Studies in Plant Science, 6

Advances in Plant Glycosides, Chemistry and Biology

C-R. Yang and O. Tanaka
(Editors)

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Studies in Plant Science, 6

Advances in Plant Glycosides, Chemistry and Biology

Proceedings of the International Symposium on Plant Glycosides, August 12-15, 1997, Kunming, China

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PREFACE

Glycosides are contained in various species of higher plants. The structural diversity of plant glycosides is caused by the large varieties and the stereochemical configurations of their sugar components. Aglycones belong to terpenoid, steroid, flavonoid, quinonoid, lignan, other simple phenolics, and isothiocyanate. Biological activities of glycosides are, in many cases, susceptible to the nature of sugar moieties, even though their aglycone is the same. Glycosides are main sources of natural medicines, cosmetics, and some functional vegetable diets. Therefore, more advanced knowledge of natural glycosides is required by the researchers and students in this field and by those who are dealing with the practical application of natural phytochemicals.

This volume records lectures, oral communications, and poster displays, which were presented during the International Symposium on Plant Glycosides, held in Kunming, Yunnan Province, China, in August 12-15, 1997. The symposium was excellently organized by Dr. Chong-Ren Yang (Kunming Institute of Botany, Chinese Academy of Sciences) as the chairman, Dr. Shi-Song Lin (Science and Technology Committee of Yunnan Prov.), Dr. Zhuang-Xin Zhang (Kunming Branch, Chinese Academy of Sciences) as the vice chairman, and other staff members of the organizing Committee.

Kunming is the most suitable place for holding this symposium with the background that Yunnan Prov. has plentiful botanical resources under a variety of climatological and geographical conditions from lower tropical to higher alpine. The chemical and biological research on plant glycosides and their application will progress towards the coming 21st century, for which this symposium has undoubtedly given a remarkable stimulation.

The contributing authors are highly appreciated for their cooperation in making the symposium fruitful and in developing this volume. Appreciation is also expressed to the host members of the Organizing Committee for their great efforts to run the symposium effectively.

Shoji Shibata, Dr. Pharm. Sc.
Professor Emeritus of University of Tokyo
Member of Japan Academy

PREFACE

In the plant kingdom, a variety of chemical constituents occur in a glycoside form (conjugation with sugar), and plant glycosides are the highly important target for modern scientific studies on utilization of plants.

Yunnan Province, China, is world-wide famous for abundant growing of a variety of plants; flora of the tropical, subtropical and temperate zones as well as flora which are characteristic of high mountain zone. These have attracted much attention of botanists and phytochemists of the world in view of plant systematics and development of new plant resources as well.

Kunming Institute of Botany, Chinese Academy of Sciences, is located in the center of this province. Phytochemists and botanists of this institute have extensively engaged in collaborative investigation of chemical constituents of the plants collected in this area, establishing a number of outstanding researches. This institute has an opened laboratory for the program of the international cooperative studies on phytochemistry, contributing to the progress of the chemistry of natural products in the world.

Based on this brilliant achievement, it is quite adequate that International Symposium on Plant Glycosides was organized and held by his institute and Scientific & Technological Committee of Yunnan Province. In this symposium, a number of up-date papers on chemistry, biochemistry and application of bioactive plant glycosides were presented by the distinguished scientists from the world.

It is my great pleasure that the proceeding of this symposium is published by Elsevier Sciences. This is undoubtedly valuable in both academic and applied field of phytochemistry.

I would like to express my sincere thanks to Professor Dr. Chong-Ren Yang, the Chairman of the Symposium who is the most admirable Chinese friend of mine for his effort on the publication. I am also deeply grateful to the members of the Institute for their cooperation.

June 16th, 1998

Osamu Tanaka, Ph.D.
Emeritus Professor of
Kunming Institute of Botany and
Hiroshima University

FOREWORD

Glycoside is an important secondary metabolite in plant kingdom, with the characters of higher polarity, higher molecular weight, instability as well as complicated chemical structure. Not only for its difficulties of isolation and purification, but also for the important biological function in plant life and the remarkable biological activities, it is attracting more and more attention and interests from botanists and phytochemists in the world. From the 80s, there have been a great advance in the researches of plant glycosides. And now, plant glycoside becomes a very important resources of natural medicine, healthy food, cosmetics and food supplementary.

The first International Symposium on Plant Glycosides (ISPG) was finally held in Kunming, Yunnan Province, China, in August 12-15, 1997, organized by Kunming Institute of Botany, Chinese Academy of Sciences, and Science and Technology Committee of Yunnan Province. More than 150 scientists from seventeen countries and areas attended this symposium. During the four days' meeting, 96 reports on plant glycosides, including structure elucidation, ethnobotany, pharmacology, quantitative evaluation, synthesis, pharmacology, biotechnology and so forth, were presented and discussed profoundly. All of these papers expressed the recent research results in the last decade on plant glycoside and its developing tendency. I believe that it must contribute a lot to the progress of this field in the future.

With the effort of all of contributors and members of the Organizing Committee, the Proceedings of this symposium is now finally published by Elsevier Sciences, the Netherlands. 54 up-date papers were presented by the distinguished scientists from the world, are collected in this proceedings.

Here, I would like to express my sincere thanks to all of the participants for their support and contribution to make the symposium successful and fruitful. Thanks all of the contributing authors for their cooperation to make the publication of this proceedings smoothly. I would also like to thank all of the following institutions and corporations for their financial support to the symposium:

The State Committee of Science and Technology of China
Chinese Academy of Sciences
Scientific and Technological Committee of Yunnan Province
Kunming Branch of Chinese Academy of Sciences
Phytochemistry Laboratory of Kunming Institute of Botany
UNESCO
Beckman Instrument, Inc. of USA
Buchi Company of Switzerland,
Hua-Yee Instrument Company of Hong Kong
EUROP Continents of France
LVMH Recherche of France
Oriental Medicine Society of Japan
Wakunaga Pharmaceutical Co. Ltd. of Japan
Sino-American Kunming Green Field Biological Product Co. Ltd.
Weihe Health and Foodstuff Ltd. Co. of China.

Also, I would like to thank my colleagues for their great effort to make everything concerning of the meeting smoothly and effectively.

I am deeply grateful to Prof. Shoji Shibata, member of International Advisory Board of ISPG, and Prof. Osamu Tanaka, Chairman of the Scientific Committee of ISPG, for their great contribution to the symposium and preparing the preface for this proceedings.

The first International Symposium on Plant Glycosides was ended, left us many unforgettable memory and also many things to do, to study and to think. The research on plant glycoside and its application will be further developed and will have a very amplitude future. I am looking forward to the opening of the second International Symposium on Plant Glycosides being held in Japan in 2000.

Chong-Ren Yang
Professor, Ph.D.
Kunming Institute of Botany
Chinese Academy of Sciences

CONTENT

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CHEMICAL IDENTIFICATION OF THE OLD DRUGS STORED IN SHOSOIN, THE IMPERIAL STORE HOUSE
AN UNIDENTIFIED DRUG SPECIMEN, N-93, IN SHOSOIN

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Among old drug specimens stored in Shosoin for more than 1200 years, only one, N-93, has been unidentified, since the first scientific investigation was made in 1948-1949 under the conduct of the late Professor Yasuhiko Asahina. The drug N-93, which had been labelled as Ginseng, was morphologically proved not to be identical with ginseng and assumed to be Fankui (防葵) or Landu (狼毒), while it was also supposed to be Niupiexiao-gen (牛皮消根イケマ) or Geshan-Baishouwu (隔山白首烏コイケマ) because of its similar appearance to theirs.[1)]

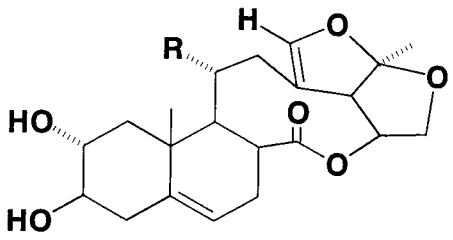
Fankui and Landu were recorded on the original drug catalogue of Shosoin (種々藥帳), but now they are lost. Landu is the root of euphobiaceous plant, *Stella chamajasme* L. The original plant of Fankui is not certain, but may be *Peucedanum japonicum* Thunb. (Umbelliferae).

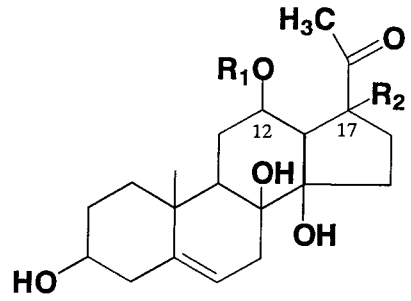
Niupiexiao-gen and Geshan-Baishouwu are the roots of asclepiadaceous medicinal plants, *Cynanchum caudatum* Maxim. and *C. wilfoldi* (Maxim.)Hemsl., respectively, but both of them were recorded neither in the original Shosoin drug catalogue nor in any other medical literatures in the early Tang Dynasty when most of the Shosoin drugs were brought from China. Moreover, by the microscopic observation, the cut section of N-93 specimen was revealed to be slightly different from that of authentic specimens of these Cynanchum drugs: N-93 has stone cells not only in the primary and secondary epidermis parts but also in the xylem, even in the part of rootspit.

The chemistry of polyhydroxypregnane ester glycosides in asclepiadaceous plants has extensively for various *Cynanchum* species gas been provided especially by H. Mitsuhashi and his coworkers.

Morphologically and chemotaxonomically, the plants of *Cynanchum* spp., which involve more than 18 Chinese medicinal plants, are divided into 2 groups: those in one group have hairy roots containing mainly 13, 14-14, 15-disecopregnane as the aglycone of ester glycosides, and those in the other group have big main roots with two or three branches downward containing 14β-pregnene as the aglycone. Some Chinese botanists proposed to classify the former group as an independent genus, *Vincetoxicum*. The original plants of Baiqian (白前)and Baiwei (白薇), *Cynanchum stauntonii* (Decne.) Schltr. ex Levl. and *C. atratum* Bge., belong to the former group containing glaucogenin A and B, and those of Niupiexiao-gen (牛皮消根) and Geshan-Baishouwu (隔山白首烏), *C. caudatum* Maxim. and *C. wilfordi* (Maxim.) Hemsl., belong to the latter group containing lineolon or desacylmetaplexigenin as the aglycone.

The glycosides isolated from the root of C. caudatum were investigated by Mitsuhashi and his collaborators [2)], and those isolated from the root of C. *auriculatum* (耳葉牛皮消) were formulated by J-J. Chen et al.[3)]





R = H	Glaucogenin A	**R1 = R2 = H**	Lineolon
R = OH	Glaucogenin B	**R1 = H, R2 = OH**	Deacylmetaplexigenin

Chart 1 Aglycones of Cynanchum glycosides

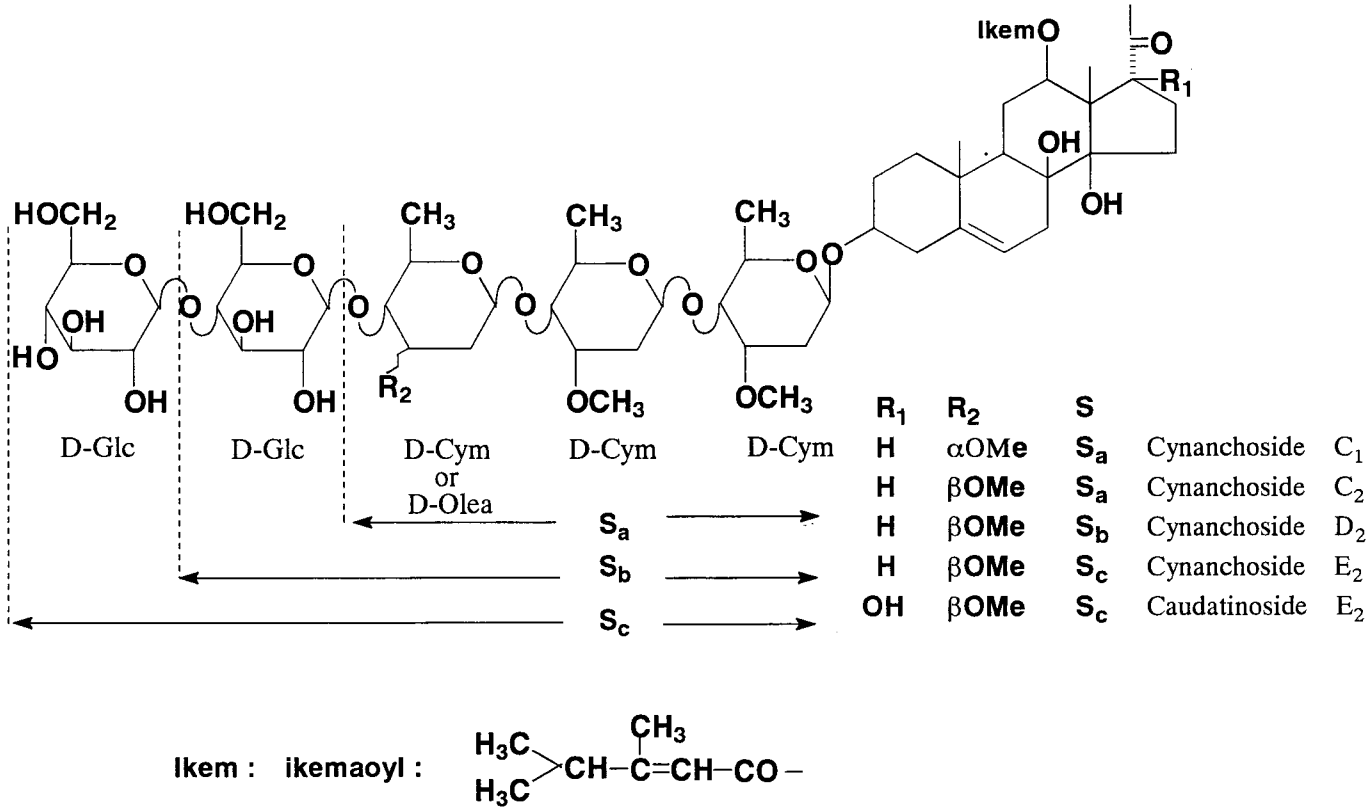


Chart 2 Glycosides isolated from the root of *Cynanchum caudatum* (牛皮消根)

K.Wada, K.Hayashi, H.Mitsuhashi, H.Bando: Chem. Pharm. Bull., **30**, 3500(1982)

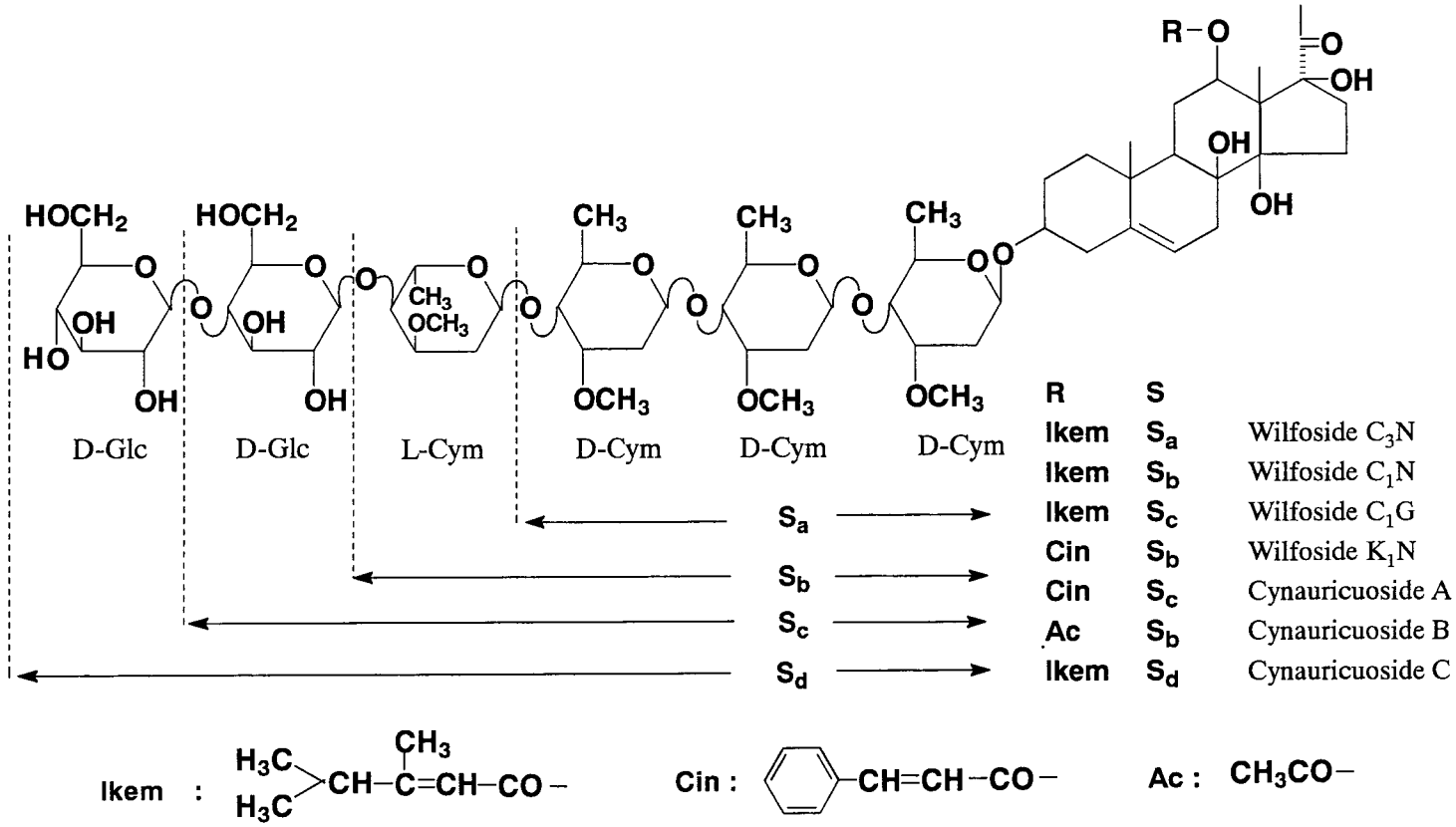


Chart 3 Glycoside isolated from the root of *Cynanchum auriculatum* (耳叶白首烏)
Chen J-J, Zhang Z-X, Zhou J.:Acta Botanica Yunnanica **12**, 197-210 (1990)

Judging by the morphological appearance, the specimen N-93, which is a cylindrical long main root, is apparently related to the latter group of Cynanchum. For the reference, the Chinese drugs on the market, which belong to *Cynanchum caudatum* group, are tabulated below:

Table 1 Chinese herb drugs of Cynanchum spp. having a thick cylindrical main root

Drug name	Botanical origin			Chemical constituent	
	Chinese name	Latin name	Japanese name	Aglycone ester	Aglycone
牛皮消根	牛皮消	*C. caudatum*	イケマ (IKema)	Cynachogenin	Lineolon
青羊参	青羊参	*C. otophyllum*		Qingyanshengenin	Desacylmetaplexigenin
	昆明杯冠藤	*C. wallichii*		Qingyanshengenin	
	(断節参)				
	麗江牛皮消	*C. likiangense*			
	戟葉鵝絨藤	*C. sibiricum*		Sibirigenin	17-α-H-lineolon
	戟葉白首烏	*C. bunqei*			
白首烏	耳葉牛皮消	*C. auriculatum*			
	隔山牛皮消	*C. wilfordi*	コイケマ (Koikema)	Caudatin	Desacylmetaplexigenin
	鵝絨藤	*C. chinese*			
	朱砂藤	*C. officinale*			
	豹葉藤	*C. decipiens*			

2,6-Dideoxysugar such as cymarose commonly exists in the sugar components of the ester glycosides of asclepiadaceous plants including *Cynanchum* spp., which can be detected by the positive Keller-Kiliani colour reaction of the extracts. Applying the above mentioned chemical findings, we performed a renewed chemical investigation to identify the specimen N-93 in comparison with known Chinese herb drugs of *Cynanchum* spp. which have cylindrical main roots.

A part of the specimen N-93 was extracted with chloroform, and a glycoside fraction (S-chf) was obtained. The residue was extracted with methanol. For the reference, the chloroform extracts of the roots of *C. caudatum* and *C. wilfordii* were prepared and named A-chf and B-chf, respectively. All the chloroform extracts, S-chf, A-chf and B-chf, gave positive Keller-Kiliani reaction and Liebermann-Burchard reaction, showing the presence of 2,6-dideoxysugar and steroidal skeleton in their structures, respectively. However, the HPTLC pattern of S-chf on a silica gel plate developed by $CHCl_3$-MeOH-H_2O (65:35:10, lower layer) was different from those of A-chf and B-chf. (Fig. 1)

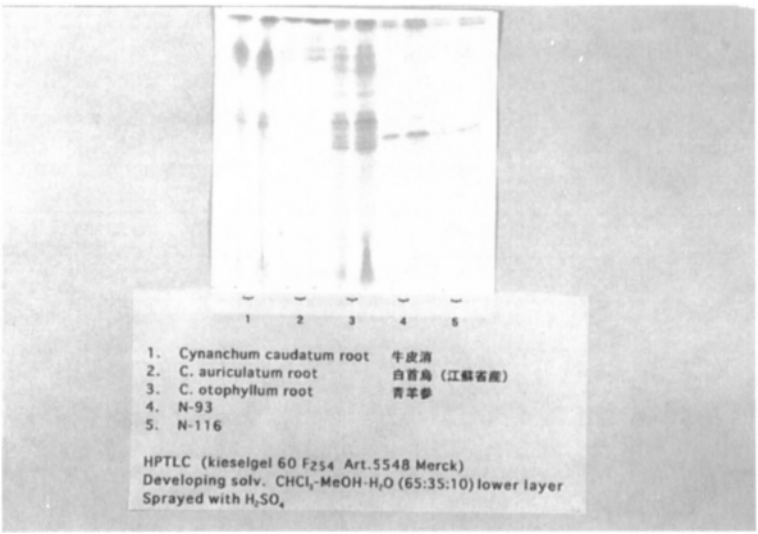


Figure 1 HPTLC Pattern for chloroform Extracts of the Roots of *Cynanchum caudatum*, *C. auriculatum* and *C. otophyllum* in comparison with the Shosoin Sample N 93 and N 116

The glycoside fraction (S-chf) of N-93 extract was subjected to acid hydrolysis. The esteric aglycone part was chromatographed over a silica gel column developed by methanol/chloroform, and 5 fractions were separated. The main fraction, purified by rechromatography and subsequent recrystallization, gave colourless prisms and was tentatively named SS-AG. SS-AG was treated with alkali, separating lineoron as the deacylated aglycone. From the sugar portion, cymarose was obtained and confirmed by TLC. (Fig. 2) The sugars, esteric aglycones and deacylated aglycones obtained from S-chf, A-chf and B-chf gave obviously different chromatographical patterns, respectively.

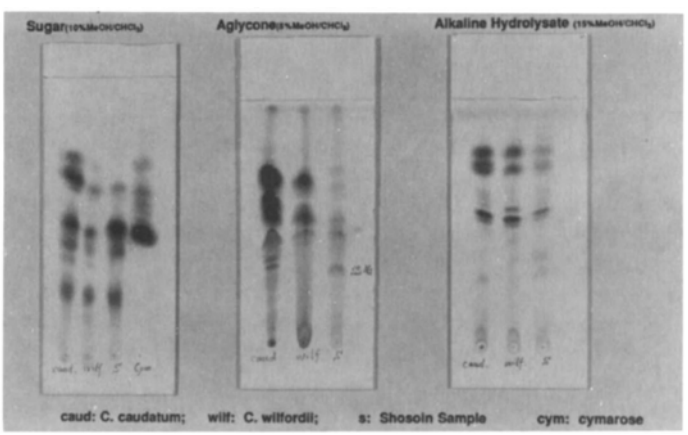


Figure 2 TLC Pattern for Sugar, Aglycone, and Alkaline hydrolysate of Aglycone of *Cynanchum caudatum*, *C. wilfordil*, and Shosoin sample

Experimental

The pulverized specimen N-93 (4.09 g) was extracted with $CHCl_3$ (60 ml) twice, each for 24 hr, at room temp., and an extract (S-chf) was obtained with a yield of 230 mg. The residue was extracted twice with MeOH (60 ml each), separating a fraction, S-me (162.1 mg). The roots of *C. caudatum* (Ikema) and *C. wilfordii* (Koikema) were extracted ass above for the reference (A-chf and B-chf).

S-chf was dissolved in $CHCl_3$ and hexane (x 40 in volume), and the mixture was allowed to stand overnight. The precipitates formed were dissolved in MeOH. The methanolic solution was added with warm 0.1N H_2SO_4 (60 ml) and allowed to react being stirred on a water bath 60_0C for 1 hr. After dilution with water (65 ml) and evaporation in vacuo below 40^0C, the residue was extracted three times with $CHCl_3$ (60 ml each) and then washed with water, 5% $NaHCO_3$ and 2N HCl, subsequently.

The esteric aglycone part (85 mg) was accumulated in the CHCl3-soluble fraction, and the sugar portion (28 mg) was concentrated in the aqueous fraction neutralized through an anion ion-exchange resin column. From a part of the aglycone portion (2 mg), treated with 5% KOH/MeOH (1 ml) and allowed to stand overnight at room temp., lineolon was proved by TLC. Some additional spots, which have UV-absorption and resist against complete hydrolysis, were shown on the TLC. Cymarose was indicated on TLC of the sugar fraction.

The esteric aglycone portion (80 mg) was chromatographed over a silica gel column developed by MeOH/$CHCl_3$, separating into 5 fractions. The main fraction, Fr.3, (18.6 mg)

was recrystallized from hexane/acetone and gave colourless prisms, SS-AG (III), with a yield of 7.2mg. SS-AG(III): m.p.>300^0, $[\alpha]_D$ -37.2^0 (C=0.285, $CHCl_3$).

^{13}C NMR (δ in ppm, C_5D_5N): 15.9 (C-18), 18.3 (C-19), 22.1(C-16), 25.1(C-11), 31.9 (C-2), 32.0 (C-21), 34.2 (C-7), 35.2 (C-15), 37.5 (C-10), 39.2 (C-1), 43.2(C-4), 44.7 (C-9), 56.2 (C-13), 60.4 (C-17), 71.6 (C-3), 73.0 (C-12), 74.7 (C-8), 87.5 (C-14), 116.1 (C-4', 6'), 118.4 (C-6), 122.1 (C-2'), 132.4 (C-3', 7'), 140.4 (C-5), 163.5 (C-5'), 165.5 (C-1'), 209.3 (C-20).

^{1}H NMR (δ in ppm, $CDCl_3$): 1.37 (3H, s, C-18Me), 1.42 (3H, s, C-19Me), 2.04 (3H, s, C-21Me), 3.51 (1H, t, J=9.3 Hz, C-17βH), 3.86 (1H, m, C-3αH), 5.33 (1H br. d, J=4.6, C-6H), 5.38 (1H, dd, J=11, 8.4, 1 Hz, C-12αH), 7.20 (2H, d, J=8, 8 Hz, C-4'H and C-6'H), 8.25 (2H, d, J=8.8 Hz, C-3'H and C-7'H).

IR γ^{KBr}_{max} cm^{-1}: 3536, 3372, 1714,1690,1612,1594, 1516,1278, 850.

UV λ^{EtOH}_{max} nm(ε): 259 (27000)

EI-MS m/z: 346($C_{21}H_{30}O_4$, M-138), 328(138-H_2O), 313(328-CH_3), 295(313-H_2O), 208(346-138), 121,93.

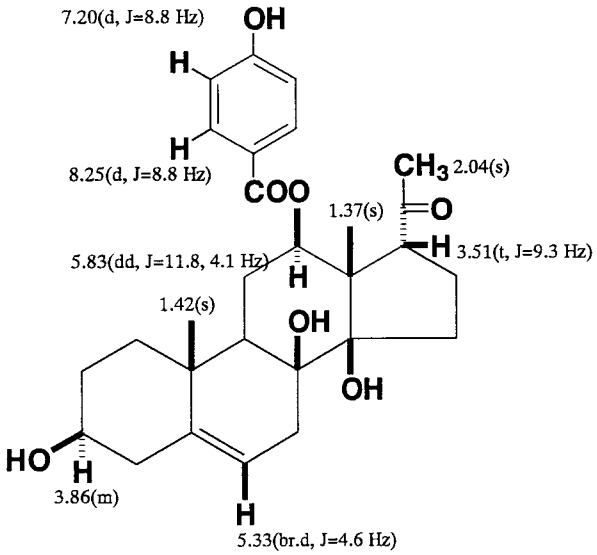
HR-EI-MS calcd. for $C_{21}H_{30}O_4$ m/z: 346.21441

Found m/z: 346.211397.

Discussion

IR, UV and ^{1}H NMR of III revealed the Presence of *P*-substituted aromatic carboxylic ester in the molecule. EI-MS of III, which gave m/z 346($C_{21}H_{30}O_4$) and m/z 208 ($C_{12}H_{16}O_3$) fragments, revealed the release of *P*-hydroxybenzoic acid and a fragment formed by the retro Diels-Alder reaction, respectively. The molecular formula of III is $C_{28}H_{36}O_7$ whose ^{13}C NMR data are corresponding to those given by cynanchogenin except their ester portions. Therefore, III is *P*-hydroxybenzoyl ester of lineolon (I), and the ester group is attached to $C_{(12)}$ from the data of ^{1}H NMR. Thus, III is formulated as to be 12β-*p*-hydroxybenzoyl-3β, 8β, 14β-trihydroxypregn-5-en-20-one.

