors controlling senescence are not known, but it appears to be stimulated by high temperatures, even when cooler weather follows (Rees, 1972).

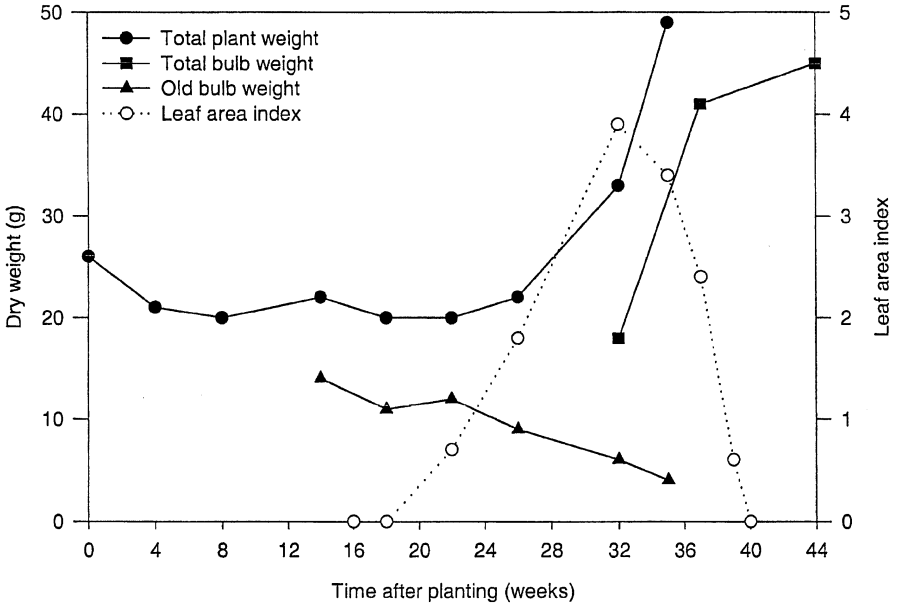

Figure 4.7 Changes in plant dry weight and leaf area index during the growing season Data for narcissus 'Golden Harvest' from Rees (1972), with permission o Academic Press Ltd.

Foliage removal

Where bulbs are lifted early, the green foliage is usually removed using acid or mechanically. Sulphuric acid (77%) can be applied as a crop spray by contractors, working to strict protocols. Crop foliage and weeds are desiccated quickly. There are no known harmful effects on subsequent crop performance. Suitable contact herbicides for desiccating narcissus, such as dinoseb, are no longer available. Mechanical methods of leaf removal ('flailing' or 'top bashing') remove the foliage at a greater or lesser distance above the bulb neck, cutting either above soil level or into the soil of the ridge tops (in which case less soil is lifted when harvesting), and depositing the excised foliage on the ground. Chain harrows may be used to remove foliage from the ridge tops, and this also help break clods before lifting. Linfield (1990) surveyed crop husbandry practices in relation to neck rot: the main correlation was that neck rot increased when foliage removal involved cutting into the ridge, close to the bulb neck, rather than cutting above the ridge, and when there was an interval of several days between flailing and lifting. Clearly, the presence of damaged shoot tissue contaminated with soil and debris might be expected to result in infection of the bulbs. It is common to remove foliage immediately in advance of the bulb lifter.

Bulb lifting

Bulbs may be harvested in two stages – in which case they are elevated to the surface and picked up manually later – or in one stage, using a 'complete harvester'.

Two-stage lifting requires considerable labor for picking up bulbs, and is used on small farms or specialist operations and where it is usual to leave bulbs in rows on the soil surface to dry naturally ('windrowing'), as is the traditional practice in south-west England. On small farms bulbs may be collected into trays, net bags, or bulk bins. One-stage lifters vary in complexity, from small tractor-mounted machines to large lifting machines which may be manned (for picking off clods by hand) or un-manned and which deposit bulbs in bulk bins or trailers (Figure 4.8). Specialised bulb lifters may be used, or machines designed for lifting potatoes or onions can be modified. Effective separation of bulbs and clods is the key element in bulb lifters. The machinery should be designed to minimize mechanical damage. Specialist machinery is available for lifting bulbs grown in beds on sandy soil.

Bulb handling for sale or re-planting

This phase includes any post-lifting fungicide treatment, drying, storage, cleaning, grading and inspection, as well as, for re-planting stocks, HWT and associated treatment. HWT was described above, under 'Pre-planting Bulb Treatments'. All these operations are key to the control of base rot in susceptible stocks. In trials with highly infested stocks, even when no fungicide was applied (at post-lifting or in HWT), the introduction of consistent optimum bulb drying and storage regimes reduced the level of base rot markedly (Hanks, 1992b, 1996b).

Ideally, diseased and damaged bulbs should be removed at all stages of bulb handling, although this is labor-intensive and impractical in most stages of handling in bulk. Diseased bulbs could include those with obvious rots as well as dry rotted bulbs (mummified bulbs and 'puffers'). There are no automated methods of detecting and removing diseased bulbs. However, 'floater-sinker'

Figure 4.8 Large unmanned bulb lifter discharging into bulk trailer (Photograph: Horticulture Research International).

methods have been tested, in which lighter, infected bulbs generally tend to float, albeit with some apparently healthy bulbs (Anon., 1980; Tompsett, 1976); because such a procedure could itself spread disease, if used HWT should follow promptly.

Bulb drying in the field

Bulbs may be elevated to the surface and left there to dry for several days. In suitable situations (in a dry, windy climate) the method is widely used and is successful, although there are several disadvantages. Drying is clearly dependent on weather, bulbs can be attacked by moulds or can re-root if conditions are damp, they are liable to sun scorch, and a reliable supply of labor is needed to pick up bulbs. When harvesting bulbs in this way, a post-lifting spray cannot conveniently be applied, although some growers are known to spray fungicide over the bulbs as they are lifted and deposited on the surface.

Post-lifting cleaning and fungicide application

Narcissus bulbs should progress rapidly from the field to drying. When bulbs are being handled in bulk containers (½- or 1-tonne bins), rapid transfer to the drying area is easy, but it does not allow soil removal, breaking up clumps of bulbs or an on-line fungicide application, although bulbs in bulk bins may conveniently receive an immediate dip treatment (in formaldehyde) before drying. When bulbs are harvested in loose bulk in trailers, they can be unloaded and passed along a series of lines, which could involve vibrating riddles and a rotating barrel riddle, for separation, soil removal and fungicide spray application *en route* to the store. Mechanised lifting can result in a high clod and stone content, and fluidised bed separators may be used to separate bulbs from soil (Zaltzman *et al.*, 1985). Some preliminary bulb grading may be needed at this stage in certain operations. Narcissus bulbs are not usually washed to remove soil, because of the danger of increasing bulb rots, but the method may be used where bulbs are lifted under wet conditions from 'sticky' soils.

In the case of cultivars susceptible to base rot, a prompt dip or spray treatment is highly beneficial. The benefits of a post-lifting cold dip in formaldehyde have been well demonstrated (e.g., Hawker, 1935; ADAS, 1973; Millar, 1978, 1979). A fungicide may be added to the formaldehyde in bulb dips. Benzimidazole fungicides (thiabendazole, benomyl) were investigated as bulb dips by Gould and Miller (1970, 1971a,b), replacing the mercurial fungicide used earlier and which could be phytotoxic (Gould *et al.*, 1961; Miller and Gould, 1967). A treatment of 1000ppm thiabendazole for 30 minutes at 25 °C one day after lifting was found to be very effective. Current recommendations in the UK are for a 15 minute dip in thiabendazole and formaldehyde at ambient temperatures, within a day of lifting bulbs. Dip treatments impose an extra burden on bulb drying and require the disposal of spent dips. Spray treatments are more economical, and several fungicides are effective used in this way (Hanks, 1994b), but the number of fungicides approved for such use is limited; in the UK thiabendazole is used. Thiabendazole sprays are usually applied via simple arrangements of conventional spray nozzles at a convenient point in the line, but ultrasonic and electrostatic sprayers have also been used effectively to give a more even or targeted spray (G.R. Hanks,

Figure 4.9 Letter box drying wall for bulbs in bulk bins. Air is blown through the slots in the wall into the pallet bases of the one tonne bins which are stacked against them (Photograph: Horticulture Research International).

unpublished data). While single treatments with thiabendazole can be highly effective, it is the cumulative effect of treatment over several years that most effectively reduces incidence of the disease, and if base rot is severe fungicide can be applied both post-lifting and in HWT (Hanks, 1992b, 1996b).

Bulb drying

Rapid and efficient bulb drying is essential for good bulb quality and especially for controlling base rot, since the pathogen does not spread effectively under dry conditions (Hawker, 1935, 1940). The method of bulb drying depends on the scale of the bulb-growing operation and on the temperature regime adopted for drying. On small farms, bulbs may be handled in small containers (e.g., bulb trays), in which case they may simply be stacked in a well ventilated or open shed, or even outdoors, allowing sufficient air spaces between stacks of containers; alternatively, to avoid problems due to unsuitable weather and to speed drying, the trays may be dried under ceiling fans in a shed or controlled temperature store. Loose bulbs can be elevated onto the floor of a bulk store fitted with air ducts for drying as used for grain or onions. Bulbs in bulk bins require a special drying facility, a 'letter box' drying wall (Figure 4.9). The bins have solid sides and a slatted pallet base, of which the fork-lift slots form the air duct for drying and ventilating bulbs via the slatted base of the bin. The bins are placed against slots in the drying wall located at appropriate positions to match the pallet bases of the bins, building up a line of bins of length and height appropriate to the facility, and closing the pallet bases at the outer ends of the rows with a temporary closer, usually a thick piece of foam rubber, so that the air flow from the letter boxes is forced upwards through

the boxes, exiting at the top of the stack. Small numbers of bins can be dried using portable fans blowing into the pallet base or using 'box tops' fitted with fans blowing downwards, or by placing bins over flow ducts in a bulk drier, but in either case an arrangement of foam rubber closers or polythene film fixed around the bins is needed to direct the air flow through the bulbs.

The forced air used to dry bulbs may be at ambient temperatures or it may be heated. A number of recommendations state that a lift of about 3 °C at temperatures of about 25 °C should be used (e.g., van Paridon, 1990), contrary to usual advice to growers in the UK (e.g., ADAS, 1988a). Price (1975a,b) showed that the incidence of rotting bulbs, and the numbers of propagules of the base rot pathogen isolated from the base plate of healthy bulbs, increased with increasing storage temperatures from 15 to 24 °C, then declining to 30 °C. In early studies of base rot, Gregory (1932) and Hawker (1935) showed that bulbs should be stored below 25 °C, while Xu *et al.* (1987) reported that the incidence of base rot was greater with temperatures >19 °C and Moore *et al.* (1979) stated that storage at 18 °C is a reasonably acceptable and practical recommendation. 'High temperature drying' of narcissus bulbs at 35 °C has been developed in the UK, and, as well as the convenience of rapid surface drying (in two to three days), it produces cleaner bulbs as the outer skins and soil contamination are more easily removed, and there is no increase in base rot due to the higher temperature (Tompsett, 1977). However, the safety of drying narcissus bulbs at 35 °C has been questioned by Linfield (1986b) on the basis of culture experiments with the base rot pathogen on solid and liquid media. On solid media, the fungus grew rapidly at temperatures of 20 or 25 °C, but growth was slower outside this range and had ceased at 40 °C, confirming the findings of McClellan (1952) that the optimum temperature for growth was 24 °C and that there was little growth at 35 °C. In liquid media, however, Linfield (1986b) found that growth of the pathogen was rapid over the range 15–35 °C, and had not ceased entirely even at 45 °C, and she argued that in a freshly-lifted bulb, conditions would be more like those of liquid culture, so that warm air drying would, initially, favour pathogen growth: the rate of moisture removal from the tissues would be more important than the drying temperature itself.

Bulb drying can be divided into first and second stages (Moore, 1980). Where high temperature drying at 35 °C is used, this is only for first-stage drying, and lower or ambient temperatures are used for second-stage drying. First-stage drying consists of the removal of surface water, and is essential for the control of surface moulds and other fungi. The rate of loss of surface water depends on the rate of air movement and its temperature, and high rates of air movement are necessary (425 m^3/h/t for bulbs in loose bulk, and up to three-times this, for bulbs in bulk bins to allow for leakage). With lower air flows, bulbs in the base of the stack dry faster than those at the top, subsequently leading to variations in bulb performance. Relative humidity should not exceed 75%. Second-stage drying extends to the removal of internal water and ensures bulbs are thoroughly dry; high rates of air movement are not needed (170 m^3/h/t for loose bulbs) and the humidity can be 80–85%. Higher ventilation and circulation rates are needed throughout storage for disease-prone cultivars such as 'Tête-à-Tête'. Robertson *et al.* (1980) developed a computer simulation of drying times based on air flows, temperature and bed depth, which was in reasonable agreement with experience in practice. Bulbs may lose 20–25% of their lifted weight during drying, cleaning,

etc., and it is important that drying does not continue to the point of excessive weight loss or desiccation.

Bulb storage

Once dry, bulbs are often stored at ambient temperatures in sheds, but it is preferable to store them at 17–18 °C: lower temperatures can slow development and can render the bulbs susceptible to HWT damage, while higher temperatures favour the development of base rot. The ambient summer temperature of the region needs to be considered in deciding whether controlled temperature storage is likely to be cost-effective. Bulb storage is in effect an extension of second-stage drying, and free movement of air around the bulbs is essential in controlling moulds and re-rooting. When bulbs are in trays or have already been transferred to net bags, no forced ventilation may be needed, but where they are held in bulk bins, continued use of fans will be needed, if only for part of the day. During this phase, bulbs can be extracted for cleaning, grading, etc.

Little information is available on the harmful effects of ethylene on narcissus bulbs. Hitchcock *et al.* (1932) reported that concentrations as low as 1.5 ppm (0.75 ppm for 'Paper White') retarded leaf and stem elongation, while 3 ppm or more caused a variety of leaf and bud distortions. Hydrogen fluoride can cause leaf scorch as an air pollutant (Spierings, 1969).

If bulbs are kept at high humidities above 30 °C, soft rot due to *Rhizopus* species can reduce narcissus bulbs to a musty mass. This can occur in the transit of bulbs or in propagation during the incubation of 'chips' (see below).

Cleaning, inspecting and grading

Once dry, bulbs are usually passed along a line involving the removal of loose skins and soil by vibrating riddles, brushes and dust extractors, bulb splitting and removal of damaged and diseased bulbs by hand, grading over a series of riddles, and collection and packing of different grades of bulbs. At all stages of bulb handling mechanical damage, which could lead to bruising, infection or poor appearance, should be minimised by reducing drops or cushioning surfaces. Trials with drops of 25 cm have shown that narcissus bulbs are susceptible to damage in the first 10 days after lifting and later, in autumn, and damage was increased by subsequently storing bulbs at ambient temperatures rather than at 17 °C (Schipper, 1971). Mechanical damage to the base plate can result in infection by the base rot fungus (Gregory, 1932; Hawker, 1935). Some studies, however, failed to show a correlation between simulated mechanical damage and the later development of bulb rots (Millar, 1978).

When grading narcissus bulbs, it is usual to bump bulbs along a series of slotted riddles of progressively larger sizes. Because narcissus bulbs are often asymmetrical or flattened on one side, round riddles (as used with tulip bulbs, for example) are considered unsuitable. Although other grades may be used, narcissus bulbs are often graded in 2 cm-wide bands, bulbs of 12–14 and 14–16 cm grades being used for sales and smaller and larger grades being used as re-planting stock (some cultivars have relatively small bulbs and other grades may apply). Machines for counting and weighing bulbs are available. Graded narcissus bulbs for sale are

usually collected in 25 kg lots in nylon mesh bags. After grading, bulb storage should continue under the conditions as before. It is usually advantageous to organize bulb handling operations so that re-planting stocks move quickly to HWT and planting, and bulbs for sales are separated and despatched promptly. Where bulbs are being sold for growing on, inspection and certification may be needed from the appropriate plant health authority. For example, in the UK samples of bulbs are subjected to dry bulb inspection by the Plant Health and Seeds Inspectorate to ensure freedom from soil.

In the case of narcissus bulbs being grown for processing, many of these steps – which are aimed at producing visually attractive, healthy flowering bulbs for the ornamentals trade – may not be applicable.

Long-term bulb storage

In commercial floriculture, long-term storage methods have been developed for narcissus bulbs to facilitate transport to the southern hemisphere or to produce very late flowers. Both warm and cool storage methods have been used (Beijer, 1957). Where bulbs are being supplied for processing, longer-than-usual storage may be necessary to suit production schedules.

In retarding narcissus bulbs by warm storage, Dutch bulbs were formerly stored at 28 °C and 70% relative humidity from lifting (in July) until shipping the following year and planting in South Africa in August (Hartsema and Blaauw, 1935). Long-term storage at extreme temperatures (–1.5 °C or 34 °C) was detrimental to the bulbs, but good results were obtained by storage at 25.5 to 31 °C followed by 10 weeks at 17 °C (Hartsema and Blaauw, 1935). Beijer (1957) obtained best results by storage at 30 °C from lifting to mid-October, followed by –0.5 °C until late-December then 25.5 °C until shipping in February-March and planting in April. For longer storage, 25.5 °C was used from late-November (Beijer, 1938). Warm storage can be prolonged for a year, making all-year-round flowering possible. This has been investigated with several standard cultivars (ADAS, 1970, 1989a; Tompsett, 1988). Bulbs were stored for several months from lifting at 26 °C and 70% relative humidity, then at 17 °C for 4 weeks before being planted and placed at 9 °C for 6 weeks; after this they were transferred to cool growing conditions. The normal cold requirement did not seem to apply, but the 9 °C period promoted root growth. The required flowering dates could be attained by 'holding back' the plants at 2–5 °C as necessary.

Long-term storage of narcissus bulbs at low temperatures was investigated by Griffiths (1936). Bulbs were stored at 1 °C from November for up to 10 months. Vegetative growth was satisfactory, although the flower buds had died, indicating that prolonged cold storage for non-cropping purposes should be satisfactory. Beijer (1957) reported that bulbs could be shipped in September following storage at 20 °C, then planted in November at 5 °C for 1 month and then at 0 °C until as long as July, flowering satisfactorily. Alternatively, bulbs can be frozen at –1.5 °C over winter and spring, planted and then stored at 7 °C for 3 weeks followed by 9 °C for 2 weeks and then 1 °C as required, flowering thereafter taking place in 4–6 weeks (ADAS, 1989a).

Transient exposure to sub-zero temperatures, during growth or after harvest, damages many flower-bulbs. Cohen *et al.* (1997) investigated the hardening of bulbs

of *Narcissus tazetta* 'Ziva' to freezing stress. Acclimatisation of bulbs by hardening at 2 °C was unsuccessful, but a single soil drench with paclobutrazol or uniconazole produced daughter bulbs that were not injured by freezing at –2 °C for 12 hours.

Transport

For shipping bulbs, well-ventilated refrigerated containers (reefers) should be used, preventing losses due to 'heating in transit' observed in earlier years. On receipt, bulbs should be stored in well-ventilated, ethylene-free stores at 13–17 °C (De Hertogh, 1989). De Hertogh *et al.* (1978) investigated hypobaric storage as an aid to shipping. Bulbs were stored for 2 weeks at 76 mm Hg and 17 °C prior to cooling: low pressure storage retarded bud growth, but eventual flowering was not affected, compared with control bulbs stored under ambient conditions.

SPECIALIST TYPES OF *NARCISSUS* BULB PRODUCTION

As well as the 'standard' narcissus and daffodil cultivars, other types of narcissus may be required, needing different growing techniques. These include Tazetta narcissus, dwarf and small-bulbed cultivars and *Narcissus* species. Integrated crop management and organic systems of growing are also considered.

Production of Tazetta *Narcissus*

Tazetta narcissus require a frost-free climate for natural-season growing. The main producing country for Tazetta bulbs is Israel (Yahel and Sandler, 1986; van der Weijden, 1988). Here, bulbs are planted in October, lifted in June and stored at ambient temperatures (25–30 °C), which retard the bulbs naturally until temperatures fall low enough to allow growth. Floral initiation takes place after lifting, in July/August, and anthesis occurs before winter if other conditions are favourable. The bulbs can be retarded by storage at 30 °C, producing late flowers in April or May (Yahel and Sandler, 1986). In northern Europe, a suitable climate exists in the Isles of Scilly, where the Tazetta cultivar 'Grand Soleil d'Or' ('Sols') is an important crop, although bulb production here is secondary to the production of the early, fragrant flowers (Veldt, 1988; Schaap, 1989). Cultural methods are usually adapted from standard practices for the region (ADAS, 1970), but are largely influenced by the local requirement to extend the flower cropping season and to maximize flower, rather than bulb, yields. These procedures include leaving crops down for several years (Tompsett, 1980b), early lifting and heat treatment (Rees and Goodway, 1970), burning-over using a tractor-mounted propane burner, and covering the crop with polythene film (Tompsett, 1980b, 1985). Similar responses have been reported for other Tazetta cultivars, including 'Paperwhites' (Imanishi, 1983; Tompsett, 1985). Although bulb production from Israeli bulbs in the Isles of Scilly is satisfactory in the first year, growth in the second year is poor, because higher temperatures are needed (Vreeburg and Korsuize, 1989). In unsuitable climates, or to enhance growth, Tazetta narcissus can be grown under protection (Kim and Lee, 1982). As well as being unusual in not having a cold requirement, narcissus of the Tazetta group characteristically

respond to ethylene or smoke treatments with faster or better flowering (Imanishi, 1983; Imanishi and Ohbiki, 1986).

Tazetta bulbs can be converted to summer crops to provide a means of bulb production where they are not naturally hardy (Tompsett, 1988; ADAS, 1989a). This involves warm-storage techniques for retarding flowering: bulbs were stored at 30 °C over winter and then for 4 weeks at 25 °C before planting outdoors in March, producing satisfactory yields in south-west England when bulbs were lifted in late-October. The technique was also used successfully in eastern England (G.R. Hanks, unpublished data). In the Netherlands, bulbs imported from Israel were stored at 30 °C from receipt until planting in spring. The best bulb yields were obtained when bulbs were planted in April to May and harvested in late-October (van der Weijden, 1988; Vreeburg and Korsuize, 1989; Vreeburg and Dop, 1990). Bulbs could be stored at 2 °C instead of 30 °C, but prolonged cold storage resulted in damage to the leaves.

While Tazetta narcissus are resistant to base rot, serious losses have been reported in Israel due to the nematode *Aphelenchoides subtenuis* (Mor and Spiegel, 1993). The nematode infects the roots and secondary infections of fungi such as *Fusarium* cause bulb rotting, giving the syndrome the name 'basal plate disease'. In the Isles of Scilly the nematode *Pratylenchus penetrans* can attack narcissus bulbs, leading to a 'root rot' in conjunction with the fungus *Nectria radicicola*.

Production of dwarf and small-bulbed cultivars and *Narcissus* species

The importance of dwarf and small-bulbed narcissus, such as Cyclamineus, Jonquilla and Triandrus types, was referred to in the section on production statistics. Many of these types have small bulbs, requiring the use of sandy soils to facilitate bulb lifting, and requiring more labor-intensive bulb handling generally. It may be appropriate to modify equipment designed for handling small bulbs like freesias or onion sets. Many are relatively 'delicate' or, like 'Tête-à-Tête', are prone to diseases such as *Penicillium* rots, skin diseases or smoulder (van der Weijden, 1989). The production of these types therefore requires extra care in pesticide use and in drying and storing bulbs, and examples have already been cited in the section on the production of standard narcissus bulbs.

There is very little commercial production of *Narcissus* species and it is limited to specialist nurseries, but bulbs of many species have been exported from Mediterranean countries, especially Portugal (Oldfield, 1989). Several *Narcissus* species are considered to be under threat as a result of over-collecting or loss of habitats (Koopowitz and Kaye, 1990). Commercial bulb companies are now very aware of the environmental implications of trading wild-collected bulbs, and, because of consumer interest in these attractive species, there would be scope for commercial production if appropriate, sustainable farming methods and stocks were available (Hanks and Mathew, 1997).

Integrated crop management and organic production

At the present time the demand for more 'environmentally friendly' or 'organic' production of ornamental crops is in its infancy, but is probably inevitable that it will increase, following trends in food crops, particularly as multiple retailers

promote more 'sympathetic' production protocols. This might also apply to plants being grown for processing for the production of pharmaceuticals, where it may be desirable to exclude pesticides for various reasons. As well as environmental reasons for encouraging less reliance on pesticides, there is the practical situation that relatively few pesticides are approved for use on horticultural crops: sales of pesticides, other than for use on major crops, are unlikely to justify the development and registration costs, and the chemical armoury of the grower of horticultural crops has decreased in recent years and may decrease further. In the Netherlands, the major bulb-growing country, the intensity of horticultural production, high use of pesticides and fertilisers to reduce losses due to pests and diseases and to increase yields, and the vulnerability of the water table and water courses have led to major restrictions on the use of agrochemicals in the bulbs industry. Raven and Stokkers (1992) and Stokkers (1992) summarised this situation, reporting that 10% of the use of pesticides in the Netherlands was used in the production of flower-bulbs (a 12-fold higher input than the average input per ha), and listed the objectives of the Dutch 'Multi-year Crop Protection Plan' (Anon., 1990). There is a need to develop integrated crop management (ICM) for narcissus crops. The setting up of experimental farms to test prototype ICM systems for bulbs was described by Raven and Stokkers (1992) and De Vroomen and Stokkers (1997). De Ruijter and Jansma (1994) described a model which optimizes production as regards environmental goals (nitrogen residues and pesticide inputs) and financial goals (income) for crops including narcissus, while Rossing *et al.* (1997) explored the options for environmentally friendly flower-bulb production systems and the prospects for pest and disease control in bulb crops in a world less dependent on agrochemicals was reviewed by Van Aartrijk (1997). A recent review of three systems for growing narcissus in the Netherlands – integrated, experimental integrated and biological – suggested there were good prospects for the integrated system (Wondergem *et al.*, 1999).

Up to now, little research has been conducted specifically on more environmentally friendly narcissus bulb production, although several possibilities are evident from previous R&D (Hanks, 1995b). These include physical, cultural and biological methods. Thus, in handling narcissus bulbs, emphasis has been laid on rapid drying and correct storage to reduce the levels of base rot (Hanks, 1992b, 1996b). Cultural methods of base rot control would include early lifting and late planting to avoid high summer soil temperatures, and there is also scope for using mulches or controlled weed growth to reduce soil temperatures, conserve water and reduce the reliance on herbicides. Koster *et al.* (1997) reported trials on the development of low-dose herbicide treatments for bulbs, involving leaving straw mulches in place to prevent weed germination, covering the soil with intercrops between bulb crops, and optimising the use of mechanical weed control, which might involve changes to bulb planting systems. In trials to control the nematode *Pratylenchus penetrans*, flooding was an effective alternative to soil sterilisation (van Beers, 1990). On a small scale, solar sterilisation may be useful (Higgins, 1999). The biological control of the base rot pathogen by antagonistic fungi has been reported by Langerak (1977), Beale and Pitt (1990, 1995), Hiltunen *et al.* (1995) and Hanks and Linfield (1997). Non-pathogenic micro-organisms (*Penicillium* species, *Trichoderma* species, *Minimedusa polyspora* and a *Streptomyces* species) inhibited pathogen growth, reduced disease development, or improved the effects of

using thiabendazole alone. Nematode levels in soils can be reduced by growing *Tagetes* and other species. In experiments in soils inoculated with *Pratylenchus penetrans* or Trichodorid nematodes, the population of *P. penetrans* was reduced by planting *Tagetes patula*, and bulb yield in a subsequent narcissus crop was increased (Conijn, 1994).

BREEDING AND PROPAGATION

Breeding *Narcissus* cultivars

Fernandes (1967) defined two sub-genera within *Narcissus, Hermione* with a base haploid number of chromosomes of 5 (or 10 or 11), and *Narcissus* with 7 (or 13), and crosses between the sub-genera result in a range of chromosome numbers. Brandham (1986, 1992; see also Kington, 1998, p.12) and Brandham and Kirton (1987) have made extensive studies of the cytogenetics of narcissus: diploid, triploid and tetraploid cultivars are common, with high ploidy levels in some species (e.g., *N. bulbocodium* is hexaploid). Brandham (1992) tabulated data for 731 cultivars. Most narcissus cultivars, interpreted as the optimum level of horticultural fitness, were tetraploids (2n = 28) (Brandham and West, 1993). Other cytogenetic studies of narcissus include those of Kalihaloo (1987), Kalihaloo and Koul (1989) and Gonzalez-Aguilera *et al.* (1988).

The bulk of narcissus breeding has been carried out by enthusiasts with the show-bench in mind, for example, in Northern Ireland, the USA, New Zealand and Australia. Commercial bulb growers identify new cultivars that may have the right characteristics for commercial exploitation, such as high rates of bulb and flower production. De Hertogh and Kamp (1986) and De Hertogh (1990) listed the desirable characters for commercial cultivars, such as sturdy stems and leaves, reliable bud opening, long-lasting flowers, fragrance, tolerance or resistance to diseases, and critical weights for floral initiation such that a double-nosed bulb will reliably produce two flowers. In some cases, breeding programs may have more specific aims. In the UK, there have been breeding programs aimed at producing yellow trumpet and large-cup flowers similar to 'Golden Harvest' or 'Carlton' but with relative resistance to base rot derived from 'St. Keverne'. One programme, concentrating on the production of early field-grown flowers, has already resulted in several cultivars being commercialised (Pollock, 1989). In another programme investigating the genetic basis of resistance to base rot (Bowes, 1992), new cultivars are presently under evaluation (Bowes *et al.*, 1996). The latter programme exploits the absolute resistance to base rot found in species such as *Narcissus jonquilla* (Linfield, 1986a, 1990, 1992a,b), rather than the relative (or field) resistance of commercial cultivars such as 'St. Keverne' (Tompsett, 1986). Breeding for resistance to base rot is hampered by the difficulties in screening seedling bulbs because of the development of adult plant resistance (Linfield and Price, 1986). Breeding for resistance to base rot currently utilizes a lengthy screening method that requires large numbers of clonal two-year-old bulbs (Bowes *et al.*, 1992). An *in vitro* assay using bulb scales is showing promise as a much faster alternative (J.H. Carder, personal communication). Narcissus pollen can be stored long-term in liquid nitrogen (Bowes, 1990).

Mutation breeding of narcissus was reported by Misra (1990). Two narcissus cultivars flowered early without leaves, after exposure to gamma radiation. Rahi *et al.* (1998) carried out experiments with *N. tazetta* 'Paper White' in which bulbs were exposed to gamma radiation and then planted in normal or alkaline soil. The performance of irradiated plants in the alkaline soil indicated possibilities for selecting salt-resistant strains.

D.O. Sage (personal communication) is developing a transformation system for narcissus as a possible route to cultivar development. Transgenic callus of narcissus 'Golden Harvest' has been produced, carrying a selectable marker and reporter gene, and attempts are being made to regenerate plants from it. Work will then concentrate on 'clean' transformation technologies for producing transgenic narcissus ultimately without selectable marker and reporter genes. The technology should then be able to approach pest and disease control by introducing resistance genes to otherwise acceptable cultivars.

Future uses of narcissus plants may require breeding for characteristics such as alkaloid or essential oil content, which have not apparently so far been attempted. Whatever the goals of narcissus breeding, there is a need to conserve genetic material for future use. Because of the huge numbers of commercial cultivars there is a danger that historical but valuable parent cultivars may be lost, while the loss of wild species (and potentially useful subspecific taxa) through over-collecting or habitat destruction has already begun (see Chapter 3, this volume). Historic cultivars and wild types need to be conserved. Koopowitz (1986) has discussed the wider implications of conserving amaryllids, including the need for a large number of each to represent the variation of the gene pool meaningfully, long generation times, specialised cultural requirements and the widespread occurrence of virus diseases.

As well as improving narcissus cultivars, narcissus genes may be useful in the production of other transgenic plants. Booth (1957, 1963) studied carotenoids in narcissus, finding the coronas to be among the richest sources of carotene. Rice contains neither β-carotene (provitamin A) nor its precursors, and Burkhardt *et al.* (1997) transformed rice by microprojectile bombardment with a cDNA coding for phytoene synthesis from narcissus.

Narcissus propagation

A major problem with commercial narcissus breeding is the long time – 15–20 years – needed to build up adequate stocks beginning with one bulb and using natural multiplication, about 1.6-fold per annum, by which it takes about 16 years to go from one to 1000 bulbs (Rees, 1969). Micropropagation is almost certain to be required, perhaps followed by a low-cost macro-propagation method, such as chipping combined with optimised field production, once reasonable numbers of bulbs have been produced (Hanks and Rees, 1979). Propagation from seed is also considered.

Micropropagation

Whereas macropropagation techniques such as chipping are simple and require minimal facilities, in many cases they will not produce the multiplication rates

required for the effective bulking of a stock in a reasonable period. Further, meristem-tip culture can be used to produce 'virus-free' plants (Stone, 1973; Phillips, 1990; Sochacki *et al.*, 1997).

Hussey (1975, 1980, 1982) reported the regeneration of plants using leaf, scale and stem explants and from callus derived from the ovary wall. Using scale tissue, usually double scale segments ('mini-chips'), shoots were produced on the abaxial surface close to the base plate, and the multicellular origins of such growth suggested that the progeny should be genetically uniform. Sub-culturing at 16-week intervals, 500–2000 bulblets could be produced from one parent bulb in 18 months. All shoots eventually became senescent and formed dormant bulblets. After a cold treatment of 8–10 weeks at 2–9 °C, bulblets sprouted and could be planted out, reaching flowering size in three or four years. Seabrook *et al.* (1976) and Seabrook and Cumming (1982) described shoot, root and callus induction on leaf base, stem and ovary segments. The most productive explants were the bases of young leaves from cold-treated bulbs, and from two leaf sections, 2620 shoots were obtained after five months and four sub-cultures (Seabrook *et al.*, 1976). Several cultivars were tested and were found to differ in vigor, but multiplication rates of up to 27-fold *per annum* were achieved. However, the plantlets transferred to soil with difficulty.

Squires and Langton (1990) carried out an evaluation of narcissus micropropagation on a commercial scale. Using an adaptation of Hussey's (1982) methods, shoots were obtained from 'mini-chips' and sub-cultured before senescence was induced by transfer to a hormone-free medium; after a cold treatment, bulblets were planted out. There were large differences between cultivars, but, on average, 7.6 shoots were obtained initially from each bulb, there was a multiplication rate of 1.8 per transfer, a rate of conversion to bulblets of 1.4, and 60% success in transplanting. From one parent bulb, starting in August and using only the inner scales (to reduce contamination), using nine transfers and planting out after 18 months for three or four years to produce flowering size bulbs, 1200 bulbs were produced, with a labor requirement of 2.6 man-minutes per bulb. Although the multiplication rate was lower than that of Seabrook *et al.* (1976), planting out bulblets rather than rooted shoots was more convenient, and a high degree of genetic stability was expected because the shoots produced had an origin similar to that of natural increase. Squires *et al.* (1991) investigated the causes of poor transplanting, and adjustments to the culture method improved the success of transplantation to 81% in some cultivars. The main constraints to better growth rates were the onset of leaf senescence both in culture and in the year after transplanting. Bulblet weights of 0.20 g were needed for reliable transplanting.

The micropropagation of narcissus was also investigated by Selby and co-workers (for review, see Harvey and Selby, 1997). In narcissus micropropagation, a large amorphous mass of achlorophyllous tissue develops at the base of the shoot-clump cultures and new leaves arise from it (Chow, 1990). Chow *et al.* (1993) and Harvey *et al.* (1994) compared the structure of the basal tissue of the shoot-clump cultures with the base plate of bulbs, and showed they were similar. Thus the leaves of such cultures do not appear to derive from callus and therefore should be true-to-type. Non-dormant bulbils that transferred to soil well could be produced from shoot clump cultures by using high sucrose levels in culture (Chow *et al.*, 1992a). Multiplication rates in culture could be increased by trimming all green

leaf tissue at alternate transfers (Chow *et al.*, 1992b). Staikidou *et al.* (1994) compared bulbil formation by single leaf explants and by shoot clump cultures. Bulbil formation on the former was slow and inefficient compared with using shoot clump cultures, but was responsive to growth regulators and so might be a useful, simple system for investigating the regulation of narcissus bulbil initiation and development.

Other reports on the micropropagation of narcissus include those of Popov and Cherkasov (1984), Paek *et al.* (1987), Kozak (1991), Langens-Gerrits and Nashimoto (1997) and Sochacki *et al.* (1997), who used various explants including those consisting of scale bases with attached base plate tissue. Infection of cultures with *Fusarium* is a problem in the tissue culture of narcissus, and Hol and van der Linde (1989) demonstrated that infection could be controlled by storing bulbs at 30 °C from lifting and giving bulb HWT at 54 °C for 1 hour before setting up cultures. Riera *et al.* (1993) reported the beneficial effect of the polyamine diaminopropane on regeneration from twin-scales in culture. Langens-Gerrits and Nashimoto (1997) demonstrated that the presence of roots on bulblets was needed to obtain good subsequent growth when they were planted out. Joung *et al.* (1997) reported regeneration from different parts of the flower stem. Santos *et al.* (1998) reported tissue culture starting with twin-scale explants of *N. bulbocodium*, in which 90% of the bulbs obtained flowered during the first growing season. There are some advantages in using a shaken liquid culture over using solid agar media. Bergoñón *et al.* (1992) described shake cultures of *N. papyraceus* (see also Chapter 7, this volume). In other tissue culture investigations with narcissus, ovule culture produced viable seeds (Lu *et al.*, 1988), as did excised placentae pollinated *in vitro* (Balatková *et al.*, 1977).

For Tazetta cultivars, successful induction of plantlets has also been reported using young stem explants (Hosoki and Asahira, 1980), stem and scale explants (Nagai, 1999a,b) and cultured twin-scale bases (Dachs *et al.*, 1979; Steinitz and Yahel, 1982; Gu and Gao, 1987), and Li and Tang (1982) reported callus induction on twin-scale segments. In the case of cultivar 'Grand Soleil d'Or', under the most appropriate conditions 80–100 explants were obtained from each parent bulb, giving 200–300 bulblets in 6 months, and 80–90% of bulblets weighing over 0.25 g with two leaves and abundant roots were transplanted successfully (Dachs *et al.*, 1979; Steinitz and Yahel, 1982). Gu and Zhang (1991) compared production from micro-propagated Tazetta narcissus derived from callus cultures with naturally produced bulblets. Micropropagated plants flowered after 4 to 5 years, and there were no differences between them or naturally derived plants in the number of flowers or in flower morphology.

Sage *et al.* (2000) produced somatic embryos from narcissus cultivars. Somatic embryos were obtained from leaf lamina, leaf base, bulb scale and, especially, stem explants. Leaf explants from shoot cultures produced somatic embryos and converted to plantlets efficiently on an appropriate medium following a cold treatment, the plantlets subsequently transferring to *ex vitro* conditions readily.

Chipping, twin-scaling and related propagation techniques

Several simple propagation methods suitable for on-farm use have been investigated for the multiplication of choice cultivars and VT stocks of narcissus. Scooping

and cross-cutting, standard techniques used with hyacinth bulbs, gave poor results with narcissus (Stone, 1973; Stone *et al.*, 1975), and leaf cuttings were unsuccessful (Alkema, 1971b). Twin-scaling and chipping were, however, successfully adapted to narcissus from methods used with other Amaryllidaceae in which the bulb is cut into a number of segments and the segments divided into single or paired scale pieces each with a piece of the base plate attached (Luyten, 1935; Traub, 1935; Everett, 1954). Only a small percentage of single scale pieces, but over 80% of paired scales, produced bulblets (Alkema, 1970, 1971a, 1975; Broertjes and Alkema, 1971). These bulb division methods rely on the destruction of the apical dominance of the existing buds, resulting in adventitious bud formation on the proximal part of the bulb scale adjacent to the base plate (Hussey, 1975; Grootaarts *et al.*, 1981).

In 'twin-scaling', flowering-size bulbs are cut into a number of longitudinal segments (often 8 or 16), each with a wedge-shaped piece of base plate; these segments are further divided by cutting off the scales in pairs, each with a conjoining piece of base plate, and 60 to 100 twin-scales can be cut from one large bulb. These pieces are incubated in a moist medium (usually damp vermiculite) for 3 months at 20 °C, when bulblets develop, usually one per 'twin-scale' (Alkema, 1970, 1975; Alkema and van Leeuwen, 1977). Typically, 80–90% of twin-scales from bulblets, and a small percentage rots. When grown-on, the bulblets flower in their third or fourth year (Mowat, 1980b). This method was used to multiply VT narcissus stocks in Scotland (Sutton and Wilson, 1987). Testing a wide range of cultivars, Fry (1978) found that one bulb could be multiplied to between seven and 41 flowering-size bulbs in 4 years, compared with six by natural increase. Practical accounts of twin-scaling include those of Flint and Hanks (1982) and Hanks and Phillips (1982).

While twin-scaling is a simple technique, it is time-consuming and the propagules are small and delicate. More robust propagules can be obtained by only partly dividing the original bulb segments, giving pieces with a few scales each (Everett, 1954; Stone, 1973), or by leaving the original 8–16 segments intact, the method known as 'chipping' (Flint, 1982) (Figure 4.10). Several practical accounts of chipping are available (e.g., Flint, 1984; Vreeburg, 1984a, 1986; ADAS, 1985c; Hanks, 1989). As well as being used for on-farm multiplication of select stocks and cultivars, chipping produces attractive round bulbs ideal for sale in 'pre-packs' and with potential for improving predictability, uniformity and mechanised handling (Vreeburg and van der Weijden, 1987a; Hanks, 1989). The method has been used extensively with the popular dwarf cultivar 'Tête-à-Tête' (Vreeburg and van der Weijden, 1987a), where 14 to 71% of bulblets flowered in their second year, and it is also successful with several *Narcissus* species (Hanks, 1987). Although earlier projections of multiplication rates now appear unduly optimistic, multiplication rates of 3- or 4-fold per annum (6.5 or 5 years from one to 1000 bulbs) seem realistic (Hanks and Rees, 1979; Hanks, 1993).

The factors affecting the productivity of twin-scaling and chipping have been reviewed by Hanks and Rees (1979) and Hanks (1993). In the following account, only the more important effects will be mentioned. It is generally recommended that bulbs for chipping should receive HWT about a week before cutting, which improves results and controls bulb scale mites (Vreeburg and van der Weijden, 1987a), but HWT immediately before chipping is harmful. Although twin-scales

Figure 4.10 Narcissus 'chips' with bulblets at the end of incubation. (Photograph: Horticulture Research International).

may form bulbils when cut at any time of the year, subsequent growth is best when propagation takes place using 'dormant' bulbs in July or August (Alkema and van Leeuwen, 1977; Hanks and Rees, 1978; Vreeburg and van der Weijden, 1987a), and this also gives propagules that are ready for planting-out at a reasonably convenient time of year. In earlier work, emphasis was on cutting many small twin-scales or chips per bulb in order to maximize multiplication, for example aiming for twin-scales weighing 0.5–0.8 g (Mowat and Chambers, 1975) or cutting 16 chips per bulb (ADAS, 1985c). However, small propagules take four or more years to produce bulbs of flowering size, and recent recommendations have been more pragmatic, sacrificing numbers for bulbil size and a quicker production of flowering-size bulbs: bulbs of 10–12 cm circumference (weighing about 40 g) are cut into eight segments only (Vreeburg and van der Weijden, 1987a; Hanks, 1989). Cutting rates can be adjusted to initial bulb size to achieve target bulb weights after a year, after which bulb rate increase is independent of cutting method (Fenlon *et al.*, 1990). While cutting bulbs by hand is the only possibility in twin-scaling, chipping machines are available which can increase throughput to about 0.5 t/day (Flint *et al.*, 1984; Zandbergen, 1984). Chipping machines are based on either a star-shaped blade operated by a pneumatic plunger or on arrangements of circular saw blades fed with bulbs or bulb halves on a conveyor. To avoid spreading pests and disease between bulbs, and to minimize other contamination, sensible hygiene should be observed when twin-scaling or chipping bulbs, including disinfecting blades (Vreeburg and van der Weijden, 1987a). Immediately following cutting, twin-scales or chips are treated by soaking in a fungicide, effective materials including products based on captan and benomyl, often used in combination (Vreeburg and van der Weijden, 1987a; Hanks, 1989; Linfield and Price, 1990).

In practice, moulds associated with bulbs may be difficult to control during incubation of the propagules, perhaps because of the presence of fungicide-resistant strains (Lyon, 1978) or due to using grossly infected bulb stocks (Hanks, 1989). Bulbil production on twin-scales is inherently variable because of the different properties of bulb scales from different parts of the bulb (Hanks, 1985).

Chips or twin-scales are usually incubated after cutting, usually by mixing with damp vermiculite and holding in trays at 20 °C for 12 weeks (Vreeburg and van der Weijden, 1987b; Hanks, 1989). These conditions are ideal for fungal growth as well as bulbil production. Attempts to scale up the treatment of machine-cut chips by using deep crates, or by omitting the medium and controlling humidity, gave promising results (G.R. Hanks, unpublished data). In the period immediately after cutting, it is important to control chip temperatures which increase as a result of wound respiration and the production of heat by the damped vermiculite (Hanks, 1989), although the exact temperature and duration of incubation is not critical (Hanks, 1986). By the end of incubation, the bulb scales should be largely depleted of reserves: bulblets over 10 mm in length grow well, but smaller ones often remain dormant (Hanks and Rees, 1979). The propagules are planted in the field following the gentle removal of the vermiculite, and in some cases after a pre-planting fungicide dip (Vreeburg and van der Weijden, 1987c). In the case of twin-scales, better growth has been reported following planting in a frost-free glasshouse than in an unheated gauze house (Mowat and Chambers, 1977). As an alternative to incubating chips, they may be planted in the field directly after cutting and fungicide treatment, but chipping should take place in July so that bulbils are produced before soil temperatures become sub-optimal, or a polythene mulch may be used to raise soil temperatures from planting to emergence (ADAS, 1987; Vreeburg and van der Weijden, 1987b).

In the field, plant growth should be maximised through careful husbandry, for example controlling weeds by using non-damaging herbicides or a straw mulch, and having a prolonged fungicide spray programme, and by using a low planting density (Vreeburg and van der Weijden, 1987c; Hanks, 1989). Planting densities quoted include 0.5 million chips per ha or 1–5 t original bulb weight/ha.

These methods would be useful in bulking supplies of bulbs for extraction, perhaps following an initial phase of micropropagation. Chipping is likely to be more practical than twin-scaling, as chips are robust, may be cut by machine, and are more uniform.

Optimised production in the field

Standard bulb production methods are designed to give the most cost-effective production of bulbs and (or) flowers, but in the case of valuable bulb stocks it may be more important to maximize bulb yields. This can be achieved by greatly reducing planting density and by adopting one-year-down growing. Planting density should be reduced so that inter-plant competition is minimised, provided there is no wind damage. In a trial of optimised bulb production, low planting densities and annual planting and lifting were effective, but there was little additional benefit of using combinations of foliar feeding, top-dressing, de-heading, irrigation or fungicidal sprays (ADAS, 1982c). However, all these methods might be considered beneficial in specific circumstances where it is desired to maximize bulb yields.

Production from seed

Production from seed is, of course, the method used by breeders, and is also important for species that form few offsets. Narcissus breeders may sow seed soon after collection, either outdoors or in a cold-frame, and germination occurs somewhat unevenly in spring. Caldwell and Wallace (1955) reported that, under natural conditions, seed of *Narcissus pseudonarcissus* germinated naturally in November or December, while Wells (1989) stated that, when seed is sown outdoors in early May soon after collection, *N. bulbocodium* types start to germinate in late-August and germination continues through the winter, with *N. pseudonarcissus* offspring perhaps not germinating until early spring. Under natural conditions, summer drought may induce dormancy in *N. pseudonarcissus* (Barkham, 1980). Rees (1972) stated that narcissus seeds have a cold requirement of many weeks, and Linfield and Price (1986) germinated seed of commercial cultivars using a cold treatment of 12 weeks at 12 °C. Thompson (1977) reported that, in *N. bulbocodium*, conditioning imbibed seed for 7 weeks at 26 °C (but not at 6 or 16 °C) gave rapid germination when subsequently moved to 5–16 °C. This suggested that seed should be sown in a warm glasshouse and moved to cooler conditions after 2 months. Hanks and Mathew (1997) reported that synchronous germination could be obtained in *N. cyclamineus, N. bulbocodium* var. *citrinus* and *N. pseudonarcissus* by keeping imbibed seed at 25–30 °C for 8–12 weeks then transferring to 15 °C.

There is little information on other aspects of the seed physiology of narcissus, although Caldwell and Wallace (1955) reported that seeds of *N. pseudonarcissus* were not light sensitive. Hanks and Mathew (1997) reported that controlling seed-borne fungi on *N. cyclamineus* by disinfection, HWT, etc., was difficult. Under optimum conditions, it takes 3 years for seed-raised plants of species such as *N. bulbocodium* and *N. triandrus* to reach flowering size, 4–5 years in other species and 7–8 years in some cultivars (Koopowitz, 1986; Oldfield, 1989). This long period needed to produce saleable bulbs is a strong disincentive to commercial production. Germination and growing systems for the commercial production of *Narcissus* species were investigated by Hanks and Mathew (1997), who showed that seedlings could be successful raised in cellular trays for sale as 'plug plants' or for growing on, a method useful for species like *N. bulbocodium* that reach flowering size quickly.

CONCLUSIONS

This chapter demonstrates that the methods for growing and handling narcissus bulbs are well documented, but that there remains considerable scope for changes in husbandry and other practices that might make the crop more cost-effectively produced when growing for processing, or which would enable it to be grown in an more environmentally friendly way. Some major research needs are evident, such as the need for cultivars resistant to base rot and virus diseases (or their vectors), for safer and (or) more effective chemicals (e.g., for bulb disinfection and the control of large narcissus fly), and for the development of mechanical or robotic bulb handling and sorting to reduce labour inputs. The transformation of narcissus bulb production seen in the UK in recent decades has demonstrated

that, with enterprise, much can be achieved, even in the face of sometimes adverse economic circumstances.

The future needs for growing narcissus bulbs for pharmaceutical and other processing purposes are uncertain. In 1995, predictions of a vast expansion of the UK narcissus area in order to produce bulbs for galanthamine extraction caused a stir in the industry (Anon., 1995; Long, 1996). At present there may be options both for the extraction of galanthamine from bulbs, and for utilising new developments in the synthesis of the compound. But the array of alkaloids present in narcissus, and the wide range of potential uses which they have, suggests there will be a need for growing narcissus bulbs as an industrial crop for many years to come.

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5 Economics of *Narcissus* bulb production

James B. Briggs

INTRODUCTION

A variety of influences determines the crops produced on any farm, and physical factors such as poor soil or difficult drainage exclude growing *Narcissus* on many farms. However, where soils and rotational requirements are favourable, the potential financial returns from narcissus bulb production can be evaluated to see whether it could be a feasible and economic enterprise. It is necessary to examine the profitability of bulb growing relative to other typical enterprises on similar land types. A farmer is only likely to enter into a contract for growing a particular crop where there is a financial incentive, or if there are perceived to be other advantages such as benefit to the rotation or spread of workload.

The expression of crop profitability in terms of 'gross margin per hectare' is widespread, and is effectively an indication of the output less the cost of variable inputs. As a rough guide, fixed costs on a typical UK arable farm split to one-third labour, one-third machinery and one-third other costs. The allocation of fixed costs to specific enterprises is more difficult, and is not widely adopted. If done at all, it will normally cover the two major elements of labour and machinery costs. Where specialist equipment is required, as for bulbs, it is not likely that this crop will be included in the rotation, as it will increase overhead costs significantly. If the equipment can be utilised for other crops, or if contractors can be used to carry out the work, or if equipment can be leased, then the bulb crop may be a realistic option.

TYPICAL GROSS MARGINS FOR *NARCISSUS* BULB PRODUCTION

Gross margins for narcissus growing are shown in Table 5.1, based on 1996 and 1999 costings for a typical 'two-year-down' narcissus enterprise in England (for background information see, for example, ADAS (1985a)). These examples refer to growing bulbs for the ornamentals industry and, in growing bulbs for processing, some circumstances may be different. This evaluation gives gross margins of £1922 and £1554 per hectare per annum for 1996 and 1999, respectively (in this narrative, the years quoted refer to the years crops were lifted). The following assumptions were made in these calculations:

1 15 t planting stock (bulbs of grade <10 cm and >16 cm) per hectare at £400/tonne. Output = 13.5 t/ha saleable bulb stock at £450/tonne. Initial stock

Table 5.1 Typical gross margin for growing *Narcissus* bulbs 'two-year-down' in the UK

	1996 (£/ha)		*1999* (£/ha)	
Output (year 2 only)				
Saleable and planting stock		12 750		12 075
Variable costs				
Year 1				
Bulbs	6000		6000	
Hot-water treatment	1088		1200	
Fertilisers	116		116	
Sprays (field)	380		374	
	7584		7690	
Year 2				
Fertilisers	20		20	
Sprays (field)	401		357	
Post-lifting spray	600		600	
Drying	300		300	
	1321	8905	1277	8967
		3845		3108
Gross margin per hectare per annum		1922		1554

prices would need to be used in the gross margin for the first cycle. Bulb prices rose sharply in the mid-1990s and saleable stock was realising £525–£550/tonne. There has, however, been a levelling out of bulb prices in the mid-1990s at £400–£450/tonne.

2 For the purposes of this exercise, it has been assumed that there were no flower sales. It is estimated that, in seven years out of ten, flower sales in the second year of 30 000 bunches/ha (ten stems per bunch) at 10 p/bunch (net of labour and marketing costs) could be achieved. On an average basis, this would add an additional £2100 to the two-year gross margin. This must be weighed against the potential loss of yield of saleable bulbs, which may be as much as 10–20%.

3 In the first year of planting, growers would probably have to buy planting stock at open market prices of £500/tonne, which would reduce the gross margin on the first crop by £1500. Once the system is established and planting stock is available from the harvested yield, the value of the stock is not important, as it is comprised of smaller bulbs that would not normally be marketed.

4 It has been assumed that bulbs will be harvested in July after natural senescence, so that chemical desiccation (using sulphuric acid) is not required.

Further details of the variable costs used in this exercise are given in Table 5.2. Bulb grading, if required, would add approximately £1000/ha to these costs. Marketing or packing costs, if applicable, would add £40 per tonne sold or £600/ha.

Most narcissus crops are grown on a 'two-year-down' basis in the UK (see also Chapter 4, this volume). The results of trials in Lincolnshire and Cornwall in the 1980s, comparing one- and two-year-down systems, showed that in the UK one-year-down growing was not economic. The introduction of a one-year-down

Table 5.2 Variable costs in growing *Narcissus* (see Table 5.1). All calculations are based on 1 ha of crop at 1999 values

1	Bulbs planted at 15 t/ha @ £400/tonne	
2	Hot-water treatment for 15 t bulbs @ £30.00/tonne + £50.00/tonne thiabendazole fungicide for control of basal rot	
3	Fertilisers: year 1	
	100 kg nitrogen @ £0.40/kg	40.00
	100 kg phosphate @ £0.43/kg	32.00
	200 kg potash @ £0.22/kg	44.00
		116.00
	Fertilisers: year 2	
	50 kg nitrogen @ £0.40/kg	20.00
4	Sprays: year 1	
	Iprodione fungicide (two sprays) 6 litres @ £16.40/litre	98.40
	Mancozeb fungicide 6 kg @ £3.30/kg	19.80
	Chlorothalonil fungicide 3 litres @ £5.50/litre	16.50
	Paraquat + diquat herbicide 8.5 litres @ £5.60/litre	47.60
	Chlorpropham + linuron herbicide 11.2 litres @ £8.80/litre	98.56
	Glyphosate herbicide 5 litres @ £3.52/litre	17.60
	Cyanazine herbicide 5.2 litres @ £14.60/litre	75.92
		374.38
	Sprays: year 2	
	Paraquat + diquat herbicide 8.5 litres @ £5.60/litre	47.60
	Chlorpropham + linuron herbicide 11.2 litres @ £8.80/litre	98.56
	Cyanazine herbicide 5.2 litres @ £14.60/litre	75.92
	Iprodione fungicide (two sprays) 6 litres @ £16.40/litre	98.40
	Chlorothalonil fungicide 3 litres @ £5.50/litre	16.50
	Mancozeb fungicide 6 kg @ £3.30/kg	19.80
		356.78
5	Post-lifting spray of thiabendazole fungicide, 30 t bulbs @ £21/tonne, for control of basal rot	
6	Bulb drying in store will be required at a fuel cost of approximately £10/tonne	

system should be considered only if rigorous disease control is necessary. To achieve gross margins from two, one-year-down crops in excess of the gross margin for a two-year-down system (with a 150% bulb weight increase), annual bulb weight increases of between 70 and 100% have to be obtained from a bulb planting rate of 25 t/ha (ADAS, 1984, 1985b, 1987a,b). This can be achieved commercially, but, given the need for consistently high yields, it would be difficult to do so. Further, in the one-year-down system all bulbs are lifted and re-planted each year, and the volume of bulbs handled each year is nearly doubled, creating logistical problems for most businesses. The use of the two-year-down growing system, therefore, became firmly established in the UK.

ACTUAL GROSS MARGINS

The figures given above represent 'typical' gross margins. Examples of actual gross margins recorded by two bulb growers in Lincolnshire, UK, are given in Table 5.3.

Table 5.3 Actual gross margins from two *Narcissus* growers

	Grower 1 (£/ha)	*Grower 2* (£/ha)
Output		
Bulbs sold	5910	3770
Bulbs re-planted	3087	4848
	8997	8618
Bulbs	3087	3158
Hot-water treatment	371[a]	440
Fertilisers	114	79
Crop sprays	408	361
Drying	173	190[b]
	4153	4228
Gross margin (£/ha)	4844	4390
Gross margin per year (£/ha)	2422	2195

Notes
[a]Grower 1, no figure given for hot-water treatment, so a typical figure for treatment without thiabendazole has been used for comparability.
[b]Grower 2, no figure given for bulb drying, so a typical figure has been used.

For Grower 1, the figures used are based on 1993, and, for Grower 2, 1995. The bulb yields for Grower 2 are particularly low, but 1995 was a dry year when bulb yield was severely reduced where irrigation was not available. It is obvious that both of these growers are operating under a lower input of pesticides than the one previously described, particularly in terms of using fungicide in hot-water treatment. However, more intensive systems to guarantee crop quality, particularly for control of basal rot, are becoming necessary, and the typical gross margin figures given above are based on such a system. The actual gross margins achieved in these examples were £2422 and £2195 per ha per annum, respectively.

THE RELATIVE PROFITABILITY OF *NARCISSUS* GROWING

Arable farming, either of combinable crops or mixed cropping, is currently carried out in most of the geographic areas suited to narcissus growing. The 1999 gross margins of the most common crops are given below, expressed as £/ha:

Winter wheat (feed)*	530 ± 100
Winter wheat (milling)*	600 ± 100
Winter oilseed rape*	450 ± 100
Winter barley (feed)*	480 ± 100
Spring barley (malting)*	460 ± 100
Winter beans*	450 ± 100
Linseed*	400 ± 100
Maincrop potatoes	2000 ± 1000
Sugar beet	900 ± 500
Bulb onions (spring-sown sets)	2090 ± 1500
Summer/autumn cauliflower	1800 ± 1300

These figures give some idea of the high variability in returns for some crops, particularly field vegetables and roots. Climatic factors, particularly rainfall, have a significant effect on market prices, notably for potatoes.

Crops marked with an asterisk in the above list are within the Integrated Administration and Control System (IACS) of agricultural support. IACS is the system for administering farm support payments within the EU. Included in this regime is the requirement for an area of arable land to be set-aside from food production, in order that support payments can be claimed on both that land and on land cropped with combinable crops such as wheat and oilseed rape. These support payments are known as Arable Area Payments, or simply area payments, and are one of the main pillars of support under the Common Agricultural Policy (CAP). The current level of gross margin is heavily reliant on these subsidies which, for the 1999 harvest (including an element of agromonetary compensation), were as follows (£/ha):

Cereals	235
Proteins	340
Oilseeds	254
Linseed	456

Obviously, changes in the level of support will alter the relative profitability of such crops. In order to be eligible for these payments, one of the requirements of the scheme is that, an area of land must be 'set-aside' and not used for food production, in return for which a subsidy of around £298/ha is payable.

FIXED COSTS

As mentioned previously, the allocation of fixed costs is not often carried out, yet different farming systems carry with them different fixed cost structures, particularly in the area of labour and machinery costings. Within each type of enterprise there will still be considerable variation due to differences in business circumstances and efficiency. The 'top 25% figures' are frequently presented to give an indication of the performance levels of the most efficient businesses. Table 5.4 (University of Nottingham Rural Business Research Unit, 1998) shows the actual results for 1996–1997 and 1997–1998 for costed farms, grouped by cropping type and farm size.

As a long-term trend, root and vegetable farms generate higher gross margins associated with high-value crops such as potatoes and sugar beet. However, exceptional grain prices, at the same time as a high area payment, gave high levels of profitability in 1996. In contrast, by the 1998 harvest, the gross margin for a cereal farm was nearer £200/ha, with many farms generating a negative net farm income. This trend has continued in 1999.

Machinery costs are usually higher on mixed cropping farms, with depreciation reflecting a higher value of capital investment in specialist equipment. The use of agricultural contractors used to be more common on mixed cropping farms, but is now an increasing trend on all types of farm, as machinery becomes more expensive. The point at which it is cheaper to use contractors differs with cropping type

Table 5.4 Actual costs of 'medium' and 'small' cereal[a] and root/vegetable[b] farms[c]

Farm type	*1996–1997* (£/ha)				*1997–1998* (£/ha)			
	Medium		*Small*		*Medium*		*Small*	
	cereals	*root/ vegetable*	*cereals*	*root/ vegetable*	*cereals*	*root/ vegetable*	*cereals*	*root/ vegetable*
Average size (ha)	311	300	69	65	392	369	69	64
Output	1022	1121	1050	1162	881	987	929	939
Gross margin	771	804	738	895	613	680	600	673
Contract	23	25	28	32	20	23	31	53
Casual labour	6	7	5	9	6	7	16	10
Regular labour[d]	87	157	79	49	126	137	69	54
Equipment depreciation	107	131	80	98	112	119	108	108
Equipment repairs	41	60	48	79	46	57	60	59
Fuel and oil	23	28	26	37	25	26	29	23
Electricity	6	9	8	11	6	7	7	7
Rent and rates	139	141	130	136	147	158	145	156
General repairs	11	23	15	11	21	22	15	11
Miscellaneous	55	61	76	78	56	65	85	94
Total	498	642	508	540	564	621	565	574
Net farm income	273	162	230	355	49	59	35	99

Notes
[a]Cereal farms: >50% area in arable crops and <5% area in root/vegetable crops.
[b]Root/vegetable farms: >50% in arable crops and >5% in root/vegetable crops.
[c]*Source*: University of Nottingham Rural Business Research Unit (1998).
[d]Labour figures exclude farmer and spouse labour.

and farm size. Rents are usually higher on land capable of growing higher value crops. Labour characteristics are not very clear from the examples given, but total labour costs are usually higher for mixed cropping.

COSTS OF BULB HUSBANDRY AND FIELD OPERATIONS

The field operations required during the growing of narcissus bulbs are similar to those for onions or potatoes. A typical sequence for a two-year crop, with standard farmers' costings (£/ha), would be:

Ploughing	37
Power harrowing	25
Fertiliser application (3×9)	27
Planting	100
Spraying (12×9)	108
Topping (defoliating)	22
Harvesting	215
Transporting to store	84
	618

BENEFITS OF INTRODUCING BULBS INTO THE ROTATION

Many farmers would welcome a new crop to grow, particularly on a contract, where, price is guaranteed for produce of acceptable quality. Potato and vegetable cropping are, however, becoming increasingly concentrated in the hands of specialists who are prepared to invest heavily in crop handling and storage equipment. The returns from these crops are also unpredictable, particularly if irrigation is not available. Further, in the UK, sugar beet production is effectively a closed shop, due to the requirement for quota from the British Sugar Corporation.

Recent changes in the relative profitability of combinable crops and alterations to support payments under the CAP have generated additional interest in alternative crops. Reform of the CAP has been necessary due to the need to compete more effectively in world markets, to reduce the cost of agricultural support and to discourage accumulation of large food surpluses produced to the detriment of the environment. A major series of negotiations in early 1999 known as 'Agenda 2000' brought agreement to revisions to the CAP which progressively reduced commodity support from the year 2000 onwards. As a result, EU farmers will be exposed to the volatility of world markets and the realities of global competition.

Potential bulb producers can be grouped into two classes, those who already grow bulbs, onions, carrots or potatoes, and those who do not grow any of these crops. The reason for this division is the existing investment in equipment which, members of the first group are already likely to have undertaken. The equipment required for bulb handling, storage, drying and hot-water treatment is described below, and such investment would not be undertaken lightly by a new grower. Farmers in the first group are likely to possess, or have access to, the necessary equipment. They will, therefore, have the higher fixed cost structure associated with these crops and, in the case of potato growers, the narcissus crop would have the following considerable agronomic and financial advantages:

1 An additional high-value crop to incorporate into the rotation (there should be a minimum of 5 years between potato crops).
2 Labour and machinery requirements dovetail well with the potato enterprise:

   Harvesting of bulbs will be complete, except where early potatoes are grown.
   Irrigation will have some overlap, but is generally earlier and for less time for bulbs.
   Bulbs can be in and out of store before potatoes go in.

3 No major pest or disease problems are common to both crops.

BUDGET CAPITAL COSTS FOR *NARCISSUS* PRODUCTION

The capital costs, at 1999 levels, for the establishment of a 10 ha narcissus bulb enterprise are presented in Table 5.5. It is assumed that total bulb yields of 350 tonne

Table 5.5 Capital costs for equipment needed in growing *Narcissus* bulbs[a]

	Item	*Size*	*Cost*
1a	*Pre-HWT warm store (bulk system)*	175 t	
	Building	19.2 m × 19.2 m × 5.5 m	£54 600
	Tunnel	19.2 m × 1.2 m × 1.5 m	£6048
	Floor	19.2 m × 18 m	£15 225
	Fans	8.27 m^3/s	£1890
	Heater	110 kW	£2730
	Total		£80 493
1b	*Pre-HWT warm store (box system)*	175 t	
	Building	21 m × 12 m × 5.5 m	£36 750
	Letterbox wall	21 m × 4 m	£11 025
	Fans	8.27 m^3/s	£1890
	Heater	110 kW	£2730
	Total		£52 395
2	*HWT system*	1.25 t/hr	
	Tanks	2 × 4 box (3 tonne)	£24 150
3	*Post-HWT cooling*	175 t	
	Building	10 m × 12 m × 6.5 m	£19 950
	Fan top system	2 × 4 box, 0.4 m^3/s	£2625
	Total		£22 575
4	*Bed former*		£4037
5	*Stone clod separator*		£23 189
6	*Bulb planter*	2.5 t/h	£5250
7	*Haulm topper*		£4725
8	*Bulb lifter*		£38 850
9	*Handling equipment*	2.5 t/h	
	Hopper, soil remover, riddle, inspection table		£23 100
	Sprayer		£1890
	Conveyor/elevator		£4998
10a	*Drying system (bulk)*	350 t	
	Building	30 m × 22 m × 5.5 m	£89 250
	Tunnel	30 m × 1.5 m × 1.2 m	£9450
	Floor	30 m × 20.8 m	£23 100
	Fans	18.6 m^3/s	£3728
	Heater	465 kW	£4410
	Total		£129 938
10b	*Drying system (box)*	350 t	
	Building	24 m × 20 m × 5.5 m	£65 100
	Letter box wall × 2	24 m × 3 m × 1 m	£9660
	Fans	37.2 m^3/s	£5040
	Heater	930 kW	£8820
	Total		£88 620
11	*Boxes*	455 × 'potato boxes'	£22 932

Note
[a]All costs (at 1999 levels) are purely budgetary estimates and will vary widely with suppliers and manufacturers.

will be achieved, with 175 tonne bulbs being retained for re-planting, each year. The capital items included are:

1 A pre-hot-water treatment warm store has been specified, both for bulk ('loose') handling and for handling bulbs in boxes (bulk bins). This function

could well be performed by the main drying store, and hence this cost could be avoided.

2 Well designed and operated hot-water treatment (HWT) equipment is essential. However, costs could be shared by the establishment of a central HWT system, used by several growers.
3 Post-HWT cooling is also essential, as is a well laid out treatment site in order to prevent re-contamination of treated stock and to ensure that bulbs are cooled rapidly following HWT. The cost of a simple building is included, though the holding of treated stocks outdoors is practiced by some growers. Holding and cooling treated stocks indoors will require suitable ventilation to avoid damage to health from the formaldehyde fumes resulting from HWT. The cooling of stocks in drying stores after HWT is not recommended.
4 Bed former.
5 Stone and clod separator.
6 Bulb planter.
7 Haulm topper.
8 Bulb lifter.
9 Handling equipment: pre-storage cleaning and post-storage riddling is taken as being relatively simple, with no great degree of grading or any bagging being included.
10 Drying equipment is included both for bulk ('loose') handling and for handling in boxes.
11 Boxes for where bulbs are handled in bulk bins rather than in loose bulk.

Whilst all items likely to be required have been identified and costed, a grower already growing root crops, potatoes or onions may well have sufficient equipment available for the handling of bulbs. Potato or onion harvesting and post-harvesting handling equipment and boxes can all be used, with minor modifications, for the bulbs. Onion driers/stores operate on very similar principles to bulb stores and could therefore be used for bulbs. Redundant potato stores could be adapted for bulb drying and storage.

ACKNOWLEDGEMENTS

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6 Alkaloids of *Narcissus*

Jaume Bastida and Francesc Viladomat

INTRODUCTION

Since the isolation of the first Amaryllidaceae alkaloid, lycorine **(1)**, *from Narcissus pseudonarcissus* by Gerrad at the end of the last century, more than 200 species and varieties belonging to this plant family have been examined for alkaloids. Although this group of alkaloids has been of minor pharmaceutical importance until recently, there has been an increased interest due to the possible applications of galanthamine **(69)**, an alkaloid isolated exclusively from species of this family. There are several reviews of Amaryllidaceae alkaloids (Ghosal *et al*., 1985b; Martin, 1987) and, likewise, this topic is regularly reviewed by the journal *Natural Products Reports* of The Royal Society of Chemistry (Lewis, 1998, and previous years). This chapter covers phytochemical studies on *Narcissus* alkaloids up to March 1999.

There has been considerable taxonomical controversy over which genera belong to the Amaryllidaceae. The revisions of Dahlgren's group (Dahlgren, 1980; Dahlgren *et al*., 1985) have contributed to clarifying this aspect. On the other hand, one of the best tools for the classification of several genera and species of this family has been the type of alkaloids that are found exclusively in Amaryllidaceae. Furthermore, it is unusual to find other types of alkaloids in Amaryllidaceae, but if present, they are always accompanied by typical Amaryllidaceae alkaloids. Up to now, only three alkaloids isolated from this family do not belong to this specific type, but to the mesembrane (*Sceletium*) type generally found in the Aizoaceae family (Jeffs, 1981), and for this reason the Amaryllidaceae alkaloids have a high chemotaxonomical value. One of these compounds, mesembrenone **(82)**, was isolated from *Narcissus pallidulus* (Bastida *et al*., 1989) (Figure 6.1).

The general characteristics of the Amaryllidaceae alkaloids can be summarised as follows:

1 A fundamental ring system composed of a C_6–C_1 and an N–C_2–C_6 building block, derived from L-phenylalanine and L-tyrosine, respectively.
2 They are moderately weak bases (pKa values of 6–9).
3 Each alkaloid contains only one nitrogen atom which is secondary, tertiary or even quaternary, and the carbon content varies from 16 to 20 atoms.

Most of the Amaryllidaceae alkaloids may be classified into nine principal skeletally homogeneous subgroups, although there are several other alkaloids with structures derived from these main molecular frameworks. Representative alkaloids from

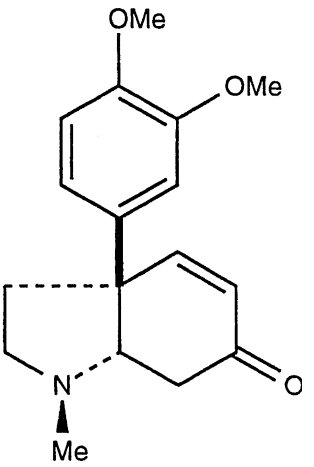


Figure 6.1 Sceletium type alkaloid mesembrenone **(82)** isolated from *N. pallidulus*.

each of these classes include: norbelladine **(83)**, lycorine **(1)**, homolycorine **(23)**, crinine **(84)**, haemanthamine **(49)**, narciclasine **(63)**, tazettine **(58)**, montanine **(85)** and galanthamine **(69)** (Figure 6.2). With the aim of unifying the numbering system of the different types, Ghosal's proposal (Ghosal *et al.*, 1985b) will be used in this chapter for the following reasons:

1 Numbering in the aromatic ring A is always the same (irrespective of the alkaloid skeletal type).
2 The benzylic position, α with respect to the heteroatom, is always 6.
3 The vicinal position with respect to the nitrogen in the pyrrolidine ring and not involved in the ring fusion is always 12.
4 Numbering in ring C is always clockwise except for homolycorine type alkaloids due to their biosynthetic process.

NARCISSUS ALKALOIDS AND THEIR OCCURRENCE

The alkaloids isolated from *Narcissus* species, classified in relation to the different ring types, are shown in Table 6.1(a-h). Table 6.2 lists the different *Narcissus* species and cultivars with their corresponding alkaloids.

BIOSYNTHETIC PATHWAYS

Work on the biosynthesis of Amaryllidaceae alkaloids reached a peak in the period 1960–1976, with a great number of studies related to the subject. However, since then, little new work has been produced apart from the isolation of compounds predicted as biosynthetic intermediaries of a certain pathway or, more recently, a new biosynthetic proposal to obtain galanthamine **(69)** which differs from the initial one.

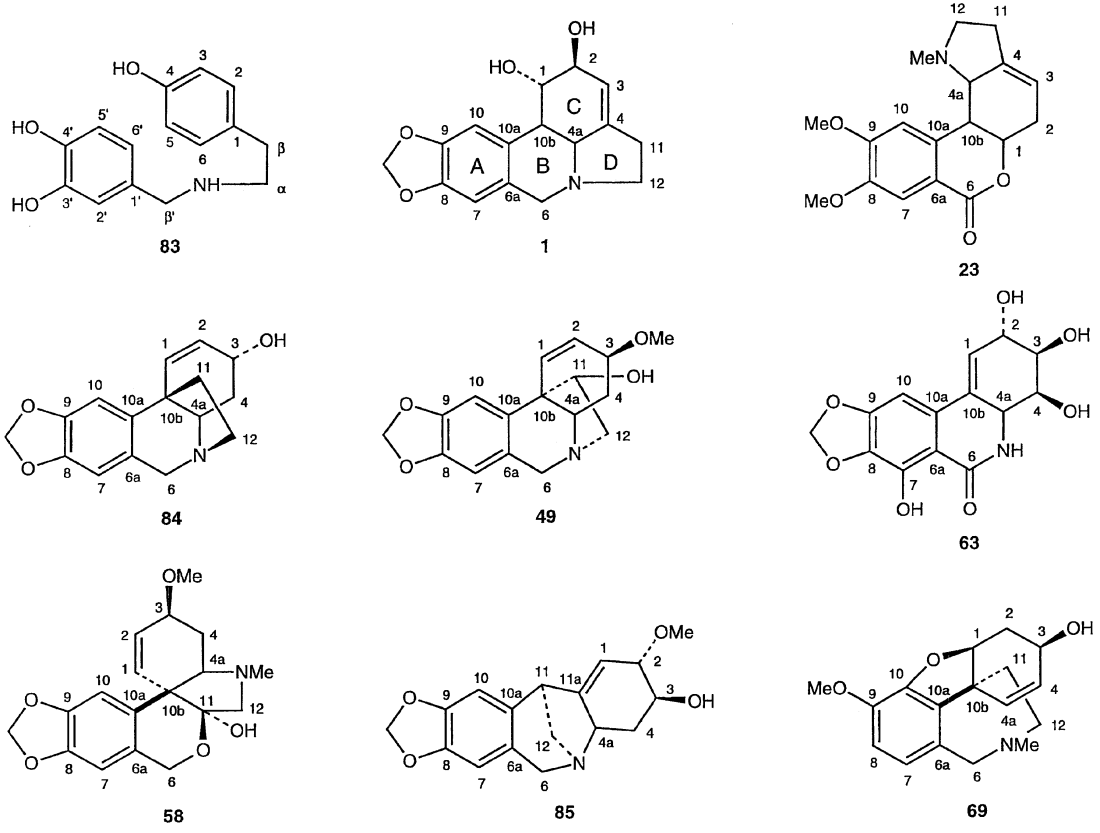
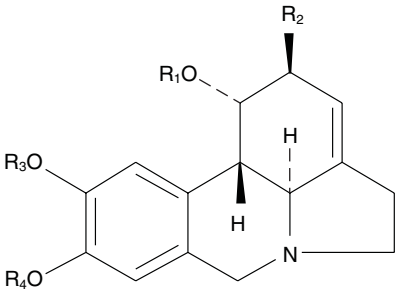


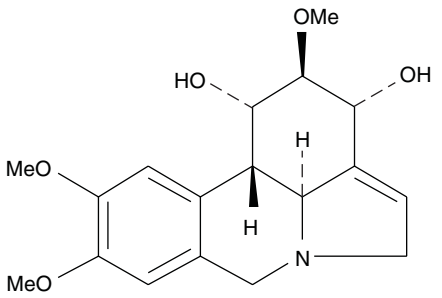
Figure 6.2 Narcissus alkaloid types.