^{1}H-NMR($CDCl_3$)



^{13}C-NMR(Py-d_5)

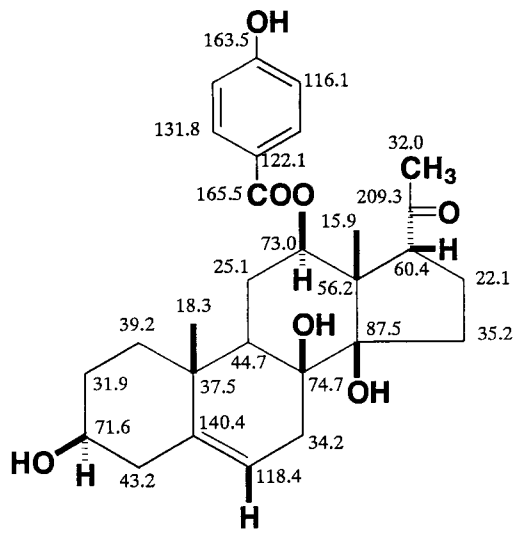


Chart 4 **NMR chemical shifts of SS-AG**

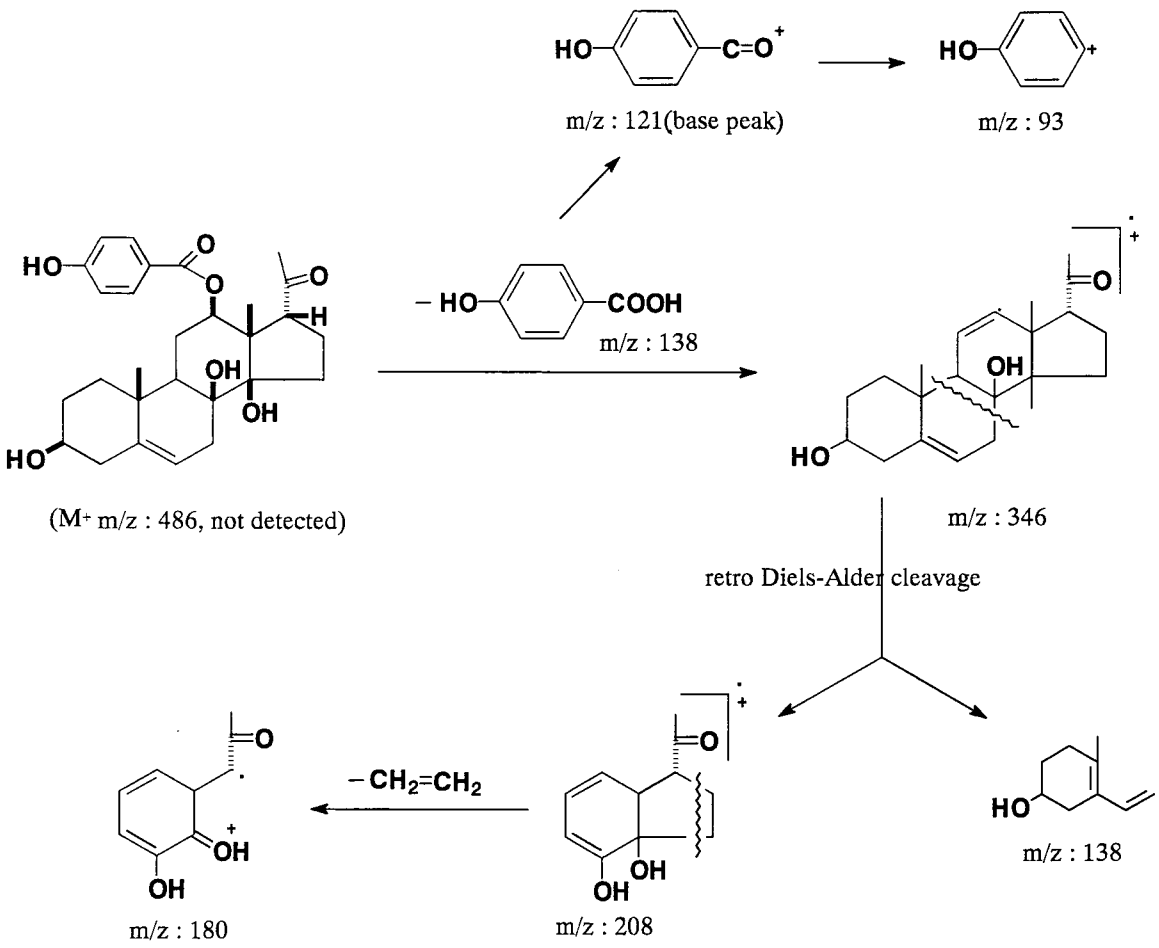
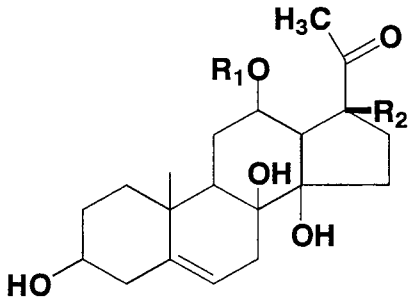
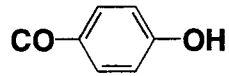


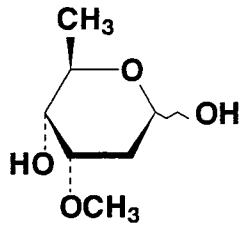
Chart 5 **Mass fragmentation of SS-AG**



	R_1	R_2	
Lineolon(I)	: H	H	
Cynanchogenin(Ⅳ)	: ikemaoyl	H	*C.caudatum*
Caudatin	: ikemaoyl	OH	*C.caudatum, C. wilfordi*
Deacylmetaplexigenin(Ⅵ)	: H	OH	
Qingyanshegenin(Ⅴ)	: *p*-hydroxybenzoyl	OH	*C.wallichii, C.otophyllum*
SS-AG(Ⅲ)	: *p*-hydroxybenzoyl	H	

p-hydroxybenzoyl : 

ikemaoyl : $CO-CH=C(CH_3)CH(CH_3)_2$



cymarose (main sugar component of the glycosides)

Chart 6 The main esteric aglycone(SS-AG) obtained from Shosoin-drug N-93 and some other aglycones of Cynanchum glycosides as the reference.

The presence of lineolon as an aglycone of the ester glycoside contained in N-93 specimen of Shosoin drug revealed that it must be the root of *Cynanchum* spp. But lineolon P-hydroxybenzoyl ester has not been found as yet as the esteric aglycone from any other herb drugs of *Cynanchum* spp. so far identified, whereas *p*-hydroxybenzoate of 17β-hydroxylineolon has been isolated from C. otophyllum[4] and C. wallichii root[5] as to be qingyanshengenin (V). Therefore, the ester glycoside (III) isolated from N-93 specimen is a new compound. Hence, the original plant of N-93 has not chemically been identified as yet, but it undoubtedly belongs to a member of *Cynanchum* spp. which has a thick cylindrical main root.

Acknowledgements I wish to thank the late Prof. H. Mitsuhashi and Dr. K. Hayashi, Research Laboratory of Tsumura Co., Ltd., and Prof. T. Okuyama, Meiji College of Pharmacy, for their kind collaboration for this investigation. This report has been submitted under the permission of the Shosoin Office, Imperial Household Agency, to whom I am deeply indebted.

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SIGNIFICANCE OF GLYCOSIDES IN FOOD MATERIALS

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Introduction

Other than the so-called nutrients such as proteins, lipids, carbohydrates, minerals and vitamins etc., secondary metabolites in food materials play important roles as fragrances, spices, sweeteners, pigments and so on. Recently, phytochemicals in food materials have attracted much attention in pharmacological activities of diets and beverages. Now, some topics of biologically active glycosides in food materials are reviewed.

Dihydroflavonol Glycosides from Leaves of *Engelhardtia chrysolepis* (Juglandaceae)

This ever green tall tree (黃杞、huang-qi in Chinese, koh-ki in Japanese) grows wild in southern region of China, and the dried and processed leaves have been locally used as a health-giving tea. The dried leaves taste slightly sweet. Kasai et al., Hiroshima University under the cooperation of Wen-Hua Zhou et al., South China Institute of Botany, Chinese Academy of Sciences, Guangzhou, searched a sweet principle, isolating the four known dihydroflavonol-rhamnosides (dihydroquercitrin); astilbin [3-O-L-rhamnoside of (+)-taxifolin (=2R, 3R-dihydroquercetin)][1], and its three diasteromers, neoastilbin, isoastilbin and neoisoastilbin (Fig. 1) [2]. Of these four isomers, neoastilbin tastes weakly sweet. Treatment of each isomer with aqueous pyridine, NaOAc-EtOH, aqueous CH_3CN or hot H_2O, afforded an equilibrated mixture of the four isomers[3, 4]. Yields of isolation of these dihydroflavonol-rhamnosides from the leaves in a preparative scale were rather low, but quantitative analysis by HPLC revealed that a total content of the four isomeric rhamnosides is nearly 4 %. This means that huang-qi tea prepared by decoction of 1 g of the leaves, contains about 40 mg of the dihydroflavonol rhamnosides.

Together with these compounds, the known compounds, eucryphin and quercitrin [1], and a new sweet glucosyl-rhamnoside of (+)-taxifolin named huangqioside E [5] were also isolated as minor constituents of the leaves (Fig. 1).

The surprisingly high content of dihydroflavonol rhamnosides (astilbin and its isomers) in the leaves prompted us to investigate biological activities of the extract, astilbin and its isomers as well as taxifolin (aglycone). The following activities have been observed by Mizutani, Kambara et al., Maruzen Pharm. Co. Ltd.[6].

Antioxidative activity: The methanolic extract as well as astilbin and (+)-taxifolin exhibited potent inhibitory activity against the autoxidation of linoleic acid (ferric thiocyanate method) which is stronger than BHT, a potent synthetic antioxidant.

Suppression of active oxygen generation: In the assay of suppression of generation of superoxide anion in xanthine - xanthine-oxidase system (NBT-reduction method), astilbin and (+)-taxifolin exhibited potent activity which was stronger than baicalein, the potent antioxidative flavonoid. The similar activities were observed for quercitrin, neo-, iso- and neoiso-astilbins and also for the extract. It was further revealed by the *in vitro* assay that astilbin and (+)-taxifolin suppressed active oxygen species such as singlet oxygen, peroxides, hydroxy radical and other radicals including superoxide anion.

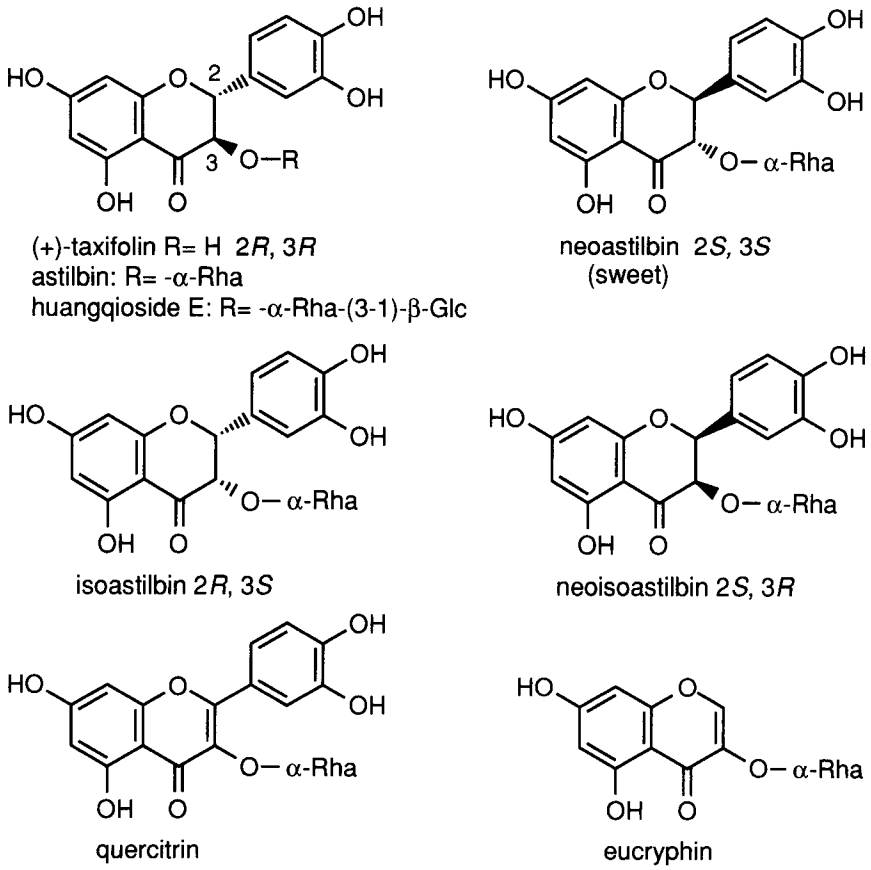


Fig.1 Phenolic constituents from leaves of *Engelhardtia chrysolepis*

Inhibition of lipid peroxidation: It has been recognized that the active oxygen species causes the peroxidation of lipids, resulting in aging, carcinogenesis and other diseases of aged people. Haraguchi's group, Fukuyama University and our group proved that (+)-taxifolin and astilbin show potent protective effects on rat microsomal and mitochondrial lipid peroxidation. Both the compounds also protect the oxidative hemolysis of human erythrocyte.

Igarashi's group, Yamagata University disclosed that on feeding the diet containing astilbin or (+)-taxifolin, peroxidized lipid level in serum and liver of rats was decreased. Further, the activities of two antioxidation enzymes, superoxide dismutase (SOD) and catalase in blood were enhanced by feeding the diet containing astilbin or (+)-taxifolin.

Antiallergic and antiinflammatory activities in vitro and in vivo: Hyaluronidase has been known to concern with allergy, inflammation, migration of cancer cells, etc. In *in vitro* assay, the extract, astilbin and (+)-taxifolin inhibited the activity of this enzyme.

The inhibition on release of b-hexosaminidase from rat basophilic leukemia (RBL-2H3) has been employed to determine an inhibitory index of histamine release. In this test, the extract and (+)-taxifolin showed the inhibitory activity.

In *in vitro* test, oral and external administration of the extract, astilbin and (+)-taxifolin exhibited suppression of picryl chloride induced contact dermatitis and carageenin induced paw

edema in mice.

Antitumor promoting activitiy: Carcinogenesis is generally composed of multistage process, initiation stage, promotion stage etc. Recently, studies on inhibitory effects of natural products on the promotion stage have attracted much attention. To prevent carcinogenesis, daily intake of the active diets and beverages may be preferable to intake of the active medicines in view of economical problems and side effects. Inhibitory activities of huang-qi extract and its constituents against tumor promotion have been investigated extensively.

A test of inhibitory effects on Epstein-Barr virus early antigen activation in Raji cells induced by a combination of n-butylic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA) has been employed (EBV-EA test) as a simple primary screening for antitumor promoting agents. In this test, significant inhibitory effect was observed for astilbin and its isomers as well as (+)-taxifolin. Of these dihydroflavonol derivatives, neoastilbin showed the strongest activity.

As *in vivo* experiments, the following tests have been conducted.

1. Two stage mouse skin carcinogenesis initiated with dimethylbenzanthracene (DMBA) and promoted with TPA: Topical application of astilbin or (+)-taxifolin exhibited significant inhibitory effect.

2. Two stage mouse skin carcinogenesis induced with DMBA and promoted with UV (B-region) irradiation : Oral administration of the extracts exhibited inhibitory effect.

3. Two stage mouse pulmonary carcinogenesis initiated with 4-nitroquinoline-N-oxide and promoted with glycerol: Oral administration of astilbin, (+)-taxifolin or the extract showed potent inhibitory activities.

Glycosides and Glycosidases in Food Materials

In living plant cells, chemical constituents and related enzymes are generally located separately from each other, while destruction of plant cells by crushing, cutting, withering, heating or freezing, results in the contact of a constituent with an enzyme, leading to the modification of a constituent. In production of frozen vegetables, materials are sometimes heated for a short time before freezing (deactivation of enzymes, named blanching process) to prevent the deterioration.

Such an enzymic modification is observed for glycosides. By destruction of plant cells, a glycoside is contacted with a corresponding glycosidase, leading to the hydrolysis into an aglycone and sugars.

It has been revealed that glycosides of monoterpenes and phenylpropanoids occur in plants as a fragrance precursors which generate fragrance principle by hydrolysis with co-occurring glycosidase. Recently, Yoshikawa, Arihara et al., Tokushima Bunri University, isolated several fragrance precursors from fresh flower-buds of Citrus unshiu. (Fig. 2) [7].

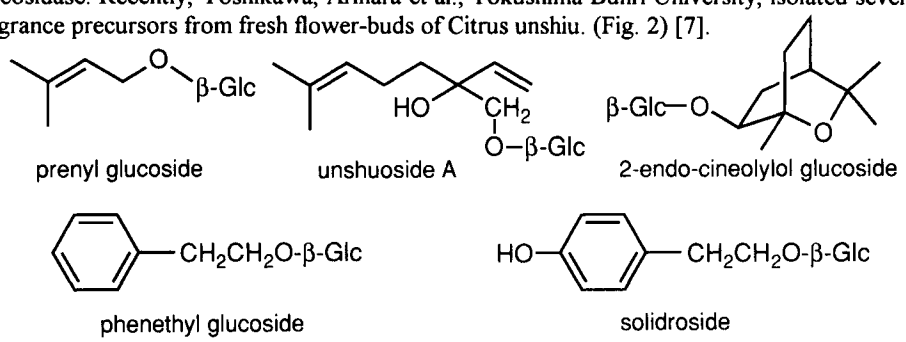


Fig.2 Fragrance precursors from fresh flower buds of *Citrus unshiu*

In case of a glycoside of an unstable aglycone which is stabilized by binding with sugar

moiety, the hydrolysis is accompanied by secondary structure modification to form an artificial aglycone (artifact). In some cases, the resulting artificial aglycones exhibit characteristic physiological activities. As to the case of food materials, the following examples have been well-known. Toxic aglycones: HCN from cyanogenic glycosides; HCHO and CH_3OH from cycasin (pseudocyanogenic glycosides from *Cycas revoluta*), goitrin from progoitrin (glucosinolate from *Brassica* spp.) (Fig. 3); a carcinogenic dienone from ptaquiloside (sesquiterpene glucoside from bracken) etc. Pungent aglycones: isothiocyanates from glucosinolates.

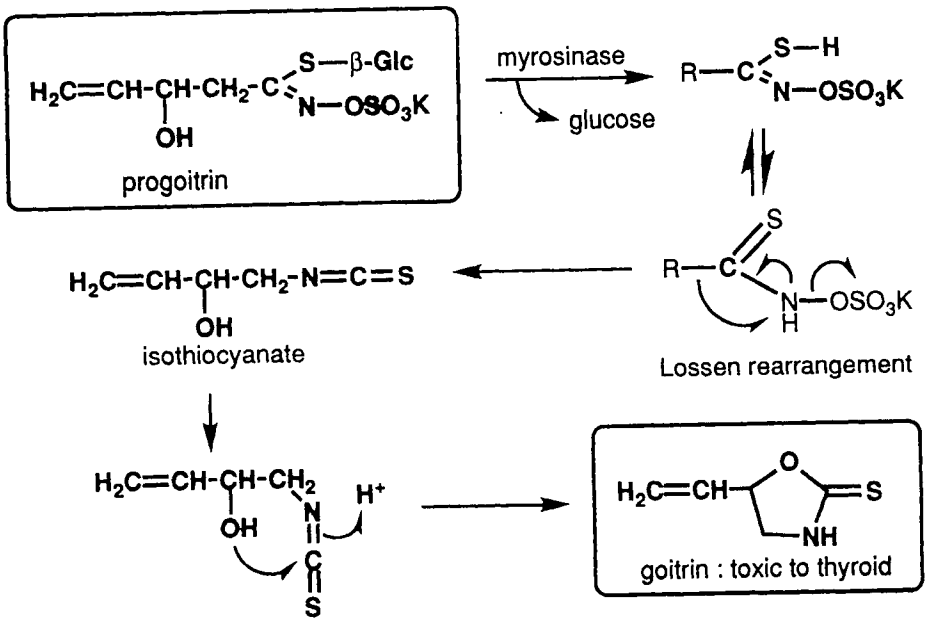


Fig. 3. Progoitrin, glucosynolate from Brassica spp.

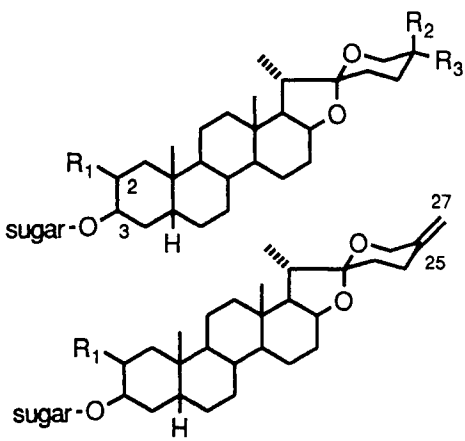
Antiyeast spirostanol-monodesmosides The deterioration of cooked foods is known to be caused by yeasts.

We have examined antiyeast saponins, and disclosed that a saponin fraction from stems of Mohave yucca which is mainly composed of spirostanol monodesmosides, exhibited potent antiyeast activity against brewing yeast (*Saccharomyces cerevisiae*), food-detriorating yeasts (*Candida famata, Hansenula anomala, Cryptpcoccus* sp., and *Pichia carsonii*) and film-forming yeasts (damaging of soy sauce) (*Pichia nakazawae, Debaryomyces hansenii* and *Zygosaccharomyces rouxii*) (Table 1). This fraction was also exhibited growth inhibition of some dematophytic yeasts and fungi.

Table 1. Antiyeast Antifungal Activity of Yucca Saponin Fraction

	MIC (μg/ml)		MIC (μg/ml)
Saccharomyces cerevisiae	62.5	*Debaryomyces hansenii* **	31.3
Candida famata *	31.3	*Zygosaccharomyces rouxii* **	31.3
Hansenula anomala *	31.3	*Candida albicans* ***	62.5
Cryptococcus laurentii *	125	*Trichophyton rubrum* ***	15.6
Kloeckera apiculata	62.5	*T. mentagrophytes* ***	31.3
Pichia nakazawae **	31.3	*Sabouraudites canis* ***	31.3
P. carsonii *	31.3	*Epidermophyton floccosum* ***	31.3

*food-deteriorating yeastt **film-forming yeast in soy sauce ***dermatophytic yeast and fungi



aglycone

sarsasapogenin (sarsa)
$R_1 = R_3 = H$, $R_2 = CH_3$
smilagenin (smila)
$R_1 = R_2 = H$, $R_3 = CH_3$
markogenin (marko)
$R_1 = OH$, $R_2 = CH_3$, $R_3 = H$

aglycone

5β-spirost-25(27)-en-3β-ol (en-ol)
$R_1 = H$
5β-spirost-24(27)-ene-2β,3β-diol
(en-diol) $R_1 = OH$

sugar moiety

1: -β-Glc-2-β-Glc
3-β-Xyl

2: -β-Gal-2-β-Glc
3-β-Xyl

3: -β-Glc-2-β-Glc
3-β-Glc

saponin	aglycone	sugar	MIC (μg/ml) *S.c.*	*C.a.*	*H.a.*
YE-1	en-ol	1	12.5	12.5	3.13
YE-2	mixture of sarsa and smila		12.5	12.5	3.13
YE-2*S*	sarsa	1			
YE-2*R*	smila	1			
YE-3	en-diol	2	100	100	50
YE-4	marko	2	100	100	100
YE-5	en-ol	3	12.5	25	6.25
YE-6	mixture of sarsa and smila		25	50	6.25
YE-6*S*	sarsa	3			
YE-6*R*	smila	3			

MIC : minimal inhibitory concentration
S.c.: Saccharomyces cerevisiae (brewers yeast)
C.a. Candida albicans (pathogenic yeast)
H.a.: Hansenula anomala (food-deriorating yeast)

Fig. 4. Saponins from Mohave yucca and their antiyeast activity.

Mohave yucca, *Yucca schidigera* (Agavaceae) which grows widely in North and Central America, has been used as a foodstuff and folk medicine. The extract of the stems is utilized as a long-lasting foaming agent in carbonated beverages and as a flavor enhancer in foods. Several spirostanol-3-O-monodesmosides which exhibited potent antiyeast activity, were isolated from the saponin fraction of the stems (Fig. 4). A fraction which is composed of furostanol-bisdesmosides, showed no antiyeast activity.

Yucca extract is recognized as safe for human food use by FDA. The extract tasteless and odorless, showing no effect on the taste of foods. The extract is now commercially utilized for extending the shelflife of cooked foods such as "sushi" and "musubi" and fermented seasonings

such as soy sauce in Japan.

Furostanol-glycoside 26-O-b-glucosidase (F26G) from Costus speciosus rhizomes: As mentioned above, spirostanol-3-O-mondesmosides from Mohave yucca exhibited potent antiyeast activity, while co-occurring furostanol-3, 26-O-bisdesmosides showed no activity. It has been mentioned that spirostanol-3-O-monodesmosides are formed from co-occurring furastanol-bisdesmosides on post-harvest treatment and storage [9].

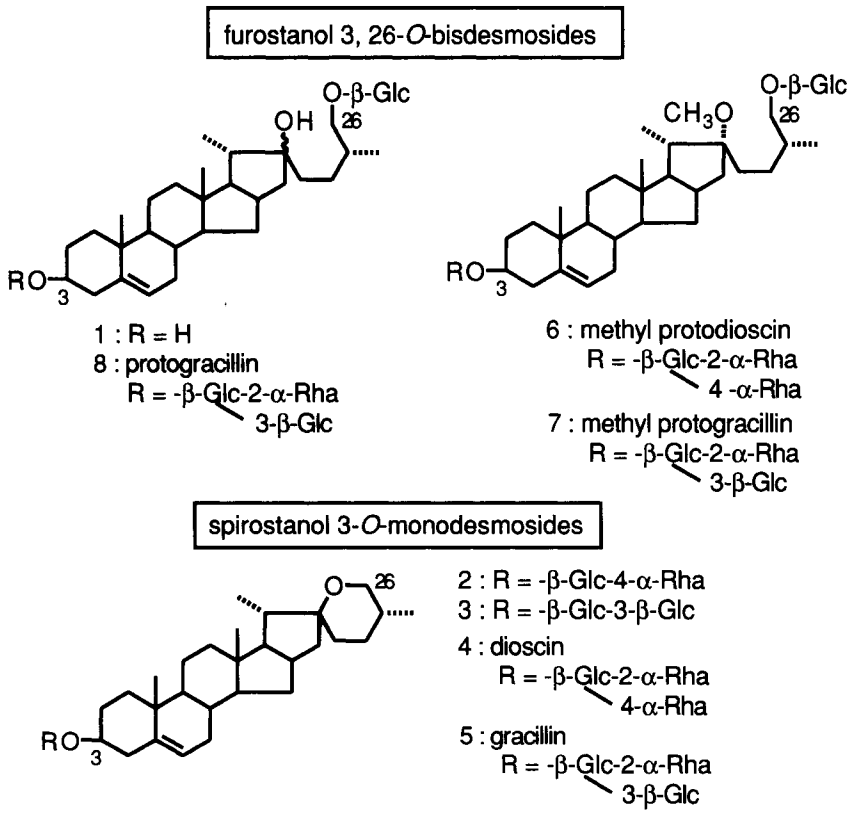


Fig. 5. Steroidal saponins of *Costus speciosus*

The selective hydrolysis of 26-O-glycoside linkage of the 3, 26-O-bisdesmosides which is followed by sponteneous cyclization of the side chain to form a spirostanol-3-O-monodesmoside, has been observed for homogenates of some plants which contain both furostanol-3, 26-O-bisdesmosides and corresponding spirostanol-3-O-monodesmosides. However, isolation and characterization of an enzyme which is responsible for this novel activity, has not been reported until recently.

Costus speciosus (Zingiberaceae) grows wild in Southeast and South Asia. From the rhizomes, a number of spirostanol-3-O-monodesmosides of diosgenin and the corresponding furostanol-3,26-O-bisdemosides have been isolated as shown in Fig. 5 [10].

From rhizomes of this plant, Ebizuka and the coworkers, Tokyo University, succeeded to isolate an enzyme with this activity named furostanol-glycoside 26-O-β-glucosidase (F26G) [11-13]. The purified F26G is highly specific for the cleavage of the 26-O-β-glucosyl linkage of

furostanol-glycosides; by this enzyme, protogracilin and protodioscin afforded gacillin and dioscin, respectively, while no hydrolysis were observed for cellobiose, gentiobiose, amygdalin, prunacin and octyl, phenyl and p-nitrophenyl β-D-glucosides.

Conclusion

Investigation of biologically active principles of traditional herbal drugs has been carried out extensively. Currently, "medicated diet" is reassessed in the world. Unidentified physiologically active substances might occur in food materials which we have daily taken without consciousness of the biological activities. In this view point, chemical and pharmacological studies of phytochemicals in food materials will be an important research subjects in the 21th century. It is noteworthy that Nohara and Yahara et al., Kumamoto University, isolated steroidal alkaloid-glycosides from potato tubers and edible fruits of tomato and egg-plant (Solanaceae) in significants yields [14]. Cytotoxic activities of steroidal saponins and steroidal alkaloid-glycosides from *Solanum* genera plants against a variety of cell lines, were currently reported by the same group [15].

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BIOACTIVE TRITERPENE GLYCOSEDES FROM SEVERAL MEDICINAL FOODSTUFFS

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In Chinese traditional medicinal books such as Shen Nong's Herbal (神農本草經), compendium of Chinese Materia Medica (本草綱目), and Dictionary of Chinese Materia Medica (中藥大辭典), a number of foods are listed as medicine and are prescribed in various traditional preparations. However, their bioactive principles and pharmacological properties are lift unclarified, except for a few examples. During the course of our characterization studies of bioactive components in natural medicines, we have isolated many triterpene and sterol glycosides with inhibitory activities of alcohol and glucose absorption, antiallergic, anti-inflammatory, hepatoprotective, antinociceptive, and adjuvant-like activities from medicinal foodstuffs.

This article focuses in the first half, our recent chemical and pharmacological studies of the triterpene glycosides with glucose-absorption inhibitory activity in a medicinal foodstuffs, sugar beet. In the second half of this article, we describe the chemical studies of the triterpene glycosides with histamine-release inhibitory activity from kidney bean.

I. Glucose Absorption Inhibitors from Sugar Beet [1)]

The roots of *Beta vulgaris* L. (Sugar beet, Chenopodiaceae) have been used industrially as a raw material for sugar. The fresh roots and leaves of this plant, Which is commonly called "Satoudaikon" or "Tensai" in Japanese, are used as a vegetable and food garnish in Japanese-style dishes. In Chinese traditional medicine, the toots of sugar beet have been known to exhibit sedative and emmenagogue-like effects In chemical studies on the constituents of sugar beet, betacyanins and phenolic compounds have been reported[2)] and recently, several oleanolic acid glycosides were isolated from the leaves of this plant. [3)]

In the course of our screening studies on the bioactive constituents of medicinal foodstuffs, [1,4)] the saponin fraction obtained from the roots of Japanese sugar beet was found to show an inhibitory effect on the increase of serum glucose levels in oral glucose-loaded rats.

i. Isolation of Betavulgarosides[1a, 1b)]

The saponin constitute of the roots were separated by the procedures shown in chart 1. In the process of extraction and purification of betavulgarosides, it was found that betavulgarosides were partly converted to their 1'''-methyl esters during extraction with methanol under reflux, while the nonsubstituted derivative of betavulgaroside III, chikusetsusaponin Iva, was obtained by extraction with water under reflux. After a preliminary examination to identify optimal extraction conditions, the fresh roots of sugar beet cultivated in Hokkaido Prefecture, Japan, were extracted with aqueous methanol and the extract was separated by reverse-phase silica-gel column chromatography (Chromatorex ODS). The methanol eluate from this chromatography was subjected to normal-phase silica-gel column chromatography to give a saponin fraction (fraction 6) with hypoglycemic activity. The saponin fraction was further separated by normal-phase silica-gel column chromatography and then HPLC to give betavulgarosides I (**1**, 0.0012% from the fresh root), II (**2**, 0.0004%)., III (**3**,

0.0013%, IV (**4**, 0.0006%), VI (**6**, 0.0001%), VII (**7**, 0.0003%), and VIII (**8**, 0.0001%). On the other hand, betavulgarosides V (**5**), IX (**9**), and X (**10**) were isolation from the leaves of sugar beet by similar separation procedure.

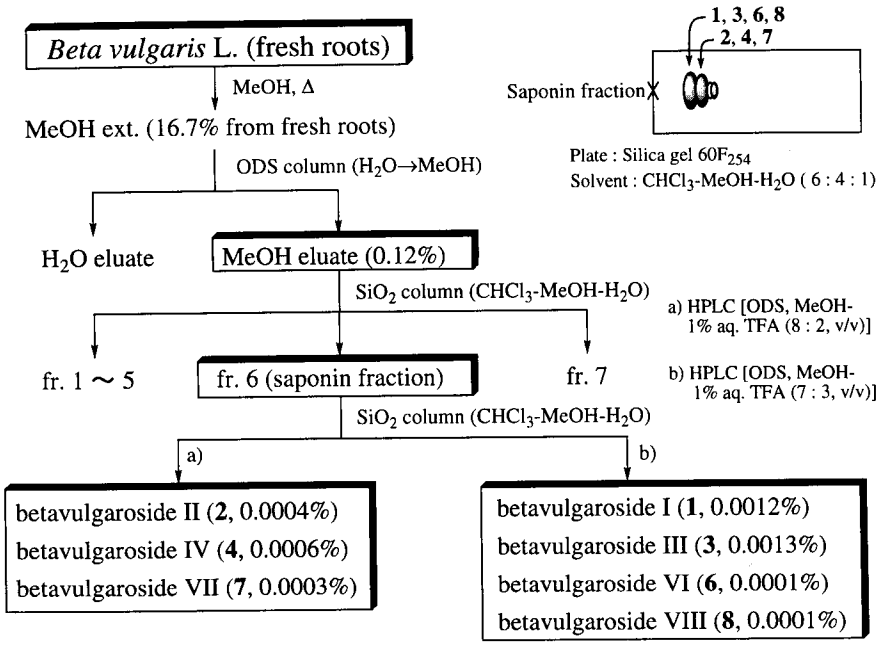


Chart 1. Isolation Procedures of Betavulgarosides

ii. Structure of Betavulgarosides [1c)]

The whole strictures of betavulgarosides (**1-10**) have been determined on the basis of physicochemical and chemical evidence, except for the stereostructure of the novel acetal-type substituent on betavulgarosides III (**3**)---IX (**9**).

The stereostructure of the acetal-type substituent was determined by the chemical correlation of betavulgaroside IV (**4**) with a known saponin momordin I (**19**), which was isolated from *Momordica cochinchinensis*.[5)] First, in order to confirm the plane structure of the acetal-type substituent analogues (**14**, **16**, **17**, **18**) were synthesized from L- and D-arabinose as shown in chart 3. Next, we carried out the chemical correlation of betavulgaroside IV (**4**) with momordin I (**19**), whose component monosaccharides were determined to be D-glucuronic acid and L-arabinose by GLC analysis of their trimethylsilyl thiazolidine derivatives. Thus the α -L-arabinopyranosyl moiety of momordin Ic (**19**) was converted to the α -L-ribopyranosyl group (**24**) *via* the 2-keto derivative(**23**). From the α -L-ribopyranosyl derivative (**25**), momo-methoxymethyl-betavulgaroside IV tetramethyl ester (**26**) was synthesized through the following successive reactions: 1) $Pb(OAc)_4$ cleavage of the 3" and 4"-diol moiety, 2) oxidation

of the aldehyde groups, and 3) diazomethane methylation. On the other hand, the 2"-epimer (**28**) was synthesized from the α -L-arabinopyranosyl derivative (**20**) to compare the physical data with those of **26**. On the basis of above-mentioned evidence, the 1"*R* and 2" *R* configurations of the acetal-type substituent were determined and consequently, the absolute stereostructures of betavulgarosides III(**3**)—IX(**9**) were also characterized as shown.

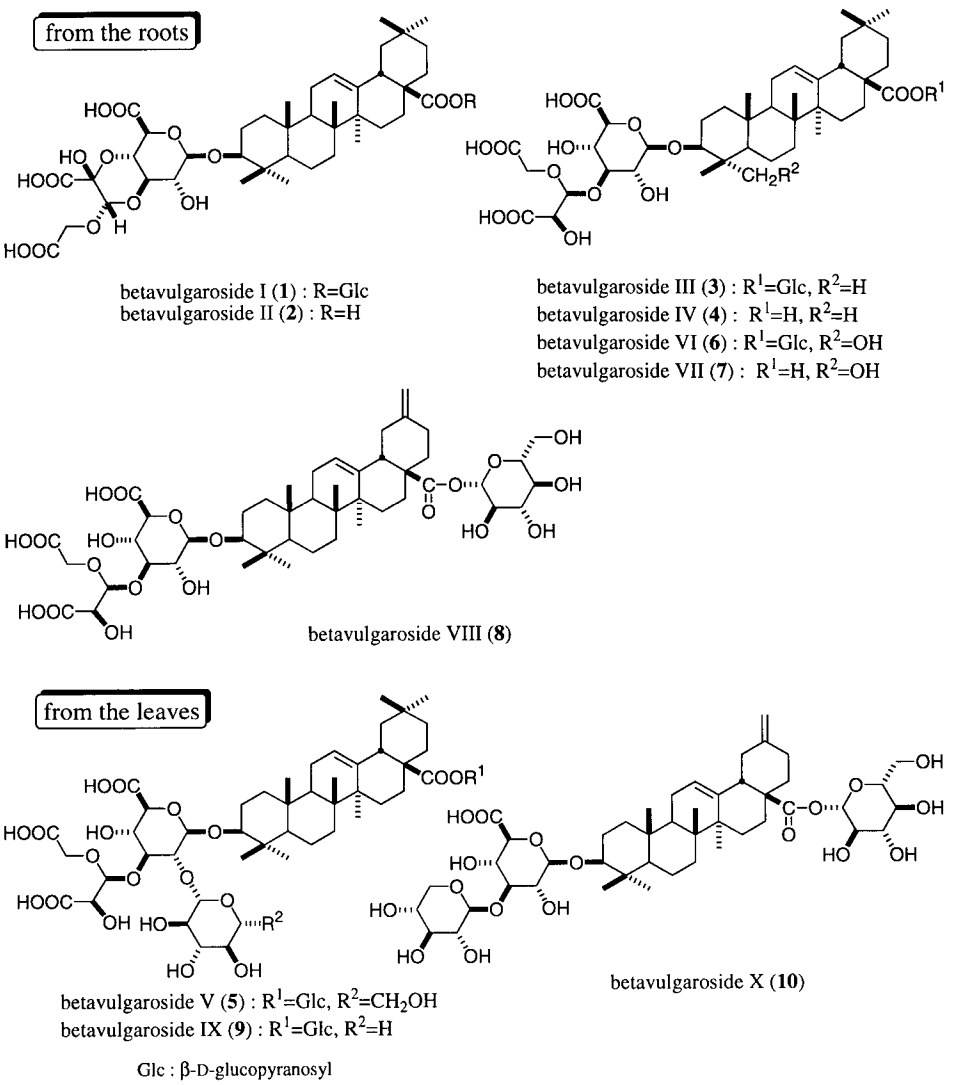
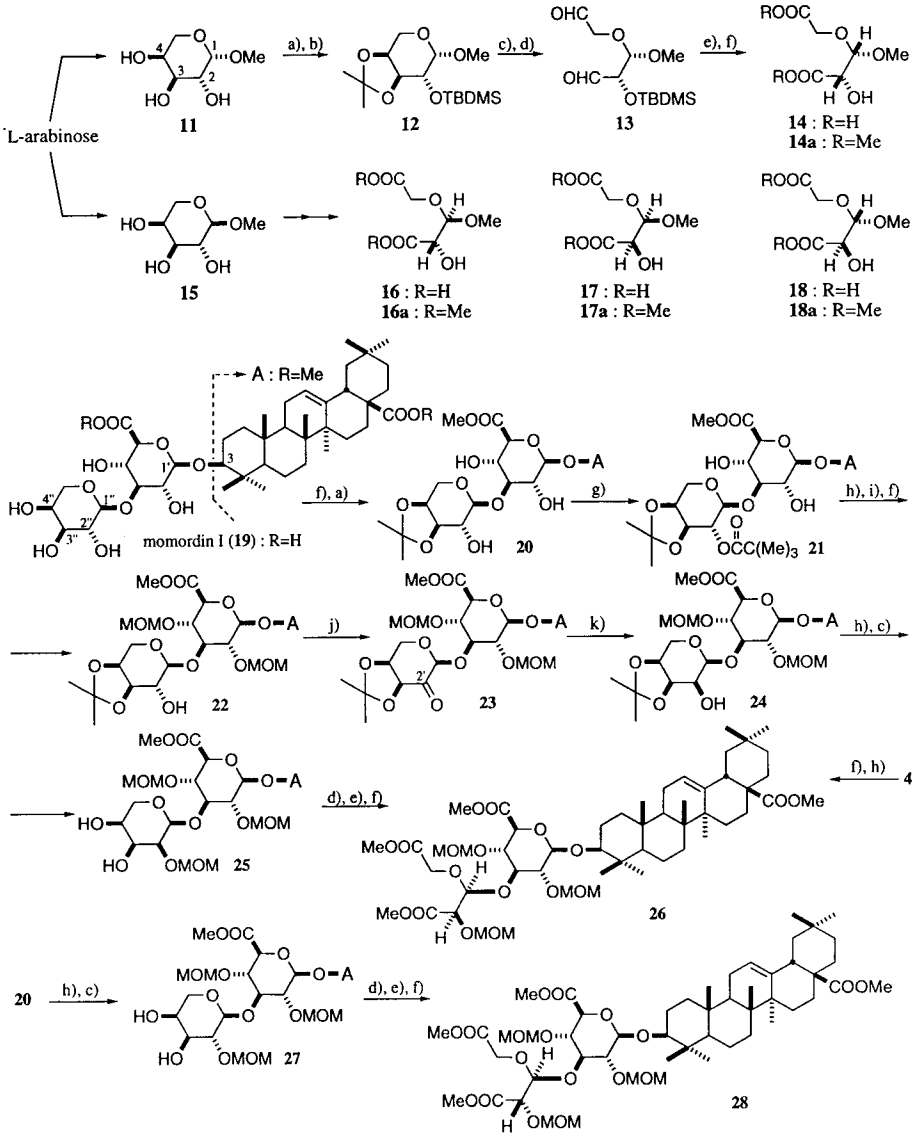


Chart 2. Structures of Betavulgarosides from Sugar Beet



a) 2,2-dimethoxypropane, p-TsOH·HO/DMF; b) TBDMS-Cl, imidazole/DMF; c) 80%aqueous AcOH; d) $Pb(OAc)_4$/benezene; e) $NaClO_2$, NH_2SO_3H/75% aqueous 1,4-dioxane; f) CH_2N_2- etherate/MeOH; g) pivaloyl chloride, DMAP/pyridine,0℃; h) MOM-Cl, *i*-Pr_2EtN/CH_2Cl_2; i) 5% NaOMe-MeOH; j) PCC/benzene; k) $NaBH_4$/MeOH

Chart 3. Chemical correlation of Betavulgaroside IV (**4**) with Momordin I (**19**)

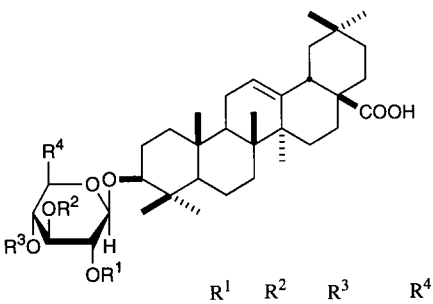
Table 1. Inhibitory Effects of the Betavulgarosides I (**1**), II (**2**), III (**3**), IV (**4**) and V (**5**), 28-*O*-Deglucosyl Betavulgaroside V (**29**), Oleanolic Acid Oligoglycosides (**29**—**37**, **39**—**42**), and Oleanolic Acid (**38**) on the Increase of Serum Glucose Levels in Glucose-loaded Rats

	Dose	n	Serum Glucose level (mg/dl)		
	(mg/kg, *p. o.*)		0.5h	1.0h	2.0h
Control (normal)	-	10	72.4±3.3	95.8±5.0	90.6±4.8
Control (glucose-loaded)	-	9	148.6±4.7	138.3±4.6	107.9±4.1
			(76.2±4.7)	(42.5±4.6)	(17.3±4.1)
Betavulgaroside I (**1**)	100	5	153.5±5.9	144.7±4.7	114.0±5.3
			(81.1±5.9)	(48.9±4.7)	(23.4±5.3)
Betavulgaroside II (**2**)	100	5	108.5±9.1**	137.7±5.4	121.8±4.9
			(36.1±9.1**)	(41.9±5.4)	(31.2±4.9)
Betavulgaroside III (**3**)	100	5	139.3±3.3	135.1±4.9	103.0±1.9
			(66.9±3.3)	(39.3±4.9)	(12.4±1.9)
Betavulgaroside IV (**4**)	100	5	111.5±5.7**	125.8±5.9	114.0±0.6
			(39.1±5.7**)	(30.0±5.9)	(23.4±0.6)
Betavulgaroside V (**5**)	100	5	147.0±3.4	138.5±3.1	108.3±5.7
			(74.6±3.4)	(42.7±3.1)	(17.7±5.7)
28-*O*-Deglucosyl betavulgaroside V (**29**)	100	6	124.1±5.9*	139.3±4.8	119.3±3.0
			(51.7±5.9*)	(43.5±4.8)	(28.7±3.0)
Oleanolic acid 3-*O*-glucuronide (**30**)	100	5	86.9±8.6**	105.0±10.3**	95.1±8.5
			(14.5±8.6**)	(9.2±10.3**)	(4.5±8.5)
Compound O (**37**)	100	5	137.0±10.3	122.7±6.7	98.3±3.9
			(64.6±10.3)	(26.9±6.7)	(7.7±3.9)
Oleanolic acid (**38**)	100	5	136.4±4.5	141.0±4.3	107.0±3.0
			(64.0±4.5)	(45.2±4.3)	(16.4±3.0)
Control (normal)		5	87.1±4.6**	101.6±7.7**	94.8±9.3*
Control (glucose-loaded)		7	149.7±5.2	137.2±4.8	115.0±3.1
			(62.6±5.2)	(35.6±4.8)	(20.2±3.1)
Momordin Ic (**32**)	100	5	98.9±2.0**	131.7±3.0	125.3±4.0
			(11.8±2.0**)	(30.1±3.0)	(30.5±4.0)
2'-*O*-β-D-Glucopyranosyl momordin Ic (**36**)	100	5	108.8±7.0**	119.7±6.1	110.6±4.0
			(21.7±7.0**)	(18.1±6.1)	(15.8±4.0)
Momordin IIc (**39**)	100	5	147.6±6.9	145.9±4.4	108.9±6.1
			(60.5±6.9)	(44.3±4.4)	(14.1±6.1)
2'-*O*-β-D-Glucopyranosyl momordin IIc (**42**)	100	5	137.4±8.2	142.5±4.1	113.1±4.4
			(50.3±8.2)	(40.9±4.1)	(18.3±4.4)
Control (normal)		5	78.8±4.2**	93.3±5.0**	84.4±4.6**
Control (sucrose-loaded)		6	152.7±7.4	138.8±4.7	104.5±3.0
			(73.9±7.4)	(45.5±4.7)	(20.1±3.0)
Stipuleanoside R_1 (**34**)	100	6	126.5±7.3**	136.7±5.7*	131.3±5.2
			(31.9±7.3**)	(28.1±5.7*)	(29.5±5.2)
35	100	6	96.8±2.9**	116.3±4.3**	101.0±2.2
			(18.0±2.9**)	(23.0±4.3**)	(16.6±2.2)
Stipuleanoside R_2 (**40**)	100	6	149.8±7.9	146.0±10.9	121.8±7.8
			(55.2±7.9)	(37.4±10.9)	(20.0±7.8)
Chikusetsusaponin IV (**41**)	100	6	114.8±6.7**	111.0±5.2**	105.0±6.6
			(36.0±6.7**)	(17.7±5.2**)	(20.6±6.6)
Control (normal)		6	63.3±2.5**	82.2±9.9	63.5±6.5*
Control (glucose-loaded)		6	127.7±5.7	102.4±4.4	92.9±8.3
			(64.4±5.7)	(20.2±4.4)	(29.4±8.3)
Oleanolic acid 3-*O*-glucoside (**31**)	100	5	95.9±4.0**	111.2±10.6	103.6±5.1*
			(32.6±4.0**)	(29.0±10.6)	(40.1±5.1*)
Oleanolic acid 3-*O*-β-D-xylopyranosyl (1→3)-β-D-glucopyranoside (**33**)	100	5	94.3±5.1**	105.2±6.9	94.1±6.9
			(31.0±5.1**)	(23.0±6.9)	(30.6±6.9)

means±S.E. (* $p<0.05$, ** $p<0.01$)

iii. Inhibitory Effects of Betavulgarosides and Related Compounds on Glucose Absorption

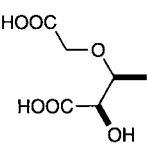
The inhibitory effects of the principal betavulgarosides (**1-5**), their prosapogenol (**29**, **30**, **37**), and the common sapogenol oleanolic acid (**38**) on the increase of serum glucose levels in oral glucose-loaded rats are summarized in Table 1.To identify the structure requirements of oleanolic acid glycosides, we examined the inhibitory activity of various oleanolic acid glycosides (**31-36**, **39-42**) obtained from *Momordica cochinchinensis* and *Panax japonicus*. As is apparent from Table 1, all oleanolic acid 3-O-momodesmosides including betavulgarosides II (**2**) and (**4**), prosapogenol (**29**, **30**), and related glycosides (**31-36**) exhibited potent inhibitory



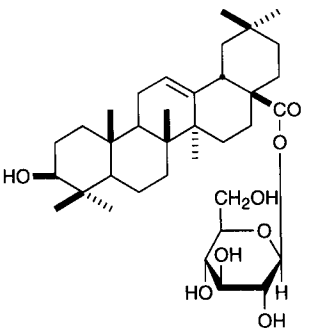
	R^1	R^2	R^3	R^4
28-*O*-deglucosyl betavulgaroside V (**29**)	Glc	AS	H	COOH
oleanolic acid 3-*O*-glucuronide (**30**)	H	H	H	COOH
31	H	H	H	CH_2OH
momordin Ic (**32**)	H	Xyl	H	COOH
33	H	Xyl	H	CH_2OH
stipuleanoside R_1 (**34**)	H	Gal	Ara (f)	COOH
35	H	H	Ara (f)	COOH
2'-*O*-glucosyl momordin Ic (**36**)	Glc	Xyl	H	COOH

Glc : β-D-glucopyranosyl
Xyl : β-D-xylopyranosyl
Gal : β-D-galactopyranosyl
Ara (f) : α-L-arabinofuranosyl

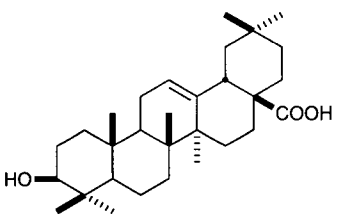
AS :



	R^1	R^2	R^3
momordin IIc (**39**)	H	Xyl	H
stipuleanoside R_2 (**40**)	H	Gal	Ara (f)
chikusetsusaponin IV (**41**)	H	H	Ara (f)
2'-*O*-glucosyl momordin IIc (**42**)	Glc	Xyl	H



compound O (**37**)



oleanolic acid (**38**)

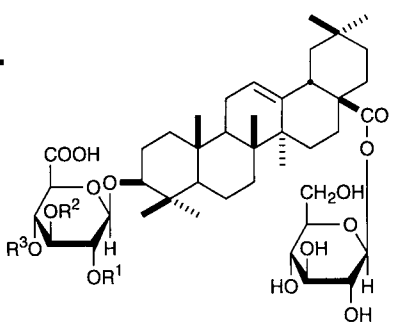


Chart 4. Structure of Oleanolic Acid Glycosides

activity after a single oral administration if 100 mg/kg. Since oleanolic acid (**38**) lacked the inhibitory activity, the 3-O-glycosede moiety of those glycosides was found to be essential for exerting the activity, Among oleanolic acid 3, 28-bisdesmosides, betavulgarosides I (**1**), III (**3**), and V (**5**) and related glycosides (**39**, **42**) showed no activity, while chikusetsusaponin IV(**41**), which possessed an α -L-arabinofuranosyl moiety at the 4'-position of the glucuronic acid part, exhibited the inhibitory activity. Oleanolic acid 28-monodesmoside compound O (**37**), also showed little activity. This evidence revealed that the 28-ester glycoside moiety significantly reduced the activity, while the 4' -O-arabinofuranosyl moiety tended to increase it.

Inhibitors on Increase of Serum Glucose Levels in Glucose-loaded Rats

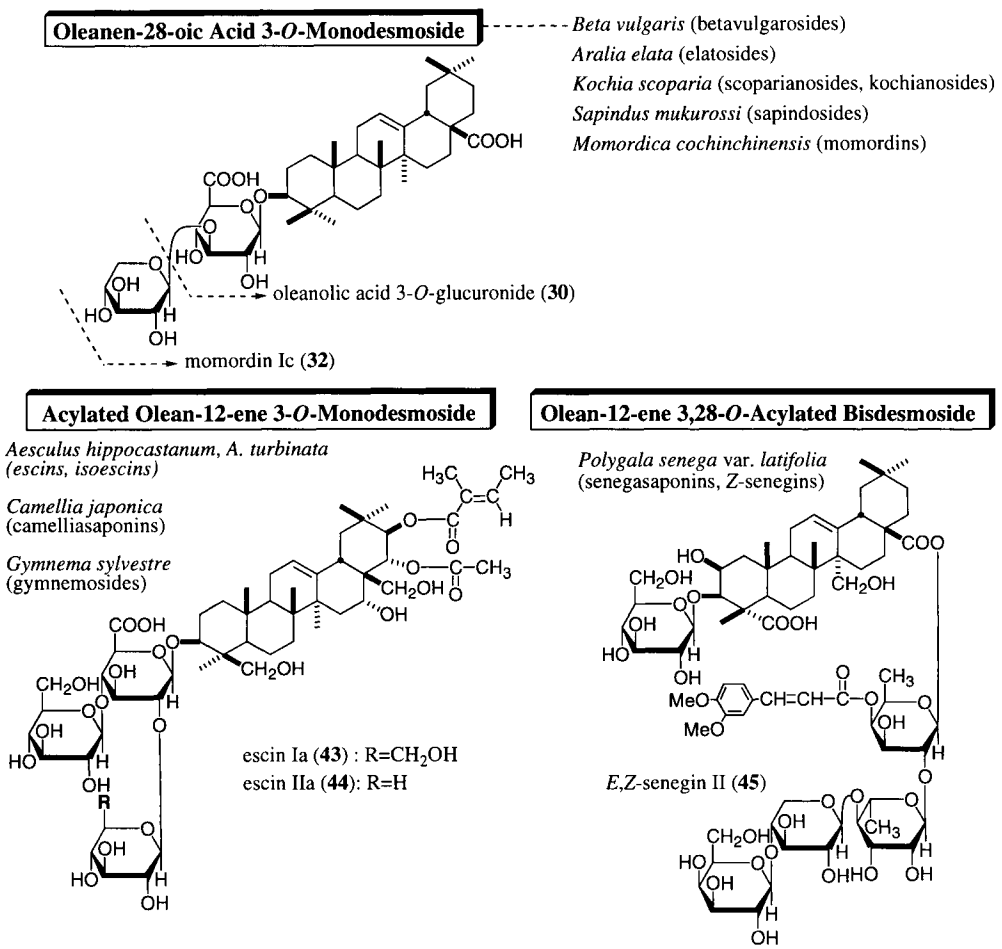


Chart 5

As our continuing studies to find the saponin constituents with inhibitory activity of

glucose absorption, we have found the saponin fractions of several medicinal foodstuffs and natural medicines to show the inhibitory activity on the increase of serum glucose levels in glucose-loaded rats(Table 2). Through bioassay-guided separation, we have isolated new active triterpene glycosides and determined their structures: elatosides A-k (the root cortex, bark and young shoots of *Aralia elata* SEEM., Araliaceae),[6] Kochianosides I-IV, scoparianosides A-C (the fruits of Kochia scoparia SCHARD., Chenopodiaceae), [7] escins Ia—VI, isoescins Ia, Ib and V(the seeds of *Aesuclus hippocastanum* L. and *A. turbinata* BLUME, Hippocastanaceae).[8] Camelliasaponins A_1---C_2 (the seeds of *Camellia japonica* L., Theaceae), [9] gymnemosides-a—-f (the leaves of *Gymnema sylvestre* R, BR., Asclepiadaceae), [10] and E, Z-senegasaponins a-c, Z-senegins II—IV (the roots of *Polygala senega* L. var. *latifolia* TORREY et GRAY, Polygalaceae). [11] Furthermore, by examination of the structure requirement for the inhibitory activity on glucose absorption, it has been characterized that the active triterpene glycosides can be classified into the following three types of structure : 1) oleanen-28-oic acid 3-O-monodesmnosides (betavulgarosides, elatosides, scoparianosides, kochianosides), 2)acylated polyhydroxyolean-12-ene 3-O-monodesmoside (escins, isoescins, camelliasaponins, gymnemosides (E, Z-senegasaponins, Z-senegins).

Table 2. Inhibitory Effects of saponin Fractions from Medicinal Foodstuffs and Natural Medicines on Increase of Serum Glucose levels in Glucose -loaded Rats

Sample	Dose (mg/kg, *p.o.*)	n	Serum Glucose (Δ mg/dl,0.5h)	Inhibition (%)
Control	200	5	77.0±8.9	--
Beta vulgaris (roots)	200	5	22.2±6.3	71.2
Aralia elata (young shoots)	200	5	17.2±6.5	77.7
Aralia elata (cortex)	200	5	44.5±8.0	42.2
Aralia elata (bark)	200	5	22.1±7.9	71.3
Kochia scoparia (fruit)	200	5	12.7±7.8	83.5
Aesculus hippocastanum (seeds)	200	5	54.2±3.2	29.6
Polygala senega var. *latifolia* (roots)	200	5	39.1±6.5	49.2
Sapindus mukurossi (bark)	200	5	48.0±9.4	37.7
Gymnema sylvestre (leaves)	200	5	56.5±5.4	26.6

means±S. E.

iv.Inhibitory Mechanisms of Triterpene Glycosides on Glucose Absorption [12]

To shed light on the action mechanisms of triterpene glycosides in their inhibitory activity on the increase of serum glucose levels in oral glucose -loaded rats, oleanolic acid 3-O-glucuronide (**30**), momordin Ic (**32**), escins Ia (**43**) and IIa (**44**), and E, Z-senegins II (**45**) were examined their pharmacological properties. All glycosides (**30**, **32**, **43**, **44**, **45**) dose dependently inhibited the increase in serum glucose levels in oral glucose-loaded rats (Chart 6). However, those glycosides showed no significant effects on serum glucose levels in normal rats, intraperitoneal glucose-loaded rats, and alloxan-induced diabetic mice. These results indicated that the glycosides had neither insulin-like activity nor insulin -releasing activity like tolbutamide, and therefore the glycosides (**30**, **32**, **43**, **44**, **45**) were seemed to affect glucose absorption the gastro-intestinal tract. As shown in Chart 7, the glycosides (**30**, **32**, **43**, **44**, **45**)

significantly suppressed gastric emptying in rats. In particular, momordin Ic (**32**) strongly suppressed gastric emptying at the dosage of 25 mg/kg, while **30**, **43**, **44**, and **45** showed significantly suppression at 50-100 mg/kg doses. The potent slowing activity of the glycosides (**30**, **32**, **43**, **44**, **45**) on gastric emptying seemed to be important factor in exhibiting inhibitory activity on the increase of serum glucose levels after oral administration of glucose. Furthermore, those glycosides (**30**, **32**, **43**, **44**, **45**) also concentration dependently inhibited glucose uptake in rat small intestinal fragments as shown in chart 8. On the basis of the above-mentioned evidence, it was assumed that the three typed if triterpene glycosides inhibited glucose absorption by suppressing the transfer of glucose from the stomach to the intestine, and by inhibiting the glucose transport system in the small intestine.

Inhibitory Effects of Oleanolic Acid 3-O-Glucuronide (**30**), Momordin Ic (**32**), Escins Ia, IIa (**43**, **44**), and E, Z-Senegin II (**45**) on Increase of Serum Glucose Levels in glucose-loaded Rats

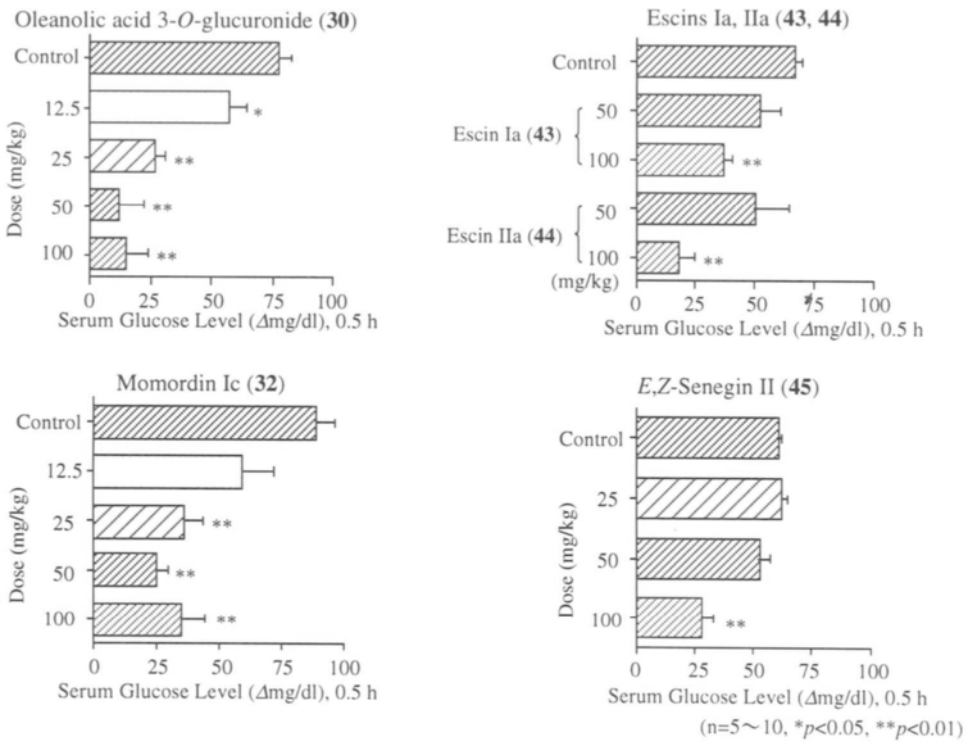


Chart 6

Inhibitory Effects of **30**, **32**, **43**, **44**, and **45** on Gastric Emptying in Rats

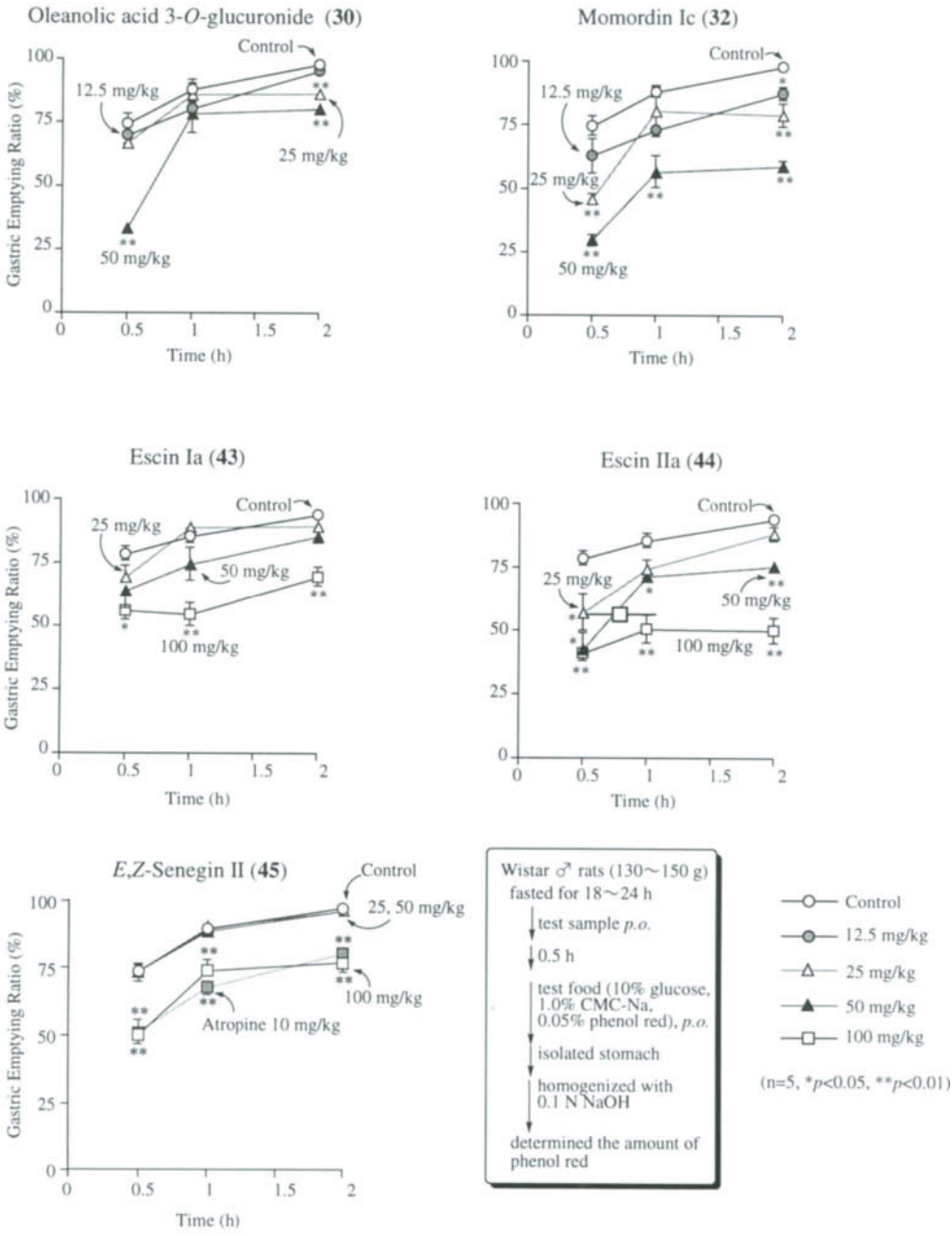


Chart 7

Inhibitory Effects of **30**, **32**, **43**, **44**, and **45** on Glucose Uptake in Rat Small Intestine Fragments (*In Vitro*)

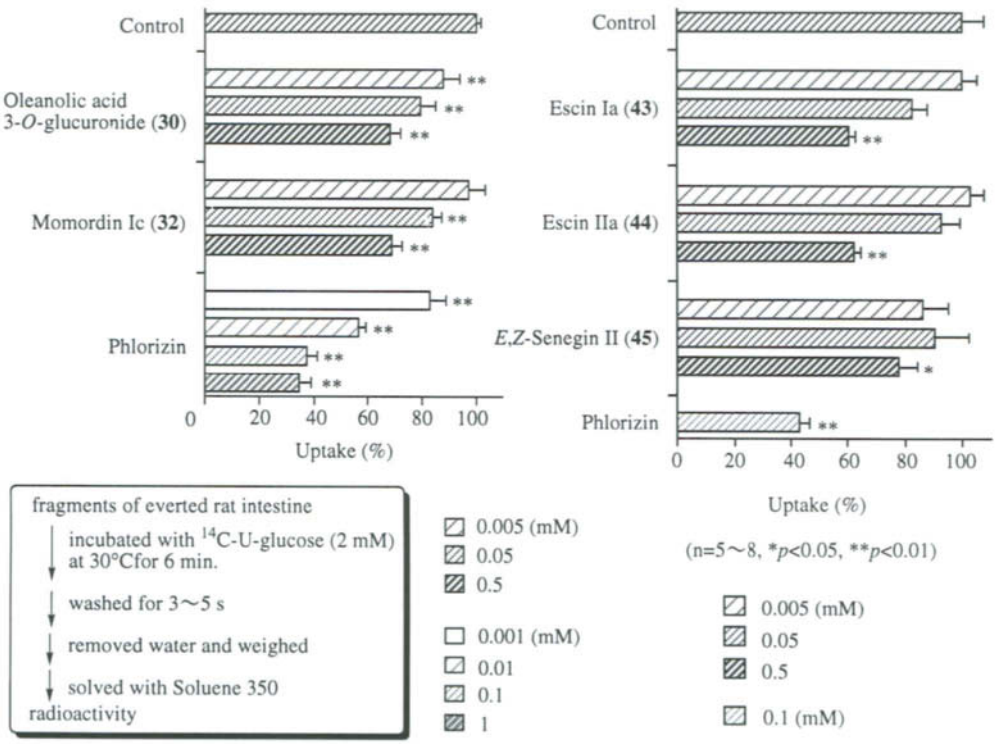


Chart 8

II. Histamine Release Inhibitors from Kidney Bean [13]

Phaseolus vulgaris L. (leguminosae, Japanese names: sandomame, ingenmame, and saitou) has been widely cultivated as a vegetable or a staple food in Asia, African, Middle and South American countries. The seeds of this plant have many common names such as kidney bean, apricot bean, french bean, navy bean, and common bean. In Chinese traditional medicine, the seeds of *Phaseolus vagaries* L. have been prescribed for nutritive, antipyretic and diuretic purposes. In regard to the chemical constituents of the seeds of P*haseolus vulgaris L.*, two oleaene -type triterpene glycosides (soyasaponin V and saoyasapogenol B-glucoside), amino acids, and glycoproteins have been isolated while several flavonoids have been reported as phytoalexins from the fungal -infected seeds. [14]

As part of our search for bioactive constituents in leguminous plant, [15] we have isolated two new olean-12 -ene-type oligoglycosides, named sandosaponins A (**46**, 0.0030%) and B (**47**, 0.0005%), together with soyasaponins I (**49**, 0.0043%) [16] and V (**48**, 0.014%) [17] and

dehydrosoyasaponin I (**50**, 0.0040%) [15e] from the seeds of Japanese *Phaseolus vulgaris L.* On the basis of chemical and physicochemical evidence, the structures of sandosaponins A(**46**) and B (**47**) have been determined as shown in Chart 9. Recently, we have isolated fur methyl - migrated 16, 17-*seco*-dammarane-type triterpene glycosides (hoveidulciosides A_1--B_2 from the seeds and fruits of *hovenia dulcis* THUNB., Rhamnaceae), [18] dammarane-type triterpene glycosides (jujubosides A_1, C, acetyljujuvoside B from the seeds of *Zizyphus jujuba* Mill. var. *spinosa*Hu, Rhamnaceae), [19] and cyanoglycosides (rhodiocyanosides A—D from the underground part of *Rhodiola quadrifida* Fisch. et. May and *R. sacra* S. H, Fu, Crassulaceae),[20] and those glycosides were found to show antiallergic activity.