Table 6.1 Narcissus alkaloid structures

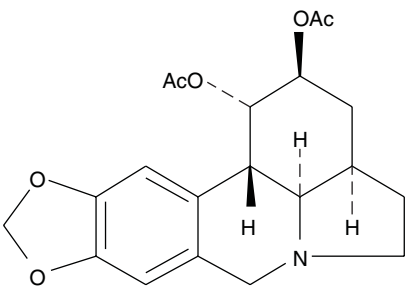
Table 6.1a Lycorine type



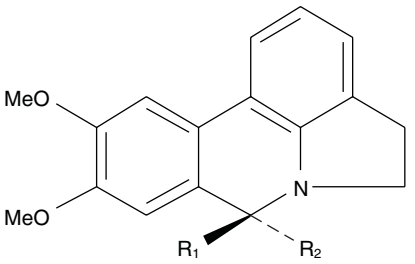
Alkaloid name		*Structure*			
		R_1	R_2	R_3	R_4
1	lycorine	H	OH	CH_2	
2	poetaminine	Ac	OH	CH_2	
3	pseudolycorine	H	OH	H	Me
4	1-*O*-acetylpseudolycorine	Ac	OH	H	Me
5	2-*O*-acetylpseudolycorine	H	OAc	H	Me
6	9-*O*-methylpseudolycorine	H	OH	Me	Me
7	galanthine	H	OMe	Me	Me
8	goleptine	H	OMe	Me	H
9	jonquilline	Ac	O	CH_2	
10	caranine	H	H	CH_2	
11	pluviine	H	H	Me	Me
12	norpluviine	H	H	Me	H
13	9-*O*-demethylpluviine	H	H	H	Me
14	1-*O*-acetyl-9-*O*-demethylpluviine	Ac	H	H	Me
15	1,9-*O*-diacetyl-9-*O*-demethylpluviine	Ac	H	Ac	Me



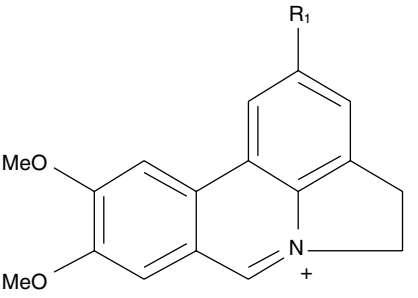
16 narcissidine



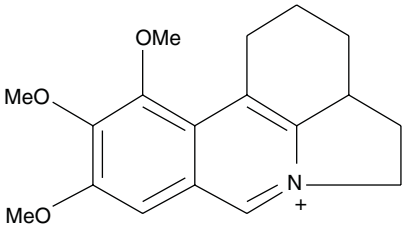
17 nartazine



18 assoanine $R_1 = R_2 = H$
19 oxoassoanine $R_1 + R_2 = O$



20 vasconine $R_1 = H$
21 tortuosine $R_1 = OMe$



22 roserine

Table 6.1b Homolycorine type

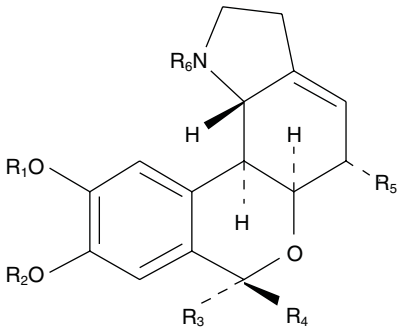
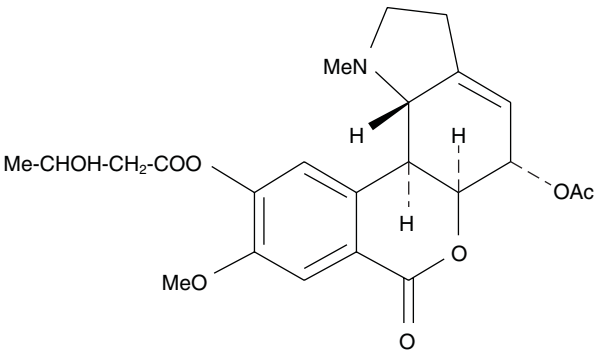
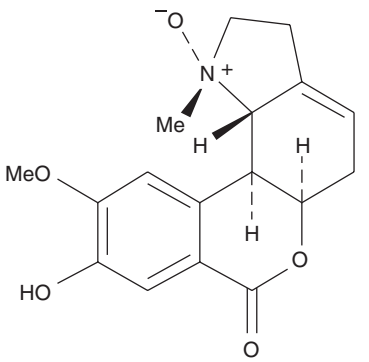


Table 6.1b Continued

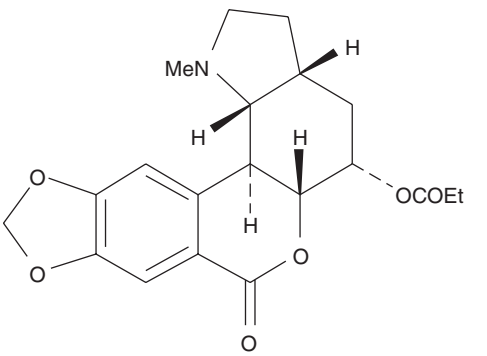
Alkaloid name		*Structure*					
		R_1	R_2	R_3	R_4	R_5	R_6
23	homolycorine	Me	Me		O	H	Me
24	8-*O*-demethylhomolycorine	Me	H		O	H	Me
25	8-*O*-demethyl-8-*O*-acetylhomolycorine	Me	Ac		O	H	Me
26	9-*O*-demethylhomolycorine	H	Me		O	H	Me
27	masonine		CH_2		O	H	Me
28	normasonine		CH_2		O	H	H
29	9-*O*-demethyl-2α-hydroxyhomolycorine	H	Me		O	OH	Me
30	hippeastrine		CH_2		O	OH	Me
31	lycorenine	Me	Me	OH	H	H	Me
32	*O*-methyllycorenine	Me	Me	OMe	H	H	Me
33	oduline		CH_2	OH	H	H	Me
34	6-*O*-methyloduline		CH_2	OMe	H	H	Me
35	2α-hydroxy-6-*O*-methyloduline		CH_2	OMe	H	OH	Me



36 dubiusine

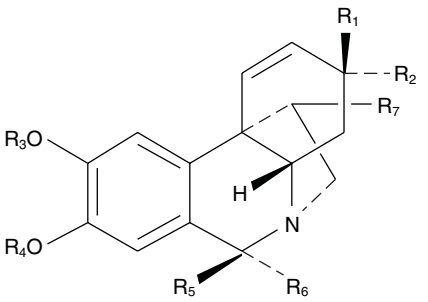


37 8-*O*-demethylhomolycorine-*N*-oxide

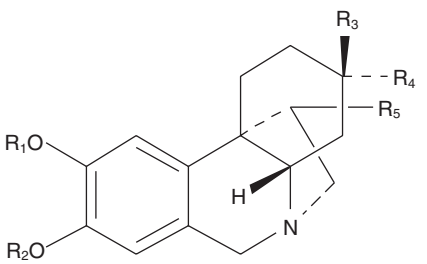


38 poetinatine

Table 6.1c Haemanthamine type

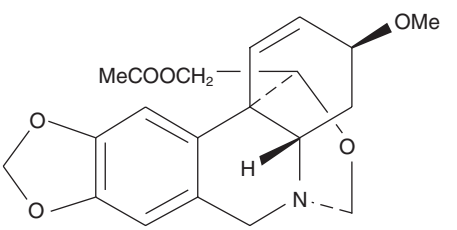


Alkaloid name		*Structure*						
		R_1	R_2	R_3	R_4	R_5	R_6	R_7
39	vittatine	OH	H	CH_2		H	H	H
40	maritidine	OH	H	Me	Me	H	H	H
41	8-*O*-demethylmaritidine	OH	H	Me	H	H	H	H
42	9-*O*-demethylmaritidine	OH	H	H	Me	H	H	H
43	*O*-methylmaritidine	OMe	H	Me	Me	H	H	H
44	papyramine	OMe	H	Me	Me	H	OH	H
45	6-epipapyramine	OMe	H	Me	Me	OH	H	H
46	*O*-methyl-6-epipapyramine	OMe	H	Me	Me	OMe	H	H
47	6α-hydroxy-3-*O*-methylepimaritidine	H	OMe	Me	Me	H	OH	H
48	6β-hydroxy-3-*O*-methylepimaritidine	H	OMe	Me	Me	OH	H	H
49	haemanthamine	OMe	H	CH_2		H	H	OH
50	11-*O*-acetylhaemanthamine	OMe	H	CH_2		H	H	OAc
51	haemanthidine	OMe	H	CH_2		H	OH	OH
52	6-epihaemanthidine	OMe	H	CH_2		OH	H	OH
53	crinamine	H	OMe	CH_2		H	H	OH
54	narcidine	OMe	H	Me	H	H	H	OH



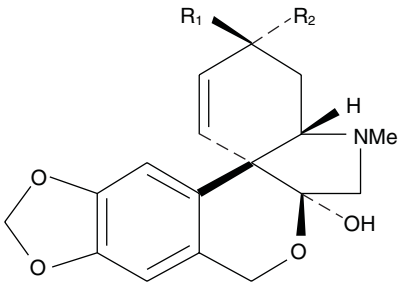
55 cantabricine $R_1 = H$, $R_2 = Me$, $R_3 = H$, $R_4 = OAc$, $R_5 = H$

56 narcimarkine $R_1 + R_2 = CH_2$, $R_3 = OMe$, $R_4 = H$, $R_5 = OCH_2CH(OH)Et$

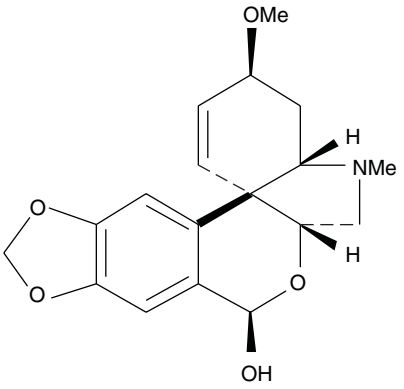


57 bujeine

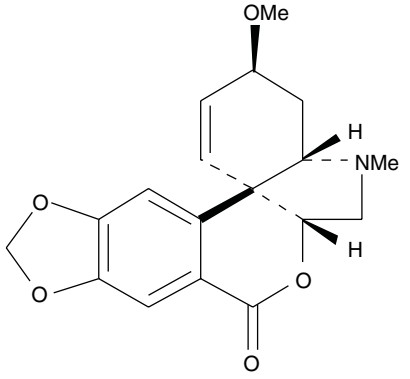
Table 6.1d Tazettine type



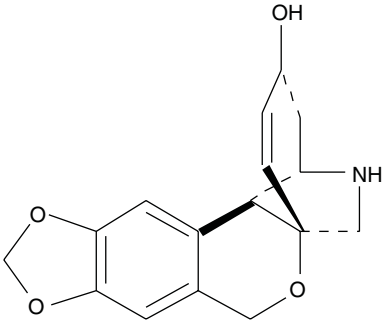
58 tazettine $R_1 = OMe$, $R_2 = H$
59 criwelline $R_1 = H$, $R_2 = OMe$



60 pretazettine

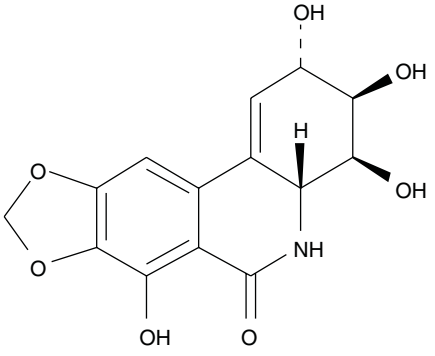


61 3-epimacronine

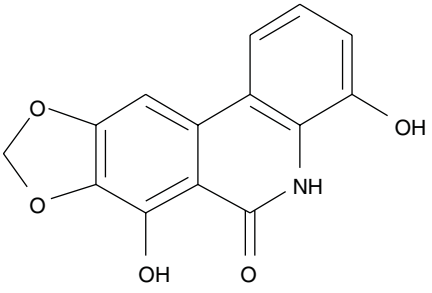


62 obesine

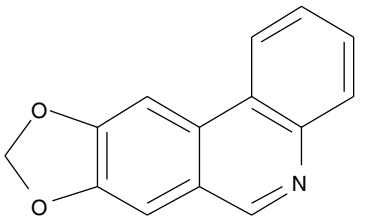
Table 6.1e Narciclasine and montanine types



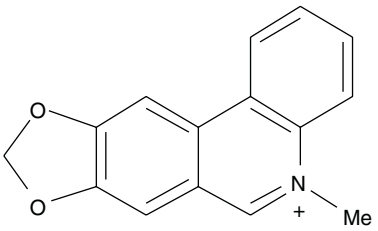
63 narciclasine



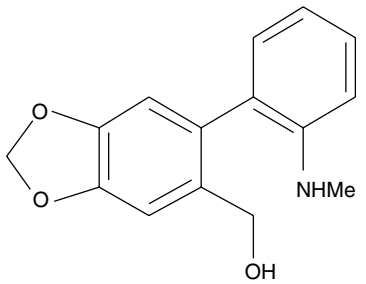
64 narciprimine



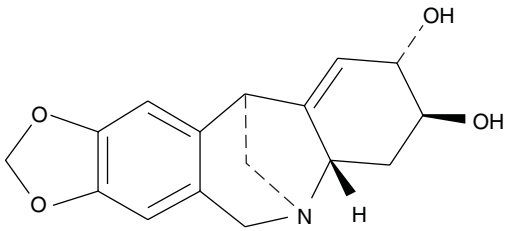
65 trisphaeridine



66 bicolorine

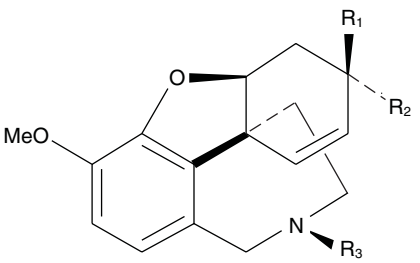


67 ismine



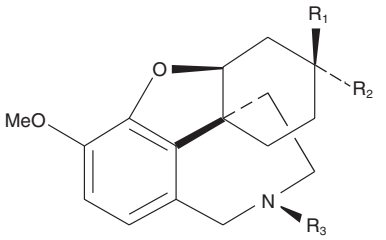
68 pancracine

Table 6.1f Galanthamine type



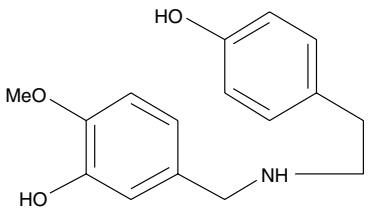
Alkaloid name		*Structure*		
		R_1	R_2	R_3
69	galanthamine	OH	H	Me
70	epigalanthamine	H	OH	Me
71	*O*-acetylgalanthamine	OAc	H	Me
72	norgalanthamine	OH	H	H
73	epinorgalanthamine	H	OH	H
74	*N*-formylnorgalanthamine	OH	H	CHO
75	narcisine	OH	H	Ac
76	narwedine	O		Me

Table 6.1f Continued



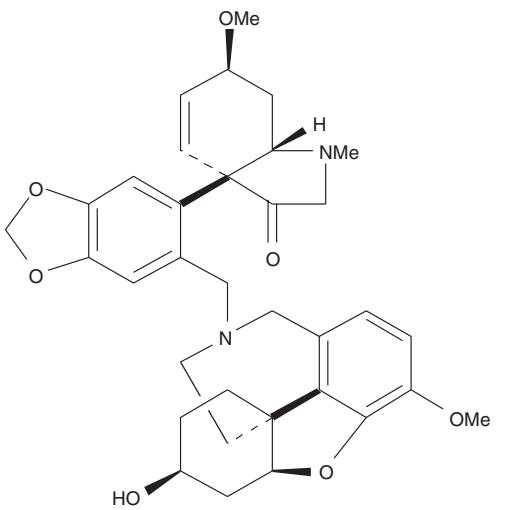
Alkaloid name		*Structure*		
		R_1	R_2	R_3
77	lycoramine	OH	H	Me
78	norlycoramine	OH	H	H
79	epinorlycoramine	H	OH	H

Table 6.1g Alkaloids without phenol oxidative coupling

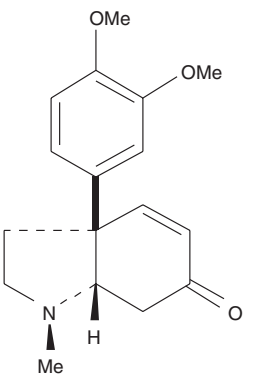


80 *O*-methylnorbelladine

Table 6.1h Miscellaneous



81 pallidiflorine (heterodimer)



82 mesembrenone (*Sceletium*)

Table 6.2 Occurrence of *Narcissus* alkaloids

*Species**	*Alkaloids*	*References*
N. angustifolius Curtis ex Haw. = *N. poeticus* L. subsp. *radiiflorus* (Salisb.) Baker	**69** galanthamine	Cherkasov *et al.*, 1988
N. assoanus Léon-Duf.	**18** assoanine **19** oxoassoanine **3** pseudolycorine **4** 1-*O*-acetylpseudolycorine **5** 2-*O*-acetylpseudolycorine	Llabrés *et al.*, 1986a Llabrés *et al.*, 1986b
N. asturiensis (Jordan) Pugsley	**49** haemanthamine **51** haemanthidine **67** ismine **61** 3-epimacronine **58** tazettine **65** trisphaeridine	Viladomat *et al.*, 1997
N. aureus Loisel. = *N. tazetta* L. subsp. *aureus* (Loisel.) Baker	**1** lycorine	Hung *et al.*, 1962
N. bicolor L.	**66** bicolorine **26** 9-*O*-demethylhomolycorine **67** ismine **61** 3-epimacronine **60** pretazettine **20** vasconine	Viladomat *et al.*, 1990
N. biflorus Curtis = *N.* × *medioluteus* Mill.	**1** lycorine	Hung *et al.*, 1962
N. bujei (Fdez. Casas) Fdez. Casas	**57** bujeine **53** crinamine **49** haemanthamine **50** 11-*O*-acetylhaemanthamine **23** homolycorine **24** 8-*O*-demethylhomolycorine **31** lycorenine **32** *O*-methyllycorenine **27** masonine **34** 6-*O*-methyloduline **58** tazettine	Labraña *et al.*, 1999
N. canaliculatus Guss.	**49** haemanthamine **63** narciclasine **58** tazettine	Boit and Döpke, 1956 Piozzi *et al.*, 1969
N. cantabricus DC.	**55** cantabricine **53** crinamine **58** tazettine **39** vittatine	Bastida *et al.*, 1995b
N. confusus Pugsley	**69** galanthamine **74** *N*-formylnorgalanthamine **49** haemanthamine **23** homolycorine **26** 9-*O*-demethylhomolycorine **60** pretazettine	Bastida *et al.*, 1987a Bastida *et al.*, 1987b
N. cyclamineus DC.	**63** narciclasine **76** narwedine	Bazhenova *et al.*, 1971 Piozzi *et al.*, 1969
N. cyclamineus DC. cv. Beryl	**7** galanthine **1** lycorine **16** narcissidine	Boit *et al.*, 1957b

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. cyclamineus DC. cv. Cairhays	**7** galanthine **51** haemanthidine **52** 6-epihaemanthidine **11** pluviine	Boit *et al.*, 1957b
N. cyclamineus DC. cv. February Gold	**23** homolycorine **77** lycoramine **31** lycorenine	Boit *et al.*, 1957b
N. cyclamineus DC. cv. Peeping Tom	**31** lycorenine **11** pluviine **58** tazettine	Boit *et al.*, 1957b
N. dubius Gouan	**36** dubiusine **29** 9-*O*-demethyl-2α-hydroxyhomolycorine **3** pseudolycorine	Bastida *et al.*, 1988a Bastida *et al.*, 1992c
N. eugeniae Fdez. Casas	**69** galanthamine **23** homolycorine **31** lycorenine	Codina *et al.*, 1988 Codina *et al.*, 1992a
N. × *gracilis* Sabine	**69** galanthamine **1** lycorine **58** tazettine	Boit *et al.*, 1957b
N. × *incomparabilis* Mill. cv. Carabiniere	**63** narciclasine	Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. Daisy Schäffer	**69** galanthamine **7** galanthine **11** pluviine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Deanna Durbin	**7** galanthine **49** haemanthamine **1** lycorine **16** narcissidine **11** pluviine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Flower Record	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Fortune	**69** galanthamine **49** haemanthamine **30** hippeastrine **33** oduline	Boit *et al.*, 1957c Gorbunova *et al.*, 1984
N. × *incomparabilis* Mill. cv. Helios	**69** galanthamine **7** galanthine **49** haemanthamine **23** homolycorine **31** lycorenine **63** narciclasine **11** pluviine	Boit *et al.*, 1957c Piozzi *et al.*, 1968, 1969
N. × *incomparabilis* Mill. cv. John Evelyn	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit *et al.*, 1957c

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. × *incomparabilis* Mill. cv. Marion Cran	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Mercato	**63** narciclasine	Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. R.O. Backhouse	**63** narciclasine	Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. Nova Scotia	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Oranje Bruid	**63** narciclasine	Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. Pluvius	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine **16** narcissidine **11** pluviine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Scarlet Elegance	**63** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. Sempre Avanti	**7** galanthine **49** haemanthamine **1** lycorine **63** narciclasine **16** narcissidine **11** pluviine	Boit *et al.*, 1957c Ceriotti *et al.*, 1967 Piozzi *et al.*, 1968, 1969
N. × *incomparabilis* Mill. cv. Suda	**69** galanthamine **7** galanthine **49** haemanthamine **31** lycorenine **11** pluviine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Toronto	**7** galanthine **49** haemanthamine **1** lycorine	Boit *et al.*, 1957c
N. × *incomparabilis* Mill. cv. Tunis	**64** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
N. × *incomparabilis* Mill. cv. Walt Disney	**64** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
N. jacetanus Fdez. Casas	**18** assoanine **19** oxoassoanine **1** lycorine **3** pseudolycorine	Bastida *et al.*, 1988b
N. jonquilla L	**69** galanthamine **77** lycoramine **63** narciclasine	Vigneau *et al.*, 1984
N. jonquilla L. cv. Golden Sceptre	**69** galanthamine **7** galanthine **8** goleptine	Boit *et al.*, 1957b Döpke, 1963b Döpke, 1963c

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
	49 haemanthamine **30** hippeastrine **23** homolycorine **9** jonquilline	Döpke, 1964 Döpke and Dalmer, 1965a
N. jonquilla L. cv. Golden Sceptre (*contd*.)	**31** lycorenine **1** lycorine **27** masonine **33** oduline **58** tazettine	
N. jonquilla L. cv. Trevithian	**69** galanthamine **31** lycorenine **1** lycorine **63** narciclasine **58** tazettine	Boit *et al.*, 1957b Piozzi *et al.*, 1969
N. leonensis Pugsley	**72** norgalanthamine **73** epinorgalanthamine **79** epinorlycoramine **1** lycorine	Bastida *et al.*, 1993
N. lobularis Hort.	**69** galanthamine **49** haemanthamine	Boit *et al.*, 1957c
N. muñozii-garmendiae Fdez. Casas	**23** homolycorine **31** lycorenine **32** *O*-methyllycorenine	Codina *et al.*, 1993
N. nivalis Graells	**69** galanthamine **72** norgalanthamine **6** 9-*O*-methylpseudolycorine	Bastida *et al.*, 1990c
N. obesus Salisb	**66** bicolorine **69** galanthamine **49** haemanthamine **67** ismine **61** 3-epimacronine **62** obesine **60** pretazettine	Viladomat *et al.*, 1992
N. × *odorus* L. var. *rugulosus*	**69** galanthamine **30** hippeastrine **23** homolycorine **1** lycorine **63** narciclasine **33** oduline **58** tazettine	Boit *et al.*, 1957b Piozzi *et al.*, 1969
N. pallidiflorus Pugsley	**49** haemanthamine **23** homolycorine **24** 8-*O*-demethylhomolycorine **67** ismine **81** pallidiflorine **60** pretazettine	Codina *et al.*, 1990
N. pallidulus Graells	**82** mesembrenone **22** roserine	Bastida *et al.*, 1989 Bastida *et al.*, 1992b
N. panizzianus Parl.	**7** galanthine **23** homolycorine	Bastida *et al.*, 1990a

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
	44 papyramine **45** 6-epipapyramine **60** pretazettine	
N. papyraceus Ker. Gawl.	**69** galanthamine **23** homolycorine **24** 8-*O*-demethylhomolycorine **37** 8-*O*-demethylhomolycorine-*N*-oxide **77** lycoramine **1** lycorine **40** maritidine **43** *O*-methylmaritidine **56** narcimarkine **44** papyramine **45** 6-epipapyramine **46** *O*-methyl-6-epipapyramine **3** pseudolycorine **58** tazettine	Döpke and Sewerin, 1981 Hung *et al.*, 1981 Suau *et al.*, 1990a
N. poeticus L.	**69** galanthamine **7** galanthine **23** homolycorine **31** lycorenine **1** lycorine **16** narcissidine **17** nartazine **68** pancracine **2** poetaminine **11** pluviine **6** 9-*O*-methylpseudolycorine	Boit and Döpke, 1956 Boit and Stender, 1954 Döpke and Sewerin, 1981 Harken *et al.*, 1976 Wildman and Brown, 1968 Willaman and Li, 1970
N. poeticus L. var. *ornatus* Hort.	**69** galanthamine **7** galanthine **49** haemanthamine **23** homolycorine **31** lycorenine **1** lycorine **2** poetaminine **38** poetinatine **58** tazettine	Boit, 1954 Boit and Döpke, 1956 Döpke, 1963a Döpke and Nguyen, 1974
N. poeticus L. cv. Actaea	**69** galanthamine **7** galanthine **31** lycorenine **1** lycorine **63** narciclasine **16** narcissidine	Boit and Döpke, 1956 Piozzi *et al.*, 1969
N. poeticus L. cv. Daphne	**69** galanthamine **23** homolycorine **31** lycorenine **1** lycorine	Boit *et al.*, 1957c
N. poeticus L. cv. Sarchedon	**69** galanthamine **7** galanthine **31** lycorenine **1** lycorine **16** narcissidine	Boit and Döpke, 1956

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. primigenius (Laínz) Fdez. Casas and Laínz	**49** haemanthamine **23** homolycorine **24** 8-*O*-demethylhomolycorine **41** 8-*O*-demethylmaritidine	Bastida *et al.*, 1994
N. pseudonarcissus L.	**80** *O*-methylnorbelladine **49** haemanthamine **23** homolycorine **1** lycorine **27** masonine	Cook *et al.*, 1954 Gude *et al.*, 1988 Willaman and Li, 1970
N. pseudonarcissus L. cv. Carlton	**69** galanthamine **71** *O*-acetylgalanthamine **72** norgalanthamine **49** haemanthamine **30** hippeastrine **23** homolycorine **77** lycoramine **79** epinorlycoramine **31** lycorenine **32** *O*-methyllycorenine **27** masonine **28** normasonine **63** narciclasine **76** narwedine **33** oduline **34** 6-*O*-methyloduline **13** 9-*O*-demethylpluviine **14** 1-*O*-acetyl-9-*O*-demethylpluviine **15** 1,9-*O*-diacetyl-9-*O*-demethylpluviine **39** vittatine	Kreh and Matusch, 1995 Kreh *et al.*, 1995a Kreh *et al.*, 1995b Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Covent Garden	**69** galanthamine **72** norgalanthamine **7** galanthine **49** haemanthamine **78** norlycoramine **31** lycorenine **1** lycorine **11** pluviine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Early Glory	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Flower Carpet	**63** narciclasine	Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Golden Harvest	**63** narciclasine	Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Grand Maître	**69** galanthamine **7** galanthine **49** haemanthamine **23** homolycorine	Boit *et al.*, 1957c

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
	1 lycorine **58** tazettine	
N. pseudonarcissus L. cv. Imperator	**69** galanthamine **7** galanthine **49** haemanthamine **23** homolycorine **31** lycorenine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. King Alfred	**18** assoanine **69** galanthamine **72** norgalanthamine **7** galanthine **49** haemanthamine **30** hippeastrine **23** homolycorine **24** 8-*O*-demethylhomolycorine **78** norlycoramine **31** lycorenine **1** lycorine **63** narciclasine **54** narcidine **16** narcissidine **33** oduline **11** pluviine **6** 9-*O*-methylpseudolycorine	Boit and Ehmke, 1956 Boit *et al.*, 1957c Fales *et al.*, 1956 Fuganti *et al.*, 1974b Harken *et al.*, 1976 Piozzi *et al.*, 1968, 1969 Tojo, 1991
N. pseudonarcissus L. cv. Magnet	**7** galanthine **49** haemanthamine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Magnificence	**69** galanthamine **72** norgalanthamine **7** galanthine **49** haemanthamine **78** norlycoramine **1** lycorine **11** pluviine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Mount Hood	**69** galanthamine **30** hippeastrine **23** homolycorine **77** lycoramine **78** norlycoramine **63** narciclasine	Bastos *et al.*, 1996 Ceriotti *et al.*, 1967 Moraes-Cerdeira *et al.*, 1997b Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Mrs. Ernst H. Krelage	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine **63** narciclasine **11** pluviine	Boit *et al.*, 1957c Ceriotti *et al.*, 1967
N. pseudonarcissus L. cv. Music Hall	**7** galanthine **49** haemanthamine **1** lycorine	Boit *et al.*, 1957c

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. pseudonarcissus L. cv. Oliver Cromwell	**69** galanthamine **7** galanthine **49** haemanthamine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. President Lebrun	**63** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Queen of Bicolors	**7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Rembrandt	**69** galanthamine **72** norgalanthamine **49** haemanthamine **78** norlycoramine **1** lycorine **63** narciclasine	Boit *et al.*, 1957c Piozzi *et al.*, 1969
N. pseudonarcissus L. cv. Rockery Beauty	**72** norgalanthamine **7** galanthine **49** haemanthamine **78** norlycoramine **16** narcissidine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Romaine	**7** galanthine **49** haemanthamine **31** lycorenine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Spring Glory	**72** norgalanthamine **7** galanthine **49** haemanthamine **78** norlycoramine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Unsurpassable	**69** galanthamine **49** haemanthamine **31** lycorenine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Victoria	**72** norgalanthamine **7** galanthine **49** haemanthamine **78** norlycoramine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
N. pseudonarcissus L. cv. Wrestler	**69** galanthamine **7** galanthine **49** haemanthamine **11** pluviine	Boit *et al.*, 1957c
N. radinganorum Fdez. Casas	**23** homolycorine **24** 8-*O*-demethylhomolycorine **42** 9-*O*-demethylmaritidine	Bastida *et al.*, 1988c
N. serotinus Löfl. ex L.	**63** narciclasine	Piozzi *et al.*, 1969

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. tazetta L.	**59** criwelline **69** galanthamine **7** galanthine **49** haemanthamine **51** haemanthidine **52** 6-epihaemanthidine **30** hippeastrine **23** homolycorine **24** 8-*O*-demethylhomolycorine **1** lycorine **43** *O*-methylmaritidine **27** masonine **63** narciclasine **75** narcisine **16** narcissidine **17** nartazine **76** narwedine **60** pretazettine **3** pseudolycorine **58** tazettine	Abdallah, 1993 Abduazimov and Yunusov, 1967 Abou-Donia *et al.*, 1989 Bi *et al.*, 1998 Boit and Döpke, 1956 Boit and Döpke, 1960 Evidente, 1991 Evidente *et al.*, 1994 Furusawa *et al.*, 1976a Piozzi *et al.*, 1968, 1969 Späth and Kahovec, 1934 Späth *et al.*, 1936 Tani *et al.*, 1981
N. tazetta L. var. *chinensis* Roem	**69** galanthamine **70** epigalanthamine **51** haemanthidine **52** 6-epihaemanthidine **23** homolycorine **77** lycoramine **31** lycorenine **1** lycorine **40** maritidine **43** *O*-methylmaritidine **47** 6α-hydroxy-3-*O*-methyl-epimaritidine **48** 6β-hydroxy-3-*O*- methyl-epimaritidine **44** papyramine **45** 6-epipapyramine **11** pluviine **60** pretazettine **3** pseudolycorine **58** tazettine	Hung *et al.*, 1966 Laing and Clark, 1974 Ma *et al.*, 1986
N. tazetta L. cv. Cragford	**49** haemanthamine **23** homolycorine **1** lycorine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. Early Perfection	**49** haemanthamine **30** hippeastrine **23** homolycorine **1** lycorine **11** pluviine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. Geranium	**69** galanthamine **49** haemanthamine	Boit and Döpke, 1956 Ceriotti *et al.*, 1967

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
N. tazetta L. cv. Geranium (*contd*.)	**23** homolycorine **1** lycorine **63** narciclasine **16** narcissidine **58** tazettine	Evidente, 1991 Moraes-Cerdeira *et al.*, 1997b
N. tazetta L. cv. La Fiancée	**7** galanthine **49** haemanthamine **23** homolycorine **1** lycorine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. Laurens Koster	**49** haemanthamine **23** homolycorine **1** lycorine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. L'Innocence	**49** haemanthamine **23** homolycorine **1** lycorine **16** narcissidine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. Scarlet Gem	**7** galanthine **49** haemanthamine **23** homolycorine **1** lycorine **58** tazettine	Boit and Döpke, 1956
N. tazetta L. cv. St. Agnes	**49** haemanthamine **23** homolycorine **1** lycorine **58** tazettine	Boit and Döpke, 1956
N. tortifolius Fdez. Casas	**36** dubiusine **69** galanthamine **23** homolycorine **24** 8-*O*-demethylhomolycorine **29** 9-*O*-demethyl-2α-hydroxyhomolycorine	Bastida *et al.*, 1990b
N. tortuosus Haworth	**1** lycorine **21** tortuosine	Bastida *et al.*, 1995a
N. triandrus L. cv. Silver Chimes	**49** haemanthamine **1** lycorine **58** tazettine	Boit *et al.*, 1957b
N. triandrus L. cv. Thalia	**49** haemanthamine **23** homolycorine **31** lycorenine **1** lycorine **63** narciclasine	Boit *et al.*, 1957b Piozzi *et al.*, 1968, 1969
N. triandrus L. cv. Tresamble	**69** galanthamine **49** haemanthamine **31** lycorenine **63** narciclasine	Boit *et al.*, 1957b Piozzi *et al.*, 1968, 1969
N. vasconicus Fdez. Casas	**23** homolycorine **25** 8-*O*-demethyl-8-*O*-acetylhomolycorine	Bastida *et al.*, 1992a

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
	1 lycorine **20** vasconine	
Narcissus L. cv. Celebrity	**63** narciclasine	Piozzi *et al.*, 1969
Narcissus L. cv. Cheerfulness	**69** galanthamine **63** narciclasine	Moraes-Cerdeira *et al.*, 1997b Piozzi *et al.*, 1969
Narcissus L. cv. Clamor	**63** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
Nancissus L. cv. Folly	**1** lycorine **58** tazettine	Abduazimov and Yunusov, 1967
Narcissus L. cv. Ice Follies	**10** caranine **69** galanthamine **49** haemanthamine **30** hippeastrine **77** lycoramine **78** norlycoramine **1** lycorine	Moraes-Cerdeira *et al.*, 1997a Moraes-Cerdeira *et al.*, 1997b
Narcissus L. cv. Inglescombe	**69** galanthamine **49** haemanthamine **30** hippeastrine **23** homolycorine **77** lycoramine **78** norlycoramine **31** lycorenine **1** lycorine **11** pluviine	Bastos *et al.*, 1996 Boit *et al.*, 1957c
Narcissus L. cv. Insulinde	**7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
Narcissus L. cv. Irene Copeland	**1** lycorine **76** narwedine	Boit *et al.*, 1957c
Narcissus L. cv Kristalli	**69** galanthamine **1** lycorine **76** narwedine **58** tazettine	Abduazimov and Yunusov, 1967
Narcissus L. cv. Livia	**10** caranine **7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit *et al.*, 1957c
Narcissus L. cv. Salome	**53** crinamine **72** norgalanthamine **30** hippeastrine **35** 2α-hydroxy-6-*O*-methyloduline **3** pseudolycorine **21** tortuosine **20** vasconine	Almanza *et al.*, 1996
Narcissus L. cv. Texas	**69** galanthamine **49** haemanthamine	Barton and Kirby, 1962 Boit *et al.*, 1957c

Table 6.2 Continued

*Species**	*Alkaloids*	*References*
Narcissus L. cv. Texas (*contd.*)	**1** lycorine **63** narciclasine **76** narwedine **11** pluviine **12** norpluviine	Ceriotti *et al.*, 1967 Kirby and Michael, 1973 Kirby and Tiwari, 1966 Piozzi *et al.*, 1969
Narcissus L. cv. Totus Albus Prior = *N. papyraceus* Ker. Gawl.	**63** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
Narcissus L. cv. Twink	**69** galanthamine **7** galanthine **49** haemanthamine **1** lycorine **11** pluviine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
Narcissus L. cv. Van Sion	**69** galanthamine **72** norgalanthamine **7** galanthine **49** haemanthamine **23** homolycorine **78** norlycoramine **31** lycorenine **1** lycorine **11** pluviine **58** tazettine	Boit and Ehmke, 1956 Boit *et al.*, 1957c
Narcissus L. cv. Verger	**63** narciclasine	Ceriotti *et al.*, 1967 Piozzi *et al.*, 1969
Narcissus sp.	**69** galanthamine **63** narciclasine **64** narciprimine **2** poetaminine	Ceriotti, 1967 Cherkasov *et al.*, 1989 Piozzi *et al.*, 1968 Willaman and Li, 1970

Note
*The taxonomical aspects are based on the works of Barra and López-González (1984a,b), Blanchard (1990), Dorda and Fernández-Casas (1984a,b, 1989, 1990, 1994), Dorda *et al.* (1991), Fernandes (1975, 1991), Fernández-Casas (1983, 1984a,b, 1986) and Pugsley (1933) for wild *Narcissus* species and hybrids, and those of Boit and Döpke (1956), Boit and Ehmke (1956), Boit *et al.* (1957b,c), Jefferson-Brown (1991), Kington (1989) and Piozzi *et al.* (1969) for *Narcissus* cultivars.

Although L-phenylalanine (L-phe) and L-tyrosine (L-tyr) are closely related in chemical structure, they are not interchangeable in plants. In the Amaryllidaceae alkaloids, L-phe serves as a primary precursor of the C_6–C_1 fragment, corresponding to ring A and the benzylic position (C-6), and L-tyr is the precursor of ring C, the two-carbon side chain (C-11 and C-12) and nitrogen, C_6–C_2–N. The conversion of L-phe to the C_6–C_1 unit requires the loss of two carbon atoms from the side chain as well as the introduction of at least two oxygenated substituents into the aromatic ring, which is performed *via* cinnamic acids. The presence of the enzyme phenylalanine ammonia lyase (PAL) has been demonstrated in Amaryllidaceae (Suhadolnik *et al.*, 1963), and the elimination of ammonia mediated by this enzyme is known to occur in an antiperiplanar manner to give *trans*-cinnamic acid, with loss of the

β-pro-S hydrogen (Wightman *et al.*, 1972). Thus, it may be expected that L-phe would be incorporated into Amaryllidaceae alkaloids with retention of the *β-pro*-R hydrogen. However, feeding experiments in *Narcissus pseudonarcissus* cv. King Alfred showed that tritium originally present at C-*β* of L-phe, whatever the configuration, was lost in the formation of several haemanthamine and homolycorine type alkaloids, which led to the conclusion that fragmentation of the cinnamic acids involves oxidation of C-*β* to ketone or acid level, the final product being protocatechuic aldehyde or its derivatives (Figure 6.3). On the other hand, L-tyr is degraded no further than tyramine before incorporation into the Amaryllidaceae alkaloids.

Thus, tyramine and protocatechuic aldehyde or its derivatives are logical components for the biosynthesis of the precursor norbelladine **(83)**. This reaction occupies a pivotal position since it represents the entry of primary metabolites into a secondary metabolic pathway. The junction of the amine and the aldehyde results in a Schiff's base, two of which have been isolated up to now: craugsodine (Ghosal *et al.*, 1986) and isocraugsodine (Ghosal *et al.*, 1988a). The existence of Schiff's bases in nature, as well as their easy conversion into the different ring-systems of the Amaryllidaceae alkaloids, allows the presumption that the initial postulate about this biosynthetic pathway was correct.

Barton and Cohen (1957) proposed that norbelladine **(83)** or related compounds could undergo oxidative coupling of phenols in Amaryllidaceae plants, once ring A had been suitably protected by methylation, resulting in the different skeletons of the Amaryllidaceae alkaloids (Figure 6.4).

Lycorine and homolycorine types

The alkaloids of this group are derivatives of the pyrrolo[de]phenanthridine alkaloids (lycorine type) and the 2-benzopirano-[3,4-g]indole alkaloids (homolycorine type), and both types originate from an *ortho-para′* phenol-oxidative coupling (Figure 6.5).

The biological conversion of cinnamic acid *via* hydroxylated cinnamic acids into the C_6–C_1 unit of norpluviine **(12)** has been used in a study of hydroxylation mechanisms in higher plants (Bowman *et al.*, 1969). When [3-^{3}H, *β*-^{14}C]cinnamic acid was fed to *Narcissus pseudonarcissus* cv. Texas, a tritium retention in norpluviine **(12)** of 28% was observed. This is very near a predicted value of 25%, resulting from *para*-hydroxylation with hydrogen migration and retention, where half the tritium would be lost in the first hydroxylation and half the remainder in the second.

In the conversion of *O*-methylnorbelladine **(80)** into lycorine **(1)**, the labeling position [3-^{3}H] on the aromatic ring of L-tyr afterwards appears at C-2 of norpluviine **(12)**, which is formed as an intermediate, the configuration of the tritium apparently being *β* (Kirby and Tiwari, 1966). This tritium is retained in subsequently formed lycorine **(1)**, which means that hydroxylation at C-2 proceeds with an inversion of configuration (Bruce and Kirby, 1968) by a mechanism involving an epoxide, with ring opening followed by allylic rearrangement of the resulting alcohol (Figure 6.6). Supporting evidence comes from the incorporation of [2*β*-^{3}H]caranine **(10)** into lycorine **(1)** in *Zephyranthes candida* (Wildman and Heimer, 1967). However, a hydroxylation of caranine **(10)** in *Clivia miniata* occurring with retention of configuration was also observed (Fuganti and Mazza, 1972b). Further, [2*α*-^{3}H; 11-^{14}C]caranine **(10)** was incorporated into lycorine **(1)** with high

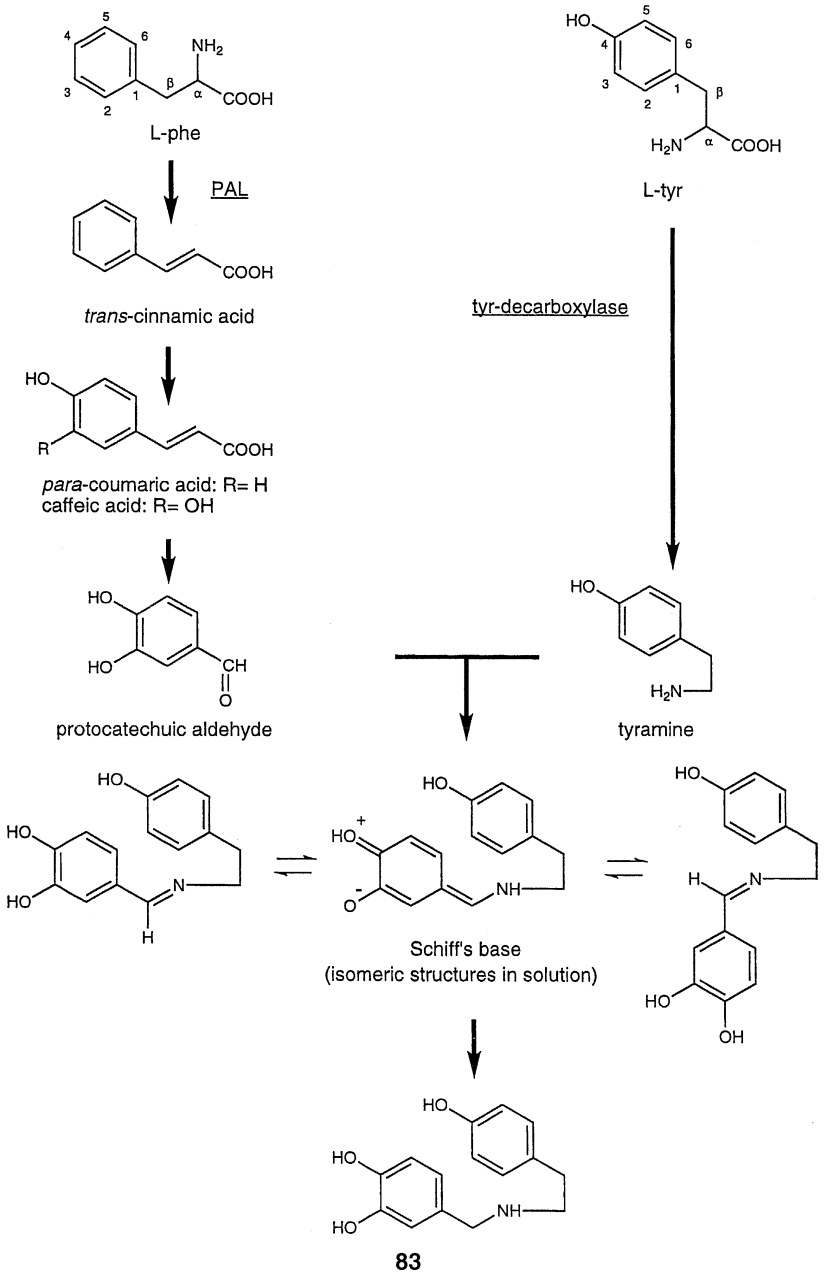


Figure 6.3 Biosynthetic pathway to norbelladine.

retention of tritium at C-2, indicating that no 2-oxo-compound can be implicated as an intermediate.

The conversion of the *O*-methoxyphenol to the methylenedioxy group may occur late in the biosynthetic pathway. Tritiated norpluviine **(12)** is converted to

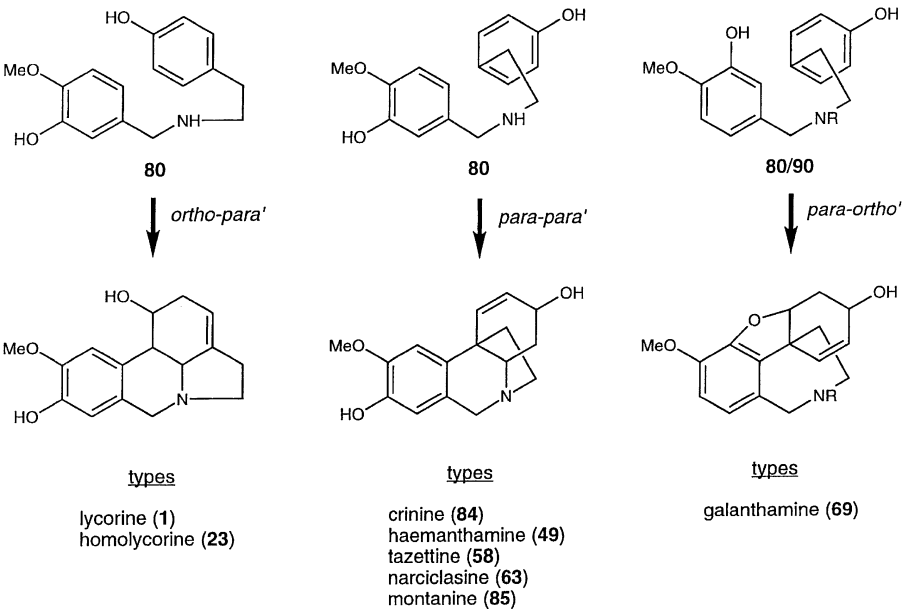


Figure 6.4 Oxidative phenyl-phenyl coupling in Amaryllidaceae alkaloids.

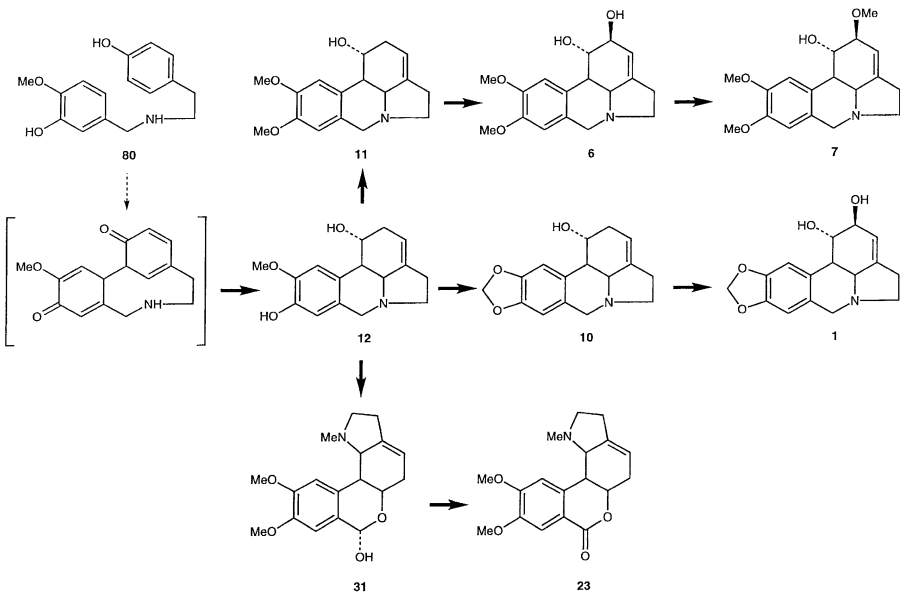


Figure 6.5 Alkaloids proceeding from an *ortho-para'* coupling.

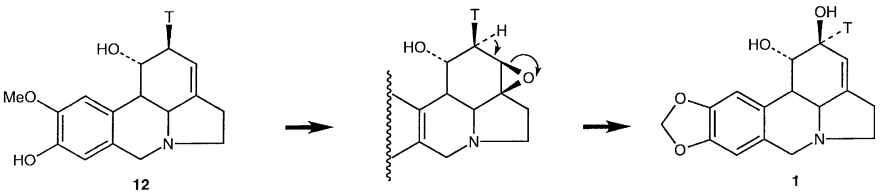


Figure 6.6 Biosynthesis of lycorine **(1)** with inversion of the configuration.

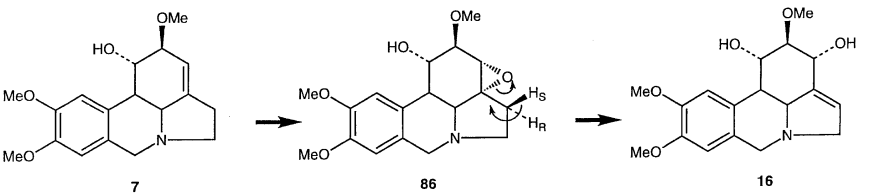


Figure 6.7 Conversion of galanthine **(7)** to narcissidine **(16)** *via* epoxide **(86)**.

tritiated lycorine **(1)** by *Narcissus* × *incomparabilis* cv. Deanna Durbin, which not only demonstrates the previously mentioned conversion but also indicates that the C-2 hydroxyl group of lycorine **(1)** is derived by allylic oxidation of either norpluviine **(12)** or caranine **(10)** (Battersby *et al.*, 1964).

Regarding the conversion of [2β-^{3}H, 8-OMe-^{14}C]pluviine **(11)** into galanthine **(7)**, in *Narcissus pseudonarcissus* cv. King Alfred, the retention of 79% of the tritium label confirms that hydroxylation of C-2 may occur with inversion of configuration (Harken *et al.*, 1976).

It was considered (Fuganti *et al.*, 1974a) that another analogous epoxide **86** could give narcissidine **(16)** in the way shown by loss of the *pro*-S hydrogen from C-11, galanthine **(7)** being a suitable substrate for epoxidation. Labelled [α-^{14}C, β-^{3}H]-*O*-methylnorbelladine **(80)**, when fed to *Narcissus* x *incomparabilis* cv. Sempre Avanti, afforded galanthine **(7)** (98% of tritium retention) and narcissidine **(16)** (46% tritium retention). Loss of hydrogen from C-11 of galanthine **(7)** was therefore stereospecific. In this decade, Kihara *et al.* (1992) have isolated a new alkaloid, incartine **(86)**, from flowers of *Lycoris incarnata*, which could be considered to be the biosynthetic intermediate of this pathway (Figure 6.7).

The biological conversion of protocatechuic aldehyde into lycorenine **(31)**, which proceeds *via O*-methylnorbelladine **(80)** and norpluviine **(12)**, first involves a reduction of the aldehyde carbonyl, and afterwards, in the generation of lycorenine **(31)**, oxidation of this same carbon atom. The absolute stereochemistry of these processes has been elucidated in subsequent experiments (Fuganti and Mazza, 1973), and the results show that hydrogen addition and removal take place on the *re*-face of the molecules concerned (Hanson, 1966), the hydrogen initially introduced being the one later removed (Fuganti and Mazza, 1971a). It is noteworthy that norpluviine **(12)**, unlike pluviine **(11)**, is converted *in Narcissus pseudonarcissus* cv. King Alfred primarily to alkaloids of the homolycorine type. Benzylic oxidation

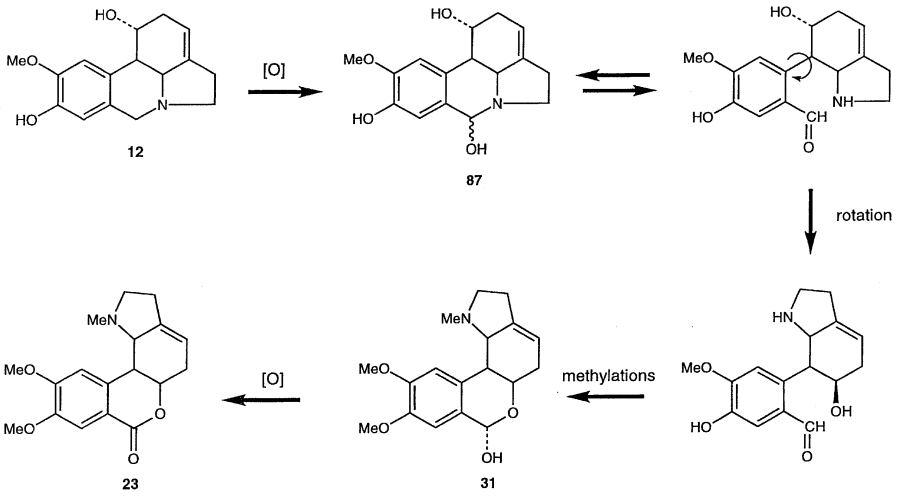


Figure 6.8 Conversion of norpluviine **(12)** to homolycorine type alkaloids.

of position 6 would afford **87**, followed by ring opening to form an amino aldehyde, and then a hemiacetal formation and methylation could provide lycorenine **(31)** (Harken *et al*., 1976), and, on subsequent oxidation, could give homolycorine **(23)** as can be seen in Figure 6.8.

Crinine, haemanthamine, tazettine, narciclasine and montanine types

This group includes the alkaloids derived from 5,10b-ethanophenanthridine (crinine and haemanthamine types), 2-benzopyrano[3,4-c]indole (tazettine type), phenanthridine (narciclasine type) and 5,11-methanomorphanthridine (montanine type), originating from a *para-para′* oxidative phenolic coupling (Figure 6.9).

Results of experiments with labelled crinine **(84)**, and less conclusively with oxovittatine, indicate that the two naturally occurring enantiomeric series, represented in Figure 6.9 by crinine **(84)** and vittatine **(39)**, are not interconvertible in *Nerine bowdenii* (Feinstein and Wildman, 1976).

Incorporation of *O*-methylnorbelladine **(80)**, labelled in the methoxy carbon and also in positions [3,5-^{3}H], into the alkaloid haemanthamine **(49)**, was without loss of tritium, half of which was sited at C-2 of **(49)**. Consideration of the possible mechanisms involved in relation to tritium retention led to the suggestion that the tritium which is expected at C-4 of **(49)** might not be stereospecific (Fuganti, 1969). The conversion of *O*-methylnorbelladine **(80)** into haemanthamine **(49)** involves loss of the *pro*-R hydrogen from the C-β of the tyramine moiety, as well as a further entry of a hydroxyl group at this site (Battersby *et al*., 1971). The subsequent benzylic oxidation results in an epimeric mixture **51/52**, which even High Performance Liquid Chromatography (HPLC) cannot separate. The epimeric forms were proposed to be interconvertible through **88a**. The biosynthetic conversion of the 5,10b-ethanophenanthridine alkaloids to the 2-benzopyrano[3,4-c]indole was demonstrated by feeding tritium-labelled alkaloids to *Sprekelia formosissima*. It was shown that

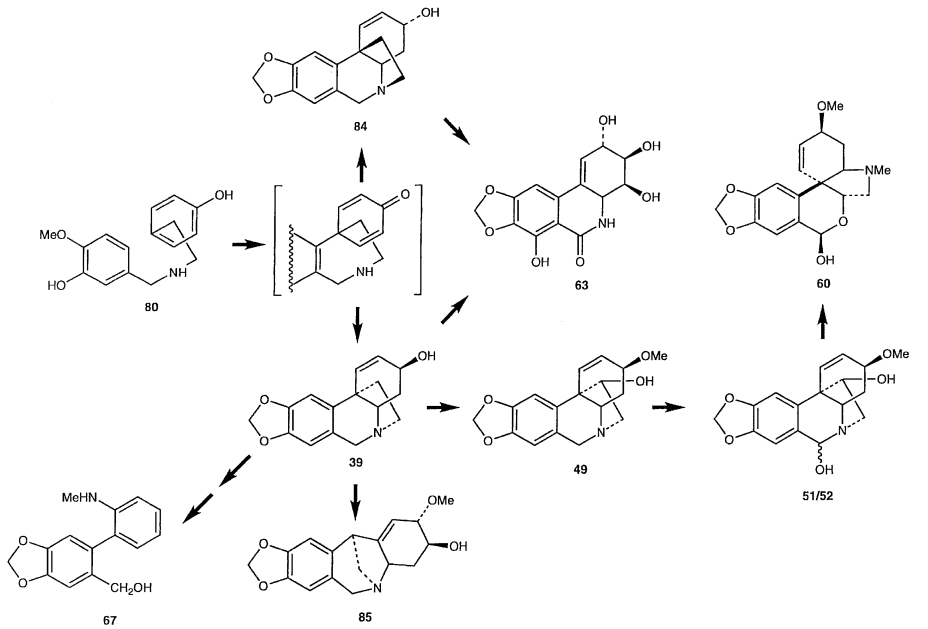


Figure 6.9 Alkaloids proceeding from a *para-para′* coupling.

this plant converts haemanthamine **(49)** to haemanthidine/epihaemanthidine **(51/52)** and subsequently to pretazettine **(60)** in an essentially irreversible manner (Fales and Wildman, 1964). This transformation was considered to proceed through **88a** or the related alkoxide anion, although this intermediate **88a** and its rotational equivalent **88b** have never been detected by spectral methods (Wildman and Bailey, 1969) (Figure 6.10).

It has also been proved that the alkaloid narciclasine **(63)** proceeds from the pathway of the biosynthesis of crinine and haemanthamine type alkaloids and not through norpluviine **(12)** and lycorine **(1)** derivatives. In fact, in view of its structural affinity to both haemanthamine **(49)** and lycorine **(1)**, narciclasine **(63)** could be derived by either pathway. When *O*-methylnorbelladine **(80)**, labelled in the methoxy carbon and in both protons of position 3 and 5 of the tyramine aromatic ring, was administered to *narcissus* plants, all four alkaloids incorporated activity. The isotopic ratio [^{3}H:^{14}C] for norpluviine **(12)** and lycorine **(1)** was, as expected, 50% that of the precursor, because of its *ortho-para′* coupling. On the contrary, in haemanthamine **(49)** the ratio was unchanged. These results prove that the methoxy group of **(80)** is completely retained in the alkaloids mentioned, providing a satisfactory internal standard, and also the degree of tritium retention is a reliable guide to the direction of phenol coupling. Narciclasine **(63)** showed an isotopic ratio (75%) higher than that of lycorine or norpluviine **(12)** though lower than that of hemanthamine **(49)**. However, the fact that more than 50% of tritium is retained suggests that *O*-methylnorbelladine **(80)** is incorporated into narciclasine **(63)** *via para-para′* phenol oxidative coupling.

O-methylnorbelladine **(80)** and vittatine **(39)** are implicated as intermediates in the biosynthesis of narciclasine **(63)** (Fuganti *et al.*, 1971; Fuganti and Mazza,

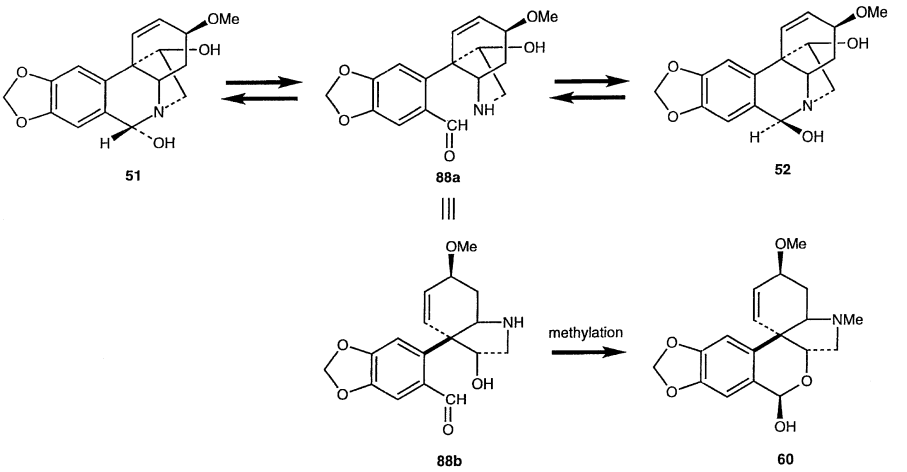


Figure 6.10 Biosynthesis of pretazettine **(60)**.

1971b, 1972a), and the loss of the ethane bridge from the latter could occur by a retro-Prins reaction on 11-hydroxyvittatine **(89)**. Strong support for this pathway was obtained by labeling studies. 11-Hydroxyvittatine **(89)** has also been proposed as an intermediate in the biosynthesis of haemanthamine **(49)** and montanine **(85)** (a 5,11-methanomorphanthridine alkaloid) following the observed specific incorporation of vittatine **(39)** into the two alkaloids in *Rhodophiala bifida* (Feinstein and Wildman, 1976) (Figure 6.11).

Fuganti and Mazza (1971b, 1972a) concluded that in the late stages of narciclasine **(63)** biosynthesis, the two-carbon bridge is lost from the oxocrinine skeleton, passing through intermediates bearing a pseudoaxial hydroxy-group at C-3 position and further hydrogen removal from this position does not occur. Noroxomaritidine was also implicated in the biosynthesis of narciclasine **(63)**, and further experiments (Fuganti, 1973) showed that it is also a precursor for ismine **(67)**.

The alkaloid ismine **(67)** has also been shown (Fuganti and Mazza, 1970) to be a transformation product of the crinine-haemanthamine series. The precursor, oxocrinine labelled with tritium in the positions 2 and 4, was administered to *Sprekelia formosissima* plants and the radioactive ismine **(67)** isolated was shown to be specifically labelled at the expected positions.

Galanthamine type

The alkaloids with a dibenzofuran nucleus (galanthamine type) are obtained from a *para-ortho′* phenyl oxidative coupling.

Although norbelladine **(83)** was shown not to be a precursor of galanthamine **(69)** in *Narcissus pseudonarcissus* cv. King Alfred, incorporation of this labelled compound has been obtained in *Leucojum aestivum* (Fuganti, 1969).

The initial studies of this pathway suggested that the phenyl oxidative coupling does not proceed from *O*-methylnorbelladine **(80)** but that the order of methyla-

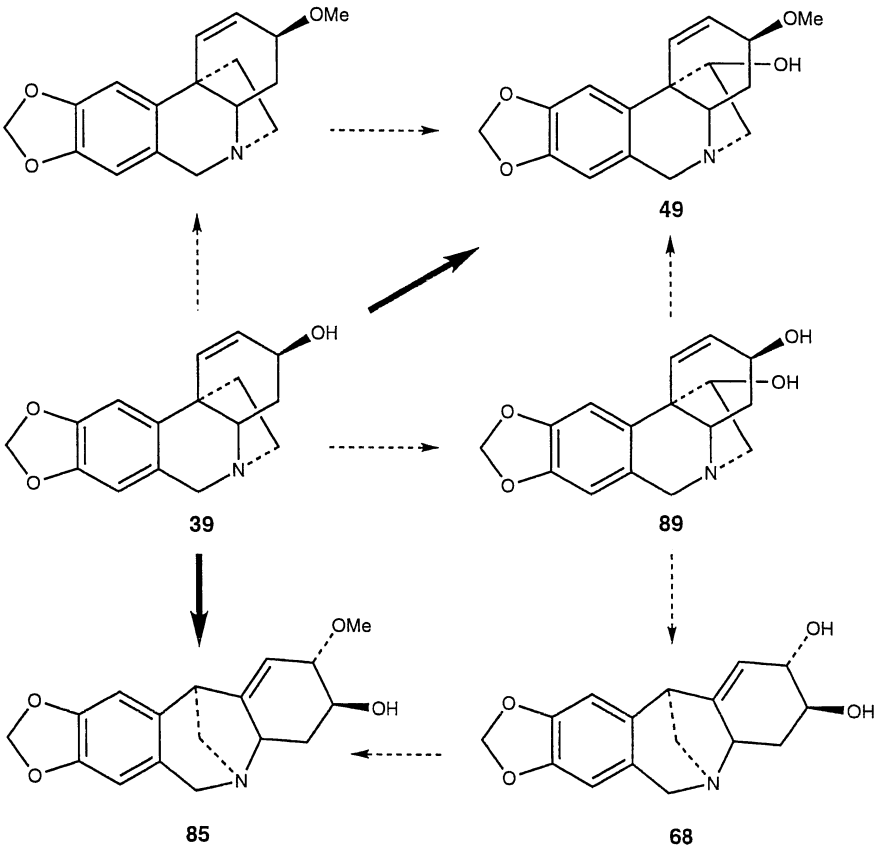


Figure 6.11 Proposed biosynthetic pathways to haemanthamine **(49)** and montanine **(85)**.

tion of the precursors should be norbelladine **(83)** --> *N*-methylnorbelladine --> *N*,*O*-dimethylnorbelladine **(90)** to give finally galanthamine **(69)** (Barton *et al.*, 1963), the conversion of **91** to narwedine **(76)** either being not reversible or, if so, enzymically controlled (Fuganti, 1969). The precursor *N*,*O*-dimethylnorbelladine **(90)** was first isolated in 1988 from *Pancratium maritimum* (Vázquez-Tato *et al.*, 1988), a species that also contains galanthamine **(69)** (Figure 6.12a).

Chlidanthine **(92)**, by analogy with the known conversion of codeine to morphine, might be expected to arise from galanthamine **(69)** by *O*-demethylation. This was shown to be true when both galanthamine **(69)** and narwedine **(76)**, with tritium labels, were incorporated into chlidanthine **(92)** (Bhandarkar and Kirby, 1970).

However, the most recent study seems to contradict the evidence set forth here. Experiments carried out with application of ^{13}C-labelled *O*-methylnorbelladine **(80)** to organs of field grown *Leucojum aestivum* have shown that the biosynthesis of galanthamine **(69)** involves the phenol oxidative coupling of *O*-methylnorbelladine **(80)** to a postulated dienone which undergoes spontaneous closure of the ether bridge to yield *N*-demethylnarwedine **(93)**, giving norgalanthamine **(72)** after stereoselective reduction. Furthermore, it was shown that norgalanthamine **(72)** is

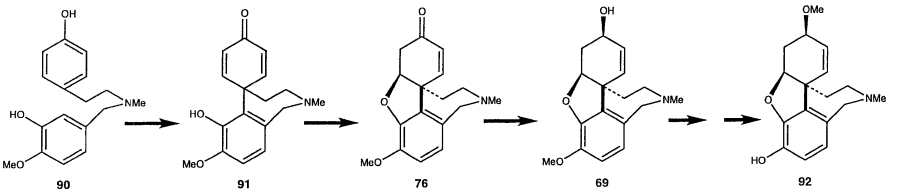


Figure 6.12a Biosynthesis of galanthamine **(69)** proposed by Barton *et al*. (1963).

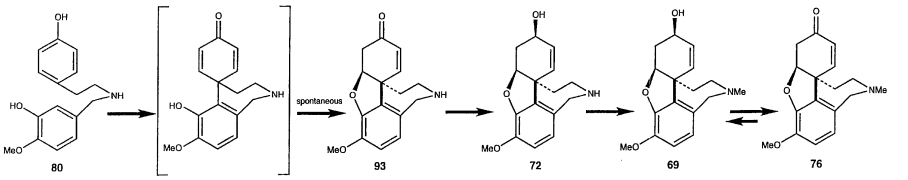


Figure 6.12b Biosynthesis of galanthamine **(69)** proposed by Eichhorn *et al*. (1998).

N-methylated to galanthamine **(69)** in the final step of biosynthesis (Eichhorn *et al*., 1998) (Figure 6.12b). In contrast with the literature, *N*,*O*-dimethylnorbelladine **(90)** was metabolised to a lesser extent in *L. aestivum* and incorporated into galanthamine **(69)** as well as norgalanthamine **(72)** at about one-third the rate of *O*-methylnorbelladine **(80)**.

According to Eichhorn *et al*. (1998), narwedine **(76)** is not the direct precursor of galanthamine **(69)**, and could possibly exist in an equilibrium with galanthamine **(69)**, a reaction catalysed by a hypothetically reversible oxido-reductase.

SPECTROSCOPY

Only Proton Nuclear Magnetic Resonance (^{1}H-NMR), Carbon^{13} Nuclear Magnetic Resonance (^{13}C-NMR) and Mass Spectrometry (MS), the three most important spectroscopic methods for the Amaryllidaceae alkaloids, will be treated here. A list of the different narcissus alkaloids and their spectroscopic properties is given in Table 6.3. The literature with the most recent spectroscopic data for these alkaloids is given, even when they were isolated from species other than *Narcissus*.

Table 6.3 Narcissus alkaloids – spectroscopic data

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
18 assoanine	$C_{17}H_{17}NO_2$	267	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Llabrés *et al.*, 1986b
19 oxoassoanine	$C_{17}H_{15}NO_3$	281	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Llabrés *et al.*, 1986b
66 bicolorine	$C_{15}H_{12}NO_2$	238	IR, MS, ^{1}H NMR, ^{13}C NMR	Viladomat *et al.*, 1990
57 bujeine	$C_{20}H_{23}NO_6$	373	IR, MS, ^{1}H NMR, ^{13}C NMR, CD	Labraña *et al.*, 1999

Table 6.3 Continued

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
55 cantabricine	$C_{18}H_{23}NO_4$	317	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1995b
10 caranine	$C_{16}H_{17}NO_3$	271	UV, IR, MS, ^{1}H NMR	Evidente *et al.*, 1986
			CD	Kuriyama *et al.*, 1967
53 crinamine	$C_{17}H_{19}NO_4$	301	UV, ^{1}H NMR	Likhitwitayawuid *et al.*, 1993
			IR, MS, CD	Viladomat *et al.*, 1994
			^{13}C NMR	Viladomat *et al.*, 1996
			X-Ray	Roques *et al.*, 1977
59 criwelline	$C_{18}H_{21}NO_5$	331	UV	Hauth and Stauffacher, 1964
			MS	Duffield *et al.*, 1965
			^{1}H NMR, ^{13}C NMR	Razafimbello *et al.*, 1996
			CD	De Angelis and Wildman, 1969a
36 dubiusine	$C_{23}H_{27}NO_8$	445	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1988a
69 galanthamine	$C_{17}H_{21}NO_3$	287	UV, IR, MS, ^{1}H NMR	Bastida *et al.*, 1987b
			^{13}C NMR	Abdallah *et al.*, 1989
			CD	De Angelis and Wildman, 1969a
			X-Ray	Peeters *et al.*, 1997
71 *O*-acetyl-galanthamine	$C_{19}H_{23}NO_4$	329	UV, MS, ^{1}H NMR, ^{13}C NMR	Kreh *et al.*, 1995b
70 epigalanthamine	$C_{17}H_{21}NO_3$	287	UV, MS	Kametani *et al.*, 1969
			IR, ^{1}H NMR	Vlahov *et al.*, 1989
			CD	De Angelis and Wildman, 1969a
72 norgalanthamine	$C_{16}H_{19}NO_3$	273	UV	Laiho and Fales, 1964
			IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1990c
			CD	Li *et al.*, 1987
			X-Ray	Roques and Lapasset, 1976
73 epinorgalanthamine	$C_{16}H_{19}NO_3$	273	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1993
74 *N*-formyl-norgalanthamine	$C_{17}H_{19}NO_4$	301	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1987b
7 galanthine	$C_{18}H_{23}NO_4$	317	UV	Kobayashi *et al.*, 1977
			MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1990a
8 goleptine	$C_{17}H_{21}NO_4$	303	IR	Döpke, 1964
49 haemanthamine	$C_{17}H_{19}NO_4$	301	UV, IR, MS	Bastida *et al.*, 1987b
			^{1}H NMR	Pabuççuoglu *et al.*, 1989
			^{13}C NMR, CD	Baudouin *et al.*, 1994
			X-Ray	Watson *et al.*, 1984
50 11-*O*-acetyl-haemanthamine	$C_{19}H_{21}NO_5$	343	IR, MS, ^{1}H NMR, ^{13}C NMR, CD	Labraña *et al.*, 1999

Table 6.3 Continued

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
51 haemanthidine	$C_{17}H_{19}NO_5$	317	UV	Irie *et al.*, 1959
			IR, MS	Trimiño *et al.*, 1989
			^{1}H NMR	Pabuççuoglu *et al.*, 1989
			^{13}C NMR	Antoun *et al.*, 1993
			CD	Wagner *et al.*, 1996
52 6-epihaemanthidine	$C_{17}H_{19}NO_5$	317	UV	Irie *et al.*, 1959
			IR, MS	Trimiño *et al.*, 1989
			^{1}H NMR	Pabuççuoglu *et al.*, 1989
			^{13}C NMR	Antoun *et al.*, 1993
			CD	Wagner *et al.*, 1996
30 hippeastrine	$C_{17}H_{17}NO_5$	315	UV	Irie *et al.*, 1959
			IR, MS, ^{1}H NMR, ^{13}C NMR	Almanza *et al.*, 1996
			CD	Wagner *et al.*, 1996
23 homolycorine	$C_{18}H_{21}NO_4$	315	UV, IR, MS, ^{1}H NMR	Bastida *et al.*, 1987a
			^{13}C NMR	Jeffs *et al.*, 1985
			CD	Wagner *et al.*, 1996
24 8-*O*-demethyl-homolycorine	$C_{17}H_{19}NO_4$	301	UV, IR, ^{1}H NMR, ^{13}C NMR	Latvala *et al.*, 1995b
			MS	Bastida *et al.*, 1988c
			CD	Wagner *et al.*, 1996
			X-Ray	Latvala *et al.*, 1995a
37 8-*O*-demethyl-homolycorine-*N*-oxide	$C_{17}H_{19}NO_5$	317	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Suau *et al.*, 1990a
25 8-*O*-demethyl-8-*O*-acetylhomolycorine	$C_{19}H_{21}NO_5$	343	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1992a
26 9-*O*-demethyl-homolycorine	$C_{17}H_{19}NO_4$	301	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1987a
29 9-*O*-demethyl-2α-hydroxy-homolycorine	$C_{17}H_{19}NO_5$	317	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1990b
67 ismine	$C_{15}H_{15}NO_3$	257	UV, IR, MS, ^{1}H NMR	Suau *et al.*, 1990b
			^{1}H NMR, ^{13}C NMR	Viladomat *et al.*, 1997
			X-Ray	Viladomat *et al.*, 1998
9 jonquilline	$C_{18}H_{17}NO_5$	327	UV, IR	Döpke and Dalmer, 1965a
77 lycoramine	$C_{17}H_{23}NO_3$	289	IR, MS, ^{1}H NMR, ^{13}C NMR	Li *et al.*, 1987
			^{13}C NMR	Kobayashi *et al.*, 1991
			^{13}C NMR	Youssef and Frahm, 1998
78 norlycoramine	$C_{16}H_{21}NO_3$	275	IR, MS, ^{1}H NMR	Kihara *et al.*, 1987
79 epinorlycoramine	$C_{16}H_{21}NO_3$	275	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1993
31 lycorenine	$C_{18}H_{23}NO_4$	317	UV	Kitagawa *et al.*, 1955
			MS	Ibuka *et al.*, 1966
			^{1}H NMR, ^{13}C NMR	Codina *et al.*, 1992a
			X-Ray	Clardy *et al.*, 1972

Table 6.3 Continued

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
32 *O*-methyllycorenine	$C_{19}H_{25}NO_4$	331	IR, MS, ^{1}H NMR, ^{13}C NMR	Codina *et al.*, 1993
			X-Ray	Labraña *et al.*, 1999
1 lycorine	$C_{16}H_{17}NO_4$	287	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Likhitwitayawuid *et al.*, 1993
			^{13}C NMR	Spohn *et al.*, 1994
			CD	Wagner *et al.*, 1996
			X-Ray	Gopalakrishna *et al.*, 1976
61 3-epimacronine	$C_{18}H_{19}NO_5$	329	IR, MS, ^{13}C NMR	Viladomat *et al.*, 1990
			^{1}H NMR	Kihara *et al.*, 1987
			CD	Wagner *et al.*, 1996
			X-Ray	Linden *et al.*, 1998
40 maritidine	$C_{17}H_{21}NO_3$	287	UV	Hung *et al.*, 1981
			IR	Tomioka *et al.*, 1977
			MS, ^{1}H NMR	Ghosal *et al.*, 1985a
			^{1}H NMR, ^{13}C NMR	Youssef and Frahm, 1998
			CD	De Angelis and Wildman, 1969b
			X-Ray	Zabel *et al.*, 1979
41 8-*O*-demethyl-maritidine	$C_{16}H_{19}NO_3$	273	IR, MS	Kihara *et al.*, 1987
			^{1}H NMR	Pabuççuoglu *et al.*, 1989
			^{13}C NMR	Bastida *et al.*, 1994
42 9-*O*-demethyl-maritidine	$C_{16}H_{19}NO_3$	273	IR, MS, ^{1}H NMR	Bastida *et al.*, 1988c
43 *O*-methylmaritidine	$C_{18}H_{23}NO_3$	301	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Suau *et al.*, 1990a
			CD	Ma *et al.*, 1986
47 6α-hydroxy-3-*O*-methylepimaritidine	$C_{18}H_{23}NO_4$	317	UV, IR, MS, ^{1}H NMR, CD	Ma *et al.*, 1986
48 6β-hydroxy-3-*O*-methylepimaritidine	$C_{18}H_{23}NO_4$	317	UV, IR, MS, ^{1}H NMR, CD	Ma *et al.*, 1986
27 masonine	$C_{17}H_{17}NO_4$	299	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Kreh and Matusch, 1995
			CD	Jeffs *et al.*, 1985
28 normasonine	$C_{16}H_{15}NO_4$	285	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Kreh and Matusch, 1995
82 mesembrenone	$C_{17}H_{21}NO_3$	287	IR, MS, ^{1}H NMR	Bastida *et al.*, 1989
			^{13}C NMR	Jeffs *et al.*, 1974
63 narciclasine	$C_{14}H_{13}NO_7$	307	UV, IR	Piozzi *et al.*, 1968
			MS, ^{1}H NMR, ^{13}C NMR	Evidente, 1991
			X-Ray	Bi *et al.*, 1998
54 narcidine	$C_{17}H_{21}NO_4$	303	UV, IR, MS, ^{1}H NMR	Tojo, 1991
56 narcimarkine	$C_{21}H_{27}NO_5$	373	IR, MS	Döpke and Sewerin, 1981
64 narciprimine	$C_{14}H_9NO_5$	271	UV, IR, ^{1}H NMR	Piozzi *et al.*, 1968
			MS	Spenglers and Trimiño, 1989

Table 6.3 Continued

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
75 narcisine	$C_{18}H_{21}NO_4$	315	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Abdallah, 1993
16 narcissidine	$C_{18}H_{23}NO_5$	333	UV IR, X-Ray MS ^{1}H NMR X-Ray	Fales and Wildman, 1958 Clardy *et al.*, 1970 Kinstle *et al.*, 1966 Kihara *et al.*, 1995 Immirzi and Fuganti, 1971
17 nartazine	$C_{20}H_{23}NO_6$	373	IR	Boit and Döpke, 1956
76 narwedine	$C_{17}H_{19}NO_3$	285	UV IR, MS, ^{1}H NMR	Kametani *et al.*, 1969 Li *et al.*, 1987
80 *O*-methyl-norbelladine	$C_{16}H_{19}NO_3$	273	IR, MS, ^{1}H NMR, ^{13}C NMR	Machocho *et al.*, 1999
62 obesine	$C_{16}H_{17}NO_4$	287	MS, ^{1}H NMR, ^{13}C NMR	Viladomat *et al.*, 1992
33 oduline	$C_{17}H_{19}NO_4$	301	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Kreh and Matusch, 1995
34 6-*O*-methyloduline	$C_{18}H_{21}NO_4$	315	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Kreh and Matusch, 1995
35 2α-hydroxy-6-*O*-methyloduline	$C_{18}H_{21}NO_5$	331	IR, MS, ^{1}H NMR, ^{13}C NMR	Almanza *et al.*, 1996
81 pallidiflorine	$C_{34}H_{40}N_2O_7$	588	IR, MS, ^{1}H NMR, ^{13}C NMR	Codina *et al.*, 1990
68 pancracine	$C_{16}H_{17}NO_4$	287	UV, MS, ^{1}H, ^{13}C NMR, CD	Ali *et al.*, 1984
44 papyramine	$C_{18}H_{23}NO_4$	317	UV IR, MS, ^{1}H NMR, ^{13}C NMR	Suau *et al.*, 1990a Bastida *et al.*, 1990a
45 6-epipapyramine	$C_{18}H_{23}NO_4$	317	UV IR, MS, ^{1}H NMR, ^{13}C NMR	Suau *et al.*, 1990a Bastida *et al.*, 1990a
46 *O*-methyl-6-epipapyramine	$C_{19}H_{25}NO_4$	331	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Suau *et al.*, 1990a
11 pluviine	$C_{17}H_{21}NO_3$	287	UV IR	Kirby and Tiwari, 1966 Boit *et al.*, 1957a
13 9-*O*-demethyl-pluviine	$C_{16}H_{19}NO_3$	273	UV, MS, ^{1}H NMR, ^{13}C NMR	Kreh *et al.*, 1995b
14 1-*O*-acetyl-9-*O*-demethylpluviine	$C_{18}H_{21}NO_4$	315	UV, MS, ^{1}H NMR, ^{13}C NMR	Kreh *et al.*, 1995b
15 1,9-*O*-diacetyl-9-*O*-demethylpluviine	$C_{20}H_{23}NO_5$	357	UV, MS, ^{1}H NMR, ^{13}C NMR	Kreh *et al.*, 1995b
12 norpluviine	$C_{16}H_{19}NO_3$	273	UV IR	Kirby and Tiwari, 1966 Sandberg and Michel, 1968
2 poetaminine	$C_{18}H_{19}NO_5$	329	UV, IR	Döpke and Dalmer, 1965b

Table 6.3 Continued

*Alkaloid**	*Formula*	*MW*	*Spectroscopic data*	*References*
38 poetinatine	$C_{20}H_{23}NO_6$	373	IR, MS, ^{1}H NMR	Döpke and Nguyen, 1974
60 pretazettine	$C_{18}H_{21}NO_5$	331	UV, IR, MS, ^{1}H NMR	Ghosal *et al.*, 1984
			CD	Wagner *et al.*, 1996
3 pseudolycorine	$C_{16}H_{19}NO_4$	289	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Llabrés *et al.*, 1986a
4 1-*O*-acetyl-pseudolycorine	$C_{18}H_{21}NO_5$	331	UV, IR, MS, ^{1}H NMR	Llabrés *et al.*, 1986a
5 2-*O*-acetyl-pseudolycorine	$C_{18}H_{21}NO_5$	331	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Llabrés *et al.*, 1986a
6 9-*O*-methyl-pseudolycorine	$C_{17}H_{21}NO_4$	303	UV, MS, ^{1}H NMR	Evidente *et al.*, 1984
			IR	Fales *et al.*, 1956
22 roserine	$C_{18}H_{22}NO_3$	300	MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1992b
58 tazettine	$C_{18}H_{21}NO_5$	331	UV	Wildman and Kaufman, 1954
			IR	Trimiño *et al.*, 1987
			MS, ^{1}H NMR,	Ghosal *et al.*, 1984
			^{13}C NMR	Crain *et al.*, 1971
			CD	Wagner *et al.*, 1996
			X-Ray	Ide *et al.*, 1996
21 tortuosine	$C_{18}H_{18}NO_3$	296	IR, MS, ^{1}H NMR, ^{13}C NMR	Bastida *et al.*, 1995a
65 trisphaeridine	$C_{14}H_9NO_2$	223	UV, IR, MS, ^{1}H NMR	Suau *et al.*, 1990b
			^{13}C NMR	Viladomat *et al.*, 1997
20 vasconine	$C_{17}H_{16}NO_2$	266	IR, MS	Bastida *et al.*, 1992a
			^{1}H NMR, ^{13}C NMR	Almanza *et al.*, 1996
39 vittatine	$C_{16}H_{17}NO_3$	271	UV, IR, MS, ^{1}H NMR, ^{13}C NMR	Vázquez-Tato *et al.*, 1988
			^{1}H NMR	Pabuççuoglu *et al.*, 1989
			^{13}C NMR	Frahm *et al.*, 1985
			CD	Wagner *et al.*, 1996

Note
* Alkaloids are listed in alphabetical order with disruption of the derivatives that follow the principal alkaloid.

Proton nuclear magnetic resonance

^{1}H-NMR spectroscopy gives important information about the different types of Amaryllidaceae alkaloids. Several early contributions about homolycorine and crinane-haemanthamine type alkaloids were made by Hawksworth *et al*. (1965) and Haugwitz *et al*. (1965). In the last decade, the routine use of 2D-NMR techniques (Correlated Spectroscopy (COSY), Nuclear Overhauser Enhancement and Exchange Spectroscopy (NOESY), Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY), etc.) has facilitated the structural assignments and the settling of their stereochemistry.

Lycorine type

This group has been subject to several ^{1}H-NMR studies, and lycorine **(1)** as well as its main derivatives have been completely assigned. The general characteristics of the ^{1}H-NMR spectra are:

1 Two singlets for the *para*-oriented aromatic protons in the range δ 6.5–7.2 ppm.
2 A unique olefinic proton around 5.5 ppm.
3 Two doublets as an AB system corresponding to the benzylic protons of C-6.
4 The deshielding observed in the β protons of positions 6 and 12 in relation to their α-homologs is due to the effect of the *cis*-lone pair of the nitrogen atom.
5 Like almost all other lycorine type examples, the alkaloids isolated from narcissus show a *trans* B/C ring junction, the coupling constant being $J_{4a,10b} \sim 11$Hz.

In the plant, the alkaloid lycorine **(1)** is particularly vulnerable to the oxidation processes, giving several ring-C aromatised products.

Homolycorine type

This group includes lactone, hemiacetal or cyclic ether alkaloids. The general traits for these types of compounds could be summarised as follows:

1 Two singlets for the *para*-oriented aromatic protons. In lactone alkaloids, differentiation of the H-7 and H-10 signals is readily made by virtue of the deshielding of H-7 by effect of the *peri*-carbonyl group.
2 The hemiacetal alkaloids always show the substituent at C-6 in α-disposition, and the benzylic proton H-6β appears as a singlet between 5–6 ppm, depending on the substituent at C-6.
3 The majority of compounds belong to a single enantiomeric series containing a *cis* B/C ring junction, which is made clear by the small size of the coupling constant $J_{1,10b}$. In the *Narcissus* genus no exception to this rule has been observed.
4 The large coupling constant between H-4a and H-10b ($J_{4a,10b} \sim 10$ Hz), is only consistent with a *trans*-diaxial relationship.
5 In general, the C ring presents a vinylic proton around 5.5 ppm.
6 The singlet corresponding to the *N*-methyl group is in the range δ 2.0–2.2 ppm, its non-existence being very unusual.
7 If position 2 is substituted by an OH, OMe or OAc group, it always displays an α-disposition.
8 The H-12α is more deshielded than H-12β as a consequence of the *cis*-lone pair of the nitrogen atom.

An interesting study of homolycorine type alkaloids with a saturated ring C has been made by Jeffs *et al*. (1988). They describe empirical correlations of *N*-methyl chemical shifts with stereochemical assignments of the B/C and C/D ring junction.

Crinine – haemanthamine types

The absolute configuration of these alkaloids, which allows both series to be differentiated, is determined through the circular dichroism spectrum. The alkaloids of narcissus belong to the haemanthamine type, while in genera such as *Brunsvigia, Boophane*, etc., the crinine type alkaloids are predominant. It is also noteworthy that the alkaloids isolated from narcissus do not show additional substitutions in the aromatic ring apart from those of C-8 and C-9. On the contrary, in the genera where crinine type alkaloids predominate, the presence of compounds with a methoxy substituent at C-7 is quite common. Thus, taking into account the previous considerations, haemanthamine type alkaloids show the following characteristics:

1 Two singlets for the *para*-oriented aromatic protons in the range δ 6.4–7.0 ppm.
2 Using $CDCl_3$ as the solvent, the magnitude of the coupling constants between each olefinic proton (H-1 and H-2) and H-3 gives information about the configuration of the C-3 substituent. Thus, in those alkaloids in which the two-carbon bridge (C-11 and C-12) was *cis* to the substituent at C-3, H-1 shows an allylic coupling with H-3 ($J_{1,3} \sim 1$–2 Hz) and H-2 a smaller coupling with H-3 ($J_{2,3} \sim 0$–1.5 Hz), as occurs in crinamine **(53)**. On the contrary, in the corresponding C-3 epimeric series, e.g. haemanthamine **(49)**, a larger coupling between H-2 and H-3 ($J_{2,3}$ 5 Hz) is shown, the coupling between H-1 and H-3 not being detectable.
3 It is frequently possible to observe an additional coupling of H-2 with the equatorial H-4β, in a W-mechanism.
4 The proton H-4α shows a large coupling with H-4a ($J_{4\alpha,4a} \sim 13$ Hz) due to their *trans*-diaxial position, characteristic of the haemanthamine series.
5 Two doublets for an AB system, corresponding to the benzylic protons of position C-6.
6 The pairs of alkaloids with a hydroxy substituent at C-6, like papyramine/6-epipapyramine **(44/45)**, haemanthidine/6-epihaemanthidine **(51/52)**, etc., appear as a mixture of epimers not separable even by HPLC.
7 Also in relation with position C-6, it is interesting to note that ismine **(67)**, a catabolic product from the haemanthamine series, shows a restricted rotation around the biarylic bond, which makes the methylenic protons at the benzylic position magnetically non-equivalent.

Tazettine type

Although tazettine **(58)** is one of the most widely distributed alkaloids in the Amaryllidaceae family, it was found to be an extraction artefact of pretazettine **(60)** (Wildman and Bailey, 1967).

The presence of an *N*-methyl group (2.4–2.5 ppm) in tazettine type alkaloids immediately distinguishes them from the haemanthamine type, from which they proceed biosynthetically. Moreover the ^{1}H-NMR spectrum always shows the signal corresponding to the methylenedioxy group.

We have also included the alkaloid obesine **(62)** in this group, although it exhibits some structural differences with the skeleton type.

Galanthamine type

Among the Amaryllidaceae alkaloids, only the galanthamine type shows an *ortho*-coupling constant between both aromatic protons of ring A. The general characteristics of their ^{1}H-NMR spectra are:

1 Two doublets for the two *ortho*-oriented aromatic protons with a coupling constant of $J_{7,8} \sim 8$Hz.
2 The assignment of the substituent stereochemistry at C-3 is made in relation with the coupling constants of the olefinic protons H-4 and H-4a. When coupling constant $J_{3,4}$ is about 5 Hz, the substituent is pseudoaxial, while if it is $\sim$0 Hz this indicates that the substituent at C-3 is pseudoequatorial.
3 Two doublets as an AB system corresponding to the benzylic protons of C-6.
4 The existence of the furan ring results in a deshielding effect in H-1.
5 This type of alkaloid often shows an *N*-methyl group but occasionally *N*-formyl or *N*-acetyl derivatives have been reported.

Carbon^{13} nuclear magnetic resonance

^{13}C-NMR spectroscopy has been extensively used for determining the carbon framework of Amaryllidaceae alkaloids, and the major contributions are due to Crain *et al*. (1971), Zetta *et al*. (1973) and Frahm *et al*. (1985). The assignments are made on the basis of chemical shifts and multiplicities of the signals (by Distorsionless Enhancement by Polarisation Transfer (DEPT) experiment). The use of 2D-NMR techniques such as Heteromolecular Multiple Quantum Correlation (HMQC) and Heteromolecular Multiple Bond Correlation (HMBC) allows the assignments to be corroborated.

The ^{13}C-NMR spectra of narcissus alkaloids can be divided in two regions. The low-field region (>90 ppm) contains signals of the carbonyl group, the olefinic and aromatic carbons as well as that of the methylenedioxy group. The other signals, corresponding to the saturated carbon resonances, are found in the high-field region, the *N*-methyl being the only characteristic group, easily recognisable by a quartet signal between 40–46 ppm.

The effect of the substituent (OH, OMe, OAc) on the carbon resonances is of considerable importance in localising the position of the functional groups.

The analysis of the spectra allows conclusions to be drawn about the following aspects:

1 The number of methine olefinic carbons.
2 The presence and nature of the nitrogen substituent.
3 The existence of a lactonic carbonyl group.
4 The presence of a quaternary carbon signal assignable to C-10b in the chemical shift range of 42–50 ppm.

It is worth noting that the montanine type alkaloids are distinguished from the other ring type systems by the downfield signal at around 150 ppm, assignable to the quaternary olefinic C-11a.

Mass Spectrometry

Extensive studies on the Mass Spectrometry of Amaryllidaceae alkaloids by electron impact were reported in the 1960s and 1970s (Fales *et al.*, 1969, 1970; Ibuka *et al.*, 1966; Onyiriuka and Jackson, 1978; Samuel, 1975). The fragmentation patterns in the Electronic Impact Mass Spectrometry (EIMS) of various skeletal types are fairly well documented and have considerable diagnostic value.

Lycorine type

The molecular ion appears as a quite intense peak, and generally suffers the loss of water, as well as C-1 and C-2 and their substituents by a retro Diels-Alder fragmentation (Figure 6.13). The loss of water is not present in the spectra of acetyl derivatives.

The ease of the loss of water from the molecular ion was found to be greatly dependent on the stereochemistry of the C-2 hydroxyl group. Thus, in the mass spectrum of lycorine **(1)**, the relative intensity is low, while in 2-epilycorine it is the base peak (Kinstle *et al.*, 1966).

Homolycorine type

In this structure type, the cleavage of the labile bonds in ring C by a retro Diels-Alder reaction is dominant, generating two fragments: one, the more characteristic, represents the pyrrolidine ring (plus substituents in position 2), and the other (a less abundant fragment) encompasses the aromatic lactone or hemilactone moiety (Figure 6.14).

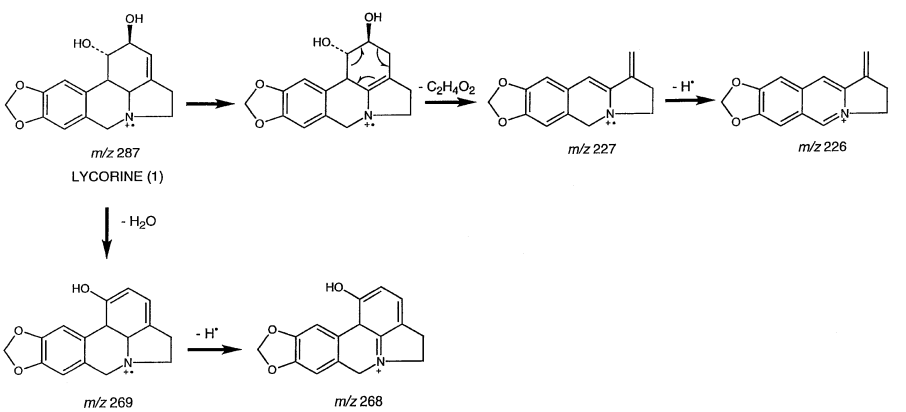


Figure 6.13 Mass fragmentation pattern of lycorine **(1)**.