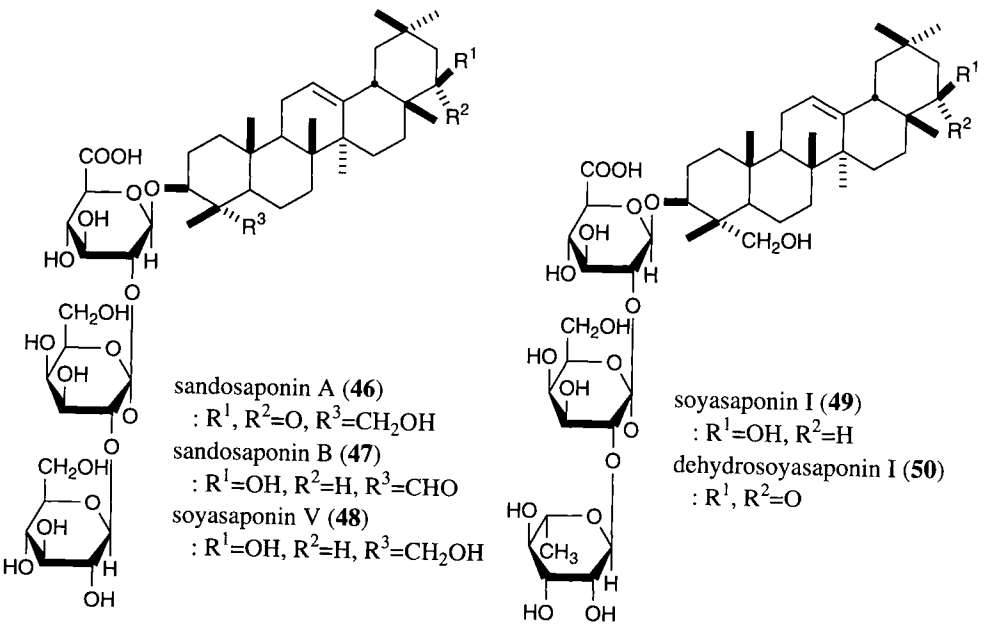


Chart 9. Structure of Saponin constituents from Kidny Bean

As an extension of this work, we have examined the inhibitory activity of sandosaponins and soyasaponins on histamine release from rat peritoneal exudate cells induced by an antigen-antibody reaction. The new saponins sandosaponins A (**46**) and B (**47**) showed more potent inhibitory activity than soyasaponin V (**48**) as shown in Table 3. In addition, dehydrosoya-saponin I (**50**) was reported to exhibit much potent inhibitory activity than soyasaponin I (**49**) on the binding of monodotyrosine chorybdotoxin to large -conductance calcium-dependent potassium channels in smooth muscle membrance.[21] It is noteworthy that replacing the 22- or 24- hydroxyl group of the soyasapogenol B moiety in soyasaponins I (**48**) and V (**49**) with a carbonyl group significantly increased the activity.

Table 3 Inhibitory Effects of Saponins (**46-50**) on Histamine Release from Rat Exudate cells Induced by an Antigen-Antibody Reaction

	Concentration M	n	Inhibition %
Sandosaponin (**46**)	10^{-6}	4	36.7±14.1
	10^{-5}	4	58.2±4.1
	10^{-4}	4	90.9±7.5
Sandosaponin (**47**)	10^{-6}	4	43.0±8.4
	10^{-5}	4	59.4±6.7
	10^{-4}	4	66.8±1.4
Soyasaponin V (**48**)	10^{-6}	4	6.0±12.8
	10^{-5}	4	10.7±5.1
	10^{-4}	4	19.3±3.5
soyasaponin I (**49**)	10^{-6}	4	4.6±9.1
	10^{-5}	4	11.2±4.7
	10^{-4}	4	62.3±5.0
dehydrosoyasaponin I (**50**)	10^{-6}	4	8.8±8.6
	10^{-5}	4	15.9±10.6
	10^{-4}	4	42.9±12.5

means±S. E.

III. Regioselective Oxidation of the Hydroxyl Group in Polyhydroxylated Triterpene by Indirect Anodic Oxidation Method [22]

Although great many oxidation methods of hydroxyl group have reported, a few methods could be applicable for selective oxidation of a hydroxyl group in polyhydroxylated compounds. Several electrochemical methods for the oxidation of hydroxyl group have been developed, in particular, indirect anodic oxidation using a suitable mediator was reported to be practical because of its low oxidative potential. However, electrochemical oxidation methods including indirect methods have been only applied to simple mono-hydroxyl compounds and it seems to need further application for complex skeletal and highly functionalized alcohols to clarify the selectivity and specificity of electrochemical reaction. During the course of our studies to develop electrochemical transformation of triterpene glycosides, [23] we have examined the indirect anodic oxidation of triterpene alcohols and saponins.

First, we examined the anodic oxidation of soyasapogenol B (**51**), using halide as a mediator in the solvent system having the dissolving ability for both of triterpene and electrolyte. As is apparent from Table 4, indirect anodic oxidations of **51** under almost all conditions (Run1-5) yielded the 3-ketone derivative (**52**) as the only oxidation product and, by exhaustive oxidation for 5h (Run 6), the 3, 22-diketone derivative (**54**) was obtained as the minor product. The electrolysis condition (Run 1) using KI in *t*-BuOH-H_2O (1:1) was found to be most effective and **52** was produced from **51** in 85.9% yield by the electrolysis for 10min. On the other hand, chemical oxidations found to give the 22-ketone derivative (soyasapogenol E, **53**). This evidence showed that the indirect anodic oxidation selectively proceeded the oxidation of 3-equatorial hydroxyl group in preference to the 22-axial hydroxyl group which was easily

oxidized by other oxidants.

Table 4. Indirect Anodic Oxidation [a] of Soyasapogenol B (**51**) [b] with Different Electrolytes and Solvent Systems

Run	Solvent (ml)	Electrolyte (mmol)	Time	Electricity (F/mol)	Product
1	*t*-BuOH-H_2O(1:1, 5.4)[d]	KI(0.48)	10min	1.3	**52**(85.9%)
2	*t*-BuOH-H_2O(1:1, 5.4)[d]	KBr(0.48)	10min	1.3	**52**(80.0%)
3	Et_2O-*t*-BuOH-H_2O(3:2:4, 5.4)[d]	KI(0.48)	1h	7.8	**52**(76.9%)
4	*n-hexane-t*-BuOH-H_2O(2:3:4, 27)[d]	KI(2.41)	2h	3.1	**52**(80.1%)
5	*n-hexane-t*-BuOH-H_2O(2:3:4, 5.4)[d]	NaI(0.48)	2h	15.5	**52**(78.8%)
6	*n-hexane-t*-BuOH-H_2O(2:3:4, 27)[d]	KI(2.41)	5h	7.7	**52**(54.2%), **53**(28.4%)
7	*n-hexane-t*-BuOH-H_2O(2:3:4, 5.4)[d]	*n*-Bu_4NI(0.48)	1h	7.8	Complex mixture

a) Constant current electrolysis (Pt anode and cathode, 13mA/cm^2) in an undivided beaker with vigorous stirring.
b) 0.022mmol of **51** for Run 1, 2, 3, 5, and 7; 0.262mmol for Run 4 and 6.
c) One phase solution. d) Two phase solution.

We next examined the extension of this oxidation method for other polyhydroxyl triterpenes (**55-58**, **65-70**) and soyasapogenol A oligoglycoside (**59**). Among them, triterpene alcohols (**55-58**) having the 3, 24-dihydroxyl moiety were found to provide the corresponding 3-ketone derivative, respectively [**60** (72.7%), **61** (92.0%), **62** (82.3%), and **63** (67.0%)] by the indirect anodic oxidation using KI in *t*-BuOH-H_2O. Furthermore, soyasapogenol A oligoglycoside (**59**) was also converted to the 3-ketone derivative (**64**) in 80.2% yield without previous protection of the hydroxyl groups in the glycosidic moiety. On the other hand, other triterpene alcohols (**65-70**), which lacked the 24-hydroxyl group, yield no product under the same anodic oxidation conditions as that for **55-58** and were recovered almost completely. These findings led us to presume that the selective anodic oxidation of the 3-hydroxyl group was specific to the triterpene alcohol having the 3.24- dihydroxyl moiety.

To shed light on the reaction pathway for the selective anodic oxidation, we examined the electrolysis of the 24-protected derivatives (**51a**, **51b**), and the 3-epimer (**71**) of **68**. In case of the 24-protected derivatives (**51a**, **51b**), the corresponding 3-ketone derivatives were not obtained at all and the starting compounds were recovered. Here again, it was confirmed that the 24-hydroxyl group was indispensable to this selective anodic oxidation. The 3a,23-dihydroxyl derivative (71) was also found to give no product by anodic oxidation. On the basis of above mentioned evidence, although some other pathways could be considered, an electrochemical process would be proposed for the present anodic oxidation.

Since the present anodic oxidation method is need no previous protection of hydroxyl groups, it may be significant to the synthesis of partially oxidized derivative from triterpene

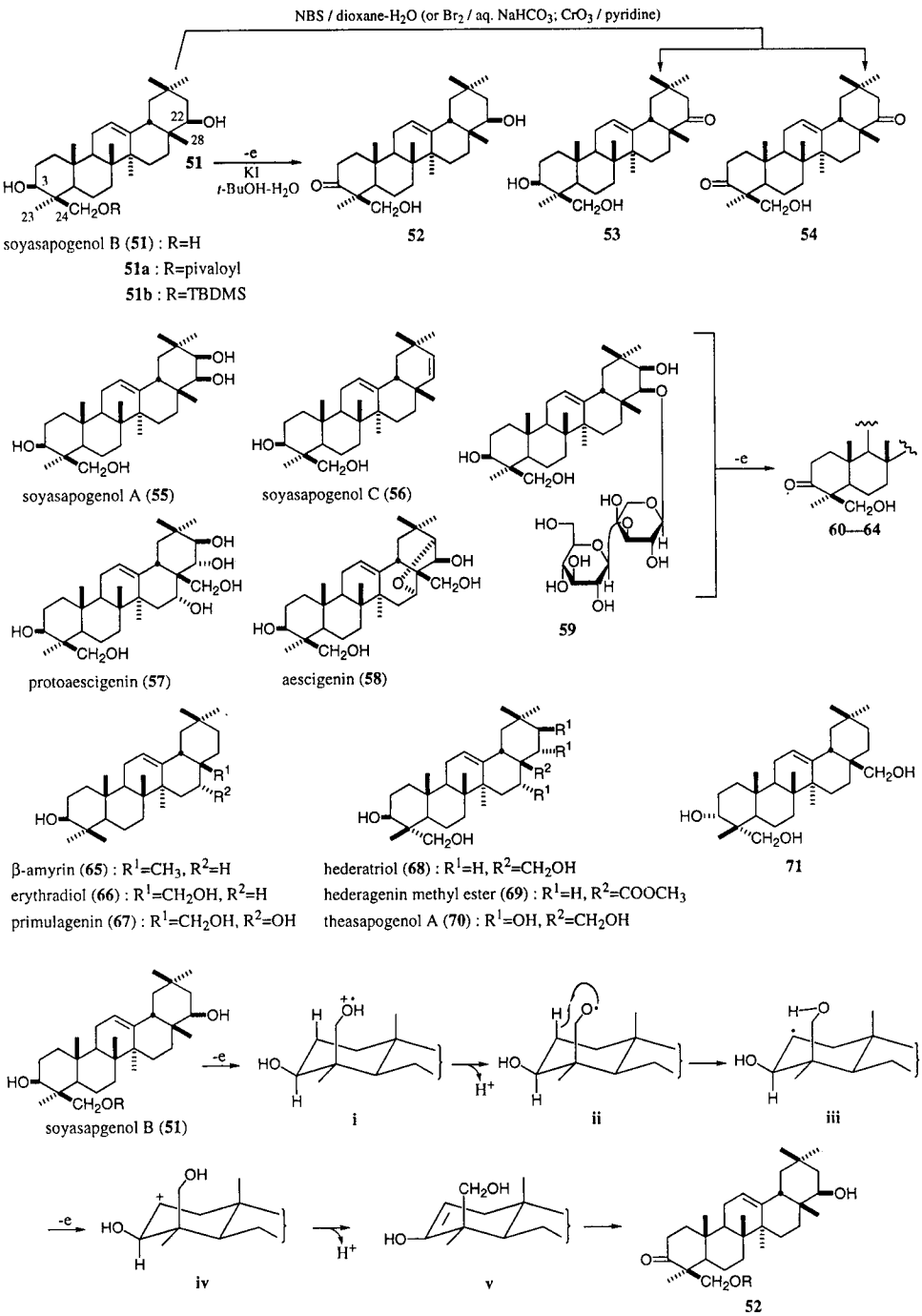

NBS / dioxane-H2O (or Br2 / aq. NaHCO3; CrO3 / pyridine)
-e
KI
t-BuOH-H2O
51
52
53
54
soyasapogenol B (51) : R=H
51a : R=pivaloyl
51b : R=TBDMS
soyasapogenol A (55)
soyasapogenol C (56)
59
60—64
protoaescigenin (57)
aescigenin (58)
β-amyrin (65) : R1=CH3, R2=H
erythradiol (66) : R1=CH2OH, R2=H
primulagenin (67) : R1=CH2OH, R2=OH
hederatriol (68) : R1=H, R2=CH2OH
hederagenin methyl ester (69) : R1=H, R2=COOCH3
theasapogenol A (70) : R1=OH, R2=CH2OH
71
soyasapgenol B (51)
i
ii
iii
iv
v
52

Chart 10

saponins.

References

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CANCER CHEMOPREVENTIVE ACTIVITIES OF *PANAX NOTOGINSENG* AND GINSENOSIDE RG1[1)]

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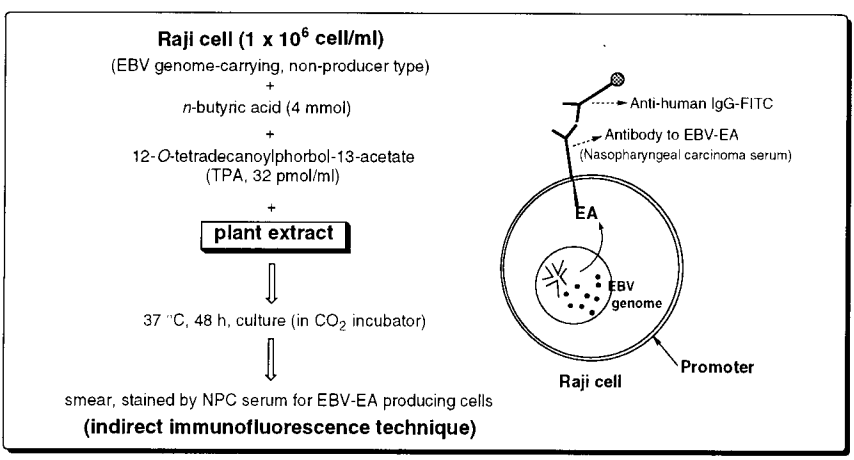
Introduction

Currently, although many kinds of anti-tumor agents are developed and the medicinal sciences make rapid progress in the treatment of cancer, cancer is the most tragic disease and one of the major cause of death in the world. Therefore, the advancement of cancer chemoprevention is very important as well as the development of cancer treatment. The mechanism of chemical carcinogenesis has been explained by either a two-stage theory or a multi-stage theory, which consist of the initiation, promotion and progression stage.[2)] In these stages, the promotion stage is long-term and reversible reaction, and the development of the inhibitors on promotion stage (anti-tumor-promoters) have been regarded as the most promissing method for the chemo- prevention of cancer. To search for possible anti-tumor promoters (cancer chemopreventive agents) from natural resources, we carried out an *in vitro* primary screening of many kind of natural products (triterpenoids[3)], flavonoids[4)], euglobals[4)], plant extracts and kampo prescriptiions[6)]) using their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by a strong tumor-promoter, 12-O-tetra-decanoylphorbol-13-acetate (TPA). As a continuation of our biological studies on the potential anti-tumor-promoters, several extracts of *Panax* plants were assayed by the synergistic primary screening system. Of these extracts, the extract of *Panax notoginseng* exhibited significant inhibitory effects on EBV-EA activation. On the other hand, the root of *P. notoginseng* (San-chi-ginseng) is well known as the Chinese traditional medicine having haemostatic, anti-hepatitis and anti-inflammatory activities, and as the major component of Chinese medicine such as "Yunnan Bai Yao" and "Pien Tze Huang". Further, ginsenoside Rg1 is one of the major saponins of this crude drug. In this paper, the results of primary screening test *in vitro* and two-stage carcinogenesis tests on mouse skin, pulmonary and hepatic tumors of an extract of *P. notoginseng* and ginsenoside Rg1 are discussed.

Inhibitory Effects on Epstein-Barr virus Early Antigen Activation



The primary screening test was carried out utilizing a short-term *in vitro* assay on EBV-EA

activation as shown in Figure 1. In this assay method, the EBV genome-carrying lymphoblastoid cells (Raji cells) were incubated in a medium containing n-butyric acid, TPA and various amounts of tested extracts. Smear were made from the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique. The EBV-EA inhibitory activity of the tested extract was compared with that of the control experiment with n-butyric acid plus TPA. In the experiments, the EBV-EA activities were ordinarily around 30%, and these values were taken as the positive control. Inhibitory effects of the *Panax* plant on the EBV-EA activation and the viabilities of Raji cells used as indicator cells in this assay method are shown in Table 1.

Table 1. Percentages of EBV-EA Induction in Presence of Extracts of *Panax* Plants with Respect to Positive Control (100%).

Sample	Concentration (μg/μl)				
	500	100	50	10	1
MeOH extracts of					
P. notoginseng	0.0[a] (60)[b]	7.6 (70)	33.4 (>80)	53.8 (>80)	91.6 (>80)
P. ginseng (white)	38.7 (70)	79.6 (>80)	100.0 (>80)	100.0 (>80)	100.0 (>80)
P. ginseng (red)	13.5 (60)	22.4 (>80)	75.3 (>80)	100.0 (>80)	100.0 (>80)
P. japonica	83.2 (50)	100.0 (>80)	100.0 (>80)	100.0 (>80)	100.0 (>80)
P. vietnamensis	----	62.1 (60)	----	84.9 (>80)	100.0 (>80)
P.quinquefolium (wild)	----	0.0 (60)	----	56.3 (>80)	92.1 (>80)

[a] Values represent relative percentages to the positive control. [b] Values in parentheses are viability percentages of Raji cells. [c] not tested

Of these extracts, *P. notoginseng* exhibited significant inhibitory effects on EBV-EA activation (100% inhibition of activation at 500 μg/ml, more than 90%, 65% and 45% inhibition of activation at 100 μg/ml, 50 μg/ml and 10 μg/ml, respectively) with high viability of Raji cells even at high concentrations. In our experiments, many natural products which strongly inhibit the TPA- induced EBV-EA activation, have been shown to act as inhibitors of tumor promotion *in vivo*. Many kinds of saponins have been isolated from the root of *P. notoginseng*, and especially, ginsenoside-Rb1, -Rb2, -Rd, -Re and -Rg1 have been isolated as major neutral saponins. Furthermore, acetylene derivatives, panaxytriol and panaxynol have been also isolated from this plant. Therefore, these saponins and acetylenes were assayed on EBV-EA activation, and these results are shown in Table 2.

Table 2. Percentages of EBV-EA Induction in Presence of Saponins and Acetylenes from *Panax notoginseng* with Respect to Positive Control (100%)

Sample	Concentration(mol ratio, compound/TPA)				
	$2.5x\ 10^3$	$1x\ 10^3$	$5x\ 10^2$	$1x\ 10^2$	1x 10
ginsenoside Rb1	0.0[a] (>80)[b]	20.1 (>80)	41.7 (>80)	71.8 (>80)	100.0 (>80)
ginsenoside Rb2	0.0 (>80)	22.6 (>80)	48.3 (>80)	78.5 (>80)	100.0 (>80)
ginsenoside Rd	0.0 (>80)	17.6 (>80)	38.0 (>80)	67.4 (>80)	94.8 (>80)
ginsenoside Re	0.0 (>80)	18.9 (>80)	40.7 (>80)	69.3 (>80)	94.4 (>80)
ginsenoside Rg1	0.0 (>80)	12.4 (>80)	32.5 (>80)	63.6 (>80)	91.0 (>80)
Sample	Concentration(mol ratio, compound/TPA)				
	$1x\ 10^3$	$1x\ 10^3$	5x 10	1x 10	1
panaxytriol	----c (0)	----c (0)	----c (0)	0.0 (20)	64.9 (>80)
panaxynol	----c (0)	----c (0)	0.0 (30)	23.3 (>80)	84.5 (>80)

[a] Values represent relative percentages to the positive control. [b] Values in parentheses are viability percentages of Raji cells. [c] not detected

Of these compounds, acetylenes showed significant inhibitory effects, but they have very strong cytotoxicities on Raji cells (0% viability at $1x10^2$ mol ratio/TPA and less than 30% viability at 5x10 mol ratio/TPA). On the other hand, ginsenoside-Rg1 exhibited the most strong inhibitory effects in these five saponins and preserved high viability even at high concentration. Further, O. Tanaka and his co-workers have reported analysis of saponins in ginsengs, and it has been cleared that the content of ginsenoside Rg1 in the root of *P. notoginseng* was more than 10 times in other *Panax* plants.[7] Therefore, it was deduced that the significant inhibitory activity of the crude extract of *P. notoginseng* is exhibited by the combination of ginsenosides with acetylenes (the inhibitory effect of ginsenosides is strongly enhanced by acetylenes).

Inhibitory Effects on Two-Stage Carcinogenesis of Mouse Skin Tumor

The results of *in vitro* assay strongly suggested that *P. notoginseng* might be valuable anti-tumor-promoters. The method of *in vivo* two-stage carcinogenesis test on mouse skin tumor is shown in Figure. 2

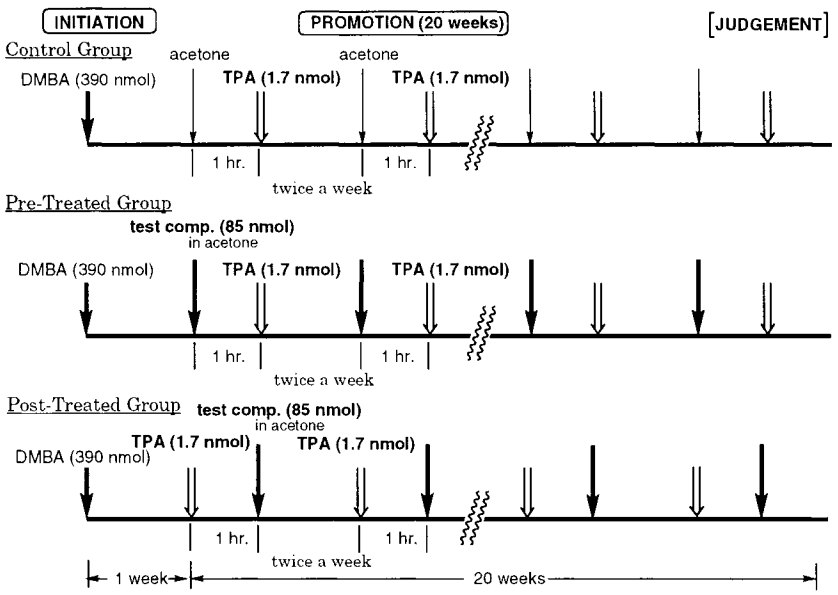


Figure 2. Method of In vivo Two-Stage Carcinogenesis Test on Mouse Skin Tumor

As shown in Figure 2, the inhibitory effects of the MeOH extract of *P. notoginseng* on two-stage carcinogenesis of mouse skin tumor under pre-treatment and post-treatment, using dimethylbenz- [a]anthracene (DMBA) as an initiator and TPA as a promoter, were investigated. The rate (%) of papilloma-bearing mice (Fig. 3-A) and the average number of papillomas per mouse (Fig. 3-B) of treated group were compared with those of the positive control group. In the positive control group, 80% of mice bore papillomas at 8 weeks of promotion and 100% of mice bore papillomas at 10 weeks of promotion, and more than 10 papillomas were formed per mouse after 20 weeks of promotion. In the group pre-treated with the extract of *P. notoginseng*, only about 45% of mice bore papillomas at 10-12 weeks of promotion, and only 5 papillomas were formed per mouse after 20 weeks of promotion. In the group post-treated, only 20% of mice bore papillomas at 11 weeks and 65% even at 20 weeks of promotion, and 2-3 papillomas were formed per mouse after 20 weeks of promotion. Therefore, the MeOH extract of *P. notoginseng* significantly delayed the formation of papillomas and reduced the

number of papillomas per mouse.

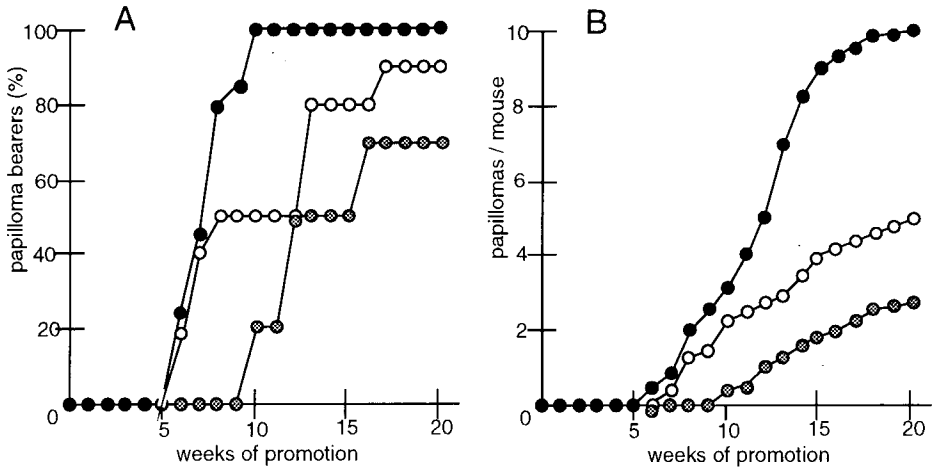


Figure 3. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of MeOH Extract of *Panax notoginseng*

All mice were treated first with BMBA (initiator, 100 μg, 390 nmol) and then with TPA (promoter, 1 μg, 1.7 nmol) given twice weekly, starting 1 week after the initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas per mouse. ●, positive control TPA alone; ○, TPA + MeOH extract of *P. notoginseng* (50 μg, pre-treatment); ⊗, TPA + MeOH extract of *P. notoginseng* (50 μg, post-treatment).

Furthermore, another two-stage carcinogenesis test on mouse skin tumor (promoter: DMBA and initiator: fumonisin B1) were carried out, and the results are shown in Table 3.

Fumonisin B1 had been isolated from the corn as one of the mycotoxins, having the similar chemical structure to sphingosine. And this mycotoxin has strong tumor-promoting activity, but the carcinogenic mechanism of this toxin would be different from that of TPA. As shown in Table 3, the extract of *P. notoginseng* also exhibited the inhibitory effects on mouse skin tumor induced by the different type promoter, fumonisin B1. The inhibitory effects of the major saponin of *P. notoginseng*, ginsenoside Rg1, were also investigated and compared with those of glycyrrhetic acid which have been known as a strong anti-tumor-promoter[8] (Fig. 4-A,B).

Table 3. Inhibition of Fumonisin B1-Induced Tumor Promotion by Oral Administration of MeOH Extract of Panax notoginseng.

weeks of promotion	% of mice bearing papillomas		average # of papillomas / mouse	
	positive control (fumonisinB1)	treated group with *P. notoginseng*	positive control (fumonisinB1)	treated group with *P. notoginseg*
2	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0
6	0.0	0.0	0.0	0.0
8	20.0	0.0	0.3	0.0
10	66.6	40.0	1.2	0.7
12	100.0	46.6	3.7	1.8
14	100.0	53.3	4.8	2.5
16	100.0	60.0	6.2	2.9
18	100.0	86.6	6.9	3.5
20	100.0	100.0	7.2	3.8

When ginsenoside Rg1 was applied continuously before each TPA treatment, it remarkably delayed the formation of papillomas in mouse skin and reduced the number of papillomas per mouse as shown in Fig.4.