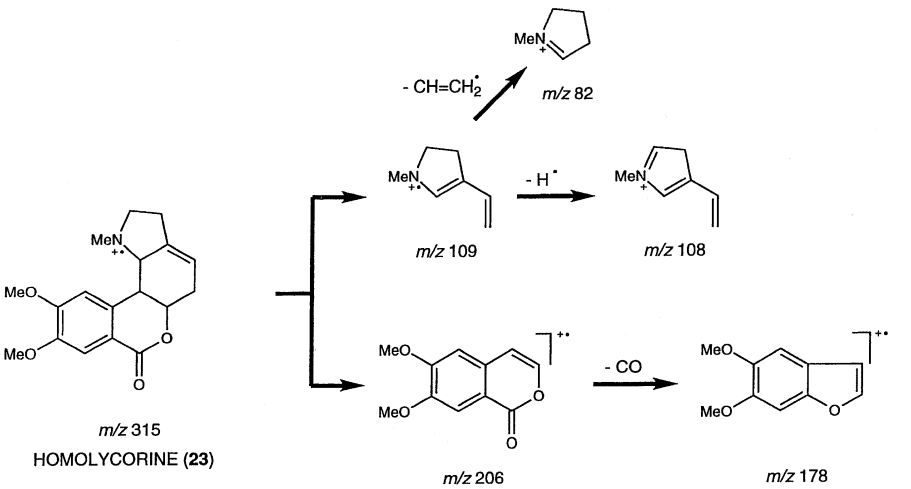


Figure 6.14 Mass fragmentation pattern of homolycorine **(23)**.

A further general and noteworthy feature is the low abundance of the molecular ion in all compounds with a double bond $\Delta^{3,4}$ (Schnoes *et al.*, 1968).

Crinine-haemanthamine types

Several general considerations should be taken into account for these types of alkaloids:

1 The stability of the molecular ion, which is almost always the base peak.
2 The important role played by the aromatic ring in the stabilisation of the ions, which is retained in all fragments of high mass while the nitrogen atom is often lost.
3 The relatively large number of nitrogen-free ions.
4 The fragmentation mechanisms are initiated by the rupture of a bond β to the nitrogen atom which implies the opening of the C-11/C-12 bridge (Longevialle *et al.*, 1973a,b).

(a) Compounds with a saturated ring C and no bridge substituent.
The configuration of the substituent on ring C plays a minor role in the fragmentation process.

(b) Compounds with a double bond ($\Delta^{1,2}$) in ring C and no bridge substituent.
The fragmentation pattern involves ruptures of C-4a/C-10b and C-3/C-4 bonds. A characteristic feature is the loss of a nitrogen-containing moiety, C_3H_5N [M-55].

(c) Compounds with a double bond ($\Delta^{1,2}$) in ring C and a hydroxyl substituent at C-11.

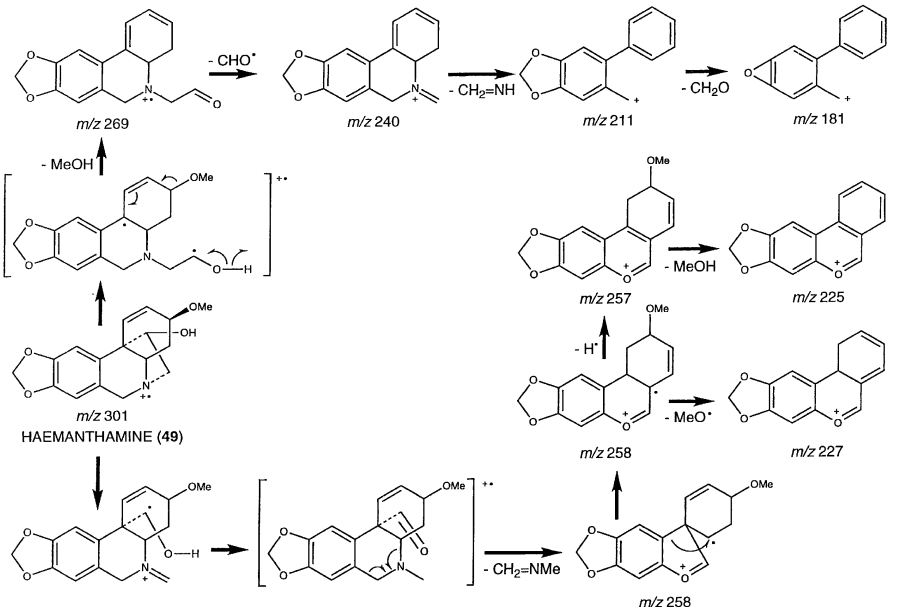


Figure 6.15 Mass fragmentation pattern of haemanthamine **(49)**.

The presence of a hydroxyl group on C-11 is responsible for dramatic changes in the fragmentation pattern (Figure 6.15), and it is profoundly influenced by the stereochemistry. There are three fundamental patterns of fragmentation:

1 Loss of CH_3OH: it is more favourable when the two-carbon bridge and the substituent on C-3 are on the same side of the molecule.
2 Loss of C_2H_6N: the relative significance of the loss of this neutral nitrogen moiety is governed by the ease with which the methanol is eliminated.
3 Loss of CHO: A peak at *m/z* [M-29] due to the loss of an aldehyde radical is present in all compounds of this type.

Tazettine type

Minor changes in stereochemistry are sufficient to cause appreciable differences in the stereoisomers in these kind of structures. Thus, in the MS of tazettine **(58)**, with a β configuration of the methoxyl group at C-3, the dominant ion occurs at *m/z* [M-84], following a C-ring fragmentation by a retro Diels-Alder process. In contrast, the mass spectrum of criwelline **(59)**, which differs only in the configuration of the mentioned methoxyl group, contains a peak of low abundance at *m/z* [M-84] (Figure 6.16). Ions occur in both steroisomers due to the successive loss of a methyl radical and water from the molecular ion (Duffield *et al.*, 1965).

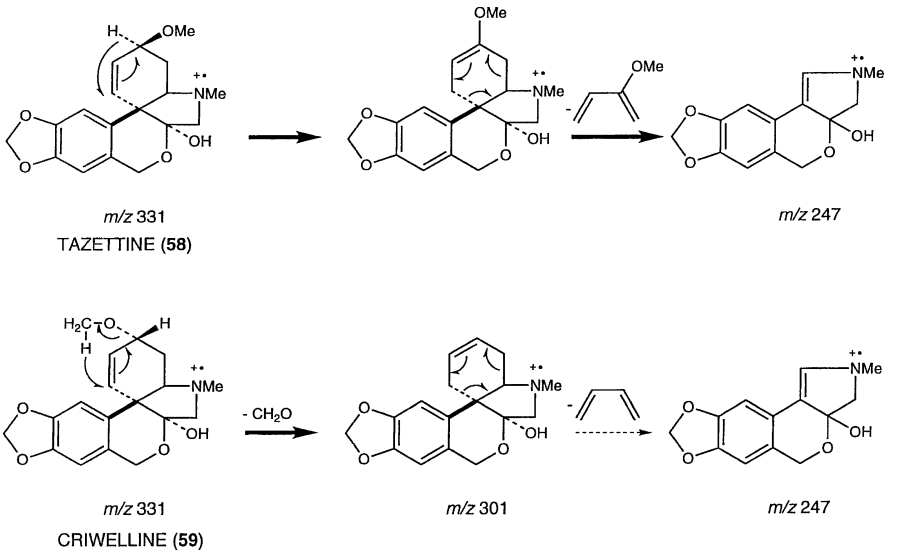


Figure 6.16 Mass fragmentation pattern of tazettine **(58)** and criwelline **(59)**.

Montanine type

The mass spectral fragmentation patterns observed for alkaloids containing the 5,11-methanomorphanthridine nucleus is very depending of the substituents at C-2 and C-3, the nature as well as their particular configuration have a very significant effect. Thus, all the compounds which possess a methoxyl group give rise to an M-31 ion.

The configuration of the C-2 substituent has a considerable effect on the extent to which the retro Diels-Alder fragmentation ion is observed (Figure 6.17). There

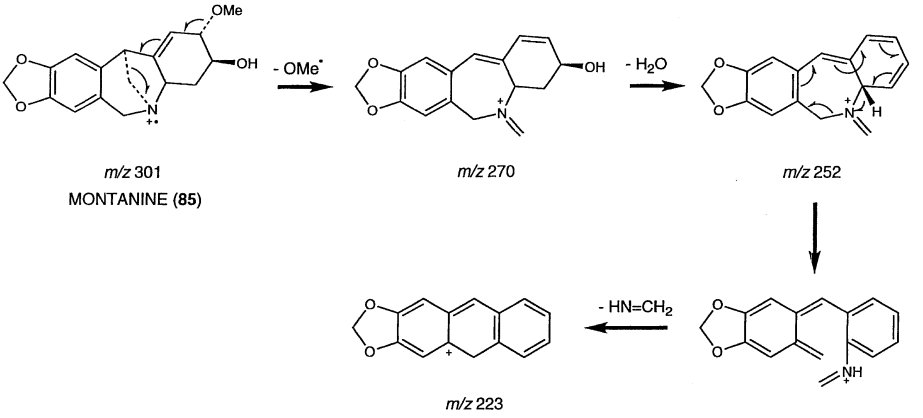


Figure 6.17 Mass fragmentation pattern of montanine **(85)**.

is a definite enhancement of this fragmentation when the C-2 substituent has an α configuration (Wildman and Brown, 1968).

Galanthamine type

In this type of structures, the intense molecular ion, as well as [M-1] peak, the breaking of ring C (losing a C_4H_6O fragment) and the elimination of elements of ring B (including the nitrogen atom) are characteristic (Figure 6.18). This behaviour is similar for the dihydro derivatives (Razakov *et al.*, 1969).

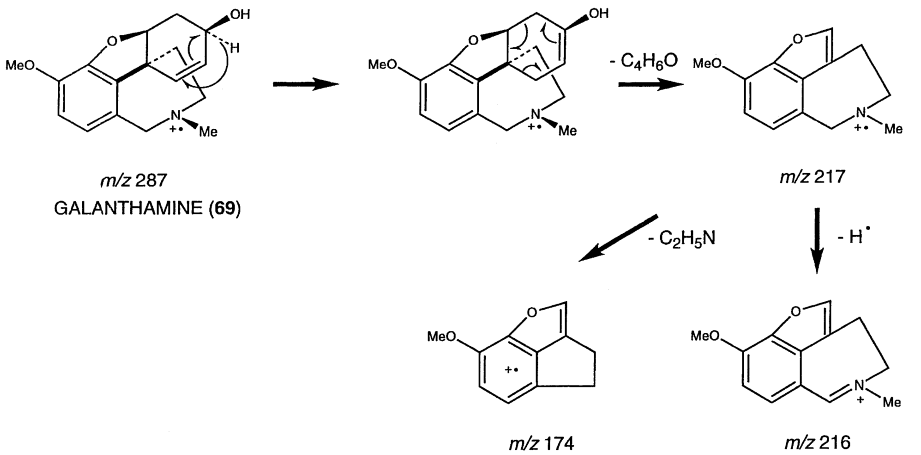


Figure 6.18 Mass fragmentation pattern of galanthamine **(69)**.

BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

Toxic and hallucinogenic effects

Plants of this genus have been used throughout history as a stimulant to induce trance and hallucinations, and as an agent in suicide. It has been known for a long time that daffodil ingestion is very dangerous, resulting in toxic symptoms in both man and animals (Jaspersen-Schib *et al.*, 1996; Wilson, 1924; Wu *et al.*, 1965). After ingestion of *N. pseudonarcissus* or *N. jonquilla* (Vigneau *et al.*, 1984), the first visible symptoms are salivation, acute abdominal pains, nausea, vomiting and diarrhoea, followed by neurological (trembling, convulsions, etc.) and cardiac sequel, and sometimes resulting in death if eaten in larger quantities. There are many cases of poisoning in which daffodil bulbs were cooked by mistake in the place of leeks or onions. The bulbs of *N. poeticus* are more dangerous than those of *N. pseudonarcissus*, being powerfully emetic and irritant. In turn, *N. papyraceus* is believed to be toxic for herbivorous mammals; in this case, the alkaloid content is five times higher in the aerial part than in the bulbs (Suau *et al.*, 1990a). The good news is that the bulb tastes awful, making it highly unlikely that anyone could keep down even one bite. In cases of massive ingestion, activated charcoal, salts and laxatives are adminis-

tered. When symptoms are severe, atropine sulphate is given by intravenous injection (Junko *et al*., 1994).

Some *Narcissus* species can produce harmful effects without being swallowed. Thus, species like *N. bulbocodium* must not be placed in confined spaces because the scent of the flowers, when present in any quantity, can produce headaches and even vomiting. The association of alkaloids with essential oils is found in oils of patchouli, juniper, orange and jonquil absolute (Maurer, 1994). In turn, *N. pseudonarcissus* shows irritant and allergenic properties on contact with animals and men (Bruynzeel, 1997; Bruynzeel *et al*., 1993; De Jong *et al*., 1998; Gonçalo *et al*., 1987; Güneser *et al*., 1996). The compounds responsible for the irritation are not known, but alkaloids are thought to be involved (Gude *et al*., 1988). Additionally, extracts of the bulbs, when applied to open wounds, can produce staggering, numbness of the whole nervous system and paralysis of the heart. The mucilage secreted by bulbs can also produce harmful effects in plant species such as rose, rice and cabbage, inhibiting seed germination and seedling growth (Bi *et al*., 1998; Van Doorn, 1998).

Traditional medicinal usage

Despite their lethal potential, the extracts of various narcissus plants have been used in traditional medicine to treat a variety of medicinal problems. This aspect is covered in Chapter 2 of this volume.

Biological activities for extracts of *Narcissus*

Several *Narcissus* extracts have shown the following activities: antiviral (Abou-Karam and Shier, 1990; Furusawa *et al*., 1973, 1975; Papas *et al*., 1973; Ramanathan *et al*., 1968; Suzuki *et al*., 1974; Vacik *et al*., 1979; Van den Berghe *et al*., 1978), prophage induction (Dornberger and Lich, 1982), antimicrobial (Dornberger and Lich, 1982; Ieven *et al*., 1979), antifungal (Chaumont and Senet, 1978; Chaumont *et al*., 1978), cytotoxic (Abou-Karam and Shier, 1990; Furusawa *et al*., 1973, 1980), antitumour (Furusawa *et al*., 1972, 1973, 1975; Papas *et al*., 1973; Suzuki *et al*., 1974; Wu *et al*., 1965), antimitotic (Ikram, 1983), hipotensive (Chiu *et al*., 1992), emetic (Wu *et al*., 1965), antifertility (Matsui *et al*., 1967), antinociceptive (Çakici *et al*., 1997), chronotropic (Chiu *et al*., 1992), pheromone (Keiser *et al*., 1975), plant growth inhibitor and allelopathic (Bi *et al*., 1998; Ceriotti, 1967; Chiu *et al*., 1992; Van Doorn, 1998).

Biological and pharmacological activities of *Narcissus* compounds

The compounds responsible for the majority of the above-mentioned activities are the alkaloids. However, the mannose-binding lectins have also received much interest recently (Barre *et al*., 1996; Van Damme *et al*., 1998).

The different pharmacological and/or biological properties exhibited by the alkaloids from the genus *Narcissus* are shown in Table 6.4, but only some of the activities of a reduced number of these alkaloids are known. The most extensively studied effect is that of non-specific inhibition. The relationship of chemical structure

and biological activity is largely unknown (Chiu *et al*., 1992), and further studies of several alkaloids still remain to be done for therapeutic purposes. The best-studied alkaloids in this group are galanthamine, lycorine, narciclasine and pretazettine, which possess a diversity of pharmacological activities.

Table 6.4 Biological and pharmacological activities of *Narcissus* alkaloids

Alkaloid	*Activity*	*References*
10 caranine	* Weak analgesic * Convulsant and hypotensive * Acetylcholinesterase inhibitor * Active against the murine P-388 lymphocytic leukaemia (as acetylcaranine)	CHCD, 1996 Pettit *et al.*, 1984
53 crinamine	* Powerful transient hypotensive in dogs * Respiratory depressant * Moderate antimalarial * Antimicrobial * Moderately active against murine Rauscher viral leukaemia * Cytotoxic against Molt 4 lymphoid and LMTK fibroblastic cell lines * Weak cytotoxic against HepG2 hepatoma	CHCD, 1996 Furusawa *et al.*, 1980 Likhitwitayawuid *et al.*, 1993 Weniger *et al.*, 1995
36 dubiusine	* Cytotoxic against non-tumoural LMTK cells * Moderately active against Molt 4 lymphoma	Weniger *et al.*, 1995
69 galanthamine	* Powerful analgesic as strong as morphine * Anticonvulsive * Hypotensive * Inductor of hypothermia in rat * Acetylcholinesterase inhibitor with peripheral and central pharmacological effects * Highly selective for acetylcholinesterase versus butyrylcholinesterase (more than 50-fold greater) * Centrally-acting acetylcholinesterase inhibitor which has shown potential for the treatment of Alzheimer's disease * Attenuates or reverses cognitive deficits induced by drugs – and lesions – in animal models * Acts as noncompetitive nicotinic receptor agonist	Bores *et al.*, 1996 Chao *et al.*, 1965 CHCD, 1996 Cozanitis, 1977 Cozanitis and Rosenberg, 1974 Cozanitis and Toivakka, 1978 Cozanitis *et al.*, 1983 Fulton and Benfield, 1996 Ghosal *et al.*, 1985b Han *et al.*, 1992 Harvey, 1995 Kewitz, 1996 Mihailova and Yamboliev, 1986 Mihailova *et al.*, 1985 Mihailova *et al.*, 1987 Mihailova *et al.*, 1989 Pereira *et al.*, 1994 Radicheva *et al.*, 1996 Riemann *et al.*, 1994 Schuh, 1976 Storch *et al.*, 1995 Suzuki *et al.*, 1974

Table 6.4 Continued

Alkaloid	*Activity*	*References*

	* Combines both physostigmine and neostigmine properties:
	  * Like physostigmine, reverses opioid-induced respiratory depression, but not the concomitant analgesia
	  * Like neostigmine, antagonizes muscle paralysis induced by *d*-tubocurarine, also antagonizes the ganglionic blockade and increases the contraction response
	* As hydrobromide has shown several central and peripheral effects:
	  * Has central effects such as antagonism of the respiratory depressant effect of morphine-like compounds
	  * Is capable of penetrating the blood-brain barrier and it is used in the treatment of the central effects of scopolamine (hyoscine) intoxication. It has certain advantages over physotigmine for this purpose
	  * Shortens REM latency, increases REM density, and reduces slow wave sleep
	  * Is able to reverse the central anticholinergic syndrome
	  * used for its anticholinesterase activity in the treatment of disturbances in the peripheral sympathetic synaptic transmission
	  * Reverses the neuromuscular blocking effect of curare-type muscle relaxants and has been used safely in anaesthesia (postsurgery) and in the treatment of various neurologic disorders (pareses, paralysis of different origins, myasthenia gravis, progressive muscular dystrophy, etc.)
	  * Affects the contraction of skeletal muscle not only at the level of neuromuscular junction, but also by enhancing the efferent impulses as a result of the reflexive excitation centre in the spinal cord
		Svensson and Nordberg, 1997
		Sweeney *et al.*, 1988
		Sweeney *et al.*, 1989
		Thomsen and Kewitz, 1990
		Vigneau *et al.*, 1984
		Weniger *et al.*, 1995
		Westra *et al.*, 1986
		Zakirov and Umarova, 1971

Table 6.4 Continued

Alkaloid	*Activity*	*References*
69 galanthamine (*contd*.)	* Is over 90% bioavailable after oral administration and has a plasma elimination half-life of approximately 6 hours. * Nausea and vomiting are the most commonly reported adverse effects; liver toxicity has not been reported to date * Energix®, a preparation composed of galanthamine, increases endurance during exercise and delays the onset of fatigue * Used in a pharmaceutical composition for treatment of alcoholism * Cytotoxic against non-tumoural LMTK cells * Produces poisoning of digestive, respiratory, neuromuscular and central nervous systems	
70 epigalanthamine	* More hypotensive and less toxic than galanthamine * Anticholinesterase activity lower than galanthamine	Bazhenova *et al.*, 1971 Ghosal *et al.*, 1985b Thomsen *et al.*, 1990 Yamboliev *et al.*, 1988 Zakirov and Umarova, 1971
72 norgalanthamine	* Cytotoxic against Molt 4 lymphoid and LMTK fibroblastic cell lines	Weniger *et al.*, 1995
74 *N*-formyl-norgalanthamine	* Moderate cytotoxic against Molt 4 lymphoma and HepG2 hepatoma * Cytotoxic against non-tumoural LMTK cells	Weniger *et al.*, 1995
7 galanthine	* Analgesic * Hypotensive * Weak cytotoxic against Molt 4 lymphoid cells	CHCD, 1996 Ghosal *et al.*, 1985b Weniger *et al.*, 1995
49 haemanthamine	* Hypertensive * Cytotoxic against a variety of cultured cells (*in vitro*) * Cytotoxic against fibroblastic LMTK cells * Moderately active against Molt 4 lymphoid cells * Moderately active against Rauscher viral leukaemia * Inhibitor of HeLa cell growth * Inhibitor of protein synthesis, blocking the peptide bond formation step on the peptidyl transferase centre of the 60S ribosomal subunit	Báez and Vázquez, 1978 Codina *et al.*, 1992b Furusawa *et al.*, 1980 Ghosal *et al.*, 1985b Jiménez *et al.*, 1975b Jiménez *et al.*, 1976 Lin *et al.*, 1995 Weniger *et al.*, 1995

Table 6.4 Continued

Alkaloid	*Activity*	*References*
	* Slightly reduces DNA synthesis, whereas RNA synthesis is practically unaffected	
51 haemanthidine	* Analgesic * Antiinflamatory * Active against A-431, KB, Lu1, Me12 and ZR-75-1 cell lines * Significantly active against LNCaP and HT cell lines	Antoun *et al.*, 1993 Çitoglu *et al.*, 1998 Tanker *et al.*, 1996
30 hippeastrine	* Antiviral. Active against *Herpes simplex* type 1 * Weak insect antifeedant * Significantly active against the LNCaP and HT cell lines * Cytotoxic against non-cancerous LMTK cells * Weak cytotoxic against Molt 4 lymphoid cells	Antoun *et al.*, 1993 Renard-Nozaki *et al.*, 1989 Weniger *et al.*, 1995
23 homolycorine	* Inductor of delayed hypersensitivity in animals * Cytotoxic against fibroblastic LMTK cells	Gude *et al.*, 1988 Weniger *et al.*, 1995
24 8-*O*-demethyl-homolycorine	* Cytotoxic against fibroblastic LMTK cells * Weak cytotoxic against Molt 4 lymphoid cells	Weniger *et al.*, 1995
29 9-*O*-demethyl-2α- hydroxy-homolycorine	* Cytotoxic against fibroblastic LMTK cells * Moderately active against Molt 4 lymphoid cells	Weniger *et al.*, 1995
67 ismine	* Cytotoxic against Molt 4 lymphoid and LMTK fibroblastic cell lines	Weniger *et al.*, 1995
77 lycoramine	* Central anticholinesterase activity stronger than galanthamine * Inhibits the formation of peptide bond in protein synthesis * Like neostigmine, antagonizes muscle paralysis induced by *d*-tubocurarine, also antagonizes the ganglionic blockade, and increases the contraction response * Produces acute poisoning of digestive, respiratory, cardiovascular, neuromuscular and central nervous systems	Chao *et al.*, 1965 Missoum *et al.*, 1997 Vigneau *et al.*, 1984
31 lycorenine	* Analgesic * Weak hypotensive * Vasodepressor action ascribed to	Codina *et al.*, 1992b Ghosal *et al.*, 1985b Miyasaka and Hiramatsu, 1980

Table 6.4 Continued

Alkaloid	*Activity*	*References*
31 lycorenine (*contd*.)	maintenance of its α-adrenergic blocking action in conjugation with the reduction of the spontaneous sympathetic nerve activity, and produce bradycardia by modifying vagal activity	Miyasaka *et al.*, 1979 Weniger *et al.*, 1995
	* Cytotoxic against murine LMTK and human HepG2 cell lines	
32 *O*-methyllycorenine	* Cytotoxic against fibroblastic LMTK cells * Weak cytotoxic against HepG2 hepatoma	Weniger *et al.*, 1995
1 lycorine	* Emetic * Analgesic * Antiinflammatory * Respiratory stimulant * Expectorant * Used to treat bronchitis and bronchial asthma * Relaxant of isolated epinephrine-precontracted pulmonary artery * Increases contractility and rate of isolated perfused heart. These effects are mediated by stimulation of β-adrenergic receptors * Specific inhibitor of ascorbic acid biosynthesis. Seems to act as a powerful inhibitor of the mitocondrial L-galactono-γ-lactone dehydrogenase * Most of the effects of lycorine on physiological processes have been ascribed to its ability to inhibit ascorbic acid biosynthesis *in vivo* * The ability to inhibit the ascorbic acid biosynthesis has made this substance a valuable tool for studying the ascorbic acid dependent reactions * Inhibitor of cyanide-resistant respiration. Ascorbic acid is needed for the synthesis of hydroxyproline-containing proteins, specifically utilised for the development of KCN-resistant respiration * Inhibitor of peroxidase enhancement, which seems related with the synthesis of hydroxyproline-containing proteins	Abbassy *et al.*, 1998 Abdalla *et al.*, 1993 Arrigoni *et al.*, 1975 Arrigoni *et al.*, 1996 Arrigoni *et al.*, 1997a Arrigoni *et al.*, 1997b Báez and Vázquez, 1978 Campbell *et al.*, 1998 Carrasco *et al.*, 1975 Chattopadhyay *et al.*, 1984 CHCD, 1996 Çitoglu *et al.*, 1998 Córdoba-Pedregosa *et al.*, 1996 Davey *et al.*, 1998 De Gara *et al.*, 1994 Del Giudice *et al.*, 1997 Evidente *et al.*, 1983a Evidente *et al.*, 1983b Evidente *et al.*, 1985 Evidente *et al.*, 1986 Gabrielsen *et al.*, 1992 Ghosal *et al.*, 1984 Ghosal *et al.*, 1985b Ghosal *et al.*, 1985c Ghosal *et al.*, 1988b Ghosal *et al.*, 1989b Ghosal *et al.*, 1990 Ieven *et al.*, 1982 Ieven *et al.*, 1983 Jiménez *et al.*, 1975b Jiménez *et al.*, 1976 Kushida *et al.*, 1997 Likhitwitayawuid *et al.*, 1993 Lin *et al.*, 1995 Liso *et al.*, 1984 Liso *et al.*, 1985 Makhkamova and Safonova, 1994 Massardo *et al.*, 1994 Papas *et al.*, 1973 Renard-Nozaki *et al.*, 1989 Schultz *et al.*, 1996

Table 6.4 Continued

Alkaloid	*Activity*	*References*
	* Powerful inhibitor of growth and cell division in higher plants, algae and yeasts, inhibiting the cell cycle during interphase. Ascorbic acid is required for cell division * Inhibitor of cell division in rat fibroblasts. Ascorbic acid is required for cell division * Inductor of flat morphology in K-ras-NRK cells (transformed fibroblasts) * Inhibitor of protein synthesis, blocking the peptide bond formation step * Plant growth inhibitor by inhibition of protein synthesis * The effects on ascorbic acid biosynthesis occur at concentrations below those at which protein synthesis is affected * Moderate antitumoural. Its mechanism of action is thought to be through inhibition of protein synthesis at the ribosomal level * Decreases the growth of several viruses through its inhibitory action on viral protein synthesis * Active against several RNA and DNA viruses * Does not inhibit the activity of reverse transcriptase * Inhibitor of DNA synthesis * Is able to differentiate between cells containing mitocondrial DNA and those lacking it. Inhibits growth of strains containing mitocondrial DNA * Cytotoxic against a variety of cultured cell lines * Inhibitor of HeLa cells growth * Antimalarial * Weak protozoicide * Inhibitor of germination of seeds and growth of roots. Lycorine-1-*O*-β-D-glucose has the reverse effect, and may also produce mitogenic activity in animal cells * Ungeremine, a natural metabolite of lycorine, is	Singh and Pant, 1980 Tanker *et al.*, 1996 Van den Berghe *et al.*, 1986 Vrijsen *et al.*, 1986 Weniger *et al.*, 1995 Yui *et al.*, 1998

Table 6.4 Continued

Alkaloid	*Activity*	*References*
1 lycorine (*contd*.)	responsible, at least in part, for the growth-inhibitory and cytotoxic effects of lycorine * Certain bacterias transform lycorine into pancrassidine (less cytotoxic than ungeremine) * The changes observed in response to stress suggest its role in protective and repair mechanisms of producer plants * Insecticide * Insect antifeedant	
61 3-epimacronine	* Weak cytotoxic against human Molt 4 and murine LMTK cell lines	Weniger *et al.*, 1995
40 maritidine	* Antineoplastic	Alarcón *et al.*, 1986 Youssef and Frahm, 1998
27 masonine	* Inductor of delayed hypersensitivity in animals	Gude *et al.*, 1988
82 mesembrenone	* Cytotoxic against Molt 4 lymphoid cells * Weak cytotoxic against fibroblastic LMTK cells	Weniger *et al.*, 1995
63 narciclasine	* Antimitotic and cell growth inhibitor * Strong tumour inhibitor. One of the most important antineoplastic Amaryllidaceae alkaloids * Active against larynx and cervix carcinomas * Inhibitor of growth of Ehrlich tumour cells * Inhibitor of HeLa cells growth * No effect has been observed towards solid tumours (sarcoma 180) * Inhibitor of protein synthesis in eukariotic ribosomes, blocking the peptide bond formation on the 60S ribosomal subunit. * Resistance to narciclasine in a mutant strain of *Saccharomyces cerevisiae* is due to an alteration on the peptidyl transferase centre * Reduces DNA synthesis, whereas RNA synthesis is practically unaffected * Enhances the uptake of uridine and stimulates the synthesis of	Abou-Donia *et al.*, 1991 Aller, 1981 Báez and Vázquez, 1978 Bi *et al.*, 1998 Carrasco *et al.*, 1975 Ceriotti *et al.*, 1967 CHCD, 1996 Evidente, 1991 Evidente *et al.*, 1986 Gabrielsen *et al.*, 1992 Ghosal *et al.*, 1989a Hua *et al.*, 1997 Jiménez *et al.*, 1975a Jiménez *et al.*, 1975b Jiménez *et al.*, 1976 Keck and Fleming, 1978 Pettit *et al.*, 1986 Pettit *et al.*, 1993 Pettit *et al.*, 1995a Pettit *et al.*, 1995b Rodríguez-Fonseca *et al.*, 1995 Veronese *et al.*, 1991

Table 6.4 Continued

Alkaloid	*Activity*	*References*
	pre-rRNA (38S) and low-molecular weight (4–5 S) RNA * Inhibitor of ascorbic acid biosynthesis *in vivo* * Inhibitor of cell division in plant tissue cultures * Inhibitor of seed germination and seedling growth in a dose-dependant manner, interacting with hormones in some physiological responses. The *O*-glucoside of narciclasine has the reverse effect * Strong antibiotic. Active against *Corynebacterium fascians* * Active against RNA-containing flaviviruses and bunyaviruses * Narciclasine-4-*O*-β-D-glucopyranoside has shown cytotoxic and antitumour activity very similar to narciclasine * The peculiar activity of narciclasine seems to arise from the functional groups and conformational freedom of its C-ring	
76 narwedine	* Hypotensive * Increases the amplitude and frequency of respiratory movements * Increases the amplitude and decreases the frequency of cardiac contractions * Decreases soporific effects of ethanol and barbiturics * Increases the analgesic effect of morphine * Protective against thiopental poisoning * Enhances the effects of caffeine, corazole, arecoline, and nicotine * Evaluated as potential agent for treatment of Alzheimer's disease * Probably acts primarily on *m*-cholinoreactive structures of the brain	Bazhenova *et al.*, 1971 Ghosal *et al.*, 1985b Szewczyk *et al.*, 1995 Zakirov and Umarova, 1971
44 papyramine	* Cytotoxic against fibroblastic LMTK cells * Weak cytotoxic against human tumoural cell lines Molt 4 and HepG2	Weniger *et al.*, 1995

Table 6.4 Continued

Alkaloid	*Activity*	*References*
60 pretazettine	* Analgesic * Anticancer activity * Active against murine Rauscher viral leukaemia * Active against spontaneous AKR T cells in mice * Active against intraperitoneally and subcutaneously implanted LLC (Lewis lung carcinomas) * Effective against Ehrlich ascites tumour, a non viral transplantable tumour * Cytotoxic against fibroblastic LMTK and Molt 4 lymphoid cell lines * Inhibitor of HeLa cell growth * Inhibitor of protein synthesis in eukariotic ribosomes, blocking the peptide bond formation on the 60S ribosomal subunit. * Slightly reduces DNA synthesis; has no effect on RNA synthesis * Active against *Herpes simplex* type 1 (HS-1) * Active against neurotropic RNA viruses * Potent inhibitor of viral reverse transcriptase of RNA tumour viruses, binding to the polymerase enzyme * Demonstrates synergistic effect in combination with standard cytotoxic drugs * In combination with ryllistine inhibits nucleic acid synthesis * Can be administrated over a long period of time without apparent toxicity	Antoun *et al.*, 1993 Báez and Vázquez, 1978 CHCD, 1996 Furusawa and Furusawa, 1986 Furusawa and Furusawa, 1988 Furusawa *et al.*, 1975 Furusawa *et al.*, 1976b Furusawa *et al.*, 1978 Furusawa *et al.*, 1979 Furusawa *et al.*, 1980 Furusawa *et al.*, 1981 Furusawa *et al.*, 1983 Gabrielsen *et al.*, 1992 Ghosal and Razdan, 1984 Ghosal *et al.*, 1985b Jiménez *et al.*, 1975b Jiménez *et al.*, 1976 Kobayashi *et al.*, 1980 Renard-Nozaki *et al.*, 1989 Rigby *et al.*, 1998 Suzuki *et al.*, 1974 Van den Berghe *et al.*, 1986 Weniger *et al.*, 1995 Zee-Cheng *et al.*, 1978
3 pseudolycorine	* Effective against murine Rauscher leukaemia in mice without apparent toxicity * Inhibitor of protein synthesis in tumour cells at the step of peptide bond formation. It has a different binding site than lycorine on the peptidyl transferase centre of the 60S ribosomal subunit * Reduces DNA synthesis. RNA synthesis is practically unaffected * Cytotoxic against human Molt 4 and murine LMTK cells	Báez and Vázquez, 1978 Furusawa *et al.*, 1971 Furusawa *et al.*, 1972 Furusawa *et al.*, 1973 Furusawa *et al.*, 1980 Gabrielsen *et al.*, 1992 Jiménez *et al.*, 1975b Jiménez *et al.*, 1976 Papas *et al.*, 1973 Renard-Nozaki *et al.*, 1989 Suzuki *et al.*, 1974 Weniger *et al.*, 1995 Zee-Cheng *et al.*, 1978

Table 6.4 Continued

Alkaloid	*Activity*	*References*
	* Moderately active against HepG2 hepatoma * Moderately active against Ehrlich ascites tumour cells * Inhibitor of HeLa cell growth * Antiviral. Active against *Herpes simplex* type 1 * Active against neurotropic RNA viruses * Does not inhibit the activity of reverse transcriptase	
5 2-*O*-acetyl-pseudolycorine	* Cytotoxic against Molt 4 lymphoid and LMTK fibroblastic cell lines * Weak cytotoxic against HepG2 hepatoma	Weniger *et al.*, 1995
58 tazettine	* Weak hypotensive * Exhibits mild activity against certain tumour cell lines * Active against the Co12 cell line * Weak cytotoxic against fibroblastic LMTK cell lines * The stereochemical rearrangement from pretazettine to tazettine inactivates the biological activity of pretazettine	Antoun *et al.*, 1993 CHCD, 1996 Codina *et al.*, 1992b Furusawa *et al.*, 1976b Furusawa *et al.*, 1980 Rigby *et al.*, 1998 Weniger *et al.*, 1995
39 vittatine	* Weak analgesic in mice * Increases the analgesic effect of morphine * Tachycardic in dogs	CHCD, 1996 Ghosal *et al.*, 1985b

Galanthamine, originally isolated from *Galanthus nivalis* in the 1940s, is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase. This enzyme is responsible for the degradation of acetylcholine at the neuromuscular junction, in peripheral and central cholinergic synapses and in parasympathetic target organs (Fulton and Benfield, 1996; Wilcock and Wilkinson, 1997). Galanthamine hydrobromide was first used by Bulgarian and Russian researchers in the 1950s and has been exploited for a variety of clinical purposes in the past. It has been used clinically for postsurgery reversal of tubocurarine-induced muscle relaxation and for treating post-polio paralysis, myasthenia gravis and other neuromuscular diseases, as well as traumatic brain injuries (Bores and Kosley, 1996; Radicheva *et al.*, 1996). Besides this, galanthamine acts as a mild analeptic, shows analgesic power as strong as morphine, and, applied in eye drops, reduces the intraocular pressure. As early as 1972, Soviet research had demonstrated that galanthamine could reverse scopolamine-induced amnesia in mice, a finding that was demonstrated in man four years later. However, this did not lead to the application of this compound in Alzheimer's disease until 1986, long after the widely accepted cholinergic hypothesis of Alzheimer's disease had been first postulated (Allain *et al.*, 1997). While the original acetylcholinesterase inhibitors, physostigmine and

tacrine, left much to be desired, galanthamine hydrobromide offers superior pharmacological profiles and increased tolerance (Ezio, 1998; Herman and Mucke, 1997a; Kewitz, 1997; Nordberg and Svensson, 1998; Rainer, 1997a). From the clinician's point of view, galanthamine is a reasonable approximation of the ideal concept of symptomatic Alzheimer's disease therapy (Rainer, 1997b; Wilcock and Wilkinson, 1997). Until very recently galanthamine could only be obtained in very small amounts from plant material (mainly from *Leucojum aestivum*) as its total chemical synthesis on an industrial scale was uneconomical (Eichhorn *et al.*, 1998; Hermann and Mucke, 1997b). This strictly limited availability of galanthamine was a severe constraint and probably the main reason for the cautious approach taken by the international pharmaceutical community. It appears that this situation has now fundamentally changed, as reflected in the increasing number of scientific reviews concerned exclusively with galanthamine and its derivatives (Bores and Kosley, 1996; Bores *et al.*, 1996; Brodaty, 1996; Fulton and Benfield, 1996; Harvey, 1995; Kewitz, 1996, 1997; Mucke, 1997a,b; Nordberg and Svensson, 1998; Svensson and Nordberg, 1997). This has resulted in much interest in the chemical synthesis of the product on an industrial scale (Czollner *et al.*, 1998; Kita *et al.*, 1998; Szewczyk *et al.*, 1995), and in production by *in vitro* tissue cultures, mainly from *Narcissus confusus* (Bergoñón *et al.*, 1996; Sellés *et al.*, 1997, 1999).

Lycorine, the most frequent and characteristic of Amaryllidaceae alkaloids, has been reported to be a powerful inhibitor of L-asc (ascorbic acid) biosynthesis (Arrigoni *et al.*, 1975; Evidente *et al.*, 1983b), and thus has been proved to be a useful tool in studying asc-dependent metabolic reactions in asc-synthesising organisms (Arrigoni *et al.*, 1997a). Lycorine is actually a powerful inhibitor of the activity of GL dehydrogenase (L-galactono-γ-lactone dehydrogenase), the terminal enzyme of asc biosynthesis (Davey *et al.*, 1998; De Gara *et al.*, 1994), which appears to be localised in the mitochondrial membrane (Arrigoni *et al.*, 1996, 1997b). Administration of lycorine induces a decrease in asc content and a simultaneous increase of dehydroascorbic acid levels in plants (De Tullio *et al.*, 1998). It has been suggested that the alkaloid could act as an inhibitor of asc biosynthesis *in vitro*, without interfering with asc utilisation in cells (Arrigoni *et al.*, 1997b). The effects of lycorine on L-asc biosynthesis have been reported to occur at concentrations below those at which protein synthesis is affected, but it seems difficult to rule out completely non-specific effects of this alkaloid since it has been reported that, at least in yeasts, lycorine is able to interact directly with mitocondrial DNA (Davey *et al.*, 1998; Del Giudice *et al.*, 1997; Massardo *et al.*, 1994). It is also well documented that lycorine is a powerful inhibitor of cell growth, cell division and organogenesis in higher plants, algae and yeasts, which seems to be related with asc levels (Arrigoni, 1994; Arrigoni *et al.*, 1997a; Córdoba-Pedregosa *et al.*, 1996; Del Giudice *et al.*, 1997; Onofri *et al.*, 1997). Lycorine also shows antitumour activity (Yui *et al.*, 1998). Its mechanism of action is thought to be through inhibition of protein synthesis at the ribosomal level (Furusawa *et al.*, 1980; Hua *et al.*, 1997). Additionally, it has antiviral (Gabrielsen *et al.*, 1992), antifeedant (Singh and Pant, 1980), and antimalarial as well as anti-inflammatory (Campbell *et al.*, 1998; Çitoglu *et al.*, 1998) activities. There has also been much recent interest in the chemical synthesis of lycorine (Hoshino *et al.*, 1996; Ishizaki *et al.*, 1998; Schultz *et al.*, 1996).

Narciclasine, an antimitotic and antitumoural alkaloid from *Narcissus* bulbs (Ceriotti, 1967), inhibits protein synthesis by directly interacting with the 60S ribosomal

subunit and inhibiting peptide bond formation by preventing binding of the 3′ terminal end of the donor substrate to the peptidyl transferase centre (Carrasco *et al.*, 1975). Narciclasine also inhibits seed germination and seedling growth of some plants in a dose-dependent manner, interacting with hormones in some physiological responses. In this way, indole-3yl-acetic acid cannot overcome the inhibition of elongation of wheat coleoptile sections caused by narciclasine. Additionally, narciclasine suppresses the gibberellin-induced α-amylase production in barley seeds and cytokinin-induced expansion and greening of excised radish cotyledons (Bi *et al.*, 1998). There are also interesting studies related to the chemical synthesis of this alkaloid (Angle and Wada, 1997; Rigby and Mateo, 1997).

The anticancer and antiviral activities exhibited by pretazettine have also stimulated considerable interest in this compound (Furusawa and Furusawa, 1988; Furusawa *et al.*, 1980; Gabrielsen *et al.*, 1992; Suzuki *et al.*, 1974) and in its chemical synthesis (Nishimata and Mori, 1998).

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7 Production of galanthamine by *Narcissus* tissues *in vitro*

Carles Codina

INTRODUCTION

Galanthamine is a morphine-like alkaloid that is a possible therapeutic agent in Alzheimer's disease because of its central cholinergic effects (Harvey, 1995). It has been shown to be competitive with other anticholinesterase compounds like tacrine or physostigmine in the treatment of the syndrome (Rainer *et al.*, 1989). In contrast with the proven hepatotoxicity of tacrine (Gauthier *et al.*, 1990), galanthamine shows only minor side effects like agitation or insommia (Thomsen *et al.*, 1990). Thus, galanthamine is considered a better therapeutic candidate for the treatment of this type of senile dementia than other acetylcholinesterase inhibitors structurally related to it (Bores *et al.*, 1996).

Galanthamine can be extracted from plants of the Amaryllidaceae family, the main natural sources being species of the genera *Galanthus* and *Leucojum* from Bulgaria and the Caucasus region. Galanthamine is difficult to obtain and extremely expensive for clinical usage. Although the total synthesis of galanthamine has been achieved (Czollner *et al.*, 1998), the stereoselectivity of its reactions and the low yields make this process economically unattractive. Therefore, as the plant remains the only valid source of galanthamine, a biotechnological approach has been considered as an alternative method for the production of the alkaloid.

Although much work has been done on the propagation of bulbous plants, very little exists on the production of secondary metabolites from them, this being restricted mainly to the production of colchicine by callus culture of *Colchicum autumnale* (Hayashi *et al.*, 1988; Yoshida *et al.*, 1988), haemanthamine by root cultures of *Zephyranthes robusta* (Furmanova and Oledzka, 1990), and bufadienolides by different tissues of *Urginea indica* (Jha *et al.*, 1991).

Investigations at the University of Barcelona with plants of the genus *Narcissus* have focused on the propagation of several species by *in vitro* liquid culture (Bergoñón *et al.*, 1992; Bergoñón, 1994; Riera, 1996; Sellés, 1996), and also on the production of alkaloids using different explants of *Narcissus confusus*, a wild species which was found to contain 0.1% of galanthamine on a fresh weight basis, as well as other alkaloids including *N*-formylnorgalanthamine, haemanthamine and tazettine (Bastida *et al.*, 1987). Experiments dealing with the production of galanthamine and related alkaloids have been performed using shoot-clumps (meristematic clusters), which were obtained from two different types of explants, of seed or bulb origin. These shoot-clumps constitute a good experimental system as they are made up of differentiated tissue, with a higher expression of secondary metabolism

and, consequently, higher production of alkaloids than cell suspension cultures. Media were based on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Cultures were maintained at 25 ± 1 °C under a photoperiod of 16 h, and they were shaken at 110 rpm. Extraction of the alkaloids was carried out as described previously (Sellés *et al.*, 1997b), and they were determined in both tissue and liquid medium by high performance liquid chromatography (Sellés *et al.*, 1997a). Results are presented of studies into the effect of cellular and tissue differentiation, growth regulators, sucrose concentration and culture vessel size on growth and production of alkaloids in *Narcissus confusus* explants.

EFFECT OF THE DEGREE OF CELLULAR AND TISSUE DIFFERENTIATION ON ALKALOID PRODUCTION

The results with organogenic plant material obtained by micropropagation, starting from both seeds and bulbs of *Narcissus confusus*, are presented. Although several experiments dealing with bud formation induced from bulb scales and from the basal part of the leaf of different species of *Narcissus* cultured *in vitro* have been published (Seabrook, 1990; Chow *et al.*, 1993), there is little information on the micropropagation of these plants via callogenesis, owing to the difficulty in obtaining callus. The plant material used to start the experiments were seeds and bulb scales of dormant *N. confusus*. The three different types of tissues obtained from seeds were called friable callus, meristematic callus, and organogenic tissue, according to the development of the explants. In the case of bulb scales, alkaloid levels were determined in the shoots obtained throughout the differentiation process, as well as in the initial scales, separated into inner, central and outer scales according to their position in the bulb.

Seed-derived plant material

It seems that the explants offering the best potential for starting callus formation come from cut ovaries kept in a medium supplemented with 20 mg/l naphthaleneacetic acid (NAA) (Seabrook, 1990). In the present case, however, callus tissue was obtained from seeds. The levels of alkaloids present in the different kinds of callus are described below.

Callus induction: alkaloids in friable callus

Callus induction was carried out as previously described (Sellés *et al.*, 1999), and the levels of alkaloids were determined in the two cellular strains showing the best growth, A, callus cultured in MS medium supplemented with 10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and B, callus maintained in MS medium supplemented with 10 mg/l picloram. During culture, some of the friable callus obtained was analysed for its alkaloid content, and the results are shown in Figure 7.1A. In both strains, the alkaloid profile was very similar. In general, the content of galanthamine was not very high at this low level of cellular differentiation, representing 15.3–24.0% of total alkaloids. *N*-formylnorgalanthamine was the major alkaloid present, reaching values of up to 80 and 110 µg/g dry weight (DW).

Although de-differentiated callus was not the best system for alkaloid production, it was interesting to observe that it had a biosynthetic capability, thus constituting a useful experimental system for studying the biosynthesis of alkaloids because of its simple structure.

Callus maintenance: alkaloids in meristematic tissue

After 6 months of maintaining callus in the same conditions, meristematic callus from both strains A and B was transferred to an MS medium without auxins to promote embryo maturation. Two months later, the callus derived from strain A showed a high number of white globular structures which seemed to be cauline tips, and even some somatic embryo-like protuberances were observed. The B strain derived callus was more compact, and some of the protuberances were elongated like the root meristem.

The alkaloid content was determined separately in callus from both A and B strains when removing the auxins from the medium. As expected, from the chemical point of view, the alkaloid profile was different in the two strains (Figure 7.1B), according to their different morphological development. While the embryogenic callus (strain A) accumulated mainly galanthamine-type alkaloids, *N*-formylnorgalanthamine being the most abundant (58% of total alkaloids), those from strain B (rhizogenic callus) contained haemanthamine as the major alkaloid (43.5% of total alkaloids). Thus, the type of cellular organisation influences the qualitative profile of alkaloids accumulated in the meristematic callus.

Callus regeneration: alkaloids in organogenic tissue

Callus from *N. confusus* has been found to maintain its regenerative capability for a period of about two years if kept in a medium supplemented with 10 mg/l of 2,4-D or picloram (Sellés, 1996). This organogenic capacity of the *N. confusus* callus is not

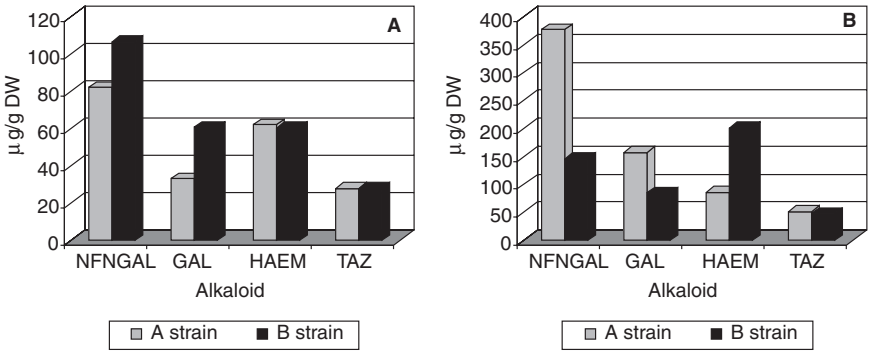

Figure 7.1 Alkaloid content in friable (A) and meristematic (B) callus from two strains of seed-derived explants (values represent means of three replicates). NFNGAL, *N*-formylnorgalanthamine; GAL, galanthamine; HAEM, haemanthamine; TAZ, tazettine.

easily lost on transfer to a regeneration medium, as happens, for instance, with *Allium cepa* (Havel and Novák, 1988).

Regeneration of the meristematic callus (strains A and B) was induced by growing it in several MS culture media supplemented with 3% of sucrose and with and without 0.5 or 1 mg/l of the cytokinins benzyladenine (BA) and kinetin. Under these conditions, the globular callus became green and developed aggregates of young shoots, whereas numerous roots were formed in the most friable callus, especially in presence of cytokinins. The alkaloid content in the organogenic tissues was then determined in two types of aggregates, shoot and root clusters.

Alkaloids in shoot clusters

These organogenic tissues were constituted by aggregates of eight to ten young shoots per explant, although they also showed meristematic buds which were far less abundant in the callus grown with kinetin. The alkaloid content of these clusters was similar, in global terms, to that of the meristematic callus, but the alkaloid profile was not the same (Figure 7.2). One can observe that, in that callus with a higher degree of differentiation, the main alkaloid was galanthamine.

Alkaloids in root clusters

After seeing that the clusters derived from the B strain treated with 0.5 mg/l of kinetin grew well in solid medium, they were transferred to a liquid medium without growth regulators and supplemented with 6% of sucrose. They were maintained for 6 weeks, and alkaloid levels were determined every two weeks (Figure 7.3A). The alkaloids were progressively released into the liquid medium throughout the experiment. In general, the removal of alkaloids was higher in the first subculture, with the exception of the alkaloids haemanthamine and galanthamine, which reached a maximum percentage in the second and third subcultures, respectively. *N*-formylnorgalanthamine was the alkaloid released to the medium in the highest proportion, which was also observed in other assays carried out in liquid medium.

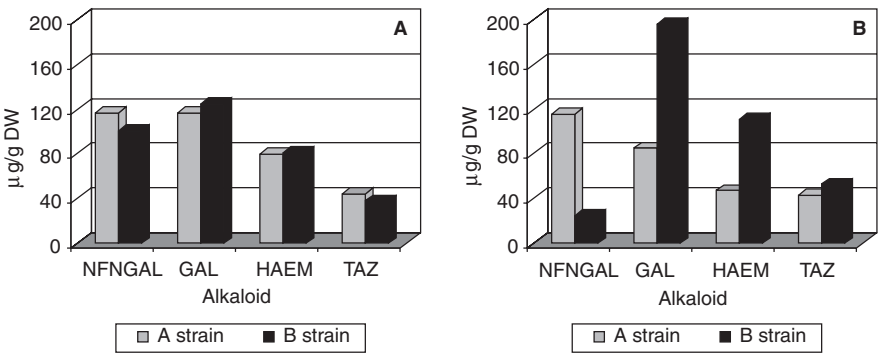

Figure 7.2 Alkaloid content in shoot clusters grown in MS solid medium supplemented with 1 mg/l of BA (A) or kinetin (B) (values represent means of three replicates). For abbreviations, see Figure 7.1.

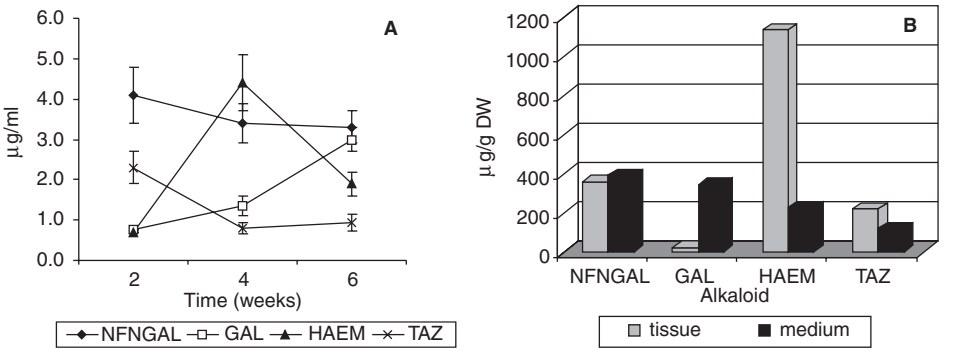

Figure 7.3 A: levels of alkaloids released by root clusters to the culture medium throughout the experiment. B: accumulation of alkaloids in both tissue (root cluster) and liquid medium at the end of the experiment (values represent means of three replicates; thin bars represent SD). For abbreviations, see Figure 7.1.

At the end of the experiment, the major alkaloids in both tissues and liquid medium were haemanthamine and *N*-formylnorgalanthamine, with a maximum content of 1.38 and 0.75 mg/g DW, respectively (Figure 7.3B). The haemanthamine type alkaloids were mainly accumulated in the tissue, whereas those of the galanthamine type were mainly released to the liquid medium, especially in the last subculture, coinciding with a remarkable necrosis of the root tissue.

In general, the kind of explant used influenced the type of alkaloid obtained *in vitro*, so most of the subsequent experiments were carried out with shoot-clumps, or with small bulblets, as galanthamine has been the main alkaloid accumulated in these explants.

In vitro cultured plant cells grown in an undifferentiated state either produce insignificant amounts of secondary metabolites or they lose their ability to produce them when maintained long-term. This is the case, for instance, in *Urginea indica* (Jha *et al.*, 1991). This loss, however, can be restored when shoots or roots are differentiated from disorganised tissue (Payne *et al.*, 1991). The present results show that embryogenic callus of *N. confusus* may accumulate nearly the same amounts of alkaloids as shoot-clumps (Table 7.1). Since the globular structures (embryogenic callus) which start alkaloid accumulation are rather simply organised,

Table 7.1 Alkaloid content (µg/g DW ± SD calculated from three replicates) at various development stages of *Narcissus confusus* tissue cultures[a]

Tissue culture	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
De-differentiated callus	0.08 ± 0.02a	0.03 ± 0.02a	0.06 ± 0.00a	0.02 ± 0.00a
Embryogenic callus	0.25 ± 0.17a	0.11 ± 0.05a	0.13 ± 0.08a	0.04 ± 0.00a
Shoot-clumps	0.10 ± 0.01a	0.14 ± 0.01a	0.08 ± 0.00a	0.03 ± 0.00a
Plantlets	0.73 ± 0.22b	1.43 ± 0.51b	1.02 ± 0.63b	0.68 ± 0.15b

Note
[a]means followed by the same letter are not significantly different from each other at $p \leq 0.05$. Abbreviations: DW, dry weight; FW, fresh weight; SD, standard deviation; NFNGAL, *N*-formylnorgalanthamine; GAL, galanthamine; HAEM, haemanthamine; TAZ, tazettine.

they might be more suitable for investigating the mechanisms triggering alkaloid accumulation than shoots or plantlets. Furthermore, somatic embryos are easier to handle biotechnologically than more complex structures. Embryogenic strains may, therefore, be of special value in further investigations of alkaloid formation in *N. confusus in vitro*.

Plantlets regenerated from either shoot-clumps or somatic embryos were found to produce alkaloids. Table 7.1 shows that only the content of galanthamine increased with tissue differentiation. A possible explanation is that the differentiation stage could influence the biosynthetic pathway of these alkaloids in a different manner. Galanthamine and *N*-formylnorgalanthamine derive from a *para-ortho* oxidative phenolic coupling, whereas haemanthamine and tazettine result from *para-para* coupling. In general, the more tissue organisation there is, the higher the alkaloid content. Plantlets regenerated after 6 months were characterised by a well formed bulb and emerging leaves, and seemed to be the best material for experiments on galanthamine production.

Bulb-derived plant material

Shoots were obtained directly from the bulbs by using the 'twin-scaling' technique (Hanks and Jones, 1986), and they were classified according to the position of the scales in the bulb: internal, central or external. The alkaloid content was analysed in the scales initially and in shoots obtained from each kind of scale (Sellés, 1996).

The different kinds of explants were maintained in the same culture conditions for 5 months as previously described (Bergoñón *et al.*, 1996), and then the alkaloids were analysed in samples from the regenerated shoots and their respective scales (Figure 7.4). In all cases, the major alkaloids were haemanthamine and galanthamine, in that order, and the alkaloid profile of the regenerated shoots was found to coincide with that of the bulb scales, both qualitatively and quantitatively.

It is important that all the shoots induced after 5 months are small shoots with the capacity to accumulate galanthamine. Therefore, these explants, after being

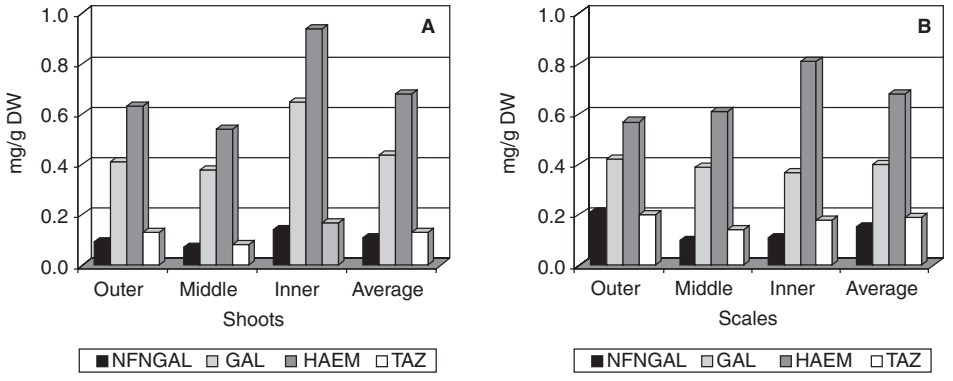

Figure 7.4 Alkaloid content in shoots (A) and their respective twin-scales (B) according to their position in the bulb (values represent means of three replicates). For abbreviations, see Figure 7.1.

subcultured and developed in a multiplication medium, also constituted good plant material for further experiments on the production of galanthamine type alkaloids.

EFFECT OF GROWTH REGULATORS ON GROWTH AND MORPHOLOGY OF EXPLANTS AND ALKALOID PRODUCTION

Although the growth of aggregates of both buds and shoots of *Narcissus* in liquid medium has been succesfully achieved without adding any kind of phytohormones (Bergoñón *et al.*, 1992), the influence of two kinds of growth regulators has been studied: cytokinins (BA and kinetin), which are responsible for both cell division and growth of the aerial part of the shoots, and paclobutrazol, an inhibitor of the synthesis of gibberellins, which has been previously tested with other bulbous plants such as *Gladiolus* (Steinitz and Lilien-Kipnis, 1989; Steinitz *et al.*, 1991; Ziv, 1992) and *Nerine* (Lilien-Kipnis *et al.*, 1992). The main action of paclobutrazol is to inhibit the growth of the aerial part of the explants, thus producing wider bulbs.

Effect of cytokinins

It has been observed that higher levels of growth regulators are needed in *Narcissus* plants than in other monocots to induce adventitious shoots (Seabrook *et al.*, 1976; Seabrook, 1990) and bulblets (Keller, 1993), but their influence in liquid medium cultures has not been previously studied.

Although the addition of cytokinins to the liquid medium is not very advisable, especially at high concentrations, because they induce vitrification of the plant tissues (Hussey, 1986), this experiment was carried out using seed-derived shoot-clumps with a few (four or five) emergent leaves (Sellés, 1996). The clusters were grown for two months in baby-food jars with MS liquid medium supplemented with 6% sucrose and cytokinin (BA or kinetin) at concentrations of 0, 1, 3, 5 or 10 mg/l. Every two weeks, coinciding with the subculturing steps, the alkaloids released to the liquid medium were measured. The alkaloids accumulated in the tissues were determined at the end of the experiment. In the present chapter, we have considered the 'total production' of alkaloids as the sum of the alkaloids released to the liquid medium during the successive subculturing steps, and those accumulated in the tissues at the end of the experiment.

Benzyladenine

In general, in the shoot-clumps growing in a liquid medium with cytokinins, the aerial part was more developed than the bulb, exhibiting narrow, intensely green leaves. Under these culture conditions the percentage of survival was around 90%, with the exception of the clusters treated with 10 mg/l of BA, 80% of which became vitrified and necrotic.

Accumulation of alkaloids in the liquid medium

The results concerning the levels of alkaloids released by the shoot-clumps to the liquid medium during the two-month experiment are shown in Figure 7.5. The

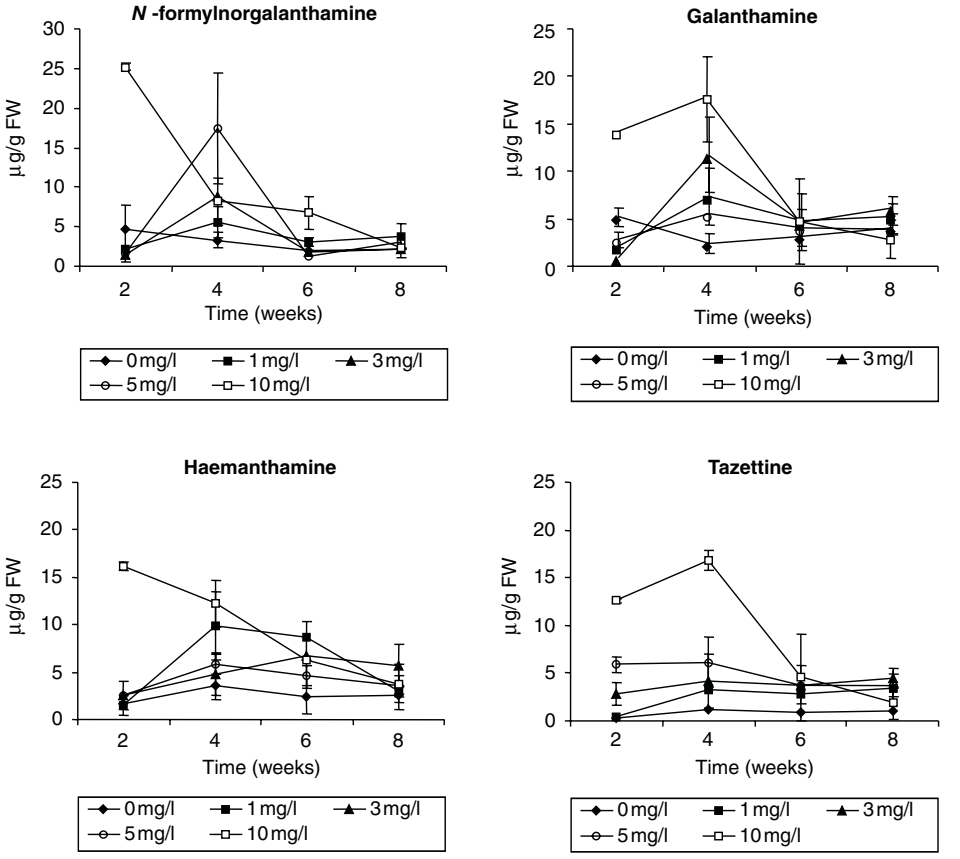

Figure 7.5 Levels of alkaloids released by shoot-clumps treated with BA into the culture medium throughout the experiment (bars represent the SD calculated from three replicates).

production of alkaloids was not very high, and it was more or less constant during the experiment. *N*-formylnorgalanthamine and haemanthamine were the main alkaloids released into the liquid medium after two weeks in the case of the explants treated with 10 mg/l of BA, and, in general, tazettine was the alkaloid with the least tendency to be released into the medium.

Accumulation of alkaloids in tissue

Eight weeks after starting the culture, the alkaloid content of the shoot-clumps was determined, as well as that of the alkaloids released to the liquid medium (Figure 7.6). In all cases, the concentration of alkaloids was higher in the tissue than in the liquid medium, optimum levels being reached in the shoot-clumps treated with 3 mg/l of BA. The major alkaloids were haemanthamine, reaching values of up to 2.17 mg/g DW, and galanthamine, reaching 1.42 mg/g DW.

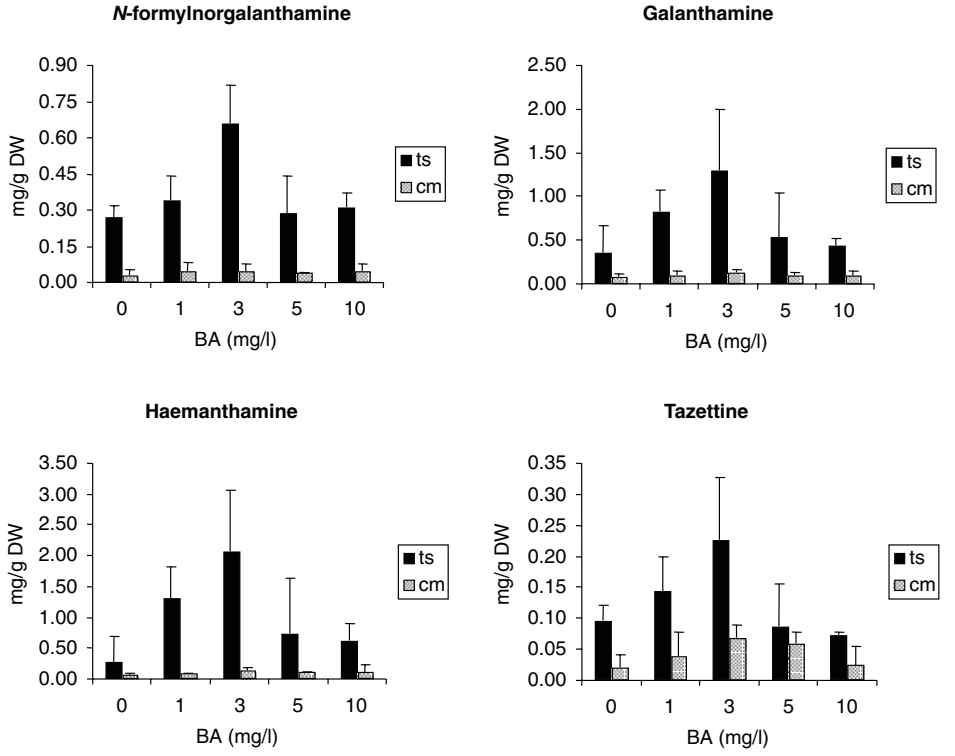

Figure 7.6 Accumulation of alkaloids in both tissue (shoot-clumps) and liquid medium at the end of the experiment with BA. Bars represent the SD calculated from three replicates. ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

Table 7.2 Total production of alkaloids (accumulation in both tissue and liquid medium) in shoot-clumps grown under different concentrations of BA (μg/g FW ± SD calculated from three replicates)[a]

BA	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
0 mg/l	284.2 ± 14.1	475.5 ± 35.1	488.8 ± 39.0	79.7 ± 5.6
1 mg/l	355.6 ± 19.4	903.7 ± 52.3	1358.5 ± 78.5	157.4 ± 8.2
3 mg/l	687.4 ± 38.7	1354.2 ± 83.3	2097.5 ± 129.1	248.7 ± 13.2
5 mg/l	347.1 ± 23.4	572.1 ± 46.9	856.1 ± 77.2	110.8 ± 6.8
10 mg/l	377.8 ± 14.3	465.7 ± 18.3	673.0 ± 30.1	115.3 ± 6.8

Note
[a]for abbreviations, see Table 7.1.

Total production of alkaloids during the experiment

The levels of alkaloids produced throughout culture are shown in Table 7.2. The highest accumulation of alkaloids was observed in the shoot-clumps treated with 3 mg/l of BA, which also displayed the best growth, followed by those treated with 1 mg/l of BA. The absence of BA coincided with the lowest accumulation of alkaloids.

Kinetin

As observed in the experiment with BA, the control shoots developed narrow leaves and a well formed bulb, as well as roots. On the contrary, bulbs subjected to high doses of kinetin (5 and 10 mg/l) had a necrotic and even vitrified appearance, like shoot-clumps treated with the same concentrations of BA.

Accumulation of alkaloids in the liquid medium

The release of alkaloids into the liquid medium of the shoot-clumps treated with kinetin was similar to that observed in the clusters grown in media supplemented with BA. However, what was notable was the high proportion of the alkaloids galanthamine and haemanthamine released into the medium by the explants treated with 10 mg/l of kinetin four weeks after starting the culture, which reached values of up to 73.6 and 95.6 µg/g fresh weight (FW), respectively (Figure 7.7).

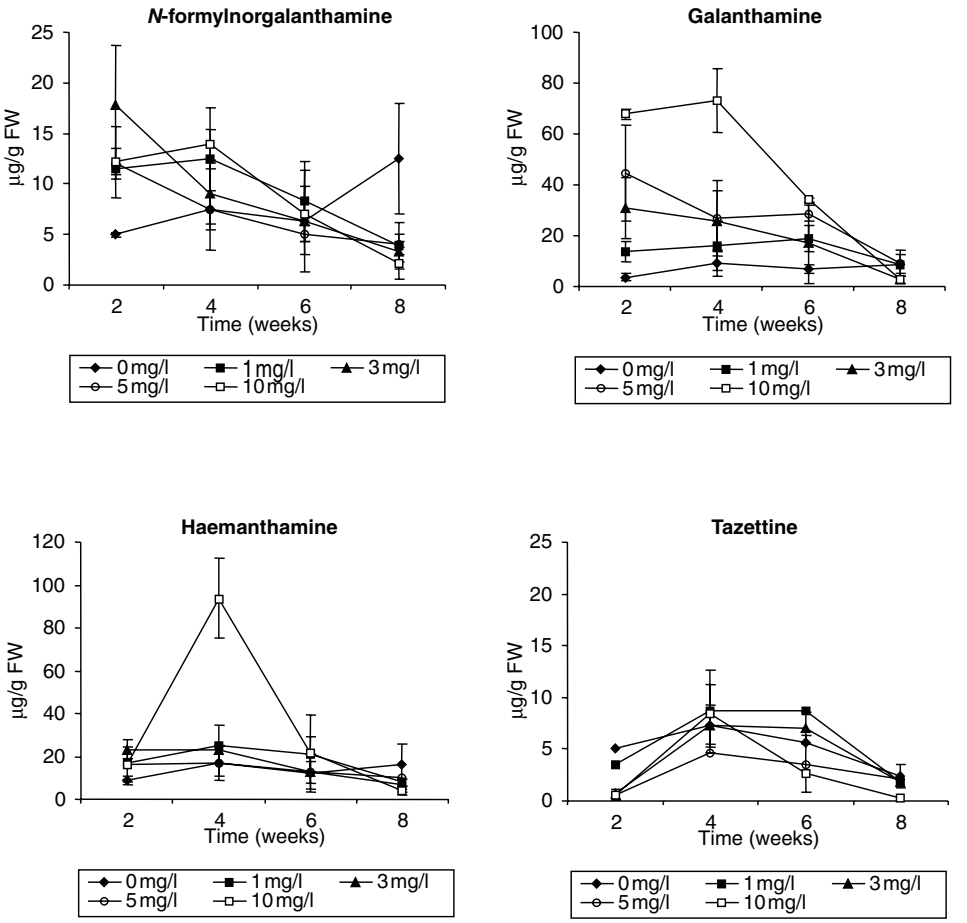

Figure 7.7 Levels of alkaloids released by shoot-clumps treated with kinetin into the culture medium throughout the experiment (bars represent the SD calculated from three replicates).

Accumulation of alkaloids in tissue

Unlike BA, kinetin, especially at high concentrations, inhibited alkaloid production in the shoot tissues (Figure 7.8), as has also been described for cultures of *Scopolia maxima* (Mantell and Smith, 1983). Thus, the maximum levels of alkaloids were found in the control shoot-clumps, with the exception of tazettine, whose maximum level was found in the shoot-clumps treated with 3 mg/l of kinetin. This growth regulator seems to have more effect on the release of alkaloids to the medium than on their accumulation in the tissues.

Total production of alkaloids during the experiment

The total content of alkaloids (those accumulated in tissue and those released into the medium) produced by the shoot-clumps treated with different concentrations of kinetin during the two-month experiment is shown in Table 7.3. Kinetin affected the alkaloid production in a negative way, so the highest levels of these compounds, especially of haemanthamine and galanthamine, were found in the control shoots.

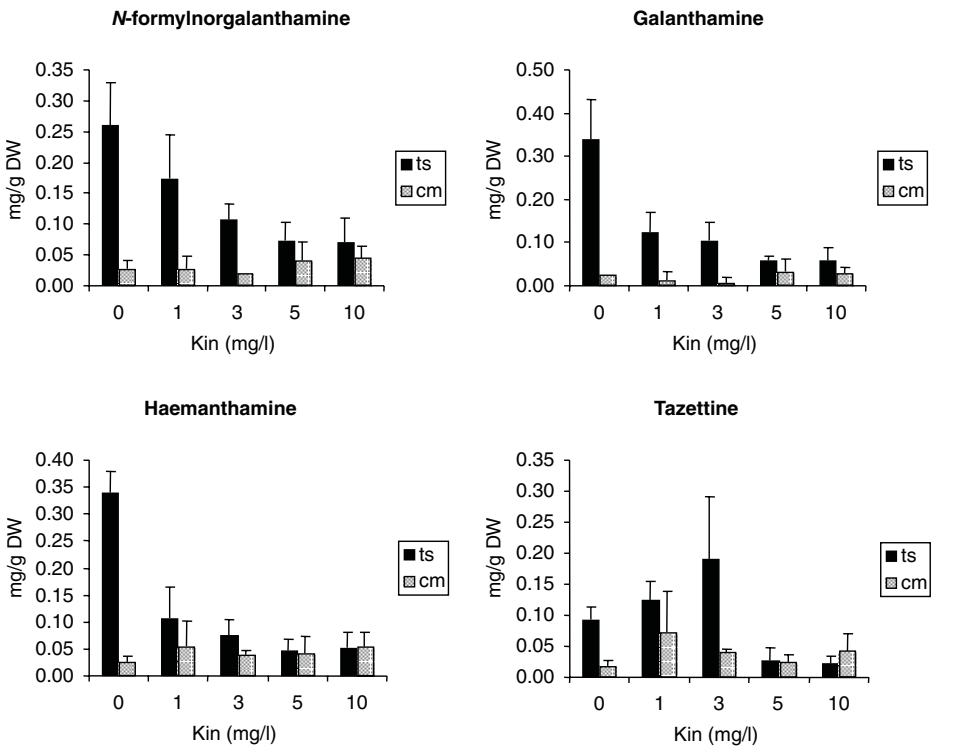

Figure 7.8 Accumulation of alkaloids in both tissue (shoot-clumps) and liquid medium at the end of the experiment with kinetin (Kin) (bars represent the SD calculated from three replicates). ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

Table 7.3 Total production of alkaloids (accumulation in both tissue and liquid medium) in shoot-clumps grown under different concentrations of kinetin (μg/g FW ± SD calculated from three replicates)[a]

Kinetin	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
0 mg/l	274.9 ± 9.5	358.9 ± 26.1	368.9 ± 55.2	139.2 ± 21.9
1 mg/l	209.1 ± 7.2	179.1 ± 15.1	173.0 ± 22.6	147.8 ± 4.3
3 mg/l	144.1 ± 6.6	182.2 ± 13.3	142.9 ± 9.0	208.6 ± 3.9
5 mg/l	95.1 ± 8.8	159.2 ± 19.9	103.4 ± 8.2	39.3 ± 1.9
10 mg/l	98.2 ± 8.3	225.8 ± 37.0	188.7 ± 38.1	32.8 ± 5.2

Note
[a]for abbreviations, see Table 7.1.

Comparing the effects of these cytokinins on explant development and alkaloid production, one can observe that shoot-clumps treated with BA showed a better development than those treated with kinetin. The best treatment for the multiplication of the explants was the concentration of 3 mg/l of BA, and the controls, with a lower number of leaves, accumulated a higher amount of dry matter. In addition, the degree of necrosis in the experiment with kinetin was higher than in the other experiments, which could be related to a higher release of alkaloids by the shoot-clumps. In comparison with the control explants, the alkaloid content, in general, was strongly influenced by the different concentrations of BA, galanthamine and haemanthamine being the predominant alkaloids produced.

The results of this experiment reveal that *Narcissus confusus* is not especially susceptible to vitrification, which is usually found when tissues are cultivated in a liquid medium and, in addition, under high concentrations of cytokinins (Hussey, 1986). This could be due to the fact that the subcultures were systematically performed every two weeks, instead of every five weeks as described for other species of this genus (Chow *et al.*, 1993). This higher frequency of subculturing could have avoided the excessive accumulation of ethylene inside the culture flasks, which is also involved in the vitrification process (Hussey, 1986).

Effect of paclobutrazol

Paclobutrazol, an inhibitor of gibberellin biosynthesis, has been used in liquid cultures with other plants belonging to the genera *Gladiolus* (Steinitz and Lilien-Kipnis, 1989), *Nerine* (Lilien-Kipnis *et al.*, 1992), *Colchicum* (Ellington, 1998), *Solanum* (Simko, 1994), *Nephrolepis* and *Philodendron* (Ziv, 1992), and its influence on both the proliferation of meristematic shoots and the lateral growth of explants has been observed.

The effect of paclobutrazol has been examined on explants with different levels of development: bulblets with emerging leaves and well formed bulbs, and shoot-clumps. The explants were obtained as previously described (Sellés, 1996), and cultured in baby-food jars with MS liquid medium supplemented with 6% sucrose and paclobutrazol at concentrations of 0, 1, 3, 5 and 10 mg/l. They were cultivated for two months at 25 ± 1 °C under a photoperiod of 16 h, and shaken at 110 rpm. The concentrations of the alkaloids accumulated in tissue were determined 8 weeks after starting the experiment, whereas the release of alkaloids to the liquid medium was measured every two weeks, coinciding with the subculturing steps.

Bulblets

As a consequence of the effect of paclobutrazol, the shoots formed during the 8-week experiment were characterised by the absence of leaves and roots, and showed an especially well-developed bulb.

Accumulation of alkaloids in the liquid medium

The release of alkaloids into the liquid medium of the explants treated with paclobutrazol decreased during the experiment (Figure 7.9). Independently of the paclobutrazol concentration, in all the experiments the main alkaloids released to the medium were haemanthamine and galanthamine, whose levels reached maximum values of 71.3 and 35.8 µg/g FW, respectively. Tazettine was not excreted into the liquid medium in the last sample. The treatment that promoted the highest release of alkaloids to the medium was 5 mg/l of paclobutrazol.

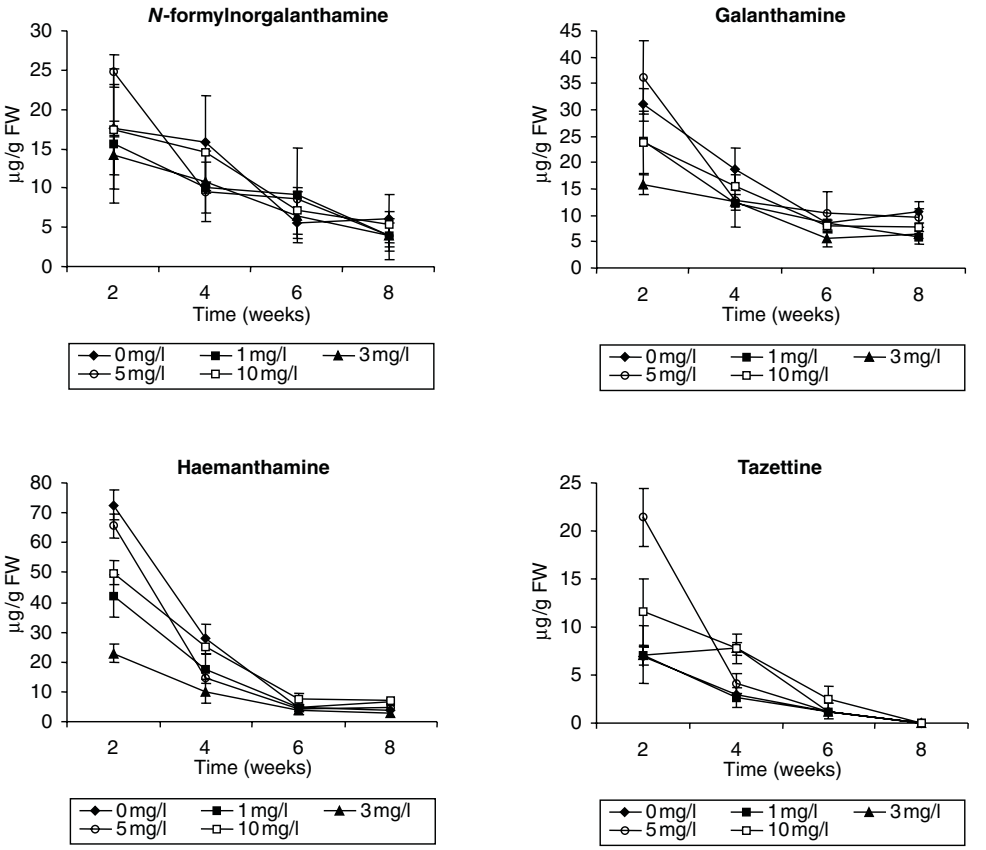

Figure 7.9 Levels of alkaloids released by bulblets treated with paclobutrazol into the culture medium throughout the experiment (bars represent the SD calculated from three replicates).

Accumulation of alkaloids in tissue

The alkaloid content in tissues at the end of the experiment was higher than that of the culture medium, haemanthamine and galanthamine being the major alkaloids (Figure 7.10). The control explants (grown without paclobutrazol) accumulated the highest concentration of alkaloids.

Total production of alkaloids during the experiment

The final alkaloid content (alkaloids accumulated in tissue and those released to the liquid medium) at the end of the experiment, after a two-month period, is shown in Table 7.4. The control explants were the most productive, showing a good appearance with narrow leaves and well developed roots.

Shoot-clumps

In this experiment, some necrosis and vitrification problems appeared from the fourth sample onwards in all the explants treated with paclobutrazol. These problems were less notable in the control shoot-clumps and those treated with the lowest

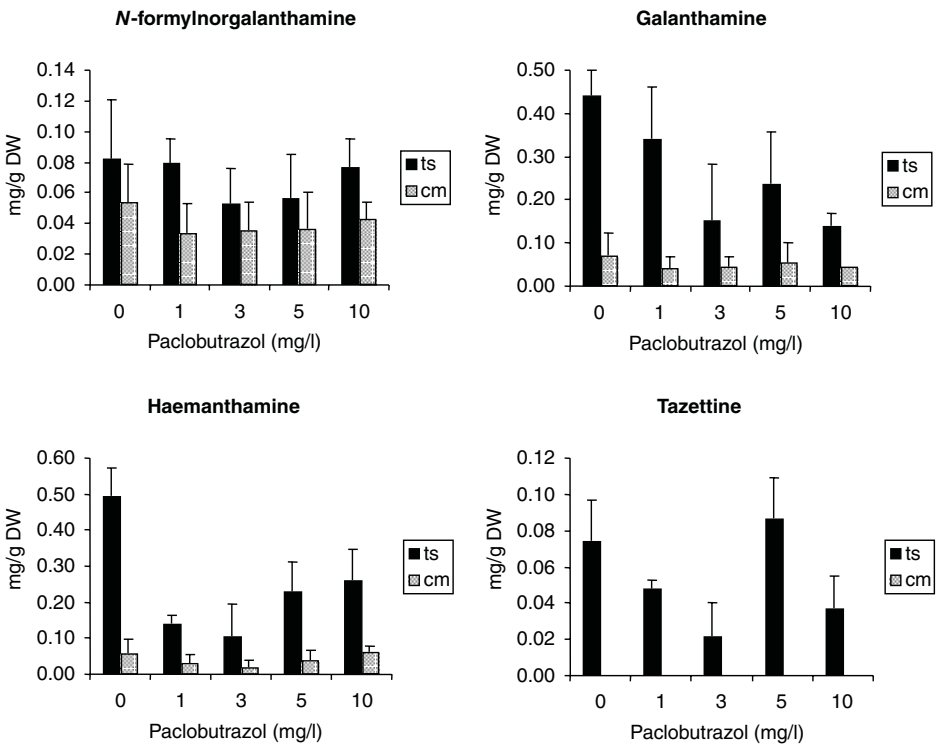

Figure 7.10 Accumulation of alkaloids in both tissue (bulblets) and liquid medium at the end of the experiment with paclobutrazol (bars represent the SD calculated from three replicates). ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

Table 7.4 Total production of alkaloids (accumulation in both tissue and liquid medium) in bulblets grown under different concentrations of paclobutrazol (µg/g FW ± SD calculated from three replicates)[a]

Paclobutrazol	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
0 mg/l	56.6 ± 5.6	118.8 ± 4.2	170.9 ± 4.8	23.8 ± 3.4
1 mg/l	52.8 ± 0.9	99.1 ± 1.2	91.1 ± 6.8	16.6 ± 1.4
3 mg/l	43.4 ± 4.4	60.3 ± 4.9	53.2 ± 2.5	11.9 ± 0.8
5 mg/l	56.6 ± 3.8	99.6 ± 2.2	123.0 ± 3.6	38.4 ± 2.7
10 mg/l	56.5 ± 0.4	72.9 ± 4.4	124.8 ± 3.4	26.8 ± 4.2

Note
[a]for abbreviations, see Table 7.1.