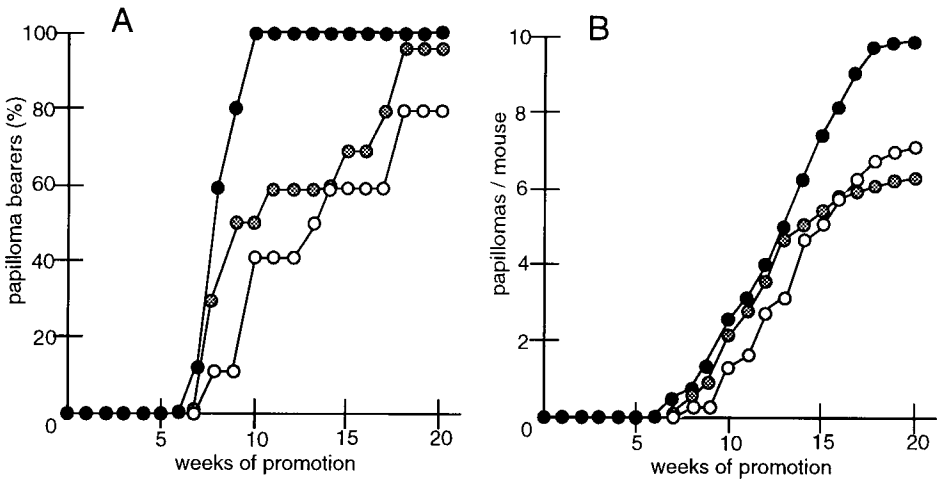


Figure 4. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of Ginsenoside Rg1 ang Glycyrrhetic Acid.

All mice were treated first with BMBA (initiator, 100 μg, 390 nmol) and then with TPA (promoter, 1 μg, 1.7 nmol) given twice weekly, starting 1 week after the initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas per mouse.●, positive control TPA alone; ○, TPA + Ginsenoside Rg1 (85 nmol); ⊗, TPA + Glycyrrhetic Acid (85 nmol)

In our experiments, these inhibitory effects of ginsenoside Rg1 are similar to those of glycyrrhetic acid, and it was also found that the ginsenoside Rg1 enhanced the weak inhibitory effects of *P. ginseng* (white-ginseng) when Rg1 was additionaly applied with the extract of white-ginseng. Therefore, it was deduced that ginsenoside Rg1 is one of the active constituents of anti-tumor-promoting activity of *P. notoginseng*.

Inhibitory Effects on Mouse Pulmonary and Hepatic Tumors

Further the inhibitory effects by oral administration on other forms of carcinogenesis were also investigated. The two-stage carcinogenesis test of the extract on pulmonary and hepatic tumors were examined as follows. In the case of pulmonary tumor, 4-nitroquinoline-N-oxide (4-NQO) was used as an initiator (by single subcutaneous injection) and 8% glycerol solution in drinking water was used as a promoter.[9, 10] A total of 75 mice were divided into five groups (group I to V) of 15 animals, and each group was traeted as shown in the foot notes of Table 4. The MeOH extract of *P. notoginseng* was dissolved in drinking water (2.5 mg / 100 ml) together with glycerol. After 25 weeks the mice were killed by cervical dislocation, and each pulmonary lobe was separated and the number of induced tumors was counted under a dissecting microscope. Both the total number of pulmonary tumors in 15 mice and the percentage of mice with tumors were markedly reduced (more than 70% inhibition in the total number of tumors and about 50% reduction in the percentage of mice with tumor after 25 weeks) by administration of the MeOH extract of *P. notoginseng* (group I), compared with those of the positive control (group II).

Table 4. Incidences of Pulmonary Tumors in Mice Treated with the MeOH Extract of *Panax notoginseng*

Group	Treatment	total No. of tumors	No.of tumor per mouse	% of mice with tumor
I.	4NQO +8% glycerol +ext of *P. notoginseng*[1] (2.5 mg/100 ml)	10	0.67	53.3
II.	4NQO+8% glycerol[2]	45	3.0	100
III.	4NQO + water[3]	1	0.06	6.7
IV.	8% glycerol alone[4]	0	0	0
V.	water alone [5]	0	0	0

[1] Initiated with 4NQO, and 8% glycerol solution including the extract of *P. notoginseng* has been drunk (for 25 weeks) as the promotion treatment instead of drinking water. [2] Initiated with 4NQO, and 8% glycerol solution has been drunk (for 25 weeks) as the promotion treatment instead of drinking water. [3] Initiated with 4-nitroquinoline-N-oxide (4NQO, 0.3 mg/mouse, subcutaneous injection), and drinking water. [4] Without initiation and 8% glycerol solution has been drunk as the promotion treatment instead of drinking water. [5] Without initiation, drinking water alone.

In the case of hepatic tumor, N-nitrosodiethylamine (DEN) was used as an initiator (by single peritoneal injection) and 0.09% phenobarbital (PB) solution in drinking water was used as a promoter. None of the mice in groups III to V had hyperplastic nodules of liver, and all of mice in the positive control group (group II) had hyperplastic nodules, a total of 47 hyperplastic nodules being formed in the 15 mice. In the treated group I, 46.6% of mice had hyperplastic nodules and a total of 23 nodules were formed in the 15 mice at 25 weeks of promotion. Therefore, oral administration of the MeOH extract of *P. notoginseng* markedly reduced the hyperplastic nodule formation (more than 50% inhibition in the total number of hyperplastic nodules and more than 50% reduction in the percentage of mice with hyperplastic nodules of liver) as shown in Table 5.

Table 5. Incidences of Hyperplasia of Liver in Mice Treated with the MeOH Extract of *Panax notoginseng*

Group	Treatment	total No. of hyper-plastic nodules	No. of hyperplastic nodules per mouse	% of mice with hyperplastic nodules (%)
I.	DEN+0.09% PB +ext of P. notoginseng[1] (2.5 mg/100 ml)	23	1.53	46.6
II.	DEN+0.09% PB[2]	47	3.13	100
III.	DEN+water[3]	0	0	0
IV.	0.09% PB alone[4]	0	0	0
V.	water alone[5]	0	0	0

[1] Initiated with DEN, and 0.09% PB solution including the extract of *P. notoginseng* has been drunk as the promotion treatment instead of drinking water(for 25 weeks). [2] Initiated with DEN, and 0.09% PB solution has been drunk as the promotion treatment instead of drinking water(for 25 weeks). [3] Initiated with N-nitrosodiethylamine (DEN, 1.8 mg/mouse, peritoneal injection), and drinking water. [4] Without initiation and 0.09% phenobarbital (PB) solution has been drunk as the promotion treatment instead of drinking water. [5] Without initiation, drinking water alone.

Further in our experiment, the MeOH extract *of P. notoginseng* exhibited the strong inhibitory effects on the liver damage of mouse induced by the dioxine derivative, tetrabromodioxine (TBDD).

These results strongly suggested that *P. notoginseng* might be a valuable anti-tumor-promoter and chemopreventive agent in chemical carcinogenesis, and that one of the active principles of *P. notoginseng* might be ginsenoside Rg1. In the case of the hepatitis or the prevention of cancer relapse, we should consider to apply the chemopreventive agents such as *P. notoginseng* to reduce the severe side actions of anticancer agents. For the application of natural products to chemoprevention, we have many problems to be solved, and one of the most important problem is the inhibitory mechanisms of these compounds on chemical carcinogenesis.

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NMR STUDIES ON SUGAR MOIETIES OF SOME NEW TRITERPENOID SAPONINS FROM *ARALIA SPINIFOLIA* AND *NOTHOPANAX DAVIDII*

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Abstract: Nine new triterpeniod saponins with oligosaccharide linkages as sugar moieties were isolated from *Aralia spinifolia* and *Nothopanax davidii*. Their structures were elucidated on the basis of spectroscopic and chemical evidence. In this paper, we would like to report their NMR studies on the sugar moieties by modern NMR techniques (COSY, RCT, NOESY, HMBC, DIFNOE, SINEPT and ^{13}C-T_1 relaxation times).

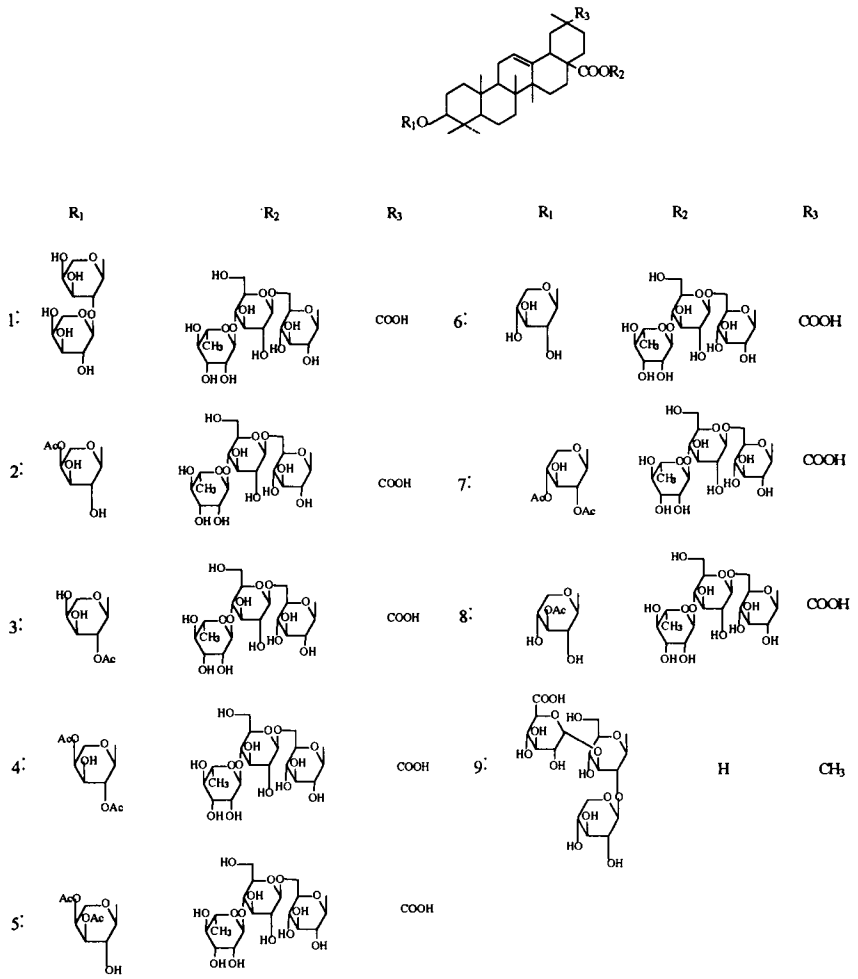
Introduction

The structural elucidation of saponins mainly included determination of structure of the aglycone, interglycosidic linkage and sequence in the oligosaccharides, and location of the oligosaccharides on the aglycone. The determination of interglycosidic linkage and sequence in the oligosaccharides was the problem of structural elucidation for saponins with the glycosidic linkages containing three or more than three monosaccharide units.

The structural complexity of oligosaccharides arises from the variable linkage positions[(1-2), (1-3), (1-4), (1-6), etc], the aromeric configurations (α or β) of glycosidic likages, and also the ring size (pyranose or furanose form) of the monosaccharide units. The structures of oligosaccharides are sometimes branched and can have non -carbohydrate substituents such as acetate, phasphate etc. These features pose fundamental difficulties associated with structure elucidation and often require time -consuming further studies even after the monosaccharide composition has been determined.

The ^{1}HNMR spectra of sugar moieties of saponin compounds are surpringly complex, due to extensive overlap of ring protons within a narrow spectral width (δ 3.0~4.2), and are often strongly coupled. Therefore, ^{1}HNMR spectra of sugar moieties of saponin compounds are usually very difficult to assign. For this reason, analysis of ^{1}HNMR spectra was generally limited to chemical shifts and scalar couplings of well resolved resonances such as anomeric protons (H-1)(δ4.4~5.6) and methyl resonances of 6-deoxy monosaccharide residues (δ1.1-1.3). Although unlike ^{1}HNMR spectra, line broading and overlapping do not seriously affect ^{13}CNMR spectra owing to the much larger interval of chemical shifts covered, the unambiguous assignments of ^{13}CNMR spectra of oligosaccharides for multi-sugar saponins are still very difficult.

During recent years, various NMR spectroscopic approaches (COSY, COLOC, HMBC, RCT, HOHAHA and DIFNOE) were widely used for structural studies of saponins compounds. Only few papers were delt with study of assignments and assignment methods of each ^{1}H and ^{13}C signal of sugar moieties of saponins by modern NMR techniques. Here, we would like to report NMR studies on sugar moieties of nine new saponins from *Aralia spinifolia* and *Nothopanax davidii* [1-8]. It is the purpose of this paper to describe how to assign each ^{1}H and ^{13}C signal of sugar moieties of saponins and to find some new conclusions of chemical shifts of ^{1}H and ^{13}C signals.



Structures of Saponins (1-9) from *Aralia spinifolia* and *Nothopanax davidii*

Results and Discussion

The methods of assignments are now demonstrated using the triterpenoid saponin **1** from *Nothpanax davidii* as an example. As described above, the anomeric proton resonances are usually well separated, they are commonly used as a spectral window in the analysis of ^{1}HNMR spectrum of sugar moieties of saponins.

The individual proton spin systems for each sugar residue were delineated by homonuclear and heteronuclear correlations as detected from COSY and multistep homonuclear relayed coherence transfer (RCT) experiments [9]. In this way triple relayed correlations were detected from anomeric protons (H_1) to H_5 via the intervening H_2 (direct coupling), H_3 (single relayed coherence transfer) and H_4 (double relayed coherence transfer). Thus, beginning from the anomeric proton (δ 6.13, Glc H_1; 5.68, Rha. H_1; 4.89, Glc'. H_1; 4.70, Ara'. H_1; 4.68, Ara. H_1), all 2-position protons of sugar moieties were easily located in the

COSY spectrum (Figure 1) , including the correlation between the weakly coupled Rha. H_1 with Rha. H_2 ($^{3J}H_1/H_2$=2.3Hz). The remaining proton spins of the rhamnose residue (H_3 through H_6) were also delineated from the COSY spectrum enabling the assignment of Rha. H_6 through Rha. H_3. These rhamnose proton assgnments were subsquently confirmed by the double and triple RCT spectra. The two arabinose anomeric protons were overlapped, but distinguished from cross-sections of COSY spectrum at the frequencies of Ara. H_2 and Ara'. H_2, respectively. The single RCT spectrum (Figure 2a) showed the coherence transfer from the sugar H_1 to H_3 protons via the intervening H_2 [10]. In such a way, most 3-positions of the sugar residues could be assigned. One ambiguity existed : the cross peaks between the two arabinose anomeric protons with their respective 3-position protons were overlapped due to the near magnetic equivalence of Ara. H_3 and Ara'. H_3, 1,3-Diaxial intraresidue NOE'S between the anomeric protons of the two arabinose with the 3-position protons appeared at exactly the same position as the arabinose H_1/H_3 relayed coherence transfer in the single RCT spectrum, also suggesting that the two arabinose H_3's were near-coincident. This was confirmed by the $^{13}C/^1H$-COSY experiment: the overlapped proton signals of Ara. H_3 and Ara'. H_3 showed correlations with two distinct carbons (δ 73.6, Ara.' And 70.3, Ara. C_3, ultimately distinguished from their relative ^{13}C-T_1 relaxation times and chemical shift analysis, vide infra). Cross-sections from the $^{13}C/^1H$-COSY spectrum at the frequencies of Ara. C_3 and Ara'. C_3 provide the chemical shifts of these two proton (δ4.09, Ara. H_3; 4.10, Ara'. H_3).

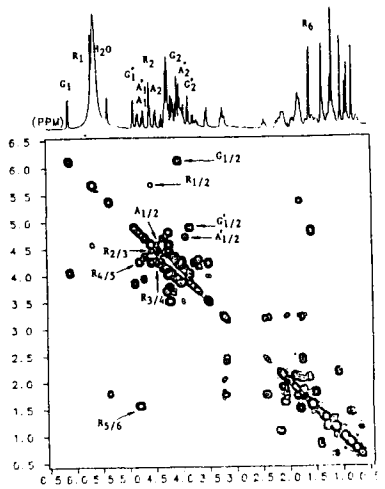


Figure 1. $^1H/^1H$ COSY spectrum of **1**; sugar anomeric to 2-position proton correlations labelled above diagnal, rhamnose H2-H6 system labelled below diagonal

With the assignments of the 3-position protons of individual sugar units, mapping of the sugar spin systems contained to the respective positions, and subsequently to the 5-position protons with double and triple RCT experiments, respectively (Figure 2b and 3). The correlation signals from the anomeric to the 4-position protons of the two glucose residues through two-step relayed coherence transfers were clear in the double RCT spectrum, but only one correlation between the anomeric and the 4-position protons of the two arabinose residues was detectable, which implied that the two 4-position proton signals of arabinoses were also overlapped. The triple relay RCT spectrum (Figure 3) also displayed all correlations between the rhamnose anomeric proton with other rhamnose protons (H_2 through H_5), which was consistent with the assignment of these protons based upon the COSY experoments [11].

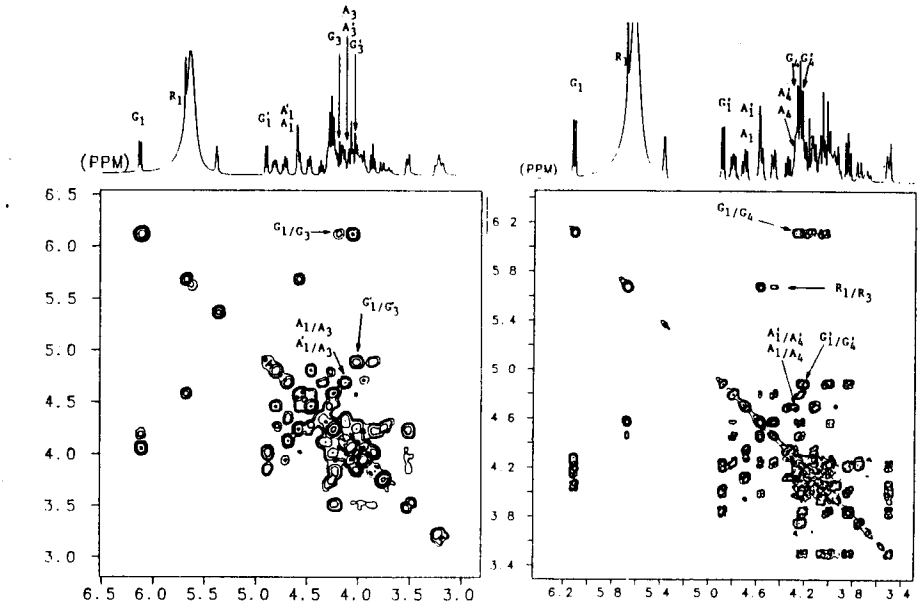


Figure 2 (a) Single RCT spectrum of **1**. Labelled peaks indicate the relayed coherence transfers between anomeric protons of sugar residues with their respective 3-position protons. (b) Double RCT spectrum of **1**. Labelled peaks indicate the relayed coherence transfers between anomeric protons with their respective 4-position protons, and the coherence transfer between Rha.H_1 with Rha.H_3.

With the COSY and RCT experiments completed, most sugar protons were assigned. The assignment of the 3-position and 5-position protons of the glucose residues as well as the 3-position protons of the arabinose units were confirmed by intraresidue NOE'S between three protons with the respective anomeric protons due to 1,3-diaxail depolar interactions observed in the NOESY experiment (vide infra, Figure 4 and 6).

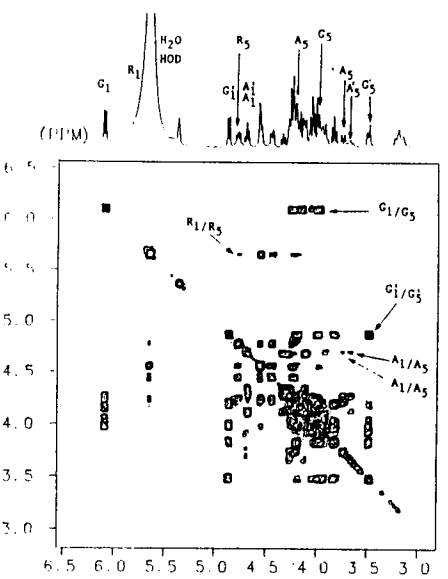


Figure 3. Triple RCT spectrum of **1**. Labelled peaks indicate correlations between anomeric protons of sugars with respective 5-position protons.[16]

The C_6 methylene protons of the two glucose units were most easily located through their respective $^{13}C/^1H$ correlation in the fixed evolution $^{13}C/^1H$ COSY spectrum [12]after the C6 resonances had been assigned. (These methylene $^{13}C/^1H$ correlations did not appear in the normal $^{13}C/^1H$-COSY spectrum, as is often a problem for methylene carbons bearing magnetically nonequivalent protons) [13]. Since C_6 of glucose and C_5 of arabinose were the only methylene carbons in the region of sugar carbon resonances, they were easily discerned by their multiplicity in the DEPT experiment (δ68.2, 66.1, 65.8, 60.3). Arabinopyrannose C_5 resonances normally appear between 65-66ppm [14], and glucose C_6 in the range of 60-62 ppm except when glucosidated (66-69ppm) [15]. Therefore, the most

downfield methylene carbon (δ68.2) was assigned to a glucosidated glucose (Glc. C6). The remaining methylene resonances were assigned as Ara.C_5 (δ65.8), Ara'C_5 (δ66.1), and Glc'C_6 (δ60.3), respectively, according to their chemical shifts. (Distinction of Ara.C_5 and Ara'.C_5 was ultimately based on T_1 relaxation times). The assignments of the methylene protons to each glucose residue were then completed by the vicinal and geminal coupling correlations in the COSY spectrum(Figure 5). With all the sugar protons assigned, a normal $^{13}C/^1H$-COSY spectrum then allowed assignments of the sugar carbon resonances to each individual sugar. The sole exceptions were distinction of carbons attached to the overlapped arabinose protons (H_1's, H_3's, and H_4's).The assignment of these carbons relied upon their relative $_{13}C$-T_1 relaxation times.

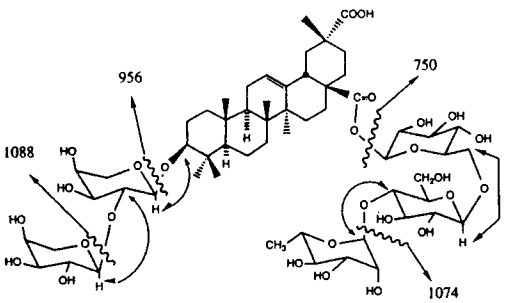


Figure 4. Mass spectral fragmentations and interglycosidic NOE's of **1** for sugar sequence and linkage site determination.

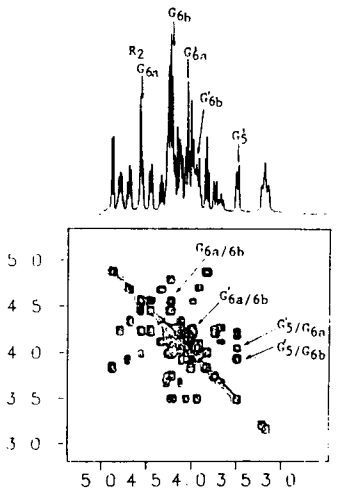


Figure 5. $^1H/^1H$-COSY spectrum of **1**. Labelled peaks indicate geminal and vicinal couplings of glucose C_6 methylene protons.

For the saponins like1, molecular mobility of the individual sugar residues increases (τ_c decreases) as the residues are located away fronm the aglycone, increasing the T_1 relaxation times [16] . Such meassurements can be particularly valuable for distinguishing terminal and internal sugar residiues in oligosaccharide chains,[13], which was the problem at hand to distinguish the arabinose units. Since the correlation times are viscosity dependent, the measurements were recorded at both ambient temperature and at 40°C. The results (Table 1) indicated that the order of T_1 relaxation times of sugar residues obtained from the higher temperature experiment was identical as that determined at room temperature, so an

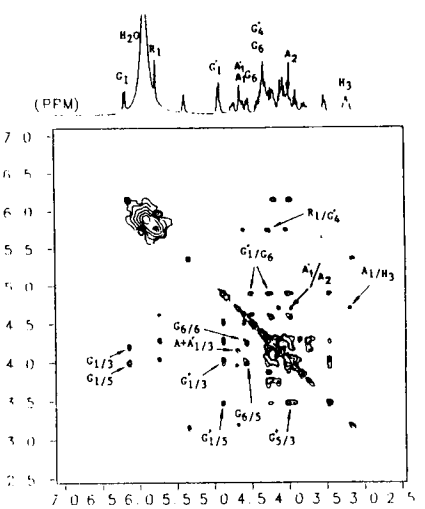


Figure 6 NOESY spectrum of **1** at 2 °C; intraresidue NOE's of sugars are labelled under diagonal and interresidue NOE's are above diagonal. At rt, the NOE between Ara'.H1 and Ara.H2 was not observed.

additional meassurement at higher temperature was not undertaken.

Table 1. ^{13}C-T_1 Relaxation Time of Sugar Units and Anomeric Carbons of **1**[a]

Sugar	T1 (sec. 23^{0}C)		T1 (sec, 40^0 C)	
	Ave. NT_1[b]	Anomeric Carbon	Ave. NT_1[b]	Anomeric Carbon
Glc	0.232	0.232	0.235	0.232
Glc'	0.242	0.240	0.245	0.242
Rha	0.261	0.259	0.266	0.261
Ara	0.341	0.312	0.319	0.314
Ara'	0.342	0.341	0.347	0.343

a) See text for specific T_1's of other arabinose carbons.

b) N= number of attached protons.

The two anomeric carbons of the arabinose units showed distinctive T_1 relaxation times: one (δ 106.7) with the longer relaxation time (0.343s, 40^0), was assigned to the terminal arabinose C_1, and the other anomeric carbon (δ 106.5, T_1=0.314s, 40^0) was assigned to the internal arabinose. Similarly, distinguishing the 5-position carbons of the two arabinose residues was achieved by the difference of their T_1 relaxation times (Ara. C_5, T_1=0.453s, Ara'. C_5, T_1=0.469s). The assignments of the 2-position carbons of the arabinose units were routine based on their chemical shifts: the carbon signal at δ71.5 with its bonded proton at δ3.96 was assigned as Ara'. C_2, while the downfield resonance (δ 74.6) with its attached proton at δ4.35 was assigned as Ara. C_2, the relative downfield chemical shift indicating a glycosidic linkage. The two overlapped arabinose 3-position protons showed correlations with carbons at δ73.6 and 70.2, which were assigned as Ara'. C_3 and Ara. C_3, respectively, based on a chemical shift analysis. Usually arabinose C_3 carbon appear at 72-73, and the relatively high field carbon resonance of Ara. C_3 results from a β-glycosidation. The two C_4 resonances of the arabinose were not resolved (δ 68.6, integration to 2 X C, inverse gated decoupling).

Similarly, ^1H and ^{13}C signals of sugar moieties of additional eight saponins were also assigned.

Conclusion

Beginning from the anomeric protons of sugar moieties of saponins, all 2-position protons of the sugar moieties were easily located in the COSY spectrum. The complete assignments of ^1H and ^{13}C signals of sugar moieties of saponin can be determined by hom-, hetero-nuclear COSY and multi-step RCT experiments, if there is at least one resonance in the spin system, such as the anomeric proton, which is well isolated and which has a reasonably large coupling to its neighbouring spin. Therefore, a slice taken through later spectra at each anomeric proton along the diagonal yields ^1H sub-spectra containing all scalar-coupled protons within that sugar residue. However, the distribution of magnetization around the spin system can be impeded by small couplings such as typically found between H_4 and H_5 of galactosyl residues. In order to circumvent the bottleneck of a small coupling, 1D and 2D versions of the relayed HOHAHA pulse sequence are of intrisic value [17].

Relative ^{13}C-T_1 relaxation times, DIFNOE, NOESY, SINEPT and HMBC experiments are valuable supplements to COSY, RCT experiments in the assignments of ^1H and ^{13}C signals of sugar moieties of saponins, and they are the method of choice when only a few assignments remain questionable after homo-, hetero- COSY and RCT have been applied. Thus, converted application of homo-, hetero- NMR approches and relative ^{13}C-T_1 relaxation times can lead to the unambiguous ^1H and ^{13}C signal assignments for sugar moieties of saponins.

Table 2. NMR Chemical Shift Assignments of **1**

Position	^{13}C Aglycone of $(C_5D_5N)^a$	- Sugar-		
		Position	$^1H(C_5D_5N)^b$	$^{13}C(C_5D_5N)^a$
1	38.1 (t)	Glc. 1	6.13 (d, 8.1Hz)	94.9 (d)
2	22.7 (t)	Glc. 2	4.60 (o)	72.9 (d)
3	88.1 (d)	Glc. 3	4.18 (o)	77.4 (d)
4	38.8 (s)	Glc. 4	4.27 (o)	69.6 (d)
5	55.1 (d)	Glc. 5	3.99 (o)	77.0 (d)
6	17.7 (t)	Glc. 6	4.58, 4.26 (o)	68.2 (t)
7	32.3 (t)	Glc'. 1	4.89 (d, 7.9 Hz)	103.8 (d)
8	39.1 (s)	Glc'. 2	3.85 (dd, 7.9, 8.1 Hz)	74.3 (d)
9	47.3 (d)	Glc'. 3	4.02 (o)	75.5 (d)
10	36.3 (s)	Glc'. 4	4.23 (o)	77.6 (d)
11	28.3 (t)	Glc'. 5	3.50 (o)	76.2 (d)
12	122.6 (d)	Glc'. 6	4.01, 3.97 (o)	60.3 (t)
13	142.9 (s)	Rha. 1	5.68 (d, 2.3 Hz)	101.9 (d)
14	41.6 (s)	Rha. 2	4.57 (o)	71.7 (d)
15	27.5 (t)	Rha. 3	4.46 (dd, 8.7, 2.9 Hz)	71.6 (d)
16	23.1 (t)	Rha. 4	4.25 (o)	69.6 (d)
17	46.3 (s)	Rha. 5	4.80 (m)	69.6 (d)
18	40.1 (d)	Rha. 6	1.58 (d, 5.9 Hz)	17.1 (q)
19	39.1 (t)			
20	41.4 (s)	Ara. 1	4.68 (d, 7.9 Hz)	106.5 (d)
21	26.0 (t)	Ara. 2	4.35 (dd, 7.4, 6.5 Hz)	74.6 (d)
22	30.8 (t)	Ara. 3	4.09 (o)	70.3 (d)
23	27.5 (q)	Ara. 4	4.28 (o)	68.6 (d)
24	16.3 (q)	Ara. 5	4.22, 3.74 (o)	65.8 (t)
25	14.9 (q)			
26	16.7 (q)	Ara. 1	4.70 (d, 7.1 Hz)	106.7 (d)
27	25.3 (q)	Ara. 2	3.96 (o)	71.5 (d)
28	175.9 (q)	Ara. 3	4.10 (o)	73.6 (d)
29	180.9 (q)	Ara. 4	4.28 (o)	68.8 (d)
30	19.2 (q)	Ara. 5	3.3-3.7 (m, 2H)	66.1 (t)

a) Carbon multiplicities from a DEPT experiment; glycosidation sites underlines.
b) " o " = overlapped.

From the assignments described above, it was found that chemical shifts of the two C_6 methylene protons of the glucose units not only shifted downfield, but also | Δδ| values of two protons became more bigger due to glycosilation (Usually, | Δδ|value of normal C_6 methylene protons of glucose units was between 0.11~0.07ppm, while glycosidated C_6 mthylene protons in the range of 0.28~0.34ppm.)(Table 3).

Tabe 3 ^{1}HNMR Chemical Shifts of Normal C_6 Methylene Protons and Glycosidated C_6 Methylene Protons of Glucose Units in Saponins **1-8**(in C_5D_5N)

Type of C_6 Methylene protons	Saponin							
	1	2	3	4	5	6	7	8
Normal C_6 Methylene Protons	4.01 3.97	4.05 4.15	4.05 4.20	4.05 4.14	4.07 4.17	4.05 4.15	4.05 4.15	4.05 4.15
Glycosidated C_6 Methylene Protons	4.26 4.58	4.35 4.65	4.30 4.65	4.35 4.70	4.32 4.65	4.35 4.65	4.35 4.65	4.34 4.15

It is evident that chemical shifts of the proton and carbon signals of the acetified monosaccharide unit involved in a chain differ from those of a monosaccharide without substitution of acetyl group . The most significant changes are associated with the carbons and protons directly involved in the acetyl linkage (C-α) and the adjacent carbons (C-β). From the above assignments (Table 4), it was found that proton signal of H-α appears between 5.1~5.9ppm (downfield shift); the C-α shifts to downfield by 0.7~2.1ppm, while the C-β shifts to highfield by 1.0~3.1ppm.

Table 4.^{13}CNMR Chemical Shifts of Acetified and Normal Monosaccharide Units

Sugars	2	3	4	5	6	7	8	10*
Ara-1	107.2	105.0	104.8	107.1				107.4
Ara-2	73.1	73.9	74.4	70.1				71.2
Ara-3	72.2	72.0	70.5	74.3				74.6
Ara-4	72.4	69.0	73.1	69.7				69.3
Ara-5	65.7	66.9	65.0	64.2				66.7
Xyl-1					107.6	104.4	107.1	
Xyl-2					73.8	74.7	73.8	
Xyl-3					78.7	74.0	79.2	
Xyl-4					71.2	72.4	69.2	
Xyl-5					67.0	64.4	66.7	

Acetification sites underlines. *Compound "10" is Liangwanoside II.

Experimental

For saponin 1, ^{1}H and ^{13}C NMR spectra were recorded in pyridine-d_5 (0.5ml with 2 drops D_2O) after exchanging hydroxy protons for deuterons for 5 X D_2O wash/lyophilization cycles, on a varian XL-400. The δ 7.55 resonance of residual [4-^{1}H] pyridine-d_4 and the δ135.5 resonance of 2-^{13}C] pyridine-d_5 were used as internal references for ^{1}H and ^{13}C, respectively; ^{1}HNMR spectrawere recorded at 400MHz and ^{13}C spectra were recorded at 100MHz. All NMR pule sequences were run using standard Varian software, version 6.1c, except the fixed evolution heteronuclear COSY spectrum which was added to the sequence library according to Reynolds program using an evolution period fixed to 20ms[12]. For the additional saponins: NMR spectra were taken on a brucker AM-500(^{1}HNMR at 500MHz and ^{13}CNMR at 125MHz) spectrometer in C_5D_5N with TMS as an internal standard.

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STRUCTURES OF FOUR NEW TRITERPENOID SAPONINS FROM THE LEAVES OF *OPLOPANAX ELATUS* NAKAI, VII.

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Key Word Index – Oplopanax elatus; Araliaceae; cirenshenoside S;cirenshenoside T; cirenshenoside U; cirenshenoside V

Abstract Four new triterpenoid saponins , named as cirenshenosides S(**1**),T(**2**), U(**3**) and V(**4**), were isolated from the leaves of *Oplopanax elatus* Nakai. Their structures were elucidated to be 28-O- α-L-rhamnopyranosyl(1 → 4)- β -D-glucopyranosyl (1 → 6)- β -D-glucopyranosyl esters of 3 β ,23-dihydroxylup-20(29)-en-28-oic acid 3-O- β -D-glucopyranoside(**1**), hederagenin3-O- β -D-glucopyranoside(**2**), 3 β -hydroxyolean-9(11),12-dien-28-oic acid 3-O- β -D-glucopyranoside(3), 3 α-hydroxyolean-12-en-23,28-dioic acid(**4**), respectively.

Introduction

Oplopanax elatus Nakai (Chinese name "Ciren 刺人参")(Araliaceae) is one Chinese herb whose roots possess tonic, antitussive, fever eliminating and blood pressure regulating effects. Cirenshen mainly grows in Changbai Mountain district of China, especially abounding in Changbai and Antu counties. As one part of our chemical studies on the triterpenoid saponins in the species of Araliaceae grown in the Northeast district of China, we have investigated the triterpenoid saponins in the leaves of *Oplopanax elatus* Nakai. In previous papers[1-3], we reported the isolation and the structure elucidation of twelve new triterpenoid saponins, named cirenshenosides A, E, F, G and H(lupene-type triterpenoid saponins), and cirenshenosides B, C, D, I, J, K and L(oleanene-type triterpenoid saponins), from the leaves of this plant. Further, we have been investigating the constituents in the leaves of this plant and isolate more triterpenoid saponins. The present paper describes the isolation and structure elucidation of four new saponins named as cirenshenosides S, T, U and V, respectively.

Result and Discussion

The dried leaves of *Oplopanax elatus* were extracted with methanol. The methanol extract was treated as experimental section to afford the crude saponins, and repeated chromatography followed by HPLC to furnish four new compounds **1-4**.

All compounds were positive in the Liebermann-Burchard reaction and showed the strong hydroxyl and ester absorption in the infrared(IR) spectra. They were all deduced to be triterpenoid saponins by analyzing the data of NMR and MS spectra.

On acid hydrolysis compound **1** gave D-glucose, L-rhamnose and an aglycone(**1a**) which was identified as 3 β ,23-dihydroxylup-20(29)-en-28-oic acid by direct comparison with an authentic sample[3,4]. The FAB-MS and NMR spectrum data proved that **1** has three β -D-glucosyl groups and one α-L-rhamnosyl group. That glycosylation shifts were observed in the chemical shifts of C-3 and C-28 signals in the ^{13}C-NMR spectrum , which were at δ 82.0 and

174.9 in **1**, while δ 73.4 and 178.9 in **1a**, suggested that **1** is the 3,28-bisdesmoside. The selective cleavage of esteric glycoside linkage of **1** gave an anomeric mixture of methyl α-L-rhamnopyranosyl(1 → 4)- β -D-glucopyranosyl1 → 6)- α and β -D-glucopyranoside(**8**)[5] . Therefore, the structure of **1** was established to be 28-O- α-L-rhamnopyranosyl(1→4)- β -D-glucopyranosyl(1→6)- β -D-glucopyranosyl ester of 3 β ,23-dihydroxylup-20(29)-en-28-oic acid 3-O- β -D-glucopyranoside.

Compound **2** gave hederagenin[6] as an aglycone and D-glucose and L-rhamnose on acid hydrolysis. By the analysis of spectral data and selective cleavage of ester-glycoside linkage in the same way, the structure of **2** was elucidated as 28-O- α-L-rhamnopyranosyl(1→4)- β -D-glucopyranosyl1→6)- β -D-glucopyranosyl ester of hederagenin 3-O- β -D-glucopyranoside.

Compound **3**, white powder $C_{54}H_{86}O_{22}$, showed maximum absorption at 280nm in the UV spectrum, which indicated that there is a conjugated diene system in the same ring in **3**. On acid hydrolysis **3** gave glucose, rhamnose and an aglycone(**3a**). **3a**, white powder, $C_{30}H_{46}O_3$, showed absorption bands due to hydroxyl, carboxylic and double bond groups in the IR spectrum . The ^{1}H-NMR spectrum of **3a** exhibited signals due to seven methyl groups. The ^{13}C-DEPT spectrum indicated that there are seven methyl, nine methylene, two methine, two carbon-carbon double bonds, one hydroxyl and one carboxylic groups. The UV data proved that the two carbon-carbon double bonds is a conjugated diene system in the same ring of **3a** . By further analyzing and comparing the spectral data with the reported[3,7] , **3a** was established to be 3 β -hydroxyolean-9(11),12-dien-28-oic acid. By the same as the above, the structure of **3** was elucidated as 28-O- α-L-rhamnopyranosyl(1 → 4)- β -D-glucopyranosyl1 → 6)- β -D-glucopyranosyl ester of 3 β -hydroxyolean-9(11),12-dien-28-oic acid 3-O- β -D-glucopyranoside.

Compound **4**, white powder, $C_{48}H_{76}O_{19}$, showed absorption bands due to carboxylic group besides hydroxyl and esteric groups in the IR spectrum. The ^{1}H-NMR spectrum of showed six tertial methyl groups and one secondary methyl groups. Its ^{13}C-NMR spectrum resembled that of cirenshenoside C(7)[1] , but **4** had a carboxylic carbon signal at δ 180.1 instead of the C-23 hydroxymethyl carbon signal at δ 71.3 in **7**, suggesting that **4** was a 23-carboxylic derivative of **7**. On acidic hydrolysis , **4** gave glucose , rhamnose and an aglycone(**4a**). On a selective cleavage reaction of esteric glycoside linkage, **4** gave **4a** as an aglycone and the same methyl oligosaccharide **8** such as **1**. **4** was methylated with diazomethane followed by reduction with lithium borohydride. The product was identified as **7** by direct comparison with an authentic specimen. Therefore, **4a** and **4** were characterized as 3 α-hydroxyolean-12-ene-23,28-dioic acid and its 28-O- α-L-rhamnopyranosyl(1 → 4)- β -D-glucopyranosyl1 → 6)- β -D-glucopyranosyl ester, respectively.

Experimental

All melting points were determined on a Kofler micro-melting point apparatus and were uncorrected. Optical rotation were measured with WZZ polarimeter at room temperature. IR spectra were recorded with 5DX-FI spectrometer, and NMR spectra with Unity-400 spectrometer. The FAB- and EI-MS were determined on a VG-7070E spectrometer. Gas liquid chromatography (GLC) was run a Shimaz GC-6A unit equipped with a flame ionization detector (FLD). Experimental conditions for sugar TMS ether: column, 5% SE-52 on chromosorb W, 3mm×2m; column temp. 179℃; injection temp. 200℃; carrier gas(N^2)

1.0kg/cm^2. Thin layer chromatography(TLC) was performed on a precoated silica gel plate(Merck), and detection was achieved by spraying 10% H_2SO_4, followed by heating. For column chromatography silica gel(Merck) and Diaion on HP-20 were used.

Extraction and Isolation of Saponins Dried leaves (2.5kg) of *Oplopanax elatus* Nakai collected in Jilin Province of China were extracted with MeOH (5,000ml×3) at room temperature and the MeOH solution was evaporated *in vacuo* to yield the MeOH extract. The residue (400g) was suspended in water (1,500ml) and was extracted with Et_2O (1,000ml×3) to give an Et_2O extract (96g) and an aqueous extract (285g) after removal of the solvent *in vacuo*. The aq. extract was subjected to column chromatography on DIAION HP-20 with sucessive elution system of H_2O, 3:7MeOH/H_2O, 7:3 MeOH/H_2O, MeOH and $CHCl_3$ to give five fractions; Fr 1(61.5g), Fr 2(10.8g), Fr 3(30.5g), Fr 4(42.6g), Fr 5(10.g). The Fr 3(20g) was chromatographed on silica gel with $CHCl_3$-MeOH-H_2O(65:35:10, v/v, lower layer) and then on ODS with 29:21 MeOH/H_2O to give **1**(220mg), **2**(180mg), **3**(210mg) and 4(185mg).

Cirenshenoside S(**1**): White powder, m.p. 220-222℃, $[\alpha]_D^{20}$-23.5 〃 (c=0.5, MeOH). IR ν_{Max}^{KBr}(cm^{-1}): 3400(OH), 1730(COOR), 1640, 880(C=CH_2). Anal. Calcd. for $C_{54}H_{88}O_{23}3H_2O$: C, 55.96; H,8.12. Found(%): C, 55.90; H,8.08. FAB-MS(m/z): 1103[M-H]$^-$, 957[M-methylpentosyl-H]$^-$, 941[M-hexosyl-H]$^-$, 795[M-hexosyl-methylpentosyl-H]$^-$, 633[M-2×hexosyl-methylpentosyl-H]$^-$, 471[M-3×hexosyl-methylpentosyl-H]$^-$. ^{1}H-NMR(C_5D_5N) δ : 0.63, 0.74, 0.75, 0.92, 1.48(3H each, s), 1.49(3H, d, J=8Hz, Rha H-6), 4.46, 4.63(1H each, m, C=CH_2), 4.73(1H, d, J=8Hz, outer Glc H-1), 4.93(1H, d, J=8Hz, C(3)-Glc H-1), 5.66(1H, br.s, Rha H-1), 6.15(1H, d, J=8Hz, inner Glc H-1). ^{13}C-NMR spectrum (C_5D_5N): see Table Ⅰ.

Cirenshenoside T(**2**): White powder, m.p. 240-242℃, $[\alpha]_D^{20}$+0.7 〃 (c=0.2, MeOH). IR ν_{Max}^{KBr}(cm^{-1}): 3400(OH), 1730(COOR). Anal. Calcd. for $C_{54}H_{88}O_{23}3H_2O$: C, 55.96; H,8.12. Found(%): C, 55.92; H,8.04. FAB-MS(m/z): 1103[M-H]$^-$, 957[M-methylpentosyl-H]$^-$, 941[M-hexosyl-H]$^-$, 795[M-hexosyl-methylpentosyl-H]$^-$, 633[M-2×hexosyl-methylpentosyl-H]$^-$, 471[M-3×hexosyl-methylpentosyl-H]$^-$. ^{1}H-NMR(C_5D_5N) δ : 0.65, 0.66, 0.74, 0.91, 0.97(3H each, s), 1.49(3H, d, J=6Hz, Rha H-6), 4.78(1H, d, J=8Hz, outer Glc H-1), 4.93(1H, d, J=8Hz, C(3)-Glc H-1), 5.19(1H, m, H-12), 5.67(1H, br.s, Rha H-1), 6.04(1H, d, J=8Hz, inner Glc H-1). ^{13}C-NMR spectrum (C_5D_5N): see Table Ⅰ.

Cirenshenoside U(**3**): White powder, m.p. 224-226℃, $[\alpha]_D^{20}$+78.6 〃 (c=0.5, MeOH). UV λ_{max}nm:280(ε =10,000). IR ν_{Max}^{KBr}(cm^{-1}): 3420(OH), 1730(COOR). Anal. Calcd. for $C_{54}H_{86}O_{22}3H_2O$: C, 56.84; H,8.07. Found(%): C, 56.75; H,8.04. FAB-MS(m/z): 1085[M-H]$^-$, 939[M-methylpentosyl-H]$^-$, 923[M-hexosyl-H]$^-$, 777[M-hexosyl-methylpentosyl-H]$^-$, 615[M-2×hexosyl-methylpentosyl-H]$^-$, 453[M-3×hexosyl-methylpentosyl-H]$^-$. ^{1}H-NMR(CD_3OD) δ : 0.65(3H×2, s), 0.83, 0.96, 0.98, 1.14, 1.20(3H each, s), 1.52(3H, d, J=6Hz, Rha H-6), 4.73(1H, d, J=7.6Hz, outer Glc H-1), 4.93(1H, d, J=8Hz, C(3)-Glc H-1), 5.49(2H, br.s, H-11 and H-12), 5.70(1H, br.s, Rha H-1), 6.08(1H, d, J=8.2Hz, inner Glc H-1). ^{13}C-NMR spectrum (C_5D_5N): see Table Ⅰ.

Cirenshenoside V(**4**): White powder, m.p. 230-232℃, $[\alpha]_D^{20}$-5.5 〃 (c=0.5, MeOH). IR ν_{Max}^{KBr}(cm^{-1}): 3400(OH), 1725,1700(COOR). Anal. Calcd. for $C_{48}H_{76}O_{19}2H_2O$: C, 58.06; H,8.06. Found(%): C, 58.02; H, 8.10. FAB-MS(m/z): 955[M-H]$^-$, 809[M-methylpentosyl-H]$^-$, 647[M-hexosyl-methylpentosyl-H]$^-$, 485[M-2×hexosyl-methylpentosyl-H]$^-$. ^{1}H-NMR(CD_3OD) δ : 0.62, 0.65, 0.83, 0.89, 0.97, 1.27(3H each, s), 1.50(3H, d, J=8Hz, Rha H-6), 4.79(1H, d, J=8Hz, outer Glc H-1), 5.22(1H, m, H-12), 5.67(1H, br.s, Rha H-1), 6.04(1H, d, J=8Hz, inner Glc H-1). ^{13}C-NMR spectrum (C_5D_5N): see Table Ⅰ.

Acid Hydrolysis of Compounds 1-4 Each compound (40mg) in 2N H_2SO_4(4ml) was heated in a boiling water bath for 2 hr, cooled and the mixtures were extracted with $CHCl_3$(4ml ×3). The aq. layer was neutralized, derivatived to TMS ether and examined on GLC: t_R(min), 10.0 and 15.0(D-glucose); 3.5 and 4.6(L-rhamnose). The $CHCl_3$ solution was dried over Na_2SO_4 followed by removal of the solvent to give **1a**(10.5mg), **2a**(14.2mg), **3a**(10.0mg) and **4a**(12.4mg), respectively.

Compound **1a**: white powder , m.p. 265-268℃. EI-MS m/z: 472[M^+], 454, 436, 395, 248,223,203, 189(base peak). ^{1}H-NMR(C_5D_5N) δ :0.91, 1.02, 1.04, 1.09, 1.78(3H each, s, tert. -CH_3), 2.23(2H, m), 2.59(1H, d, J=12Hz), 2.74(1H, br. t), 3.45, 4.19(1H each, m, C=CH_2), 3.71, 4.18(1H each, d, J=10Hz), 4.77, 4.94(1H each, s). ^{13}C-NMR spectrum(C_5D_5N): see Table Ⅰ. 1a was identified as 3 β ,23-dihydroxylup-20(29)-en-28-oic acid by the comparision of the NMR spectral data with reported values.

Compound **2a**: colorless needles($CHCl_3$-MeOH), m.p. 330-334 ℃(decomposed), was identified as hederagenin by m.p. and co-TLC with the authentic sample

Compound **3a**: colorless needles($CHCl_3$-MeOH), m.p. 296-298℃. U V λ_{max}nm: 279(ε =10,000). EI-MS m/z: 454, 436, 379. ^{1}H-NMR(C_5D_5N) δ :0.67, 0.69, 0.78, 0.91, 0.95, 1.21, 1.20(3H each, s, tert. -CH_3), 5.36(1H d, J=5.6Hz), 5.40(1H , d, J=5.5Hz).

Compound **4a**: colorless needles(hexane-EtOAc), m..p. 282-284℃. EI-MS m/z: 486[M^+], 468,440, 422, 248, 220, 203,187, 175. ^{1}H-NMR(C_5D_5N) δ : 0.70, 0.72, 0.88, 0.91, 0.93, 1.25(3H each, s, tert. -CH_3), 2.98(1H, br. s, H-18), 3.50(1H, br. , H-3), 5.22(1H, m, H-12). ^{13}C-NMR spectrum(C_5D_5N): see Table Ⅰ. **4a** was identified as 3α-hydroxyolean-12-en-23,28-dioic acid by the comparision of the NMR spectral data with reported values.

Selective Cleavage of Esteric Glycoside Linkage A solution of a sample (40mg) and LiI(40mg) in anhydrous MeOH(4ml) containing 2,6-lutidine (1ml) was refluxed for 24 hr under N_2. After cooling, the reaction mixture was diluted with 50% aq. MeOH and deionized with Amberlite MB-3 resin followed by removal of the sovent *in vacuo*. The residue was chromatographed on silica gel($CHCl_3$-MeOH-H_2O=6:4:1, v/v), to give a prosapogenin or an aglycone and a methyl oligosaccharide(**8**), the latter being identified as an anomeric mixture of methyl α-L-rhamnopyranosyl(1 → 4)-β-D-glucopyranosyl(1 → 6)- α- and -β-D-glucopyranosides.

Conversion of **4** to **7** by reduction with $LiBH_4$ Treatment of **4**(40mg) in MeOH (2ml) with CH_2N_2 ether was a methyl ester of **4**. The methyl ester of **4** was heated at 60 with $LiBH_4$(35mg) in EtOAc(4ml) for 96 hr. After decomposition of the excess reagent with acetone (ml) followed by removal of then solvent *in vacuo*, the residue was purified by silica gel chromatography with CHCl*3*-MeOH-H_2O=30:10:1, v/v, to yield **7**(6mg).

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Table I ^{13}C-NMR Signals of Compounds **1-8** in C_5D_5N

	1	**2**	**3**	**5**	**4**	**7**	**1a**	**2a**	**3a**	**6**	**4a**
Aglycone											
C-1	38.9	38.7	37.0	37.1	33.0	32.9	38.6	38.7	38.0	32.2	32.8
2	26.0	25.8	27.1	27.2	26.0	26.5	27.9	28.1	27.1	27.0	26.1
3	82.0	82.2	88.2	80.6	72.8	75.7	73.4	73.3	78.7	75.2	72.9
4	43.5	43.4	38.4	37.9	51.7	40.7	48.7	42.6	38.3	41.0	51.8
5	47.7	47.7	51.5	51.2	44.8	43.6	42.9	48.7	51.0	39.6	44.7
6	18.1	18.2	18.0	18.2	21.8	18.4	18.6	19.1	18.3	18.2	21.3
7	34.3	32.5	32.1	32.1	32.5	32.6	34.5	33.6	32.6	32.0	32.4
8	41.1	39.9	39.2	38.6	40.4	41.3	41.1	39.9	39.4	41.3	40.5
9	49.7	48.1	154.9	153.9	48.1	48.1	49.7	48.1	154.5	155.8	48.1
10	37.0	36.9	40.8	40.7	37.2	37.3	37.6	37.5	40.5	39.1	37.4
11	21.1	23.8	115.3	115.9	23.8	23.8	21.1	24.0	115.0	115.5	24.3
12	25.9	122.9	120.0	120.7	122.8	123.0	26.1	120.0	120.1	121.0	122.5
13	38.3	144.1	145.0	147.2	144.2	144.2	39.1	144.1	144.6	145.3	144.1
14	42.7	42.1	42.6	42.7	41.7	42.2	42.9	42.0	42.3	43.1	41.9
15	30.1	28.3	26.7	25.6	28.2	28.2	30.3	28.1	26.3	27.5	28.3
16	32.2	23.3	23.5	24.3	23.2	23.4	32.8	23.5	23.4	23.9	23.2
17	56.9	47.0	45.9	32.1	47.0	47.0	56.6	47.0	45.6	46.4	47.5
18	47.4	41.6	39.6	45.6	41.7	41.7	47.7	41.9	39.3	40.1	41.7
19	50.9	46.2	45.7	46.8	46.0	46.2	49.7	46.2	45.5	46.2	46.0
20	150.8	30.7	30.2	31.1	30.7	30.7	151.3	30.8	30.0	30.6	30.9
21	30.8	34.0	33.4	34.6	33.9	34.0	31.2	34.0	33.2	33.8	34.0
22	36.8	32.8	31.8	36.9	31.7	29.9	37.3	32.8	31.5	29.9	31.8
23	64.5	64.8	27.9	28.7	180.1	71.3	67.8	68.2	27.5	71.2	180.0
24	13.5	13.6	16.4	16.8	18.2	18.2	12.9	13.2	16.0	18.2	18.0
25	17.4	16.2	20.0	20.0	16.0	15.9	16.8	15.9	19.8	25.7	16.0
26	16.4	17.6	20.4	21.0	17.8	17.6	16.4	17.1	20.2	21.0	17.9
27	14.8	26.1	24.9	28.1	26.0	26.1	14.9	26.1	24.7	20.4	26.0
28	174.9	176.5	176.1	25.3	176.5	176.5	178.9	180.0	179.8	176.6	180.5
29	110.0	33.1	32.4	33.2	33.0	33.0	109.7	33.1	32.1	32.8	33.1
30	19.4	23.7	23.1	23.7	23.7	23.7	19.4	23.7	23.0	23.6	23.7

	1	**2**	**3**	**6**	**4**	**7**	**8**		
							8a	**8b**	
Sugar at C-3 of algycone									
Glc-1	105.7	105.8	106.3						
2	75.9	75.8	75.3						
3	78.3	78.7	78.3						
4	71.6	71.6	71.3						
5	78.7	78.3	77.8						
6	62.8	62.8	62.5						
Sugars at C-28 of aglycone									
Glc-1	95.2	95.6	95.2	95.6	95.6	95.6	101.0	105.5	
2	74.0	74.0	73.5	73.9	73.9	74.0	73.6	74.9	
3	78.7	78.7	78.2	78.6	78.7	78.7	75.0	78.2	
4	70.8	70.9	70.4	70.8	70.8	70.9	72.2	71.4	
5	78.0	78.0	77.5	77.9	78.0	78.0	72.5	76.8	
6	69.4	69.2	68.7	69.1	69.1	69.2	70.1	69.9	
Glc-1	105.1	104.8	104.3	104.7	104.9	104.8	104.8	104.6	
2	75.3	75.3	74.8	75.2	75.3	75.3	75.0	75.0	
3	76.4	76.5	76.0	77.1	76.5	76.5	76.2	76.2	
4	78.1	78.2	77.7	78.3	78.1	78.3	78.1	78.1	
5	77.1	77.1	76.6	76.5	77.1	77.1	76.8	76.8	
6	61.3	61.3	60.8	61.2	61.2	61.3	61.1	61.1	
Rha-1	102.7	102.7	102.3	102.7	102.7	102.7	102.4	102.4	
2	72.6	72.6	72.1	72.5	72.6	72.5	72.4	72.4	
3	72.7	72.7	72.3	72.7	72.7	72.7	72.5	72.5	
4	73.9	73.8	73.4	73.9	73.8	73.8	73.6	73.6	
5	70.3	70.3	69.8	70.3	70.3	70.3	70.1	70.1	
6	18.5	18.5	18.0	18.4	18.5	18.5	18.2	18.2	
OMe								55.0	56.7

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7. Jesus G., Braaulio M. F., Antonio G. G., Pedro G., Melchos G. H. and Jose M. M. (1984) *Phytochemistry* **23**, 1471-1473

RECENT ADVANCES IN STUDIES ON GLYCOSIDES OF ARALIACEOUS PLANTS

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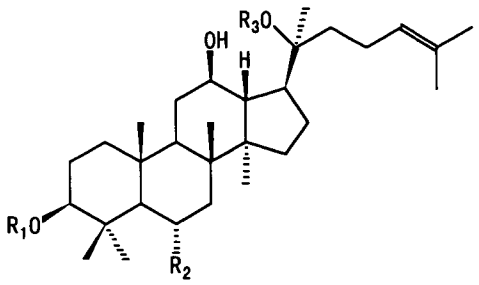
Introduction

Many plants of Araliaceous family have been used as traditional drugs, such as *Panax ginseng*, for a long time in many countries. Araliaceous plants were always important objects in the field of research on natural glycosides. Up to the end of 1980s, the chemical constituents of many Araliaceous plants had been thoroughly investigated with the advent of the very efficient chromatographic techniques and newer spectroscopic methods, e.g. ^{13}C-NMR spectroscopy and soft-ion mass spectrometry, namely FD-MS and FAB-MS. It is discovered that glycosides, especially triterpenoid glycosides as the main biologically active constituents, are widely distributed in these plants. The structures and most of biological actions of these glycosides are well known. The classical era of the chemistry of triterpenoid glycosides seems to have ended at the end of 1980s. But triterpenoid glycosides have been attached much attention in recent years because of their newly discovered biological activities. Many novel glycosides have been isolated and elucidated from some Araliaceous plants. This review incorporates newly discovered structures and biological activities reported during the period 1990 to 1997.

Reports of New Glycosides

Ginseng, the most famous oriental drug in the world has long been used as a tonic or a drug of longevity in Chinese traditional medicine. A number of triterpenoid glycosides derived from protopanaxatriol, protopanaxadiol and oleanolic acid have been isolated and identified. Two new glycosides named ginsenoside-F_5 (**1**) and ginsenoside Rd_2 were isolated from the leaves with the aim of searching for dammarane saponin with biological activity of reducing the toxicity induced by some anticancer drugs[1,65]. Ginsenoside F_5 is the first example of protopanaxatriol-type saponin with arabinofuranose from the taxon within *P. ginseng* species. The study indicates that arabinopyranoside and arabinofuranoside coexist not only in protopanaxadiol-type but also in protopanaxatriol-type in the same plant. In the course of search for new natural sources of biologically active saponin of reducing side-effects induced by prednisone, another new dammarane saponin, ginsenoside-I_a (**2**), was isolated from the same source as mentioned above[2]. It is the first protopanaxatriol-type glycoside from *P. ginseng* with a glucose linked to the C-3 position.



R_1=H, R_2=OH, R_3=glc[6]—ara(fur) Ginsenoside F_5 (1)

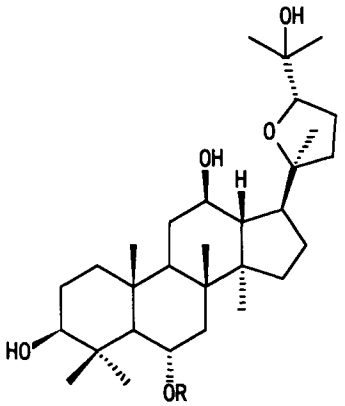
R_1=glc, R_2=OH, R_3=glc Ginsenoside Ia (2)

P. ginseng and its two congeners, *P.notoginseng*, *P. quinquefolium* are well known and have been widely used in many countries of the world, especially in Asia and North America. Previously, South Yunnan of China and North Vietnam seemed to be the southern limit of the distribution of the *Panax* genus. However, a wild *Panax* species named *Panax vietnamensis* Ha et Grushv., was discovered in Central Vietnam in 1973. This plant was a folk medicine of the South Annamitic Range in Central Vietnam and has been regarded as a life-saving plant drug used for treatment of many serious diseases and for enhancing physical strength.

From rhizomes and roots of *P. vietnamensis*, commonly known as Vietnamese Ginseng, thirty-seven triterpenoid glycosides have so far been isolated and elucidated, including twenty-three known compounds and fourteen new glycosides[3-5]. The new compounds are vina-ginsenoside-R1 to -R14 (**3-16**). The known compounds are ginsenoside-Rh_1, 20(R)-ginsenoside- Rh_1, ginsenosides-Rg_1, -Re, -Rd, -Rb_3, -Rb_2, -Rb_1, pseudo-ginsenoside-RS_1, notoginsenosides-R1 and Fa, pseudo-ginsenoside-RT_4, 24(S)- pseudo-ginsenoside-F_{11}, majonosides-R1 and -R2, ginsenoside-Ro, hemsloside-Ma, 20-gluco-ginsenoside-Rf, ginsenoside-Rc, notoginsenoside-R_6, quinquenoside-R_1, gypenoside XVII, majoroside F1.

Among the new saponins, vina-ginsenoside-R3 is the first naturally occuring glycoside of dammarenediol II, while vina-ginsenoside-R5 and -R6, two ocotillol-type saponin, are two other examples of saponins having the rare α -glycosyl linkage. The underground part of this plant contains mainly dammarane saponins and a small amount of oleanolic acid saponins. The yield of octotillol-type saponins, especially majonoside-R2, is more than 5% and *ca.* half of the total yield saponin. This characteristic saponin composition seems to be significant as far as the search for further taxonomical correlation of this plant to other *Panax* spp. is concerned. Moreover, the high content of ocotillol-type saponins has also stimulated the study of this plant with a view to discovering new pharmacological actions and medicinal uses. Vietnamese Ginseng has already been subjected to pharmacological and clinical study and has been widely used in Vietnam. Besides ginseng-like pharmacological actions, it also exhibits markedly

antibacterial effects on *Streptococci* and is apparently effective in the treatment of some throat, cough, etc.