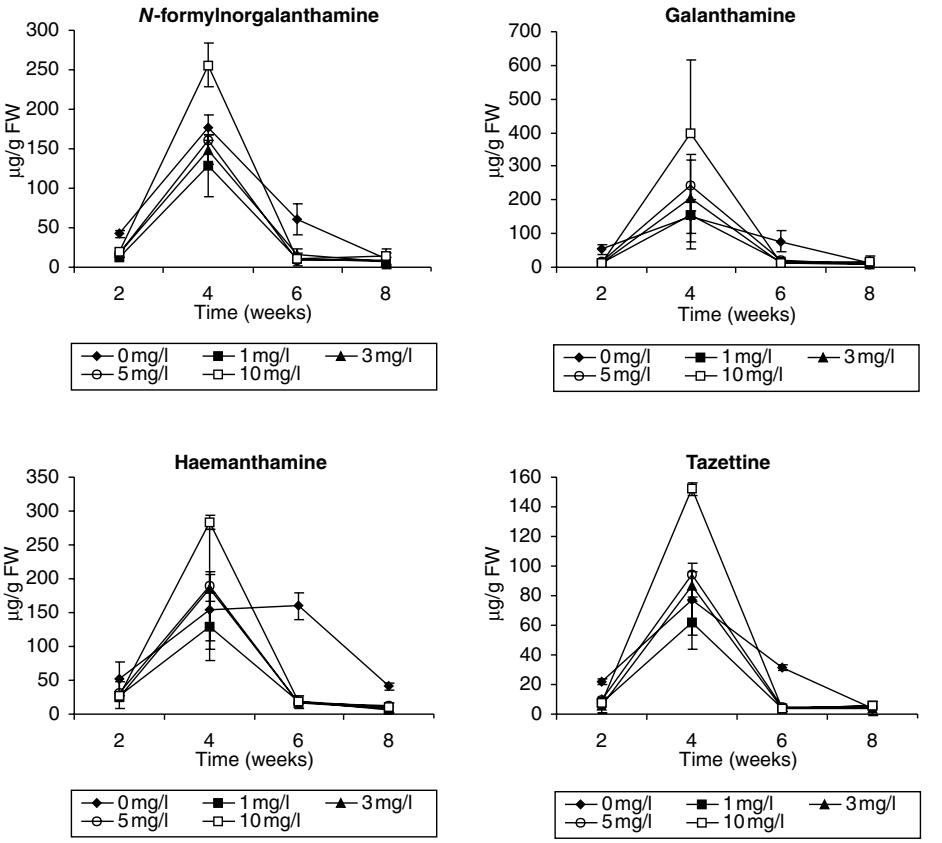

Figure 7.11 Levels of alkaloids released by shoot-clumps treated with paclobutrazol into the culture medium throughout the experiment (bars represent the SD calculated from three replicates).

concentration of the growth regulator. The control shoot-clumps had a good appearance, each one showing about eight to nine shoots at the end of the experiment. The lower growth index of the explants treated with the highest doses of paclobutrazol agrees with observations in *Gladiolus* (Ziv, 1992).

Accumulation of alkaloids in the liquid medium

Although the release of alkaloids into the medium increased until the fourth week of culture, from then on, and coinciding with the necrosis and vitrification of the tissues, excretion decreased drastically (Figure 7.11). As in the bulblets, the main alkaloids released to the medium were galanthamine and haemanthamine, reaching values of 404.2 and 295.5 µg/g FW, respectively. In general, the levels of alkaloids released by the shoot-clumps into the liquid medium were higher than those released by the bulblets.

Accumulation of alkaloids in tissue

In the control shoot-clumps, or in those treated with low doses of paclobutrazol, the alkaloids were accumulated mainly in the tissue, whereas at concentrations of 5 mg/l or more of this growth regulator the alkaloids were mainly released to the culture medium (Figure 7.12). This fact could be related to the degree of vitrification, which was proportional to the concentration of paclobutrazol.

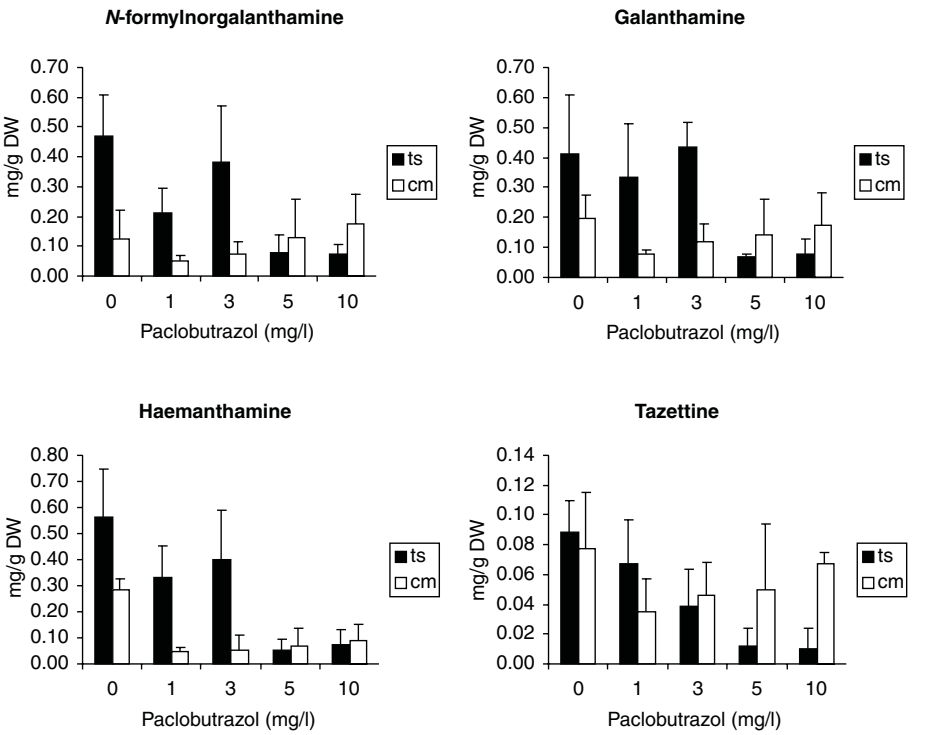

Figure 7.12 Accumulation of alkaloids in both tissue (shoot-clumps) and liquid medium at the end of the experiment with paclobutrazol (bars represent the SD calculated from three replicates). ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

Table 7.5 Total production of alkaloids (accumulation in both tissue and liquid medium) in shoot-clumps grown under different concentrations of paclobutrazol (μg/g FW ± SD calculated from three replicates)[a]

Paclobutrazol	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
0 mg/l	298.7 ± 12.4	329.4 ± 77.1	418.4 ± 43.4	149.2 ± 24.1
1 mg/l	159.5 ± 31.2	208.4 ± 72.4	177.9 ± 33.4	81.1 ± 15.0
3 mg/l	198.3 ± 17.1	268.4 ± 102.2	231.8 ± 16.2	109.9 ± 3.4
5 mg/l	208.6 ± 6.6	307.4 ± 108.7	243.5 ± 106.1	119.2 ± 4.6
10 mg/l	320.5 ± 25.0	465.1 ± 221.1	346.0 ± 60.2	177.4 ± 3.8

Note
[a]for abbreviations, see Table 7.1.

Total production of alkaloids during the experiment

The total production of alkaloids in the shoot-clumps treated with 1, 3 or 5 mg/l of paclobutrazol was lower than in the controls (Table 7.5). Nevertheless, the highest production took place in those treated with 10 mg/l of paclobutrazol. Galanthamine was the main alkaloid produced under the influence of paclobutrazol, whereas the control shoot-clumps accumulated mainly haemanthamine.

By comparing the effect of paclobutrazol on both the morphology of the explants and the production of alkaloids, one can observe that the bulblets were better developed than the shoot-clumps, as the latter became necrotic. In general, growth was higher in the explants (bulblets and shoot-clumps) treated with paclobutrazol, as was also observed in *Gladiolus* (Ziv, 1992), although the differences among the different treatments were small. In both kinds of explants, the presence of paclobutrazol in the medium, promoted the development of adventitious shoots and, in general, inhibited the synthesis of alkaloids in comparison with the control explants. The excretion of alkaloids into the liquid medium was higher in shoot-clumps than in bulblets.

EFFECT OF SUCROSE CONCENTRATION ON GROWTH AND PRODUCTION OF ALKALOIDS

The importance of sucrose, a source of carbon essential for the growth and development of bulbs, has previously been demonstrated in plants belonging to the genera *Allium* (Keller, 1993), *Lilium* (Takayama and Misawa, 1979), *Tulipa* (Taeb and Alderson, 1990), *Narcissus* (Chow *et al.*, 1992) and *Gladiolus* (Steinitz *et al.*, 1991). With the exception of *Gladiolus*, these experiments were carried out in solid agar media. The present experiments were performed in liquid medium with shoot-clumps derived from both bulbs and seeds of *Narcissus confusus*, which were treated with different concentrations of sucrose, 30, 60, 90, 120, 150 and 180 g/l (Sellés *et al.*, 1997b). The experiment took place over two weeks, as the secondary metabolites are usually formed at the end of the growth period, when the nutrients of the medium become limiting.

Effect on growth and morphology of the explants

The different origin of the shoot-clumps used in this experiment (bulbs or seeds) did not seem to influence significantly the growth of the explants, although the shoot-clumps derived from bulbs were slightly bigger. In both cases, the clusters formed in the treatment with 90 g/l of sucrose exhibited the best growth index of weight, showing a well formed bulb and emerging intensely green leaves. The shoot-clumps treated with concentrations of sucrose higher than 90 g/l showed pale leaves, with a tendency to vitrification. In addition, the tissue of the base of the bulb was yellowish, sometimes showing necrotic areas, and the emergence of leaves was less vigorous. Apparently, high concentrations of sucrose (≥ 150 g/l) became toxic to the explants derived from both strains of shoot-clumps (coming from bulbs or seeds), and caused shoot dormancy.

Effect on alkaloid production

Although the two strains showed a similar growth and morphology, they exhibited different behaviour in relation to the production of alkaloids.

Bulb-derived shoot-clumps

The effect of sucrose concentrations on the production of alkaloids in bulb-derived shoot-clumps is shown in Figure 7.13. The main alkaloid was haemanthamine, with a value of 0.87 mg/g DW in the treatment with the lowest sucrose concentration (3%), whereas the minor alkaloid was tazettine, with a value of 0.15 mg/g DW in the same treatment. The maximum accumulation of galanthamine took place in the treatments with the highest and lowest doses of sucrose (3 and 18%), reaching practically the same value, 50 mg/g DW. The maximum production of *N*-formylnorgalanthamine occurred in the explants treated with 30 g/l of sucrose.

In general, the qualitative profile of the alkaloids in the different treatments with sucrose was very similar. From the quantitative point of view, however, an increase in the source of carbon led to a corresponding decrease in the production of alkaloids, the minimum value thus being obtained in the treatment with 90 g/l of sucrose. At higher concentrations of sucrose, the production of alkaloids increased, but without reaching the maximum values observed in the treatment with 30 g/l of sucrose. In all cases, the alkaloids were mainly accumulated in the tissue, a very small proportion being released to the culture medium (Figure 7.13).

Seed-derived shoot-clumps

The production of alkaloids in the seed-derived shoot-clumps and treated with different doses of sucrose are shown in Figure 7.14. In this case, one can observe that the accumulation of galanthamine type alkaloids was higher in the medium culture than in the tissue, except in the treatments with 90 and 120 g/l of sucrose. In the case of the haemanthamine type alkaloids, the percentage accumulated in the tissue was generally higher than that released to the liquid medium.

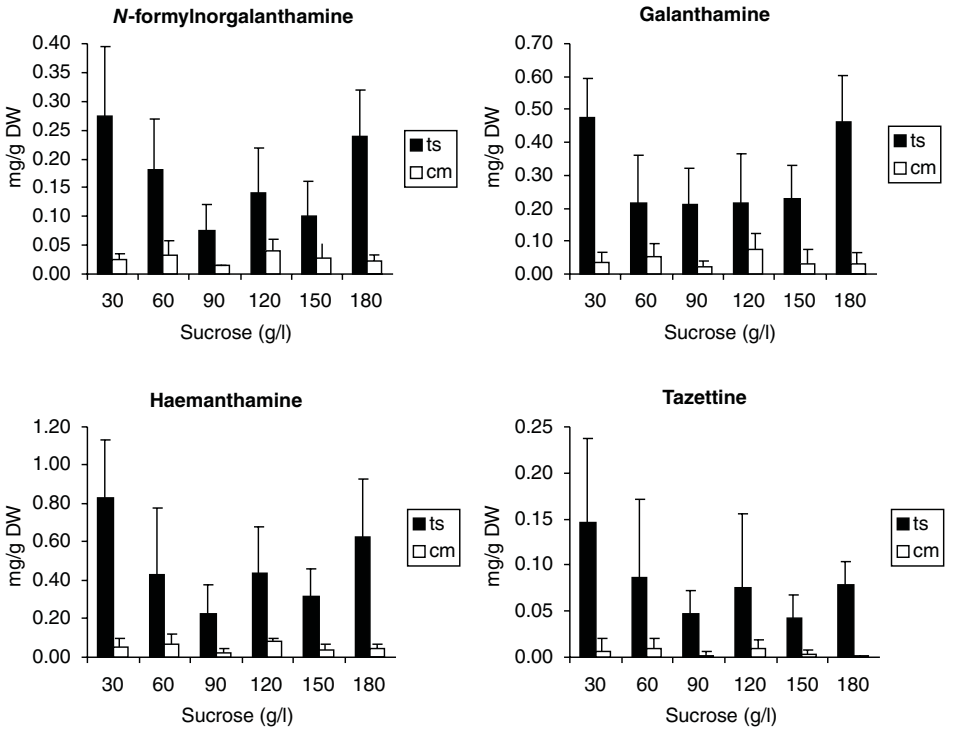

Figure 7.13 Alkaloid production in bulb-derived shoot-clumps grown under different sucrose concentrations. Bars represent the SD calculated from three replicates. ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

As in the case of the shoot-clumps derived from twin-scales, the maximum accumulation of alkaloids took place at low concentrations of sucrose, and it decreased progressively as the source of carbon increased, especially in the case of the galanthamine type alkaloids. Nonetheless, the alkaloid profile is not exactly the same as that observed in the bulb-derived shoot-clumps. In this case, the major alkaloids were *N*-formylnorgalanthamine and galanthamine, reaching a maximum accumulation in the treatment with 30 g/l of sucrose.

Comparing the effect of the sucrose on alkaloid production in the two kinds of explants used in this experiment, it is observed that generally the shoot-clumps derived from seeds produced a higher amount of alkaloids than those derived from bulbs (Table 7.6). The highest content of galanthamine was 0.61 mg/g DW in the seed-derived shoot-clumps treated with 3% of sucrose. Likewise, the release of alkaloids to the culture medium was also higher in the seed-derived shoot-clumps than in those derived from bulbs. Although the causes are unknown, there are several factors that might be influential, for instance, the initial physiological state of the inoculum.

The results obtained in the two strains show that the production of alkaloids does not seem to be directly related to the growth of the explants, as the maximum

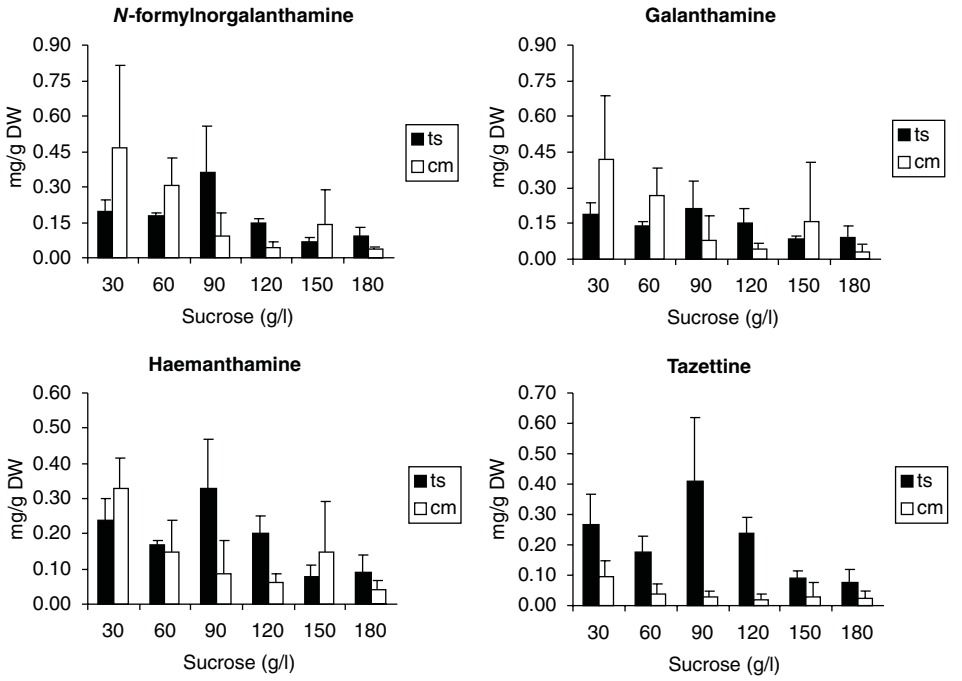

Figure 7.14 Alkaloid production in seed-derived shoot-clumps grown under different sucrose concentrations. Bars represent the SD calculated from three replicates. ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

Table 7.6 Effect of sucrose on alkaloid production (accumulation in both tissue and liquid medium) in shoot-clumps obtained from bulbs and seeds (values are % DW)[a]

Sucrose (g/l)	*Bulb-derived shoot-clumps*				*Seed-derived shoot-clumps*			
	NFNGAL	*GAL*	*HAEM*	*TAZ*	*NFNGAL*	*GAL*	*HAEM*	*TAZ*
30	0.028	0.028	0.082	0.015	0.065	0.061	0.057	0.037
60	0.018	0.021	0.041	0.009	0.048	0.040	0.031	0.021
90	0.008	0.021	0.022	0.005	0.045	0.030	0.041	0.043
120	0.014	0.021	0.043	0.008	0.020	0.018	0.025	0.025
150	0.009	0.022	0.032	0.004	0.021	0.023	0.023	0.012
180	0.024	0.047	0.061	0.009	0.012	0.010	0.011	0.008

Note
[a] for abbreviations, see Table 7.1.

growth index (at 90 g/l of sucrose) does not coincide with the maximum concentration of alkaloids (at 30 g/l of sucrose). Although both strains showed similar growth, the seed-derived shoot-clumps constituted a better kind of plant material for alkaloid production than those obtained from adult bulbs.

EFFECT OF CULTURE VESSEL SIZE ON GROWTH AND ALKALOID PRODUCTION

After observing that the best treatment for the growth of the explants in liquid medium was that of 90 g/l of sucrose, an experiment to produce alkaloids during a longer period of time (two months) was designed. Unlike the previous experiments, this was performed with small plantlets obtained from a strain of seed-derived organogenic callus, and two sizes of culture flasks (250 and 500 ml) were used to check the possible influence of this factor on the growth and morphology of explants and alkaloid production (Sellés, 1996). The selected plantlets showed a well formed bulb (*ca.* 0.9–1.3 cm), with emerging leaves (2–3 cm in length) and small shoots at the base of the bulb. They were cultured in a liquid MS medium supplemented with 90 g/l of sucrose. The 250 ml and 500 ml erlenmeyer flasks contained 50 ml and 100 ml of liquid medium, respectively. The samples were analysed every two weeks, coinciding with subculturing, and the morphology of the plantlets and the accumulation of alkaloids were determined.

Effect on growth and morphology of the explants

In general, plantlets grown in both sizes of vessels showed well formed bulbs, with leaves up to 8–10 cm in length. The number of shoots developed by the end of the experiment normally coincided with the number of small buds of the initial explant, and they showed good development with apical dominance of the central shoot. Some vitrification and necrosis were observed during the experiment in about 7% of the explants. These problems were more notable in the 500 ml flasks, probably because the explants were practically submerged in the liquid culture in these vessels which prejudiced foliar development and promoted the growth of bulbs.

Effect on alkaloid production

In the first sample, the excretion of alkaloids to the culture medium was not determined, only accumulation in the tissue.

250 ml flasks experiment

Figure 7.15 shows the results of the alkaloids produced by the plantlets cultured in the 250 ml flasks. In general, no significant fluctuations in the alkaloid production were observed during liquid culture, although the highest levels were observed in the first subculturing step. The main alkaloids were found to be galanthamine and haemanthamine, which reached total (tissue and liquid culture) levels of 1.54 and 1.51 mg/g DW, respectively. The highest accumulation of *N*-formylnorgalanthamine took place four weeks after starting the experiment, reaching 1.01 mg/g DW. The total levels of tazettine were found to be more or less constant throughout the experiment, but they were always lower than those accumulated by the explants before starting the assay.

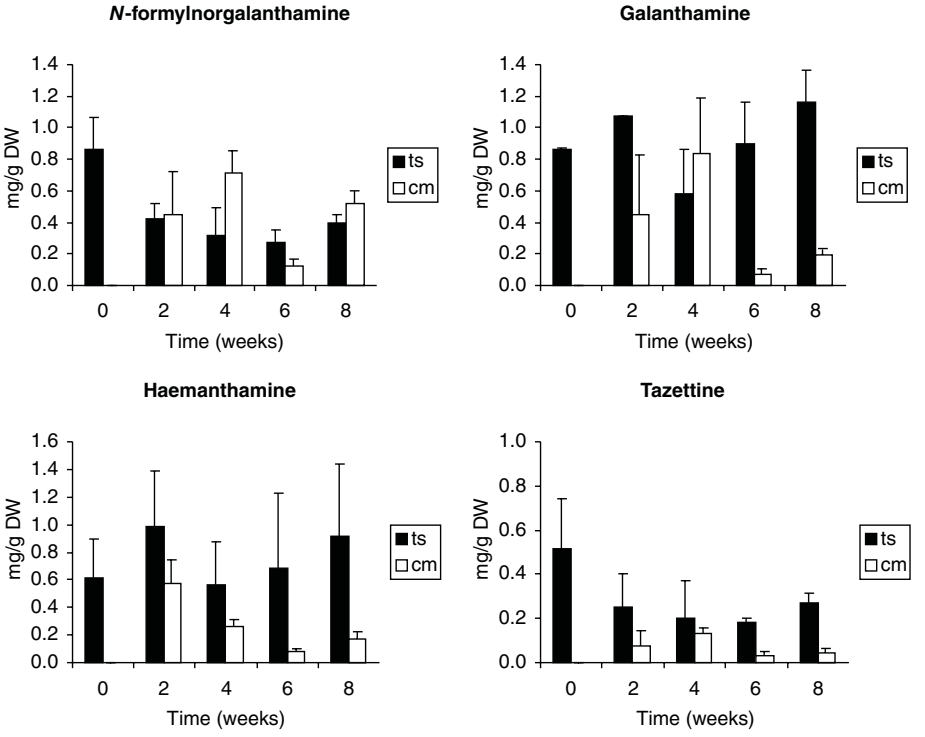

Figure 7.15 Alkaloid production by plantlets grown in 250 ml flasks. Bars represent the SD calculated from three replicates. ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

In general, the accumulation of alkaloids was always higher in the tissue than in the culture medium, except in the case of the galanthamine type alkaloids, especially four weeks after starting the experiment in the liquid medium culture. From the beginning, *N*-formylnorgalanthamine was the alkaloid most susceptible to being released into the culture medium. In all the cases, the maximum concentration of alkaloids in tissue occurred in the final step of the experiment. This was probably due to the fact that the plantlets kept in this kind of flask were close to the limit of exponential growth, when the accumulation of secondary plant metabolites is usually at its highest (Maldonado-Mendoza *et al.*, 1993).

500 ml flasks experiment

As in the former experiment, plantlets cultivated in 500 ml flasks produced galanthamine and haemanthamine as the major alkaloids, also showing maximum concentration two weeks after starting the experiment, with total (tissue and culture medium) values of 1.24 and 1.32 mg/g DW, respectively (Figure 7.16). In addition, *N*-formylnorgalanthamine and tazettine also reached maximum levels in the same period, 1.18 and 0.38 mg/g DW, respectively.

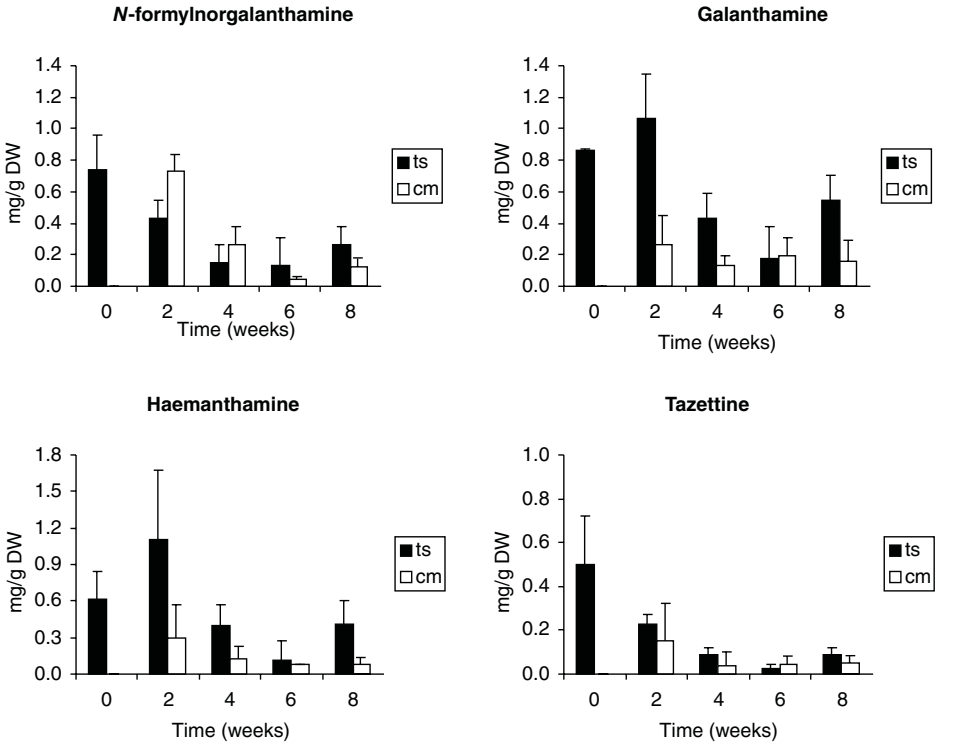

Figure 7.16 Alkaloid production by plantlets grown in 500 ml flasks. Bars represent the SD calculated from three replicates. ts, alkaloids detected in the tissue; cm, alkaloids released to the culture medium.

As in the 250 ml flask experiment, the main alkaloid released to the medium culture in the 500 ml flasks was found to be *N*-formylnorgalanthamine, especially in the first subculturing steps. In general, there was a higher accumulation of alkaloids in the tissue than in the liquid medium. Nonetheless, the maximum concentration of alkaloids in this case did not take place at the end of the experiment, probably because the plantlets still had room to grow in the bigger flasks, thus being far from their maximum potential growth.

Comparing the effect of the size of the culture vessels on the alkaloid production in *N. confusus* plantlets one can first observe that the alkaloid profile of the explants is not always the same (Figure 7.17). Thus, in 250 ml flasks the total concentration of alkaloids stayed more or less constant throughout the experiment, with minor fluctuations. The main alkaloids were found to be galanthamine and haemanthamine, showing the highest levels two weeks after starting the culture. The accumulation of *N*-formylnorgalanthamine and tazettine was more or less constant throughout the experiment, with the exception of the minimum levels corresponding to 6 weeks of culture. The plantlets grown in 500 ml flasks exhibited a similar behaviour, and except for tazettine, all the alkaloids reached maximum and minimum concentrations two and six weeks after starting the culture, respectively.

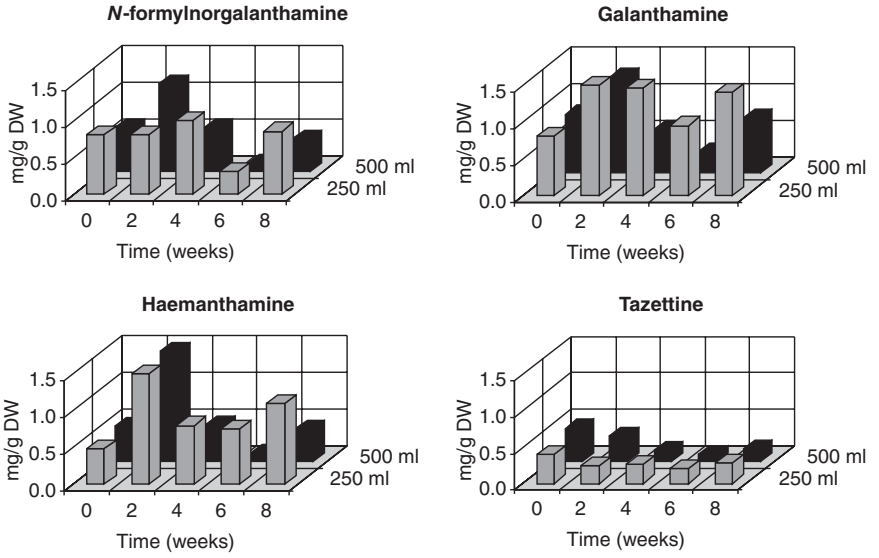

Figure 7.17 Comparison between the total production of alkaloids (detected in the tissue and released to the liquid medium) by plantlets grown in two different flask sizes throughout the experiment.

The maximum concentration of alkaloids in both sizes of flasks was observed in the first subculture. This could be due to the fact that the explants have to adapt to new environmental conditions at an early stage, when transferred from a solid medium with growth regulators to a liquid one without phytohormones and with a higher than normal concentration of sucrose. This stressful situation could be responsible for a higher synthesis of alkaloids.

In general, the accumulation of alkaloids was higher in 250 ml than 500 ml flasks, although the maximum levels were similar in both vessels. From a morphological point of view, the plantlets obtained at the end of the experiment appeared similar, although the leaf development of the explants cultured in 500 ml flasks was slightly poorer. As the shaking speed was the same in both cases, the larger flasks had more atmospheric oxygen and, therefore, better aerated plantlets, producing bulblets that were less stressed and with lower levels of alkaloids.

CONCLUSIONS

The culture of shoot-clumps of *Narcissus confusus* in liquid medium has been found to be a good experimental system for the production of galanthamine and related alkaloids. The culture of this kind of explant is improved by the addition of the cytokinins BA or kinetin at low concentrations (1–3 mg/l). The accumulation of galanthamine in tissues is higher in shoot-clumps treated with BA, whereas its release into the culture medium takes place more when kinetin is present. Another growth regulator, paclobutrazol, affected the two kinds of explants (bulblets and

shoot-clumps) in different ways, although it did not contribute to an increase of alkaloid accumulation. Sucrose is required for both the growth of the explants and alkaloid production. Nonetheless, independently of the origin of the explant used, bulbs or seeds, the optimal concentration of sucrose to obtain biomass (90 g/l) does not coincide with the best conditions to induce higher alkaloid accumulation (30 g/l). In general, the culture of shoot-clumps in liquid medium has been found to be stable throughout the experiments (up to two months), the galanthamine-type alkaloids being released into the culture medium in a higher proportion, which facilitates their extraction. Obviously, apart from these culture conditions, other parameters involved in the biosynthesis of galanthamine, as well as technological conditions, have still to be studied before considering the possibility of scaling-up production using biotechnological methods.

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8 *Narcissus* and other Amaryllidaceae as sources of galanthamine

O.A. Cherkasov and O.N. Tolkachev

INTRODUCTION

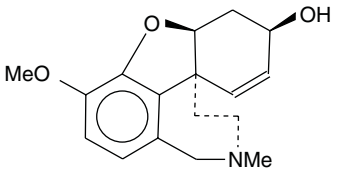
The Amaryllidaceae family contains 65 genera and over 860 species. Amaryllidaceae species are widely distributed in the tropical, sub-tropical and temperate zones of both hemispheres, and a vast diversity of species is characteristic of the flora of the South African Cape region, South and Central America and the Mediterranean coasts. They possess highly decorative characteristics, and have been used from ancient times in floriculture and medicine (Artyushenko, 1970; Khamidkhodzhaev, 1984).

Amaryllid plants are very important as ornamentals. They are popular for growing in parks and gardens, as farm crops and as indoor and greenhouse plants for winter and spring colour (Artyushenko, 1963; Tsytsin, 1960; Petrov, 1975).

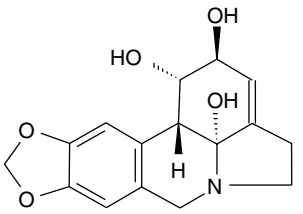
All Amaryllidaceae are rich in alkaloids, the principal biologically active components of plant extracts. Today over 200 alkaloids have been isolated from plants of this family. Incidents of animals being poisoned by Amaryllidaceae, due to the alkaloids contained, have been recorded in the literature: poisonous examples include *Galanthus* and *Leucojum* species (Gusynin, 1955). The presence of alkaloids in plants is believed to be a protective adaptation, which in Amaryllidaceae is connected with the seasonal cycle of development, many species growing in early spring when other genera are only just starting to grow.

Amaryllidaceae have attracted the attention of scientists as the sources of novel compounds, including potentially valuable alkaloids of medicinal importance. The chemistry of Amaryllidaceae alkaloids has been reviewed in several reviews and monographs (Cook and Loudon, 1952; Cherkasov, 1977; Döpke, 1976, 1978; Wildman, 1960, 1968; Fuganti, 1975; Grundon, 1984, 1985, 1987; Jeffs, 1990; Lewis, 1990, 1997; Medvedeva *et al.*, 1994; Abduazimov, 1993; Polt, 1996; Ruan, 1988; Yunusov, 1981). The absolute configuration and ring conformation of (–)-galanthamine have recently been determined by crystallographic methods (Peeters *et al.*, 1997).

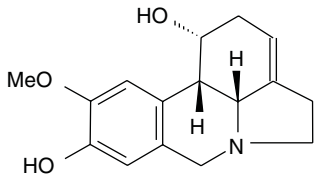
Galanthamine (Figure 8.1), also known as Galanthin and Nivaline Jilkon (Negwer, 1978), is the most important pharmaceutically active compound among the Amaryllidaceae alkaloids, and it is used in medicine as the hydrobromide. It is an anticholinesterase agent of low toxicity used in medicine for the treatment of myasthenia, myopathy, neuritis, residual phenomena after poliomyelitis anterior acuta (infantile paralysis), psychogenic 'spinal impotence', spastic pareses and progressive muscular dystrophy, and as the antagonist of muscular relaxants in



Galanthamine



Lycorine



Fortucine

Figure 8.1 Formulae of galanthamine, lycorine and fortucine.

the case of surgical interventions (Klyuev and Babayan, 1979; Kovanev *et al.*, 1967; Krylov, 1999; Mashkovsky and Kruglova-L'vova, 1951; Mashkovsky, 1955, 1984; Saev and Tenev, 1963; Sokolov and Zamotaev, 1989; Turova and Sapozhnikova, 1982). It counteracts the sedative, hypnotic or respiratory effects of benzazepines and is used for treatment of schizophrenia (Snorrason, 1996). Galanthamine is a selective acetylcholinesterase inhibitor which was recently clinically trialled for the treatment of Alzheimer's disease (Fulton and Benfield, 1996; Selles *et al.*, 1997b). The biogenetic precursor of galanthamine, (±)-narwedine, has been studied as a respiratory stimulator (Bazhenova *et al.*, 1972). Narwedine was shown to inhibit the action of narcotics and hypnotics, and to increase the effect of analgesics.

Lycorine (Figure 8.1), also known as Narcissin and Galanthidin (Negwer, 1978), and dihydrolycorine have antiarrhythmic action (Aliev, 1972), and lycorine hydrochloride is used in medicine in Russia as a strong broncholytic (Klyuev and Babayan, 1979). Some alkaloids of this group possess an inhibitory activity on herpes simplex virus (Renard-Nozaki *et al.*, 1989). The pharmacological activity of Amaryllidaceae alkaloids has been reviewed by Ruan (1988) and Evidente *et al.* (1986).

A number of galanthamine derivatives have been obtained. A study of structure–activity relationships revealed potent *N*-methyl-galanthamine hydroxide, which is more than 20 times as active as galanthamine in anticholinesterase activity (Abdusamatov, 1968, 1972). Three-dimentional quantitative structure–activity relationship studies of galanthamine and its analogues have been carried out. From the calculation of the lowest energy conformations, it was shown that the dominant factor was the steric effect, whereas the electrostatic effect of the large substituent decreased activity (Luo *et al.*, 1995).

SOURCES OF GALANTHAMINE AND LYCORINE USED IN MEDICINE

Galanthamine and lycorine have so far attracted most interest for their medical applications. Galanthamine was isolated for the first time from *Galanthus woronowii* Losinsk. by Proskurnina and Yakovleva (1952, 1955), and was afterwards found in many other species of Amaryllidaceae, including *Leucojum aestivum* L., *Ungernia victoris* Vved. and *Pancratium* and *Sternbergia* species (Abdusamatov, 1972; Boit, 1961; Cherkasov, 1975, 1976, 1977; Cherkasov *et al.*, 1984a,b, 1985, 1986, 1988, 1989; Cherkasov and Tolkachev, 1996; Gorbunova *et al.*, 1978; Maisuradze *et al.*, 1984, 1985; Sadykov and Khodzhimatov, 1988). On a commercial scale, galanthamine has been produced from *Galanthus nivalis* L. in Bulgaria under the name 'Nivalin'. In the former Soviet Union galanthamine was produced from leaves of *G. woronowii* in Russia and from leaves of *Ungernia victoris* in Uzbekistan (Abdusamatov, 1968, 1972; Cherkasov *et al.*, 1984a; Khamidkhodzhaev, 1967, 1977, 1982). *U. victoris* is an endemic plant growing widely in Tajikistan in the Gissar mountain region, the natural area of which is limited (Cherkasov *et al.*, 1984a). Leaves of the plant contain 0.05–0.15% galanthamine, while its content in bulbs varies from 0.05% up to 0.40–0.60% in some populations. The limited supply of *U. victoris* has been exhausted by commercial harvesting. Attempts at the cultivation of *Ungernia* were unsuccessful because of its slow regeneration: it would require about 45–50 years to restore the former production from small bulbs or from seeds. As the natural supply of the plant material does not satisfy the total demands of the pharmaceutical industry, a search for alternative natural sources of galanthamine is of great importance (Cherkasov, 1977; Cherkasov *et al.*, 1984a; Khamidkhodzhaev, 1967, 1984).

Measurement of the galanthamine levels in Amaryllidaceae has been carried out by a number of groups in Russia (e.g., Patudin *et al.*, 1978; Gorbunova *et al.*, 1978; Sadykov and Khodzhimatov, 1988). The concentration of galanthamine in Amaryllidaceae has been found to vary widely between the species from the 18 genera studied, from trace amounts to 0.5%. In *Leucojum vernum, Galanthus elwesii, G. nivalis, Ungernia victoris* and *Narcissus*, galanthamine was found to be the principal component of the alkaloid fraction, making up from 30 to 50% of the alkaloid fraction and corresponding to 0.05–0.5% on a dry weight basis (Cherkasov, 1975; Gizba *et al.*, 1982).

Galanthamine is usually accompanied by lycorine in plants. Lycorine has been isolated from various species of the Amaryllidaceae family, including *Amaryllis belladonna* var. *purpurea, Cooperanthes (Cooperia* × *Zaphyranthes) hortensis, Crinum defixum, C. laurentii, C. powelliti* var. *krelagei, C. yemense (latifolium), Eustephia yuyuensis,*

Galanthus elwesii, G. nivalis var. *gracilis, G. woronowii, Haemanthus katherinas, Hippeastrum hybridum* 'Salmon Joy', *H. rutilum, Hymenocallis amancaes, H. calathina, H. rotata (lacera), Ismene (Hymenocallis) hybridum* 'Sulphur Queen', *Leucojum aestivum, L. vernum, Lycoris albiflora, L. aurea, L. incarnata, L. radiata, Narcissus* × *gracillis, N.*×*incomparabilis, N. lobularis, N. 'odorus rugulosus'*, hybrids derived from *N. jonquilla, N. poeticus, N. pseudonarcissus, N. tazetta* and *N. triandrus, Nerine flexuosa, N. undulata, Pancratium illyricum, Sternbergia fischeriana, Ungernia victoris, Vallota purpurea, Zephyranthes andersonii (andersoniana)* and *Z. rosea* (Abdusamatov *et al.*, 1969; Boit, 1961; Cherkasov, 1976, 1977; Kadyrov *et al.*, 1980; Medvedeva *et al.*, 1994; Popova, 1982; Ruan, 1988). In *Pancratium trianthum* and *Hymenocallis littoralis* from the Upper Volta and Ivory Coast, lycorine was the predominant alkaloid (Frederik, 1982). Interconversion of ungiminorine and lycorine was found in *Ungernia severtsovii*. Towards the end of the growing period, the level of lycorine in roots and bulbs increased, while that of ungiminorine decreased. At the time of leaf development this was reversed (Smirnova *et al.*, 1965).

SEARCHING FOR NOVEL SOURCES OF GALANTHAMINE

Amaryllidaceae in the flora of the Russian Federation and adjoining regions

Amaryllidaceae are relict species, believed to have been widespread in periods of tropical climate and retreating in glacial periods. Representatives of the Amaryllidaceae growing in temperate climates are thought to be the most advanced species. Some Amaryllidaceae occur in European Russia and the adjacent countries, and they are found in the Central Asia region (Artyushenko, 1970). *Galanthus* and *Leucojum* are found throughout Russia and the adjoining countries (Khokhryakov, 1966), and are traditionally grown in gardens and greenhouses.

The Amaryllidaceae family is represented in the flora of the Russian Federation and adjoining regions by seven genera: *Leucojum* L., *Ixiolirion* Herb., *Narcissus* L., *Pancratium* L., *Galanthus* L., *Ungernia* Bge. and *Sternbergia* Waldst. and Kit. (Artyushenko, 1965; Kalashnikov, 1970; Komarov *et al.*, 1935).

Leucojum has ten species found from Ireland and North Africa to the Crimea and Caucasus, including two species in southern Ukraine, the Transcaucasian region and northern Caucasus. *Ixiolirion* has five species growing widely in Asia, while two of them are found in the Caucasus, Central Asia and eastern Siberia. *Narcissus* includes 25 to 30 species distributed in Europe and Central Asia. *Pancratium* contains 14 species, one of them growing in the region of the Black Sea coast. *Galanthus* species are found in southern and central Europe and Asia Minor. The majority of the species, 16 among a total of 25 to 27, grow widely in the Caucasus. *Ungernia* species (six populations) are found in central Asia (Artyushenko, 1970; Vvedensky, 1935, 1963; Korotkova and Khamidkhodzhaev, 1976). *Sternbergia* comprises three or four species distributed in the Black Sea region, the eastern Caucasus and Turkmenistan.

Amaryllid species are perennial herbs, and their alkaloids occur mainly in the storage organs. In most Amaryllidaceae the above-ground parts of the radical leaves occur in files, the leaf bases forming the bulbs. Species of Amaryllidaceae

have from two to 15 leaves which are 7 to 80 cm long. Bulb diameters vary from 1 cm in *Galanthus* species to 12 cm in *Ungernia*. The inflorescences of the majority of Amaryllidaceae species are borne on leafless stems with between one (*Galanthus* sp.) and 25 florets (*Ungernia*). The florets occur in umbels or corymbs; the perianth is petalloid with six free or joined segments, there are six stamens in two whorls, and the fruit is a capsule (Artyushenko, 1970; Kalashnikov, 1970; Khamidkhodzhaev, 1967, 1977). Unlike the other genera, *Ixiolirion* species have a tuberous storage organ derived from an enlarged stem base, and the leaves are borne on the aerial part of the stem.

Collecting galanthamine-containing plants

Because of the need to exploit Amaryllidaceae species which have a limited natural distribution – some species have been included in the 'Red Book' of the USSR and the Russian Federation (Takhtadjan, 1975, 1981; Borodin, 1978; Golovanov, 1988) – a programme of collection and cultivation of suitable species was started by the All-Russian Institute of Medicinal and Aromatic Plants (VILAR). A comparative biological and chemical study of the Amaryllidaceae in the flora of Russia and the adjoining territories was carried out to find species suitable for commercial exploitation. The collection of plant material was started in 1975. Seeds of Amaryllidaceae plants were obtained from '*delectus seminum*' (seed exchanges via air-mail), while seedling material of narcissus was obtained from commercial flower growers and from botanical gardens. Specimens from the flora of Russia and the adjoining territories were collected from their respective regions by expeditions organised by VILAR and its regional experimental agricultural stations. Some of the experimental results are given below.

Galanthus woronowii containing up to 0.78% galanthamine was found in Adjaria in a canyon of the Chakrizkaly river (Patudin *et al.*, 1978). It is distributed in the area of the Caucasus as far as the Turkish border. However, large-scale cropping was not practical, because the plant has such a low weight. Another galanthamine-containing representative of the family is *Leucojum aestivum* which grows widely on the sea-coasts of the Caucasus, occupying a vast area in Colkhida and also in Ukraine and Moldavia (Transcarpathia). *L. aestivum* contains tenths or hundredths parts of one per cent of galanthamine, depending on where it is collected (Cherkasov, 1975). *Galanthus nivalis* was collected in Ukraine in the Carpathian Mountains, the basin of the Dnieper River. *Leucojum vernum* and *Narcissus angustifolius* were collected in Ukraine. *Ungernia victoris* was reported in the region of Uzbekistan and Tajikistan (Korotkova and Adylov, 1960).

Early in 1975, the collection of galanthamine-containing plants consisted of seven species and 245 populations and forms, including ten populations of *Galanthus woronowii*, two of *G. nivalis*, ten of *Leucojum vernum*, 48 of *L. aestivum*, four of *Ungernia victoris* and 163 of *Narcissus* (including 115 varieties; see below) (Cherkasov *et al.*, 1984b; Kiselev *et al.*, 1977).

Species suitable for commercial exploitation

During studies on the species mentioned above, the concentration of galanthamine was the main factor taken into consideration. Also considered was

information on biomass accumulation and growth cycles, and the influence of climatic and geographic parameters. High galanthamine levels were not always accompanied by other desirable characteristics. Over 1100 specimens of wild species and cultivars, both aerial and underground parts, were examined for galanthamine content.

The best combination of characteristics (high galanthamine content and high biomass production) was found in *Ungernia victoris*, natural areas of which are presently exploited for the commercial production of galanthamine. However, attempts to cultivate *U. victoris*, a species suited for growing on hillsides, met with difficulties. *U. victoris* grows widely in the Gissar mountain range of Tajikistan. It had an alkaloid level of 0.05–0.60% in bulbs and 0.05–0.15% in leaves. However, the maximum accumulation of total alkaloids and galanthamine in the green parts of the plant was found in the phase of early growth in spring, when leaf length did not exceed 1–5 cm. Thus, the weight of green mass in wild plants was insufficient for commercial exploitation (Abduazimov and Yunusov, 1960; Abdusamatov, 1972; Abdusamatov *et al.*, 1963; Khamidkhodzhaev, 1967, 1977, 1982, 1984; Cherkasov *et al.*, 1984b).

Galanthus woronowii is characterised by a high content of galanthamine in the majority of wild populations and cultivars with 0.10–0.9% in bulbs and 0.05–0.70% in leaves. However, *Galanthus* species have no prospects for commercial culture, as the plant has a low mass, is easily damaged during mechanical cultivation, and does not adapt well to novel climatic conditions (Kovtun *et al.*, 1978; Patudin *et al.*, 1978).

Leucojum vernum grows widely in the Karpaty region and Ukraine, while over 30 populations of *L. aestivum* are distributed in the Transcaucausian region. They contain from trace amounts up to 0.15–0.18% of galanthamine in bulbs and leaves. The leaves of *L. aestivum* populations (from Abkhazia) were found to be the most potentially useful, as a source for drug production, because in some populations there was 0.30% galanthamine in bulbs and 0.34% galanthamine in leaves (Cherkasov, 1975; Cherkasov *et al.*, 1984b; Gizba *et al.*, 1982). The biology of seed germination and seedling growth in *L. aestivum* has also been studied (Cherkasov, 1980). The dynamics of galanthamine accumulation in *L. aestivum* was studied by Stefanov *et al.* (1974), and it was found that maximum concentrations of the alkaloid occurred in the phase of bud formation. The natural area of growth and supply of this species, used in Bulgaria for the production of galanthamine, has been determined by Stojanov and Savchev (1964). An experimental study on the introduction of *Galanthus, Leucojum, Narcissus* and *Ungernia* in the Moscow region has been carried out by Kiselev *et al.* (1977).

Narcissus species and cultivars as prospective sources for galanthamine production

Narcissus have been used for decoration since ancient times in Iran, Greece, Rome and Egypt, and has been described in mythological sources. Its garden forms have been systematically studied by the present authors for galanthamine content. There are about 60 species of *Narcissus* growing widely in Europe and in the Mediterranean region. In 'The Flora of USSR' (Komarov *et al.*, 1935; Kuvaev and Khamidkhodzhaev, 1989) about 20 species were described, and 'The Classified

List and International Register of Daffodil Names' (RHS, 1975) contains over 9000 names. Narcissus populations growing in the Caucasus are believed to be natural forms. Narcissus species possess a considerable vegetative mass, exceeding that of *Galanthus woronowii*.

Galanthamine content was determined for 118 varieties of *Narcissus*. In 33 garden cultivars, the level of galanthamine exceeded 0.1% of the dry weight of the plant. In wild populations, the concentration was almost zero.

A collection of *Narcissus* species and cultivars has been maintained for a number of years in the introduction plots of VILAR. Analysis of the alkaloid content of bulbs of 26 narcissus revealed that galanthamine was present in all samples (Cherkasov *et al.*, 1985). Of 19 *Narcissus* varieties, 15 contained foliar galanthamine. The cultivar 'Favourite' contained 0.15% on a dry weight basis, whereas in other cultivars the galanthamine content ranged from trace levels to 0.09%, fluctuating from year to year (Cherkasov *et al.*, 1986). At flowering time, leaves and bulbs of narcissus cultivars growing in the field contained 0.02–0.10 and 0.05–0.10% galanthamine (dry weight basis), respectively. The alkaloid contents in five greenhouse cultivars were correspondingly 0.10–0.20 and 0.05–0.20% (dry weight). Thus, after harvesting the flowers, the leaves may be used for galanthamine production (Maisuradze *et al.*, 1985). Of 81 cultivars of narcissus, 72 contained galanthamine in both leaves and bulbs. Galanthamine was reported for the first time in 79 narcissus cultivars (Cherkasov *et al.*, 1988, 1993). The galanthamine concentration in the crude drug was dependent on the plant variety, period of harvest, weather conditions, etc. The highest level of galanthamine was found in leaves of eight populations, up to 0.2–0.5%. The highest galanthamine content was found at the phase of bud formation when the growth rate was the highest.

Narcissus 'Fortune' was proposed as a prospective subject for cultivation for galanthamine extraction, as it was characterised by a consistency of chemical composition. This cultivar is widely used in commercial floriculture (Cherkasov *et al.*, 1989; Maisuradze *et al.*, 1985; Zaitseva and Novikova, 1977). The alkaloid composition of leaves and bulbs, and the dynamics of galanthamine accumulation during the growing period, were studied for 'Fortune'. Galanthamine, gemanthamine and a novel alkaloid named fortucine (Figure 8.1) were identified among the five alkaloids isolated. The structure of fortucine was elucidated by nuclear magnetic resonance and mass spectrometry, the latter distinguishing its structure from the earlier known lycorine-type alkaloids by the atypical cissoid fusion of the B/C rings (Gorbunova *et al.*, 1984; Tokhtabaeva *et al.*, 1987). The seed germination of *Narcissus* species has been studied by Cherkasov (1982).

Drying regimes for narcissus leaves have been studied, and the optimal parameters for galanthamine production were determined (80 °C, 0.77 kg/m^2/h) (Voroshilov *et al.*, 1987, 1989).

Cell cultures of the above plants were also studied. They were proposed for clonal and accelerated reproduction of rare species (Popov and Cherkasov, 1983, 1984). It has been shown that 'shoot-clump' cultures of *Narcissus confusus* in liquid medium were capable of producing galanthamine (Bastida *et al.*, 1996). Treatment with sucrose increased the growth of the cultures and affected galanthamine biosynthesis (Selles *et al.*, 1997b).

ANALYTICAL METHODS OF GALANTHAMINE DETERMINATION IN AMARYLLIDACEAE EXTRACTS

Earlier methods for galanthamine determination in plant material used paper chromatography (Vulkova and Kolusheva, 1964) and spectrophotometry (Asoeva and Bergeichik, 1967; Vulkova, 1959; Volodina *et al.*, 1970; Kalashnikov *et al.*, 1980; Kuznetsov *et al.*, 1969). However, a direct spectrophotometric determination of galanthamine is limited in its usefulness, as the compound has a low specific absorption index and occurs in the plant at low concentrations. Kolusheva and Vulkova (1966) have used spectrophotometric methods for the estimation of galanthamine (Nivaline) in ampoule solutions.

A polarographic method of galanthamine determination, proposed for measuring the compound in leaves of *Ungernia victoris*, was found experimentally to have low accuracy (Volodina *et al.*, 1970).

Methods of quantitative determination based on chromatographic or chromato-extractive separation and photo-colorimetric or photometric estimation of galanthamine in leaves of *Ungernia victoris* have been reported (USSR Pharmacopoeia, 11th edition), which were later modified and adapted for the quantitative analysis of *Narcissus* 'Fortune' (Tokhtabaeva, 1987), *N. poeticus* (Popova and Karpenko, 1989), other garden narcissus varieties and other Amaryllids such as *Galanthus* and *Leucojum*. In the method of Tokhtabaeva (1987), plant material was extracted in an acetone – water – 25% ammonia mixture. After transfer to chloroform the extract was separated by thin-layer chromatography on silica gel using chloroform – ethyl acetate – 25% ammonia as solvent. The detected zone was treated with tropaeolin 000 Nr 2 (Orange II) and the complex with the alkaloid was extracted with chloroform. The optical density of the solutions was measured photocolorimetrically. The method was used in the analytical control of galanthamine hydrobromide production. A semi-quantitative express method was also developed (Tokhtabaeva, 1987).

Recently, a high-performance liquid chromatography method has been published for the separation and quantitative determination of galanthamine and other Amaryllidaceae alkaloids in plant extracts and tissue cultures of *Narcissus confusus* (Selles *et al.*, 1997a). Capillary gas chromatography was used for the determination of the distribution of galanthamine and other alkaloids in *Narcissus* 'Ice Follies' (Moraes-Cerdeira *et al.*, 1997a,b).

A method of galanthamine extraction from leaves of *Narcissus* 'Fortune' with aqueous organic solvents has been elaborated with the application of mathematical models for the optimisation of the process (Rusakova *et al.*, 1986). An efficient large-scale technological method of galanthamine hydrobromide production has been worked out utilising available solvents and materials (Tolstykh *et al.*, 1991, 1992). Shakirov *et al.* (1969) have developed an ion exchange method of galanthamine hydrobromide production at a commercial scale from *Ungernia victoris* Vved. in 80% yield with the application of Ku-1 resin.

CONCLUSIONS

The alkaloids galanthamine and lycorine are valuable constituents of Amaryllidaceae species widely used in medicine. Recent progress in treatment of

Alzheimer's disease has attracted scientists for new investigations in this field. Comparative studies of various genera of this family have been carried out, and prospective species were found for commercial exploitation, among them *Leucojum, Ungernia* and *Narcissus*. The technological procedures for their production have been elaborated.

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9 Studies on galanthamine extraction from *Narcissus* and other Amaryllidaceae

Mirko Kreh

INTRODUCTION

When in 1947 Proskurnina and Yakovleva isolated a novel alkaloid, which they named galanthamine, from *Galanthus woronowii* (Caucasian snowdrop), they could not have foreseen the relevance of their findings for the pharmaceutical industry. Success in the treatment of poliomyelitis with galanthamine was soon reported, and the alkaloid became a routine part of the Soviet stock of medicines. As early as 1960, it was reported that galanthamine was an inhibitor of acetylcholinesterase, similar to the natural product physostigmine and the synthetic product neostigmine (Schulz, 1960).

Medicinal and pharmacological tests led to a huge number of potential uses for galanthamine. Among the variety of indications cited in the literature (ABDA, 1993) are described analgesic effects comparable with morphine (Cozanitis and Rosenberg, 1983), compensation of the effect of opiates on respiration (Paskov *et al.*, 1963), effects on alcohol abuse (Opitz, 1991) and effects in treating Alzheimer's disease (Han *et al.*, 1991).

Until the 1960s, galanthamine was isolated from natural supplies of snowdrop, *Galanthus nivalis* (Chimiko-Pharmazevtitschen Zavod, 1959), but commercial production from *Ungernia* species was also described (Cherkasov *et al.*, 1986). Nowadays, galanthamine is also isolated from *Leucojum aestivum* (summer snowflake) (Gorinova *et al.*, 1993), although a number of promising chemical syntheses have been reported (Czollner *et al.*, 1998).

The promising pharmacological properties of galanthamine, and the shrinking natural sources of the species mentioned, made it of interest to find new sources of galanthamine within the Amaryllidaceae family. The research reported here is based on a doctoral thesis presented to Marburg University (Kreh, 1995).

SELECTION AND SCREENING OF PLANT MATERIAL

The search for galanthamine was restricted to the Amaryllidaceae, because this family has a very specific alkaloid metabolism. In the literature data concerning galanthamine and the alkaloid content of species of Amaryllidaceae are found in many publications (e.g., Fuganti, 1975; Cherkasov, 1977; Cherkasov *et al.*, 1984, 1986, 1988, 1989; Tanahashi *et al.*, 1990). The concentrations reported for assay often differ for the same species. From the literature it was not possible to decide

whether these differences in galanthamine content resulted from extracting plants at different stages of development or from using plants cultivated in different regions, or were simply due to the low purity of the isolated alkaloid.

The screening described below was restricted to bulb tissue, because bulbs are the usual commodity traded and, unlike the aerial parts of the plants, are available in large quantities. The genus *Narcissus* was of particular interest, since it is indigenous to Europe and material can easily be obtained in quantity from large daffodil cultivars. *Narcissus* have been described as sources for natural galanthamine several times (Cherkasov, 1977; Cherkasov *et al.*, 1984, 1986, 1988, 1989). Looking specifically for plants which would be available in large quantities, and therefore at a low price, the screening was restricted mainly to the 20 daffodil cultivars most commonly grown in the Netherlands (Erhardt, 1993).

The plant material was obtained from cultivars grown around Hillegom and on Texel Island, the Netherlands. The identity of the plants was checked during the flowering period by studying their anatomical and microscopic characteristics. The daffodil cultivars used were identified according to 'The International Daffodil Checklist' of the Royal Horticultural Society (Kington, 1989), and, as far as possible, from reference plants and photographs.

High-performance liquid chromatography (HPLC) (Kreh, 1995) and gas chromatography (Kreh, 1995; Kreh *et al.*, 1995) were found to be suitable analytical methods for the analysis of alkaloids from Amaryllidaceae. For the quantitative galanthamine screening described below, the following HPLC method was used:

Stationary phase: LiChrospher® 60 RP-select B, 5 µm, 250×4 mm column (Merck)
Eluent A: 2.0 g sodium dodecylsulphate, 2000 ml water, 400 ml acetonitrile and 290 ml 0.05 M phosphoric acid
Eluent B: acetonitrile
Gradient: 0–25 min 25:75 A:B, 30–45 min linear to 100:0, flow 1 ml/min

For a detailed description of sample preparation, analytical equipment and quantitative evaluation, see Kreh (1995).

The results of the HPLC screening are given in Table 9.1. From the plants tested, several narcissus cultivars were found to be rich in galanthamine. However, in the case of the *Galanthus* and *Leucojum* bulbs tested, only low concentrations of galanthamine were found. Differences between these values and those reported in the literature might result from the occurrence of different chemical races (Schulz, 1960) or from the effects of growing on different soils (see below and Gorinova *et al.*, 1993). Such differences were also found in screening *Narcissus* 'Fortune' from Texel Island and from Hillegom, which have different soil types.

Narcissus 'Carlton', 'Gigantic Star', 'Ice Follies' and 'Fortune' were found to be rich in alkaloids and galanthamine. All of these belong to the group of 'large-cupped' daffodils (Division 2; Kington, 1989), which might be interesting for a further search for narcissus rich in galanthamine.

For all the subsequent studies *Narcissus* 'Carlton' (Figure 9.1) was used, since it fulfils nearly all the requirements for galanthamine production on a large scale. It has been cultivated for many years in large amounts in the Netherlands and

Table 9.1 Results of galanthamine screening: percentage content of total alkaloids and galanthamine expressed on a fresh (FW) or dry (DW) weight basis

Species or cultivar	*% Alkaloids*		*% Galanthamine*	
	FW	*DW*	*FW*	*DW*
Chlidanthus fragrans	0.371	0.531	0.0017	0.0054
Crinum × *powellii*	0.200	0.231	0	0
Elisena longipetala	0.090	0.162	0	0
Galanthus elwesii	0.113	0.290	0.0084	0.0213
Galanthus nivalis	0.071	0.124	0.0013	0.0031
Ixolirion pallasii	0.003	0.007	0	0
Leucojum aestivum	0.033	0.236	0.0009	0.0065
Leucojum vernum	0.034	0.107	0.0001	0.0001
Narcissus 'Barrett Browning'	0.127	0.263	0.0047	0.0096
Narcissus 'Brougshane'	0.172	0.503	0.0004	0.0012
Narcissus 'Carlton'	0.216	0.555	0.0725	0.1880
Narcissus 'Dick Wilden'	0.099	0.230	0.0008	0.0019
Narcissus 'Dutch Master'	0.176	0.524	0.0001	0.0001
Narcissus 'February Gold'	0.105	0.660	0.0118	0.0780
Narcissus 'Flower Drift'	0.109	0.254	0.0163	0.0381
Narcissus 'Flower Record'	0.177	0.927	0.0150	0.0800
Narcissus 'Fortune' (from Hillegom)	0.140	0.310	0.0224	0.0488
Narcissus 'Fortune' (from Texel)	0.174	0.583	0.0208	0.0695
Narcissus 'Gigantic Star'	0.157	0.650	0.0290	0.1201
Narcissus 'Golden Harvest'	0.161	0.407	0	0
Narcissus 'Ice Follies'	0.090	0.264	0.0250	0.0740
Narcissus 'Minnow'	0.028	0.079	0.0006	0.0018
Narcissus 'Salome'	0.073	0.139	0.0020	0.0038
Narcissus 'Tête-à-Tête'	0.066	0.168	0	0
Narcissus 'Unsurpassable'	0.225	0.591	0.0004	0.0010
Narcissus 'Van Sion'	0.089	0.206	0.0037	0.0086
Narcissus 'Verger'	0.055	0.161	0	0
Narcissus 'Yellow Sun'	0.138	0.480	0.0035	0.0123
Nerine bowdenii	0.220	0.241	0	0
Sprekelia formosissima	0.011	0.021	0	0
Zephyranthes robusta	0.070	0.090	0.0033	0.0150

the UK with 354 ha in 1988/1989, it was the most cultivated narcissus in the Netherlands, and therefore easy to obtain and cheap (Erhardt, 1993). *Narcissus* 'Carlton' is not especially susceptible to diseases and climate, special winter protection is not necessary, and it has a high rate of multiplication (Jefferson-Brown, 1991).

Since nearly all Amaryllidaceae alkaloids previously tested had interesting pharmacological and biological properties, it was the intention to isolate as many different alkaloids as possible from *Narcissus* 'Carlton'. Using preparative HPLC, twenty-nine alkaloids were isolated from the bulbs, in addition to galanthamine. The structures of the then unknown natural products 1-*O*-acetyl-10-norpluviine, 10-norpluviine, *O*-methyloduline, *N*-demethylmasonine, 1,10-diacetyl-10-norpluviine and *O*-acetylgalanthamine were elucidated by modern spectroscopic techniques as well as by conventional analytical methods (Kreh, 1995).

Figure 9.1 Narcissus 'Carlton'.

CONTENT AND DISTRIBUTION OF GALANTHAMINE IN *NARCISSUS* 'CARLTON' THROUGH THE YEAR

It is obvious from the growth pattern of narcissus that the amount of plant material available for galanthamine extraction varies greatly through the year, so it was presumed that galanthamine content within the plant might also vary during the year. It was already known that bulbs of narcissus harvested in March had significantly lower anti-tumour activity than bulbs from the same variety harvested between July and December (Fitzgerald *et al.*, 1958). To determine if there is an optimum time for the extraction of 'Carlton' bulbs for galanthamine, bulbs were planted in the old botanical garden of Marburg University. Over a twelve month period, samples were taken up to twice per week and tested for alkaloid and galanthamine content. The key developmental dates for these plants were:

Bulbs planted:	27 September
First leaves emerged:	24 February
Start of flowering:	30 March
Crop in full flower:	19 April
Capsule dehiscence:	20 June
Leaves died down:	18 August

Using a large number of samples and with HPLC as a highly specific analytical technique, significant results were obtained. Besides the quantitative determination of galanthamine content, analytical HPLC gave very informative findings in

relation to the qualitative and quantitative composition of the other alkaloids extracted.

Galanthamine content of the bulb

Figure 9.2 shows the content of galanthamine in the bulb of *Narcissus* 'Carlton'. Galanthamine content decreased slightly over the first few samples (in November). Three months after planting, galanthamine content increased over about four

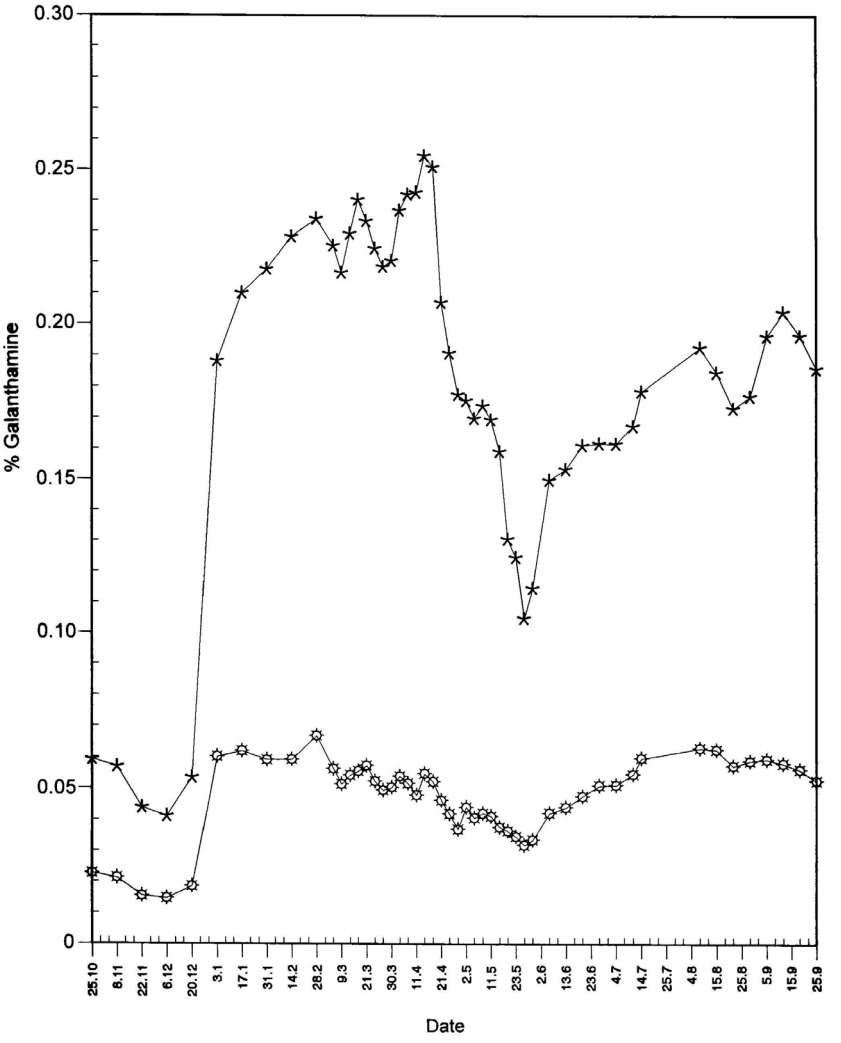


Figure 9.2 Galanthamine content of bulbs of *Narcissus* 'Carlton' over one year. Upper curve calculated on a dry weight basis; lower curve calculated on a fresh weight basis.

weeks by more than 400%, calculated on a fresh weight basis, peaking in April. The galanthamine content of the bulb decreased from the middle of April until the beginning of June, and increased again in parallel with leaf senescence. Therefore the optimum time for harvesting and extracting bulbs would be between the end of July and the beginning of August. Bulbs intended for galanthamine extraction should not be lifted earlier than this.

Although there are changes in galanthamine content of the bulbs through the growth cycle, it is not known whether some of the differences observed during the first weeks of the study were due to the change in location of the bulbs, which were obtained from Sint Maarten, the Netherlands (with a sandy soil) and grown at Marburg (in a loamy soil).

Galanthamine content of the aerial parts

During this experiment, the aerial parts of the plants were investigated as well as the bulb. Figure 9.3 shows the galanthamine content in the whole aerial parts of *Narcissus* 'Carlton'. With the growth of the leaves in spring, galanthamine content increased dramatically until just before the start of flowering at the end of March, and decreased slowly until the end of the flowering period. It then remained more or less constant for some weeks, reaching the lowest level when the leaves had completely died down.

The galanthamine content of the aerial parts was significantly lower than that of the bulb, calculated on a fresh weight basis, but it was still in a range which would make it useful for technical extraction. However, the removal of the leaves of the growing crop would not be practical because of the likelihood of subsequent rotting of the bulbs.

Location of galanthamine in the plant

Several plants of *Narcissus* 'Carlton' were separated during the flowering period into bulb, roots, leaves, scape (stem) and flower. These parts made up the following percentages of the whole plant: bulb, 39.8%; roots, 3.5%; leaves, 29.2%; scape, 21.9% and flower, 3.8%. After the natural opening of the capsules, the seeds of several plants were also collected. All plant parts were tested for their content of alkaloids and galanthamine.

Figure 9.4 shows the results of the galanthamine assay. The results demonstrated that the bulb is clearly the most interesting part of narcissus for galanthamine extraction, although all parts of the plant contain alkaloids and, in particular, galanthamine. It can be calculated from the weights of the different plant parts that bulbs planted in September (average weight, 78 g) contained an absolute amount of galanthamine which is higher than in the whole plant during its flowering period (average weight, 140 g). In summer, the absolute amount of galanthamine increased again, when the new bulb units ('daughter-bulbs') were growing.

Analytical HPLC allows an overview about the composition of the alkaloids in the individual plant parts (Figure 9.5). Galanthamine is the main component only in the bulb. In the scape, leaves and seed haemanthamine prevails, while in the flower and root both galanthamine and haemanthamine are prominent.

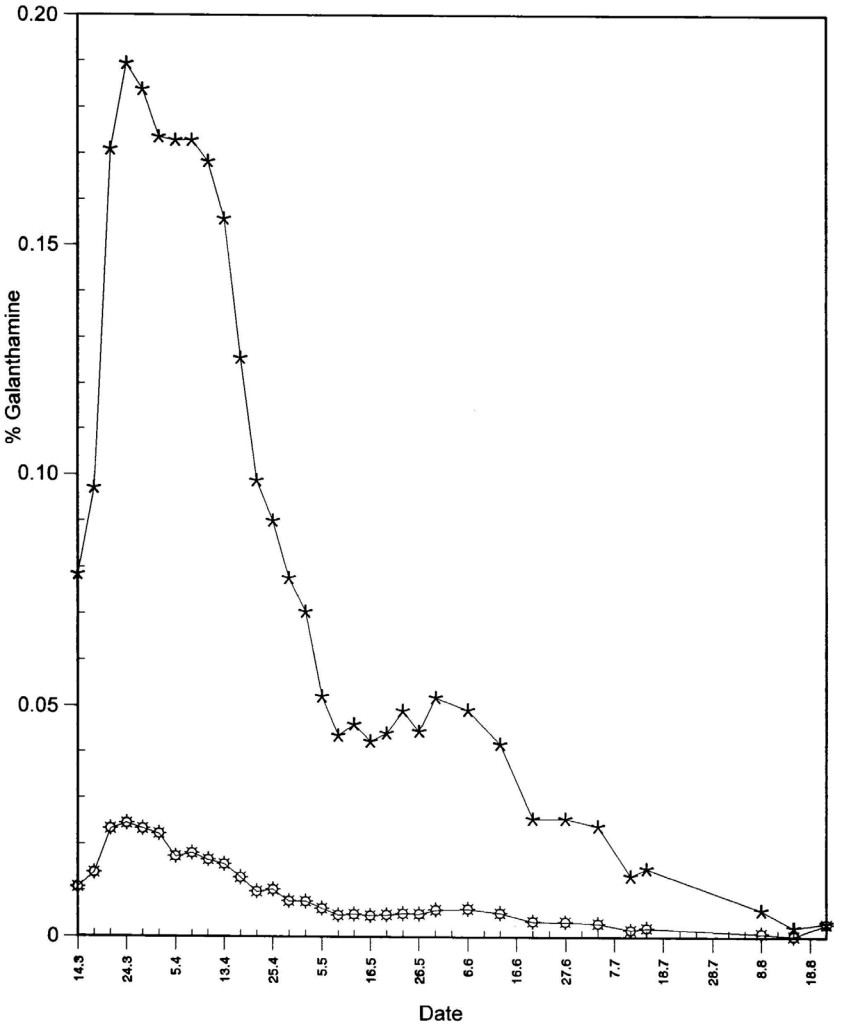


Figure 9.3 Galanthamine content of aerial parts of *Narcissus* 'Carlton' over one year. Upper curve calculated on a dry weight basis; lower curve calculated on a fresh weight basis.

EFFECT OF FERTILISERS ON THE GALANTHAMINE CONTENT OF NARCISSUS 'CARLTON'

The effect of different fertilisers on narcissus growth is well known among bulb growers. Potassium helps to develop strong bulbs and good flowers, phosphorus leads to good root development and strong plants, and nitrogen supports the growth of the leaves and the development of the bulb after leaf senescence. Too

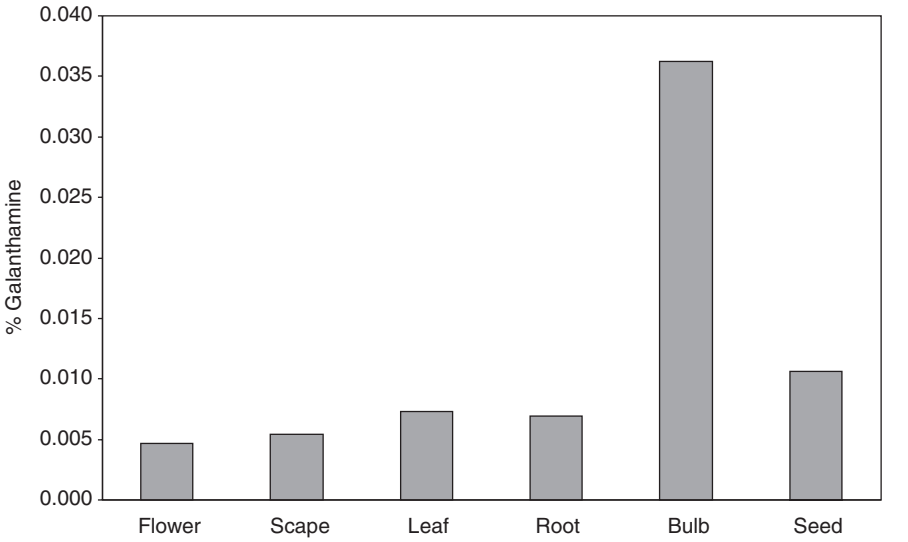


Figure 9.4 Galanthamine content of the different plant organs of *Narcissus* 'Carlton' on a fresh weight basis. Determined for flowering plants on 27 April, except for seed which was analysed after opening of the capsules (27 June).

much nitrogen, however, leads to 'soft' bulbs, which are sensitive to plant diseases and often rot (Erhardt, 1993). It was possible that the different screening results obtained for the galanthamine content of *Narcissus* 'Fortune' from Hillegom and Texel Island, the Netherlands (see Table 9.1) were a result of using different fertilisers or different soil types. Therefore, the effect of different commercial fertilisers on the galanthamine content of *Narcissus* 'Carlton' was examined.

Plots of narcissus were laid out in the old botanical garden of Marburg University. Analysis of the soil gave results typical of sandy, loamy soil (pH 6.3 and nutrient levels (mg/100 g soil) of 41 P_2O_5, 16 K_2O, 11 Mg and 0.15 soluble N). One of the plots was treated with 500 g N fertiliser per m^2 ('Kalk-Ammon-Salpeter', 27% N, of which 13.5% nitrate-nitrogen and 13.5% ammonium-nitrogen), and another with 500 g K-Mg fertiliser per m^2 (30% K_2O, 10% MgO). Untreated control plots were also grown. These amounts of fertiliser were found to be excessive, and several plants did not flower during the first year and the bulbs rotted. However, nearly all of the plants developed normally in the second year. Figure 9.6 shows the results of the galanthamine determination in the second year. The use of either nitrogen or potassium/magnesium fertiliser increased the galanthamine content of bulbs and leaves significantly over the control. An increase in galanthamine content by 70% was achieved in the case of the nitrogen fertiliser, and of 113% after using potassium/magnesium fertiliser. The results clearly demonstrated the effect of fertilisers on galanthamine content. Not only was the total amount of alkaloids markedly increased by fertiliser treatment, but also the percentage of galanthamine in the alkaloid fraction.

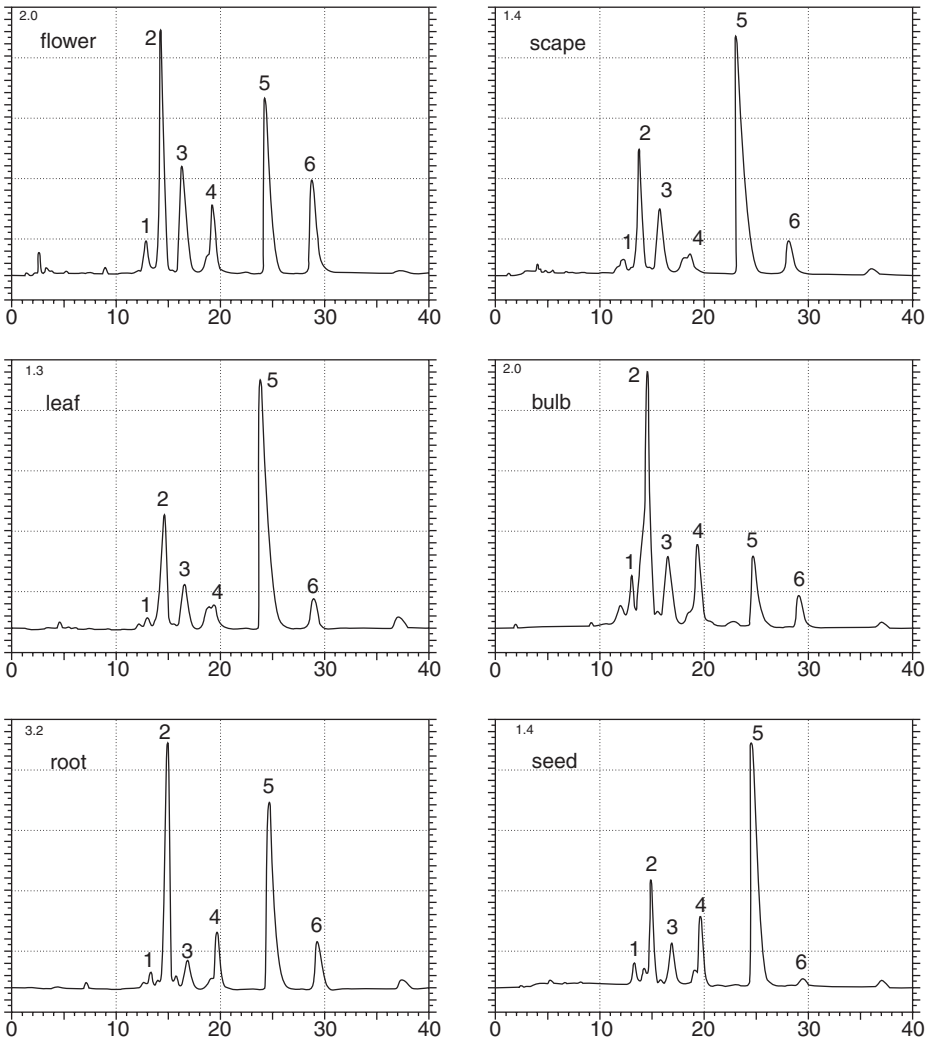


Figure 9.5 HPLC chromatograms of the alkaloids obtained from different parts of flowering *Narcissus* 'Carlton'. 1 = *N*-demethylgalanthamine; 2 = galanthamine; 3 = lycorenine; 4 = *O*-methyloduline; 5 = haemanthamine; 6 = homolycorine.

ASPECTS OF A POTENTIAL TECHNICAL EXTRACTION OF GALANTHAMINE

Narcissus 'Carlton' was identified as a potential source for technical galanthamine extraction. The well-known procedures for the production of alkaloids such as cocaine, morphine, atropine, chinine, etc., all include, as one step, a typical acid/base extraction (Schwyzer, 1931), as do the described procedures for the extraction of galanthamine (Chimiko-Pharmazevtitschen Zavod, 1963; Proskurnina-Shapiro and Yakovleva, 1966a,b; Paskov and Ivanova, 1967). The desired substance

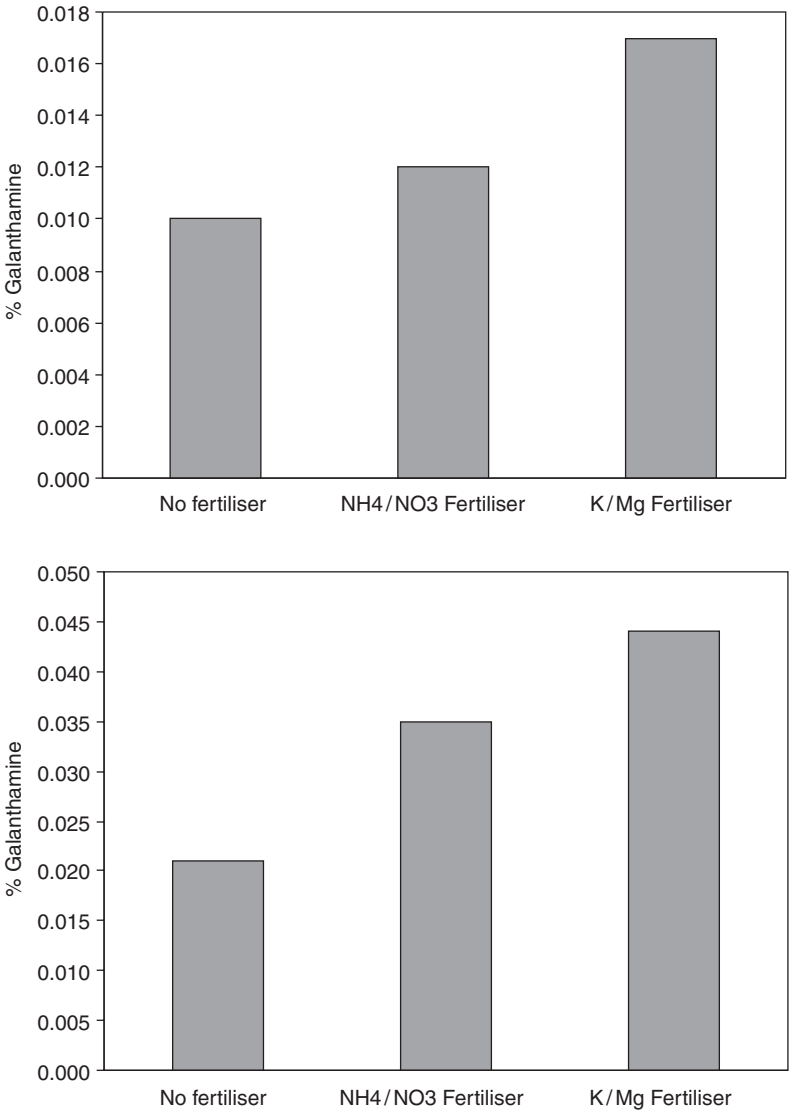


Figure 9.6 Galanthamine content in leaf (above) and bulb (below) of *Narcissus* 'Carlton' following the application of different fertilisers. All values calculated on a fresh weight basis.

is crystallised or precipitated from the raw alkaloid fraction with a suitable solvent or reagent.

To find a profitable extraction method for galanthamine, the following factors were taken into consideration:

- Isolation using a simple procedure without expensive steps such as chromatography.
- The isolation method has to guarantee a maximum yield of the desired substance.

- High purity of the resulting product.
- No drying of the plant material in the process, since it could be shown that drying the bulbs decreases galanthamine content.
- No use of expensive and toxic solvents during the entire procedure.

Optimum solvent for extraction

In the acidic plant sap galanthamine occurs in the form of its salts. A solvent was required that dissolves these salts or, after alkalisation of the plant material, the free galanthamine base. Tests were carried out using acetone, ethanol, methanol, isopropanol, chloroform, dichloromethane, diethylether, ethylacetate, n-heptane, n-hexane and n-pentane as the primary solvent for extraction and, following acid/base work-up, for obtaining the raw alkaloids (before extraction with the non-water-miscible solvents the material was made alkaline using sodium carbonate). The alkaloids were analysed by HPLC (see examples in Figure 9.7).

The polar, water-miscible solvents tested led to a quantitative isolation of galanthamine even without alkalisation of the plant material. These solvents are easily miscible with the crudely crushed plant material. The main disadvantages were that the compounds extracted in parallel with the alkaloids caused persistent emulsions during the following work-up, and that the procedure showed no preference for galanthamine within the alkaloid fraction.

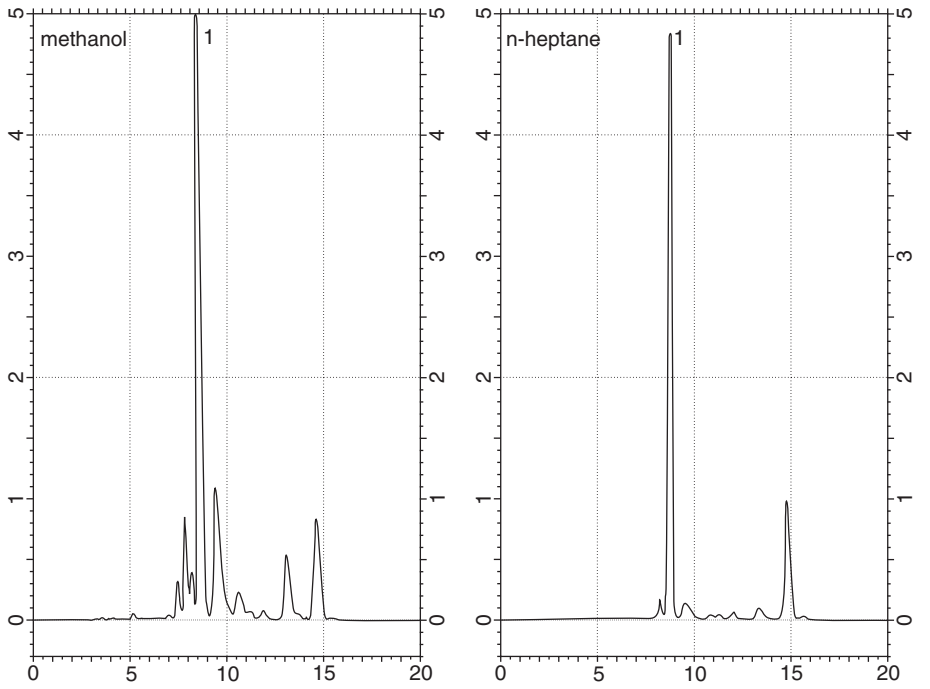


Figure 9.7 HPLC chromatograms of the alkaloid fractions obtained by using methanol (left) and n-heptane (right) for primary extraction of bulbs; peak 1 = galanthamine.

The apolar, non-water-miscible solvents tested also led, after alkalisation of the plant material, to a quantitative extraction of galanthamine. However, plant material and solvent had to be mixed vigorously, and thorough homogenisation of the bulbs greatly facilitated the process; without this, the sticky, wet plant material forms lumps into which the solvent cannot penetrate. The main advantage of some of the apolar solvents is a selectivity for galanthamine within the alkaloid fraction. For example, using chloroform for primary extraction, alkaloids were completely extracted. Using n-heptane, n-hexane or n-pentane for extraction, an alkaloid fraction was obtained in which galanthamine and haemanthamine were significantly enriched. For the following tests n-heptane was chosen, since it is less toxic and less inflammable than n-hexane or n-pentane.

The solubility of the galanthamine base is remarkable: it can be extracted with polar solvents such as methanol as well as with apolar solvents such as n-heptane. A possible explanation for this is the formation of intramolecular hydrogen bonds between the hydroxyl proton and the oxygen atom of the ether group, which makes the molecule less hydrophilic for the solvent in apolar solvents (Carroll, 1990). In polar solvents intermolecular hydrogen bonds can be formed which explain its solubility in this group of solvents.

Optimum pH for extraction

Since alkaloids may be very different in their basicity, the optimum pH value for the following liquid-liquid separations was studied. The crude alkaloid fraction was obtained by extraction with chloroform after alkalisation of the crushed plant material with sodium carbonate solution. The organic phase was extracted with diluted sulphuric acid and the resulting aqueous phase was adjusted to pH 5.0 with diluted ammonia. The aqueous phase was extracted five times with diethyl ether. The pH of the aqueous solution was adjusted to pH 6.0 and the extraction was repeated five times using diethyl ether. The procedure was repeated in steps of one pH unit until pH 12.0 was reached. The resultant ether fractions (mainly alkaloids) were analysed by HPLC. It was shown that, at pH 5, galanthamine was not found in relevant amounts in the diethyl ether fraction. However many other substances, including several alkaloids, could be separated at this pH value. At pH values >6.0, galanthamine was found in larger amounts in the organic phase. At pH values >9.0, galanthamine was extracted quantitatively from the aqueous phase, in contrast to several very basic alkaloids which still remained in the aqueous phase at pH 9.0. It was concluded that, for a technical extraction of galanthamine from *Narcissus* 'Carlton', other alkaloids should be removed at pH 5.0 by extraction with an organic solvent, before a quantitative extraction of galanthamine from the aqueous phase at pH 9.0 with the organic solvent, is carried out.

Selective crystallisation of galanthamine from the alkaloid mixture

A series of tests with many commercially available solvents was conducted to find a suitable solvent for the crystallisation of pure galanthamine from the alkaloid

fraction of *Narcissus* 'Carlton'. The best results were achieved using toluene and isopropanol. By crystallisation from isopropanol, galanthamine was obtained at a very high purity.

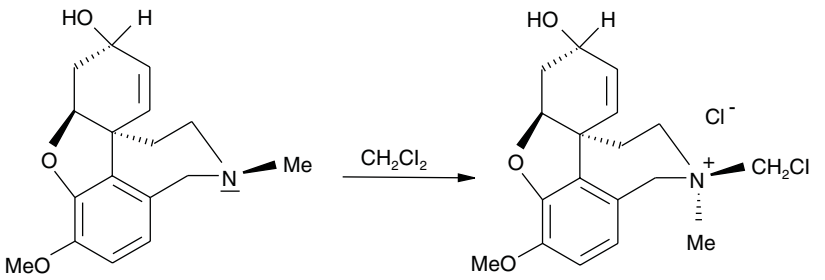
Precipitation as galanthamine hydrobromide

After precipitation of galanthamine from the alkaloid fraction using isopropanol, a significant amount of galanthamine remained in solution. To isolate the remaining galanthamine many experiments were carried out to precipitate galanthamine as various salts. However, none of the experiments proved better than the precipitation of galanthamine hydrobromide from a solution in acetone. This method is described in all patents concerning the isolation of galanthamine from plant material (Chimiko-Pharmazevtitschen Zavod, 1963; Proskurnina-Shapiro and Yakovleva, 1966a,b; Paskov and Ivanova, 1967).

Formation of potential artefacts during the extraction process

Potential consequences of the use of dichloromethane

During efforts to find the best solvent for extraction of the plant material, it was found that the use of dichloromethane should be completely avoided. Using dichloromethane led to the formation of the quaternary ammonium salt (–)-*N*-(chloromethyl)-galanthaminium chloride **(2)** in considerable quantities as an artefact (Kreh *et al.*, 1994). This compound was derived from galanthamine **(1)** by *N*-chloromethylation with the solvent dichloromethane:



(1) **(2)**

Under the same conditions using chloroform no reaction product was obtained.

Potential consequences of an excessive use of acid

During the extraction procedure it was intended to use sulphuric acid. To test for the potential degradation of galanthamine in this medium a sample of galanthamine was treated for 48 hours with 2% sulphuric acid at room temperature. In the HPLC chromatogram of the resulting mixture only extremely small amounts of degradation products were observed. However, it was of interest to know which potential degradation products would be formed under extreme conditions.

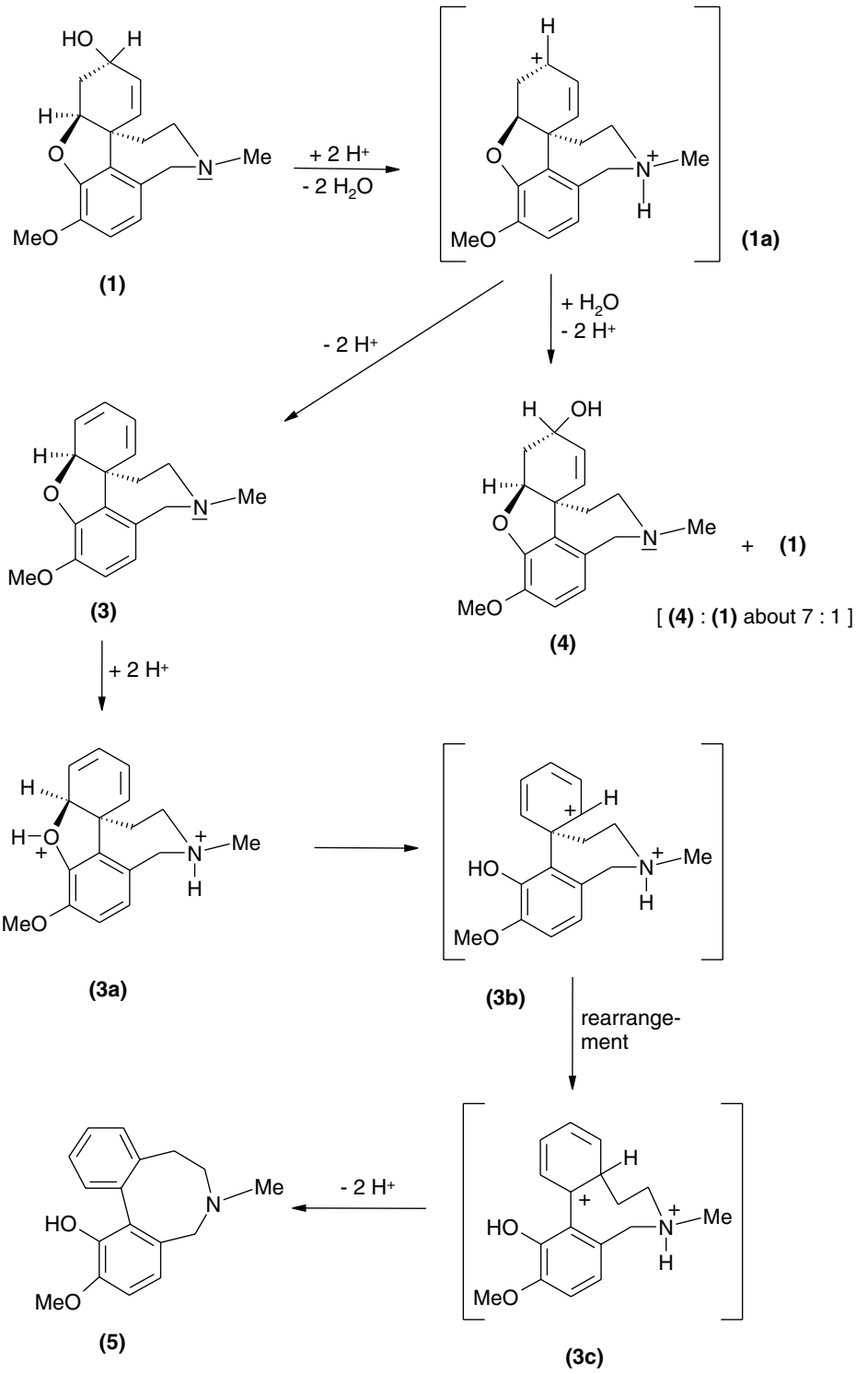
To obtain greater amounts of degradation products the experiment was repeated with 10% sulphuric acid at 70 °C. The components of the resulting solution were separated by preparative HPLC.

It was found that the excessive use of sulphuric acid led to an epimerisation of galanthamine **(1)** at the C_3-atom and the formation of epigalanthamine **(4)**. This should be taken into consideration when literature reporting the isolation of epigalanthamine from plant material is found. Another compound isolated from the reaction mixture was 3,4-anhydro-galanthamine **(3)**. The compound was not stable over a three week period. The formation of a second aromatic unit derived from galanthamine was found in the nuclear magnetic resonance (NMR) spectrum of a third degradation product which was isolated. This can occur only if a rearrangement in the galanthamine molecule takes place. A similar reaction is known after treatment of morphine with acid (apomorphine rearrangement). However, the methyl ether bond of galanthamine was not broken by sulphuric acid. The resulting molecule was identified as 6-*O*-methyl-apogalanthamine **(5)**. The scheme on the following page shows the probable mechanism of the apogalanthamine rearrangement which leads to the formation of 6-*O*-methyl-apogalanthamine.

CONCLUSIONS

Thirty commercially available species and cultivars from the Amaryllidaceae family were tested for their galanthamine content. Several large-cupped daffodil cultivars were found to be rich in galanthamine, in particular *Narcissus* 'Carlton', which is cultivated on a large scale in the Netherlands and the UK. This cultivar proved to be a suitable subject for further studies on a potential galanthamine extraction. In this cultivar galanthamine was found to be present in all parts of the plant. The highest content was found in the bulb, which is convenient for technical extraction. Galanthamine concentration in the plant fluctuated strongly during the year. The best time for harvesting the bulbs would be a short time after the usual lifting period in the Netherlands. The content of galanthamine in bulbs could be increased by applying fertilisers.

For isolation of galanthamine on a technical scale the following procedure is recommended. The bulbs are thoroughly homogenised with dry sodium carbonate and extracted with petroleum spirit (which is cheaper than n-pentane, n-hexane or n-heptane). The organic phase is concentrated by evaporation and extracted with diluted sulphuric acid. The acidic phase is adjusted to pH 5.0 with ammonia solution immediately after extraction, and separated from accompanying substances by extraction with diethyl ether. The aqueous phase is adjusted to pH 9.0 and extracted again with diethyl ether. The ether extract is dried and dissolved in warm isopropanol. Crude galanthamine base crystallises during cooling, using a seed crystal if necessary. Pure galanthamine is obtained by re-crystallisation of the crude product from isopropanol. Galanthamine remaining in the mother liquids of the crystallisations is obtained after evaporating the remaining solution in isopropanol, dissolving the residue in acetone and precipitation with ethanolic hydrogen bromide solution. The procedure is simple, relatively fast and consists of


HO
H
H
O
N
Me
MeO
(1)
+ 2 H+
- 2 H2O
H
+
O
N+
Me
H
MeO
(1a)
+ H2O
- 2 H+
- 2 H+
H
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MeO
(3a)
+
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N+
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N+
Me
H
MeO
(3c)
- 2 H+
HO
N
Me
MeO
(5)

only a few steps. Expensive purification procedures such as chromatography could be completely avoided, and the use of problematic reagents is very limited. This method has been tested with 96 kg of bulbs of *Narcissus* 'Carlton', leading to a further patent (Hille *et al.*, 1996). Galanthamine can be obtained in even higher purity than the drug currently available on the market.

ACKNOWLEDGEMENTS

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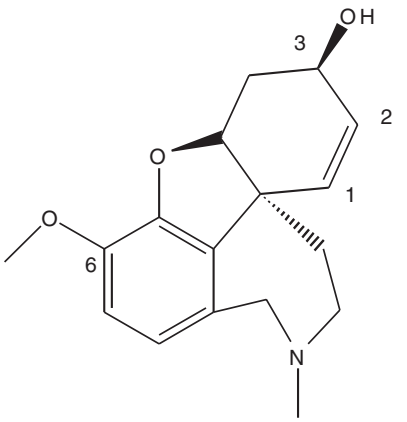
10 Galanthamine production from *Narcissus*: agronomic and related considerations

Rita M. Moraes

INTRODUCTION

Alzheimer's dementia (AD) is an ultimately fatal degenerative disease of the central nervous system, affecting four million Americans. According to the Reagan Research Institute and the Alzheimer's Association, families spend US$100 billion yearly caring for these patients. Furthermore, as the population ages, the number of Americans suffering from AD is expected to reach 6.2 million in the year 2015 (Cutler *et al.*, 1994). Galanthamine **(1)**, an alkaloid found in species of the Amaryllidaceae, has been recommended for the treatment of AD (Davis, 1987) and is currently in phase III clinical trials. Positive results have prompted the trial's sponsors, Shire Pharmaceuticals Group and Janssen Pharmaceutica Inc., to proceed with clinical development. As a selective and reversible inhibitor of acetylcholinesterase, this compound has the ability to cross the blood-brain barrier and act within the central nervous system (Mucke, 1997).

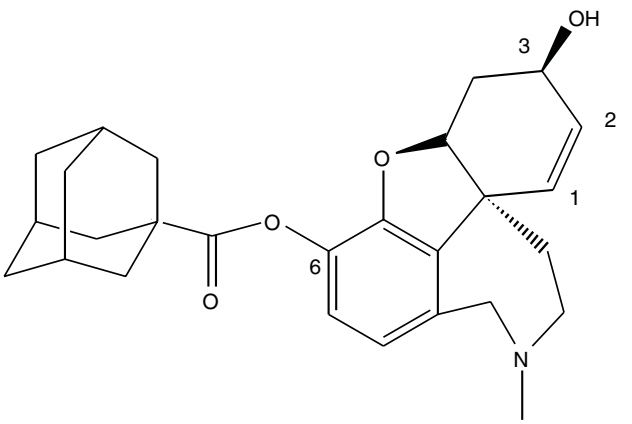


Galanthamine, **1**

In eastern Europe, galanthamine has long been used clinically as a recovery agent after anaesthesia (Schuh, 1976), and has been recently tested in a prodrug form to counteract the sedative, hypnotic and respiratory side effects of benzodiazepine therapy for schizophrenia (Snorrason, 1996). Other therapeutic uses include relief of jet lag (Davis, 1996), fatigue syndrome (Snorrason, 1994), male impotence

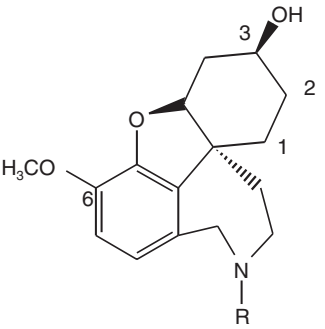
(Katz and Taneck, 1993) and nicotine (Moormann and Werner, 1997) and alcohol dependence (Opitz, 1996). Approval for the treatment of AD, together with its current uses, will create a strong and stable demand for galanthamine, even though other competitive anticholinesterase drugs are available. Recent estimates of the retail price of galanthamine are approximately US$50 000 per kilogram (Shieh and Carlson, 1994). Under present conditions, the projected demand for galanthamine would not be met by the current supply without significantly increasing its price. Most commercially available galanthamine is extracted from wild-harvested *Leucojum aestivum* in Bulgaria and Russia (Poulev *et al.*, 1993).

Research on the chemical synthesis of galanthamine has been aimed at total synthesis and also at the creation of more potent derivatives to minimise the dependence on natural resources (Bores and Kosley, 1996). Han *et al.* (1992) and Kosley *et al.* (1998) have prepared series of semi-synthetic derivatives. The adamantyl ester **(2)** is the most promising compound due to better affinity, selectivity for acetylcholinesterase and more favourable pharmacokinetics, thus reducing the amount of drug required to treat each AD patient. Research on total synthesis has improved galanthamine yields. However, the yield is still considered moderate (45 to 50%) and too low for economical commercial application (Czollner *et al.*, 1998; Eichhorn *et al.*, 1998).

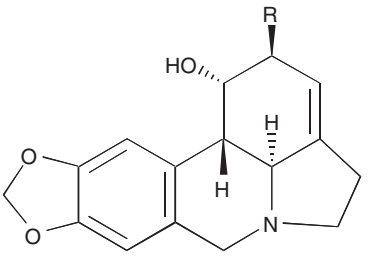


Adamantyl-ester galanthamine, **2**

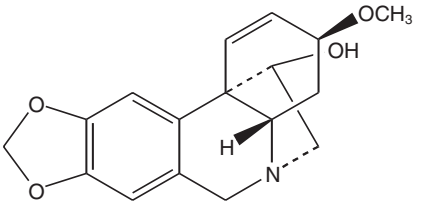
In addition to galanthamine, there is great interest in several other Amaryllidaceae alkaloids due to their wide range of biological activities, including antiviral, antimalarial and anticancer properties (Gabrielsen *et al.*, 1992; Likhiywitayawuid *et al.*, 1993). Pretazettine **(11)** is a highly cytotoxic compound against human lymphoid neoplasm. It has also been used in combination with DNA-binding alkylating agents in the treatment of the Rauscher leukaemia virus (Furusawa *et al.*, 1978). Narciclasine **(9)** has anticancer, antimitotic and antiviral activity (Evidente *et al.*, 1986; Pettit *et al.*, 1990). Lycorine **(6)** and lycoricidine possess plant growth-inhibiting properties and antiviral activity (Ieven *et al.*, 1983). According to Jimenez *et al.* (1976), dihydrolycorine, haemanthamine **(7)**, lycorine, narciclasine and pretazettine are protein inhibitors in eukaryotic organisms due to their binding to the 60S ribosomal subunit. Lycorine inhibits translation at the termination step (Vrijsen *et al.*, 1985).



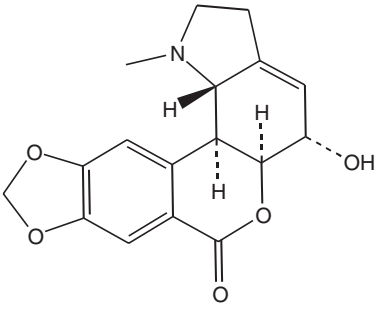
Lycoramine **3**, R=CH_3
Demethyl-lycoramine **4**, R=H



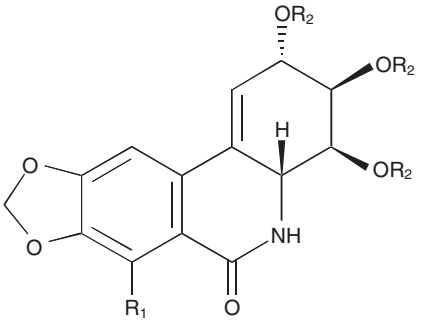
Caranine **5**, R=H
Lycorine **6**, R=OH



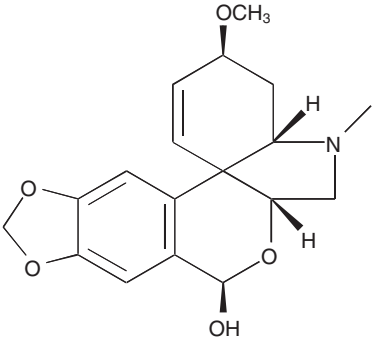
Haemanthamine **7**



Hippeastrine **8**



Narciclasine **9**, R_1=OH
R_2=H
Lycoricidine **10**, R_1=R_2=H



Pretazettine **11**

Structural formulae of the Amaryllidaceae alkaloids

At the National Center for Natural Products Research, the potential of *Narcissus* cultivars as sources of galanthamine and related alkaloids has been recognised, and research has been initiated on the agronomic factors that affect galanthamine content in them. *Narcissus* has two important advantages over *Leucojum aestivum*: firstly,

bulbs of many *Narcissus* cultivars are available in commercial quantities, offering the possibility of establishing large-scale cultivation for medicinal purposes in a short time; secondly, a comprehensive body of information already exists regarding narcissus propagation, physiology, breeding and cultivation for flower production (e.g., Rees, 1992; Hanks, 1993). In this chapter, the results of studies on planting density and depth, bulb size, and alkaloid distribution within the bulb and at different stages of bulb development, are reported. Agronomically important aspects of narcissus production for medicinal purposes will also be discussed.

AMARYLLIDACEAE FOR THE SUPPLY OF GALANTHAMINE

The present commercial sources for galanthamine are wild populations of *Leucojum* and *Galanthus*. The sourcing and cultivating of medicinal species are requirements for the progression of some natural products into pharmaceuticals. Treatment of large patient populations requires a reliable supply of the active compound at an affordable price. Therefore, the cultivation of medicinal plants is a growing segment of the pharmaceutical industry, and there is an urgent need for high-quality biomass from non-wild sources to benefit consumers and to protect the environment. Palevitch (1991) has reported the advantages of medicinal plant cultivation over collection from the wild. These are: quality products with botanical source assurance, a stable market for consumers with increasing availability of plant material, and less fluctuation in supply.

Extensive surveys seeking richer galanthamine sources have been carried out on most of the Old World taxa of the Amaryllidaceae, but New World amaryllids, especially the numerous neotropical genera, have rarely been examined. Poulev *et al.* (1993) reported that *Phaedranassa megistophylla*, a species native to Peru, contains 7.4% galanthamine on a dry weight basis. It appears to be a very promising source, since this concentration is several times higher than the highest concentration reported for any other source of galanthamine.

Galanthamine is not only found in Amaryllidaceae *sensu stricto*: low concentrations of galanthamine have been detected in the closely related families of Agavaceae, Haemodoraceae and Hypoxidaceae using an enzyme immunoassay procedure (Poulev *et al.*, 1993). Using a different method (Bastos *et al.*, 1996), most of the findings of Poulev *et al.* (1993) have been confirmed, and in a survey of *Narcissus,* more than 80 taxa were analysed. Extracts from dormant bulbs of *Narcissus* 'Inglescombe' had the highest galanthamine content, 173.7 mg per 100 g of dry weight (0.17%). A wide genotypically fixed variation in alkaloid content among cultivars was found (Bastos *et al.*, 1996; Moraes-Cerdeira *et al.*, 1997b).

ALKALOID CONTENT OF DIFFERENT PARTS OF THE BULB

The importance of galanthamine as a therapeutic drug, and the biological value of other narcissus alkaloids, prompted the study of the distribution of several alkaloids in specific bulb parts (Moraes-Cerdeira *et al.*, 1997a). Rees (1969) described the narcissus bulb as a complex branching system composed of several 'bulb units'. Each bulb unit consists of a shoot apex enclosed by bulb scales and leaf bases acting

Table 10.1 Distribution of *Narcissus* alkaloids in different bulb parts

Part of bulb	*Weights* (g)		*% total dry wt.*	*Narcissus alkaloids* (mg/100 g *dry weight*)							
	Fresh	*Dry*		*Total*	*1*[a]	*2*	*3*	*4*	*5*	*6*	*7*
Basal plates	166.7	47.3	8.3	461.2	168.5	40.9	37.1	10.8	113.7	75.6	14.6
Outer scales	484.3	186.8	32.9	148.5	59.8	16.0	22.0	6.3	25.0	18.7	trace
Inner scales	738.1	268.6	47.4	196.7	78.8	9.9	9.9	2.4	63.2	32.5	trace
Leaves	85.7	16.2	3.0	455.3	132.7	7.7	1.3	20.1	226.5	67.0	trace
Flowers	87.9	28.3	4.9	223.6	68.3	2.7	trace	30.5	73.0	49.1	trace
Bulbil	77.3	19.8	3.5	258.7	105.5	6.2	4.6	13.2	85.6	43.6	trace

Note
[a]1 = galanthamine, 2 = lycoramine, 3 = *N*-demethyl-lycoramine, 4 = caranine, 5 = haemanthamine, 6 = lycorine, and 7 = hippeastrine.

as storage organs. The scales are held together concentrically by the base plate, a solid, stem-like tissue at the base of the bulb. The shoot apices initiate scales, leaves, stems, flowers and new bulb units.

The study of Moraes-Cerdeira *et al*. (1997a) revealed that the basal plate has the highest content of galanthamine and of the other dibenzofuran alkaloids, lycoramine and *N*-demethyl-lycoramine (Table 10.1). Galanthamine concentrations were higher in the inner bulb parts. Lycorine, a highly toxic, norpluvine-derived alkaloid, is similar in its distribution to galanthamine, with a higher concentration in the inner parts. Galanthamine, lycoramine and *N*-demethyl-lycoramine have the same biogenetic precursor, but lycoramine and *N*-demethyl-lycoramine are found primarily in the scales. Therefore, no pattern of alkaloid distribution was found relating to organ specificity and biogenetic origin.

Meristematic tissues contributed one-third (33.5%) of the total alkaloids of the bulbs. Scales had 66.5% of the total content in 80% of the total dry weight of the bulb (Table 10.1). The active growth of narcissus bulbs leads to an increased ratio of meristematic tissue to scale (storage tissue) through growth that depletes the starch-containing tissues. Therefore, treatments stimulating growth enrich galanthamine and alkaloids like lycorine **(6)**, haemanthamine **(7)** and the caranine **(5)** content of the biomass, but it may also decrease the content of lycoramine **(3)** and *N*-demethyl-lycoramine **(4)**.

Data from our laboratory on different growth stages of *Narcissus* 'Inglescombe' (Figure 10.1) suggested that plants produced more galanthamine during the growing period, between emergence and anthesis, reaching as high as 287 mg per 100 g dry weight (C.L. Burandt, Jr. *et al.*, unpublished data). Galanthamine content decreased after anthesis to 173 mg per 100 g dry weight, a stage marked by scale growth and leaf senescence. As starch content increased, galanthamine appeared to become diluted by a metabolic shift towards starch production. Both studies described above indicated that agronomic factors which stimulate cell growth may enhance galanthamine content by 50% or more on a dry weight basis. Therefore, narcissus biomass for galanthamine production should be harvested in April and May at full foliage in an 18-month growing cycle. Alternatively, it may be even more economical to lift bulbs after a 24-month crop cycle, then force bulb emergence and growth by storing the bulbs in a controlled environment at high humidity and low temperature.

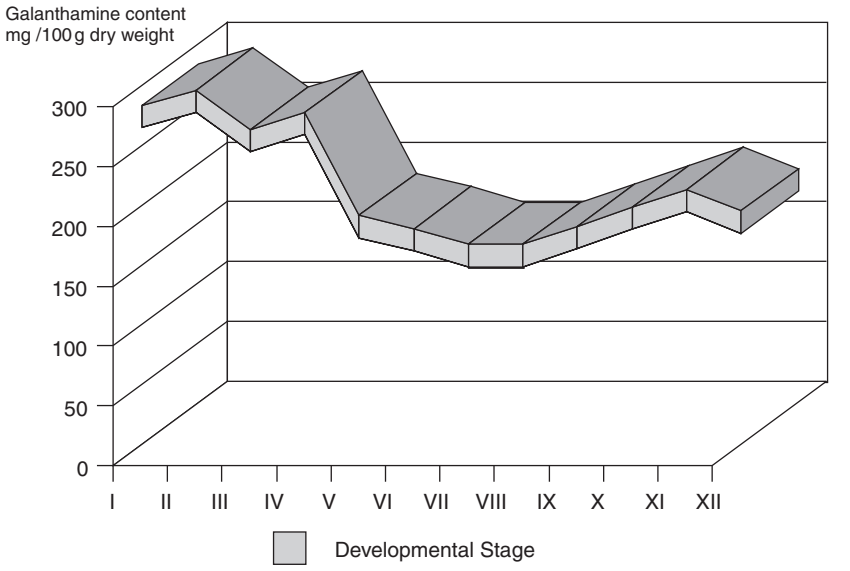


Figure 10.1 The effect of developmental stages of *Narcissus* 'Inglescombe' on galanthamine content during the 1994 growing season (unpublished data): I, pre-emergence (1 February); II, emergence, pre-bud stage (26 February); III, post-emergence, early bud stage (10 March); IV, anthesis (23 March); V, post-anthesis, 'fruiting' (25 April); VI, senescing (2 June); VII, senescent (6 July); VIII, senescent (28 July); IX, senescent (29 August); X, root re-growth (4 October); XI, shoot re-growth (4 November); XII, underground growth (10 December).

AGRONOMIC FACTORS AFFECTING DRUG YIELD

Genetic and environmental factors strongly influence biomass growth and its content of secondary compounds. To achieve maximum yields of any active compound such as galanthamine, optimum agronomic practices should be established. The agronomic practices include the identification of high-yielding cultivars, optimum growing conditions (climate, soil type, planting density, fertilisation and irrigation), pest management (weeds, insects and disease), and appropriate mechanisation for cultivation, harvesting, drying and grinding.

As mentioned above, a survey of more than 80 *Narcissus* taxa indicated that *Narcissus* 'Inglescombe' had the highest galanthamine content (Bastos *et al.*, 1996). However, the current supply of 'Inglescombe' bulbs is small and requires a mass propagation programme to increase stocks for extensive cultivation.

Propagation

The natural rate of narcissus bulb multiplication is low: the average rate of increase is 1.6 per annum, or 1 to 1000 in 16 years (Rees, 1969). Faster methods of propagation are available, such as twin-scaling (Hanks and Rees, 1979; Fenlon

et al., 1990), chipping (Flint and Alderson, 1986), and micropropagation (Squires and Langton, 1990). Mechanised chipping (Hanks, 1989) is a large-scale propagation method for abundant low-priced stocks of narcissus. In terms of achieving high numbers of propagules, the micropropagation procedure can reach 2.5 million in five years, much greater than the numbers produced by twin-scaling or chipping. However, due to the high cost of the facilities and the intensive labour required, micropropagation remains complementary to chipping and twin-scaling in the ornamentals industry. Currently, micropropagation is only used to a very limited extent on narcissus, to multiply small stocks of elite, virus-free lines and cultivars, and for germplasm propagation in breeding programmes. A combination of available propagation methods may be the wisest strategy to increase narcissus bulb stocks for galanthamine production. However, the magnitude of the losses, economic and otherwise, that AD causes to patients, families and society, may justify the high cost of micropropagating narcissus bulbs for medicinal purposes. Therefore, micropropagation may be more affordable in the pharmaceutical industry than in the ornamentals industry. If so, micropropagation could rapidly increase propagule stocks of high-yielding cultivars such as 'Inglescombe', improving biomass quality for better extractable yields.

Growth and biomass yield

As long as the supply of galanthamine relies on natural sources, there will be an increasing need to optimise biomass production and extraction to achieve maximum yields. A research programme on galanthamine supply from natural sources has both short- and long-term strategies. The short-term strategy includes a procedure with three fundamental steps, which will indicate the feasibility of pilot-scale production system. The first step consists of the identification of taxa found in abundance that could become a reliable source for establishing a production system. The second step is the optimisation of growing conditions and agronomic practices to maximise field production of the abundant source. The third step constitutes the development of a large-scale extraction and separation method to improve the yields of galanthamine isolated.

The long-term strategy consists of selecting high-yielding *Narcissus* taxa and other Amaryllidaceae, maintaining germplasm collections, mass propagating elite plants, and field studies to enhance yields in an already established production system. At the National Center for Natural Products Research, four widely available *Narcissus* cultivars, 'Geranium', 'Mount Hood', 'Cheerfulness' and 'Ice Follies', have been identified and evaluated for agronomic factors affecting their yield. The results indicated that planting depth, bulb size, planting density and the removal of flower buds did not influence drug content. However, these factors did influence bulb growth, and therefore drug yield per unit of cultivation area (Table 10.2) (Moraes-Cerdeira *et al.*, 1997b). 'Cheerfulness' and 'Geranium' exhibited good growth but low galanthamine content, thus showing little promise for drug production.

Planting density is a complex issue. Under dense plantings, the decreased growth rate reduces the yield per plant, but the biomass yield per unit area of land increases (Rees *et al.*, 1973). Galanthamine content is unaffected by high-density plantings. Therefore, depending on the growth increase, planting density may

Table 10.2 The effect of planting depth on bulb growth and galanthamine yield of four *Narcissus* cultivars

Cultivar	*Growth %*				*Galanthamine* (mg/100 g *DW*)			
	Planting depth (cm)				*Planting depth* (cm)			
	20	*15*	*10*	*means*	*20*	*15*	*10*	*means*
Geranium	37.4	20.7	0.0	19.4	6.2	6.1	8.7	7.0
Ice Follies	69.3	38.0	38.5	48.7	60.5	69.4	65.6	65.1
Mount Hood	12.8	1.7	0.9	5.1	44.2	47.7	58.7	50.2
Cheerfulness	45.0	33.0	42.5	42.5	4.9	3.9	6.4	5.1
means	41.1	23.4	22.2		28.9	31.7	34.8	
LSD[a]			9.7				11.2	

Note
[a]Least significant difference at $p < 0.05$

increase galanthamine yields per unit of production area. The economics of high-density versus low-density plantings in terms of galanthamine yield depends on the price of land, stock and labour (Moraes-Cerdeira *et al.*, 1997b). The four *Narcissus* cultivars studied showed a fixed coefficient of variation for galanthamine content, reflecting a stable and predictable drug content; the enhancement of galanthamine yield per cultivated area will therefore depend solely on biomass growth. Increasing planting density may also have a negative effect on pest and disease control but a positive one on competition from weeds.

Field production

In pharmaceutical development, the primary concerns are safety, efficacy and drug purity. Therefore, one should expect the agronomic practices in narcissus for galanthamine production to be different from narcissus grown as an ornamental. The purpose of the crop has a profound impact on cultural practices, and one example is the use of pesticides. In natural products, the use of less pesticides is a safety issue to those taking the drug in their therapy. Under this proviso, crop rotation may be a required agronomic practice for drug production to decrease pest-related problems, as well as the use of pesticides. Inter-plant distance also has an impact on disease control and pesticides application. In narcissus, the infestation of healthy bulbs with basal rot may reach 60% in dense plantings. However, healthy bulbs planted at a distance of 15 cm apart from infected bulbs showed only 6% infection (Linfield, 1993). On the other hand, dense plantings may decrease the damaging effects of weed competition. Weeds reduce crop yield by reducing growth, however weeds also decrease biomass quality due to the presence of adulterants that cannot be separated after harvest in the drying and grinding process.

Several studies have been done on alkaloid biosynthesis regarding gene regulation, the effects of different environmental conditions controlling growth and the formation of the secondary metabolites (McKnight *et al.*, 1991; Burnett *et al.*, 1993; Lopez-Meyer and Nessler, 1997). These studies revealed that most alkaloids such as galanthamine are found in higher concentrations in actively growing tissues (Moraes-Cerdeira *et al.*, 1997a; Lopez-Meyer *et al.*, 1994; Vincent *et al.*, 1997).

In order to reach maximum yields of galanthamine, bulb lifting might be early in the spring, when meristematic activity is at a maximum. Harvesting in early spring may reduce herbicide application and adulteration problems caused by weeds in heavily infested fields. On the contrary, lifting dormant bulbs may facilitate biomass-handling processes, but it will require post-harvest treatments to promote bulb sprouting to enhance galanthamine yield.

CONSIDERATIONS AND PERSPECTIVES FOR SHORT-TERM GALANTHAMINE PRODUCTION FROM *NARCISSUS*

The world's largest producer of narcissus is the UK, with a production area of about 4500 ha (Rees, 1992). Other major producers include the Netherlands, US and Poland (Hanks, 1993). *Narcissus* 'Ice Follies' is a fast growing and popular cultivar, ranking third in flower bulb sales according to the International Bulb Society, Pasadena, California. In the Netherlands, the cultivated area of *Narcissus* 'Ice Follies' in 1998/99 was given as 101 hectares (Anon., 1999). However, no equivalent data have been found on production areas of 'Ice Follies' in the other major producer countries. Therefore, we have no information on the overall commercial availability of bulbs of this cultivar, but as it is among the best selling cultivars one can expect a reasonable amount of available stock with which to initiate pilot-scale production. Additionally, 'Ice Follies' contains reasonable amounts of galanthamine: the average content extracted from dormant bulbs is 70 mg per 100 g dry weight (Moraes-Cerdeira *et al.*, 1997b).

The recommended daily dose of galanthamine to treat an AD patient varies between 30 and 50 mg (Dal-Bianco *et al.*, 1991). Therefore, a single patient would need around 20 g of galanthamine per year. Today, there are 4 million AD patients in the US alone. To treat 30% of these patients with galanthamine hydrobromide, 24 000 kg of the compound would be required per year. Aiming for a 30% market is considered a standard goal for pharmaceutical companies to invest into a new product.

Under Mississippi climatic conditions, *Narcissus* 'Ice Follies' produced 14 300 kg of bulbs per hectare in one growing cycle (Moraes-Cerdeira *et al.*, 1997b), representing a growth increase of 70%. Based on this example of bulb production and using commercial scale drug isolation of 65% efficiency, the cultivated area necessary to treat 30% of the US patients in one year would be 10 018 ha. This estimated area reflects the bulb yield of *Narcissus* 'Ice Follies' grown in Mississippi under suboptimal climatic conditions (Anderson, 1989) and 36.82% dry weight of the fresh bulbs with 70 mg of galanthamine per 100 g of dry biomass. Under the UK conditions, narcissus bulbs are typically planted at a rate of around 17.5 t/ha, and a weight increase of at least 120% would be expected for an average variety after two years, a total yield of about 38.5 t/ha (Hanks, 1993). Such high-density plantings would be a suitable growing system for a cheap cultivar with a low yield of galanthamine, and on this basis an area of 13 636 ha would be needed to produce the same amount of galanthamine as in the example above for 'Ice Follies' growing in Mississippi, but for a two-year demand to treat 30% of AD patients. Using chipped bulbs (eight chips per bulb) and planting 2 t of chipped bulbs per ha, a reasonable expectation would be a nine-fold increase in weight and number of bulbs after two

years, a yield of 18 t/ha (Langton and Hanks, 1993). This would be a more suitable growing system for a high galanthamine content cultivar which was expensive, and in short supply. An area of 7164 ha would be needed to equal the previous production of galanthamine for a two-year drug supply to treat 30% of the US Alzheimer patients using high yielding cultivars such as *Narcissus* 'Inglescombe' (174 mg per 100 g of dry weight at bulb dormant stage). Cultivation of high yielding bulbs would decrease all production requirements and costs (plantation size, handling, storage, extraction and waste products). Furthermore, harvesting 'Inglescombe' during active growth (284 mg per 100 g dry weight) would yield 63% more galanthamine than dormant bulbs and would decrease costs even more.

Another strategy to reduce the cultivation area would be the semi-synthetic approach, extracting galanthamine analogues (lycoramine, demethyl-lycoramine, narwedine and demethylnarwedine) and chemically converting these to galanthamine. Following the first promising results of galanthamine in clinical trials, many semi-synthetic derivatives of galanthamine were synthesised. A series of carbamates of 6-demethylgalanthamine were found 1000 times more potent than galanthamine as cholinesterase inhibitors (Bores and Kosley, 1996). Several esters were also prepared and among these, the adamantyl ester **(2)** is the most promising derivative. In addition, the adamantyl ester has greater selectivity and a better oral pharmacokinetic profile, and thus a higher oral therapeutic index, compared with galanthamine.

The economically viable production of galanthamine, directly or by semi-synthesis from natural resources for pharmaceutical purposes, calls for the co-ordinated contribution of many scientists in a multidisciplinary effort. Treatment of AD patients with galanthamine will require the production of large quantities of narcissus biomass of high galanthamine content. Substantial research is needed to define the most favourable agronomic practices and to optimise extraction and isolation of the pure compound.

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11 Extraction and quantitative analysis of Amaryllidaceae alkaloids

N.P. Dhammika Nanayakkara and Jairo K. Bastos

INTRODUCTION

Amaryllidaceae alkaloids represent a diverse class of natural bases that occur in different species of the family Amaryllidaceae. Owing to their wide spectrum of useful biological activities, these compounds have attracted the attention of both chemists and biologists. Many Amaryllidaceae alkaloids have been isolated and identified, and a number of methods to quantify them have been developed. Galanthamine, a common alkaloid in this family (Figure 11.1), has shown cholinesterase inhibitory activity and is currently undergoing clinical trials for the treatment of Alzheimer's disease. Another member of this family, pancratistatin, has shown a highly characteristic differential cytotoxicity profile against a panel of human cancer cell lines and strong activity against RNA viruses. This compound is undergoing development towards human clinical trials. In an effort to find new sources of galanthamine (Tanahashi *et al.*, 1990; Poulev *et al.*, 1993; Bastos *et al.*, 1996) and pancratistatin (Pettit *et al.*, 1995a,b), several species of Amaryllidaceae have been examined.

In the early years, quantitative analytical studies of Amaryllidaceae alkaloids were carried out using gravimetric methods. During the last four decades, several other analytical procedures have been described. Most of these methods were developed to quantify galanthamine in biological samples and natural sources. Several sensitive, practical methods to analyse most common alkaloids of this class in natural sources, simultaneously and quantitatively, were also reported. In this chapter, procedures published over the last four decades are reviewed.

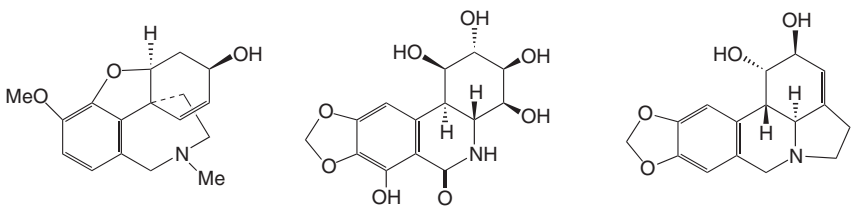

Figure 11.1 Chemical structures of Amaryllidaceae alkaloids with important biological activities.

EXTRACTION OF AMARYLLIDACEAE ALKALOIDS

In quantitative analytical studies, alkaloids are usually extracted using the traditional method. Plant material is typically dried and ground prior to extraction. However, fresh plant materials are often used for this purpose, the water content of which fluctuates with season, age, tissue type and storage conditions. For this reason, quantitative data generated for fresh plant samples collected and processed under different conditions may not be reliable for comparative purposes. Traditionally, dried ground plant material, or fresh plant material after maceration, is extracted with ethanol or a dilute acid solution. To remove lipophilic non-alkaloids, the dilute acid extract is directly partitioned with an organic solvent, whereas the ethanol extract must be evaporated and reconstituted in dilute aqueous acid prior to partitioning. The use of fresh plant material can present problems in the evaporation process, as large amounts of water and partially water-soluble polysaccharides, especially in bulbs, make evaporation of the ethanol extract difficult. After the removal of ethanol, the concentrated extract is diluted with an acid solution and partitioned with an organic solvent. The polysaccharides can also interfere with solvent extraction processes, tending to make thick emulsions and causing incomplete extraction of non-alkaloids and alkaloids in the subsequent steps. After the removal of lipophilic constituents, the aqueous layer is basified and alkaloids are extracted into an organic solvent.

In quantitative studies, where small samples are involved, problems due to emulsions in the solvent extraction process can be overcome by centrifugation (Davey *et al.*, 1998; Bastos *et al.*, 1996). However, a thick interface between organic and aqueous layers may cause incomplete removal of the target compounds. The error caused by this can be corrected by the addition of an internal standard at the beginning of extraction, and by carrying out recovery studies for the substrates and the internal standard (Claessens *et al.*, 1983; Tencheva *et al.*, 1987; Bastos *et al.*, 1996).

Queckenberg and Frahm (1994) developed a supercritical fluid extraction procedure to remove the alkaloid fraction selectively from methanol extracts. The re-extraction of dried methanol extract of Amaryllidaceae plants with supercritical nitrous oxide (N_2O) in the presence of a modifier (methanol containing ammonia gas) efficiently removed the alkaloids, which were subsequently analysed by chromatographic methods (Queckenberg and Frahm, 1993; Queckenberg *et al.*, 1996). Zhu *et al.* (1993) developed a supercritical fluid extraction method to isolate lycorine from bulbs of Amaryllidaceae, and optimum conditions for the separation were determined.

The procedure for the extraction of alkaloids (and their metabolites) from biological fluids or tissues is somewhat similar to that used for plant materials. However, due to the complex nature of the biological samples, more steps are involved (Bickel *et al.*, 1991a,b; Tencheva *et al.*, 1987). Recently, Bores *et al.* (1996) used a solid phase extraction procedure to separate galanthamine and its metabolite, 6-*O*-demethylgalanthamine, from biological samples. This procedure involves the application of biological fluids containing test compounds to reversed-phase cartridges and selective elution of the compounds of interest with appropriate solvents.

In many instances, the separation method can be simplified based on the selectivity of the detection mode. In studies where highly specific detection techniques (e.g., immunoassay) were used, minimum sample processing was necessary (Tanahashi *et al.*, 1990; Poulev *et al.*, 1993).

QUANTITATIVE ANALYSIS OF AMARYLLIDACEAE ALKALOIDS

Gas chromatography

Packed column-gas chromatography (PC-GC)

In early studies, the feasibility of gas chromatography for the analysis of alkaloids was explored using packed columns. Lloyd *et al.* (1960) analysed several underivatised Amaryllidaceae alkaloids by PC-GC, and single component sharp peaks for these compounds were observed in chromatograms. Yamaguchi *et al.* (1962) reported the detection of galanthamine, lycoramine and tazettine by PC-GC under similar conditions. However, this method was not applicable to non-volatile alkaloids, such as lycorine, and other hydroxylated alkaloids showed significant peak tailing, especially with polar liquid surfaces.

Takagi *et al.* (1968a) described the GC analysis of twelve Amaryllidaceae alkaloids, using several commercially available polar and non-polar stationary phases. The test compounds were converted to their trimethylsilyl derivatives prior to analysis. The trimethylsilyl derivatives of these compounds showed symmetrical and relatively sharp peaks, even for non-volatile alkaloids such as lycorine. Multiple peaks for lycorenine were observed under these conditions, presumably due to decomposition. These authors later applied this method to determine the quantitative changes in lycoramine and galanthamine content in *Lycoris radiata* through a period of one year (Takagi *et al.*, 1968b). In a subsequent study (Takagi *et al.*, 1969), the lycorenine content in *L. radiata* was determined quantitatively by converting it into tetrahydrohomolycorenine and analysing the trimethylsilyl ether derivative by PC-GC.

Packed column-gas chromatography-mass spectrometry (PC-GC-MS)

Millington *et al.* (1972) analysed the alkaloid fraction of *Crinum glaucum* after trimethylsilyl derivatisation by PC-GC-MS in electron impact mode. In addition to the identification of ambeline and lycorine, several new alkaloids were recognised. In a subsequent report (Onyiriuka and Jackson, 1978), the same group used PC-GC-MS in electron impact mode, in combination with other mass spectral data and nuclear magnetic resonance (NMR) spectroscopic information, to detect and propose chemical structures for several new compounds in alkaloid fractions of *Crinum ornatum* and *C. natans*.

Capillary gas chromatography (CGC)

A CGC method coupled with nitrogen phosphorus detection was also used for the quantitative analysis of Amaryllidaceae alkaloids in plant extracts (Bastos *et al.*, 1996). In this study, baseline resolution for eleven reference alkaloids

and an internal standard was achieved using a 15 m capillary column within a 15 minute run time. Good linearity of response was observed for all the reference compounds in a concentration range of 8–500 µg/ml, and concentrations around 10 µg/ml could be quantified reproducibly using this method. Quantitative analysis was carried out using 500 mg of dried plant material, and this method was used to quantify alkaloids in different plant parts (Moraes-Cerdeira *et al.*, 1997a), and to evaluate the galanthamine content of several *Narcissus* cultivars grown under experimental conditions (Moraes-Cerdeira *et al.*, 1997b).

Capillary gas chromatography-mass spectrometry (CGC-MS)

Kreh *et al.* (1995a) studied the application of CGC-MS in the detection and identification of underivatised Amaryllidaceae alkaloids. Fifteen reference alkaloids were subjected to CGC-MS in electron impact or chemical ionisation mode, and their stability under column conditions was studied. All but two compounds were found to be stable, with haemanthamine and lycorenine undergoing partial decomposition during analysis. Derivatisation with trimethylsilylating agents failed to improve either the sensitivity or the resolution of the analysis. Using this method, they were able to detect and propose the structures of several new compounds in *Narcissus* 'Carlton' (Kreh *et al.*, 1995b).

High performance liquid chromatography (HPLC) for the determination of Amaryllidaceae alkaloids in natural sources

High performance liquid chromatography (HPLC)

Westwood *et al.* (1981) investigated the feasibility of coupling HPLC with a circular dichroism (CD) spectrometer to analyse optically active compounds with suitable chromophores selectively. By using stopped-flow techniques, full CD spectra of optically active compounds were recorded. An alkaloid fraction of *Crinum glaucum* was chromatographed using a reversed-phase C-8 column and a mixture of methanol-water with a trace of ammonia as the solvent. One of the major compounds in the extract, ambelline, was detected through a comparison of the on-line recorded CD spectrum with that obtained under standard conditions.

Evidente *et al.* (1983) developed a rapid quantitative analytical procedure for lycorine using a C-18 reversed-phase column and acetonitrile:0.01 M ammonium carbonate (47:53) as the solvent. Lycorine was analysed as the sulphate salt, and was detected using an ultra-violet (UV) detector at 290 nm. The detection limit for lycorine was 5 ng. These authors applied the method to quantify lycorine in crude acid extracts of bulbs and leaves of *Sternbergia lutea*. Davey *et al.* (1998) adapted this system under semi-preparative and analytical conditions to purify and quantify lycorine from acid extracts of *Crinum asiaticum*.

Bruno *et al.* (1985) described an HPLC method to determine lycorine and tazettine in the bulbs of *Narcissus tazetta*. The alkaloid fraction was separated on a LiChrosorb-CN® column using methanol:water (25:75) containing 1% Pick reagent as the mobile phase. Compounds were detected by fluorescence, using an

excitation wavelength of 290 nm and an emission wavelength of 320 nm. The detection limit was less than 5 ng/ml.

Könükol and Şener (1992) described a reversed-phase HPLC procedure to quantify lycorine and crinine simultaneously in bulbs of *Pancratium maritimum*. A reversed-phase C-18 column and a mobile phase containing a mixture of chloroform:methanol (90:10), with UV detection at 292 nm, were used in this procedure.

Another reversed-phase method was reported by Sellés *et al.* (1997), who quantitatively evaluated galanthamine, *N*-formylgalanthamine, haemanthamine and tazettine in wild populations and tissue cultures of *Narcissus confusus*. They used a C-18 column and a mobile phase consisting of water (containing phosphoric acid, pH 3) (solvent A) and acetonitrile (solvent B) in a 60:40 ratio. Peak broadening and tailing were minimised by the addition of 10 and 12 mM of octanesulphonic acid to solvents A and B, respectively. Compounds were detected using a diode array UV detector operating at 280 nm.

High Performance Liquid Chromatography (HPLC) – Thin Layer Chromatography (TLC)

Queckenberg and Frahm (1993) developed a procedure wherein reversed-phase HPLC was coupled with automated multiple development (AMD) on normal phase silica to detect and quantify Amaryllidaceae alkaloids in natural sources. At first, conditions such as stationary phase, base solvent, modifier, buffer (pH and concentration), gradient profile, flow rate, temperature and sample size and solvent were optimised to achieve the best HPLC resolution for 20 reference Amaryllidaceae alkaloids. Under optimum HPLC conditions, the eluent from the HPLC column was applied to silica TLC plates and subjected to AMD. Compounds on the developed TLC plate were detected and quantified by different spectroscopic and chemical methods. The UV spectra of individual bands were used in combination with retention data to confirm the identity of each compound. Densitometric evaluation by a TLC scanner in the absorption/reflectance mode was used to quantify individual bands. The detection limit for tazettine by this method was 0.2 ng. These authors applied the method to re-investigate the alkaloid extract of *Amaryllis belladona* (Queckenberg *et al.*, 1996). This led to the identification of nine alkaloids, in addition to five previously known from this species, and three more were tentatively identified.

High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS)

By coupling MS and HPLC, Eckers *et al.* (1980) analysed an alkaloid fraction of *Crinum glaucum*. The extract was separated by reversed-phase HPLC using an ODS 5 μ Spheresorb® column and water:acetonitrile:ammonia (20:79.7:0.3) as the mobile phase. The eluent was subjected to MS with a moving belt interface, and total ion current in electron impact (EI) mode was recorded. Two alkaloids, lycorine and ambelline, were identified on the basis of their EI spectra and retention times. Another compound, criglaucine, of uncertain structure, was also detected.

High performance liquid chromatography (HPLC) for the determination of galanthamine and its metabolites in biological fluids and tissues

High performance liquid chromatography (HPLC)

In addition to the analysis of plant material, several HPLC methods were developed to screen galanthamine and its metabolites in biological fluids. Claessens *et al.* (1983) initially reported an HPLC method for the quantitative determination of galanthamine in biological fluids using a normal phase silica column with hexane-dichloromethane containing ethanolamine as the mobile phase. The minimum detectable concentration was 5 ng/ml, and the standard deviation varied between 18.9 and 2.5% for the concentration range 10–100 ng/ml. Subsequently, a reversed-phase HPLC method was described, using a C-18 column and acetonitrile-pentanesulfonic acid buffer in water as the solvent (Claessens *et al.*, 1988). In this method, body fluids were pre-processed by a preparative isotachophoresis prior to chromatographic analysis. Satisfactory recoveries, excellent resolution and good sensitivity were achieved. In both these procedures, a UV detector operating at 235 nm was used to detect the test compounds.

Tencheva *et al.* (1987) and Tencheva and Budevski (1987) developed a reversed-phase HPLC method for the quantitative determination of galanthamine and its metabolites, epigalanthamine and galanthaminone, in human body fluids. They used a C-8 column and a methanol:water (40:60) mobile phase modified with dibutylamine adjusted to pH 7 with 85% phosphoric acid. Codeine was used as an internal standard. The test compounds were detected by fixed-wavelength UV detector (280 nm). The detection limit was 0.05 µg/ml. This method was also subsequently used to quantify galanthamine in several native populations of *Leucojum aestivum* (Gorinova *et al.*, 1993).

A somewhat similar HPLC method was reported by Bickel *et al.* (1991a) to determine galanthamine and epigalanthamine in plasma and tissues of mice. A reversed-phase C-8 column and a mobile phase containing acetonitrile, tetrahydrofuran, water and di-*n*-butylamine at pH 7, adjusted with 85% phosphoric acid, were employed in this procedure. Codeine was used as an internal standard, and a fluorescence detector, set to 290 and 320 nm for excitation and emission frequencies, respectively, was used to detect the test compounds.

High performance liquid chromatography – mass spectrometry (HPLC-MS)

An HPLC-MS procedure for the determination of galanthamine and its metabolite, 6-*O*-demethylgalanthamine in biological samples was described by Bores *et al.* (1996). In this procedure, a reversed-phase HPLC system was utilised with a mass spectrometer operating both in the multiple reaction monitoring mode (using a heated nebuliser interface) and in the selected ion monitoring mode (with an electrospray interface).

Thin layer (TLC) and paper chromatography (PC)

Several thin layer and paper chromatographic methods have been developed for the quantitative and qualitative analysis of Amaryllidaceae alkaloids.

Sandberg and Michel (1963) analysed the alkaloid profiles in different parts of *Pancratium maritimum* collected from different geographic areas using a two-dimensional TLC procedure. Separation of the alkaloids was carried out on 10 × 10 cm silica gel plates using mobile phases diethylether: methanol: diethylamine (85:10:5) and chloroform:methanol:diethylamine (92:3:5) in the two directions. Iodoplatinate reagent was used for visualisation. A total of fifty-two alkaloids, ranging from 14 to 37 per specimen, were detected in these samples, and their relative percentages were estimated. Five of them were isolated and identified as lycorine, haemanthidine, tazettine, vittatine and hordenine, and four more new compounds were also partially characterised.

TLC on alumina was employed by Asoeva and Vergeichik (1967) to separate and quantify alkaloids in *Galanthus krasnovii*. Benzene:ethanol was used as the mobile phase, and the content of galanthamine and four other unidentified alkaloids were determined. Leifertova and Brazdova (1967) analysed *G. nivalis* collected from different regions by TLC and PC, and seven compounds, including galanthamine, lycorine and tazettine, were detected.

A quantitative and qualitative study of the alkaloid composition of wild and introduced *Leucojum aestivum* populations was carried out by Stefanov (1977). Alkaloid fractions were separated by TLC on silica gel using methanol:diethylether:diethanolamine (5:90:5) as the mobile phase, and six compounds (galanthamine, galanthaminone, lycorine, lycorenine, nivalidine and an unidentified alkaloid) were determined quantitatively.

The alkaloid fraction of common snowdrop bulbs (*Galanthus nivalis*) was studied by TLC on silica gel using chloroform:acetone:diethylamine (50:40:10), *n*-hexane: chloroform:acetone:diethylamine (80:25:30:5) and toluene:acetone:chloroform: diethylamine (45:25:25:5) as mobile phases (Kalashnikov, 1969). Dragendroff reagent was used as the visualising agent and six alkaloids, including galanthamine and lycorine, were detected.

Wurst *et al.* (1980) developed a quantitative TLC procedure to determine the galanthamine content in extracts of *Leucojum aestivum*. The alkaloid fraction was separated by TLC on silica gel using diethylether:methanol:diethylamine (80:15:5) as the mobile phase, and the compounds were detected using a TLC scanner operating at 288 nm. The detection limit for galanthamine by this procedure was about 0.2 µg, and good linearity of response and reproducibility were obtained.

Hong *et al.* (1981) developed TLC and PC procedures to identify galanthamine and lycoramine. In TLC, the best results were obtained on alumina by using either cyclohexane:chloroform:diethylamine (5:4:1) or benzene:ethyl acetate:diethylamine (7:2:1) as mobile phases. In PC, the paper was treated with a buffer at pH 4.4, saturated with water vapour prior to use, and chloroform was used as the solvent.

Dobronravova *et al.* (1982) studied the chromatographic behaviour of halide salts of some alkaloids on alumina. TLC of hydrochloride salts of lycorine and galanthamine showed two spots after spraying with Dragendroff reagent, one major and the other near the start, presumably due to decomposition. The sensitivity of detection for these compounds by Dragendroff reagent was 500 µg.

Immunoassays

Immunoassay provides a sensitive and specific tool for many analytical tasks. Tanahashi *et al.* (1990) developed a radioimmunoassay procedure for quantifying galanthamine. The antiserum was raised in rabbits against a conjugate of galanthamine-2-*O*-hemisuccinate-bovine serum albumin. This was highly specific for galanthamine, and showed practically no cross reactivity against other common Amaryllidaceae alkaloids. However, other minor cross-reactive materials were present in a crude extract of *Leucojum aestivum*. This procedure was very sensitive (measuring range 0.5–100 ng), and was used to determine the galanthamine content of crude extracts of several *L. aestivum* samples, as well as of a number of South African Amaryllidaceae.

The same group subsequently replaced the radio-labelled antigen with enzyme-labelled antigen, thereby avoiding the use of radioactive material (Poulev *et al.*, 1993). This enzyme immunoassay procedure was easier to perform and more sensitive by a factor of 100, and required only a very small amount (1–12 mg) of plant material. By using this method, galanthamine contents of 1000 individual *Leucojum aestivum* plants and of more than 130 herbarium samples of Amaryllidaceae and closely related plant families were determined. Preliminary investigation showed that this method could be applied to analyse galanthamine in biological fluids, and the results generated by this method were in good agreement with those obtained by an HPLC method. This method was later used to determine the galanthamine content in bulbs and callus of two *Pancratium* species (Sarg *et al.*, 1996).

Quantitative determination of galanthamine by acetylcholinesterase inhibition

Ghous and Townshend (1998) reported a method to determine galanthamine quantitatively by measuring its inhibition of acetylcholinesterase immobilised on controlled pore glass. The determination was carried out by a flow injection procedure where galanthamine and substrate, acetylthiocholine, were injected to coincide in the buffer stream, which then passed through the immobilised acetylcholinesterase column. Active enzyme cleaves the substrate to a chromogen reactive product. The eluent was mixed with a chromogen (5,5′-dithiobis-(2-nitrobenzoic acid)) solution and the absorbance was measured at 405 nm. Under optimised conditions, the response was linear over the range 5×10^{-7} to 6×10^{-6} M. The limit of detection was 5×10^{-7} M. Another group (Nikol'skaya *et al.*, 1989; Kugusheva *et al.*, 1992) also reported a procedure to determine galanthamine based on the same principle, using enzyme-containing membranes.

Spectrophotometric and fluorometric determination

Several simple spectrophotometric procedures have been developed to quantify galanthamine. These methods involved the measurement of absorbance in the UV range of galanthamine as a free base (λ 290 nm) (Bagdasarova, 1984) or in the visible range as a complex with other reagents (Kuznetsov *et al.*, 1969; Pavlov and Ponomarev, 1981; Tokhtabaeva, 1987). Kolusheva and Vulkova (1966) studied the

UV spectra of galanthamine, lycorine and nivalidine. All three compounds had similar UV spectra with maxima at 222 and 286 nm. A quantitative spectrophotometric method was developed for the determination of galanthamine at 286 nm.

The relationship between the fluorescence characteristics and chemical structure of galanthamine, lycorenine, lycorine, tazettine and vittatine was studied by Bruno and De Laurentis (1982). All these compounds had emission maxima at about 320 nm and excitation maxima at 290 nm. A linearity between the fluorescence intensity and concentration over 1–6 μM/l was observed, and the detection limit was 0.05 μg/ml.

Burgudjiev and Grinberg (1993) analysed the UV and fluorescence spectra of galanthamine base and its hydrobromide in water, ethanol and *n*-hexane, and limits of detection for UV spectrophotometry and fluorometry were established.

Yamboliev and Mikhailova (1985) described a spectrofluorometric method for the determination of galanthamine in biological fluids. Galanthamine was extracted using an organic solvent and re-extracted into a 0.1% aqueous sulphuric acid solution. The concentration of galanthamine was determined by spectrofluorometry using excitation and emission wavelengths of 286 and 315 nm, respectively. The fluorescence signal was linear in the range 0.05–15.0 μg/ml, and the detection limit was 0.05 μg/ml. However, due to insufficient specificity, the data on urine, bile and other pigmented fluids were unreliable.

Several spectrophotometric and fluorimetric procedures have also been described to determine lycorine in Amaryllidaceae species. In these, lycorine was first separated by chromatographic methods and was quantitatively measured spectrophotometrically at 292 nm (Volodina *et al.*, 1972, 1973; El-Din *et al.*, 1983; Makhkmova and Safonova, 1994) or fluorimetrically at excitation and emission wavelengths of 292 and 330 nm, respectively (El-Din *et al.*, 1983).

Electrophoretic methods

Gheorghiu *et al.* (1962) analysed the alkaloid extracts of *Leucojum vernum* and *L. aestivum* by a two-dimensional electrophoresis method. The analysis was carried out in alkaline medium and two compounds were detected and separated.

Mikhno and Levitskaya (1971) described a paper electrophoresis method for the detection and quantification of galanthamine and securinine in biological material. The alkaloids were separated by paper electrophoresis at pH 2 and were detected with Dragendroff visualising agent. Galanthamine and securinine were eluted from the paper with 0.1N HCl and were quantitatively determined by spectrophotometry at 289 and 256 nm, respectively.

Davey *et al.* (1998) reported a micellar electrokinetic capillary chromatographic method to analyse ascorbic acid and lycorine simultaneously in tissue extracts of *Crinum asiaticum*. Ascorbic acid and lycorine were extracted from the plant material using 3% metaphosphoric acid. Analysis utilised a 41 cm total length (34 cm to detector) × 50 μm fused silica capillary with a borate buffer (pH 9.0) and 50 mM sodium dodecyl sulphate as background electrolyte (applied voltage of +20 kV). Compounds were detected at 185 nm by fixed-wavelength detector or at 202 and 267 nm by dual-wavelength detector. A linear detector response was observed for lycorine between 17 and 700 μM. The detection limits at 185 and 202 nm were 17 and 1 μM, respectively.

Polarographic methods

Occasionally, polarographic methods have been used to determine Amaryllidaceae alkaloids quantitatively. Volodina *et al.* (1970) determined the polarographic constants of galanthamine, lycorine, pancratine, hordenine and dl-nawardine isolated from *Ungernia victoris*, and used this procedure to quantify galanthamine isolated from a chloroform extract of this species by preparative TLC. In a subsequent study, Volodina *et al.* (1976) examined nine alkaloids isolated from *Ungernia* polarographically. They found that, within certain limits, there was a linear relation between the maximum current and the concentration that could be used in analytical studies. Recently, Meng *et al.* (1998) reported an oscillopolarographic titration method to determine galanthamine hydrobromide content in drug form, using silver nitrate as the titrant, with potassium nitrate and sulphosalicylic acid as supporting electrolytes.

Counter current chromatography (CCC)

Ma *et al.* (1994) described a pH-zone-refining CCC procedure for the separation of crinine, powelline and crinamidine from *Crinum moorei* using a multilayer coil planet centrifuge. Methyl *tert*-butyl ether containing triethylamine (5–10 mM) and water containing HCl (5–10 mM) were used as the solvent system. The separation was performed by using either the organic (displacement mode) or the aqueous phase (reverse-displacement mode) as the mobile phase. Compounds were eluted as an irregular rectangular peak and were separated into three plateaus by a UV detector or by pH measurement of the fractions. TLC analysis of the fractions showed a successful separation of the compounds with very narrow mixing zones. Results observed in both displacement and reverse-displacement modes were similar; however, the compounds were eluted in the reverse order.

Gravimetric methods

Amico *et al.* (1980) used a gravimetric method to quantify lycorine in various parts of *Sternbergia lutea* during the different stages of its life cycle. The crude alkaloid fraction obtained after traditional base neutral separation was crystallised from 90% ethanol to yield lycorine in crystalline form. Lycorine was separated and its percentage based on dry weight was calculated.

Variations in the formation of pancratistatin and related isocarbostyrils in *Hymenocallis littoralis* were studied by Pettit *et al.* (1995a) by a gravimetric method. Pancratistatin, narciclasine, 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine were isolated from *H. littoralis* bulbs by a series of extraction, precipitation and separation procedures, and the variation in their content during a period of one year was determined.

CONCLUSIONS

A number of methods have been developed during the last four decades to analyse Amaryllidaceae alkaloids quantitatively. In recent years, reversed-phase HPLC

Table 11.1 GC and HPLC procedures for the identification and quantitation of Amaryllidaceae alkaloids

Method	*Test Compounds*[a] *(Internal standard)*	*Stationary phase or column*	*Mobile phase (modifier)*	*Detector*	*Detection limit*	*References*
PC-GC	Galanthine, Acetylcaranine, Lycorenine, Galanthamine, Crinine, Powelline, Tazettine, Belladine	SE-30	Argon	FID		Lloyd *et al.*, 1960
PC-GC	Galanthamine, Lycoramine, Tazettine	SE-30				Yamaguchi *et al.*, 1962
PC-GC	Norpluviine, Lycorine, Lycoramine, Galanthamine, Buphanamine, Vitattine, Crinine, Tazettine, Haemanthamine, Crinamidine, Hippeastrine, Undulatine, Homolycorine (Chrysene)	SE-30, XF-1105, XE-60, XF-1150, ECNSS-S, NGS, PEG-20M, EGSS-X, HI-EFF 8B	Nitrogen	FID		Takagi *et al.*, 1968a
PC-GC	Lycoramine, Galanthamine	HI-EFF 8B	Nitrogen	FID		Takagi *et al.*, 1968b
PC-GC-MS	Ambelline, Criwelline, Crinamine, Lycorine, Criglaucine, Criglaucidine	OV1	Helium	Ion current (MS)		Millington *et al.*, 1972
PC-GC-MS	Lycorine, Ornazidine, Ornazamine, Crinatine	OV-17		Ion current (MS)		Onyiriuka *et al.*, 1978
CGC-MS	Galanthamine, Lycoramine, *N*-Demethyl-galanthamine, *epi*-Norlycoramine, *O*-Methyloduline, Vittatine, Narwedine, Demethylpluviine, 10-*O*-Demethylpluviine, Oduline, Lycorenine, Haemanthamine, Masonine, Homolycorine, *N*-Demethyl-masonine	DB-1, DB-5	Helium	Ion current (MS)		Kreh *et al.*, 1995a

CGC	Galanthamine, Lycoramine, *N*-Demethyl-lycoramine, *O*-Methylmaritidine, Caranine, Pluviine, Haemanthamine, Lycorine, Homolycorine, Hippearstrine, Narcissidine (Deoxydihydrocodeine)	DB-1	Helium	FID/NPD		Bastos *et al.*, 1996
HPLC	Ambelline	C-8	Methanol, Water (Ammonia)	Circular dichroism (CD)		Westwood *et al.*, 1981
HPLC	Lycorine	Perkin-Elmer C18/10	Acetonitrile, Aqueous ammonium carbonate	UV (290 nm)	5 ng	Evidente *et al.*, 1983
HPLC	Lycorine, Tazettine	LiChrosorb-CN	Methanol, Water (Pick reagent)	Fluorescence (excitation 290/emission 320 nm)	<5 ng	Bruno *et al.*, 1985
HPLC	Lycorine, Crinine	μ Bondapak C18 (10 μ)	Methanol, Water	UV (292 nm)		Könükol *et al.*, 1992
HPLC	Galanthamine, *N*-Formylgalanthamine, Haemanthamine, Tazettine	Spherisorb ODS 2C18 (5 µm)	Water (phosphoric acid), Acetonitrile			Sellés *et al.*, 1997
HPLC/ TLC	Lycorine, Flexinine, Cherylline, Crinine, 6-Hydroxycrinine, Ungeremine, Powelline, Crinamine, Ambelline, Buphanisine, 6-Hydroxybuphanisine, Undulatine, Tazettine, Criwelline, Ismine, Anhydrolycorine-7-one, 8,9-Methylenedioxyphenanthridine, Pratorimine, Pratosin, Hippadine	*m*-Bondapak C18, Hiber C18, Eurospher C 18, Nucleosil E 18 AB Silicagel 60 HPTLC plates	Methanol, Tetrahydrofuran, Aqueous ammonium acetate, Acetonitrile (Acetic acid) Ethyl acetate, Diisopropylether, Methanol	TLC Scanner, UV (280 nm)	0.2 ng	Queckenberg *et al.*, 1993

Table 11.1 Continued

Method	*Test Compounds*[a] *(Internal standard)*	*Stationary phase or column*	*Mobile phase (modifier)*	*Detector*	*Detection limit*	*References*
LC/MS	Lycorine, Ambelline, Criglaucine	ODS 5 µ Spheresorb	Water, Acetonitrile, Ammonia	Ion current (MS)		Eckers *et al.*, 1976
HPLC	Galanthamine (Phenacetin)	CP^{tm} Micro Spher Si 5 µm	*n*-Hexane-Dichloro-methane-Ethanol-amine	UV (235 nm)	5 ng/ml	Claessens *et al.*, 1983
HPLC	Galanthamine	Novapack C-18 (4 µ)	Acetonitrile-Pentane-sulfonic acid buffer	UV (235 nm)	10 ng/ml	Claessens *et al.*, 1988
HPLC	Galanthamine, Epigalanthamine, Galanthaminone (Codeine)	Hibar-LiChrosorb RP-8 (5 µm)	Methanol-Water (Dibutylamine, 85% Phosphoric acid)	UV (280 nm)	0.05 µg/ml	Tencheva *et al.*, 1987a
HPLC	Galanthamine, Epiganthamine (Codeine)	Intersil-C-8 silica (5 µm)	Acetonitrile, Tetra-hydrofuran, Water (di-*n*-Butylamine, 85% Phosphoric acid)	Fluorescence (excitation 290/emission 320 nm)	1 ng/200 µl	Bickel *et al.*, 1991

Note
[a]Reference compounds or those detected.

and CGC have emerged as the most accurate and practical analytical procedures to determine these compounds in natural sources and in biological samples. HPLC was the most widely used technique for this purpose, especially when only one constituent was quantified. Due to the presence of at least one aromatic ring in their chemical structures, Amaryllidaceae alkaloids can be conveniently quantified with high sensitivity by a UV or a fluorescence detector. This method is not always suitable for the routine analysis of Amaryllidaceae alkaloids in natural sources. Usually, alkaloid fractions of Amaryllidaceae contain complex mixtures of compounds with diverse chemical structures and polarities. It is often difficult to achieve good resolution of these mixtures with reasonable peak shapes, even with complex solvent programming. The resolution and peak shapes for a mixture of standards also appear to vary widely under identical experimental conditions for commercially available reversed-phase columns with similar specifications from different suppliers.

CGC appears to be a better technique for the simultaneous quantification of several different alkaloids in natural sources. Amaryllidaceae alkaloids can be analysed by this method without any prior derivatisation. However, the method is not applicable to quaternary alkaloids and alkaloid *N*-oxides. Compounds usually show very sharp peaks and can be detected using a flame ionisation detector (FID), a nitrogen phosphorus detector (NPD) or mass spectrometry (MS). A very high resolution, even for complex mixtures of alkaloids, can be achieved with relatively short capillary columns within a reasonable experimental time. Two of the most common Amaryllidaceae alkaloids, haemanthamine and lycorine, were reported to undergo partial decomposition under GC experimental conditions. These compounds still appear as relatively sharp single peaks, with good concentration/response linearity in a wide range of concentrations, and can be quantified reproducibly. These analyses can be performed with little sample preparation, and are suitable for routine quantitative analysis of Amaryllidaceae alkaloids in natural sources. Useful information on these two analytical procedures is summarised in Table 11.1.

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12 Synthesis of galanthamine and related compounds

V.N. Bulavka and O.N. Tolkachev

INTRODUCTION

(–)-Galanthamine is the principal representative alkaloid from the Amaryllidaceae family, possessing a characteristic benzofuro[3a,3,2-ef][2]benzazepine skeleton. It is an anticholinesterase agent of low toxicity used in medicine for the treatment of a number of conditions (Mashkovsky and Kruglova-L'vova, 1951; Mashkovsky, 1955). It is under evaluation for the treatment of Alzheimer's disease (Fulton and Benfield, 1996; Moraes-Cerdeira *et al.*, 1997).

Galanthamine hydrobromide was produced commercially as 'Nivalin' in Bulgaria from *Galanthus nivalis* L. and later from *Leucojum aestivum* cultivars, and in the former USSR from leaves of *Ungernia victoris* L. collected in the mid-Asia regions. The limited plant sources available in the Russian flora for commercial exploitation, the low concentration of the alkaloid in plant material and the labour-consuming character of the chemical processes, led to a need for alternative routes of galanthamine production. At present, synthetic methods seem to have assumed practical significance.

The chemistry of Amaryllidaceae alkaloids has been extensively reviewed (Abduazimov, 1993; Cook and Loudon, 1952; Fuganti, 1975; Grundon, 1984, 1985, 1987; Jeffs, 1990; Lewis, 1990, 1997; Polt, 1996). Today about 20 natural alkaloids of the galanthamine type, isolated from species of Amaryllidaceae, are known. Up to now, five alkaloids – galanthamine, narwedine, lycoramine, sanguinine and *N*-norgalanthamine – have been obtained by total synthesis.

CHEMICAL PROPERTIES OF GALANTHAMINE

(–)-Galanthamine ((–)-**1**) is a strong base, which on interaction with alkylhalogenides produces quaternary ammonium salts. The oxidation of (–)-**1** with MnO_2 in mild condition leads to the ketone **2**, isolated in optically inactive form (Barton and Kirby, 1962; Combes and Lefebvre, 1962). Its (–)-enantiomer is the natural alkaloid known as narwedine (Figure 12.1). Narwedine readily undergoes racemisation in alcoholic solutions, especially in the presence of organic bases and acids, or spontaneously without any additives (Barton and Kirby, 1960, 1962). The mechanism of this process has been explained by the formation of tautomeric dihydrofuranoic and hydroxyspirodienone **(3)** forms, which are converted in a

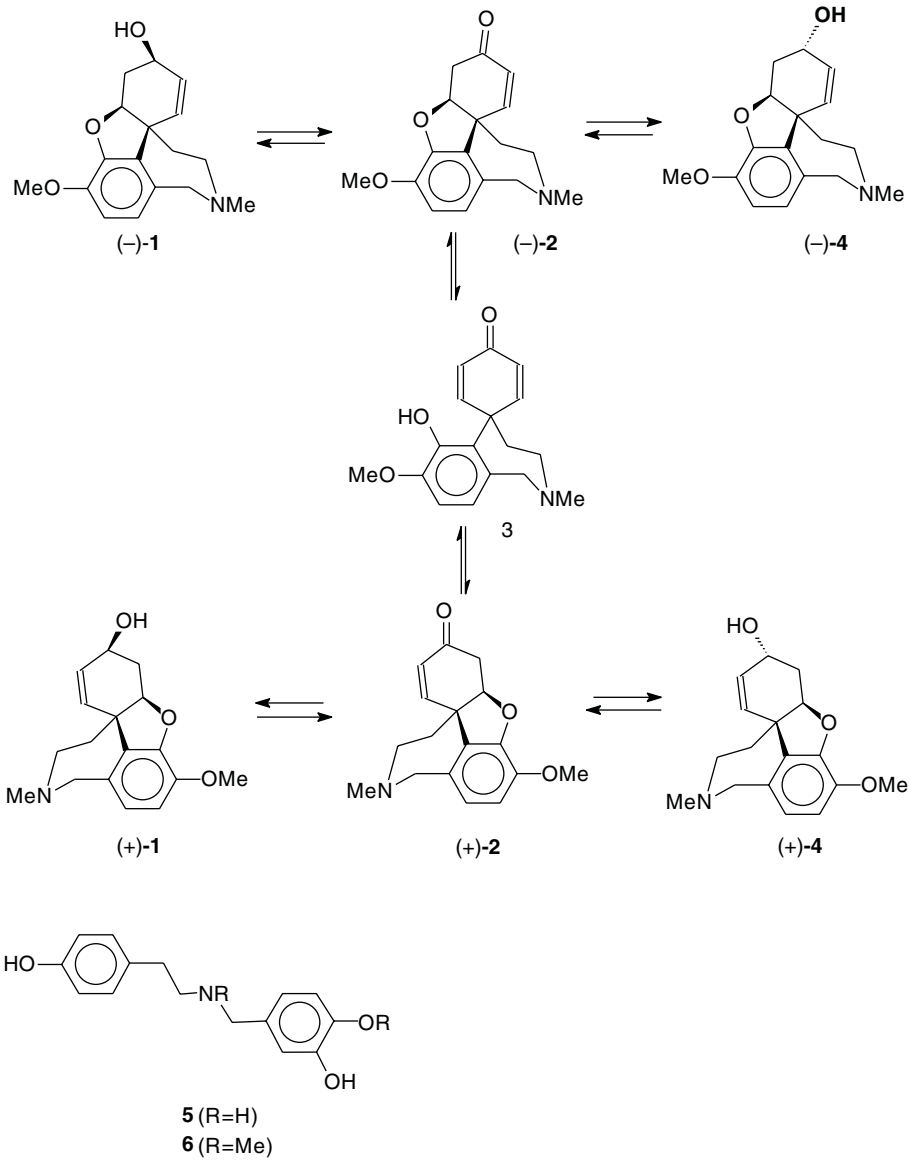


Figure 12.1 (Above) The equilibrium process between narwedine hydrohydienone **(3)** and its enantiomers **(2)** and oxidative/reductive interconversion between **(2)** and galanthamine **(1)** and *epi*-galanthamine **(4)** enantiomers. (Below) Norbelladine **(5)** and *N,O*-dimethylnorbelladine **(6)**.

equilibrium process as shown in Figure 12.1. Enantiomeric (–)-**2**, on reduction with different agents, produces a mixture of (–)-**1** and (–)-*epi*-galanthamine ((–)-**4**). The key compound in the biosynthesis of (–)-**1** and related Amaryllidaceae alkaloids is norbelladine (**5**, R = H; Figure 12.1), which is formed from tyrosine (Barton and Cohen, 1957; Barton *et al.*, 1961, 1962, 1963; Schutte, 1969).

THE SYNTHESIS OF ALKALOIDS OF THE GALANTHAMINE GROUP

The first synthesis confirming the structure of galanthamine

Galanthamine was isolated for the first time from *Galanthus woronowii* and its preliminary structure was proposed from data on the chemical degradation of its molecule using classical methods (Proskurnina and Yakovleva, 1952, 1955). The final confirmation of the structures of galanthamine, narwedine and lycoramine were made by their total synthesis. The first biomimetic route of (±)-**2**, and after its resolution, of (+)-**1**, (–)-**1** and (–)-lycoramine synthesis, proposed by Barton and Kirby (1960, 1962), included the oxidation of diphenolic substrates such as *O*,*N*-dimethyl-norbelladine (**6**, R = Me), the biogenetic precursor of **1**, or its derivatives (Figure 12.2). The synthesis was carried out starting from 4-hydroxybenzaldehyde **(7)** through cyanohydrine **(8)** and 4-hydroxyphenylacetic acid **(9)** (34%), which, after *O*-benzylation into **10** (67%), was converted into the acid chloride **11** (Figure 12.2). The coupling component was obtained from 3-benzyloxy-4-methoxybenzaldehyde **(12)** via the intermediate Schiff base **(13)**, with a subsequent reduction to 3-benzyloxy-4-methoxy-*N*-methyl-benzylamine **(14)** (68% yield). With the acid chloride **11**, the latter yielded the corresponding arylacetamide **15** (85%). The $LiAlH_4$ reduction of **15** (88%) and Pd/C hydrogenolysis of the intermediate tertiary amine **16** produced the desired compound **6** in 76% yield. Oxidation with different agents produced the target ketone (±)-**2** in very low yield (0.1–1.4%). The best results were obtained on $K_3[Fe(CN)_6]$ oxidation (0.92–1.4%). The final $LiAlH_4$ reduction afforded a mixture of (±)-**1** and (±)-**4** in the ratio 3:2 (*ca.* 100%), from which the first compound was isolated in crystalline form (39%). Aluminium isopropoxide reduction produced preferentially the *epi*-isomer (±)-**4** (60%). The total yield of (±)-**2** was 0.18%, and of (±)-**1**, only 0.07%.

Also proposed was a method of conversion of the ketone (±)-**2** into enantiomeric (–)-**1** via a step of dynamic resolution of (±)-**2** to (+)-**2** in the presence of optically active additives (–)-**1**, (–)-**4** or (–)-lycoramine, and the $LiAlH_4$ reduction of (+)-**2** to yield a mixture of (+)-**1** and (+)-**4**. The latter were used for seeding during the conversion of the racemate to (–)-**2**. A similar $LiAlH_4$ reduction of (–)-**2** produced the target (–)-**1** in 58% yield. The subsequent hydrogenation over Pd/C afforded (–)-lycoramine (Barton and Kirby, 1960, 1962). Recently, a modified version of the pilot plant method of dynamic resolution of (±)-narwedine has been applied (Shieh and Carlson, 1994; Czollner *et al.*, 1998).

Synthesis of narwedine via palladium-directed coupling

The synthesis was modified by Holton *et al.* (1988) as follows (Figure 12.3). A hydroxy group in 3-hydroxy-4-methoxybenzaldehyde **(18)** was protected by the action of NaH in $OP(NMe_2)_3$, N_2, then $MeSCH_2Cl$; the intermediate *O*-methylthiomethyl derivative **(19)** (Holton and Davis, 1977), on condensation with 4-hydroxyphenethylamine **(17)**, gave the Schiff base **20**, which, after $NaBH_4$ reduction to **21**, hydroxymethylation and $NaB(CN)H_3$ reduction of **22**, gave the *N*-methyl-derivative **23** in 90% overall yield. With $LiPdCl_4$ in MeOH-$(i\text{-}Pr)_2NH$ (–78 °C), **23** produced the palladium derivative (±)-**24** as a diastereomeric mixture (95%). Oxidation with $(CF_3COO)_3Tl$ (–10 °C) gave the expected compound (±)-**25**, which

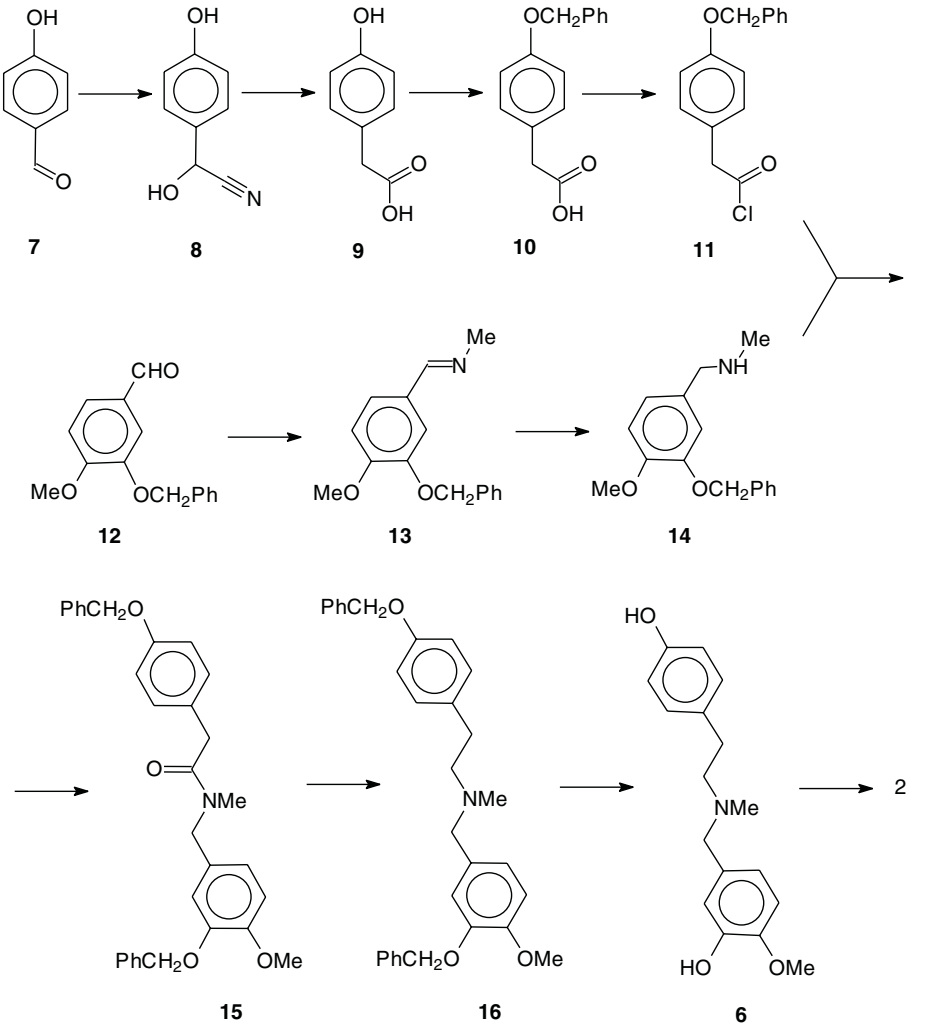


Figure 12.2 Synthesis of narwedine **(2)** via *N,O*-dimethylnorbelladine **(6)** (Barton and Kirby, 1960, 1962).

on hydrolysis produced (±)-**26** (98%). (±)-**25** on keeping at 25 °C in the presence of Ph_3P (14 h) yielded (±)-**2** (51%). Thus the total yield of (±)-**2** was 43.6%.