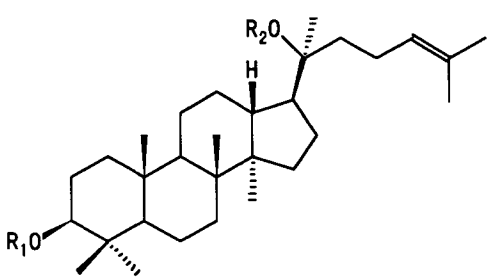


R=glc^2—rha (6-Ac) Vina-ginsenoside-R1 (**3**)

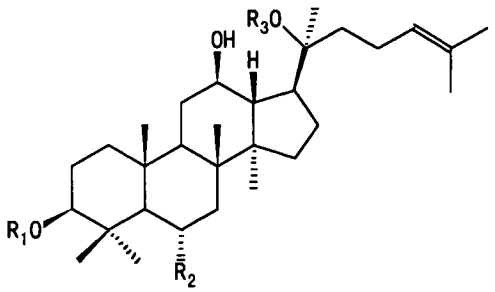
R=glc^2—xyl (6-Ac) Vina-ginsenoside-R2 (**4**)

R=glc^2—xyl^4— α -glc Vina-ginsenoside-R5 (**7**)

R=glc^2—xyl (6-α -glc) Vina-ginsenoside-R6 (**8**)

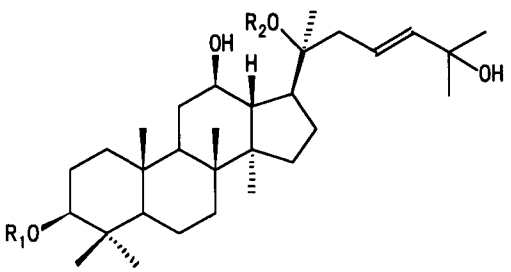


R_1=glc^2—glc, R_2=glc Vina-ginsenoside-R3 (**5**)

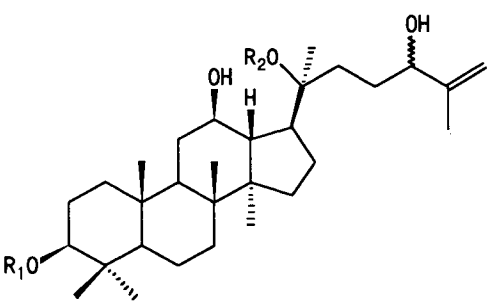


R_1=glc^2—glc^2—xyl, R_2=H, R_3=glc Vina-ginsenoside-R7 (**9**)

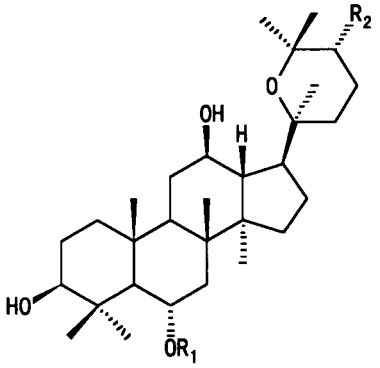
R_1=glc^2—glc, R_2=OH, R_3=glc Vina-ginsenoside-R4 (**6**)



R_1=glc^2—glc, R_2=glc Vina-ginsenoside-R8 (**10**)

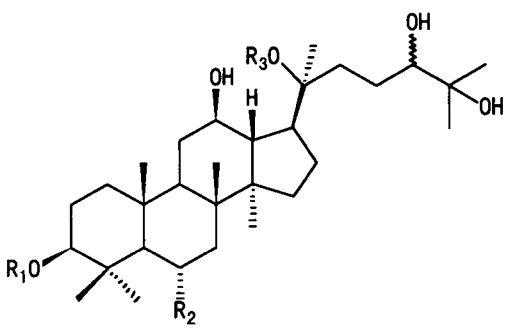


R_1=glc^2—glc, R_2=glc 24(S) - Vina-ginsenoside-R9 (**11**)



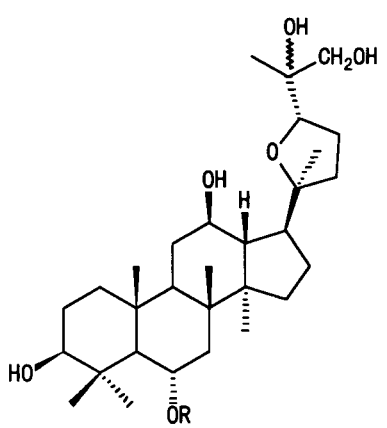
R_1=glc, R_2=OH Vina-ginsenoside-R10 (**12**)

R_1=glc^2—xyl, R_2=OH Vina-ginsenoside-R11 (**13**)



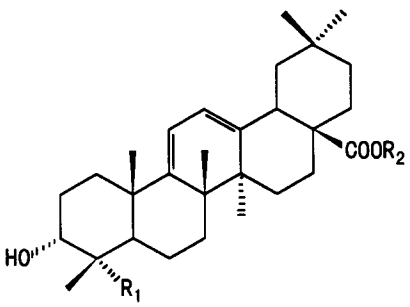
R_1=H, R_2=-O-glc, R_3=H Vina-ginsenoside-R12 (**14**)

R_1=glc^2—glc, R_2=H, R_3=glc Vina-ginsenoside-R13 (**15**)

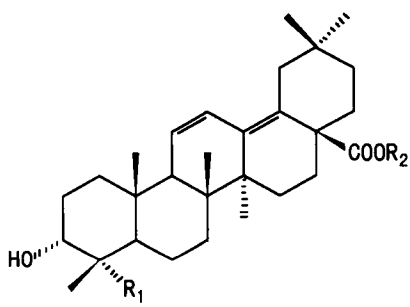


R=glc^2—xyl Vina-ginsenoside-R14 (**16**)

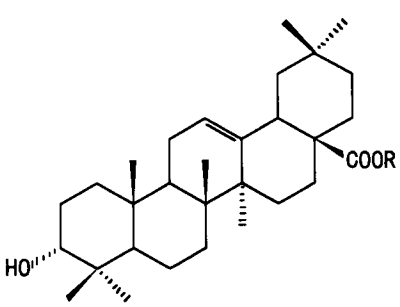
Oplopanax elatus Nakai, Araliaceae, one of traditional Chinese medicines, is used for enhancing physical strength, relieving cough, reducing fever and regulating blood pressure. From leaves of this plant, six new triterpenoid glycosides were isolated and elucidated as cirensenoside I (**17**), cirensenosides J-K (**18-20**), cirensenosides O and P (**21-22**)[6,7].



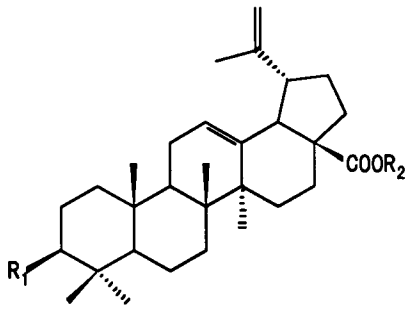
R_1=CHO, R_2=glc^6—glc^4—rha, Cirensenoside I (**17**)
R_1= CH_2OH, R_2=glc^6—glc^4—rha, Cirensenoside J (**18**)



R_1=CHO, R_2=glc^6—glc^4—rha, Cirensenoside K (**19**)
R_1= CH_2OH, R_2=glc^6—glc^4—rha, Cirensenoside L (**20**)

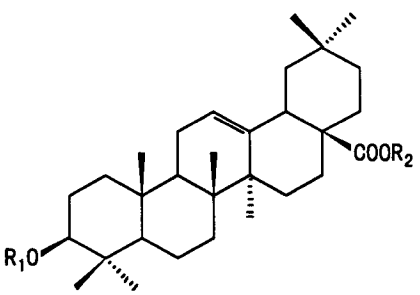


R=glc^6—glc^4—rha, Cirensenoside O (**21**)



R_1=O-glc, R_2=glc^6—glc Cirensenoside P (**22**)

With the aim of search for biologically active principles of having preventive and therapeutic effects for ethanol intoxication from Chinese traditional medicines, *Aralia elata* Seem. was investigated by Yoshikawa et al. Elatosides A-D were isolated and elucidated from fresh bark.



R_1=glcA^3(xyl^2)—gal, R_2=H Elatoside A (**23**)

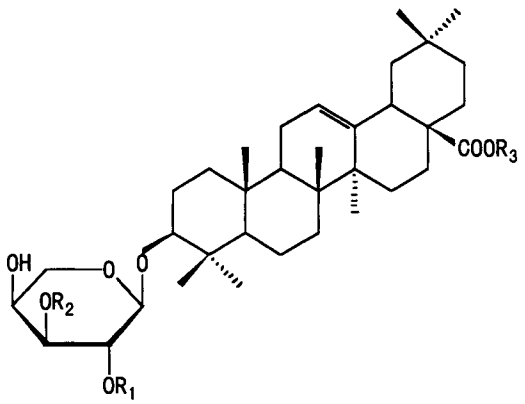
R_1=glcA^3(gal^2)—gal, R_2=H Elatoside B (**24**)

R_1=glcA^3(xyl^2)—gal, R_2=glc Elatoside C (**25**)

R_1=glcA^3(gal^2)—gal, R_2=glc Elatoside D (**26**)

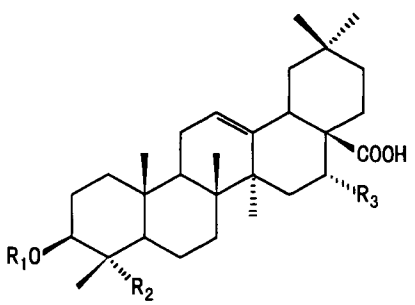
It was found that the 3-O-glucuronide moiety and the 28-carboxyl group in oleanolic acid glucuronide saponin were required to exert the inhibitory activity. Elatoside A and B were found to show potent inhibitory activity on ethanol absorption.

Research on hypoglycemic principles from the root cortex and the young shoot of *Aralia elata* resulted in the isolation and elucidation of elatoside E-K (**27-33**)[8-10].



R_1=xyl, R_2=glc, R_3=H Elatoside E (**27**)

R_1=xyl, R_2=glc, R_3=glc Elatoside F (**28**)



R_1=glcA, R_2=CH_2OH, R_3=OH Elatoside G (**29**)

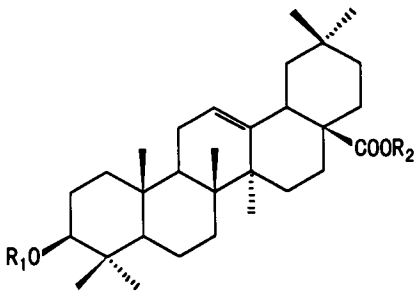
R_1=glcA^3—glc, R_2=CH_3, R_3=OH Elatoside H (**30**)

R_1=glcA^3—glc, R_2=CH_3, R_3=H Elatoside I (**31**)

|2
glc

R_1=glc^3—glc, R_2=CH_2OH, R_3=H Elatoside J (**32**)

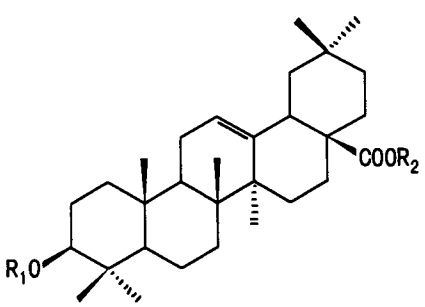
|2
glc



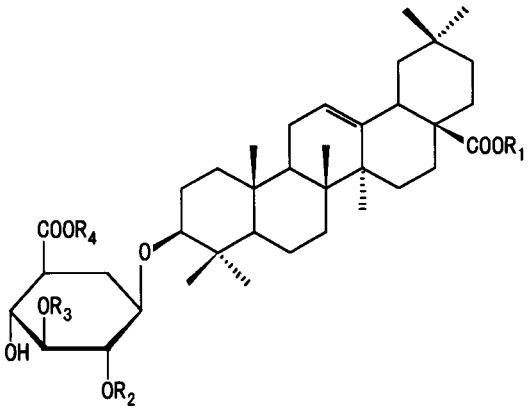
R_1=glcA^3—glc, R_2=glc (Elatoside K (**33**))
|^2
xyl

Other new natural triterpenoid glycosides with trivial names and sources are listed as follows:

Name	Source	Reference
Aradecoside A (**34**)	*Aralia decais*	[11]
Taibaienoside Ⅵ (**35**)	*Aralia taibaiensis*	[12]
Armatoside (**36**)	*Aralia armata*	[13]
Congmuyenoside A-B (**37-38**)	*Aralia elata*	[14]
Papyrioside LA-LH (**39-46**)	*Tetrapanax papyriferum*	[15,16]

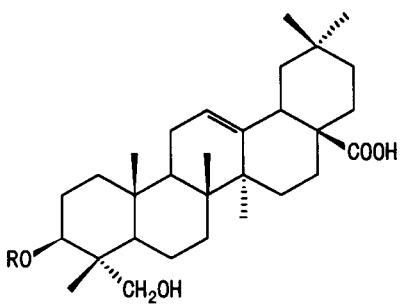


R_1=glc^3—xyl, R_2=glc^6—glc (**34**)
|^2
glc



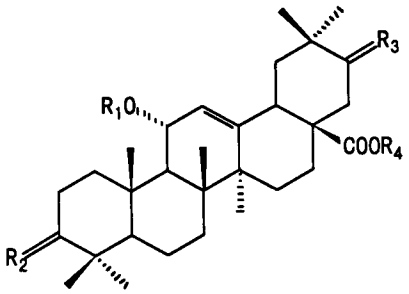
R_1=H, R_2=xyl, R_3=glc **(35)**

R_1=glc, R_2=glc^2—ara(fur), R_3=H **(36)**



R=glc^3—glc (2 → glc) Congmuyenoside A **(37)**

R=glc^3—glc^3—glc (2 → glc) Congmuyenoside B **(38)**

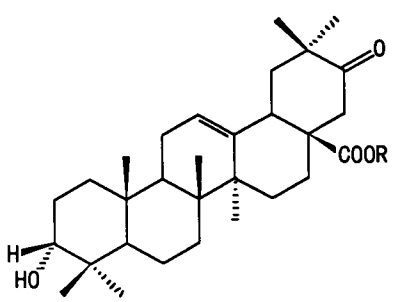


R_1=H, R_2=O, R_3=O, R_4=glc^6—glc^4—rha (**39**)
Ac (6)

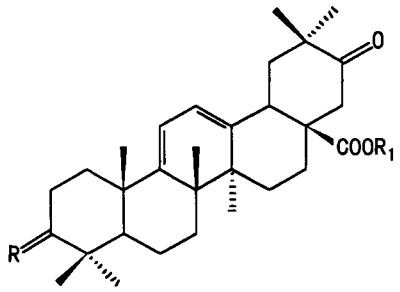
R_1=Me, R_2=O, R_3=O, R_4=glc^6—glc^4—rha (**40**)
Ac (6)

R_1=Me, R_2= H, OH , R_3=O, R_4=glc^6—glc^4—rha (**41**)
Ac (6)

R_1=Me, R_2=O, R_3= H, OH , R_4=glc^6—glc^4—rha (**42**)



R=glc^6—glc^4—rha (**43**)
R=glc^6—glc^4—rha (**44**)
Ac (6)



R=  , R_1=glc^6—glc^4—rha (**45**)

R=O, R_1=glc^6—glc^4—rha (**46**)

Biological Activity

It is well known that Araliaceous plants are rich in triterpenoid glycosides which possess a wide range of biological properties. In recent years, some known and new structural glycosides have been investigated and many new biological activities described.

Action on Cardiovascular System

Many ginsenosides were proven to possess anti-arrhythmic activity. The relationship between structures of ginsenosides and their anti-arrhythmic activities was clarified by Chen and his colleagues[66]. Ginsenoside-Rb_1 was studied for its blockade action on calcium channel[17]. Rb_1 at concentration of 250 μ g/ml significantly inhibited the activity of single type B Ca^{2+} channel on single ventricular myocardiocytes of Wistar rats. Rb_1 decreased the open-state probability and shortened the open time of Ca^{2+} channel, without influencing the amplitude of the ionic current flow through the opening Ca^{2+} channel. By using the methods of observation under electron microscopy and electron spin resonance spectroscopy, antioxidation damage action of Rb_1 on cultured myocardiocytes of rats was studied[18]. The results indicated that xanthine-xanthine oxidase destroyed the myofilaments and mitochondria and increased the amount of free radicals. Rb_1 at concentration of 30 μ g/ml in the medium could apparently antagonize all of the variations induced by xanthine-xanthine oxidase.

Effect of panaxadiol saponin (PDS) on phorbol ester induced change of protein kinase C activity in cardiomyocytes of rats was investigated[19]. Partially purified protein kinase C was incubated with PDS at concentration of 1 to 1000 μ g/ml for ten minutes *in vitro*. The activity of kinase C was inhibited in a dose-dependent manner by PDS. In cardiomyocytes preincubated with PDS at concentration of 250, 500, 1000 μ g/ml, respectively, for ten minutes, PMA-induced decrease of cytosol protein kinase C activity and increase of membrane protein kinase C activity were greatly inhibited in the same manner. PDS not only inhibited protein kinase C *in vitro*, but also inhibited activation of protein kinase C in cardiomyocytes.

It was reported that ginsenosides exerted a protective effects on calf aortic endothelial cells

(ECs) damage against lipid oxidation, and might play an important role in antiatherosclerosis through its protective effects on ECs[20]. Ginsenosides at concentration of 40 μ g/ml could reduce the concentration of MDA in the cultured calf aortic endothelial cells, while the 6-keto-$PGF_{1\alpha}$ content in the medium was reduced and the morphologic damage of the Ecs was alleviated.

Jiang et al. reported that Ginsenosides-Re, -Rg_1, -Rg_2 and-Rh had both calcium channel blockade and anti-free-radical actions, -Rf had blockade action on L type channel in cultured myocardiocytes of rats with the cell attached configuration of patch-clamp technique and electron spin resonance method[21]. Ginsenoside-Re could coordinately promote the colony formation and increase ^{3}H-TdR incorporation of bone marrow cell[22].

It was found that in rats pretreated with *Panax quinquefolium* saponin (PQS) at doses of 50 and 100mg/kg ig once every day for seven days, the scope of myocardial necrosis decreased significantly at 24h after injecting isoprealine, and serum CK and LDH activity also decreased significantly. The levels of serum FFA and myocardial tissue LPO decreased significantly in animals[23]. PQS also showed antioxidation effect on the myocardium injury induced by doxorubicin in rats. The action may be related to the increase of the glutathione peroxidase and SOD activities[24]. PQS could protect cultured rat cardiac cells from the oxidative damage induced by xanthine-xanthine oxidase[25]. In addition, PQS could significantly decrease LPO content of liver tissue and serum in rats with hyperlipidemia and increase glutathione peroxidase activity[26].

A study on PQS on myocardial blood flow and cardiac oxygen metabolism in dogs indicated that PQS at a dose of 50mg/kg iv could significantly increase myocardial blood flow, decrease coronary resistance and cardiac oxygen consumption. It also caused drop of myocardial minute oxygen consumption index[27]. PQS at concentration of 0.03-3mg/ml could inhibit the contractility of papillary muscle of guinea pigs, and on depolarized sample of papillary muscle with high potassium, PQS could increase this contractility. Ginsenoside-Re (10mg/kg), -Rb_3 (30mg/kg) could inhibit the hemodynamic indication of rats, but pseudoginsenoside-F_{11} (10mg/kg) acted the other way round[28]. Rb_3 from leaves of *P. quinquefolium* at a dose of 30mg/kg could decrease the MAP $\pm dp/dt_{max}$ and LVSP of anesthetic rat hearts *in vivo*. 300mg/L of Rb_3 could decrease the open time and open-state probability of L, B and T type calcium channel[29]. Pseudoginsenoside-F_{11} at dose of 10mg/kg could increase Bp, LVSP and $\pm dp/dt_{max}$ of anesthetized rats significantly. When -F_{11} was injected into lateral ventricle and posterior hypothalamic nucleus, it elevated blood pressure of rats significantly in comparison with that of saline control group[30]. At concentration of 3, 10, 30mg/L, respectively, -F_{11} increased the amplitude of the action potential, overshooting, threshold, the maximum diastolic potential, the maximum rate of depolarization and action potential duration at 10%, 50% levels of depolarization in cultivated rat cardiac cells in dose-dependent manner. The effect of -F_{11} (10mg/L) was antagonized by verapamil (2 μ mol/L), but was similar to Bay K 8644[31]. In

addition, PQS showed effect of prevention and treatment on the atherosclerosis[32].

Huang, et al. reported that *Panax notoginseng* saponin had protective effects on myocardial ischemia and reperfusion injury in conscious rabbits[33]. *Panax notoginseng* saponin at dose of 50 and 100mg/kg significantly decreased the activities of creratine phosphokinase (CPK) and lactate dehydrogenase (LDH), and the abnormal changes of ECG during ischemic and reperfusion periods, reduced the size of myocardial ischemic area. Panaxatriol saponins from *Panax notoginseng* showed protective effects on myocardial ischemic-reperfused injury and arrhythmias in the Langendorff's perfused rat heart[34]. The panaxatriol saponin was also found to exert remarked antiarrhythmic activities on coronary artery induced ischemic and reperfused arrythmias in rats. For iv $CaCl_2$-Ach induced a trial fibrillation and/or flutter in mice, the saponin produced a significant protective effect[35]. The panaxatriol saponin was shown to increase the duration of action potential (APD_{30}, APD_{50}, APD_{90}) in sheep cardiac Purkinje firbers at concentration of 2.5 and 5.0 μ g/ml. At concentration of 1.25 to 10.0 μ g/ml. the saponin depressed the delayed rectifier current in time- and dose-dependent manner[36]. In addition, the saponin was found to inhibit experimental thrombosis significantly at dose of 50 to 200mg/kg in rats. This action may be due to its inhibitory action on platelet aggregation and TAX_2 release[37].

The study on influence of *Acanthopanax senticosus* saponin on blood flow dynamics of isolated working heart showed the saponin had calcium channel blockade action dose-dependently. At concentration of 50, 100 and 200 μ g/ml, the saponin reduced the myocardial contractility immediately, after washing out, the action disappeared[38].

Acanthopanax sentcosides b, C_1 and C_3 were proved to be antagonist on calcium channel[39-41].

Action on Immunomodulation

Chang, et al. reported that proliferation of thymocytes could be promoted by the supernatant from cultured rat hippocampus cells, and proliferation of the lymphocytes could be enhanced by injection of *Panax quinquefolium* saponin into the hippocampus[42].

The male rats were given ip panaxatriol saponin from *P. ginseng* 5mg/d 24h before and after whole-body irradiation with 5 Gy X-ray for fourteen days. It was found that significant increase in the number of WBC, thymocyte and splenocyte and the decrease in thymic and splenic weight following irradiation was also abated by panaxatriol saponin. Meanwhile, there was also a tendency of increase in the number of peripheral WBC, thymocyte and splenocyte in the panaxatriol group. The investigation suggested that the saponin had not only a radioprotective effect on immune organs in the irradiated body, but also an enhancing effect on the defense functions in the normal body[43].

It has been well documented that the immune function declines with age in the human and animals. The possible cause for the decline are the inability of lymphocytes to proliferation in response to mitogenic stimulation and the decrease of IL-2 production. Ginsenoside Rg_1 was

shown to selectively enhance the proliferation of lymphocytes and the production of IL-2 in aged rats, and was found to promote IL-2 gene expression which showed increase of IL-2 mRNA and IL-2 protein contents. Under the same conditions, no marked influence was observed in the studies on effect of Rg_1 on the immune function of young adult rats, suggesting Rg_1 is an immunoregulator rather than a purely immunopotentiating agent[44]. Using methods of fluorescence flow cytometry and Western blot analysis, Rg_1 was found to enhance the expression of IL-2 receptor α chain and inhibit the release of soluble IL-2 receptor. In *in vitro* experiment, Rg_1 showed no influence on Con A-induced increase of cytoplasmic free calcium concentration, but significantly increase the levels of intracellular cAMP and cGMP in aged rats, suggesting that Rg_1 enhances immune function in old rats might be mediated by increase of cAMP and cGMP contents, resulting in IL-2 gene expression and splenocyte proliferation[48].

Subcutaneous administration of ginsenosides from stems and leaves of *P. ginseng* at dosage of 50mg/kg for four days could resist the elevation and the reduction of cAMP-phosphodiesterase activity in activated T cells from traumatized mice. The results is related to corrective effect of ginsenosides on the suppression of T cells function after trauma[45]. The ginsenoside could also enhance IL-2 and IL-2R gene transcription expression after trauma[46]. Ginsenosides could promote indirectly the immune function of rat splenocytes via hippocampus[47].

The RNA dot hybridization with human IL-6 cDNA showed that panaxatriol saponin enhanced the interleukine-6 mRNA concentration within PHA activated peripheral blood mononuclear cell by about four folds, suggesting that panaxatiol saponin could promote the IL-6 gene transcription of lymphoid tissue[49].

Araloside from *Aralia elata* were studied for their effects on allergic reaction[50]. Aralosides inhibited significantly PCA reaction when it was administered ig before and after sensitization. Some suppressive effect on PCA reaction was shown when it was given ig before challenge, however, the difference between the two groups of rats was not significant statistically. It had marked suppressive effect on Forssman cutaneous vasculitis, and the adjuvant arthritis. The delayed type hypersensitivity reaction induced by tuberculin in rats was distinctly suppressed by administration of aralosides both before and after sensitization as well as the challenge, however, the suppressive effect was more marked when administration of aralosides was before and after challenge than before and after sensitization. When araloside was given ig administered before and after sensitization it had no apparent effect upon Arthus reaction, however, when it was administered before challenge Arthus reaction was noticeably inhibited. It had significant suppressive effect on reversible passive Arthus reaction. Aralosides had no effect on the content of immune complex in serum of rats with Arthus reaction.

Action on Nerve System and Antiaging Activity

Ginsenoside Rb_1 and Rg_1 were studied for the nootropic mechanism in mice[51]. Weaning

mice were supplied drinking water containing Rb_1 and Rg_1 0.125 or 0.25mg/ml for four weeks. Rb_1 (28.6 and 56.1mg/kg) and Rg_1 (27.4 and 53.9mg/kg) were found to accelerate young mice body and brain development as well as facilitate memory acquisition in step down and step through avoidance response test. With the technique of quantitative measurement synapses, Rb_1 and Rg_1 were found that administration for four weeks can increase synapse number in the hippocampal CA3 region of mice. Rb_1 and Rg_1 were also found to prolong the duration of neuronal life and provided partial protection against the excitotoxic effect of glutamate in primary hippocampal cultures of rats[52]. Since excitotoxic neuronal injury of glutamate is considered to be caused by the increase of intracellular Ca^{2+} concentrations, the effects of Rb_1 and Rg_1 on $[Ca^{2+}]i$ elevated by glutamate were measured in cultured hippocampal cells using Fura-2/AM as a calcium indicator. Rb_1 and Rg_1 could selectively inhibit the high level glutamate (500 μ mol/L) induced increase of $[Ca^{2+}]i$, suggesting that the neuroprotective activities of Rb_1 and Rg_1 were mediated by blocking calcium over-influx into neuronal cells. Ginsenoside-Rb_1 was shown to increase Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+} ATPase activities at low dose in rat cerebral cortical synaptosomes. The decrease of $[Ca^{2+}]i$ by Rb_1 may be attributed to increase of ATPase activity[64].

After the administration of ginsenoside Rg_1, the expression of c-*fos* gene and protein was decreased in the hippocampus of aged rats, but dose-dependently increased in young and aged rats. Furthermore, Rg_1 increased the level of cAMP in the hippocampus of both young and old rats[53]. The membrane fluidity of neonatal cells was shown to be significantly higher (η 1.485 ±0.211) than that in young cells (η 2.220±0.169), and that in young cells was significantly higher than that in old cells (η 2.842±0.1430). No significant difference in fluidity, neither between fetal and neonatal cortical cells nor between young and adult ones was observed. When treated with Rg_1(10, 20, 40mg/kg), the membrane fluidity of old cortical cells significantly increased (η 2.670±0.108, 2.381±0.123, 2.000±0.101)[54]. The nitric oxide content and nitric oxide synthase activity in old rats were shown to be significantly higher than those of young and adult rats. When treated with Rg_1 (10, 20, 40mg/kg), the nitric oxide content and nitric oxide synthase activity in old rats decreased. the inhibitory effect of Rg_1 on nitric oxide synthase was found to be dose-dependent in the range of 10 to 40mg/kg. The optimal reduction in nitric oxide content and nitric oxide synthase activity induced by Rg_1 occurred at 40mg/kg for old rats. In view of the close relationship of nitric oxide content and nitric oxide synthase activity with aging, the inhibitory effect of Rg_1 on nitric oxide synthase activity might provide an explanation for its antiaging function[55].

Antitumor Activity

The effect of ginsenosides from stems and leaves of *P. ginseng* on gastric carcinoma cells (BGC-823) cultured *in vitro* was reported[56]. The growing speed and mitotic ability of the gastric carcinoma cells were inhibited, the concentration of glycogen in the cells was increased, and the activity of mucopolysaccharide and ACP in the cells were decreased. Ginsenoside Rh_2

inhibited the growth of B_{16} melanoma cells at the concentration of 10 μ g/ml *in vitro*. The melanin synthesis of Rh_2 in treated B_{16} cells was increased. Porphologically, the Rh_2 induced B_{16} cells turned to be epithelioid cells. B_{16} became dendrite shaped morphologically at higher concentration of Rh_2. Flow cytometry demonstrated that the B_{16} cells treated with Rh_2 were blocked at G_1 phase[57].

Other Activities

Total ginsenosides from stems and leaves of *P. ginseng* were studied for antiviral activities against HSV-1, HSV-2,AdV-3 and VSV. The ginsenosides could suppress the lesion of cultured cells induced by virus. Ginsenosides-Rb_1, Rb_2, Rb_3, Ro and Re also showed antiviral activities. A cream containing the ginsenosides was used in the treatment of herpes lebialis infected by HSV-1 and twenty-seven of thirty-one (87.1%) cases were cured[58,59]. It was found that the total ginsenosides mentioned above showed inhibitory effect on sperm abnormalities induced by mitomycin C in mice. The frequency of abnormal sperms induced by mitomycin C was reduced significantly by ip injection of the ginsenosides and mitomycin C at the same time[60].

Elatoside A and B from Aralia elata were found to show potent inhibitory activity on ethanol absorption after a single oral administration at the dose of 100mg/kg. Particularly, elatoside A inhibited ethanol absorption in a dose dependent manner at 25-100mg/kg[9]. Elatoside A, E, G, H and I were found to exhibit potent hypoglycemic activity in the oral glucose tolerance test in rats[8,10].

Acanthopanax senticosus saponin isolated from the leaves at dose of 100, 200mg/kg ip could decrease various cases of experimental hyperglycemias induced by injection of adrenaline, glucose and alloxan, without affecting the levels of blood sugar in normal mice[61].

Ginsenoside Re was found to enhanced the membrane fluidity of both juvenile and mature red blood cells[62]. Another study on the effect of Re on membrane fluidity of normal and transformed cells showed the effect of Re on both-direction adjustment of cells membrane fluidity[63].

Ginsenosides could significantly reduce the side effects of prednisone, and ginsenoside-Re was proven to be the major effective constituent[67,68].

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NEW DAMMARANE SAPONINS FROM VIETNAMESE GINSENG

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Key Word Index--Vietnamese ginseng, *Panax vietnamensis*, dammarane saponins, vina-ginsenosides- R15 -- R24.

Abstract *Panax vietnamensis* Ha *et* Grushv., Araliaceae, is a new *Panax* species discovered in Central Vietnam in 1973. The plant, which is now commonly known as Vietnamese ginseng, has become an important medicinal plant of Vietnam. From the underground part of *P. vietnamensis* growing wild, twenty-four known saponins and fourteen new dammarane saponins named vina-ginsenosides-R1 -- R14 were previously isolated and identified . In our course of further study on the saponin composition of *P. vietnamensis*, ten more new dammarane saponins, vina-ginsenosides-R15 -- R24, were isolated along with two known saponins, pseudo-ginsenoside-RC_1 and gypenoside-IX. The structure of the new saponins was elucidated based on chemical and spectroscopic evidence.

Introduction

Panax ginseng C. A. Meyer (ginseng) and its two congeners, *P. notoginseng* (Burk.) F. H. Chen(Sanchi ginseng), and *P. quinquefolium* L. (American ginseng), are well-known medicinal plants which have been widely used in the world. For years, new *Panax* spp. have been an objective of the search for similar sources of the valuable medicine.

In 1973, a wild *Panax* species was discovered at Ngoc Lay, Kon Tum Province, Central Vietnam, at the elevation of 1,800 m above sea level.[1] Subsequent investigations revealed that it was a secret medicine of the Sedang ethnic group living in high mountains of the Truong Son Range and has been regarded as a life-saving herb used for treatment of many serious diseases and enhancement of physical strength. In 1985, the plant was designated as *Panax vietnamensis* Ha *et* Grushv.,[2,3] a new *Panax* species, and readily turned out to be an important medicinal plant of Vietnam with the common name Vietnamese ginseng.

In our previous papers, we reported the identification and structure elucidation of twenty-four known saponins and fourteen new saponins named vina-ginsenosides -R1 -- R14 which were isolated from the underground part of *P. vietnamensis*.[4,5,6,7] We now summarize the result of our further study on the saponin composition of Vietnamese ginseng, which led to the isolation and structure elucidation of two known saponins, pseudo-ginsenoside-RC_1 (**1**) and gypenoside-IX (**2**), and ten new dammarane saponins named vina-ginsenosides-R15 -- R24 (**3** - **12**, respectively).

Results and Discussion

The extraction and isolation of minor saponins from Vietnamese ginseng was summarized in Figure 1.