Hence, the concept of using metal-substituted substrate-induced *o*-diphenol oxidation in galanthamine synthesis was successful. However, the use of toxic, expensive and scarce compounds means that the scheme is unsuitable for large-scale application.

Synthesis of galanthamine and *N*-norgalanthamine via belladine-derived amide oxidation

A significant achievement in the synthesis of **1** was found in the work of Kametani *et al.*, 1969a,b and Kametani, 1972a, shown on Figure 12.4. 5-Benzyloxy-2-bromo-

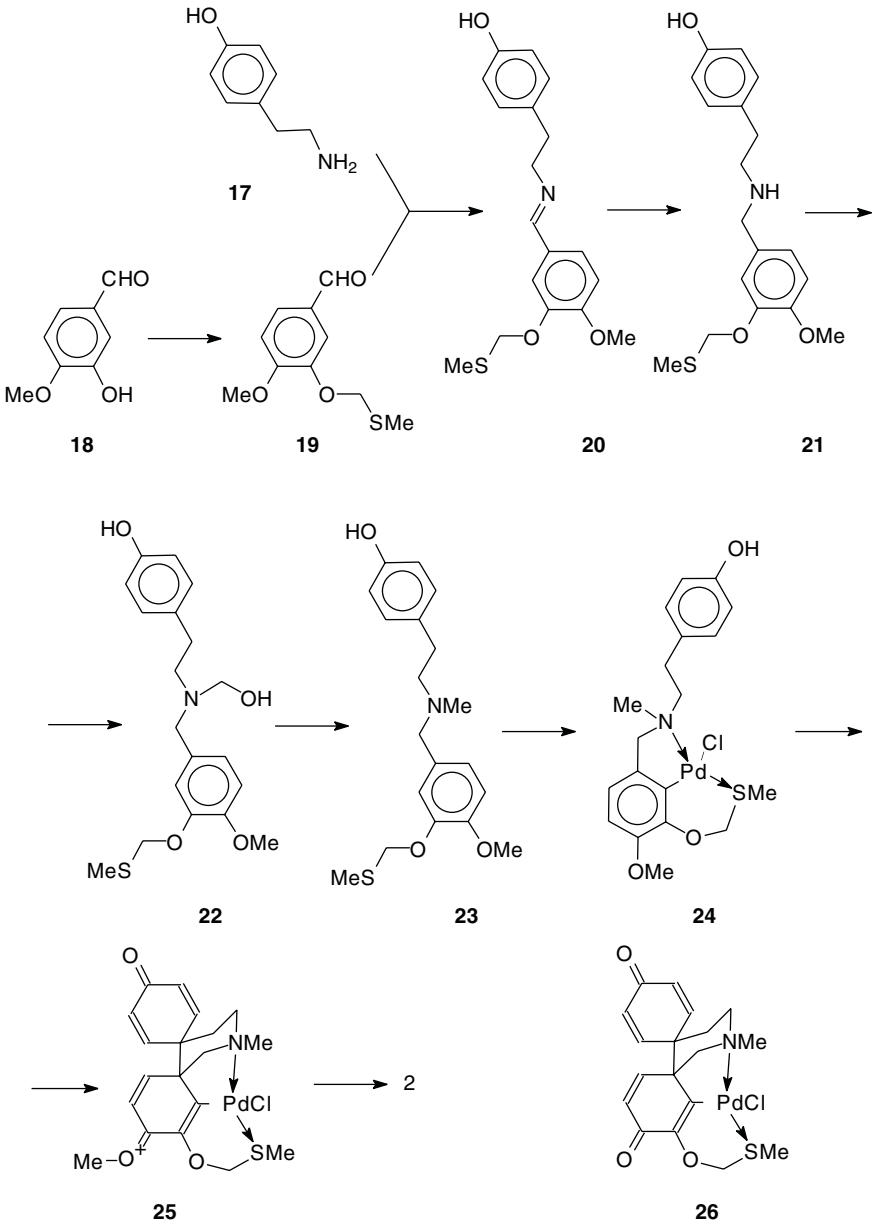


Figure 12.3 Synthesis of narwedine **(2)** via palladium-containing intermediate **(24)** (Holton *et al.*, 1988).

4-methoxy-benzaldehyde **(29)** was oxidised with silver oxide into the corresponding acid **30** (89%), which was then transformed into the acid chloride **31** (52%). The coupling component **28** was obtained from 4-benzyloxyphenylacetic acid **(10)** via the acid chloride **11**, *N*-methyl acetamide **(27)** (91%) and final reduction to **28**

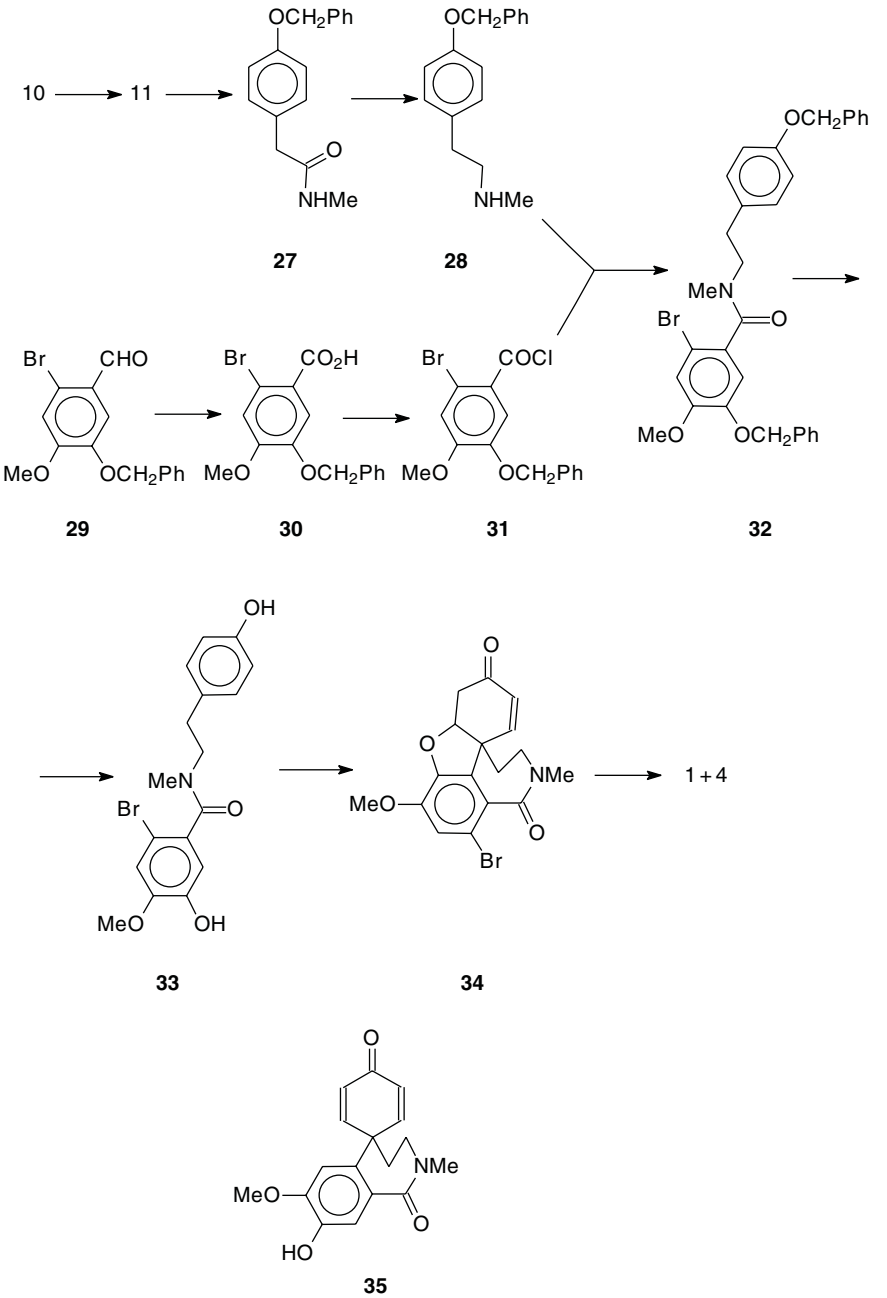


Figure 12.4 Synthesis of galanthamine **(1)** via belladine-type benzamide **(33)** (Kametani *et al*., 1969a,b; Kametani, 1972a).

with $LiAlH_4$ in 82% yield. Coupling **31** with **28** produced the corresponding benzamide **32**, which on acidic hydrolysis afforded the 4-hydroxy compound **33** in 63% yield. The $K_3Fe(CN)_6$ oxidation of **33** produced the key compound (±)-**34**

(40%), together with the debrominated spiro-dienone **35** (0.4%). Ketone (±)-**34**, on $LiAlH_4$ reduction, produced a mixture of (±)-**1** (50%) and (±)-**4** (40%), which were separated chromatographically. Thus, the total yield in the synthesis of (±)-**1** as shown in Figure 12.4 was 9.4%. The use of the amide synthone **33** was successful in the oxidation reaction. The yield from cyclocondensation at this step was 29 times higher than in case of tertiary amine oxidation used in Barton and Kirby's scheme. However, taking into consideration that from the starting material **10** (not a com-

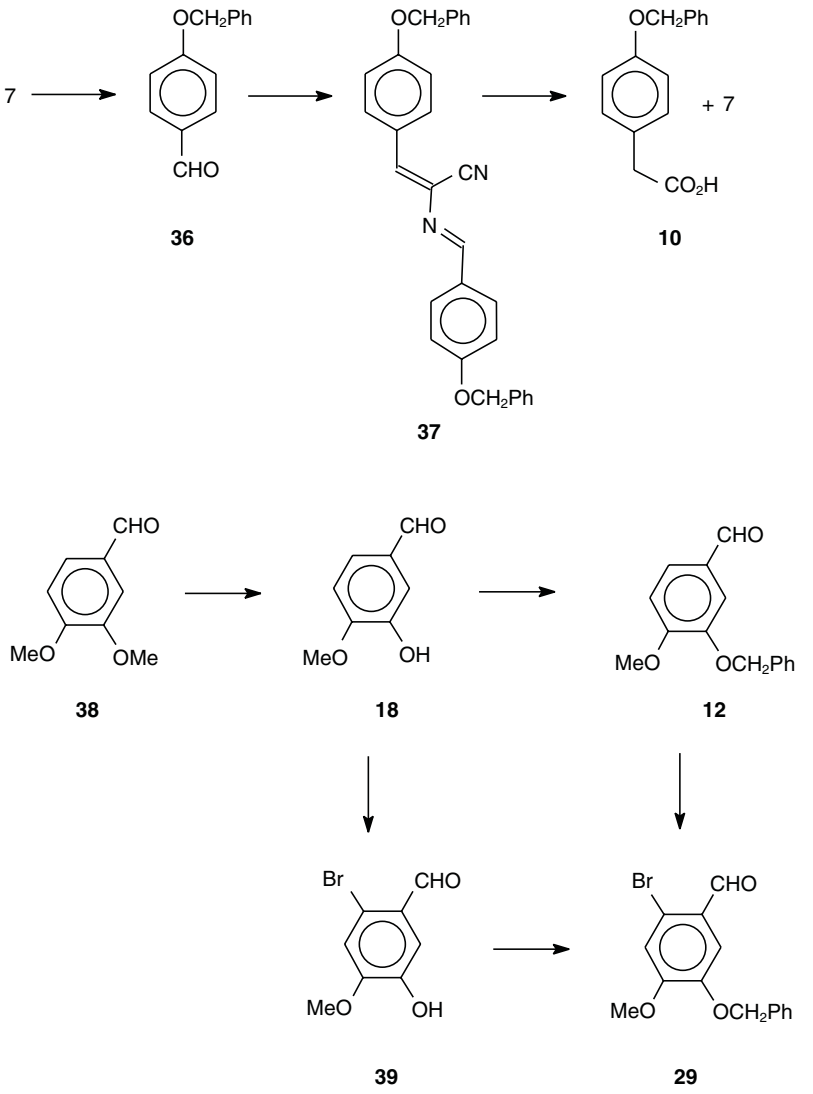


Figure 12.5 Synthesis of 4-benzyloxyphenylacetic acid **(10)** (above; Vaghani and Merchant, 1961) and of 2-bromo-4-methoxy-5-benzyloxy-benzaldehyde **(29)** (below; see references in text).

mercially available compound) these authors obtained a 23% yield in three stages, the yield of (±)-**1** from **7** (which is commercially available) is 2.14% (30 times higher).

Kametani *et al.* (1969a,b) used the following route for the synthesis of 4-benzyloxyphenylacetic acid **(10)** (Figure 12.5). **10** was obtained from **7** through the aldehyde **36** and the intermediate imino-propionitrile of structure **37** (Vaghani and Merchant, 1961). However, in this scheme, the total yield of **10** was not indicated, and only half of the starting material was inserted into the target compound. Another component, 2-bromo-4-methoxy-5-benzyloxy-benzaldehyde **(29)**, was obtained in three steps starting from 3,4-dimethoxybenzaldehyde **(38)** by 3-monodemethylation with concentrated H_2SO_4 to isovanilline **(18)** (61%) (Brossi *et al.*, 1967), and bromination with bromine in acetic acid to give the expected compound **39** in 67% yield (Henry and Sharp, 1930; Raiford and Ravely, 1940). The total yield of **29** after *O*-benzylation was about 33% (Jackson and Martin, 1966), which after total conversion should exceed 54%. Recently, a more advanced route (Figure 12.5) was proposed from isovanilline **(18)**, which after *O*-benzylation to **12** (98%) and bromination in acetic acid in the presence of NaOAc produced **29** in 79% yield (Bolton *et al.*, 1987), or a total 47% yield (77% taking into consideration a complete conversion of products on the hydrolysis reaction step).

Similarly, Kametani *et al.* (1973) synthesised (±)-**1** via (±)-*N*-norgalanthamine ((±)-**44**) (Figure 12.6). The substrate for oxidation was obtained in three steps from the acid **30** via the acylchloride **31**, coupled with the amine **40** to produce the amide **41**, which was debenzylated to **42** in 72% total yield. On $K_3[Fe(CN)_6]$ oxidation, the key compound (±)-**43** was obtained in only 0.7% yield. The latter was reduced with $LiAlH_4$ to (±)-**44** in 32% yield. The final formaldehyde-formic acid methylation of (±)-**44** produced the desired (±)-**1** in 70% yield, corresponding to 0.11% total yield.

In another synthesis of (±)-**1**, Kametani *et al.* (1971a) used the intermediate isomeric amide **48** (a positional carbonyl isomer of **33**). Starting from 4-benzyloxyphenylacetic acid **(10)** converted to acyl chloride **(11)**, and the bromoamine **46** obtained from bromoaldehyde **(29)** in two steps without isolation of the intermediate **45** (75%), the desired amide **47** was produced in 72.3% yield (Figure 12.6). After elimination of protective benzyl groups with hydrobromic acid, the resulting dihydroxy-amide **48** was oxidised with $K_3[Fe(CN)_6]$ to afford a tetracyclic amide of structure (±)-**49** in low yield (1.9%). A subsequent $LiAlH_4$ reduction afforded 12.3% of (±)-**1** and 4.9% of (±)-**4**. Thus, the total yield of the title compound according to this scheme was 0.095%, which was 99 times lower than that according to the alternative route via the intermediate **33**.

The oxidation of unprotected amide **52** (obtained from **28** and **50** via **51**) with $VOCl_3$ produced the expected cyclic compound (±)-**53** in 2% yield (Figure 12.7), which was reduced to (±)-**1** and (±)-**4**, with (±)-**1** isolated in 61% yield. The 0.77% total yield of the latter is 12 times lower than that shown according to the route in Figure 12.4 with the application of bromo amide (Kametani *et al.*, 1971b; Kametani, 1972b).

In another modification of (±)-**1** synthesis, Kametani *et al.* (1972) used the isomeric 2-bromo derivative **54** as a starting material (Figure 12.8). The latter was transformed via **55** (63%) and **56** (86%) into the acid chloride **57**, and coupled with the methylamine derivative **28** to give the amide **58** (37%). Hydrochloric acid debenzylation produced diphenol **59** (77%), which on irradiation with a mercury lamp afforded the key compound (±)-**53** in only 1% yield. $LiAlH_4$ reduction as

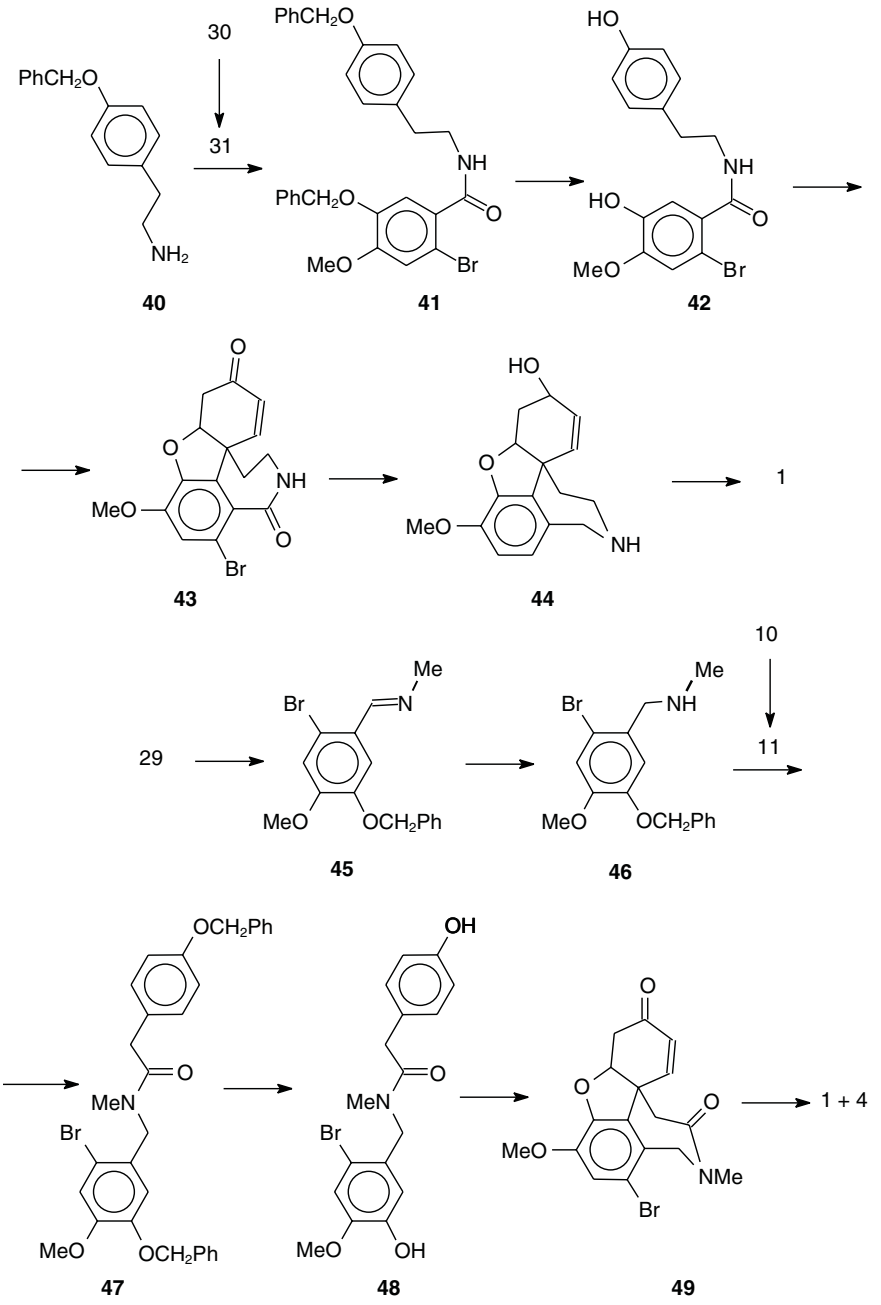


Figure 12.6 Synthesis of galanthamine **(1)** via *N*-norgalanthamine **(44)** (above; Kametani *et al*., 1973) and via belladine-type phenylacetamide **(48)** (below; Kametani *et al*., 1971a).

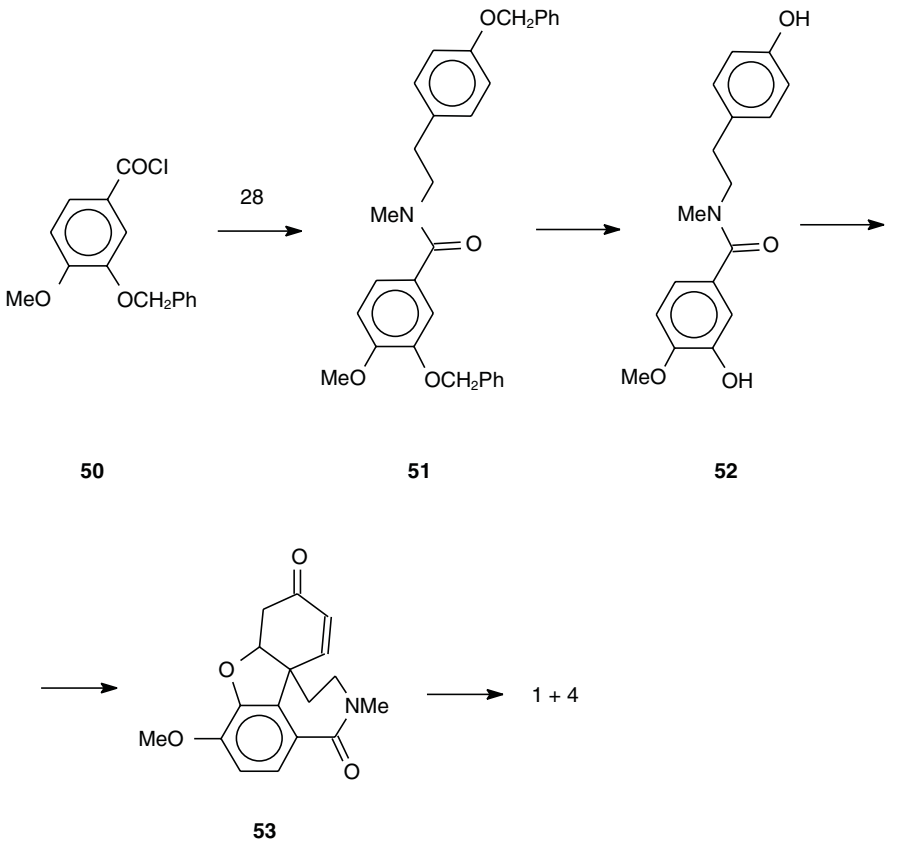


Figure 12.7 Synthesis of galanthamine **(1)** via non-brominated belladine-type benzamide **(52)** (Kametani, 1972b; Kametani *et al*., 1971b).

above produced a mixture of (±)-**1** and (±)-**4**. A total yield of 0.093% was obtained using this scheme, which was 40 times lower than in the case of (±)-**1** synthesis via isomeric bromoamide. Thus Kametani's group demonstrated the necessity of blocking the *para*-position to the hydroxy group by bromination in the benzyl moiety in the phenolic coupling process.

Subsequently, Bulgarian and German scientists tried to improve the synthesis of **1**. Vlahov *et al.* (1978), in a modified route of the synthesis, used 4-methoxybenzylation for hydroxyl group protection and subsequent de-protection in mild conditions. Thus, intermediate amides **60** and **61** were transformed to **33** and **48**, respectively (Figure 12.9). However, the final cyclocondensation of **48** to **49** (as in figure 12.6) proceeded with low yields. In another variation of the synthesis by the same authors, in order to avoid the use of $LiAlH_4$ reduction of the amide **62**, the following succession was applied: a transformation of **62** to thioamide **63** by P_2S_5-pyridine treatment (58% yield) with subsequent $NaBH_4$-$CoCl_2$ reduction to the substituted *N*-methyl-phenethylamine **(64)** in 80% yield. The oxidative cyclocondensation of the amide **33** produced the desired racemic bromo-ketone (±)-**34** in a

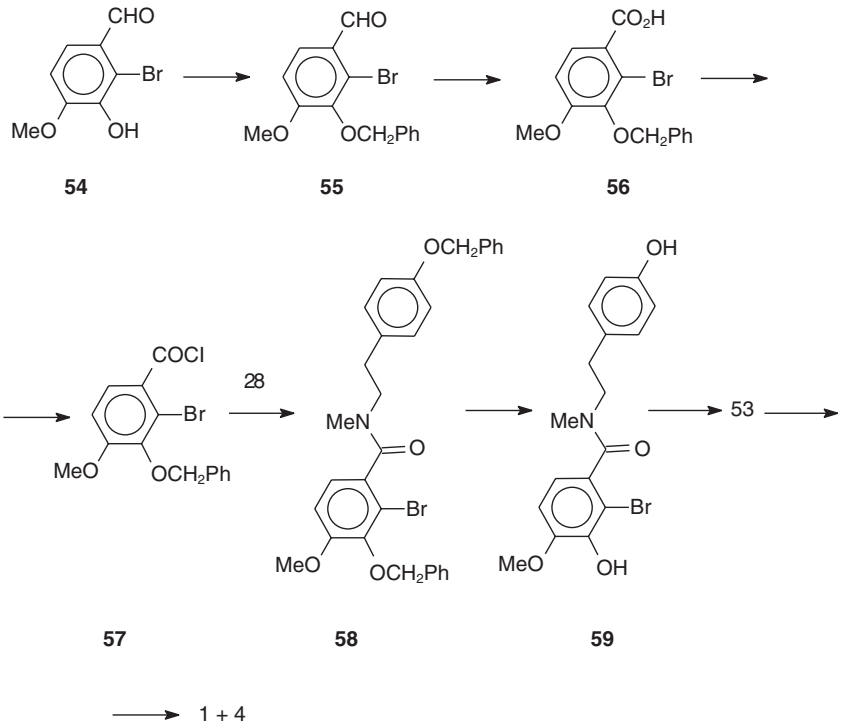


Figure 12.8 Synthesis of galanthamine **(1)** via photochemical substitution of bromine in belladine-type benzamide **(59)** (Kametani *et al*., 1972).

yield after recrystallisation below 15% (Vlahov *et al.*, 1978, 1989). The Bulgarian group also used microbiological reduction of (–)-**34** to (–)-bromogalanthaminone ((–)-**65**) with *Nematospora coryli* and to (–)-bromo-*epi*-galanthamine ((–)-**66**) with *Septomyxa affinis* (Vlahov *et al.*, 1984) (Figure 12.9).

Synthesis of enantiomeric galanthamine starting from chiral synthone

Shimizu *et al.* (1977, 1978) (Figure 12.10) have synthesised (+)-**1** starting from its natural precursor L-(+)-tyrosine methyl ester **(67)**, which, after condensation with 3,5-dibenzyloxy-4-methoxybenzaldehyde **(68)** and $NaBH_4$ reduction, without the isolation of the corresponding Schiff base, was converted into L-(+)-*N*-(3,5-dibenzyloxy-4-methoxybenzyl)tyrosine methyl ester (L-(+)-**69**). Its trifluoroacetyl derivative L-(–)-**70** was debenzylated by Pd/C hydrogenolysis (100%) and the trihydroxy compound L-(–)-**71** was oxidised with Mn(III) acetylacetonate in acetonitrile into the tetracyclic (+)-**72** (34%); its (–)-stereomer (–)-**72** was not isolated. The hydroxy group was phosphorylated with diethylphosphoryl chloride to produce (+)-**73** and its (–)-stereomer ((–)-**73**) (13:1), with the former isolated in 81% yield. $NaBH_4$ reduction followed, with trifluoroacetyl group elimination giving a mixture of the epimeric alcohols (+)-**74** (axial) and (+)-**74** (equatorial) (4:1). The key compound (+)-**74** (axial) was isolated in 21% yield on formaldehyde-formic acid methylation,

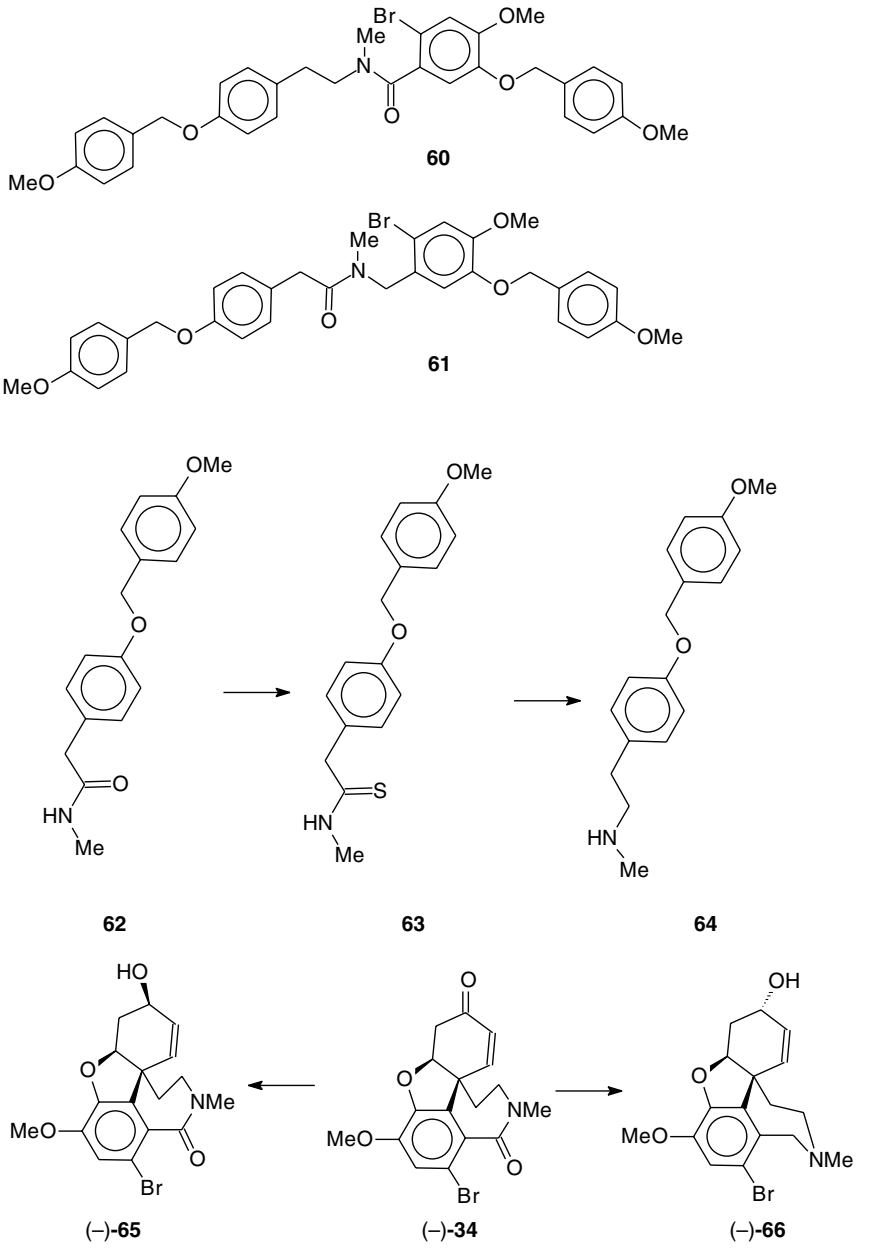


Figure 12.9 (Above) 4-Methoxybenzyl-protected intermediates for the synthesis of galanthamine **(1)** (Vlahov *et al*., 1978, 1989). (Below) Microbiological reduction of bromo-narwedinone **(34)** (Vlahov *et al*., 1984).

and subsequent aminolysis produced (+)-carboxamide ((+)-**75**) (37%). The *O*-acetyl derivative **76** (89%) was transformed into the cyano derivative **77** ($POCl_3$), and $LiAlH_4$ reduction afforded benzazepine of the structure (+)-**78** in 42% yield. The

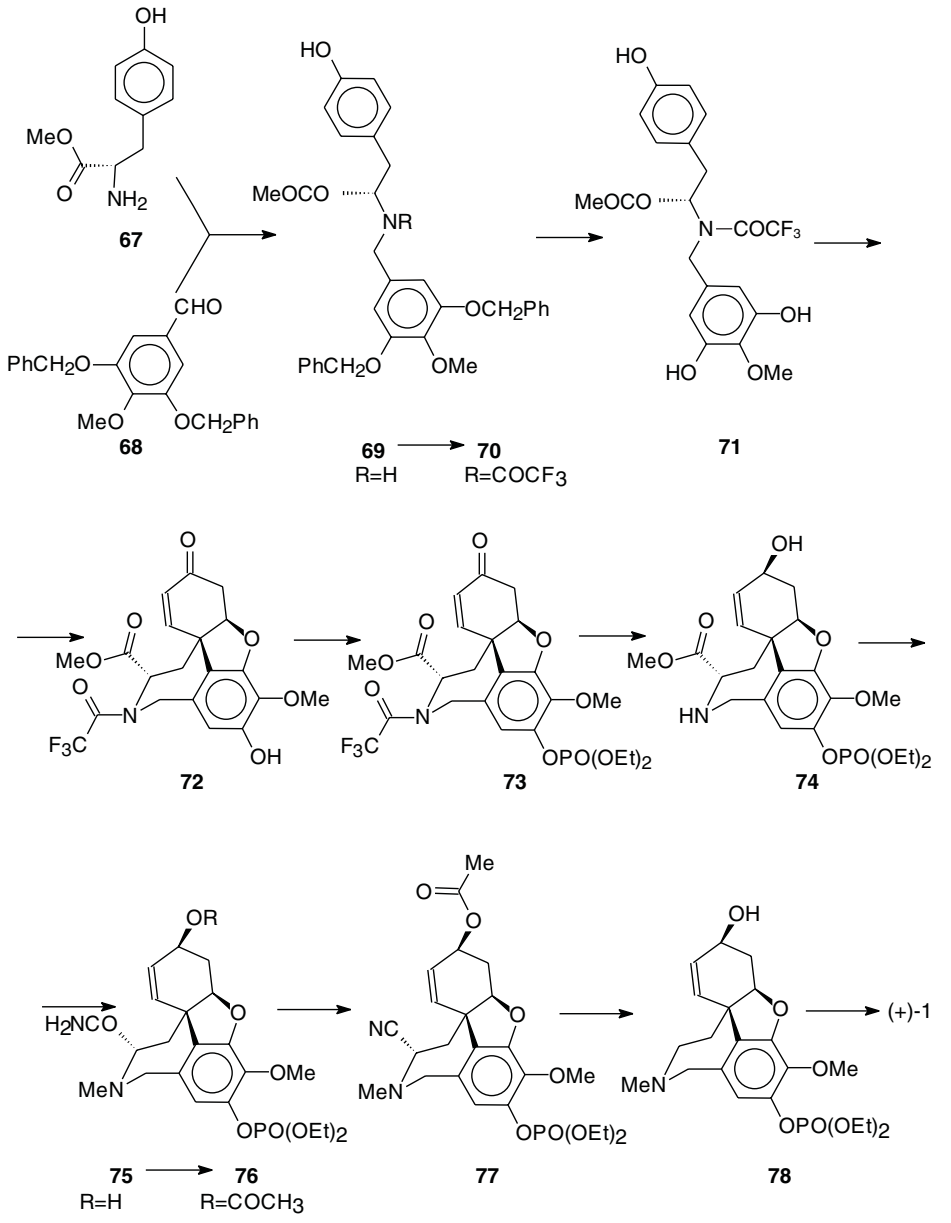


Figure 12.10 Synthesis of enantiomeric galanthamine **(1)** (Shimizu *et al*., 1977, 1978).

final protection group elimination with Na-liquid NH_3 gave the expected compound (+)-**1** in 72% yield (0.63% total yield).

Despite the low total yield of galanthamine, this work was of a great importance because of its prospects for further improvement. Thus, the tyrosine hydroxyl group was not protected, and this shows that the protection and de-protection of

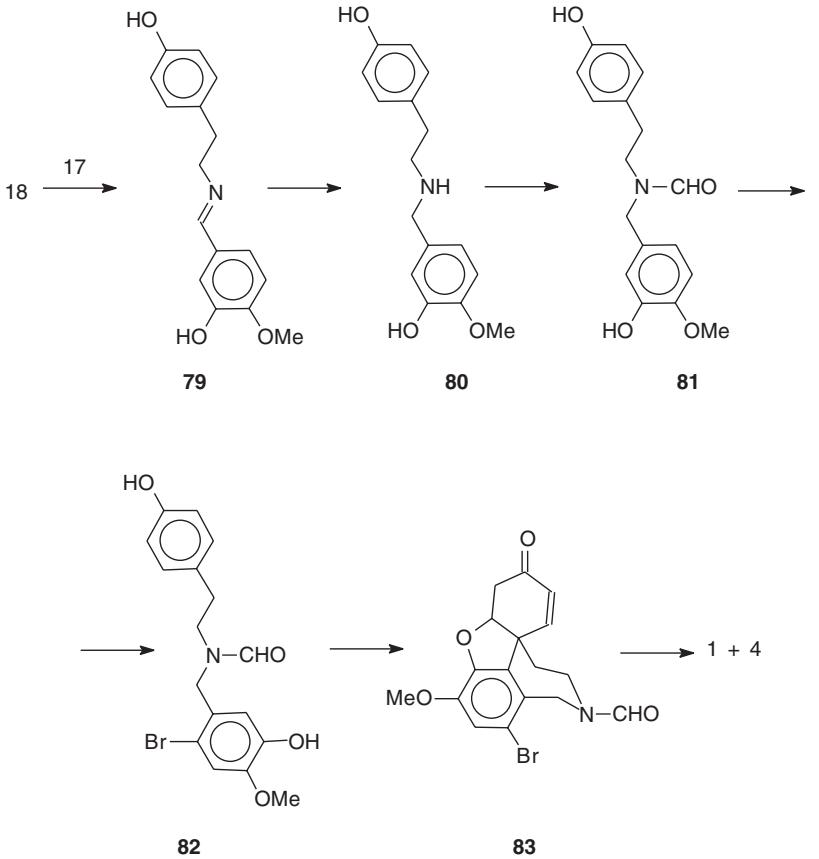


Figure 12.11 Synthesis of galanthamine **(1)** via *N*-formylnorbelladine derivative **(82)** (Szewcyzk *et al*., 1988, 1995).

the phenolic hydroxyl groups was not necessary. The amide modification of the *N*-methyl part of the substrate, and the successful oxidation in homogeneous conditions, were also important methodological findings.

Galanthamine synthesis via *N*-formylnorbelladine derivative

The logical continuation of the syntheses shown in Figures 12.5 to 12.10 was realised in the improved method of galanthamine synthesis proposed by Szewczyk *et al.* (1988, 1995) (Figure 12.11). This involved a six-step scheme starting from 4-hydroxyphenethylamine **(17)** and isovanilline **(18)** via the Schiff base **79**, which was reduced without isolation using $NaBH_4$ to a secondary amine **80** in 99% yield. The latter, on treatment with ethyl formate, gave the corresponding formamide **81** in 80% yield, which on bromination with bromine in chloroform – methanol mixture (–65 °C) afforded the bromo-formamide **82** (85%). $K_3[Fe(CN)_6]$ oxidation gave the expected benzazepine **83** (21%). The final $LiAlH_4$ reduction produced a mixture

of 53% (±)-**1** and 31% (±)-**4**. The total yield of (±)-**1** according to this scheme was 7.5%, which exceeded by 3.5 times the results published in the work of Kametani *et al.* (1969a,b). Taking into account that isovanilline **(18)** is usually obtained from veratric aldehyde **(38)** in 61% yield (100% conversion) (Brossi *et al.*, 1967), in this scheme Szewczyk *et al.* (1988, 1995) used all commercially available compounds. The synthesis seems to be simple in realisation on a preparative scale, but its basic deficiency is the use of the inflammable $LiAlH_4$.

Galanthamine synthesis developed by the Russian group

A comparative analysis of the known approaches to galanthamine synthesis has revealed the best route to the production of the key compound containing the carbonyl moiety as the formamido group, shown in Figure 12.11. The failing of the majority of known methods is the low yields obtained. The present authors have used the following strategy in a large-scale approach to its synthesis: the use of cheap and commercially available materials, a minimum number of steps, and the use of synthones with unsubstituted hydroxy groups. We have proposed the synthesis of (±)-**1** from 4-hydroxyphenethylamine **(17)**, or its *O*-alkyl derivatives, and 2-bromo-5-hydroxy-4-methoxy-benzaldehyde **(39)** (Bulavka, 1993; Bulavka *et al.*, 1990, 1991, 1993, 1994a,b,c, 1999; Bulavka and Tolkachev, 1995) (Figure 12.12).

The known methods of parent compound production were modified. Thus, 4-hydroxy- and 4-alkoxyphenethylamines (**86**, R = H, Me, Et, Pr, $PhCH_2$) were obtained from the corresponding 4-substituted benzaldehydes **(84)** via substituted 2′-nitrostyrenes **(85)** through zinc dust reduction in diluted hydrochloric acid or in a mixture of hydrochloric and acetic acids. The method was optimised to produce the expected 4-hydroxy- and 4-alkoxyphenethylamines in 80–98% yields in one operation (Bulavka *et al.*, 1993, 1994a,c), higher yields than in earlier published methods. Primary amines **86** afforded formamides **87** (90–95%) with formic acid. **87** (R = Me) was reduced to *N*-methylated amine **91** with $NaBH_4$ – acetic acid (74%) and alternatively with zinc dust and sulphuric acid in tetrahydrofouran (67%) (Bulavka, 1993; Bulavka *et al.*, 1994c). The alternative method of *N*-methyl-4-methoxyphenethylamine and *N*-methyl-4-hydroxyphenethylamine production starting from 4-methoxyacetophenone **(88)** via *N*-methyl-4-methoxyphenylthioacetamide **(89)** was also studied. The Willgerodt-Kindler reaction of **88** and $MeNH_2$ (160–170 °C in ampoules) gave a complex mixture of products, from which **89** (10.4%) was isolated together with the by-product *N*-methyl-4-methoxyphenylacetamide (21.9%). The method was modified as follows: interaction of 4-methoxyacetophenone **(88)** with methylamine in the presence of dehydrating agents gave the expected Schiff base **90** in 77% yield, which was then transformed to desired thioamide **89** in 21.5% yield. The reaction of **88** with methylamine hydrochloride and sulphur in dimethylformamide (100 °C, sodium acetate, or 60–70 °C, 4-toluenesulfonic acid – Et_3N) gave **89** in 57–58% yields. The reaction of **88** with MeNHCHO and sulphur (170–180 °C) afforded the expected **89** in 30–35% yield. Subsequent reduction of **89** with zinc dust and hydrochloric acid afforded *N*-methyl-4-methoxyphenethylamine **(91)** (91%), which was demethylated with hydrochloric acid at 170 °C to *N*-methyl-4-hydroxyphenethylamine **(92)** (98%) (Bulavka, 1993; Bulavka *et al.*, 1999).

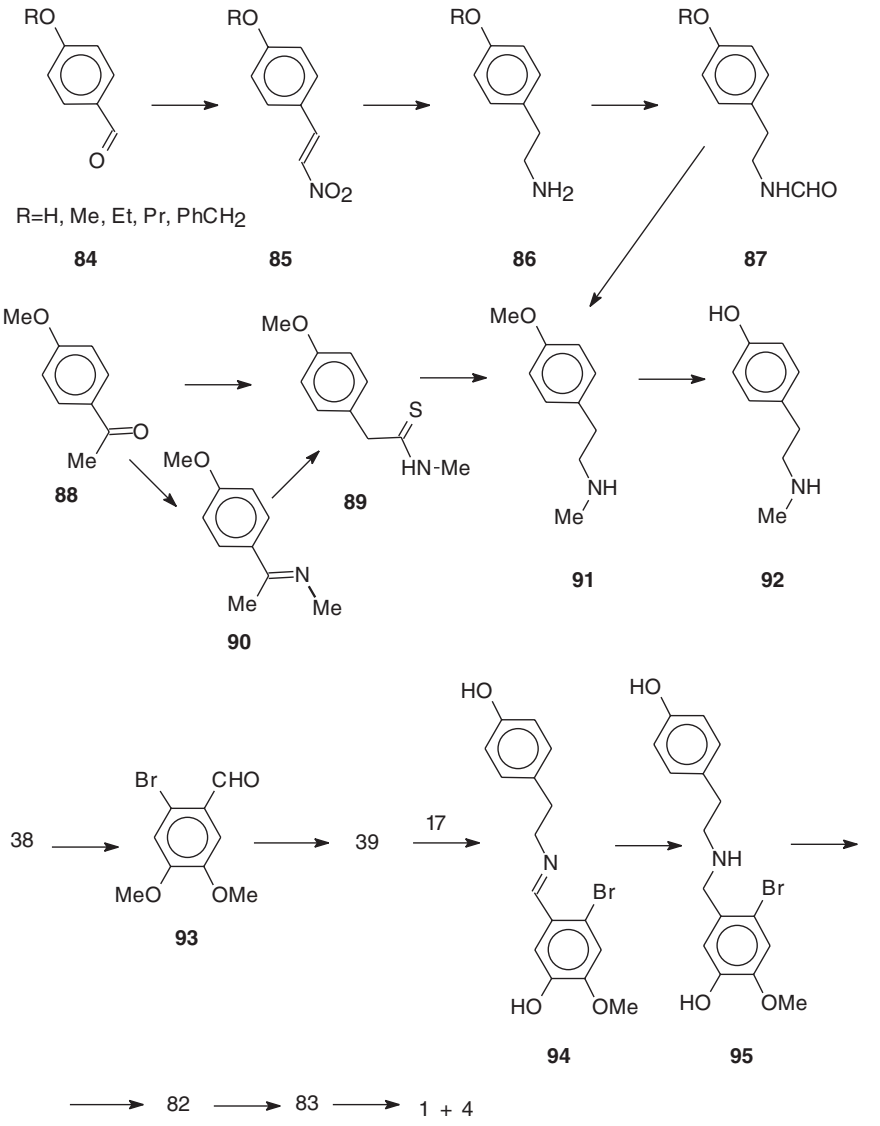


Figure 12.12 Synthesis of phenylethylamine intermediates (above; Bulavka, 1993; Bulavka *et al.*, 1994a,c, 1999) and galanthamine **(1)** (below; Bulavka, 1993; Bulavka *et al.*, 1990, 1994b; Bulavka and Tolkachev, 1995).

Owing to the low yields of **89**, preference was given to the route of (±)-**1** synthesis via the intermediate Schiff base from **17** and **39**. The latter was obtained from 3,4-dimethoxy-benzaldehyde **(38)** which was brominated regioselectively with bromine in dichloromethane to produce 2-bromo-4,5-dimethoxy-benzaldehyde **(93)** in 98% yield. The latter was selectively O-demethylated with sulphuric acid to afford 2-bromo-5-hydroxy-4-methoxy-benzaldehyde **(39)**. The last reaction

was optimised to produce the key compound in 80% yield (92% conversion) (Bulavka *et al.*, 1990). Isomeric 2-bromo-3-hydroxy-4-methoxy-benzaldehyde **(54)**, suitable for photochemical cyclisation (as on Figure 12.8), was also obtained by highly regioselective bromination of isovanilline with *N*-bromosuccinimide (Bulavka *et al.*, 1991).

Synthesis of 2-bromo-*O*-methylnorbelladine **(95)** was carried out according to the scheme shown on Figure 12.12 via the Schiff base **94**, preferably without its isolation. The reductive amination of **39** with **17** (molecular sieves 4 Å, then $NaBH_4$ in MeOH) yielded the secondary amine **95** (85–96%), which after N-formylation (89%, or 99% conversion) to **82** (Bulavka *et al.*, 1994b) was oxidised with Mn(III) acetylacetonate in acetonitrile to **83** (25%) (Bulavka and Tolkachev, 1995), and then converted to (±)-**1** and (±)-**2** (53% and 31%). The total yield of (±)-**1** was 9.5%, higher than in the previous scheme.

Industrial synthesis of galanthamine – recent modifications

Czollner *et al.* (1997, 1998) recently modified the method of oxidation, optimising the phenol coupling process to 45–50%. The final product was obtained as the optically active compound. Phenolic oxidation of the formyl-tyramine derivative **82** in a two-phase liquid system was proposed, while (±)-8-bromo-*N*-formylnornarwedine **(83)** was obtained in 26% yield (Henshilwood and Johnson, 1996). A similar route was described in a patent of Tiffin *et al.* (1997). Compound **83** was asymmetrically reduced to enantio-enriched (–)-galanthamine in 36% yield and 50% enantiomeric excess with a hydride agent formed *in situ* from $LiAlH_4$, (–)-*N*-methyl-ephedrine and 2-(ethylamino)pyridine (Dyer *et al.*, 1996; Czollner *et al.*, 1996). Using complex hydrides ($NaBH_4/CeCl_3$, $LiAlH_4/AlCl_3$, etc., –78 °C in tetrahydrofuran), narwedine was recently reduced to (–)-galanthamine in high yield (99.5%) (Shieh and Carlson, 1995). Carrying out an oxidative cyclisation of the biogenetic precursor *O*-methyl-norbelladine in the presence of extract from *Narcissus* bulbs produced (–)-galanthamine in yields 3 times higher than that obtained in the absence of precursor (Bannister and McCague, 1997). In a similar route, Chaplin *et al.* (1997a) used *N*-methylation of compound **95** before a phenolic coupling reaction (30%). Palladium-catalysed debromination produced racemic narwedine in 84% yield. Chaplin *et al.* (1997b) have also patented a resolution of racemic bromo-narwedine as dibenzoyltartrate and reduction to (–)-galanthamine with L-selectride.

Galanthamine synthesis by electrochemical oxidation of belladine-type amides

Vlahov *et al.* (1980a,b) undertook a broad experimental study of the electrochemical oxidative transformation of bis-alkoxy- or benzyloxy-substituted bromo-amides into derivatives of narwedine and cyclodienones, intermediates in the synthesis of **1** (Figure 12.13). They synthesised a series of substituted bromoamides **96–98** (R′, R″ = Me, $PhCH_2$) and **102**. Anodic oxidation of these compounds (+1.30 V in MeCN, ≤ 0 °C) gave only trace amounts of the desired enones (**34** or **49**). The main compounds isolated were dienones (**99–100** or **103**) (25–60%), together with

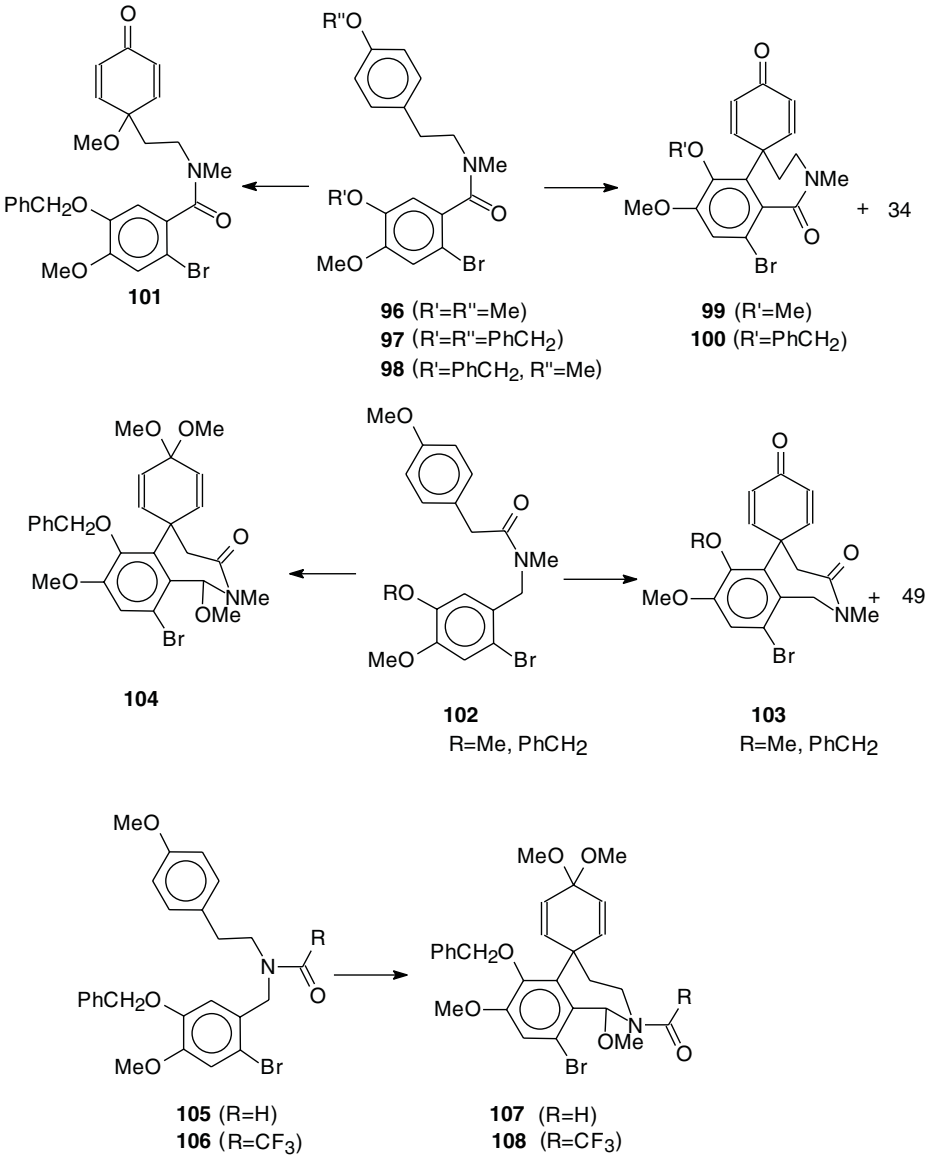


Figure 12.13 Synthesis of narwedine-type enones and dienones by electrochemical oxidation of belladine-type amides (Vlahov *et al.*, 1980a,b, 1984).

unreacted starting materials. Yields were dependent on the character of substitution in the substrate molecules. In the case of benzyloxy-substituted compounds, yields of the expected compounds reached 25–40%.

The incomplete transformation of amides **96–98** and **102** to the key compounds **99–100** and **103** was explained by the existence of starting compounds as a

mixture of E- and Z-forms. On anodic oxidation of the corresponding *N*-formyl and *N*-trifluoroacetyl derivatives **105** and **106** (R = CHO and $COCF_3$) in the presence of MeCN and 2% MeOH, a high rate of conversion of the parent compounds took place (80–100%), while the main reaction products were isolated as ketals (**107** and **108**, R = CHO and $COCF_3$) and the benzylic methylene group underwent oxidative methoxylation (Vlahov *et al.*, 1984; Krikorian *et al.*, 1984). The oxidation of amide **102** in MeCN and 33% MeOH resulted in 30% of methoxylated ketal **104**, while in the same conditions amide **98** formed methoxycyclohexadienone **101** (Vlahov *et al.*, 1984). The electrochemical oxidation reaction was thoroughly studied in respect of the effects of substituents and reaction conditions on the reaction products and their yields. It was shown that, owing to the high lability of the spirodienones produced, they could not be converted to the expected dihydrofuran derivatives.

Synthesis of alkaloids of the galanthamine group using hypervalent iodine (III) oxidation agent

Kita *et al.* (1998) extended the phenol-coupling reaction using a hypervalent iodine (III) reagent for the synthesis of galanthamine-type Amaryllidaceae alkaloids. As a result, total syntheses of (±)-sanguinine, (±)-galanthamine, (±)-narwedine, (±)-lycoramine and (±)-norgalanthamine were accomplished (Figures 12.14, 12.15 and 12.16).

The starting material 3,4-dihydroxybenzoic acid **(109)**, on esterification to methyl ester **110** (96%), and following protection of hydroxy groups with diphenyldichloromethane as the cyclic diphenylketal **111** and $LiAlH_4$ reduction to the alcohol **112**, was brominated with *N*-bromosuccinimide to afford **113** in 98% yield (over the three stages). The bromoalcohol **113** was then converted ($NaOCH_3$ – $Me_3SiOSiMe_3$) to trimethylsilanyl derivative **114** in 56% yield, which was oxidised with active MnO_2 to the corresponding aldehyde **115** (86%). Aldehyde **115** with tyramine **(17)** in methanol formed the Schiff base **116**, which on $NaBH_4$ reduction to norbelladine derivative **117** and acylation (without isolation of the latter) with trifluoroacetic anhydride, was converted to amide **118** in 96% yield (over the three stages). Phenolic oxidation of **118** with phenyliodine (III) bis(trifluoroacetate) in CF_3CH_2OH (–40 °C, N_2) led to dienone **119** in 36% yield. The latter underwent complete de-protection in trifluoroacetic acid medium to form enone **120** in 100% yield. Methylation with dimethyl sulphate gave *N*-demethyl-*N*-trifluoroacetyl-narwedine **121** in quantitative yield (Kita *et al.*, 1998) (Figure 12.14).

The same authors used compounds **121** and **120** (without isolation) for the preparation of alkaloids of the galanthamine group (Figure 12.15). Thus, **121** on hydrolysis with K_2CO_3 in aqueous methanol formed *N*-demethylnarwedine **(122)**, which, without isolation, was methylated with formaldehyde and formic acid to **2** (100%), reduced with L-Selectride (–78 °C in tetrahydrofuran) to **1** (100%), and, following hydrogenation over Pd/C, to lycoramine **(123)** (100%). According to another route **121** was hydrolysed to **122** and, without isolation, was reduced with L-Selectride to *N*-norgalanthamine **(44)** in 82% yield. Sanguinine was synthesised as follows: **119** was de-protected and **120**, without isolation, afforded **124** in 72% yield on treatment with imidazole and tertiary butyldimethylsilyl chloride. Reduction

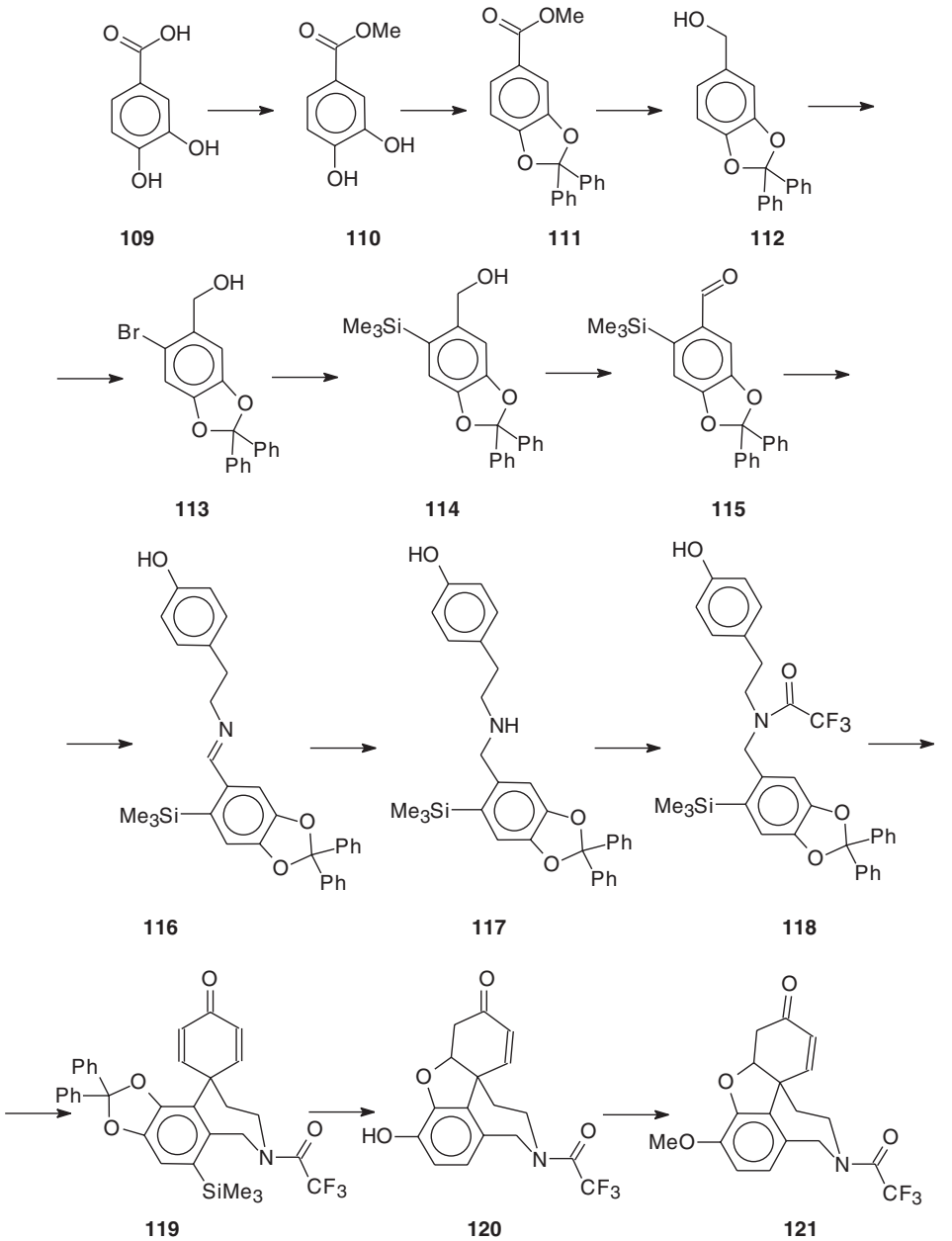


Figure 12.14 Synthesis of *N*,*O*-didemethyl-*N*-trifluoroacetylnarwedine **(120)** and *N*-demethyl-*N*-trifluoroacetyl-narwedine **(121)** (Kita *et al*., 1998).

of **124** with L-Selectride in tetrahydrofuran at –78 °C with following hydrolysis gave **125**, which on methylation with formaldehyde and formic acid gave sanguinine **(126)** in 68% yield.

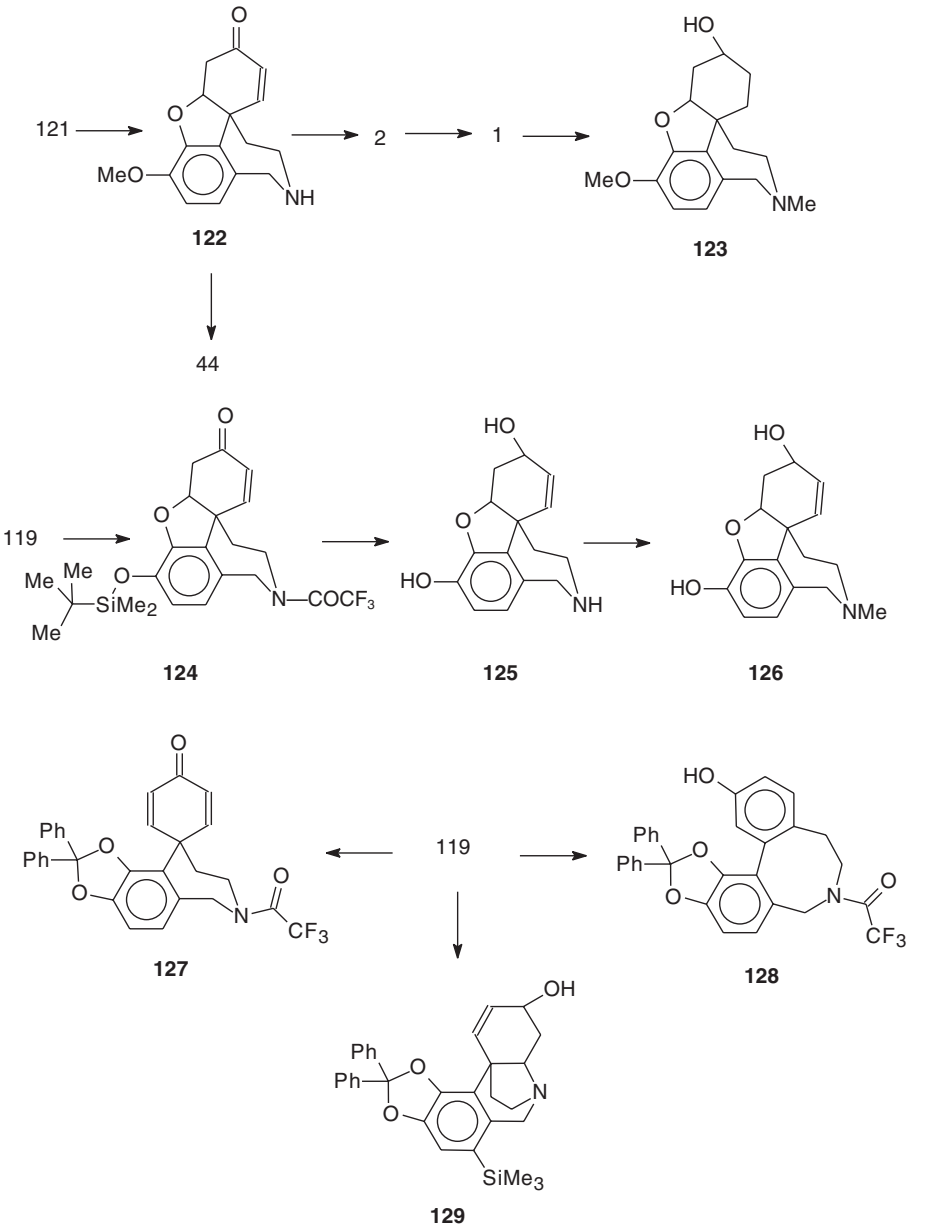


Figure 12.15 Synthesis of narwedine **(2)**, galanthamine **(1)**, lycoramine **(123)** and sanguinine **(126)** (above) and reactions of trimethylsilanyl-substituted narwedine-type dienone **(119)** (below) (Kita *et al*., 1998).

The dienone **119** was found easily to undergo various transformations (Figure 12.15). Thus, on trifluoroacetic acydolysis it produced narwedine-type enone **120** only. On treatment with concentrated HCl or BCl_3, **119** selectively lost only the trimethylsilanyl group with formation of dienone **127**. In a medium of 5N HCl in

ethanol or BBr_3, **119** underwent elimination of the trimethylsilanyl group and dienone-phenolic rearrangement, with the formation of apogalanthamine-type compound **128**. On alkaline hydrolysis with K_2CO_3 in aqueous methanol, deacylation of **119** accompanied cyclisation to crinine-type Amaryllidaceae alkaloids **129**.

A number of norbelladine-type amides **(118)** and similar compounds of the structure **130–133** were synthesised, and their oxidation process with phenyliodine (III) bis(trifluoroacetate) was thoroughly studied (Figures 12.14 and 12.16). The main products were found to be trialkylsilanylsubstituted dienones **134** (32%), **135** (46%), **136** (37%) and **137** (28%). In the case of **118** and **131**, the oxidation was accompanied by the formation of by-products, dienones **138** (9%) and **139** (12%), respectively. The amide **140** on oxidation under similar conditions produced only the trimethylsilanyl substituted compound **141**, in 26% yield.

Thus, the most easily removable group for catechol hydroxyls was the diphenylmethylene group, and the transformation of dienone to narwedine-type enone was successfully achieved only in the case of **119**. The enone **120** formed was a suitable intermediate for galanthamine type synthesis.

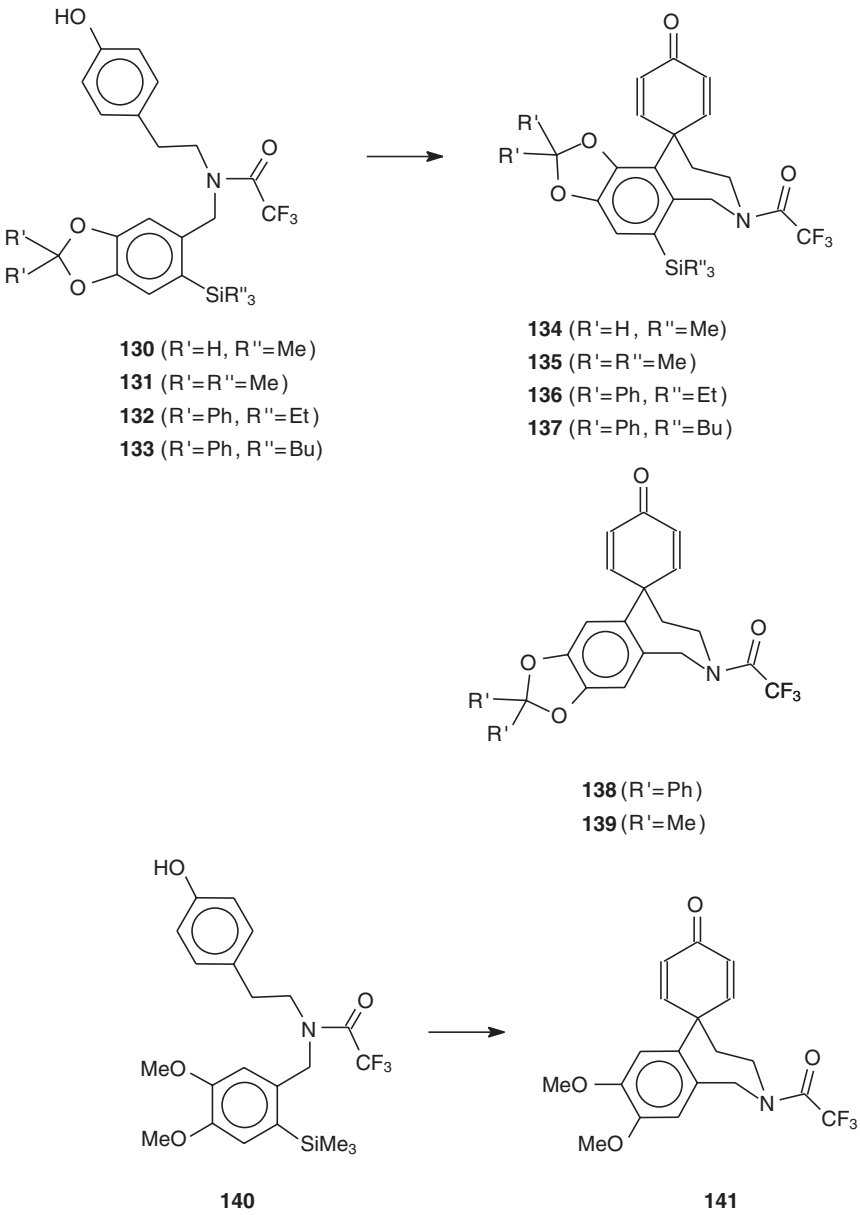
Synthesis of galanthamine and lycoramine analogues

By means of photochemical cyclisation of substituted enamidobenzamides spirocyclohexyl-isoquinolines, analogues of (±)-galanthamine and (±)-lycoramine have been produced (Missoum *et al.*, 1997). Various chemically modified galanthamine derivatives (N-C_1-C_{12}-alkylene substituted analogues) have been obtained recently as cholinesterase inhibitors (Thal *et al.*, 1997).

Classical approaches to lycoramine synthesis

Several multi-step syntheses of (±)-lycoramine have been also published. They are not actually biomimetic, and do not include galanthamine or narwedine intermediates. Two main strategic approaches were used. The first strategy was based on step-by-step construction of a lycoramine system, containing the properly substituted benzene nucleus. Thus, Hazama *et al.* (1968) offered the 23-step synthesis from 3-ethoxy-2-hydroxybenzaldehyde in 0.027% total yield. Misaka *et al.* (1967, 1968), in a 20-step synthesis from 2,3-dimethoxybenzaldehyde, reached a 0.39% total yield. Martin and Garrison (1981, 1982) published the 15-step synthesis from 2-hydroxy-3-methoxybenzaldehyde, with a 17.35% total yield. A successful 13-step synthesis from 3,4-dimethoxycinnamonitrile, in the scheme of Sanchez *et al.* (1984), was realised with over 22.64% total yield.

In the second strategic approach, the substituted aromatic and cycloaliphatic moieties underwent simultaneous modification. Thus, Schultz *et al.* (1977) have used methyl 3-hydroxy-4-methoxybenzoate and 3-ethoxy-2-cyclohexenone as synthones in a 16-step synthesis with 8% total yield. Ackland and Pinhey (1987a) used cycloaliphatic synthones, 4-ethoxycarbonyl-cyclohexadienones mixture, prepared in two stages from 2-trimethylsilyloxy-1,3-butadiene and methylpropiolate, and tributyl-(2,3-dimethoxyphenyl)stannane, in a 14-step synthesis with 8% total yield (Ackland and Pinhey, 1987b). Parker and Kim (1992) carried out an eight-step synthesis starting from cycloaliphatic synthone *trans*-2-bromo-4-(tert-butyldimeth-



130 (R'=H, R''=Me)
131 (R'=R''=Me)
132 (R'=Ph, R''=Et)
133 (R'=Ph, R''=Bu)

134 (R'=H, R''=Me)
135 (R'=R''=Me)
136 (R'=Ph, R''=Et)
137 (R'=Ph, R''=Bu)

138 (R'=Ph)
139 (R'=Me)

140 **141**

Figure 12.16 Oxidative cyclisation reactions of trimethylsilanyl-substituted norbelladine-type trifluoroacetamides to benzazepine spirodienones (Kita *et al*., 1998).

ylsilanyl)oxy-cyclohexanone and 2-bromo-3-hydroxy-4-methoxybenzaldehyde **(54)** with a 2.7% total yield. Ishizaki *et al*. (1993), in a ten-step synthesis from guajacol and benzoquinone ethylene monoketal, obtained a 11.3% total yield. The recently published synthesis of Essamkaoui *et al*. (1996) contained only six steps, starting from allylcyclohexenylveratrol.

CONCLUSIONS

A comparative analysis of the known schemes of the total synthesis of galanthamine has shown that biomimetic methods are preferable over classical synthetic approaches because they are carried out in the most economical way, with a minimum of steps, with maximum yields and simple realisation. In a single scheme, the production of different natural members of the galanthamine series is possible.

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13 Compounds from the genus *Narcissus*: pharmacology, pharmacokinetics and toxicology

David Brown

INTRODUCTION

The genus *Narcissus* has yielded several useful or potentially useful compounds, although just one of them, galanthamine, has been investigated in any great detail. As with most modern phytopharmaceuticals, identification, purification and investigation of the active principles have only been accomplished after many years, perhaps centuries, of folk medicine use as whole plant material or crude extracts. For the earliest account of a clinical use of galanthamine one has to go back to ancient Greek times. Homer's Odyssey has it that the attempts of Circe to poison Odysseus were foiled when Hermes gave Odysseus an antidotal root drawn from the earth, with a black root and milk-like flower, which was difficult for mortals to dig up and which the gods called 'moly'. On the basis of descriptions of their symptoms (memory loss, hallucinations and delusions that they had been turned into pigs) it has been suggested that the drug which Circe had used to poison Odysseus' men was a powerful, centrally acting, anticholinergic drug called stramonium, derived from the common plant *Datura stramonium*. The story is told much more eloquently by Plaitakis and Duvoisin (1983) who postulate that the antidote was derived from the common snowdrop, *Galanthus nivalis*, which contains the anticholinesterase, galanthamine. This is based on the description of the antidote or 'moly' given by Homer and by later Greek texts and recognition that both plants (*Datura* and *Galanthus*) would have been a resource native to the area. The authors state that if all this were true, then it represents the oldest recorded use of an anticholinesterase to reverse anticholinergic intoxication.

Of course, these accounts refer to snowdrop, not narcissus, but galanthamine is a derivative common to both. Originally isolated from *Galanthus nivalis* and used clinically under the name 'Nivalin', galanthamine can also be derived from *Narcissus* cultivars (Moraes-Cerdeira *et al.*, 1997) and represents the most pharmacologically interesting and to date, clinically useful compound, to be derived from the genus. There is at the time of writing, considerable interest in galanthamine as a potential treatment for Alzheimer's disease. This chapter reviews the pharmacology, pharmacokinetics and toxicology of galanthamine in animals and man. The rationale behind its various putative clinical uses is also discussed. Reference is also made to another interesting compound, pretazettine, which has been investigated as an adjunct to cancer chemotherapy. Clinical (phase 2 and 3) trials with galanthamine in Alzheimer's disease are discussed in the following chapter of this volume.

PHARMACOLOGY OF GALANTHAMINE

Galanthamine was first isolated in Russia in 1947 and its structure was determined in Japan some five years later (Prokurnina and Yakovleva, 1952; Uyeo and Kobayashi, 1953). A milestone in the development of galanthamine came in the early 1960s, when the compound was first recognised as a reversible inhibitor of acetylcholinesterase (Boissier *et al.*, 1960; Irwin and Smith, 1960a). Soon after this, evidence was provided that the drug could cross the blood-brain barrier (Nesterenko, 1964).

In the 1960s and 1970s, the drug was used in eastern bloc countries for a range of neurological disorders (see Clinical Applications, below) but because of the cold war, much of the early literature remained obscure. This, and the fact that galanthamine was available only as a natural product from limited Bulgarian and Turkish sources, explains why Western investigators were slow to appreciate and investigate its potential.

Galanthamine, a tertiary alkaloid (see Figure 13.1), is a selective and reversible inhibitor of anticholinesterase, which is an enzyme responsible for the degradation of acetylcholine at the neuromuscular junction. The detailed animal pharmacology has been reviewed elsewhere (Harvey, 1995). Essentially, galanthamine has been shown to inhibit both dog skeletal muscle *in vitro* and cat and mouse brain anticholinesterase *in vivo* and *in vitro* in micromolar quantities. When compared

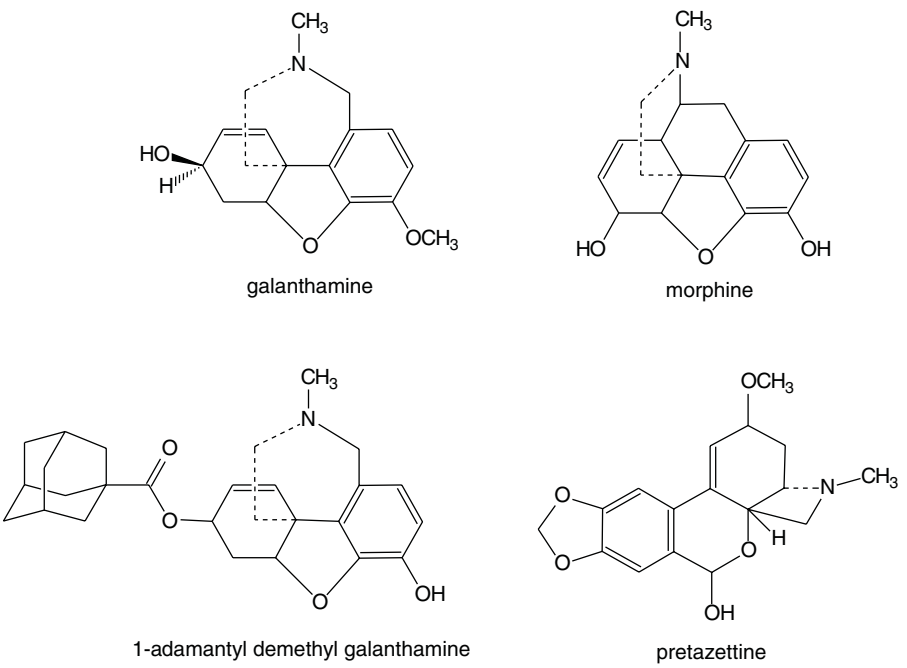

Figure 13.1 Chemical structures for galanthamine, morphine, 1-adamantyl demethyl galanthamine and pretazettine.

with neostigmine the activity of galanthamine against human erythrocyte, human serum and dog skeletal muscle cholinesterases was 400–1000 times less potent (Boissier and Lesbros, 1962), although functional studies demonstrated a potency reduction of only 10–20 times, suggesting that galanthamine may be able to potentiate the actions of acetylcholine also. The pharmacological explanation of this remains obscure. When the relative potencies of galanthamine, neostigmine and pyridostigmine in reversing pancuronium-induced muscle paralysis were studied in anaesthetised rats, galanthamine was 30 times less potent than neostigmine and 12 times weaker than pyridostigmine (Cozanitis *et al.*, 1981). The dose-response curve for galanthamine was shallower than those of the other two drugs, indicating some as yet unexplained differences in mode of action. Comparative *in vitro* studies have shown that human erythrocyte anticholinesterase inhibition by galanthamine is essentially reversible, whereas that of tacrine, a rival treatment for Alzheimer's disease, is not (Tonkopii *et al.*, 1976). In these early experiments, tacrine was considerably more potent than galanthamine against animal acetylcholinesterase, but in more recent studies, galanthamine was longer acting against mouse brain acetylcholinesterase *in vivo* (Sweeney *et al.*, 1989; Tonkopii and Padinker, 1995).

Galanthamine also inhibits human erythrocyte anticholinesterase selectively, having a much smaller effect (from 60 to 100 times less) on butyrylcholinesterase both *in vitro* and *in vivo* (Thomsen and Kewitz, 1990). This property may be important, as more potent inhibition of the latter might increase toxicity in the form of neurological side effects – notably prolonged neuromuscular and respiratory distress – particularly if the baseline activity of this enzyme is already depressed by liver disease or a hereditary defect (Pacheco *et al.*, 1995) or the drug is administered long-term, as is likely in Alzheimer's disease (Thomsen and Kewitz, 1990). This observation is also relevant to the selection of drugs for the treatment of Alzheimer's disease, where it is known that physostigmine inhibits both acetyl- and butyrylcholinesterase, while tacrine may have more effect on the latter than on the former (Thomsen *et al.*, 1991b). Inhibition of erythrocyte anticholinesterase may also be used to relate pharmacological activity to therapeutic effect, and thus optimise therapy in Alzheimer's disease. For example, a therapeutic window of dosing that stabilises erythrocyte acetylcholinesterase activity at 30–36% of baseline has been proposed (Becker *et al.*, 1991).

Directly relevant to experimental findings in animals is the observation that the pro-cholinergic activity of galanthamine was considerable in both human, post-mortem brain tissue – particularly in the frontal cortex compared with the hippocampus – and in fresh cortex samples obtained from operations to remove brain tumours (Thomsen *et al.*, 1991a). In these experiments, tacrine was three times as potent as galanthamine and physostigmine some 200 times more effective. In spite of these differences, the oral daily doses required to achieve equivalent cognitive benefit are approximately 30 and 160 mg/day for galanthamine and tacrine, respectively.

Galanthamine has also been shown to inhibit acetylcholinesterase in human volunteers and patients with Alzheimer's disease (Thomsen and Kewitz, 1990; Thomsen *et al.*, 1990a, 1991b), although the peripheral (erythrocyte) activity was ten times that of the brain activity. Baraka and Harik (1977) found that galanthamine (0.5 mg/kg, intravenous (IV)) reversed scopolamine-induced central anticholinergic

syndrome – drowsiness, disorientation, short-term memory impairment – in ten healthy volunteers. Although no objective measurements of cognitive function were made, electro-encephalogram (EEG) monitoring in two subjects showed changes matching the observed changes in consciousness. Scopolamine replaced the dominant awake alpha rhythm with slow, disorganised activity; galanthamine promptly restored the EEG pattern to normal.

PHARMACOKINETICS OF GALANTHAMINE

The reader is referred elsewhere for detailed reviews of the pharmacokinetics of galanthamine in species other than man (Harvey, 1995; Bores and Kosley, 1996; Bickel *et al.*, 1991a). Animal data are reported here where they serve to amplify what is known in man. For a more detailed description of the human data, see Kewitz (1997).

Galanthamine is commonly administered as the hydrobromide salt, and will be abbreviated to galanthamine throughout this review, unless other salts were used. In general, the pharmacokinetics of galanthamine are first-order and linear over a wide dose range. Table 13.1 contains a summary of values determined for key pharmacokinetic parameters in man. Galanthamine has been assayed in various body fluids using a variety of techniques, including high-performance liquid chromatography (HPLC) and enzyme immunoassay. For details of these, see individual references.

Table 13.1 Galanthamine pharmacokinetics: summary of human data[a]

Parameter	*Value/comments*
Overall kinetics	Linear, two-compartment, first order
Relative oral bioavailability (from solution or tablets)	85–100%
Time to peak serum level (T_{max}) after tablets	52–120 min
Absorption half-life ($t_{1/2\ abs}$)	20 min
Volume of distribution (Vd)	1.76–2.90 litre/kg
Distribution half-life ($t_{1/2}\alpha$)	6.6–9.6 min
Elimination half-life ($t_{1/2}\beta$)	4.4–8.1 h
Mean serum clearance	250–340 ml/h/kg
Mean renal clearance	82–84 ml/h/kg
Galanthamine excreted unchanged in urine	30–50%
Dose excreted in first 24 hours	60%
Metabolites (see Figure 13.2):	
O-demethyl galanthamine (iii)	20% as glucuronide
N-demethyl galanthamine (iv)	5%
Epigalanthamine (v)	<2%
Galanthaminone (ii)	trace

Note
[a]Ranges represent lowest and highest mean values quoted in the literature; see text for references.

Absorption

Galanthamine had almost complete (85–100%) oral bioavailability in man, when formulated either as a solution or tablets (Mihailova *et al.*, 1989; Bickel *et al.*, 1991b). In one study (Bickel *et al.*, 1991b), the time to peak plasma concentration (T_{max}) was shorter with a solution (15 minutes against 52 minutes). The half-life of the absorption process was 20.4 minutes. In another study (Mihailova *et al.*, 1989), absorption from oral tablets was slower, with a T_{max} in healthy volunteers of approximately 120 minutes. The bioavailability data indicate that there is no significant first-pass effect.

Therapeutically relevant drug concentrations, corresponding to 30–60% inhibition of erythrocyte acetylcholinesterase (as recommended by Becker *et al.*, 1991) were reached within 30–44 minutes of administration.

Distribution

Data from studies in anaesthetised cats indicate that galanthamine has a large volume of distribution but does not bind to plasma proteins significantly (Mihailova *et al.*, 1985). Single dose (0.3 mg/kg, IV) studies of galanthamine have been carried out in eight female patients undergoing gynaecological surgery (Westra *et al.*, 1986). Galanthamine was given at the end of surgery to reverse pancuronium-induced neuromuscular blockade. Serum levels were determined by HPLC. Mean peak levels of 543 ± 47 ng/ml were observed at approximately 2 minutes. The serum level versus time curve showed biexponential decay after the maximum was reached, indicating extensive distribution in a two-compartment model. A mean distribution half-life ($t_{1/2}\alpha$) of 0.11 hours and an elimination half-life ($t_{1/2}\beta$) of 4.4 hours were calculated. The latter was considerably longer than the half-lives of neostigmine (80 minutes) and pyridostigmine (46 minutes). The volume of distribution at steady state was large (mean: 1.76 litre/kg, with a 95% confidence interval (CI) of 1.22–2.30 litre/kg), indicating accumulation by various tissues. The mean total serum clearance was 322.2 ml/h/kg and mean renal clearance was 81.6 ml/h/kg.

Mihailova *et al.* (1989) administered single doses of 10 mg (0.11–0.18 mg/kg) subcutaneously or orally, in an open crossover fashion, to eight male volunteers. By these routes, the T_{max} was prolonged (2 hours in each case) and peak serum levels were 1.1–1.5 µg/ml and 1.0–1.4 µg/ml, respectively. The authors concluded that the subcutaneous and oral formulations were essentially bioequivalent. These values are higher than one might expect on the basis of the results of Westra *et al.* (1986) who gave, on average, three times the dose of galanthamine intravenously. The discrepancy is difficult to explain on the basis of different assay methods employed in the two studies alone. Perhaps there are as yet unexplained differences in age, sex or clinical status that determine the pharmacokinetics of galanthamine. The $t_{1/2\beta}$ values were 5.7 and 5.3 hours after oral and subcutaneous doses, respectively.

Thomsen *et al.* (1990b) reported a pharmacokinetic study in one Alzheimer's patient and one volunteer. Repeated daily doses of 30–50 mg galanthamine resulted in steady state plasma concentrations of 50–150 ng/ml. In the volunteer, IV injections of 10 mg of the drug produced a peak plasma concentration of

182 ng/ml. The half-life was reported to be 8–10 hours. These values are closer to those reported by Westra *et al.* (1986). In a further study in eight male volunteers, Bickel *et al*. (1991b) observed a maximum plasma concentration of 30–60 ng/ml after oral administration of 10 mg galanthamine and 50–100 ng/ml after the same dose given by constant rate IV infusion over 30 minutes. Galanthamine showed linear, first order pharmacokinetics. The mean values for $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 0.16 hours and 5.68 hours, respectively. The mean total clearance was 340 ml/h/kg and renal clearance was 84 ml/h/kg. A steady state volume of distribution of 2.64 litre/kg (95% CI: 2.41–2.90 litre/kg) was calculated. Twenty-five percent of the dose was excreted unchanged in the urine. Little evidence of metabolism was found: negligible amounts of epigalanthamine and galanthaminone were detected in blood and urine.

Most recently, Kewitz (1997) reported data from a 15-week clinical trial of galanthamine in 34 patients with Alzheimer's disease, whose mean age was 75 years. A mean volume of distribution of 2.9 litre/kg, a half-life of 8.1 hours and a plasma clearance of 250 ml/h/kg were calculated. As might be expected and certainly should be remembered, clearance was 30% less in this elderly population than in healthy young volunteers.

There is no direct *in vivo* evidence that galanthamine itself enters the human brain. However, examination of its structure (see Figure 13.1) reveals a tertiary nitrogen atom in common with many molecules that can penetrate the blood-brain barrier. Indirectly, there is biochemical evidence from experiments in rats (Mihailova and Yomboliev, 1986). One and 3 mg/kg intravenous doses produced peak levels of 5 and 6.3 μg/g of brain tissue, respectively, 10–15 minutes after injection, declining to about 10% between 1 and 3 hours. In mice, parenteral administration of galanthamine resulted in accumulation in brain, liver and kidney tissue (Bickel *et al.*, 1991a).

Correlation of galanthamine pharmacokinetics with erythrocyte cholinesterase inhibition

The inhibition of acetylcholinesterase in human erythrocytes correlates closely with the pharmacokinetics of galanthamine. A median maximal value of 53% inhibition was reported after a single, intravenous dose of 10 mg (Bickel *et al.*, 1991b). The *in vitro* and *ex vivo* concentration responses were essentially identical, indicating that galanthamine alone, and not its metabolites, was responsible for inhibition of cholinesterase. In a previous study (Thomsen *et al.*, 1990a), erythrocyte acetylcholinesterase activity returned to normal some 30 hours after both single dose and chronic administration at therapeutic doses (10–40 mg daily), illustrating the reversible nature of the inhibition of the enzyme by galanthamine.

Kewitz (1997) recently presented data to show the relationship between the serum level of galanthamine, enzyme inhibition in erythrocytes and central nervous system (CNS) effects. Simultaneous EEG recordings were made in a healthy young male volunteer, during an IV infusion of 15 mg galanthamine. The power density of the alpha-one frequency band of the EEG at the occipital region began 7 minutes after starting the infusion, when 40% of the enzyme activity had been blocked. Power density fluctuated in synchrony with enzyme activity as the galanthamine levels fell after the infusion was stopped.

Metabolism and excretion

Clearly, the metabolism of any drug that is used in an elderly population needs to be characterised, given the potential effects of age on liver function.

In the study by Westra *et al.* (1986), approximately 30% of the intravenous galanthamine dose was excreted in the urine unchanged. Biliary excretion was negligible and there was no evidence of conjugation with glucuronide or sulphation. Mihailova *et al.* (1989) detected the metabolites epigalanthamine (the stereo isomer of galanthamine – see Figure 13.2) and galanthaminone in plasma and urine; these metabolites possess only very weak anticholinesterase activity (<1%; Thomsen *et al.*, 1990a). Almost complete recovery of galanthamine and its metabolites occurred within 72 hours.

From their experiments, Bickel *et al.* (1991b) concluded that metabolism was not extensive. Individual metabolic capacity was variable, but on average, 50% of the administered dose was excreted in urine unchanged; at least 60% of a dose was excreted in urine in the first 24 hours after dosing, either unchanged or as metabolites. Galanthamine could not be detected at 72 hours. No data are available

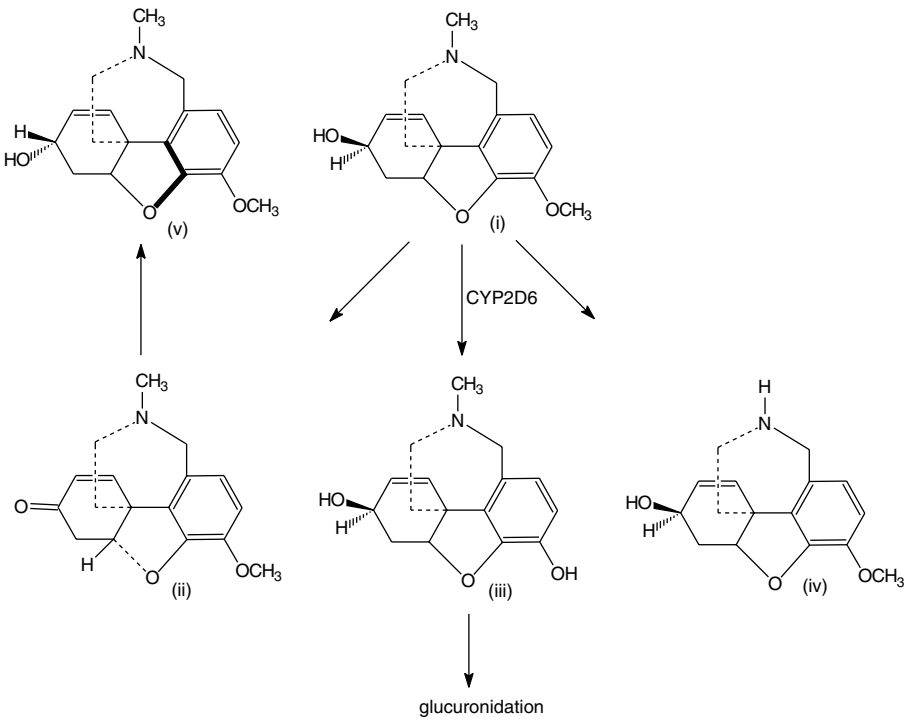

Figure 13.2 Galanthamine metabolic pathway (Bachus cited in Kewitz, 1997)
(i) Galanthamine.
(ii) Galanthaminone.
(iii) *O*-demethyl galanthamine.
(iv) *N*-demethyl galanthamine.
(v) Epigalanthamine.
CYP2D6: hepatic cytochrome isoenzyme CYP2D6.
See text for further details.

on the excretion of galanthamine or its metabolites in bile or the intestinal tract. The notion that galanthamine is not extensively metabolised is also supported by animal data (Harvey, 1995).

More recently, Bachus (1995) showed that in male volunteers, 20% of a dose of galanthamine appeared in urine as *O*-demethylgalanthamine glucuronide, 5% as *N*-demethylgalanthamine and < 2% as epigalanthamine resulting from the intermediate metabolite galanthaminone, which itself was present in traces only. *O*-demethylgalanthamine is 3–10 times more active than galanthamine, but rapid glucuronidation reduces this by a factor of at least one hundred. All the other galanthamine metabolites identified thus far have little anticholinesterase activity. *O*-demethylation is catalysed by the cytochrome isoenzyme CYP2D6. This knowledge should allow the avoidance of concomitant drugs that are also metabolised predominantly by this isoenzyme (such as beta-blockers, haloperidol, morphine, phenothiazines and some tricyclic and selective serotonin reuptake inhibitor antidepressants) and thus decrease the potential for drug interactions through metabolism induction or inhibition. Fluoxetine, haloperidol, morphine and quinidine are known to inhibit CYP2D6, but the problem may be largely theoretical. Co-adminstration of therapeutic doses of quinidine, a potent inhibitor of CYP2D6, to four healthy volunteers completely suppressed *O*-demethylation but led to only a modest (20%) rise in galanthamine serum levels. Kewitz (1997) claimed that this rise was unlikely to cause toxicity and concluded that co-administration of other drugs inhibiting the same enzyme would do little damage. Neither would problems be likely in poor metabolisers, deficient in CYP2D6 (5–10% of Caucasians and 1% of Asians), however caution is advisable. No comment was made on the likely outcome if other interacting drugs were used in poor metabolisers or in those with kidney dysfunction. Potential inhibitors of the isoenzyme CYP3A4 should also be viewed with caution, as this enzyme also plays a part in galanthamine metabolism.

Comparison with other relevant drugs

Galanthamine pharmacokinetics appear to be more attractive than those of some, but not all, other anticholinesterases currently under investigation for the treatment of Alzheimer's disease. Physostigmine has poor oral bioavailability (approx. 10%) and a short half life (<1 hour). Tacrine too has poor oral availability (<5%) and non-linear kinetics with a dose dependent half-life of 1.6–2.9 hours. Tacrine has also been associated with acute hepatitis. The pharmacokinetics of rivastigmine, donepezil and metrifonate allow once or twice daily dosing.

CLINICAL APPLICATIONS OF GALANTHAMINE

It is difficult to gain a clear picture of how the initial human trials with galanthamine were progressed after its isolation in 1947. It is known that, that the compound was first used in human trials by Bulgarian and Russian researchers in the 1950s, but as Mucke (1997) points out, the development of what today appears to be a useful drug was slowed considerably by the fact that the main research thrust came from behind the Iron Curtain. Clinical research that was published appeared in eastern European journals which, during the Cold War,

were internationally inaccessible. Western research conducted at this time merely paralleled and often duplicated the eastern European effort, rather than building upon it. Add to this the fact that early preparations of galanthamine were obtained by liquid carbon dioxide extraction of plant tissue and were therefore only available in small and often unreliable quantities of uncertain purity. Although a full synthesis of galanthamine was published in 1960 (Barton and Kirby, 1960), full-scale industrial synthesis was shown to be possible at a much later date, implying that a supply of predictable and reproducible purity was not used in early trials.

Historically, the considerable interest in galanthamine is illustrated by the fact that patents have been filed for its use in conditions as diverse as nicotine addiction, alleviation of benzodiazepine side effects, male erectile dysfunction, chronic fatigue syndrome, bipolar and panic disorders and acute mania (Mucke, 1997). There is a rational basis to many of the therapeutic uses to which galanthamine has been put. They may be divided into those, where, reversal of excessive anticholinergic activity is required, and those where, cholinergic activity is deficient.

Uses of galanthamine where reversal of excessive anticholinergic activity is required

Reversal of drug-induced paralysis in anaesthetics

The ability of galanthamine to reverse tubocurarine-induced muscle paralysis was discovered almost half a century ago (Mashkovskii, 1955). The drug has been used extensively to reverse the effects of curare and other muscle relaxants after surgery in eastern European countries, but published reports are largely anecdotal (see review by Paskov, 1986). Use has been rare in the West, where reports are fewer but better documented (Mayrhofer, 1966; Wislicki, 1967; Cozanitis, 1971; Baraka and Cozanitis, 1973). Typically, an intravenous dose of galanthamine is given to induce spontaneous respiration and awakening. Sometimes a second dose may be given, 5–10 minutes after the first. It is claimed that galanthamine has fewer cholinergic side effects than neostigmine, thus reducing the need for compensatory atropine, and that galanthamine speeds recovery from respiratory depression caused by morphine analgesia, but these effects are not fully substantiated.

For example, Cozanitis (1971) reported the successful use of galanthamine given intravenously in graded doses to a maximum of 20 mg in 40 surgical patients, to reverse the effects of alcuronium, pancuronium, gallamine and tubocurarine. The recovery rate was slower than that usually seen with neostigmine; atropine was not necessary. Side effects included increased pulse rate in most patients, increased salivation, dizziness and injection site reactions in some patients. Galanthamine was judged to be approximately 20 times less potent than neostigmine (Baraka and Cozanitis, 1973).

Uses of galanthamine where cholinergic activity is deficient

Research effort in this area has focused almost exclusively on investigating galanthamine as a potential treatment for Alzheimer's disease (AD). Phase 2 and Phase 3 clinical trials with galanthamine in this indication are reviewed in the following chapter of this volume.

Early work in animals

Galanthamine reversed the amnesia induced in mice by the injection of the anticholinergic agent, scopolamine (Chaplygina and Ilyutchenok, 1976). The drug was also shown to reverse the cholinergic deficit induced in rats by pre-treatment with the neurotoxin ibotinic acid (Sweeney *et al.*, 1988). In a notable series of experiments, Sweeney and co-workers produced lesions in the nucleus basalis magnocellularis of mice; this region is a major site of cholinergic enervation to the fronto-parietal cortex, and lesions here produce significant deficits in choline acetyltransferase activity which are linked to deficits in spatial memory. Intraperitoneal administration of galanthamine attenuated these deficits (Sweeney *et al.*, 1989), and improved memory in swim tasks (Sweeney *et al.*, 1988) and passive avoidance tests (Sweeney *et al.*, 1990); repeated doses remained effective, indicating that tolerance to the drug did not develop. Control animals given sham lesions showed impaired performance. At relatively high doses (1–4 mg/kg) galanthamine impaired the performance of control animals markedly, although an explanation for this was not forthcoming (Sweeney *et al.*, 1990).

Other workers confirmed some of this work: for example, Yonkov and Georgiev (1990) showed that galanthamine enhanced the retention of learned behaviour in both active and passive avoidance tests in rats. Chopin and Brierly (1992) showed that galanthamine was capable of reversing scopolamine-induced poor performance in a passive avoidance test in rats at very similar doses to tacrine (0.3–10 mg/kg administered intraperitoneally), a measure of the effect on memory. The doses used in these experiments were far in excess of those used in man to treat AD. Fishkin *et al.* (1993) showed that intraperitoneal galanthamine (1.25–5 mg/kg) significantly attenuated scopolamine-induced learning and memory deficits in rats, compared to controls as measured by T maze and Morris water maze experiments.

An interesting hypothesis, relevant to the pathophysiology of AD, is that in addition to inhibition of acetylcholinesterase, inhibitors of this enzyme may activate normal processing of the amyloid precursor protein which accumulates in the disease producing amyloid plaques which are a striking feature of post-mortem brain samples (Giacobini *et al.*, 1996). Whether or not galanthamine provides such neuroprotection is unknown, and definitive data will come only from long-term trials in man, where biopsy or post-mortem samples are available for study.

Early work in man

Early reports from eastern Europe claimed that recovery of consciousness after surgical anaesthesia was faster if galanthamine was used instead of neostigmine as a curare antagonist (Paskov, 1986). The mechanism remains obscure; it might be due to a direct central stimulant action or the ability to antagonise the action of opiate analgesics. The results from a study in conscious volunteers indicate that galanthamine may act as a mild central stimulant (Cozanitis and Toivakka, 1991).

Galanthamine crosses the blood-brain barrier readily, and because of this and the *in vitro* data discussed above, it was soon proposed as a potential treatment for brain diseases where cholinergic transmission appeared to be deficient – notably AD. In this disease, destruction of cholinergic neurones in the brain, notably the

cortex and hippocampus, results in reduced levels of acetylcholine which are thought to be associated with loss of memory and disrupted cognition. Drugs that preserve acetylcholine by inhibiting its destruction by cholinesterase, such as neostigmine, rivastigmine, tacrine, metrifonate and galanthamine, can reduce AD symptoms and delay, but not halt, disease progression (Mucke, 1997). Several small trials and what amount to anecdotal reports provide an impression that galanthamine is useful in this respect and, because of this early promise, several larger and more carefully controlled trials are underway. This work is reviewed in the next chapter of this volume. Specific, non-AD applications are described below.

Neurological injury

Paskov (1986) reviews the use of galanthamine to treat a wide range of neurological disorders. Most of the evidence is anecdotal and unclear, but it is logical to expect that if the neurological injury involves a cholinergic pathway resulting in a reversible deficit or imbalance of acetylcholine, then galanthamine may be of use.

Reversal of pathological paralysis

In the 1960s, galanthamine was used, again in eastern European countries, to assist recovery from paralysis associated with poliomyelitis, neuromuscular disorders or neuromuscular diseases. Trigeminal neuralgia was managed with oral and intramuscular administration and occasionally by infiltration. Doses of 15–25 mg/day were typically used (Kilimov, 1961a). Galanthamine was reported to produce motor function improvement in 15 of 25 children with muscular dystrophy (Pernov *et al.*, 1961) and in patients with myasthenia and neuromuscular dystrophy (Pestel, 1961). Sixteen of 20 patients with brain injuries (not specified) showed improvement on galanthamine (Kilimov, 1961b). Revelli and Grasso (1962) noted varying degrees of benefit in 49 of 52 patients suffering from post-febrile polio during a 3-month trial of the drug at doses of 2.5–10 mg/day. Gopel and Bertram (1971) reported the use of galanthamine in an heterogeneous group of patients studied over a 30-month period, using subcutaneous doses of galanthamine in the range 1.25–25 mg daily. Beneficial effects were seen in the following disorders, some of which were resistant to conventional therapy: facial paralysis (22 of 22); peripheral neuropathy (38 of 46); paralysis of central origin (11 of 12); progressive myodystrophy (1 of 2); poly/dermatomyositis (3 of 3) and multiple sclerosis (4 of 4). The authors stated that the drug was well tolerated.

Gujral (1965) studied 100 patients with post-polio spinal paralysis, seven with muscular dystrophy and two with facial paralysis due to lower motor neurones. Galanthamine was administered as a series of courses of 40 subcutaneous injections, repeated after a 6- to 8-week interval, up to a maximum of three courses per patient. Total daily doses were related to age. Drug treatment was combined with physiotherapy; this may have confounded the results, as physiotherapy was already proven to be effective and was not administered uniformly. In any event, the results were far from conclusive. Only modest number of patients showed improvement with galanthamine. The author concluded that the drug was most likely to have a beneficial effect in early cases of post-polio paralysis (improvement

in muscle tone and isometric contractions) and at an early, rather than late, phase of treatment (5–15 days of the first course). No improvement was seen where the muscle showed zero power from the onset or in chronic cases. Very little improvement was seen in four of the seven cases of pseudo-hypertrophic muscular dystrophy and none in the remaining patients. Some improvement in the two children with facial paralysis was noted. Galanthamine was described as being well tolerated; mild reactions included salivation, nausea and abdominal pain that resolved on decreasing the dose.

Improvement in motor function was observed when galanthamine was used as part of a programme of therapies used to treat polyneuropathy-associated Guillain-Barre syndrome (Kuyumdzhieva *et al.*, 1996).

Use in poisoning

Reports in animal, and later in human, studies, of the reversal of respiratory depression caused by opiate analgesics are tempered by observations that cholinergic side effects are likely at the doses required and that only temporary reversal is achieved (Tassonyi *et al.*, 1976). However, galanthamine has been used to reverse CNS effects in several cases of drug overdose, including scopolamine (Cozanitis, 1977), Mandrax (a combination of diphenhydramine, a drug with marked anticholinergic properties and methaqualone, an hypnotic), and dextromoramide (a narcotic analgesic related to methadone, which caused marked respiratory depression) (Cozanitis and Toivakka, 1974).

Analgesia

Poultices of the leaves and bulbs of the Amaryllidaceae have been reported in herbal lore for hundreds of years as being used to treat painful neurological conditions such as facial neuralgia; this treatment was said to provide analgesic and curative effects (Rainer, 1997).

Despite its apparent ability to reverse the respiratory depression seen with morphine-like analgesics, galanthamine does not appear to antagonise opiate-induced analgesia. Indeed, the drug is structurally related to morphine (see Figure 13.1). Evidence from standard laboratory tests suggests that the drug may have mild analgesic properties (Cozanitis *et al.*, 1983). Suggestions that galanthamine may stimulate opioid receptors directly needs substantiation by direct, radioligand binding studies.

Migraine

Ikonomoff (1968) was the first to report the use of galanthamine in the management of migraine patients. Of 140 cases involving galanthamine (in the form of the commercially available product 'Nivalin'), 25 (18%) were headache free for 6 years, and a further 64 (46%) had shown some improvement. In 42 patients, spontaneous epistaxis resolved and in 56 patients, subcutaneous ecchymosis ceased when galanthamine was used. Twenty-two of 59 female patients with dysmenorrhoea accompanying their migraine experienced resolution of period pains. Neostigmine was found to be superior in a similar range of patients. The author

reasoned that the efficacy of these two drugs could not be explained on the basis of their anticholinesterase activity alone, and suggested (but presented no evidence for) a range of actions, including beneficial effects on blood vessel tissue and a reduction of capillary permeability. Treatment, given twice daily, with an optimum total daily dose of 250 mg, was well tolerated, and no patient had to discontinue therapy because of side effects.

Mania and schizophrenia

Disturbances in the balance between neurotransmitters may be manipulated to improve a lot of patients suffering from mania and schizophrenia where, complex imbalances often occur. Snorrason and Stefansson (1991) have used this argument to justify treating manic patients with galanthamine. There is minimal detail in this report but galanthamine, 10 mg, three times a day, was observed to improve symptoms rapidly when given to a 74 year old lady who was unresponsive to lithium and where other agents were contraindicated because of neuroleptic malignant syndrome. The patient's condition deteriorated when galanthamine was stopped. The authors referred to ten other patients where the drug was of benefit. Galanthamine has been associated with a slow improvement in psychomotor function deficits in 18 of 30 patients with schizophrenia over a 3- to 4-week period (Vovin *et al.*, 1991).

Raised intraocular pressure

One paper mentions experiments where, galanthamine reduced intraocular pressure when applied in an eye drop formulation to rabbits (Agarawal and Gupta, 1990). The effect was slow and peaked at 2 hours. Physostigmine is an established drug for the treatment of glaucoma and it is interesting that galanthamine might also prove useful. There are no published trials of this use in man.

Chronic fatigue syndrome (CFS)

Several symptoms of CFS such as sleep disturbances, poor concentration and generalised fatigue are similar to the side effects of anticholinergic drugs; these can be reversed with physostigmine, a cholinesterase inhibitor similar to galanthamine. The latter has been shown to shorten rapid eye movement (REM) latency, increase REM density and reduce slow wave sleep, modify poor concentration, memory disturbances and reverse behavioural changes seen in Alzheimer's disease. In addition, galanthamine can elevate plasma cortisol levels, whereas patients suffering from glucocorticoid deficiency have similar symptoms to those with CFS. These observations have prompted suggestions that galanthamine may be of use in this syndrome. Thirty-three patients were enrolled in an 8-week, double-blind, placebo-controlled study involving galanthamine (Snorrason, 1993). Patients in the active group received total divided, daily doses of 10–50 mg. The following symptoms were improved significantly in the galanthamine group: sleep disturbances, fatigue, myalgia, anxiety, immediate and delayed recall; improvements in cognition were also observed. Similar results were published three years later by the same author (Snorrason *et al.*, 1996), highlighting marked improvements in

sleep disturbances as a potential benefit of the drug. Nausea was the most common side effect, reversible on drug withdrawal.

TOXICOLOGY AND TOXICITY OF GALANTHAMINE

Toxicological data derived from animals appear to be of limited use in predicting safety in the intended human population, but some data are of interest. The reader is referred to reviews on this topic for further information (Harvey, 1995; Mucke, 1997).

Animal toxicology

Acute toxicity is manifested mainly as exaggerated cholinergic effects that may be reversed with atropine. Death occurs through respiratory depression, whereas cardiac function is relatively unaffected. An acute LD_{50} of 5.2 mg/kg was determined in mice after IV administration (Friess *et al.*, 1961). The latter may have been an underestimate, as a value of almost double this was obtained when galanthamine was co-administered with 4-aminopyridine, a drug that enhances the release of acetylcholine from nerve terminals (Micov and Georgiev, 1986). A chronic toxicological study of the combination, with galanthamine doses up to 2 mg/kg daily, produced no remarkable changes in blood or major organs (Micov and Georgiev, 1986). As expected, the oral LD_{50} was higher than the IV value, at 18.7 mg/kg (Umarova *et al.*, 1965). Chronic oral doses (0.5 mg/kg/day) were shown to reduce respiratory volume and to desynchronise EEG patterns in rabbits, but not in other species, at this low dose. Maternal and embryo-toxicity were observed in rats and rabbits at doses of 10% of the LD_{50} (Paskov, 1986).

Toxicity in man

As an inhibitor of acetylcholinesterase, one might expect cholinergic side effects to be a feature of the side effect profile of galanthamine. Indeed, these have been reported following human consumption when narcissus bulbs have been mistaken for onions, or the leaves and flowers have been eaten (Vigneau *et al.*, 1984). Early reports of successful use in anaesthesia to reverse neuromuscular junction blocking agents mentioned few autonomic side effects – which is probably a reflection on their anecdotal and enthusiastic nature. More recent and carefully controlled studies confirm the suspicion that autonomic effects are indeed to be expected, but that these are dose related. Altered electrocardiogram (ECG), blurred vision, hypersalivation, nausea, vomiting and dizziness have been noted in conscious volunteers given 20 mg doses. Interestingly, galanthamine produced mild eosinophilia after injection. According to the authors, this might explain the local tissue reaction noted on injection of the drug (Cozanitis and Toivakka, 1971). No changes in blood sugar were observed in conscious volunteers (Cozanitis and Toivakka, 1971; Riemann *et al.*, 1994) and in patients after surgery (Cozanitis *et al.*, 1973b). At a relatively high dose of 25 mg, three out of nine surgical patients had sufficient bradycardia to require reversal with atropine (De Angelis and Walts, 1972).

Galanthamine causes bradycardia and might be expected to have a negative effect on blood pressure. However, when the drug has been tested in animals at larger doses than those used therapeutically in man, species-specific differences have been observed. A significant fall in blood pressure was noted in anaesthetised dogs (Irwin and Smith, 1960a,b), but a significant rise was noted in anaesthetised rats (Chrusciel and Varagic, 1966). In the latter experiment, physostigmine but not neostigmine produced a similar effect, suggesting a central mechanism, but the effect was not blocked by autonomic ganglion blocking drugs such as hexamethonium. Galanthamine-induced hypertension was attenuated by drugs capable of blocking the central action of the drug, such as adrenoreceptor antagonists, nicotine and atropine. Galanthamine produced no hypertensive response in pithed rats. With the exception of the lack of effect of the sympathetic ganglion blockers, the evidence points toward a central stimulant action, at least in anaesthetised rats.

Mild bradycardia was noted as a side effect in a trial of galanthamine in 14 surgical patients. A dose of 20 mg produced a reduction in mean pulse rate to 69 from 72 beats per minute (Cozanitis *et al.*, 1973a), but this had no appreciable effect on blood pressure. Six patients had more marked bradycardia, down to 55 beats per minute, associated with minor arrhythmias. When the drug was administered with atropine, mild bradycardia persisted, but only two patients showed anything like the more dramatic falls in pulse rate described above. In a trial by Khmelevsky and Gadalov (1980), galanthamine without atropine produced significant bradycardia in just two of 40 surgical patients. As with other cholinergic drugs, one should be alert to the possibility of cardiac complications in patients with pre-existing disease and in those suffering from Parkinson's disease (often co-morbid with AD) where other treatments may interact. Very limited evidence suggests that galanthamine may be administered with selegiline or muscarinic receptor blockers with appropriate caution (Losev and Kamenetski, 1985; Rainer, 1997) but there is no evidence to suggest that co-administration is safe with other drug classes that are likely to interact. The effects of galanthamine are likely to be additive with other anticholinesterases and cholinergics (including those with cholinergic side effects such as the non-selective mono-amine oxidase inhibitors) and the drug would be expected to antagonise and be opposed by anticholinergic drugs such as the antiparkinsonian agents. Thus, it is feasible that a centrally-acting anticholinergic drug might oppose the efficacy of galanthamine in AD, while one which acts peripherally might reduce unwanted side effects.

As with other drugs used in anaesthesia, it is important to know if galanthamine affects respiration. Cozanitis *et al.* (1972) used cine-bronchography to research the effect of galanthamine on the bronchial tree in five asthmatic volunteers. Parenteral doses of 20 mg produced no suspicious changes and blood gas composition was not altered.

The ability of galanthamine, in common with some other cholinergic drugs, to stimulate raised serum levels of cortisol was noted quite early on in the use of the drug in anaesthesiology (Cozanitis *et al.*, 1973b; Cozanitis, 1974). Typically, a single 20 mg dose of galanthamine produced a 48% increase in plasma cortisol from 0.54 to 0.8 μM/litre. No effect was observed with neostigmine, leading the authors to suggest that galanthamine was working centrally. A later study by the same group (Cozanitis *et al.*, 1980) demonstrated that galanthamine was capable of

stimulating the release of adrenocorticotrophic hormone (ACTH) in eight surgical patients. Again, neostigmine failed to produce this effect. Although a central action seems likely, the exact mechanism remains obscure.

No changes in liver function tests were observed in these early studies. Reviewing clinical trials to date, there is virtually no evidence that galanthamine has any liver toxicity.

As a result of its proposed use in patients with AD, attempts have been made to characterise the CNS toxicity of galanthamine. Because of a previous observation that galanthamine was a mild CNS stimulant (Cozanitis and Toivakka, 1971), the same authors investigated the epileptogenic potential of galanthamine in 18 epileptics controlled with phenytoin (Cozanitis *et al.*, 1973b). Six patients showed increased abnormal EEG activity with one patient showing marked increases in spike and paroxysmal wave activity; no seizures were precipitated. EEG changes indicating CNS stimulation have also been reported in cats (Kostowski and Gumulka, 1968), but a series of experiments in rabbits indicated that high doses (1 mg/kg) could suppress epileptic activity induced by instillation of penicillin into the dorsal hippocampus (Losev and Tkachenko, 1986).

Onset of REM sleep is known to be controlled by cholinergic mechanisms in the brain stem. Studies have shown that galanthamine reduces the latency of REM sleep and the overall duration of slow-wave non-REM sleep after single, 10 or 15 mg doses of galanthamine in healthy volunteers (Rieman *et al.*, 1994). The result is typical of other cholinergic agents and mimics some of the sleep abnormalities seen in major depressive disorders. The relevance of this to the use of galanthamine in AD, where patients can become clinically depressed, is unclear.

Safety data from clinical trials in Alzheimer's disease

The therapeutic activity of galanthamine, studied in a variety of clinical trials involving patients with AD, is discussed in the next chapter. Side effects were not reported uniformly and in some cases it is not clear whether they were omitted from the report or did not occur. The following side effects have been observed under clinical trial conditions.

In a small trial involving four Alzheimer's patients, who received galanthamine 15, 30 and 45 mg daily in an escalating regimen over 2–3 months, galanthamine was well-tolerated at the two lower doses, but only one patient could tolerate 45 mg/day; the others experienced agitation and insomnia (Thomsen *et al.*, 1990a). Dal-Bianco *et al.* (1991) described a preliminary study where six Alzheimer's patients were given galanthamine (30–50 mg daily) for up to 16 months without apparent adverse effects. However, this too was a small trial; larger studies show that at therapeutic doses, cholinergic effects are an annoying problem with galanthamine. They are usually mild, consisting of nausea and vomiting, which can be minimised by careful, stepwise upward titration over 1–2 weeks. Conventional antiemetics such as metoclopramide or domperidone may be useful in short courses lasting a few days.

In a placebo-controlled trial, 44 patients received galanthamine 20–50 mg/day in two or three divided doses for 10 weeks (Kewitz and Davis, 1994). The most common adverse events were nausea (16%) and vomiting (19%). Abdominal pain, diarrhoea, agitation and dizziness each occurred in 4% of patients. Kewitz *et al.*

(1994) go on to mention some patients with light-headedness, agitation and sleep disturbances (incidences were not reported), but there were no withdrawals due to adverse effects. Liver function tests and serum creatinine remained normal throughout.

DERIVATIVES OF GALANTHAMINE

As discussed previously, galanthamine is not an ideal drug; cholinergic side effects such as nausea and vomiting occur in significant number of patients at doses accepted as therapeutic. Consequently, there has been a degree of research interest in developing compounds with fewer side effects, and reports of semi- or wholly synthetic galanthamine derivatives are beginning to appear. The complete chemical synthesis of galanthamine was achieved and reported almost 40 years ago (Barton and Kirby, 1960) and an economic production process has recently been developed which should reduce the cost and guarantee a supply of parent compound with a consistent level of purity (Holton *et al.*, 1998). Bores and Kosley (1996) provide an extensive review of attempts to improve upon the safety, efficacy and pharmacokinetic profile of galanthamine through the synthesis of analogues. Preclinical assessment of the 6-ester derivatives of 6-demethylgalanthamine showed most promise, particularly the adamantyl ester (see Figure 13.1). This compound, like other 6-ester derivatives appears to act as a prodrug. After oral administration and absorption, rapid hydrolysis takes place, presumably by non-specific blood and tissue esterases, yielding the potent acetylcholine esterase inhibitor, 6-demethylgalanthamine – see Figure 13.2 (Bores *et al.*, 1996). This appears to be five times as potent as galanthamine at inhibiting mouse brain acetylcholinesterase (Han *et al.*, 1991, 1992), and compared with galanthamine it is a more selective inhibitor of acetylcholinesterase rather than butyrylcholinesterase. Galanthamine had better overall bioavailability than any of the ester analogues, including the adamantyl ester, in rats; but the adamantyl ester was judged to have the most favourable pharmacokinetics (i.e., slower onset and sustained levels of 6-demethylgalanthamine, combined with the greater potency of the latter towards acetylcholinesterase). The compound also had a higher oral therapeutic index, higher brain levels and greater activity *in vivo*, as demonstrated by greater efficacy at causing hypothermia (Bores *et al.*, 1996). The authors concluded that because of its composite profile, including duration of action, oral therapeutic index and pharmacokinetics, the adamantyl ester was the best therapeutic candidate for the treatment of AD. This hypothesis awaits confirmation from experiments with the compound in higher species and man.

PRETAZETTINE

Compounds in *Narcissus* species have joined the long line of phytochemicals that have been investigated for their anti-tumour activity. Several early reports, most of them originating from a single research group in Honolulu, mention a compound isolated from the bulbs of *Narcissus tazetta* called pretazettine (Furusawa *et al.*, 1976a). This agent is one of many that have been investigated for their ability to

inhibit protein synthesis and prevent the growth of cancer cells and viruses (see Figure 13.1).

During antiviral screening of medicinal plants from the Pacific area, Furasawa and co-workers found that the alkaloid pretazettine was active against Rauscher viral leukaemia and spontaneous T-cell leukaemia in mice (Furusawa *et al.*, 1976b, 1979). Pretazettine was inhibitory towards murine retroviruses in cell cultures and *in vivo*. The compound was shown to inhibit viral reverse transcriptase (Furasawa *et al.*, 1978), and was cytotoxic due to a specific inhibitory action on cellular protein rather than nucleic acid synthesis and therefore active against non-viral transplantable tumours. Later studies established that pretazettine was active against Ehrlich ascites carcinoma – a non-viral, transplantable tumour – in mice (Furusawa *et al.*, 1981) and that the compound enhanced the antitumour activity of a standard combination of adriamycin, 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and cyclophosphamide – the so-called ABC regimen – when used as an adjuvant to the latter. Significant survival rates were seen in inoculated animals. The authors also demonstrated that pretazettine could inhibit protein synthesis, but not nucleic acid synthesis in human carcinoma cells *in vitro*. A further interesting finding was that adriamycin pre-treated, human carcinoma cells were more sensitive to pretazettine; therefore the drug could be used at lower doses which were not inhibitory when pretazettine was used on its own. This suggested to the authors that the compound might be useful as adjuvant therapy, eradicating residual tumour cells by preventing the synthesis of proteins, notably DNA polymerase beta, necessary for DNA repair.

In addition to the above, preliminary experiments suggested that pretazettine was effective against intraperitoneally inoculated Lewis-lung carcinoma – another non-viral, transplantable tumour – in mice (Furusawa and Furusawa, 1983). Pretazettine was also studied when the inoculation was subcutaneous in singeneic mice, more resistant to chemotherapy (Furusawa and Furusawa, 1986). In the latter model, pretazettine was inhibitory to pulmonary metastases but not growth of the primary tumour or in prolonging life span. The compound was more effective against the primary tumour when combined with standard cytotoxic agents such as adriamycin, cisplatin, 5-fluorouracil, methotrexate and vincristine; these were not effective when administered individually. This indicates that pretazettine may be useful as an adjuvant to induce positive effects from otherwise inactive agents. In allogenic mice, pretazettine inhibited metastases and also prolonged life span.

The same group has also investigated the activity of pretazettine against spontaneous T-cell leukaemia in mice compared with that of some established antileukaemic agents. The activity of pretazettine was shown to be superior to methotrexate, 6-thioguanine and adriamycin, but inferior to vincristine. Combination with vincristine, 6-thioguanine and adriamycin was synergistic whereas, combination with methotrexate was not. Pretazettine was also shown to reverse the leukaemia enhancing effect of cyclosporine in this animal model at the pre-leukaemic stage, although the mechanism was unclear (Furusawa and Furusawa, 1988). The implications of this work for the treatment of similar cancers in man await confirmation by independent study.

Pretazettine has been shown to be active against selected RNA-containing flaviviruses (Japanese encephalitis, yellow fever and dengue) and bunyaviruses

(Punto Toro and Rift Valley fever) in organ culture. Activity in an animal model was not studied (Gabrielsen *et al.*, 1992). This activity may reflect a general ability to inhibit protein synthesis during viral replication.

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14 Galanthamine: clinical trials in Alzheimer's disease

David Brown

INTRODUCTION

The isolation, and subsequent chemical, pharmacological and toxicological characterisation of galanthamine, is described in the previous chapter of this volume, together with a summary of trials in indications other than Alzheimer's Disease (AD). This chapter reviews the early trials of galanthamine in AD and provides detail, where it is available, of ongoing clinical research with the compound. For a review of the animal work underpinning this application, the reader is referred to the previous chapter.

The development of galanthamine has been slow and clinically multi-faceted, but it is far from a dinosaur drug. At the time of writing, at least three pharmaceutical companies are developing the drug for the AD market on an international basis. Waldheim Pharmazeutika (Austria) has developed a method of synthesis that is feasible on an industrial scale. Shire Pharmaceuticals (England) has filed patents and is conducting extensive clinical trials with an allied company, Janssen Pharmaceutica (Belgium). The understandable reluctance of these companies to reveal unpublished data for inclusion in this review is evidence of the highly competitive atmosphere surrounding galanthamine.

BRIEF OVERVIEW OF ALZHEIMER'S DISEASE

AD is the most common form of dementia, accounting for 50–70% of all cases (Rossor, 1996) and is an important cause of morbidity and premature death in the elderly, worldwide. Typically, patients experience a slow but inexorable decline in memory and cognitive function, which eventually leads to complete dependency on family and professional carers and death, on average 4–6 years from diagnosis with a spread of 2–10 years. Early symptoms include short-term memory loss, progressing to confusion and disorientation with intermittent periods of lucidity, which often make the disease more painful to bear for patients and carers alike. As AD progresses, gross personality changes and emotional disintegration may occur. In long-standing disease, the patient may be mute, inattentive and completely incapable of self-care. Late psychiatric symptoms include hallucinations, agitation and aggression. Death commonly results from the complications of immobility such as bronchopneumonia; a definitive diagnosis of AD requires post-mortem

examination of a brain biopsy, although clinical diagnoses are frequently used to decide upon management.

It is estimated that over half a million people in the UK, 1.5 million in Japan and 4 million in the USA have AD (Office of Population Census and Surveys, 1989). This burden is likely to increase as the average age of these populations increases (Jorm *et al.*, 1991). The disease already places huge demands on health and social service budgets: for example, the UK spends in excess of £1000 million (Gray and Fenn, 1993) and the USA some 50 times this amount (Office of Technology Assessment, 1987). It is not surprising, then, that so much effort has been put into research to understand AD and to find effective therapies. Comprehension of this complex disease is far from complete and, presently, realistic treatment objectives are to minimise either the degree or rate of cognitive decline and preserve functional ability.

ANTICHOLINESTERASES IN ALZHEIMER'S DISEASE

AD is characterizsed by depletion of a number of brain neurotransmitters, including acetylcholine (Mucke, 1997; Davies, 1983). By the late 1960s, the role of acetylcholine in the formation and maintenance of memory was firmly established. Seminal experiments demonstrated that a transient AD-like syndrome could be produced in laboratory animals and man with the centrally penetrating agent, scopolamine (Gruber *et al.*, 1967; Crow and Grove-White, 1973). Furthermore, AD patients were shown to be more sensitive to cholinergic blockade than control individuals (Sunderland *et al.*, 1987). Davies and Maloney (1976) were the first to report the selective loss of cholinergic neurones in AD patients and this finding was soon corroborated (Perry *et al.*, 1978). Workers were able to localise the cholinergic deficit to those brain areas associated with cortical activation, notably the nucleus basalis, an important centre for cortical enervation (Whitehouse *et al.*, 1981). This deficit is common to the vast majority of AD patients studied. Bartus *et al.* (1982) finally advanced the cholinergic hypothesis of AD, based on accumulated experimental and clinical evidence. In this hypothesis, the primary defect is dysfunctional synthesis and secretion of acetylcholine from the pre-synapse, rather than alteration in catabolism by cholinesterase in the synaptic cleft (see Figure 14.1). Consequently, intra-synaptic levels of acetylcholine are reduced, resulting in impairment of signal transmission to cortical regions. While acknowledging the multifactorial nature of the biochemical disorders in brain chemistry in AD patients (levels of noradrenaline, dopamine, serotonin, gamma-amino butyric acid, glutamate, somatostatin and substance P may also be reduced) and the likely role of amyloid protein plaque development in its pathophysiology, the cholinergic hypothesis remains a strong one on which to base intervention strategies to slow the mental deterioration associated with the disease (Unni, 1998). The abundance of experimental and clinical data, which forms this basis, means that of all the possible mechanisms, it is the best understood. Secondary benefits which might result from cholinergic restoration include an increase in local blood flow, thus easing vascular dementia (Geaney *et al.*, 1990) and promotion of normal processing of the amyloid precursor proteins, thus reducing amyloid plaque formation (Giacobini *et al.*, 1996).

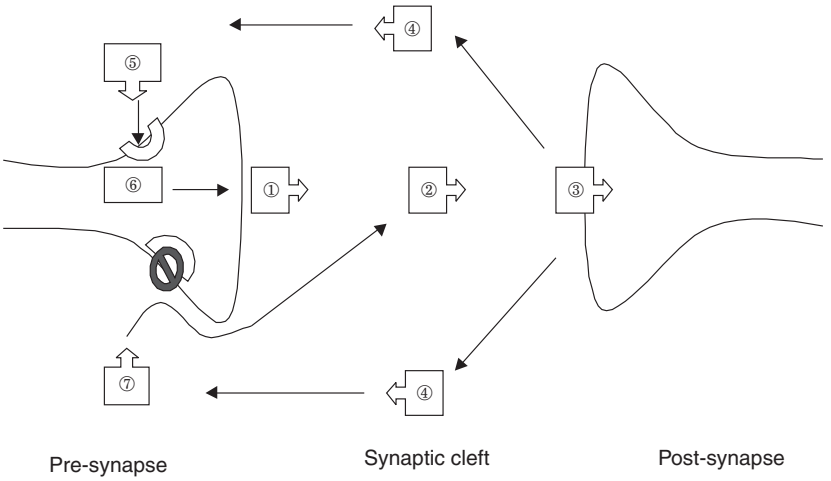

Figure 14.1 Schematic representation of the action of galanthamine.
① Acetylcholine is synthesised and released by pre-synaptic tissue and diffuses across the synaptic cleft ②.
③ In health, sufficient acetylcholine reaches post-synaptic tissue, where it interacts with receptors to ensure effective nerve transmission. In Alzheimer's disease, according to the cholinergic hypothesis, acetylcholine levels are reduced and transmission is less effective.
④ Receptor binding is reversible; released acetylcholine diffuses back into the synaptic cleft and is degraded by pre-synaptic acetylcholinesterase ⑤, producing choline ⑥, which is recycled to produce more acetylcholine.
Galanthamine ⊘ binds reversibly with acetylcholinesterase, inhibiting the catabolism of acetylcholine, which is then free to replenish and sustain more effective levels in the synaptic cleft ⑦.

Based on this hypothesis, the aim should be to restore intrasynaptic levels of acetylcholine in AD patients to those seen in health. Various ways of doing this are reviewed elsewhere (Mucke, 1997; Bartus *et al.*, 1985; Coyle *et al.*, 1983). The method which holds most promise and therefore has found most favour is to prevent catabolism of existing intrasynaptic acetlycholine by inhibiting the enzyme responsible – acetylcholinesterase. A number of anticholinesterase drugs have been investigated: physostigmine (plus two longer acting derivatives, heptylphysostigmine and phenserine), tetrahydroaminoacridine (tacrine), velnacrine, metrifonate, donepezil and rivastigmine, the last two having been licensed in the UK for AD in 1997 and 1998, respectively. None of them, including galanthamine, is ideal. For example, tacrine, a well-researched drug with which galanthamine is often compared, has met with a modicum of success, but the frequency of side effects, notably hepatotoxicity (Davies and Powchik, 1995), restricts its use and mandates special patient monitoring.

Early trials with galanthamine were at best inconclusive and of an anecdotal nature, but several centres are now working with galanthamine in thoroughly designed clinical trials, which should give us a clearer idea of the therapeutic potential of the drug in this distressing disease.

A successful cholinergic therapy for AD would have to have the following properties:

- Ability to cross the blood-brain barrier after oral or parenteral administration.
- Adequate and predictable absorbtion after oral administration.
- Unaffected by first-pass metabolism.
- High affinity for brain acetylcholinesterase.
- Ready reversibility of binding to brain acetylcholinesterase.
- Selectivity for acetylcholinesterase rather than butyrylcholinesterase (present mainly outside the central nervous system).
- A pharmacokinetic profile allowing once or twice daily dosing.
- Few side effects, and those that do occur should be well characterised and reversible on drug cessation.

Clinical studies to date indicate that galanthamine fulfils at least some of the criteria on this wish-list.

CLINICAL TRIALS WITH GALANTHAMINE

Early clinical trials

Galanthamine was evaluated in the treatment of patients with AD in a few small trials that were essentially pilot studies. Most were non-comparative, open-label, with low patient numbers making statistical interpretation difficult or impossible. The results are often available only in abstract form and interesting data, such as subjective impressions, side effects and withdrawals, are often omitted. Furthermore, the diagnostic tests used to assess response to therapy have differed from study to study, making objective comparisons difficult. Table 14.1 contains definitions of the main tests used in galanthamine trials. Table 14.2 summarises the main points of galanthamine trials reported so far. Galanthamine is commonly administered as the hydrobromide salt.

The first study to be published came from Austria (Rainer *et al.*, 1989). The trial involved ten patients with symptoms indicating Alzheimer-type dementia. Nine remained evaluable, eight females and one male; the reason for withdrawal of one patient was not given. The age range of the nine evaluable patients was 61–89 years (mean, 77 years). The mean duration of disease was 2.7 years. Patients were initiated on 15 mg galanthamine hydrobromide orally, increasing to 30 mg/day after 1 week and continuing for 7 weeks. While the pre- to post-treatment comparison did not reach statistical significance in most psychometric and neuropsychiatric tests, some improvements in clinical symptoms were observed. Patients showed improvement in some standard psychological tests of cognitive function and memory conventionally used on AD patients, notably tests of global memory performance, attention and concentration (six of nine patients) and visual-motor shape perception, visual memory and attentive concentration (six of nine patients). There was an improvement in speed but not the degree of performance in some patients. Three patients experienced improved quality of life and day-to-day performance. No adverse effects were noted.

Table 14.1 Key instruments used to assess AD symptoms in trials with galanthamine

Instrument (reference)	*Description*
MMSE (Folstein *et al.*, 1975)	Mini Mental State Examination: standardised and validated test of cognitive function, covering memory, orientation, language and praxis. On a scale of 0–30, mild to moderate cognitive impairment is indicated by a score between 26 and 10. Less than 10 implies severe impairment. Score is influenced by age, socio-economic and educational status.
ADAS-Cog (Rosen *et al.*, 1984)	Alzheimer's Disease Assessment Scale – cognitive sub-scale: standardised scale, examining aspects of cognitive performance including memory, language and praxis. On a scale of 0–70, cognitive impairment is directly related to score. Typically, patients with mild to moderate disease progress by approximately 7–11 units per year.
CIBIC-Plus (Jann, 1998)	Clinician's Interview-based Impression of Change scale: non-standardised scale assessing changes in patient performance in general, cognitive, behavioural and daily living activities; ranging from 1 – markedly improved, to 7 – markedly worse.

Around the same time, a German group (Thomsen and Kewitz, 1989; Thomsen *et al.*, 1990) reported on a 60-year-old female, suffering from advanced AD with a five-year history, who was given oral galanthamine hydrobromide in divided doses varying between 15 and 55 mg daily over a 120-day period. This included a 14-day washout period to examine the effects of drug withdrawal. Perhaps due to the advanced nature of her disease, the results of standard psychometric tests, including the widely accepted Mini Mental State Examination (MMSE) (Folstein *et al.*, 1975; see Table 14.1), showed no appreciable improvement with therapy but neither was clinical deterioration observed over the extended period. Her doctor and spouse noted subjective improvement in the patient's appearance and performance in daily tasks. Interestingly, cholinesterase inhibition in the patient's erythrocytes was monitored throughout treatment, and these improvements corresponded with high-dose phases of therapy and a cholinesterase inhibition of 50–70%. Incidentally, the technique of titrating individual galanthamine doses to achieve a target level of erythrocyte cholinesterase inhibition was used in some, but not all, subsequent clinical trials. Galanthamine appeared to be well tolerated, without side effects except for transient tachycardia, excitation and headache that appeared upon re-introduction of the drug after the washout period.

A second Austrian study (Dal-Bianco *et al.*, 1991) examined 18 patients with the diagnosis of possible AD, made according to criteria defined by the National Institute of Neurological and Communicative Disorders and Stroke with the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA or simply NINCDS criteria; McKhann *et al.*, 1984). Eighteen individuals, aged between 53 and 83 years, were given galanthamine, 30–50 mg/day for periods ranging from 2–16 months. In this rather incompletely reported study, there were no

Table 14.2 Summary of human trials with galanthamine in Alzheimer's disease

Reference	*Country*	*Patients (m = male, f = female)*	*Design/tests used*[a]	*Galanthamine daily dosage and duration*	*Major outcomes*	*Side effects*	*Comments*
Rainer *et al.*, 1989	Austria	10, but 9 remaining evaluable, with AD-type dementia. 8 f, 1 m, mean age 77 years (range 61–89)	Open label, tests unspecified	15–30 mg, for 7 weeks	No statistically significant improvements in psychometric and neuropsychometric tests. Some individual improvements in memory, attention, concentration and quality of life	None reported	Pilot study
Thomsen and Kewitz, 1989	Germany	1f with advanced AD, aged 60 years	Open label, MMSE	15–55 mg, for 120 days	Case study. No significant change in MMSE; subjective improvement in patient appearance and performance of daily tasks	Transient tachycardia, excitation, headache	Pilot study. Most improvement noted with higher doses and greater erythrocyte cholinesterase inhibition. Effects reversed during washout
Dal-Bianco *et al.*, 1991	Austria	18 with 'possible' AD (NINCDS), aged 53–83 years	Open label. Tests unspecified-'standard neurophysiological tests'	30–50 mg, for 2–16 months. 10 patients remaining at 6 months, 6 patients remaining at 12 months	No statistically significant improvements. At 12 months, the 6 remaining patients showed positive changes in everyday function and emotional stability	None reported	Pilot study. Effects of galanthamine reversed on withdrawal
Wilcock *et al.*, 1993	England	19 with probable AD (NINCDS). 7 withdrew, leaving 12 evaluable patients: 5 m (mean age 58 years; 7 f mean age 67 years)	Open label, crossover. MMSE ADAS-Cog CGIC	Low dose: 30–40 mg; high dose: 45–60 mg. 6 week treatments separated by a 3-week washout	Non-statistically significant gains in MMSE score. Significant improvements in CGIC (<1.7 points) and ADAS-Cog (<6 points) compared to baseline	7 withdrawals due to mainly gastro-intestinal side effects on high dose regimen	Pilot study. Deterioration noted during washout period. 7 stable after 9 months, 4 stable after 12 months
Rainer *et al.*, 1994	Austria	58 with mild/moderate AD	Single blind. MMSE ADAS-Cog	20–50 mg for 3 weeks	Improvements in MMSE (1.5 points) and ADAS-Cog (4.21 points) compared with baseline	Mainly anti-cholinergic: nausea, vomiting, agitation, insomnia, depression, abdominal cramps, urinary incontinence	Pilot recruitment study. Statistical analyses not provided

Berzewski *et al.*, 1994	International	141 with mild/moderate, probable AD (NINCDS)	Multicentre. Randomised, double-blind, placebo controlled after initial recruitment of responders. MMSE ADAS-Cog CGIC	Initial 3-week dose titration phase, then 20–50 mg best dose or placebo for 10 weeks	Mean improvement in ADAS-Cog of 5.14, MMSE 1.72 after 3 weeks dose adjustment phase. At 13 weeks, further improvement (1.7 points) in ADAS-Cog. Patients taking placebo deteriorated by 1.4 points. MMSE improved by 1.7 at 3 weeks and 2.5 at 13 weeks; placebo score dropped 1.7 points below baseline. Changes in CGIC described as favourable on galanthamine	Nausea and vomiting dose-related: mean dose 29.4 mg: 21%; 34.7 mg: 29%; 37.9 mg: 63%. Diarrhoea, 4%; abdominal cramps, 4%, weight loss, 1.2%, anorexia, 3%. 78% tolerated individual best dose	No significant correlation between dose and cognitive improvement
Kewitz *et al.*, 1994	Germany	95 (60–87 years) with mild/moderate primary degenerative dementia; recruited after a positive initial response under single blind conditions	Multicentre. Double-blind, placebo controlled. ADAS-Cog	20–50 mg for 10 weeks	Mean improvement in ADAS-Cog of 1.33 with galanthamine and deterioration of 0.81 points with placebo. Results not statistically significant. Significant improvement in physicians global evaluation on galanthamine	Nausea and vomiting at start of therapy. Light headedness, agitation and sleep disturbance. Blood chemistry normal	Pilot study. Number of patients on which analyses based unspecified
Wilcock and Wilkinson, 1997	England	235 with mild/moderate AD (NINCDS)	Randomised, double-blind, placebo controlled after initial 1/2 week dose adjustment. ADAS-Cog CIBIC Plus	22.5, 30.0 or 45.0 mg for 10 weeks	In patients remaining at 12 weeks, galanthamine attenuated decline in ADAS-Cog at all 3 doses. In the 30 mg/day group, difference from placebo (3.4 points) statistically, highly significant, on intention to treat basis. Positive trends in CIBIC Plus also observed	Incidence of nausea dose-related: 0, 13, 18, 35% in placebo, 22.5, 30.0 and 45.0 mg galanthamine groups. The groups had 8, 19, 12 and 38% withdrawals respectively. Adverse events occurred mainly during dose escalation. No changes in liver function tests	Interim report of on-going trial. Some evidence of dose-response correlation. Authors suggest that 30 mg/day may be the optimum, to balance side effects with efficacy
Wesnes *et al.*, 1998	England	30 with AD (diagnostic criteria not reported)	Randomised, double-blind, parallel group. Battery of attentional tests	After 4-week dose titration, either 30 or 40 mg for 12 weeks	'Significant' improvement in attentional tasks starting as early as 2 weeks	Not reported	Pilot study. Improvements reversed on stopping drug. No statistical tests reported

Notes
[a]ADAS-Cog: Alzheimer's Disease Assessment Scale – Cognitive Subscale (a positive number indicates decline and a negative number, improvement); CIBIC Plus: Clinician Interview Based Impression of Change; CGIC: Clinical Global Impression of Change; MMSE: Mini-Mental State Examination.

statistically significant improvements in non-standard neuropsychological tests of verbal, non-verbal, language, motor or attention functions at 2 months, nor at 6 months in ten patients still receiving the drug. At 12 months, six patients still taking galanthamine showed positive changes in everyday function and/or emotional stability, as noted by their carers. These patients appeared to deteriorate during drug withdrawal periods of unspecified length. No adverse events were reported. This pilot study suggested that there may be a sub-group of AD patients responding to galanthamine, whose pathology had a major component of cholinergic deficit.

In an effort to determine any relationship between dose and clinical benefit, Wilcock *et al.* (1993) treated 19 probable AD patients (as defined by NINCDS criteria) with either low dose (30–40 mg) or high dose (45–60 mg) daily doses of galanthamine for two, six-week periods, separated by a three-week washout. Seven patients withdrew due to unacceptable side effects (mainly gastrointestinal, in the high-dose group) leaving 12 evaluable patients: five males with a mean age of 58 years (range 48–74 years) and seven females of mean age 67 years (range 55–79 years). Patients were assessed by a battery of recognised tests of neuropsychological and mental function, including the MMSE and the Alzheimer's Disease Assessment Scale (ADAS, or if referring to the cognitive section alone, then ADAS-Cog, after Rosen *et al.* (1984); see Table 14.1). While a trend towards improvement was observed in several of these tests which, curiously, was most marked during the lower rather than higher dose regimen, the sole statistically significant change was an improvement in the cognitive component of the ADAS. Some deterioration was seen during the washout phase. A clinical, but statistically insignificant, improvement was recorded in the MMSE during the low-dose regimen; no clinically or statistically significant changes were observed in the Functional Life Scale, the Digit Span test or the Relatives' Stress Scale. Four patients extended from the trial on galanthamine remained stable for 12 months; three others were stable for 9 months.

These early pilot studies do provide an impression of benefit for galanthamine in AD; however, larger clinical trials were clearly needed to examine issues such as what dose to use in relation to disease severity, duration of therapy and medium to long-term side effect profile.

Later clinical trials

As with earlier work, interpretation of the results is made difficult by the nature of the publications. Most trials are reported in abstracts of symposium proceedings and, while the most important information is present, qualifying data on side effects, withdrawals, dose adjustments and assessment techniques are lacking.

Rainer *et al.* (1994) recruited 58 patients with AD who were treated with galanthamine, 20–50 mg daily for 3 weeks, during a single-blind preliminary investigation to find galanthamine responders for entry in a subsequent placebo-controlled trial. Galanthamine produced a mean decline (which represents a favourable effect) in the ADAS-Cog of 4.21 (from 32.72 to 28.51), while the MMSE score increased to 20.15 from 18.65 (a favourable result, indicating improvement in cognitive function). As with any such short-term open study, improvement might have been a practice, rather than a drug effect.

Berzewski *et al.* (1994) reported in abstract a multi-centre, international, placebo-controlled trial involving 141 patients with mild or moderate probable AD (according to NINCDS criteria) who were started on a 3-week, single blind dose titration phase of 20 mg galanthamine daily. Doses were titrated upwards in 10 mg steps, every 3 days until individual best-doses were reached, aiming for inhibition of erythrocyte cholinesterase by at least 40% of pre-therapy baseline. The upper dose limit was 50 mg/day. These patients showed a mean dose-related improvement of 5.14 ± 0.53 ADAS-Cog points, which is similar to that reported by Wilcock *et al.* (1993). Patients were then randomised to continue galanthamine at a personal best dose or switched to placebo for the next 10-week, double-blind phase. The results were encouraging. At the end of the study, those on galanthamine had improved by a further 1.66 ADAS-Cog points, but those on placebo had deteriorated by an average of 1.40 points. The MMSE scores showed a similar trend, with modest improvements during the optimisation phase (mean increase: 1.72 points) which rose to 2.50 points at the end of the trial. The placebo group dropped an average of 1.70 points below baseline. Standard neuropsychiatric tests reflecting drug effects in both cognitive and global evaluation scales showed the same favourable trend in 72% of patients treated with galanthamine – particularly the assessment of clinical global impression (effectiveness, tolerance and acceptance by the patient). Cholinergic side effects were mild, transient and dose-related. For example, the incidence of nausea and vomiting was 21, 29 and 63% in subgroups of patients taking average galanthamine doses of 29.4, 34.7 and 37.9 mg/day. Other cholinergic side effects were diarrhoea and abdominal cramps (4% each), weight loss (1.2%) and anorexia (3%). The study failed to demonstrate a statistically significant relationship between dose and cognitive improvement, although a trend was evident.

Kewitz *et al.* (1994) reported a multi-centre, placebo-controlled, double-blind trial of the safety and efficacy of galanthamine in 95 patients, aged 60–87 years, with mild to moderate primary degenerative dementia. These patients had demonstrated a primary response to galanthamine under single blind conditions. Galanthamine was started at 10 mg twice a day and was increased to a maximum of 50 mg/day during the 3 weeks of dose optimisation. While there were no significant changes between performance on placebo and galanthamine in terms of performance on the ADAS-Cog scale (–0.81 for placebo, +1.33 for galanthamine), there was a significant improvement in the physicians' global evaluation demonstrating significantly less deterioration in patients receiving galanthamine. Nausea and vomiting were the most common side effects, occurring usually at the start of therapy and tending to resolve with continued treatment. Light-headedness, agitation and sleep disturbances were also noted. No alterations in blood chemistry or liver function were noted. Some withdrawals due to gastrointestinal symptoms were noted, but these were not quantified in this abstract.

Wilcock and Wilkinson (1997) reported the interim results of an on-going, randomised, double-blind study. After an initial adjustment phase lasting up to 14 days, 235 patients with mild to moderate AD, as judged by the NINCDS criteria, received placebo or 22.5, 30.0 or 45.0 mg/day of galanthamine, in three divided doses with food. Therapeutic effects were measured by the ADAS-Cog scale and the CIBIC Plus scale (global Clinician Interview Based Impression of Change

(Jann, 1998); see Table 14.1). Adverse events were recorded and haematological and biochemical parameters were monitored. Patients were assessed at baseline and at 6 and 12 weeks during treatment. An analysis of patients remaining in the trial at 12 weeks showed that the cognitive performance of placebo-treated patients had deteriorated during the treatment phase, but this was attenuated in all the galanthamine groups, with mean ADAS-Cog scores one or two points greater than zero. A trend in relationship to dose was apparent, although no statistical analysis of this was reported. The differences between the placebo and active scores for the 30 mg and 45 mg/day groups were 3.8 and 3.9 points, respectively. Positive trends on the CIBIC Plus scale were also associated with galanthamine. On an intention-to-treat basis, the 30 mg/day dosage produced a statistically significant rise in ADAS-Cog score of 3.4 points from placebo. Side effects were mostly related to the cholinergic action of the drug, and occurred during the initial dose adjustment phase. The incidence of nausea was 0, 13, 18 and 35% in the placebo, 22.5, 30 and 45 mg/day groups, respectively; the corresponding incidences for vomiting were 6, 19, 7 and 17%, respectively. Overall, galanthamine was described as being well tolerated, although 38% of patients in the 45 mg/day regimen (compared with 8, 19 and 12% of patients taking placebo, 22.5 mg or 30 mg doses, respectively) withdrew due to cholinergic side effects, mainly during the dose-escalation phase. This is an indication of the likely maximum dose at which galanthamine may be tolerated or, at least, a warning that if such high doses are to be used, a more cautious dose escalation technique should be employed. No abnormalities in liver function tests were observed. The authors concluded that patients received optimal benefit from 30 mg/day, and that a longer titration period to achieve this dosage might avoid the cholinergic symptoms of more rapid initiation.

Wesnes *et al.* (1998) published a brief report focusing on the effect that galanthamine might have on the attention deficit seen in AD patients. They described a double-blind, parallel group trial in 30 patients randomised to receive either 30 or 40 mg/day of galanthamine for 12 weeks, following a dose titration period of 4 weeks. Attention deficit was measured using an attentional sub-battery of tests of cognition. No statistical analysis was reported, but improvements described as significant were observed in all three attention tests used, starting early on in therapy, increasing with time, but which rapidly declined on stopping galanthamine. The attention tests used in this trial were not part of the more usual ADAS-Cog test battery, and the authors pointed out that the tests they used may provide important additional information on the use of drugs for AD.

Longitudinal studies

Patients with AH may live for many years and clearly, the long-term efficacy and safety of a therapeutic agent used in the disease is of great importance. As yet, few data are available for galanthamine. Recently, the interim results from an open, 3-year follow-up evaluation of patients using galanthamine have been published (Rainer and Mucke, 1996). Patients receiving galanthamine with or without additional non-anticholineterase therapy (data reported on 21), such as antidepressants and nootropics, showed a significantly lower rate of cognitive decline than those taking placebo (data reported on 23), as shown by the mean ADAS-Cog scores. The degree of cognitive stabilisation achieved during the first year was a

good indicator of 3-year outcome. No clinically significant disturbances in blood biochemistry or liver function tests were observed.

SUMMARY

The results of early trials of galanthamine compare well with those of later studies, but one should remember that today's criteria for classification of patients with AD are much more precise. It has been estimated that early inclusion criteria may have been only 85% specific for AD (Wade *et al.*, 1987). Most of the early studies reported here are really no more than pilots, and leave much to be desired; however, they were conducted by three, independent research groups and the results attracted sufficient attention to prime the planning of more extensive and sophisticated trials. Such trials are at present ongoing and results have not yet been published.

One reason why the anticholinesterase approach is only partially successful in AD is the multifactorial nature of the neuropharmacological deficits seen in the disease (Perry, 1987). As AD appears to be a disorder involving deficits in a number of neurotransmitters, it seems logical to assume, and on the basis of the evidence, correct, that the strategy of acetylcholine preservation slows the progression of the disease but does not halt it. Secondly, a multifactorial approach, involving several pharamcological interventions at once (for example, combined strategies to boost levels of noradrenaline, serotonin, gamma-aminobutyric acid, somatostain and corticotrophin releasing factor, all of which have been shown to be deficient in AD), may hold more promise.

From the trial data so far, it would appear that there is a sub-group of patients who respond well to galanthamine, and in whom the cholinergic deficit is a major component of their disease.

The side effect profile of galanthamine appears favourable, particularly when compared with tacrine, where hepatotoxicity is a particular worry (Knapp *et al.*, 1994; Wagstaff and McTavish, 1994) and metrifonate, where clinical trials have recently been suspended because of concerns about muscle weakness (personal communication, Bayer UK). Anticholinergic side effects, particularly nausea and vomiting, may be a problem, and measures to minimise these, such as slow upward titration of the initial dose and co-administration of antiemetics, should be investigated further.

Galanthamine is approved by the Austrian authorities for use in AD. However, there remains at present a lack of controlled, double-blind trials of sufficient quality and length to define clearly the role of this interesting agent. Head-to-head comparisons with other agents such as tacrine have not been performed, and more information is also required on long-term use, dose response, use in old age (where renal and hepatic impairment are common), and potential interactions with other drugs used in co-morbid conditions (such as Parkinson's disease, agitation, anxiety, aggression and depression).

In terms of pharmaceutical development, a once-daily dosage form would be a useful advance, having obvious applications in elderly or forgetful patients and at least one transdermal, controlled release patch is under development (Morierty, 1995). An alternative is to synthesise galanthamine analogues with extended half-life and improved efficacy.

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15 Screening of Amaryllidaceae for biological activities: acetylcholinesterase inhibitors in *Narcissus*

Kornkanok Ingkaninan, Hubertus Irth and Rob Verpoorte

INTRODUCTION

Narcissus are well-known cultivated plants belonging to the Amaryllidaceae family. Tyler *et al.* (1988) classified them as poisonous plants, since ingestion of narcissus bulbs produces severe gastroenteritis and nervous symptoms. Narcissus plants contain similar alkaloids which can also be found in other members of the Amaryllidaceae. The Amaryllidaceae alkaloids have been shown to possess a wide spectrum of biological activities such as anticholinergic and analgesic (Harvey, 1995), antimalarial (Likhitwitayawuid *et al.*, 1993), antiviral (Gabrielsen *et al.*, 1992; Lewis, 1996) and anti-neoplastic (Jimenez *et al.*, 1976; Ghosal *et al.*, 1988; Pettit *et al.*, 1990, 1993). However, galanthamine is so far the only Amaryllidaceae alkaloid that has gained a wide spread commercial application because of its inhibitory activity for acetylcholinesterase (AChE).

GALANTHAMINE

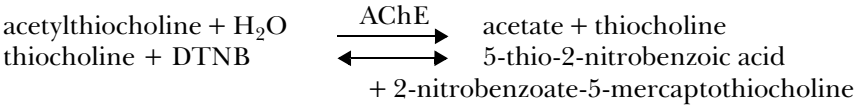
Galanthamine is a tertiary amine alkaloid belonging to the phenanthrene group. It was first isolated from bulbs of Caucasian snowdrop, *Galanthus woronowi*, in 1952 (Proskurnina and Yakovleva, 1952). It has also been found in various species of *Narcissus* (Paskov, 1986; Bastida *et al.*, 1990). The compound was first applied for medical purpose by Bulgarian and Russian researchers in the 1950s. In 1955, Mashkovskii reported that galanthamine could reverse tubocurarine-induced muscle paralysis and potentiate the actions of acetylcholine on skeletal muscles. It was then used as a potent natural cholinergic substance showing strong AChE inhibitory activity in both the central and peripheral system, and for the reversal of the neuromuscular blockade caused by various curare-like agents (Irwin and Smith, 1960; Cozanitis, 1971; Baraka and Cozanitis, 1973; Cozanitis and Rosenberg, 1974; Krivoi, 1988). This drug has been available for clinical use as Nivalin® (Pharmachim, Sofia, Bulgaria) and Galanthamine® (Medexport, Moscow, USSR). It was not until 1986, after the cholinergic hypothesis of Alzheimer's disease was

postulated, that the application of galanthamine in Alzheimer's disease was studied. Based on the cholinergic hypothesis (Perry, 1986), memory impairments in patients suffering from this disease result from a defect in the cholinergic system. One approach to the treatment for this disease is to enhance the acetylcholine level in the brain by AChE inhibitors (Winblad *et al.*, 1993). Galanthamine hydrobromide is now being developed for Alzheimer's disease. It has shown clear evidence for being suitable for the treatment of mild and moderate Alzheimer's disease. At least three corporate pharmaceutical companies are involved in developing galanthamine for the Alzheimer's disease market: Waldheim Pharmazeutika (Austria) for developing a method of synthesis, Shire Pharmaceuticals (UK) for clinical studies, and Janssen Pharmaceutica (Belgium) for final registration and marketing. The preclinical and clinical studies of galanthamine were reviewed by Mucke (1997a,b) and Rainer (1997), respectively.

Galanthamine is commercially isolated from *Leucojum aestivum* (Amaryllidaceae) (Paskov, 1986). Although the chemical synthesis of galanthamine was accomplished in 1960 (Barton and Kirby, 1960), it was only very recently that an industrial scale synthesis of galanthamine hydrobromide was established (Czollner *et al.*, 1996). Several analytical techniques have been described to quantify galanthamine in natural sources. Kreh *et al.* (1995) and Bastos *et al.* (1996) reported capillary column gas chromatographic methods for quantification and identification of galanthamine and other Amaryllidaceae alkaloids. A radioimmunoassay developed by Tanahashi *et al.* (1990) provided specific and precise quantitation of galanthamine in unpurified plant extracts. Three years later, Poulev *et al.* (1993) established a more sensitive enzyme immunoassay for the quantitation of fmol amounts of galanthamine.

SCREENING ASSAYS FOR ACETYLCHOLINESTERASE INHIBITORS

Several assays have been established for the detection of AChE activity and its inhibitors. The colorimetric method developed by Ellman *et al.* (1961) is the most widely used. This assay is based on the enzymatic hydrolysis of acetylthiocholine iodide (ATCI) to yield thiocholine. When thiocholine reacts with 5,5′-dithiobis-2-nitrobenzoate (DTNB), it will produce the yellow product of 5-thio-2-nitrobenzoic acid which can be detected at 405 nm by a spectrometer.



This spectrometric assay has been used for the screening of plant extracts for anti-AChE activity (Park *et al.*, 1996; Kim *et al.*, 1999). The use of a 96-well plate reader for measuring the AChE activity, reported by Ashour *et al.* (1987), allowed the rapid screening of series of samples.

A radiometric technique for the detection of AChE activity based on hydrolysis of [^{3}H] labelled acetylcholine was reported by Johnson and Russell (1975). Guilarte *et al.* (1983) developed a simpler method using [^{14}C]sodium bicarbonate. The acetic acid formed from the enzymatic hydrolysis of acetylcholine reacts with

[^{14}C]sodium bicarbonate to generate [^{14}C]carbon dioxide, which is measured using an ionisation chamber system. Compared with Ellman's colorimetric method, these radiometric techniques are more sensitive. However, the radiometric method is an endpoint measurement and cannot be used for kinetic studies. Furthermore, the limited linear range of the method demands the proper calibration of the activity prior to incubation.

A fluorometric method has also been developed based on the reaction of thiocholine produced by the enzyme from acetylthiocholine with the fluorogenic compound *N*-(4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl) maleimide (CPM) (Parvari *et al.*, 1983). This assay combines the specificity and the technical advantages of the Ellman technique with a wide linear range of accuracy and a limit of detection which is 100-fold lower than that of the radiometric method. Roger *et al.* (1991) developed the optical sensor for anti-AChE using immobilised fluorescein isothiocyanate (FITC)-tagged eel electric organ AChE on quartz fibres for monitoring enzyme activity. This biosensor is reusable, sensitive and easy to operate. It shows potential adaptability to field use as the instrument is portable.

Recently, Pasini *et al.* (1998) reported a chemiluminescent (CL) method for a 384-well microtiter format assay for high throughput screening of AChE inhibitors. The CL detection of AChE was based on coupled enzymatic reactions involving choline oxidase and horseradish peroxidase as the indicator enzymes. The reaction leads to light emission and luminol is used for the detection of the peroxide formed. This method had a detection limit 1000-fold lower than that of the colorimetric method.

THE SEARCH FOR AChE INHIBITORS FROM NATURAL SOURCES

Nature is a rich source of biological and chemical diversity. It has been shown many times that natural products could be developed as drug candidates. The unique and complex structures of natural products such as paclitaxel cannot be obtained easily by chemical synthesis. The well-known AChE inhibitors, galanthamine and physostigmine, are also obtained from plants. The clinical studies of AChE inhibitors for Alzheimer's disease are still ongoing. Until now, no drug of choice has been decided upon. Therefore, the search for new AChE inhibitors from natural sources is of great interest. However, in searching for new leads from crude natural products extracts, one might encounter the problem of isolation of known active compounds or compounds that give aspecific effects on the bioassays used. A strategy of 'dereplication' of active compounds, or rapid identification of known active compounds at an early stage, is important in the lead finding process. One of the approaches for dereplication is to couple a separation technique with a bioactivity detection method. The known active compounds can be rapidly identified by the aid of data analysis and the unknown active fraction can be further investigated.

Narcissus plants are a potential source for AChE inhibitors as they contain a variety of compounds, especially alkaloids, the group of compounds that shows many biological activities including AChE inhibitory effect. Since narcissus are cultivated plants, there could be a large variation of chemical constituents among different cultivars. Figure 15.1 shows the inhibitory effect of the methanol extracts

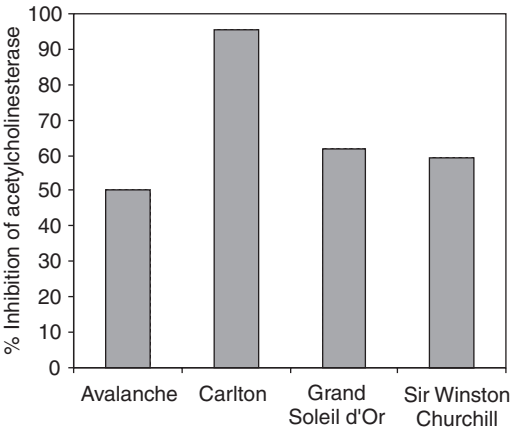


Figure 15.1 Percentage of inhibition effect of methanol extracts from some narcissus cultivars at the concentration of 0.1 mg/ml measured with the microplate assay.

from some narcissus cultivars tested in our laboratory. The microtiter plate assay used was according to the method of Ellman *et al.* (1961), as described by Ingkaninan *et al.* (2000a). All extracts showed an inhibitory activity for AChE. However, the activity might be due to the known active compound, galanthamine. A dereplication step for the rapid identification of galanthamine in crude extracts is necessary. The extracts also contain other unknown, active compounds that will be selected for further studies.

IDENTIFICATION OF AChE INHIBITORS BY HPLC WITH ON-LINE COUPLED UV, MS AND BIOCHEMICAL DETECTION

Recently, strategies for the on-line coupling of biochemical detection to separation methods such as high-performance liquid chromatography (HPLC) have been developed (Irth *et al.*, 1995). This technique allows the simultaneous separation and detection of bioactive compounds from complex mixtures such as natural products. To obtain additional information on the compounds separated, the on-line system can also be coupled with other detection methods such as mass spectrometry (MS) and ultraviolet (UV) or photodiode array (PDA) detection. In a natural products-based drug discovery programme, this technique can be useful for the detection of new active compounds in the presence of the already known active compounds. It opens the possibility for the rapid screening of galanthamine-containing plants for other AChE inhibitors.

The colorimetric assay for AChE as reported by Ellman *et al.* (1961) is suitable for development into a continuous-flow biochemical detection system. AChE from electric eels is inexpensive and has satisfactory stability at room temperature. The assay reagents, DTNB and ATCI, are also commercially available. The short reaction time of the assay, approximately 2 min, made it possible to develop it for continuous-flow biochemical detection system without causing much band-broadening.

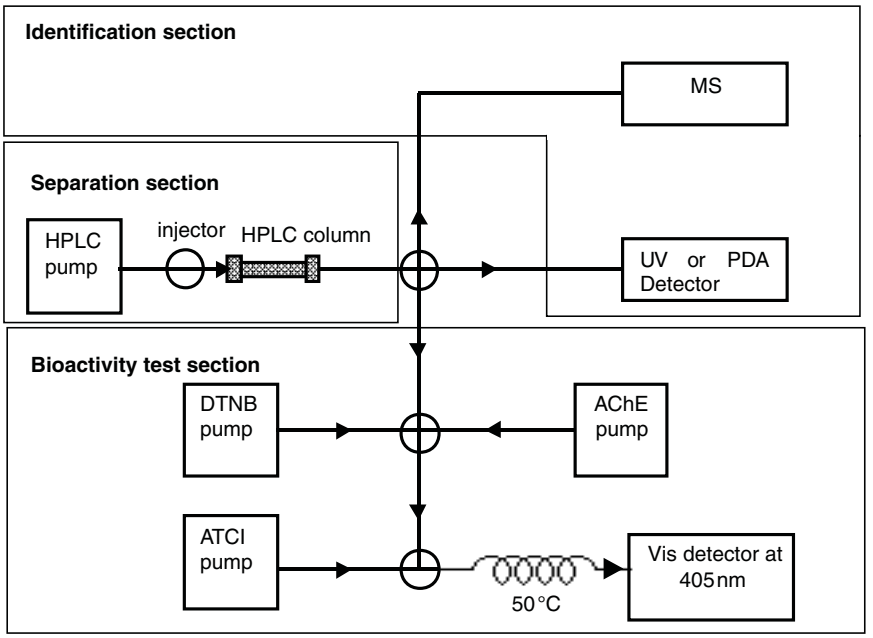


Figure 15.2 Scheme of the on-line HPLC-UV-MS-bioactivity detection for acetylcholinesterase inhibitors.

Figure 15.2 shows the scheme of the on-line HPLC-UV-MS-biochemical detection system developed in our laboratory (Ingkaninan *et al.*, 2000a). The separation is performed in an HPLC column. The eluate is split into three flows: the major flow travels to a UV detector, while two minor flows go to the MS and the biochemical detection system. In the biochemical detection system, the eluate is mixed with substrate (ATCI), enzyme (AChE) and DTNB for approximately 2 min in the reaction coil. A spectrometer set at 405 nm detects the yellow product obtained from the enzymatic reaction. Any inhibitory activity from the HPLC eluate will result in a negative peak on the biochemical detector. After determination of the delay times of the three detection lines, the results of these detections can be related and thus UV spectra and molecular weight of the active compounds can be determined. In this way, known inhibitors such as galanthamine can easily be recognised.

The strongly AChE inhibiting extract from narcissus 'Carlton' (Figure 15.1) was fractionated by means of centrifugal partition chromatography (CPC) and the active fraction was injected into the on-line HPLC-UV-MS-biochemical detection system (Ingkaninan *et al.*, 2000a). The chromatogram from two detectors and the mass spectra are shown in Figure 15.3. Although the chromatogram from the HPLC is very complex, the biochemical detection system showed a high selectivity for the AChE inhibitors. Two broad negative peaks from the biochemical detector proved the presence of at least two inhibitors in the extract. The retention time of the main active peak and the molecular weight derived from MS data ([M + H] of 288) corresponded to those of galanthamine, a well-known AChE inhibitor.

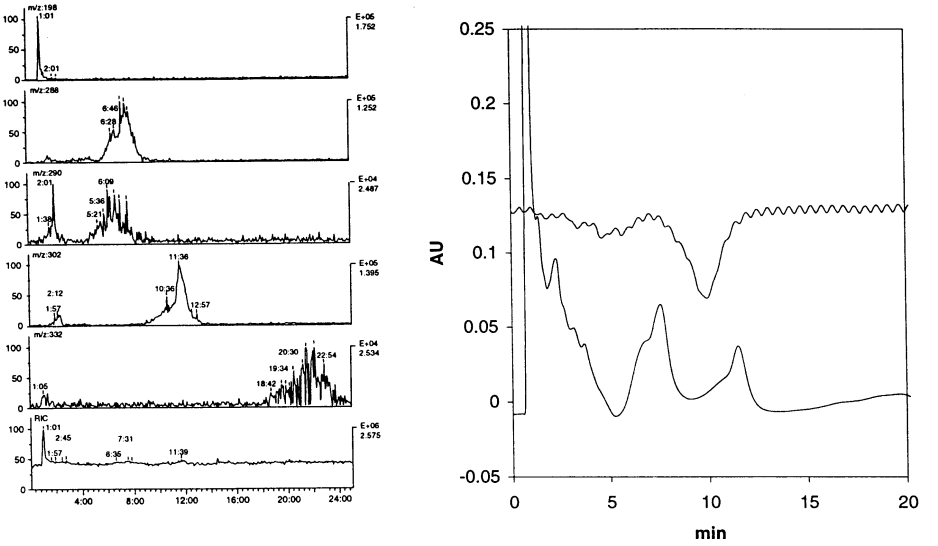


Figure 15.3 The spectra and the chromatograms obtained after injection of the fraction from narcissus 'Carlton' extract into the on-line HPLC-UV-MS-bioactivity detection system. Left: ESI-MS. Right: Chromatograms from the biochemical detection set at 405 nm (upper line) and from the UV set at 215 nm (lower line). The delay time of the peaks between the biochemical detection and the UV was 2.3 ± 0.1 min and the delay time of the peaks between the biochemical detector and the MS was 2.1 ± 0.1 min.

However, MS showed that there was another molecule present in the same peak at [M + H] of 290. It would be interesting to identify this compound further and test it for inhibitory effect. The minor peak in the chromatogram detected by the biochemical detector was caused by an unknown AChE inhibitor. Further studies should, therefore, focus on the isolation and identification of this active compound.

A second example is a study of the extract from narcissus 'Sir Winston Churchill' (Ingkaninan *et al.*, 2000b). From the microplate assay, this extract showed much less activity than that of narcissus 'Carlton' (Figure 15.1). The extract was fractionated by the same CPC procedure as that of narcissus 'Carlton'. The active fraction was also obtained at the same retention. However, when the active fraction was injected into the on-line system, no activity corresponding to galanthamine was found. The activity was from another compound with a different molecular weight and retention time. As this extract possibly contains new AChE inhibitors, it was chosen for further investigation. The active fraction was further separated by another CPC run and the fractions obtained were injected into the on-line system (Figure 15.4).

The active peak from the biochemical detector at 15.2 min corresponded to the UV peak at 12.9 min and the MS peak at 13.1 min. However, MS spectra showed that there were two compounds present at 13.1 min having molecular weights of 265 ([M + H] of 266) and 317 ([M + H] of 318). These two compounds were isolated by preparative HPLC and tested for AChE inhibitory activity by the

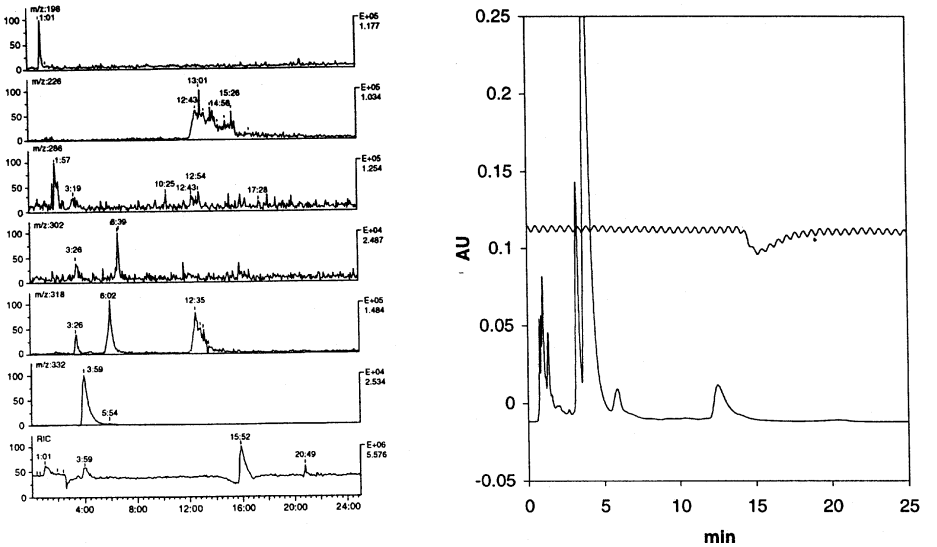


Figure 15.4 The spectra and the chromatograms obtained after injection of the fraction from narcissus 'Sir Winston Churchill' extract into the on-line HPLC-UV-MS-bioactivity detection system. Left: ESI-MS. Right: Chromatograms from the biochemical detection set at 405 nm (upper line) and from the UV set at 215 nm (lower line). The delay time of the peaks between the biochemical detection and the UV was 2.3 ± 0.1 min and the delay time of the peaks between the biochemical detector and the MS was 2.1 ± 0.1 min.

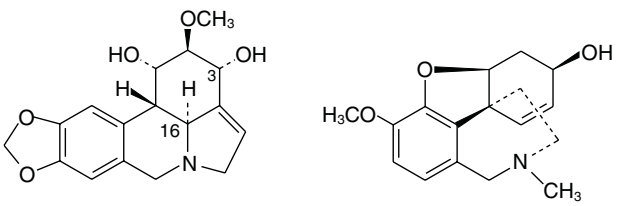


Figure 15.5 Structure of ungiminorine and galanthamine.

microplate assay. The inhibitory activity was found only in the compound with molecular weight of 265. This active compound was identified as an alkaloid, ungiminorine (Figure 15.5). This compound was first isolated by Normatov *et al.* (1965). The IC_{50} value of ungiminorine was 86 ± 7 μM while that of galanthamine was 0.98 ± 0.07 μM. Although this compound shows much lower AChE inhibitory activity compared with that of galanthamine, it clearly demonstrates the usefulness of the on-line HPLC-UV-MS-biochemical detection for the search of new leads from natural products.

FUTURE PERSPECTIVES

It is to be noted that in the on-line HPLC-UV-MS-biochemical detection, only a small amount, i.e., one-thirtieth of the eluate from the HPLC, was split to the biochemical detector. Therefore, it is possible to apply the on-line system for a preparative separation technique such as preparative HPLC or CPC and on-line fraction collection can be performed. In both separation techniques, gradient elution might be useful for broad range separation. Furthermore, to gain more information for the identification of active peaks, a PDA and an NMR can be coupled to this on-line system.

Although the Ellman colorimetric method used in this on-line biochemical detection system is not the most sensitive, it is the simplest and the most inexpensive screening method for AChE inhibitors. In natural products-based research, where milligrams of new leads are still needed for the structure elucidation, selectivity for the bioactive compounds is more important than sensitivity. However, the sensitivity of the on-line biochemical detection could be increased by using a fluorometric method. Some of the fluorescent probes such as *N*-[4-[7-(diethylamino)-4-metylcoumarin-3-yl]phenyl]maleimide (CPM), which forms a fluorescent product when it reacts with thiocholine (Berman and Leonard, 1990) or the substrate, 7-acetoxy-*N*-metylquinolinium iodide (Menger and Johnston, 1991), have been used in an ultrasensitive assay for acetylcholinesterase and are commercially available. These fluorescent probes could be further developed for the on-line biochemical detection system.

Another method that could be useful for the screening of AChE inhibitors from natural products is thin-layer chromatography (TLC). Some TLC methods using an enzyme, a substrate and a dye as the spraying reagent have been reported (Bunyan, 1964; Menn and McBain, 1966; Kiely *et al.*, 1991). The inhibition zone from the TLC indicates the inhibitory activity of the test compound to the enzyme. When complex mixtures such as crude plant extracts are tested, the presence of known inhibitors in the extract will be rapidly identified. This technique can be helpful in the selection of extracts to be further investigated for new AChE inhibitors.

In conclusion, narcissus cultivars are potential sources for AChE inhibitors. As these plants commonly contain the known active compound galanthamine, the dereplication step of galanthamine is crucial. This step can be done by using the on-line HPLC-UV-MS-biochemical detection that has already been developed, or by other techniques that could lead to the rapid separation and identification of the active compounds from complex mixtures. From our studies, it is clear that screening on the level of cultivars using such methodology may result in new compounds with AChE inhibitory activity.

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16 *Narcissus* lectins

Els J.M. Van Damme and Willy J. Peumans

INTRODUCTION

Many plants accumulate so-called 'lectins', 'agglutinins' or 'haemagglutinins' in their seeds and vegetative tissues. Lectins can be defined as carbohydrate-binding proteins which recognise and bind specifically and reversibly to certain mono- or oligosaccharides without altering the structure of the bound ligand (Peumans and Van Damme, 1995; Van Damme *et al.*, 1998a). In this way lectins are also able to interact with glycans of different glycoconjugates such as glycoproteins, glycolipids and oligo- or polysaccharides.

Since the discovery of the first lectin in extracts from castor beans by Stillmark (1888), numerous agglutinins have been detected. The rapid progress made in biochemical research, and especially the development of very efficient and highly specific affinity chromatography techniques for the purification of lectins, has resulted in the isolation of a steadily increasing number of agglutinins from varying sources. At present over two hundred plant lectins have been isolated and characterised in some detail with respect to their molecular structure, biochemical properties and carbohydrate-binding specificity. Evidence has accumulated that lectins occur in seeds as well as in vegetative tissues of many plant species, and are widespread in a large number of plant families belonging to all major taxonomic groups (Van Damme *et al.*, 1998b). However, because of the obvious differences in molecular structure and sugar specificity between the plant lectins that have been characterised thus far, one has to conclude that plant lectins have only a single common property, namely their ability to recognise and bind to carbohydrates.

Until recently, plant lectins have been considered as a very heterogeneous group of proteins widely differing from each other with respect to their biochemical properties and biological activities. Poor insight into taxonomic relationships within the heterogeneous group of plant lectins was mainly due to the lack of detailed sequence information. Therefore, the rapid progress in molecular cloning and structural analysis of numerous lectins and lectin genes during the last fifteen years provided, a powerful means to re-address the question of the classification of plant lectins. Based on the available sequence data seven distinct families of structurally and evolutionary related proteins are distinguished, which comprise the great majority of all currently known lectins. These seven lectin families are the legume lectins, the chitin-binding lectins containing hevein domains, the monocot mannose-binding lectins, the type 2 ribosome-inactivating proteins, the amaranthins, the jacalin-related lectins and the Cucurbitaceae phloem lectins (Van Damme *et al.*, 1998a).

This chapter will focus on the lectins present in different species and cultivars of *Narcissus* (daffodil). As will be shown below, narcissus lectin has been studied in detail and is shown to be a typical representative of the family of monocot mannose-binding lectins. After a brief description of the occurrence of the lectin in different *Narcissus* species and cultivars and the purification of the lectins therefrom, the most important findings on the molecular characteristics, carbohydrate-binding properties and biological activities of the narcissus lectin will be summarised. Furthermore, we will elaborate on the possible function of the lectin *in planta* as well as on possible applications of the lectin in biomedical and glycoconjugate research. Finally, a comparison of the narcissus lectin to other known plant lectins will be made.

OCCURRENCE OF LECTINS IN DIFFERENT *NARCISSUS* SPECIES AND CULTIVARS

The occurrence, isolation and characterisation of a lectin in bulbs of *Narcissus* was first described in 1988 (Van Damme *et al.*, 1988). Since then lectin activity has been detected in more than 25 species and varieties of *Narcissus* (Table 16.1; Van Damme and Peumans, 1990a).

Table 16.1 Narcissus Species and Cultivars with Lectin Activity[a]

Botanical species, etc	
Narcissus × *medioluteus (N. biflorus)*	
N. bulbocodium subsp. *bulbocodium* var. *conspicuus*	
N. jonquilla	
N. nanus (N. lobularis)	
N. × *maclaeyi*	
N. moschatus	
N. nanus	
N. obvallaris	
N. poeticus var. *physaloides*	
N. pumilus	
N. poeticus var. *recurvus*	
N. tazetta	
Cultivars derived wholly or partly from:	
N. poeticus:	'Glory of Lisse'
	'Horace'
	'Ornatus' (*N. ornatus*)
N. cyclamineus:	'Bartley'
	'Peeping Tom'
N. jonquilla:	'Bobbysoxer'
	'Rugulosus' (*N. odorus rugulosus*)
N. pseudonarcissus:	'Carlton'
	'Codlins and Cream'
	'Colleen Bawn'
	'Empress'
	'Queen of Spain'
	'W.P. Milner'
Other:	'Double Campernelle'

Note
[a]Van Damme and Peumans (1990a); names in parenthesis are synonyms.

PURIFICATION OF *NARCISSUS* LECTINS

Since preliminary experiments with crude extracts from *Narcissus* indicated that they contained an agglutinating factor that can be inhibited only by mannose, a purification scheme based on affinity chromatography on immobilised mannose was developed to purify the lectin. Although the affinity purified lectin was virtually pure, as could be judged from sodium dodecyl sulphate – polyacryl amide gel electrophoresis (SDS-PAGE), additional hydrophobic interactions chromatography and ion exchange chromatography were included to ensure complete purity of the lectin (Van Damme *et al.*, 1988). The same protocol was used to purify the lectin from different species and cultivars of *Narcissus*. Furthermore, the protocol was also applicable for the purification of the lectin from different tissues of narcissus, e.g., bulbs, leaves and ovaries.

CHARACTERISATION OF *NARCISSUS* LECTINS

Molecular structure

The molecular structure of narcissus lectin was determined using SDS-PAGE, gel filtration and ultracentrifugation. Upon SDS-PAGE the lectins isolated from all *Narcissus* species and cultivars yielded a single polypeptide band of 12.5 kDa. Since the results were identical when electrophoresis was conducted either in the presence or absence of β-mercaptoethanol, it can be concluded that the lectin subunits are not held together by disulphide bonds (Van Damme *et al.*, 1988).

Gel filtration experiments on a Superose 12 column using a phosphate buffer containing 0.2 M mannose (to avoid binding of the lectin to the matrix) demonstrated that the lectins isolated from narcissus elute with an apparent molecular mass of 25 kDa, indicating that they are probably dimers. Because aspecific interactions of lectins with the gel filtration matrix often occur and cannot be abolished completely by the addition of the specific sugar to the running buffer, the molecular mass was also determined by ultracentrifugation of the lectins. Since the molecular mass of the lectin was calculated to be 36 kDa after centrifugation, the lectin may be a trimer (Van Damme *et al.*, 1995).

It should be indicated here that all lectins isolated from different species and cultivars of *Narcissus* show the same molecular structure and biochemical characteristics (E. Van Damme, unpublished data).

Detailed analyses of the narcissus lectins have shown that the amino acid composition of these lectins is typified by high contents of asparagine/aspartic acid, threonine, glycine, serine, glutamine/glutamic acid and leucine. The lectins contained no amino sugar and only low levels of neutral sugars, most probably contaminants, indicating that the lectins are not glycosylated (Van Damme *et al.*, 1988, 1991a).

Narcissus lectins are complex mixtures of isolectins

When the purified lectins from different *Narcissus* species were analysed by high resolution ion exchange chromatography it became evident that they all yielded a

very complex elution pattern, indicating that the lectin preparations are mixtures of isoforms (Van Damme *et al.*, 1988; Van Damme and Peumans, 1990a). The occurrence of multiple isolectins was confirmed by isoelectric focusing, where the purified lectins yielded an extremely complex pattern of polypeptide bands of different intensity, indicating that many isolectins were present, some in higher concentrations than others. Molecular cloning and sequence analysis of different cDNA clones encoding the lectin from *Narcissus* 'Fortune', combined with Southern blot analysis, further revealed that the different isoforms were encoded by different genes which differed slightly from each other in their sequences (see below; Van Damme *et al.*, 1992).

A detailed study of the isolectin composition in different species and cultivars of *Narcissus* revealed pronounced inter- and intraspecies differences in the isolectin patterns. Furthermore, analyses of lectin preparations isolated from different tissues at different developmental stages indicated that the isolectin composition is both tissue-specific and developmentally regulated. Finally, it was shown that related cultivars show similar isolectin patterns (Van Damme and Peumans, 1990a).

Developmental regulation of lectin concentration

A detailed study of the developmental changes and tissue distribution of the lectin in *Narcissus* 'Carlton' using a very sensitive enzyme-linked immunosorbent assay (ELISA) has shown that the lectin occurs in almost all plant tissues, where it is present in a very high concentration at the beginning of the growing season (Van Damme and Peumans, 1990b).

In the bulb, the lectin accounts for 10 to 15% of the total tissue protein during the dormant phase. However, as the shoot starts to grow the lectin concentration in the bulb rapidly decreases. At flowering time almost all lectin has disappeared from the bulb. By the end of the growing season the outer bulb scales have been degraded. By this time new bulb units have been formed inside the bulb, and by the end of the growing season they accumulate high concentrations of lectin as they expand.

High lectin concentrations are also found in the aerial plant parts of narcissus. However, the lectin concentrations in the aerial parts are about one order of magnitude lower than in the bulb. As the shoot emerges from the bulb, lectin concentrations in leaves, stems and flower parts gradually decrease. By flowering time almost all lectin has disappeared from these tissues.

Molecular cloning of *Narcissus* lectin

In order to clone the narcissus lectin a cDNA library was constructed from poly(A)-rich RNA isolated from young developing ovaries, which are known to contain high concentrations of lectin. Screening of the cDNA library constructed with mRNA isolated from *Narcissus* 'Fortune', using a previously isolated cDNA encoding snowdrop (*Galanthus nivalis*) lectin (Van Damme *et al.*, 1991b), resulted in the isolation of multiple lectin cDNA clones (Van Damme *et al.*, 1992). Although the lectin clones showed a high degree of overall sequence homology within their coding region, they clearly differed from each other in their nucleotide sequences and deduced amino acid sequences. All cDNA sequences contained an open

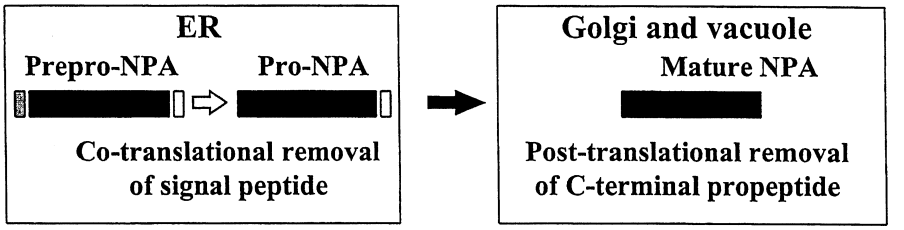

Figure 16.1 Schematic representation of the biosynthesis, processing and topogenesis of the narcissus lectin (NPA). The primary translation product Prepro-NPA is processed in the endoplasmic reticulum (ER) by co-translational removal of a signal peptide. Upon transport of Pro-NPA to the vacuole a C-terminal propeptide is removed, resulting in mature NPA.

reading frame encoding a preprolectin. This precursor contained, besides the coding sequence of the mature lectin of 109 amino acids, a signal peptide (24 amino acids) and a C-terminal peptide (30 or 38 amino acids) which are co- and post-translationally removed, respectively (Figure 16.1; Van Damme and Peumans, 1988). The calculated molecular mass of the mature narcissus lectin polypeptides varied between 11.6 and 11.9 kDa, which is in good agreement with the molecular mass of 12.5 kDa determined by SDS-PAGE (Van Damme *et al.*, 1988).

Interestingly, some differences in the deduced amino acid sequences of the different cDNA clones resulted in different charges along the lectin polypeptides, resulting in isoelectric points ranging from 3.66 to 4.24 for the mature polypeptides encoding the narcissus lectin. These results indicated that the different cDNA clones encoded isolectins with different isoelectric points. Hence the detailed sequence analysis of different cDNA clones explained the occurrence of multiple isolectins at the molecular level. Furthermore, since Southern blot analysis of genomic DNA isolated from narcissus yielded numerous restriction fragments hybridising with the lectin cDNA probe, it was evident that the expression of the isolectins is under the control of a family of closely related lectin genes (Van Damme *et al.*, 1992).

Three-dimensional structure of the *Narcissus* lectin

A detailed analysis of the sequence encoding the mature narcissus lectin revealed that the sequence shows strong homology with the sequence of snowdrop lectin, and likewise is also composed of three very homologous domains (Figure 16.2), each of which contains a carbohydrate-binding site (see below).

Molecular modelling of the deduced amino acid sequence of the mature narcissus lectin (derived from the cDNA sequence) using the co-ordinates of the related snowdrop lectin as a model, allowed the determination of the three-dimensional structure of the narcissus lectin (Barre *et al.*, 1996). Like the snowdrop lectin, the three-dimensional structure of the narcissus lectin (Figure 16.3) corresponds to a β-barrel built up of three antiparallel four-stranded β-sheets (domains) interconnected by loops (Hester *et al.*, 1995). A detailed comparison of the snowdrop and narcissus lectin sequences revealed that the residues forming the

LECNPA1 (A)

```
                                  1
MAKTSFLILATIFLGVITLPSCLS ▼ DNILYSGDTLSTGQFLSYGSYVFIMQEDCNLVLY   34
DVDKPIWATNTGGLSSDCHLSMQTDGNLVVYSPQNKAIWASNTDGENGHFVCILQKDRNV   94
VIYGTDRWATGTYTGAVGIPESPASEKYPTSGKITPTSEKYPTTGKIKLVTGK         147
```

(B)

```
     1          6 82              89  91  93  95  97                    109
  I  D N I L Y S G H F V C I L Q K D R N V V I Y - G T D R - - W A T G T Y T G
     7         12 50              57  59  61  63  65                     81
 II  G D T L S T S D C H L S M Q T D G N L V V Y S P Q N K A I W A S N T D G E N
     13        18 19              26  28  30  32  34                     49
III  G Q F L S Y G S Y V F I M Q E D C N L V L Y D - V D K P I W A T N T G G L S
```

Figure 16.2 (A) Deduced amino acid sequence of the narcissus lectin precursor encoded by cDNA clone LECNPA1. The arrow indicates the cleavage site of the signal peptide. (B) Internal sequence similarity of different segments of the mature narcissus lectin sequence of 109 amino acids. The amino acids composing the carbohydrate-binding site are indicated in bold.

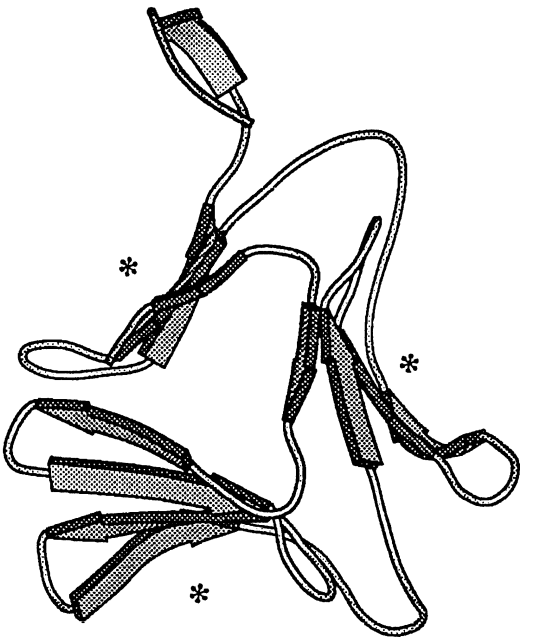

Figure 16.3 Three-dimensional structure of the narcissus lectin monomer. Strands of antiparallel β-sheet are represented by arrows. Asterisks indicate the location of the monosaccharide binding site.

mannose-binding sites are perfectly conserved and that the narcissus lectin subunits also contain three functional carbohydrate-binding sites. Hence, as in snowdrop, the narcissus lectin monomer possesses three mannose-binding sites, each composed of four amino acid residues, Gln, Asp, Asn and Tyr, that bind O2

(Asp and Asn), O3 (Gln) and O4 (Tyr) of mannose through a network of four hydrogen bonds. Another hydrophobic residue, namely Val, interacts with C3 and C4 of mannose through hydrophobic interactions.

Recently the narcissus lectin has been crystallised, and its crystal structure in complex with α1,3 mannobiose has been determined by X-ray crystallography at 2 Å resolution (Sauerborn *et al.*, 1999).

CARBOHYDRATE-BINDING PROPERTIES

The carbohydrate-binding specificity of the narcissus lectin was assessed by quantitative inhibition, sugar hapten inhibition assays using a series of simple sugars, and affinity chromatography of glycoconjugates on the immobilised lectin (Kaku *et al.*, 1990). Of all monosaccharides tested, only D-mannose was inhibitory in hapten inhibition assays. A more detailed study of the carbohydrate-binding specificity of narcissus lectin revealed that it had the highest affinity for both terminal and internal α1,6-linked mannosyl residues. The narcissus lectin also strongly precipitates several yeast mannans (e.g., *Saccharomyces cerevisae* and *Pichea pastoris* mannans containing multiple D-mannosyl side chains attached to the α1,6-linked mannose backbone) but does not precipitate α-D-glucans. Since oligosaccharides are better inhibitors than the methyl-α-D-mannoside (e.g., α1,6-linked mannotriose being twice as good an inhibitor as Manα1,6Manα-O-Me, and ten times better than methyl-α-D-mannoside), it can be concluded that the lectin possesses an extended binding site complementary to at least three 1,6-linked α-mannosyl units (Kaku *et al.*, 1990). Glycosylasparagine glycopeptides containing α1,6-Man units were retarded on a column with the immobilised narcissus lectin. However, glycopeptides with hybrid type glycans were not retarded. Therefore the lectin will prove to be a useful tool for the detection and preliminary characterisation of glycoconjugates.

It should be indicated that the analyses of the carbohydrate-binding specificity of the narcissus lectin were performed with a total lectin preparation containing several isolectins. Affinity chromatography experiments suggested differences in affinity of different isolectins for a mannose-Sepharose 4B column. Indeed, affinity chromatography of the lectin from *Narcissus* 'Fortune' on a column of immobilised mannose and elution of the lectin with a linear gradient of increasing mannose (0–0.2 M) revealed that some isolectins desorbed from the column at low concentrations of mannose, whereas other isolectins were still retained on the column after washing with 0.2 M mannose (Van Damme *et al.*, 1990a). These results clearly suggest differences in affinity for mannose among the different isoforms of the narcissus lectin.

BIOLOGICAL ACTIVITIES

The narcissus lectins readily agglutinate rabbit erythrocytes but are completely inactive towards human red blood cells, irrespective of the blood group. Agglutination of the rabbit red blood cells is enhanced after treatment of the erythrocytes with trypsin. The minimum concentrations required for agglutination of

untreated and trypsin-treated rabbit red blood cells were 1.25 and 0.25 μg/ml, respectively (Van Damme *et al.*, 1988, 1991a).

Analysis of the mitogenic activity of the narcissus lectin towards human lymphocytes revealed that the lectin is virtually non-mitogenic towards human mononuclear cells within the concentration range of 1–200 μg/ml (Kilpatrick *et al.*, 1990).

In vitro tests demonstrated that the mannose-specific narcissus lectin shows activity against human immunodeficiency virus (HIV). The narcissus lectin inhibits infection of MT-4 cells by HIV-1, HIV-2 and simian immunodeficiency virus (50% effective concentration being 0.5–0.6 μg/ml; Balzarini *et al.*, 1991; Weiler *et al.*, 1990) at concentrations comparable to those for dextran sulphate inhibition of these viruses. Unlike dextran sulphate, the narcissus lectin did not inhibit the replication of other enveloped viruses except that of human cytomegalovirus. Furthermore, the lectin suppresses syncytium formation between persistently HIV-1 or HIV-2 infected HUT-78 cells and uninfected MOLT-4 cells (Balzarini *et al.*, 1991). The narcissus lectin was also shown to prevent rabies virus attachment to susceptible cells, and affect rubella virus multiplication after the attachment step (Marchetti *et al.*, 1995).

The lectin was also tested for its toxicity to insects when incorporated into an artificial diet, as part of a search for the possible function of the lectin in the plant. The narcissus lectin shows antimetabolic effects towards nymphal stages of the rice brown planthopper *Nilaparvata lugens* (Powell *et al.*, 1995) and the peach-potato aphid *Myzus persicae* (Sauvion *et al.*, 1996). Insect feeding trials with the narcissus lectin showed that it exhibits a significant antimetabolic effect towards third instar nymphs of the rice brown planthopper, although it is less active than the *Galanthus nivalis* lectin, the LC_{50} for the narcissus lectin being 11 μM compared with 4 μM for the snowdrop lectin (Powell *et al.*, 1995). Similarly, addition of the narcissus lectin to artificial diets in a concentration range of 10–1500 μg/ml to test the toxicity and growth-inhibitory effects on nymphal development of the peach-potato aphid revealed that, although narcissus lectin does not induce significant mortality, its addition to the diet at 1500 μg/ml resulted in growth inhibition (Sauvion *et al.*, 1996).

PHYSIOLOGICAL ROLE OF *NARCISSUS* LECTIN IN *PLANTA*

The concentration of lectin in various tissues throughout the life cycle of narcissus was studied by Van Damme and Peumans (1990b). This study revealed that the lectin is present in almost all plant tissues, representing as much as 10% of the total protein content during certain stages of development. This is in contrast to most of the other plant lectins studied thus far, since their distribution is in most cases confined to one or a few tissues and their concentrations are much lower. Since in narcissus, as in other Amaryllidaceae species, the changes in lectin content of the old and new bulb units coincides with the loss and accumulation of storage compounds, it has been suggested that these lectins may function as storage proteins which are rapidly degraded as the shoot starts to grow. Furthermore, these lectins are present in large quantities in a typical storage organ, the bulb, supporting this hypothesis. Most probably these lectins must be considered as storage proteins which, as well as their storage role, have an additional carbohydrate-binding

activity. Since the lectin is present only at certain stages of development and occurs in almost all plant tissues, it might also play an active role in plant defence. For example, the recent discovery that snowdrop and narcissus lectins exhibit toxicity towards some insects certainly points in this direction. At present the Amaryllidaceae bulb lectins are considered as storage proteins that can also be mobilised as defence proteins whenever the plant is attacked by phytophagous invertebrates (Peumans and Van Damme, 1995; Van Damme *et al.*, 1998a). The occurrence of multiple isoforms may equip the protein with a broad spectrum of biological activity.

APPLICATIONS OF *NARCISSUS* LECTINS

Because of their unique and exclusive carbohydrate-binding specificity, lectins have become very useful tools in glycoconjugate research. In this respect, plant lectins are often used for the isolation and fractionation of glycoproteins and for the study of oligosaccharides and glycopeptides (Osawa and Tsuji, 1987; Cummings, 1997; Peumans and Van Damme, 1998). Furthermore, lectins are important probes in histochemistry and histopathology for the detection of specific carbohydrates on cells or in tissue sections (Schumacher *et al.*, 1991). An important prerequisite for the successful application and exploitation of a lectin is its commercial availability. At present the purified narcissus lectin and preparations derived therefrom (immobilised lectin, labelled lectin) are available from at least four companies (EY Laboratories Inc., Sigma Chemical Company, Vector Laboratories Ltd. and Leuven Bioproducts).

Hitherto, the narcissus lectin has been successfully applied in the purification of glycoproteins and characterisation of glycopeptides on the surface of cells and/or proteins. For instance, it was shown that the immobilised narcissus lectin can be used for the purification of human α_2-macroglobulin (Van Leuven *et al.*, 1993).

As already mentioned above, the narcissus lectins have a strong inhibitory effect on the infection of target cells by retroviruses including HIV and cytomegalovirus *in vitro*, which makes them very interesting probes in the study of surface components of some viruses. Based on the highly specific interaction between glycoprotein-120 (gp120) and the narcissus lectin, an ELISA was developed to determine the concentration of gp120 in HIV-infected CEM cells *in vitro* (Weiler *et al.*, 1991).

COMPARISON WITH OTHER LECTINS

The narcissus lectin is a typical representative of the group of monocot mannose-binding lectins which was first studied in snowdrop (*Galanthus nivalis*) (Van Damme *et al.*, 1987). Later it was shown that lectins with similar characteristics and biological properties also occur in other species of Amaryllidaceae, e.g., *Narcissus, Hippeastrum* and *Clivia* (Van Damme *et al.*, 1988, 1994). It is now clear that this group of lectins is not confined to Amaryllidaceae species, since examples of lectins isolated from species belonging to the plant families Alliaceae, Orchidaceae, Araceae, Bromeliaceae and Liliaceae clearly show DNA and peptide sequence similarity to the Amaryllidaceae lectins. Hence all these lectins are now considered as members of the family of monocot mannose-binding lectins (Van Damme *et al.*, 1995, 1998a,b).

All monocot mannose-binding lectins are composed of mature lectin polypeptides of 11–14 kDa. Like the narcissus lectin, many other monocot mannose-binding lectins are complex mixtures of isolectins resulting from the expression of a family of closely related genes (Van Damme *et al.*, 1998a). All DNA sequences encoding these lectins show a high degree of homology, resulting in a highly conserved three-dimensional structure for the different lectins (Barre *et al.*, 1996; Hester *et al.*, 1995; Chantalat *et al.*, 1996; Wright *et al.*, 1997).

Hapten-inhibition assays with simple sugars have demonstrated that all currently known monocot mannose-binding lectins are exclusively inhibited by mannose (Van Damme *et al.*, 1995). However, the mannose concentrations required for an efficient inhibition are high (IC_{50} = 20–200 mM), suggesting that these lectins have a low affinity for the monosaccharide. The ability of the monocot mannose-binding lectins to distinguish D-mannose from D-glucose units distinguishes this family of lectins from the mannose/glucose-binding lectins belonging to the family of legume lectins and from the mannose/maltose-binding lectins of the family of jacalin-related lectins (Van Damme *et al.*, 1998a).

Despite the fact that all monocot mannose-binding lectins will react with mannose, they differ in their fine carbohydrate-binding specificity and interaction with oligo- and polysaccharides containing D-mannosyl groups. In their immobilised form the lectins will also show different chromatographic behaviour towards glycosyl-asparagine glycopeptides (Kaku *et al.*, 1990, 1991).

GENERAL CONCLUSIONS

At present the lectin is one of the very few proteins from narcissus that has been studied in detail. The lectin represents an important fraction of the total protein content, at least in resting bulbs. Developmental regulation of the lectin concentration and toxicity of the lectin towards insects indicates a possible involvement of the lectin in storage metabolism and/or plant defence.

ACKNOWLEDGEMENTS

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17 *Narcissus* in perfumery

Christian Remy

HISTORY

The olfactory qualities of the narcissus flower have made it a valuable component of luxury perfumes since time immemorial. Originally the flower perfume was extracted by the method of enfleurage, in which the substance was absorbed by animal fat. The fat was then washed with alcohol, filtered and concentrated to obtain a material called *absolue des pommades*. Later, extraction was carried out by using a solvent, generally hexane, which allowed better extraction and faster processing. This procedure, equally applicable to other flowers such as rose, jasmin, orange-blossom, etc., was perfected towards the middle of the nineteenth century, but it was at the start of the twentieth century that it began to be used on an industrial scale.

When first used, wild narcissus flowers were harvested in Provence, in the hinterland of the Côte d'Azur, where the growing of lavender, lavandin and sage is now predominant. Narcissus grow in grassy areas (Figure 17.1), and the gradual decline in livestock farming in the region and its replacement by arable farming resulted in the scarcity of the flower, to a point at which its harvesting was no longer profitable. From 1950, it has been necessary to look elsewhere for supplies. The manufacturers in Grasse, who already knew the southern Auvergne well for its lichen (tree moss) growing on forest pine, also turned towards this area for narcissus (*Narcissus poeticus*) and Jonquil (*N. jonquilla*). Jonquils are used in perfumery as well as narcissus, but in smaller amounts. The Auvergne is traditionally a land of flower, herb and mushroom gathering, indeed the very phrase 'flower gathering' evokes a complimentary, almost trivial activity, or in any case an activity on the fringes of agriculture. But it is nothing of the sort here, if one takes into account the volumes involved and their commercial importance. In the Auvergne, flower gathering must be considered as a vital source of revenue, and a real part of the overall rural economy of the region. But there was a time when flower gathering was done just for the sake of it, as much as for sale, selling at the fair what was surplus to the family's needs.

A major change came about at the end of the Second World War, when there was a rise in flower gathering specifically for sale. In many cases, and especially with medicinal plants, this was linked with the development of the pharmaceutical industry, but other crops have remained stable right to today. It was at this time that plant collection was organised into a network, leading to the advent of collectors and gathering teams. As well as narcissus, they also gather violets, wild

Figure 17.1 Narcissus (*Narcissus poeticus*) can be seen like white sheets in pastures. On the right of the photograph there are some Jonquils (*N. jonquilla*), which generally flower 2–3 weeks before the narcissus. Both flowers can be found in the same area, but they never blend.

anemone, arnica, St. John's wort, wild pansy, mallow, burdock, cat's foot, foxglove, balm and gentian. Today, all these products have become part of a more modest flower gathering operation, sometimes very small scale, and among some of the principal products its scale is strictly controlled: bilberry and mushroom on the one hand, and tree moss, narcissus and jonquils on the other. The last three products are virtually the only materials of the region destined for use in perfumery.

The narcissus, the jewel in the crown of the region's plants, has become its emblem. In the last days of May, the meadows suddenly become white in successive waves, reaching ground as far as 1500 metres above sea level. Narcissus generally grow in damp meadows, and the intensity of the white covering that forms in the fields can reliably indicate the presence of water or a stream from a distance.

FLOWER GATHERING

Thirty years ago, some flower picking was still done by hand, but even at that time the use of the 'comb' (*le peigne*) was becoming more common (Figure 17.2). The comb was a kind of rake with cutting teeth, and it was designed to allow the collection of just the flower head on its own, without the stalk. In fact, it is only the centre of the flower itself that is of interest in perfumery. Gradually, one saw appearance of a larger, improved comb, a cart mounted on wheels (*le chariot*). The cart is somewhat cumbersome, but is more efficient than the comb on large areas lush with flowers; however, it is not particularly suited to all situations, and the comb continues to be

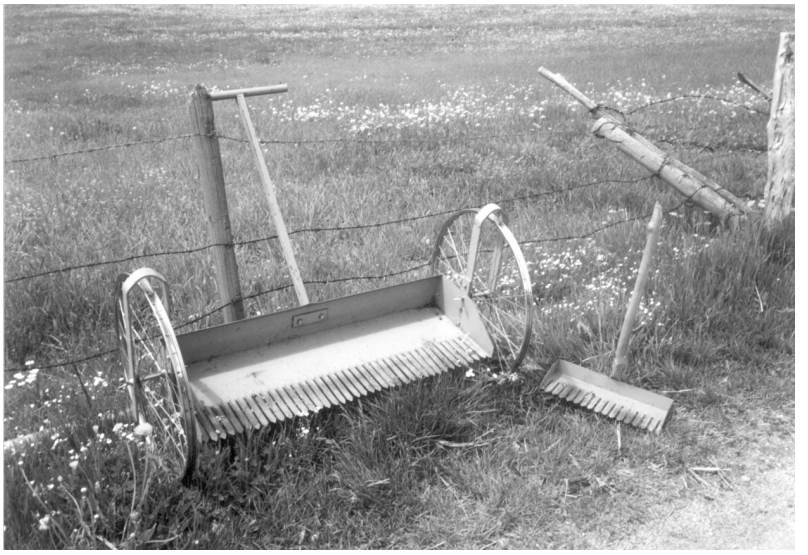

Figure 17.2 Flower collecting implements: the comb (right) and the cart (left).

used for small isolated patches of flowers or on sloping terrain where agility is needed. The principle of the cutting teeth is the same on both tools. The cart allows the cutting of three-four times as many flowers as the comb.

As stated above, narcissus grow in meadows, which can apparently lead to conflicts of interest. Unlike most forests where tree moss is collected, and which are public places, the meadows are privately owned. Meadows are always carefully fenced, since they are used for livestock farming, and this systematic fencing, added to the temperament of the Auvergne farmers, reinforces the fact of ownership. Two situations are possible:

- Where the owner has authorised access to his field for picking (possibly sharing the profit with the picker).
- Where access has been prohibited since the owner plans to crop it himself, because he believes that, in view of his decision not to exploit the field, there is no reason for somebody else to earn money from it.

This reasoning perhaps appears to have an odd logic, but it is still frequently encountered. If it is only a small part of a field that is covered with flowers, it is very tempting to leave it to waste! These conflicts can sometimes lead to violence, and, each year, some settling of scores are ended by lead shot. Sometimes there is even clandestine picking, at night. Thus, a landowner who one evening sees before his very eyes a field of white flowers, can find a green field the next morning!

As one can imagine, one of the problems posed by the gathering of narcissus is the fragility of the flower. More so than in the case of tree moss, which is not perishable, narcissus gathering demands a network of strict organisation because

collection is carried out in an area 200 km across and 500 km from Grasse. It is therefore practically impossible for a flower destined to be treated at Grasse to arrive in a satisfactory state of freshness. Picking, collection and transport by truck takes two to three days, and the flowers are largely degraded during this length of time. For this reason, a central extraction plant has been set up right in the heart of the collection area, allowing the treatment of flowers on the same day that they are collected. Thanks to this proximity, the quality of a product has been transformed and greatly improved. The central facility alone processes 50–60% of the total flowers collected each year.

QUANTITIES

The gathering of narcissus flowers is, as is the case for most plant material, subject to the weather. This situation is perhaps accentuated by the fact that flowering takes place in mountainous regions, at altitudes between 600 and 1500 metres. Thus, cold weather persisting in the preceding months, or late frosts, can jeopardise flowering. Collection may equally be spoilt in years with strong rain, because of the excessive, rapid growth of grass, practically covering the narcissus flowers and making cropping impossible in some places. However, it is true that, even if the flowers are poor, in theory there will be more than enough to satisfy the needs of the perfume industry. The problem is simply that the pickers, after having evaluated the density of flowering and having carried out a rapid calculation of profitability by the hour, decide to crop . . . or not to crop. Their decision will depend on whether collection levels that year will be normal or low. The quantities gathered will also be adjusted according to the needs indicated by the industry. This is why, with medium sized crop being between 300 and 400 tonnes, quantities can vary between 200 and 600 tonnes in different years. These are the weights of flowers alone, excluding the stems and leaves.

EXTRACTION

Narcissus flowers are fragile and delicate. It is desirable to treat them as soon as possible after collection, in order to preserve their olfactory qualities. While waiting for treatment, the flowers are spread out on a cement slab at the factory (Figure 17.3). They are stored for as little time as possible, in a thin layer which is turned over constantly to avoid fermentation starting. As is the case for the flowers of rose, jasmin, orange blossom and many others, they are treated by extraction with solvents (hexane being the usual solvent), in order to obtain a viscous product called a 'concrete' (Figure 17.4). The concrete contains a high proportion of vegetable waxes that render it useless, in that state, in perfumes. It is, therefore, necessary to remove these waxes in order to render the product soluble in alcohol. The concrete is a thick substance that hardens on cooling, and it has to be heated in a bain-marie to soften before being processed. The concrete is subjected to a second transformation through washing with alcohol in order to separate the waxes and to obtain the final product, referred to as an 'absolute'. It is the absolute that is used in the manufacture of perfumes. The narcissus belongs to the category of noble

Figure 17.3 Narcissus flowers spread out to dry, and a drum holding 20 kg of concrete, the equivalent of 10 tonnes of flowers.

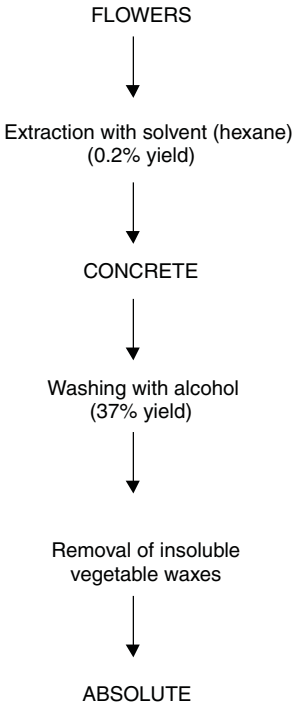

Figure 17.4 Scheme for the production of narcissus absolute.

perfumes, a title shared by other flowers such as rose, jasmin and bitter orange blossom.

The low productivity of absolute from the narcissus makes it an expensive product that cannot be used other than in luxury perfumes:

- 1000 kg of flowers provides about 2 kg of concrete (0.2%).
- From this amount of concrete, after removing the waxes, 750 g of absolute is obtained.

It therefore takes a total of 1300–1400 kg of flowers to obtain 1 kg of absolute. However, it is not only its price that determines the value of narcissus absolute, but also the olfactory quality, and these factors determine the success of a perfume.

OLFACTORY DESCRIPTION

It is always difficult to describe an olfactory sensation in words. Our vocabulary is just not adapted to it, whatever language we use. It is also very difficult to know if one particular scent is perceived in the same manner by two different people. All this makes the description of a scent a difficult and hazardous exercise. In the case of narcissus, an olfactory description is so much more difficult since this absolute is almost a perfume by itself. Its scent is rich and complex. In it one can sometimes be reminded of the elusive fragrance of iris, rose, jasmin, tuberose, ylang-ylang, orange blossom, storax and oak moss. All these brought together form a scent that is intense and lasting, flowery and exciting at the same time.

The main components of the volatile part of narcissus absolute are listed in Table 17.1.

Table 17.1 Main Components of the Volatile Part of Narcissus Absolute

Compound	*% of Volatile*
α-terpineol	23.7
Methyl trans-isoeugenol	20.0
Benzyl benzoate	19.4
Coumarin	8.9
Benzyl alcohol	5.0
δ-3-carene	3.4
Phenylethyl alcohol	2.2
Ethyl palmitate	2.2
Cinnamyl alcohol	2.0
Phenylpropyl acetate	1.7
1,8-cineole	1.5
β-caryophyllene	1.0
Benzyl acetate	0.7
Isoeugenol	0.6
cis-3-hexenyl acetate	0.5
Cinnamyl cinnamate	0.5
Total	91.3

CONCLUSION

The use of narcissus in perfumery has to take account of practical realities. Modern perfume making is very cost-conscious, and much emphasis is placed on the appearance of the packaging. Perfumes have to be sold in the face of ever increasing competition, and the advertising budget necessary for launching a perfume swallows up considerable sums. Then, far too often, manufacturers economise on the quality of the perfume. The imagination of the perfumier is also constrained, and much of the time he is forced to exclude the more expensive components from his formula. In perfumery, these are often the choicest ingredients.

This is why one has to say that the use of narcissus in perfumery is rather in decline. One can hope, in the years ahead, to see a reversal of this trend, when it will be realised afresh that the success of a perfume depends on its quality. The secret of the really great perfumes is that they endure all fashion trends.

All the information in this chapter has been based on experience gained at Laboratoire Monique Remy. Some additional sources of information may be found in the "Further reading" section.

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18 Harmful effects due to *Narcissus* and its constituents

Celia G. Julian and Peter W. Bowers

INTRODUCTION

The genus *Narcissus* has been grown commercially in the far south-west of England for more than a hundred years. The warm climate and small sheltered fields (particularly on the Isles of Scilly) have helped this industry, for which significant expansion appears likely in the next few years. Many hundreds of seasonal workers are employed, the larger labour force being needed for 6–8 weeks in the spring when the flowers are picked, bunched and packed for distribution. A smaller number of seasonal workers are employed in summer for lifting and cleaning the bulbs for re-planting or sale. Thus, any adverse effects from contact with narcissus (daffodil) can influence a potentially large number of workers and have a significant sequel.

At present, more than 1180 ha are planted with narcissus in south-west England, which includes a significant expansion within the last two years. This provides a considerable revenue for Cornwall and the Isles of Scilly, areas which otherwise rely on farming, fishing and tourism and which have a high rate of unemployment. Cornwall accounts for about 35% of the bulb acreage in the UK, other significant areas being in Lincolnshire, Norfolk and Scotland.

EFFECTS ASSOCIATED WITH HANDLING *NARCISSUS* CROPS

Handling flowers

'Daffodil itch' or 'lily rash' associated with picking narcissus is well recognised and was first described by Walsh in 1910 in relation to flower pickers on the Isles of Scilly. The name 'lily rash' arose as narcissus were originally thought of as lilies, although they are now placed in the Amaryllidaceae because they have an inferior ovary. An eczematous rash develops from direct contact between the skin of the picker and sap from the flower stem. Granulomatous sores may develop at any point of direct trauma and paronychia can occur. These changes may be quite disabling, but as the season is short and the rash therefore self-limiting, the pickers rarely present to a doctor. Hands and wrists are the most commonly affected areas, but the rash may also appear beneath the chin, on the forearms, in the axillae and on the genitalia.

The way in which the flowers are picked and collected accounts for the distribution of the rash. Picking takes place when the flowers are still in bud. The picker

Figure 18.1 Daffodil picking: snapping off the flower stem.

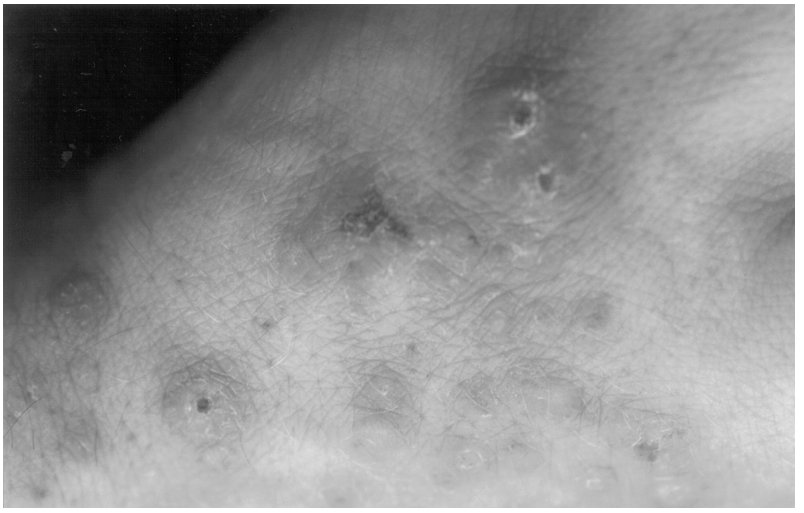

Figure 18.2 Granulomatous rash on the wrist. (*See Colour plate 3*)

slides a hand down to the base of the flower stem and then snaps, cuts or pulls off the stalk (Figure 18.1). During this process, the finger webs are particularly vulnerable to trauma by the ends of the daffodil leaves or by stalks from previously picked flowers. The base of the stalk continues to grow after the flowers have been cropped, resulting in an increasing hazard to the pickers as each row may be sequentially picked up to four times, with more protruding stalks on each occasion. As the flowers are gathered, sap drips out from the stems onto the wrist and forearm, causing further rash (Figure 18.2).

Figure 18.3 Daffodil picking: gathering up the bunches.

On the mainland daffodils are cropped mainly in the January to March period. The flowers are picked by one hand and transferred to the other until ten have been collected. The ends of the stems are then levelled against the palm of the hand and the bunch is secured with an elastic band from a supply kept in a pot at the waist. The picker straddles the row of plants and works along it, laying the bunches down in groups of three or five for collection on return. Working back down the row, the bunches are gathered in the crooks of the arms and under the chin and armpits (Figure 18.3), to be deposited in a personally labelled tray. This is a rapid procedure, as an experienced operator is capable of collecting in excess of 2000 bunches per day (20 000 flowers). The money earned is related directly to the number of bunches picked. Workers are told at the beginning of the day the price payable per bunch. They therefore have a financial incentive to hold on to as many bunches as possible. This practice is reflected in the more extensive distribution of the rash.

On the Isles of Scilly, the earlier flowering Tazetta varieties are grown, cropping from October onwards. The method of flower picking is the same as on the mainland, but the flowers are carried by tractor to sheds for bunching and packing. The pickers are paid an hourly rate, rather than by the number of bunches gathered, and do not need to hold large quantities of flowers in close bodily contact. On the Islands, the primary daffodil rash is therefore usually confined to the hands and wrists. In both groups of workers, secondary rash may develop on the face (Figure 18.4), or on the scrotum in men as a result of transfer of daffodil sap by direct contact with the hand while urinating.

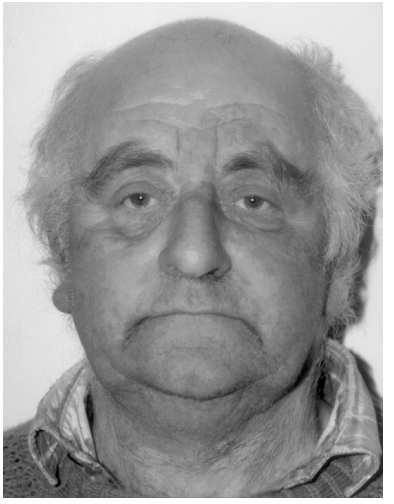

Figure 18.4 Secondary facial rash. (*See Colour plate 4*)

Apart from 'daffodil itch', there are other medical problems associated with picking narcissus flowers. The workers are perpetually bent forwards, with their backs to the wind, and are prone to backache. All pickers run the risk of having repetitive strain injury to the wrist, from the twisting action as the flower stem is plucked from the plant. The more inexperienced pickers sometimes wear inappropriate clothing, and can develop chilblains on the backs of their thighs and buttocks (Julian and Bowers, 1997).

Handling bulbs

Bulb lifting takes place during a six week period in June/July. Between 12 and 20 tonnes of bulbs are planted per hectare. After two or three years, depending on the variety, the yield of bulbs should be at least twice the planted weight, bulb quality is at its peak, and the bulbs are harvested. With a total UK crop area of over 4000 ha, huge numbers of bulbs are involved. In Lincolnshire, where the land was reclaimed from the sea and there are no large stones in the soil, the lifting process is often largely mechanical. Lifting machines (complete harvesters) lift the bulbs and transfer them to a hopper, removing much of the soil in the process, although workers on the lifting machine may remove clods. The bulbs are then transported to sheds for grading and further treatment. In Cornwall, where the soil contains large stones and the fields are often small and steeply sloping, the lifting process is only partly mechanical, the bulbs being elevated by a lifting machine to the surface where they are left to dry for several days before being gathered into baskets by hand and transferred to large boxes. Seasonal workers are again employed to pick up the bulbs, making the procedure more labour-intensive in Cornwall than in Lincolnshire. In Cornwall the bulbs are dry when picked and are handled relatively little, and in practice this gives few problems to those that collect them.

Prevention of daffodil rash

Protective clothing is an important aspect in the prevention of daffodil rash. Experienced workers are well aware that they should minimise any contact between daffodils and the skin. Flower picking often takes place during wet and windy weather. Waterproof clothing is essential to prevent sap soaking through to the skin as the bunches are collected. The majority of pickers invest in good rubber, or even nitrile, gloves, and are aware that they must avoid any gap between the end of the glove and the sleeve of their jacket, and elastic bands or neoprene cuffs are utilised for this. Bulb lifting takes place in the summer months, generally under drier conditions. The workers universally wear gloves, but need encouragement to provide adequate protection from the sun by wearing a hat and applying sunscreen regularly.

The rash resulting from direct contact between daffodil sap and the skin of susceptible individuals is mainly a primary irritant dermatitis. Experienced workers, picking up to 20 000 flowers per day, have potential contact with a large volume of sap. The amount of sap produced varies between cultivars, and also within the same cultivar under different conditions, as direct observations confirmed (Julian and Bowers, 1997). More sap is produced during wet weather. Pickers commented to the present authors that the ability of a daffodil to produce an irritant rash varies with the variety. The highly scented, multi-flowered 'Soleil d'Or' and 'Paperwhite' Tazetta narcissi, grown almost exclusively on the Isles of Scilly, rarely cause any problem. The trumpet varieties 'King Alfred' and 'Princeps' were mentioned specifically by older flower farmers on the Isles of Scilly. These cultivars are not grown in quantity at the present time, but were notorious in the past for the problems they caused. Pickers are aware of these differences: when gathering Tazetta narcissus they hold the flower stems facing inwards, but when picking standard varieties they turn round the stems, to avoid sap dripping onto their clothing.

Constituents of *Narcissus* sap

Narcissus sap contains calcium oxalate crystals, which are polymorphic and may be multi-faceted cubes or needle-shaped (Figure 18.5). The needle-shaped crystals are grouped in bundles known as raphides. A study by Sakai *et al.* (1984), which did not include narcissus, demonstrated an increase in irritancy in species with long raphides. With the electron microscope, it was possible to determine the presence of barbs and grooves in the crystal structure. Their presence was associated with irritancy. In their absence, only crystals with a length exceeding 180 μm produced irritation in Sakai's study. The relationship between crystal appearance and sap viscosity to irritancy in a number of narcissus cultivars is the subject of a current study by the present authors.

In narcissus the calcium oxalate crystals are contained within 'slime vessels'. These are formed by the elongation of normal parenchymatous cells and the subsequent breakdown of their dividing cell walls. As well as calcium oxalate crystals, they contain mucilage and alkaloids. When the flower stalk is twisted off during picking, the slime vessel is ruptured and its contents extruded by the force of turgidity of the adjacent intact cells (J.J. Beijer, personal communication). In this way, calcium oxalate crystals are able to penetrate intact skin (van der Werff, 1959).

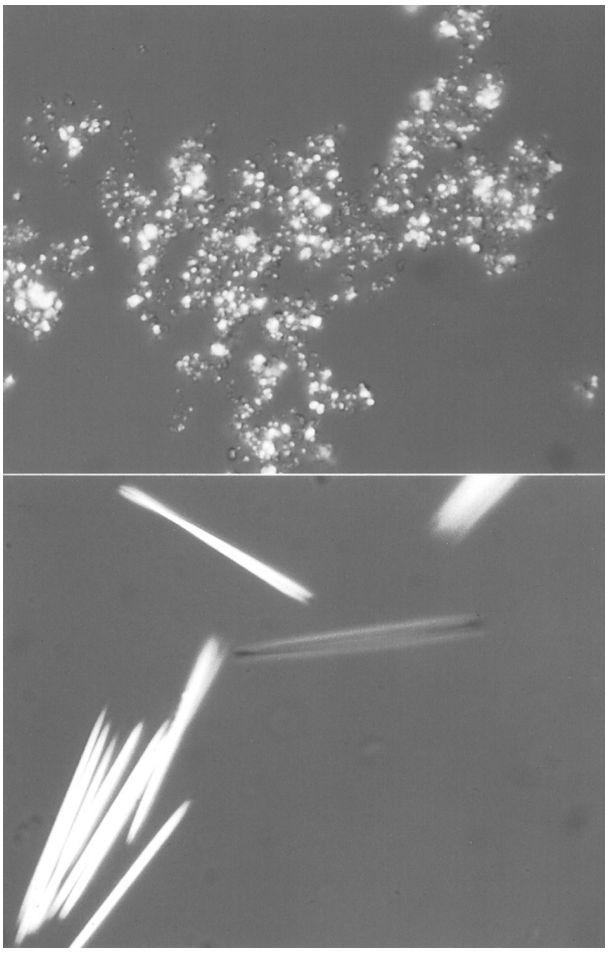

Figure 18.5 Polymorphic calcium oxalate crystals. The needle-shaped crystals are *ca.* 100 μm in length. (*See Colour plate 5*)

The alkaloids found in the slime vessels may be responsible for the rarely occurring allergic reactions to narcissus occasionally reported (Hausen and Oestmann, 1988). Traumatisation of the skin by calcium oxalate crystals allows the penetration of these substances from the slime vessels. At least 15 different alkaloids have been isolated from narcissus (Barton *et al.*, 1963), but only two of these were found to be capable of producing a weakly positive allergic response when injected into sensitised guinea pigs (Gude *et al.*, 1988). These alkaloids, homolycorin and masonin, were not detected in the stems or leaves of narcissus: their highest concentration was found in the bulbs, although sap extracted from bulbs produced minimal allergic effect. It would seem from this work that narcissus has very little ability to produce an allergic response. Additionally, a Finnish study produced only two

patients with allergic contact dermatitis to narcissus over a fourteen-year period (Lamminpää *et al.*, 1966). Both were gardeners with many years' exposure, and produced positive patch tests to a 1% concentration of narcissus plant extract. During discussions with workers in the Cornish narcissus industry, the present authors met a farm owner who developed an immediate facial erythema and swelling on entering the packing sheds, as a result of sensitisation from previous handling of the flowers.

Pesticides

Pesticides are important in the flower industry and worldwide have been noted to produce increasing skin problems in agricultural workers (Lisi *et al.*, 1987). This is predominantly a contact dermatitis where protective measures are poor. It has been reported in the tulip bulb industry, after use of the fungicide fluazinam. In this case the bulbs were handled after spraying, contrary to the manufacturer's instructions (Bruynzeel *et al.*, 1995). This fungicide is not used in the daffodil bulb industry in the UK, where the list of fungicides includes chlorothalonil, carbendazim, iprodione, vinclozolin, benomyl and mancozeb, and no particular problems have been observed with these. On one farm where we have observed bulb processing, carbendazim is currently used, but all bulb dipping takes place in tanks outside, not in bulb sheds. The same material is also used as a spray in the field to control fungal foliar diseases and delay leaf senescence. Fungicides may be present on the leaves at the time of picking, but field workers questioned by us did not report any particular problem with this. However, some farmers reported they were unable to spray the crops because of the severity of the facial rash which they develop while doing so. This is allergic dermatitis to airborne particles which contain the fungicide to which they have become sensitised. No specific product was mentioned.

Conclusions

Our study of those involved in the narcissus industry has shown that most harmful effects are produced as a result of direct contact between irritant sap and the skin. Many pickers are affected, but they have developed ingenious strategies to avoid this contact and allow them to work. The season is short and the condition self-limiting, so they rarely present to a doctor and the problem is under-reported.

OTHER HARMFUL EFFECTS OF NARCISSUS

Ingestion by humans

A rare but harmful effect of narcissus is toxicity as a result of the inadvertent ingestion of the bulbs (Venner and Gibbons, 1995). When mistaken for small onions and included in a stew, acute vomiting resulted from the effects of toxic alkaloids, including lycorine which is stable to heat. Recovery was rapid, due to the early onset of severe emesis. This probably accounts for human toxicity being limited to vomiting, abdominal cramps, shivering and diarrhoea. The onset of

symptoms is rapid, but usually resolves spontaneously within 3 hours (Litovitz and Fahay, 1982). In a second report (Vigneau *et al.*, 1984), nausea persisted in one subject for 10 days. However, all involved were completely recovered 15 days later.

Effects on other cut-flowers

The presence of daffodils in a vase with other cut-flowers has a deleterious effect on them (Barendse, 1974; van Doorn, 1998). This effect was very noticeable with tulips, whose flower stems droop and wilt within a few days, as was shown experimentally by Gugenhan (1970) who studied the effects of placing narcissus and tulip in separate vases and together. Narcissus and tulip kept separately remained normal, but within 6 days tulips placed with narcissus had deteriorated. While the narcissus still looked healthy, the tulips had aged, with mottling of the leaves and curvature and wilting of the flower stems. A similar but lesser effect was seen if tulips were placed in water previously occupied by narcissus, suggesting that the daffodil sap, rather than the actual flowers, was harmful. This effect was noted by Sytsema and Barendse (1975) to vary between cultivars, the sap of 'Carlton' being particularly aggressive. From a study with different flower foods, Terfrüchte (1981) reported that one sort could prevent this damage to tulip flowers. It would, however, seem advisable to confine all cut-flowers of narcissus to their own vase and refrain from mixing them with other species.

Blankenship and Richardson (1987) attempted to identify the component of narcissus sap responsible for causing damage in tulips. Over a 2-year period they studied the effects of ethylene, ethylene inhibitors and auxins, and concluded that the major senescence effect of stem curvature was caused by auxins. Auxins have been isolated both from the plant and from the water in which narcissus have been placed, and may be released into the water to produce their deleterious effect on flowers of other species. In their earlier study of narcissus auxins, Edelbluth and Kaldewey (1976) had found that auxin-inhibitory activity occurred in diffusates of stem segments, buds and flowers, and could be removed by washing to reveal auxin activity. Since narciclasine is present in narcissus in considerable quantities and is strongly inhibitory to cereal seedling growth (Ceriotti, 1967; Bi *et al.*, 1998), this could be the material interfering in the bioassays used to detect auxins. Van Doorn (1998) later investigated the deleterious effects of narcissus mucilage on cut-flowers of tulip and rose. Fractionating the narcissus mucilage, this author suggested that the effect on roses was due to the sugar and polysaccharide fraction, which resulted in increased bacterial growth and blocked water uptake, while the effect on tulips was due to the toxicity of a fraction that contained several alkaloids.

ACKNOWLEDGEMENTS

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19 Review of pharmaceutical patents from the genus *Narcissus*

James R. Murray

INTRODUCTION

This chapter is to give the reader a flavour of the richness of concepts associated with the *Narcissus* genus whilst bowing to the practicalities of reporting complicated patent processes that evolve over a period of years. No attempt is made to give definitive lists of published versus granted patents, as such lists would, by necessity, be out of date by the time of publication of this book. Instead, the chapter uses the listings of published patents and applications as of mid-September 1999 as a basis of a comprehensive review of the areas of research and development of products and projects associated with the cultivation and utilisation of narcissus (daffodil) plants and their constituents.

On undertaking the search for patents published around the genus *Narcissus*, 28 were identified under the title 'narcissus', 11 under 'daffodil' and 55 under 'galanthamine'. As galanthamine was originally extracted from the Caucasian snowdrop it was, perhaps, not surprising that only two of the 39 patents identified under 'narcissus' or 'daffodil' referred to galanthamine. Although not strictly related to *Narcissus* species, these patents are included here for completeness. The 'narcissus' and 'daffodil' titles are mainly related to use in cosmetic agents and fragrances, and many of these patents are from Japan and without equivalents outside Japan. These patents refer to synthetic molecules designed to mimic narcissus fragrance, as well as extracts of the plant. The galanthamine patents cover manufacture, intermediates used in the production of galanthamine, analogues of galanthamine, and the use of the cholinesterase inhibitory and nicotinic agonist pharmacological activities of the compounds in the clinic. Such clinical indications range from the original Eastern European folklore use of galanthamine in the management of patients with infantile paralysis (poliomyelitis) to the treatment of erectile dysfunction!

NARCISSUS AND DAFFODIL PATENTS

Patents related to non-therapeutic uses

A fascinating array of patents describing the utilisation of agents derived from daffodils, to mimic the fragrance of daffodils, or for agents or methods used to influence daffodils themselves, was uncovered. Who could fail to be intrigued by the grappling shovel with twin concave blades mounted on handles, operated with

the aid of a foot pedal that has been developed for optimisation of bulb, corm or tuber planting? Or by the fact that it is possible, by fusion bonding a laminate of two sheets of hot-melt type adhesives with dry pressed flowers (including daffodils as an example) in between, to form a decorative sheet-laminated fabric?

Oscillating digging shears for bulb lifting, with the front undercutting shear oscillating in the opposite phase to the following lift section, and processes to turn waste flower bulbs into chipboard by a pressing treatment at high temperatures and pressures (utilising the starch, cellulose and water in the bulbs), featured with an intriguing family of patents relating to optical and electronic uses, including endoscope diagnostic systems, in the patent search relating to the title 'narcissus'. The latter optical patents referred, in fact, to 'the narcissus type radiation effect' – clearly not related to the genus but more to the original Narcissus himself! These patents bloated the original 'narcissus' hit list by 15!

Other patents relating to the inhibition of stem elongation in flowering bulbous plants using 1-amino-cyclopropane-1–1carboxylic acid synthase inhibitors, or to methods of protecting foliage from browsing deer by spraying with deer-repellant extracts of narcissus bulbs, contrasted somewhat with a series of patents for hair and skin cosmetics containing waxes (Krause *et al.*, 1998), 'sedative essential oil comprising of narcissus absolute oil' (Kanebo Ltd., 1994) or a 'new' ortho-methyl cinnamic acid phenylethyl ester perfume (Hofmannor *et al.*, 1989) said to have a 'flowery aroma'. Such 'flowery aromas', if containing terpenic fragments, can further be protected from degradation, according to another patent, by adding rosemary and/or sage components! Other fragrances include 6-acyloxy-hexanoate esters (Ochsner, 1986) and 4-hydroxy or alkanoyl 3-ethoxy benzyl alkyl ethers, which have a 'narcissus aroma' (Ochsner, 1985). The plant-derived waxes are hydrophobic and biodegradable, and can be used as hair lotions, soaps or shampoos. The waxes are extracted from the flowers and form a protective film on the skin or hair. They are also said to improve 'combability' of hair. Daffodil fragrances are variously described as 'warm', 'fruity' or 'floral' and are often used to augment 'woody' or 'musk' aspects of perfumes.

Lectins derived from a number of plants, including the genus *Narcissus*, have an anti-nematode activity, causing mortality, reduced larval weight and/or delayed development, whilst being non-toxic to animals and birds. They can be used to protect a variety of crops including grain, cotton, potatoes, sugar cane, tomatoes, etc. (Birch *et al.*, 1995).

It was not surprising to find patents protecting the use of a medium containing abscisic acid in facilitating the multiplication of bulbs by tissue culture, or the method of encouraging vegetative bulb multiplication by cutting the bulb prior to deposition into substrate. The problems of policing some of these patents may be fraught with difficulties!

Therapeutic uses

The therapeutic uses of agents derived from daffodils under the narcissus/daffodil titles also included those that could be regarded as 'cosmeceuticals'. However, of particular interest are patents referring to the potential anti-viral activity of a mannose-specific lectin from, for example, *Narcissus pseudonarcissus*. A vaccine, produced from antibodies raised *in vivo* or *in vitro* to the lectin, is particularly relevant

to certain RNA viruses, which contain glycoproteins with mannose linkages, including Human Immunodeficiency Virus (HIV) and Human T lympho-tropic Virus (HTLV), either as a therapy or as a diagnostic (Forrest *et al.*, 1991).

In addition, the use of bulb extract with vinegar has been claimed for the treatment of fungal diseases of the skin, in particular, tinea pedis (Sugimoto, 1994).

Topical treatments derived from hot-water extracts of ground daffodil bulbs are claimed to dilate peripheral veins, improve blood flow to improve metabolism and thus complexion, and also to prevent skin ageing and damage to skin by ultraviolet light (Kobayashi, 1982).

PATENTS RELATED TO GALANTHAMINE

Galanthamine patents fall into four groups – production, formulation and use patents and galanthamine analogues. The parent molecule has been well categorised many years ago, so compound patents on the molecule itself do not feature in the literature. However, compound patents around analogues and derivatives of galanthamine and precursors of galanthine do.

Production patents

Introduction

Galanthamine was first extracted from members of the snowdrop family (hence *galanth*amine from the genus *Galanthus*). Commercially, daffodil bulbs are far more appropriate, as they are available in bulk supplies. Extraction from daffodils in the UK has been particularly relevant, as some 70% of the world's supply of daffodil bulbs come from the British Isles, with growers in the east of England playing a major role. Handling large volumes of bulbs has its problems. McFarlan Smith (Meconic Ltd.) in Edinburgh now has one of the largest plant extraction units in Europe dedicated to galanthamine extraction.

Clearly with bulb production being rather a slow process, the extraction method does lack some flexibility, and much effort has been put into exploring methods of synthetic production. Unfortunately, the galanthamine molecule has three chiral centres with eight possible enantiomers. In practice, one of these centres is effectively 'locked' and thus four stereoisomers are possible. However, the plant produces only one stereoisomer, the (–)-galanthamine, and it is this isomer that is most active pharmacologically. Synthetic production problems lie in producing the one active isomer and in overcoming a crucial oxidative phenyl-coupling step that has a particularly low yield.

Extraction patents

Given that extraction technologies contain as much art as science, it is not surprising that only a few patents exist around this method of production. However, such patents do exist, particularly from Eastern European authors, with the starting material sometimes involving sources other than daffodils. For example, the roots

and bulbs of the 'sunflower *Galanthus Kzasnovii*' (*sic*) are said to give an extraction galanthamine yield of 0.5% (dry weight) (Asoyeva, 1968).

Galanthamine can also be extracted from the summer snowflake *Leucojum aestivum* (Proskurnina, 1961), using an 8% ammonium aqueous solution plus dichloroethane or other organic solvents followed by a sulphuric acid treatment, with subsequent crystalline precipitation of other alkaloids such as lycorine and thebaine. Further treatment of the liquor with chloroform allows for extraction of the galanthamine itself by vacuum distillation.

The snowdrop (Proskyrni, 1960) is the starting material in another patent, the final liquor being treated with a solution of hydrobromic acid and acetone to form galanthamine hydrobromide. Ground, fresh amaryllis bulbs are moistened with 7% ammonium hydroxide and extracted with dichloroethane in another patent process file that leads to a yellow oily liquid which crystallises on standing (Asoyeva, 1963). Another variant of the *Leucojum aestivum* source involves treatments with aluminium oxide in the final stages of extraction (Ordzhonikidze Chem-Pharm, 1962).

A Japanese patent file describes the production of galanthamine, utilising clay or ion-exchange resins, from *Lycoris squamigera* (Shionogi & Co. Ltd., 1960). Extraction from the leaves and flowers of *Galanthus nivalis* (the common snowdrop) is the subject of another patent, but the practicalities of isolating from plants that are not available commercially in sufficient biomass limit the value of such approaches (Chimiko Pharmazetitschen Zavod, 1968). Production from *Ungernia victoris* (Aleksandrova *et al.*, 1994) in tissue culture is also cited as a commercially viable process. The claim that by first bringing the extract of bulb derived material to pH4 can lead to a pure form of the alkaloid galanthamine, is made in yet another publication (Hille *et al.*, 1996).

Production of synthetic galanthamine

The chiral nature of the galanthamine molecule with its three asymmetric carbon atoms has led to considerable problems in synthetic manufacture. Even though processes have been described several decades ago, commercialisation has, until recently, been severely limited by low yields. Obtaining an optimal starting point for (–)-galanthamine has been the subject of much research. In particular, narwedine has been the subject of several patent filings as a potential means of addressing the problem. For example, the preparation of a single (–)-narwedine enantiomer by seeding a solution of racemic narwedine with single enantiomer (–)-narwedine in the absence of amine base, plus the reduction of the resultant (–)-narwedine to the single (–)-galanthamine enantiomer is claimed, with the advantage that such (–)-narwedine prepared by this method is configurationally stable to racemisation in the solid state and can be produced on a large scale with very high purity (Potter and Tiffen, 1998).

Another approach has sought to limit the problems of possible contamination with unwanted epi-galanthamine compounds by incorporating specific reducing agents in the process of converting (–)-narwedine to (–)-galanthamine (Carlsson and Shieh, 1995).

Attempts to increase the yields in the phenolic coupling stage of the preparation of new and known narwedine derivatives include a method in which oxidation

occurs in a two-phase liquid system comprising aqueous base and organic solvent of dielectric constant of less than 4.8. The process improves the yield of sufficiently pure products in the organic phase to allow recovery by evaporation, avoiding relatively expensive chromatographic purification. These products can then readily be converted to corresponding galanthamine structures (Henshilwood and Johnson, 1996). Other filings from the same group describe asymmetric transformation of racemic narwedine-type compounds by reacting these racemates with an enantiomerically enriched acid to form a diastereomeric salt (Chaplin *et al.*, 1997b).

A method for 'easily' preparing narwedine, lycoramine, norgalanthamine and sanguinine by the preparation of intramolecularly coupled compound comprising of a phenol derivative with a supervalent iodine reagent is described elsewhere (Dyer *et al.*, 1996). As far as galanthamine itself is concerned, similar techniques can be used to prepare specific enantiomers, for example by seeding a supersaturated solution of racemic galanthamine salt with an enantiomerically enriched form of the salt, with or without the utilisation of an achiral counter-ion (Kagaku Gijutsu Shinko Jigyodan, 1999).

Of particular interest, as it has already led to a commercially available source of galanthamine, is an Austrian patent which embraces methods of producing new or known galanthamine products from benzaldehyde and phenethylamine derivatives. By reacting these together and reducing the product, an *N*-benzylphenylamine derivative is formed. This is then subjected to oxidative cyclisation and the product is then reduced (Tiffen, 1997).

A fascinating method of 'feeding' galanthamine precursors (derivatives) to plant extract to allow the enzymatic conversion by the plant material of the oxidative cyclisation precursor, is described. The object is to enhance yields of optically pure forms over and above those expected from straightforward extraction techniques (Czollner *et al.*, 1995).

Formulation patents

These patents fall into two categories – those related to combination therapy and those describing galenic presentation forms.

The combination of an acetyl-cholinesterase inhibitor with a muscarinic agonist (citing galanthamine as one of the preferred cholinesterase inhibitors with a variety of possible muscarinic agonists quoted) has been claimed to be useful in treating central and peripheral nervous system diseases (Bannister and McCague, 1997).

A fast-dissolving galanthamine hydrobromide tablet is the subject of published patent applications. The tablet includes a spray-dried mixture of lactose monohydrate and microcrystalline cellulose (75:25) as a diluent with a new disintegrant (Callahan and Schwarz, 1999).

Transdermal delivery of galanthamine is claimed to give better control of release of drug over a longer period of time (at least 24 hours), steadier serum levels and higher therapeutic effects at lower dosages with better patient acceptability (De Conde and Gilis, 1997). The system utilises a reservoir layer of active agent in a polymer matrix plus a penetration enhancer. This homogenous reservoir mixture is coated onto a backing layer and then covered with a protective layer. Interestingly, the same company has filings around the recovery of the active agent from unused or discarded transdermal therapeutic systems using solvent

extraction (Deurer and Hille, 1994). Another transdermal delivery system containing pergolide free base or its mesylate or hydrochloride salt is also described (Asmussen *et al.*, 1997).

An oral sustained release composition, in the form of a tablet or capsule, incorporates the use of active ingredient particles coated with an enteric protection agent, polyvinyl pyrrolidone (Fischer *et al.*, 1998).

New galanthamine analogues or derivatives

Benzazepine analogues and diaza-bi cycloalkanes (Davis and Goodman, 1994), methylapogalanthamine hydrochloride (Czollner *et al.*, 1997) (the latter having hypotensive properties), 1-aminomethyl-1-phenyl-4-hydroxy-cyclohexane derivatives said to be analgesic in activity (Abdusamatov, 1963), spiro-benzazepines (also analgesic and sedative) (Grelan Pharmaceutical KK, 1975) and 6-*O*-demethylgalanthamine (Grelan Pharmaceutical Co., 1975) are amongst a whole host of analogues and other salts (Davis *et al.*, 1995a,b,c; Chaplin *et al.*, 1997a; Christen *et al.*, 1997) that are claimed to be more potent, or less toxic or more brain-specific cholinesterase inhibitors, than galanthamine itself. Some of these patents cover not only the novelty of the molecules, but also method(s) of production.

Specific production patents include means of 'producing galanthamine derivatives by condensing and reducing a benzaldehyde derivative and a tyramine derivative, oxidatively cyclising the protected product and reducing the keto group' (Czollner *et al.*, 1996) and another by a ring closure method (Grelan Pharmaceutical Co., 1972).

Use patents

Patents discussed under this heading are often broader in scope than just being related to galanthamine. Analogues, and even cholinesterase inhibitors in general, are often also included in these claims.

The original uses of crude concoctions of snowdrop bulbs and, later on, of more highly purified materials, included an array of neuro-muscular disorders, such as poliomyelitis and myaesthaenia gravis, as well as more vague neurological conditions. The discovery of the reduction of levels of choline acetyl transferase in the brains of Alzheimer's Disease victims led to the search for compounds that could cross the blood-brain barrier and boost cholinergic activity in the basal nuclei, hippocampus and cortex. Tacrine was the first acetyl cholinesterase, to be developed for such a therapeutic use in Alzheimer's Disease, but it has a greater affinity for butyryl than for acetyl cholinesterase, and may lead to raised liver enzyme activity that can seriously restrict its use. Galanthamine is one of a number of so-called second generation inhibitors that do not have adverse activity on the liver, readily pass the blood-brain barrier, and are more specific inhibitors of the acetyl form of cholinesterase. In addition, galanthamine has nicotinic activities, which could well differentiate it from other second-generation cholinesterase inhibitors in the clinic.

The major indication for galanthamine at present is Alzheimer's Disease. Oral (10–2000 mg/day), parenteral (0.1 to 4 mg/kg) and intracerebroventricular administration via an implanted reservoir (0.01–5.0 mg/kg/day) are claimed to improve

cognitive function (Davis, 1987). A later patent was taken out by the same author for the same indication and routes of administration, but covering new derivatives of galanthamine or their analogues (Davis and Joullie, 1988).

Patients with Down's Syndrome (trisomy of chromosome 21) can develop histological changes resembling those seen in the brains of Alzheimer's patients. Learning ability in patients with Down's Syndrome may be improved by galanthamine (Fransits and Mucke, 1997).

One prolific investigator from Iceland has taken out a series of patents addressing the treatment of mania (Snorrason, 1994), fatigue syndromes (Snorrason, 1992a), various forms of arthritis (Snorrason and Murray, 1997), attention deficit disorders (Murray and Snorrason, 1999a) and proteolytic diseases (Murray and Snorrason, 1999b), as well as methods for countering the side-effects of benzodiazepine therapy (Snorrason, 1992b,c). Acute mania is a difficult condition to treat and often requires high doses of neuroleptics given for a number of days. The author claims galanthamine to be a faster treatment than other drugs. Stress is placed within the patent on the activity of galanthamine at nicotinic receptors.

Fatigue is a common enough symptom in primary care medicine, but its clinical importance cannot be over-estimated. It can be a major aspect of significant morbidity. Fatigue associated with viral infections, especially the Epstein Barr virus, can be prolonged and debilitating. Mononucleosis in late-teens or the University years can result in long periods of marked fatigue leading to considerable impairment of performance. In its severe form – myalgic encephalitis (ME) or Chronic Fatigue Syndrome (CFS) – patients experience debilitating fatigue lasting for more than 50% of the day over a period of six months or more. Such patients are usually in the 'bread-winning' years of life and may be unable to work for several years and can even be dependent upon a wheelchair. No effective treatment presently exists for this condition. Galanthamine is claimed as a treatment for fatigue, muscle pains and sleep disturbances in CFS. Claims are made for galanthamine, or its derivatives, to be effective in the treatment of various fatigue syndromes, including those associated with HIV infections and pre-eclampsia.

Cholinesterases also have proteolytic enzyme activity. Two patents from the Icelandic author, working with a British colleague, address the potential for galanthamine to have a positive effect on disease conditions in which proteolytic enzymes appear to play an important role. The first addresses the role of raised proteolytic enzyme and cholinesterase activity in the synovial fluid in the joints of patients with rheumatoid arthritis. Claims are made for galanthamine in the treatment of rheumatoid and osteo-arthritis, arthritis associated with auto-immune disease, psoriasis, chronic inflammatory bowel disease, ankylosing spondylitis, as well as a range of other joint disorders. Such treatment, if successful, could be expected to be disease-modifying rather than simply palliative. The second patent addresses head-on the concept that proteolytic activity in other diseases is associated with acetyl cholinesterase activity, and that this could be blocked or inhibited by treatment with galanthamine, epigalanthamine or norgalanthamine. Such diseases would include psoriasis, Crohn's Disease and ulcerative colitis.

Attention deficits and hyperactivity disorders (ADHD) are thought to affect 5% of children in the USA and Europe. Moreover, such behavioural problems can be carried into adult life where they may manifest themselves as alcohol or drug abuse and other anti-social behavioural traits. Current therapy in childhood

includes the use of methylphenidate and amphetamines. Claims for the modification of these behavioral problems if proved in the clinic could lead to an effective therapy that avoids the use of potentially habit forming drugs. (Another patent from different authors claims ADHD, but also the rarer and more bizarre Tourette's syndrome, as an indication for galanthamine (Davis, 1999).)

Counteraction of sedation, hypnosis or respiratory depression seen during benzodiazepine therapy, especially when high doses are given, can be achieved by co-administering galanthamine or its analogues or derivatives without interfering with the anxiolytic, anti-psychotic, anti-convulsant or muscle relaxant activities of the benzodiazepines. Particular emphasis is made in one of two patents from the same authors stressing the advantage of such combined therapy in patients with schizophrenia, but the patent also includes those suffering from anxiety, anxiety neurosis, panic disorders and agitating depression, as well as other psychiatric conditions.

Patients suffering from schizophrenia may experience improvement in states of apathy and aboulia when treated with 2 mg of amizil 1 to 3 times daily in conjunction with injections 30 minutes later of 2 ml of 0.5% galanthamine solution once or twice a day according to another patent (Kamenetski and Losev, 1985).

Alcohol and nicotine dependency can both be favourably influenced by galanthamine therapy according to two patents (Oplitz, 1991; Moormann and Moormann, 1994), who particularly stress the possibility of reducing the desire for alcohol or nicotine in addicted patients. These patents particularly, but not only, mention the transdermal delivery of galanthamine in such cases.

Given the early use of galanthamine it is not surprising, but very pleasing, to find a reference to treating spastic forms of cerebral paralysis in children with galanthamine and other drugs prior to bio-training sessions. Motor function was improved by 46% when galanthamine was used, compared with 33% for bio-training without galanthamine or 14% when galanthamine was used alone (Bogdanov *et al.*, 1984).

Electrodiagnostic methods for determining the degree of damage to a nerve can be improved in terms of accuracy by administering to the patient 1 ml of 1% galanthamine in solution a half to one hour prior to testing (Korletyanu, 1986).

Administering a cholinesterase inhibitor, such as galanthamine, either alone or in combination with levodopa, reduces rigidity, improves muscle function and alleviates dementia in Parkinson's Disease (Hutchinson, 1998).

Acute di:phenyl-hydramine poisoning can be treated more effectively, quicker and in a shorter period of time with riboxin (inosine) given together with galanthamine (Afanasev and Lukin, 1993).

A bio-stimulant composed of a lyophilised hydrosylate of royal jelly, creatinine, a calcium-magnesium salt of inositol phosphoric acid and galanthamine hydrochloride is claimed to be useful for patients recovering from severe or chronic illnesses. The combination is also claimed to help persons such as sportsmen or soldiers under great physical stress by acting on 'the energy balance of the body, improving the activity of the central and peripheral nervous systems and of cardiac and striated muscle' (Kirov *et al.*, 1983).

Another patent covers the alleviation of jet-lag, whereby galanthamine is used as a means of resetting a person's internal clock. The compound is given as an attention increasing agent to travellers prior to departure. The drug should be

given at a time between 4 a.m. and 3 p.m. of the time zone to be visited. To 'reset the clock', galanthamine should be administered at least 8 hours prior to the desired time of sleep. The authors believe such therapy could also be relevant to shift workers (Davis, 1996).

CONCLUSIONS

The powers of the genus *Narcissus* can be seen to be many and varied. Each plant should have its specific medical use, and clearly cognitive disorders and Alzheimer's Disease in particular must be the candidates for the daffodil. However, various forms of sexual dysfunction that can lead to severe strain on otherwise meaningful partnerships, and cause considerable distress to the sufferer, are not uncommon and can also be addressed by the noble daffodil. Physiological erectile impotence has been the subject of much recent research and has led to effective, if presently expensive, therapies. However, the use of galanthamine for erectile dysfunction due to physiological causes secondary to other disease states, disorders of the urogenital or endocrine systems, due to drug therapy (e.g., anti-hypertensives, anti-depressant and anti-psychotic drugs) or related to psychiatric problems, has been advocated (Katz, 1993). In addition, the Japanese patent that advocates the use of crude extracts of plants, such as *Lamium album, Sesamum indicum* and *Narcissus tazetta*, together with a number of fatty acids and surfactants to improve humectant properties, elasticity and smoothness of ageing skin would add to the surmise that daffodils could provide means of addressing the failings of advancing years (Taisho Pharm Co. Ltd., 1993). In any event, strong pharmacological reasoning, if backed by effective clinical studies, could well mean that the *Narcissus* genus will play an important role in medical and cosmetic practice well into the new millennium.

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For further alkaloids, readers should also consult Tables 6.1, 6.3 and 6.4; for further species and cultivars, Table 6.2; and for further biological and pharmacological effects, Table 6.4.

Narcissus and Daffodil is the first book to provide a complete overview of the genus *Narcissus*. While it has been prized as an ornamental plant in western Europe for several hundred years, it has only recently attracted attention there as a source of potentially valuable pharmaceuticals. However, in eastern European countries, *Narcissus* and other Amaryllidaceae have been valued as a source of galanthamine for several decades.

The book begins with an introduction to the biology of *Narcissus*, followed by a consideration of the folklore associated with the genus. The difficult subject of the classification of *Narcissus* is covered, with a new, practical conspectus of the recognised taxa. Commercial bulb production and the economics of bulb production are discussed. Included is a comprehensive coverage of the alkaloids of *Narcissus*, aspects of galanthamine sources, production, extraction and analysis, and galanthamine synthesis. Consideration is given to the pharmacological aspects of narcissus alkaloids, clinical trials of galanthamine in Alzheimer's disease and important aspects of narcissus chemistry such as acetylcholinesterase inhibitors, lectins and narcissus sap. The use of *Narcissus* in perfumery is also described and the volume concludes with a review of pharmaceutical patents.

Presenting original research material and information not readily accessible outside eastern Europe, *Narcissus and Daffodil* contains over 1,700 references, making it a valuable text for graduates and researchers both in academia and industry. It will be of particular importance for people working in the fields of horticulture, agriculture, medicine, pharmacy, pharmacology, perfume chemistry and drug manufacture.

About the Editor

Gordon R. Hanks started his career at the former Glasshouse Crops Research Institute, Littlehampton, UK, researching bulb crop physiology and agronomy. He now works at Horticulture Research International's Kirton site, situated near the heart of England's main bulb-growing region. His current research interests include foliar diseases of narcissus and predictive crop models for narcissus.

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Roland Hardman, BPharm BSc (Chemistry) PhD (London) FRPharmS, has had experience in retail, industrial and academic pharmacy. He has held academic posts in universities in Nottingham, UK; Ibadan, Nigeria (Head of Pharmacy Department); and Bath, UK (Reader and Head of Pharmacognosy) and is now retired. He has over 150 research and related publications to his name and has also held patents on the processing of plants and Plant Breeders' Rights. For some years he represented the UK on the committee for the Utilization of Medicinal and Aromatic Plants of the Economic Union and from 1988–1995 he was the President of the Section for Study of Medicinal and Aromatic Plants of the *Fédération Internationale Pharmaceutique*. Dr Hardman keeps in touch with the latest developments in the field through his work as a consultant.

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