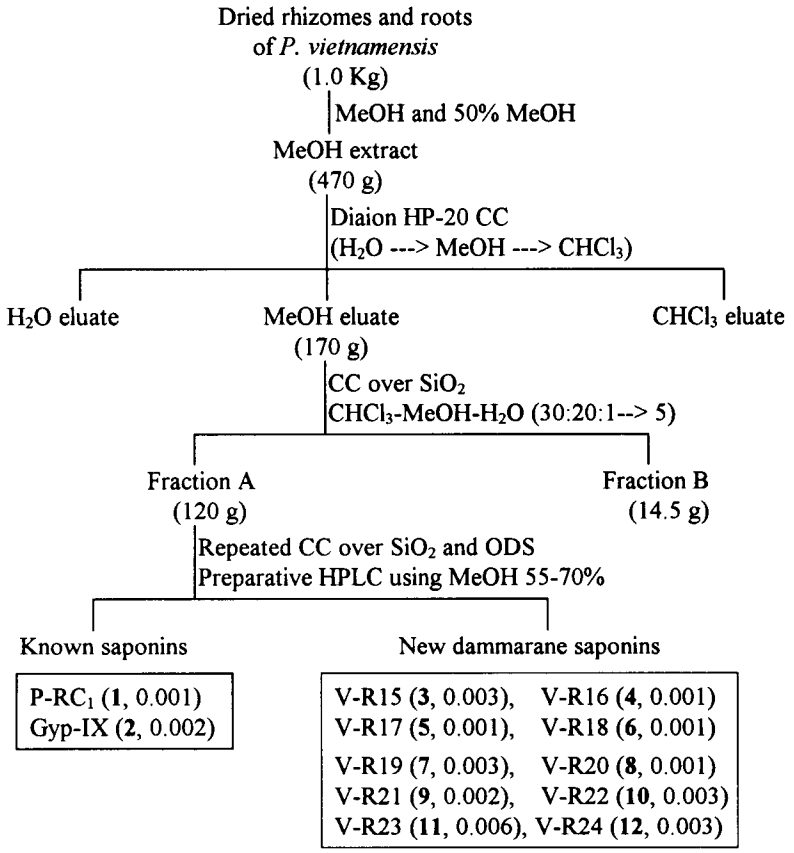


Figure 1. Extraction and Isolation of Saponins from Rhizomes and Roots of *Panax vietnamensis*.

( ) : yield %; Gyp: gypenoside, P: pseudo-ginsenoside, V: vina-ginsenoside

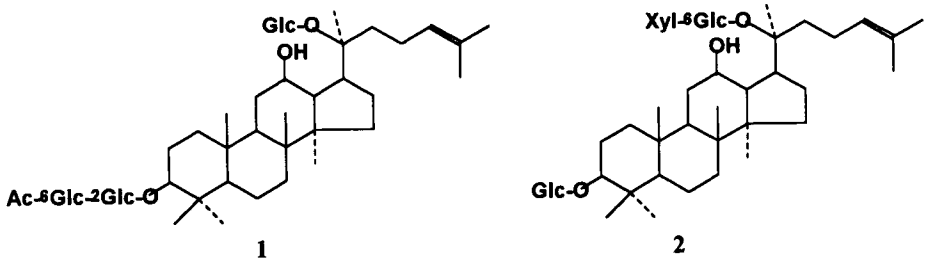
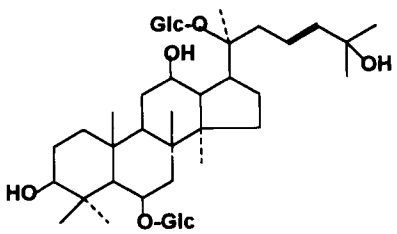
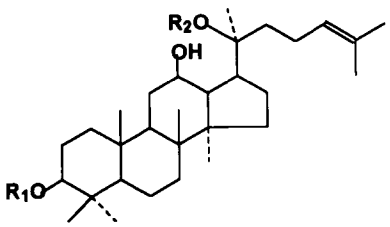


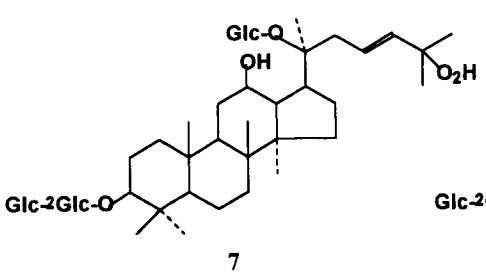
Figure 2. Known Dammarane Saponins from *Panax vietnamensis*.

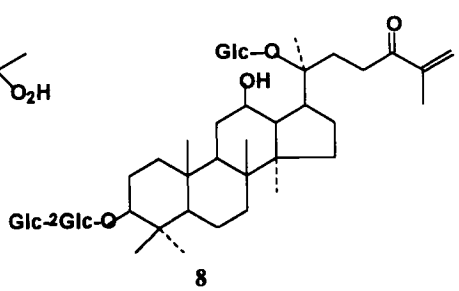
Two more known saponins, pseudo-ginsenoside-RC_1 (**1**) and gypenoside-IX (**2**) (Fig. 2), and ten new minor dammarane saponins were isolated from Vietnamese ginseng and structurally identified. The new saponins were named vina-ginsenosides-R15 - R24 (**3** - **12**, respectively). Their structures (Fig. 3) were established based on chemical and spectroscopic evidence. Various techniques of one and two-dimentional NMR including ^1H-^1H COSY, HETCOR, HSQC, HMBC, NOESY, ROESY *etc* . . .were used for the structure elucidation of the new saponins. Some examples of the structure elucidation are briefly given below.





	R_1	R_2
4	-Glc^2-Xyl	-Glc
5	-Glc^2-Glc	-Ara
6	-Glc^2-Glc	-Xyl





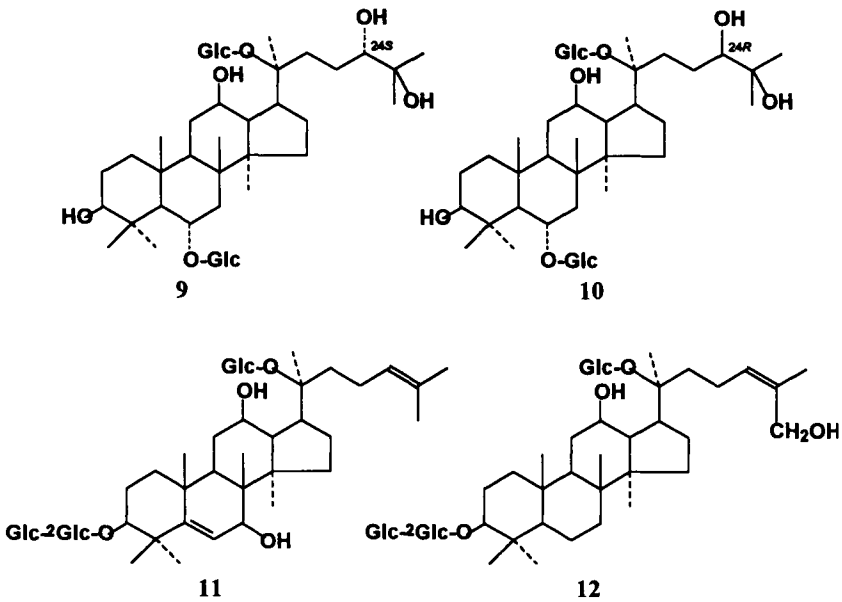
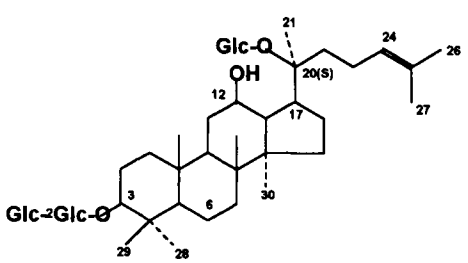


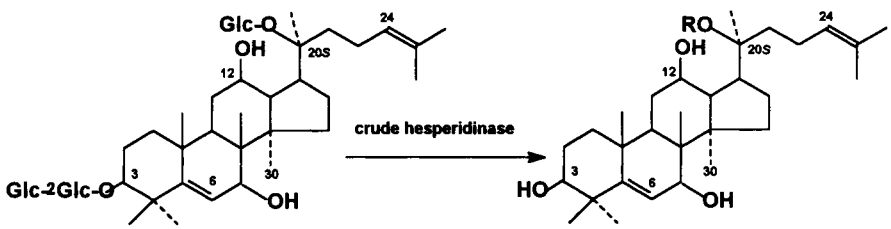
Figure 3. New Dammarane Saponins from *Panax vietnamensis*.
(Ara: α-L-arabinopyranosyl; Rha: α-L-rhamnopyranosyl
Glc: β-D-glucopyranosyl; Xyl: β-D-xylopyranosyl)

Vina-ginsenoside-R20 (**8**) has the molecular formula $C_{48}H_{80}O_{19}$ by high resolution FABMS. The UV spectrum of **8** showed an absorption 254 nm and its IR spectrum exhibited absorption bands at 1668, 1631 and 1078 cm^{-1}, which implied the presence of a keton group conjugated by a double bond. The ^{1}H- and ^{13}C NMR spectra of **8** revealed the presence of seven quartenary methyl groups and one methylen group. The ^{13}C NMR spectrum of **8** compared with that of ginsenoside-Rd (**13**) showed the similarity of chemical shifts except for those due to C-22 to C-27 of the side chain. The keto group and methylen group of **8** must therefore be located at C-24 and the double bond between C-25 and C-26. This was further confirmed by the HMBC spectrum of **8** which showed correlations between the methyl protons signal of C-27 with carbon signals due to C-24 and C-26. Consequently, the structure of **8** was determined as shown.



13

The high resolution FABMS analysis of vina-ginsenoside-R23 (**11**) revealed the molecular formula $C_{48}H_{80}O_{19}$. On enzymatic hydrolysis with crude hesperidinase, **11** gave an aglycone (**11a**) and a prosapogenin. The ^1H- and ^{13}C NMR spectra of **11a**, whose molecular formula is $C_{30}H_{50}O_4$, showed the presence of eight quartenary methyl groups, three secondary hydroxyl groups, one tertiary hydroxyl group and two trisubstituted double bonds. One double bond with carbon signals at δ_C 126.2 and 130.6 can easily be ascribable to the double bond between C-24 and C-25 which commonly occurs in dammarane-type triterpenes. The NMR spectra revealed the remainning double bond must be located in the ring part. An examination of **11a** by means of ^1H, ^{13}C-NMR, ^1H-^1H COSY, HETCOR and COLOC spectra led to the structure formation of **11a** as shown and an unambiguous assignment for its NMR chemical shifts. The stereochemistry at C-7 of **11** was determined by means of ROESY. Spatial correlations were observed between H-7 signal (δ 4.7, br s) with those due to H-6 (δ 5.9, br s) and C-30 methyl protons (δ 1.09, s). The hybroxyl group at C-27 must therefore have β-orientation. The structure of **11** was then determined by a comparison of ^{13}C chemical shifts of **11** and ginsenoside-Rd (**13**), which showed similarity of the sugar moiety.



11 **11a** R = -H

Prosapogenin R = -Glc

The molecular formula of vina-ginsenoside-R24 (**12**) was analyzed for $C_{48}H_{82}O_{19}$ by high resolution FABMS. The ^1H- and ^{13}C NMR spectra of **12** revealed the presence of seven quartenary methyl groups, and a primary hydroxyl group (δ 4.46, ABq, 11.5 Hz). A comparison of the ^{13}C NMR spectrum of **12** with that of ginsenoside-Rd (**13**) showed the similarity of their chemical shifts except for those due to C-24 to C-27 of the side chain. The primary hydroxyl group could therefore be located at C-26 or C-27. However, an NOEDS experiment indicated that it must be located at C-27. Irradiation at the proton signal of C-24 (δ 5.56, br s) showed an NOE on the signal assignable to the methyl protons of C-26 (δ 1.93, s). Accordingly, the structure of **12** was determined as shown.

Acknowledgements

We would like to thank Prof. S. Shibata, Meiji College of Pharmacy, Tokyo, Japan, for his continuing encouragement and valuable advices. Thanks are also due to the Japan Society for Promotion of Sciences and Fujisawa Foundation, Osaka, Japan, for their financial supports. One of the authors, N. M. Duc, thanks Prof. Guo-wei Qin, Shanghai Institute of Materia Medica, and the Regional Network for the Chemistry of Natural Products in Southeast Asia for their kind assistance and support.

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FORMATION OF GINSENOSIDE-PROTEIN CONJUGATES

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Key Word Index -- Ginsenoside-protein conjugate; MALDI/MS; gel electrophoresis

Abstract-Ginsenoside Rb1 was conjugated with bovine serum albumin by NaIO4. MALDI/MS spectrum of the conjugates revealed that most abundant conjugate had 10.2 moles of hapten(ginsenoside) and hapten number ranged from 0.6 moles to 24.6 moles. Ginsenoside-protein conjugate formation was also proved using ginsenoside Rc-HSA conjugate by PAGE gelelectrophoresis. With mercaptoethanol hapten number of conjugate seemed increased but solubility decreased. Monoclonal antibody formation is under investigation with ginsenoside-BSA conjugate.

Introduction

Ginsenosides has been considered as a potent active molecules and reached around 38 kinds of molecules[1, 2]. Thus total saponin content and ginsenosides pattern are used for only official index of ginseng products. The structural change of ginsenosides were reported by microbes[3, 4] and processing[5]. Chemical treatments were used for producing new ginsenosides having biological activity such as monoglycosides[6] and as degraded compounds[7]. For the radioimmunoassay of ginsenosides Rg1-26C-BSA conjugate was made[8]. Synthesis of new compounds based on ginsenosides is hardly seen. Recently Solamargine, a steroidal glycoside from the fresh fruit of *Solanum khasianum* was directly conjugated with bovine serum albumin(BSA) for producing monoclonal antibody and the ratio of hapten to BSA in the conjugate was determined by matrix assisted desorption ionization mass spectrometry[9]. We applied the same method to produce ginsenoside-protein conjugate and measured number of ginsenosides bound.

Results and Discussion

Rb1-BSA conjugate formation

Ginsenoside Rb1-carrier protein (bovine serum albumin) conjugates were synthesized as previously reported[9]. The probable chemical reaction steps were shown in Fig. 1. Precise chemical structures of binding site are obscure yet.

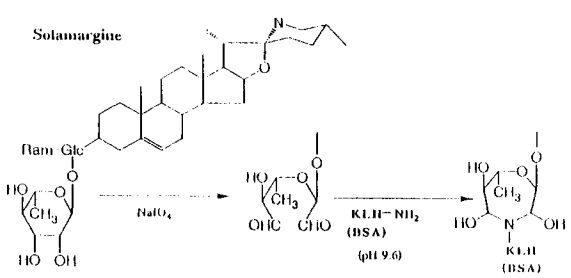

Fig. 1. Synthetic pathway of solamargine-carrier protein conjugate.

Molecular weights of ginsenoside Rb1-BSA conjugates were scanned by MALDI/TOF mass spectrometry(Fig. 2). A broad frequency peak$(M+H)^+$ of Rb1-BSA conjugates appeared from 67141(m/z) to 93660 with the maximum frequency at 77489 (mean of three times). The number of Rb1 moles per BSA ranged from 0.6 to 24.6 and 10.2 at the maximum frequency. MALDI/TOF mass spectrum of BSA was shown in Fig. 3. Molecular weight of BSA by MALDI/TOF MS was 66381 (mean of three measurements) close to theoretical one 66431.

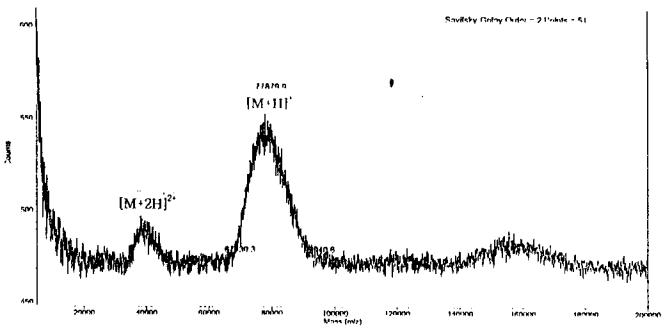

Fig. 2. Spectrum of MALDI/MS of ginsenoside Rb1-BSA conjugate.

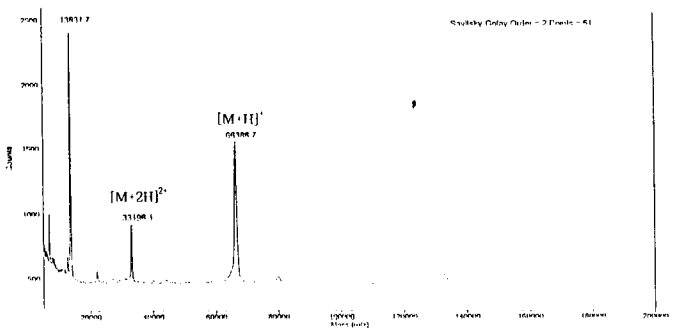

Fig. 3. Spectrum of MALDI/MS of BSA.

In this direct method number of hapten was much greater than Rg1-BSA conjugate synthesized by multi-step method[8] though the binding site is different(Table 1). In this direct method ginsenoside Rb1 showed much greater number of hapten than solamargine(Table 1). Hapten density may affect antibody formation.

Table 1. Number of hapten(HN) in glycoside-BSA conjugate

Conjugation Method	Glycoside	Binding position in glycoside	HN Range	HN Most abundant	Reference
NaIO4 pH9.6	G-Rb1	sugar	0.624.5	10.2	
Aldehyde-ester-azide	G-Rg1	26C	34	-	Sankawa et al. 1982
NaIO4 pH9.6	Solamagine	sugar	115	12	Ishiyama et al. 1996

Effect of protein structure on ginsenoside-protein conjugate formation

Carrier protein(BSA and HSA) was treated with mercaptoethanol and then allowed to make conjugate with ginsenosides(Rb1 and Rc). During the synthesis white precipitation appeared much earlier with mercaptoethanol treatment than without mercaptoethanol treatment. Polyacrylamide gel electrophoretogram of ginsenoside protein conjugates was shown in Fig. 4. Ginsenoside Rb1-BSA conjugate showed three main groups. It was same as Rc-human serum albumin(HRc in Fig. 4).

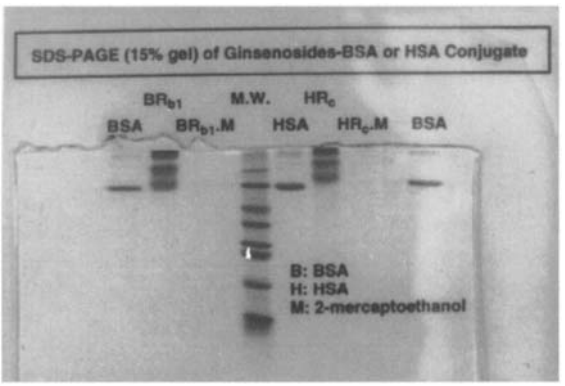

Fig. 4. Gel electrophoretogram(PAGE 15%) of ginsenoside-protein conjugates.

BSA seems better to make conjugate than HSA. When the structure of BSA or HSA was changed by mercaptoethanol no band appeared(BRb1M or HRcM) in Fig. 4. It seems to be due to extremely low solubility. When the 9% of gel was used ginsenoside-protein conjugate showed broad bands(Fig. 5 and 6). It is appeared well that Rb1-BSA conjugate seemed to make larger molecules than Rc-HSA conjugate when electrophoresis continued till maximum time(Fig. 6). Ginsenoside-BSA conjugate is under the investigation of antibody formation.

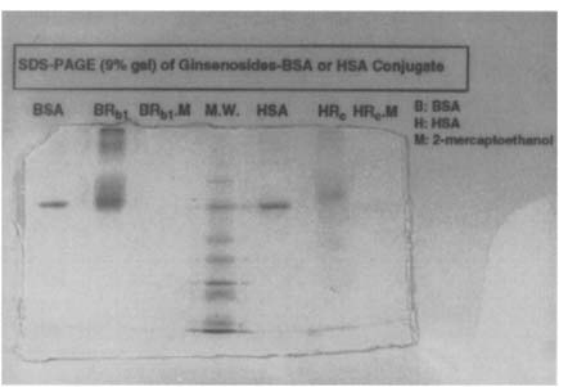

Fig. 5. Gel electrophoretogram(PAGE 9%) of ginsenoside-protein conjugates.

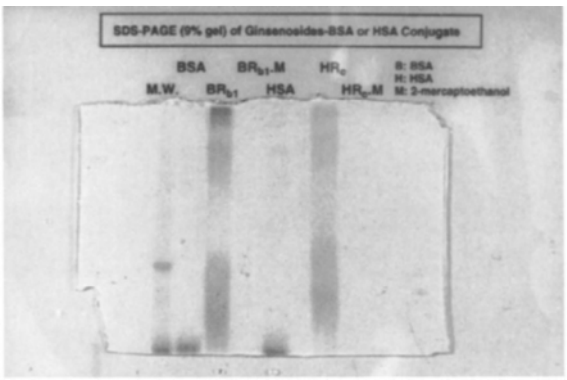

Fig. 6. Gel electrophoretogram(PAGE 9%) of ginsenoside-protein conjugates.

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BIOLOGICAL TEST ON *SCHEFFLERA* GLYCOSIDES AND GINSENG GLYCOSIDES BY RADIO-LIGAND RECEPTOR BINDING ASSAYS

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Abstract -- *Schefflera bodinieri* was studied for its activity in the central nervous system by bioassay guided isolation in conjunction with receptor binding assays. In preliminary screening, ethanol extracts of leaves and roots of *S. bodinieri* were shown to have strong binding affinity to α1- and α2-adrenergic, 5HT-1, 5HT-2, opiate, Ca^{+2} channel, sulphonylureas, dopamine 1 and 2, histamine 1, $GABA_A$ and $GABA_B$ receptors. During chemical investigation, thirteen compounds were obtained from these extracts. Nine of these isolated compounds together with ginsenosides Rb1, Rc and Re were further tested by receptor binding assays. Results showed that three compounds were able to selectively bind to muscarine receptors, three compounds to Ca^{+2}-channel receptors, two to 5HT-2 receptors and one to dopamine-2 receptors at μM level. In the drug-interaction studies, five compounds were found to enhance binding affinity of agonists or antagonists to the 5HT-1A, opiate, adenosine 1 and histamine 1 receptors. These observations suggest the possibility of interactions between multiple substances at the receptor level.

Introduction

Several species of *Schefflera* (Araliaceae) are used as folk remedies for the treatment of pain, rheumatic arthritis, bone fracture, sprains, lumbago and stomachache in the southwestern part of China and in other Asian countries [1]. In order to search for plant ingredients that are active to the central nervous system, *Schefflera bodinieri* (Levi.) Rehd. was studied by bioassay guided isolation in conjunction with receptor binding assays. Preliminary screening studies showed that ethanol extracts of *S. bodinieri* leaves and roots possessed strong binding affinity to α1- and α2-adrenergic, 5HT-1, 5HT-2, opiate, Ca^{+2} channel, sulphonylureas, dopamine 1 and 2, histamine 1, $GABA_A$ and $GABA_B$ receptors [2]. Further chemical investigations revealed that plant glycosides were the major constituents of this species. Thirteen compounds were isolated from these extracts which included eight pentacyclic triterpene glycosides, two pentacyclic triterpenes, one tetracyclic triterpene glucoside and two oligosaccharides [3-5]. In the present study, these isolated compounds together with ginsenosides Rb1, Rc and Re were tested by receptor binding assays. Moreover, interactions between these isolated compounds and agonists or antagonists of 5HT-1A, opiate, adenosine 1 and histamine 1 receptors were examined.

Results and Discussion

Twelve compounds, namely 3-oxo-20-demethylisoaleuritolic-14 (15)-ene-28, 29-dioic acid (**1**), 3α-hydroxyl-20-demethylisoaleuritolic-14(15)-ene-28,30-dioic acid (**2**), 28-O-[α-L-rhamno-pyranosyl(1→4)-O-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside of 3-oxo-isopolygalic-13(14)-ene-28-acid (**3**), 28-O-[α-L-rhamnopyranosyl (1→4)-β-D-glucopyranosyl(1→6)-]-β-D-gluco-pyranoside of 3β-hydroxyl-isopolygalic-13(14)-ene-28-acid (**4**), 28-O-[α-L-rhamopyranosyl(1→4)-β-D-glucopyranosyl(1→6)-]-β-D-glucopyranoside of 3β-hydroxyl-8-demethylisoaleuritolic-14(15)-ene-28-acid (**5**), 28-O-[α-L-rhamnopyranosyl(1→4)-O-β-D-glucopyranosyl(1→6)-]-β-D-gluco-pyranoside of 3-oxo-20-demethylisoaleuritic-14(15)-ene-28,29-

dioic acid (**6**), D-sorbitol (**7**), α-L-rhamnopyranosyl(1→4)-O-β-D-glucopyranosyl(1-6)-β-D-glucopyranoside (**8**), Stigmasterol-3-O-β-D-glucose (**9**), ginsenoside-Rb1 (**10**), ginsenoside-Rc (**11**) and ginsenoside-Re (**12**) were tested by ligand receptor binding assays as described in experimental section. The receptors tested were α1, α2, β-adrenoceptors, 5HT1A, 5HT1C, 5HT2, dopamine 1, dopamine 2, muscarinic, adenosine 1, histamine 1, Na^+/K^+ATPase, Ca^{2+}-channel (DHP), sulphonylureas and opiate receptors. Of the 12 compounds tested, 8 compounds showed binding activities to some receptors at μM level, Compounds **1, 6, 7** were found to selectively bind to muscarine receptors, **8, 10, 11** to Ca^{+2}-channel receptors, **8** and **9** to 5HT-2 receptors and **4** to dopamine-2 receptors. The IC_{50} values of these compounds are given in Table 1.

Table 1 Binding activity of the compounds tested

Compound	Receptor	IC_{50} (μM)* ± SEM (n=10)
compound **1**	muscarinic	0.91 ± 0.15
compound **4**	dopamine 2	1.83 ± 0.36
compound **6**	muscarinic	3.57 ± 1.41
compound **7**	muscarinic	3.24 ± 1.22
compound **8**	Ca^{2+}-channel	8.03 ± 2.01
	5HT2	3.81 ± 1.34
compound **9**	5HT2	8.04 ± 2.31
ginsenoside Rb1 **10**	Ca^{2+}-channel	4.23 ± 1.64
ginsenoside Rc **11**	Ca^{2+}-channel	4.46 ± 0.62

*: The IC_{50} of positive control agents in the present study were 0.16 nM for atropine (muscarinic receptor), 5.62 nM for spiperone (5HT2 receptor), 2.00 nM for nitrendipine (Ca^{2+}-channel receptor) and 2.08 nM for butaclamol (dopamine 2 receptor).

The majority of these isolated compounds are either structurally related or biosynthetically related so that the influence of the structure changes on their biological activities would be of interest to medicinal chemists. For instance, compound **1** is the aglycone of compound **6** and compound **8** is the sugar moiety of compound **6,** both **1** and **6** were able to bind to muscarinic receptor (Table 1). Interestingly, the aglycone form (IC_{50} 0.9 μM) was more active than its glycoside (IC_{50} 3.5 μM). On the other hand, the sugar moiety was inactive toward the muscarinic receptor but to the Ca^{2+}-channel receptor (IC_{50} 8.0 μM) and 5HT2 receptor (IC_{50} 3.8 μM).

Compounds **4** and **5** are isomers; they differ only in the position of the double bond, i.e., a double bond in the C_{13}-C_{14} position for Compound **4** versus the C_{14}-C_{15} position for Compound **5**. Compound **4** was able to bind to the dopamine 2 receptor with an IC_{50} value of 1.8 μM (Table 1), whilst compound **5** was inactive to all the receptors tested.

Triterpenoids and their glycosides showed various biological activities in the central nervous system. Ginsenosides, isolated from the same plant family as *Schefflera* glycosides, were reported to be the main active principles of ginseng, responsible for its physiological and pharmacological effects in nervous systems. In the present study, three ginseng glycosides were tested by ligand receptor binding assays with activity demonstrated by ginsenoside Rb1 and Rc on the Ca^{2+}-channel receptor (respective IC_{50} values at 4.2 and 4.4 μM, Table 1). The findings in this study substantiate partial activities of ginsenosides at the receptor level.

Compounds **1, 4, 5, 6, 7, 8** were further investigated for their ability to interact with a number of agonists or antagonists at the opiate, 5HT1A, histamine 1 and adenosine 1 receptors by the

approach described in the experimental section. Compound **7** did not affect the IC_{50} values of any of the four receptor specific binding agents and no compounds were found to affect the binding of 8-OH DPAT to 5HT1A receptors under the present conditions. The shifts in the IC_{50} values of these receptor specific binding agents in the presence of the isolated compounds are given in Table 2.

Table 2 Changes in the IC_{50} values of the agonists or antagonists of opiate, histamine 1 and adenosine 1 receptors by plant ingredients

Receptor	Specific Binding Agent	IC_{50} (nM) ± SEM (n=10) (Specific Binding Agent Alone)	Test Compound Added	IC_{50}(nM) ± SEM (n=10) (Specific Binding Agent Plus Test Compound)
opiate	naloxone	1.02 ± 0.17	**1**	0.63 ± 0.08**
			4	0.14 ± 0.02**
			5	0.35 ± 0.06**
			6	0.70 ± 0.05**
			8	0.40 ± 0.05**
histamine 1	pyrilamine	7.41 ± 0.77	**4**	0.40 ± 0.06**
			5	0.56 ± 0.07**
			8	0.16 ± 0.03**
adenosine 1	CHA	14.1 ± 0.72	**6**	10.0 ± 1.12**

**: $P < 0.01$ when comparing with the IC_{50} value of specific binding agent alone

Synergistic activity exhibited by similar plant extracts has been reported in previous *in-vivo* studies, e.g., ethanol leaf extract of *Schefflera arboricola* produced a significant analgesic effect when 100 g/kg of such extract was given subcutaneously along with 5 mg/kg of morphine hydrochloride [6]. In the present study, compounds **1, 4, 5, 6, 8** were found to interact with naloxone, an antagonist of the opiate receptors, by increasing the binding affinity of naloxone. This finding suggests that the synergistic effect of the extract might be triggered by the plant ingredients interacting with either endogenous or exogenous substances so that binding to the opiate receptors was enhanced.

It is necessary to mention that there are a large number of constituents in the plant extract which exert their actions in a complex manner. Therefore, it is not surprising that isolated compounds do not represent the full effects of the plant itself, and conversely, individual compounds may have certain effects which the extract does not show markedly. The present findings suggest the possibility of interactions between plant ingredients at the receptor level.

Experimental

Plant material.- Schefflera bodinieri was collected on Jinfo Mountain, Sichuan province, P.R. China in November, 1990 and identified by Professor Z. Liu at that institute. The voucher specimens were kept in the herbarium of the Institute of Medicinal Plant Cultivation, Sichuan province, P.R.China.

Biological tests on isolated compounds. The isolated compounds were tested by 15 receptor binding assays including α1, α2, β-adrenoceptors, 5HT1A, 5HT1C, 5HT2, dopamine 1, dopamine

2, adenosine 1, histamine 1, Na^+/K^+ATPase, Ca^{2+}-channel, sulphonylureas, opiate and muscarinic receptors. The methods utilised were described in details in our previous paper [2].

Interaction studies: In order to examine the possibility of interaction between the isolated plant ingredients and specific binding agents to receptors, compounds **1, 4, 5, 6, 7, 8** were tested using the protocol cited [2] with slight modifications. Ligand receptor binding assays were conducted with adenosine 1, histamine 1, opiate and 5HT1A receptors. The selected agonists or antagonists of these receptors were N^6-cyclohexyladenosine (CHA, adenosine 1 receptor), pyrilamine (histamine 1 receptor), naloxone (opiate receptor), and 8-hydroxy-dipropylaminotetralin (8-OH DPAT, 5HT1A receptor). In each assay, a single isolated compound at a constant concentration (10^{-5} M) was added to a mixture of the target receptor tissue and the required [^{3}H]-ligand which was followed by the addition of a control compound employed for that particular assay at a concentration range of 10^{-6}M-10^{-11}M. The IC_{50} values were reported for these control compounds alone and these compounds in the present of one isolated compounds.

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HAIRY ROOT CULTURE OF *PANAX JAPONICUS* VAR. *MAJOR* AND ITS SAPONIN FORMATION

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Key words: *Panax japonicus* var. *major*, hairy roots, saponin, oligosaccharins

Abstract: A hairy root strain has been established from the root explants of *P. japonicus* var. *major* by using a wild strain pRi 15834 of *Agrobacterium rhizogenes*. Experimental data on the time-course of hairy root growth and saponin formation in suspension culture indicate that both the dry weight of the hairy roots and total saponin yield reached their maximum values on the 30th day. It means that saponin formation proceeds almost in parallel with hairy root growth. The oligosaccharins DC-DP7, DC-DP8 DC-DP9 from *Dendrobium candidum*, and PP-DP6, PP-DP7, PP-DP8 from *Paris polyphylla* var. *yunnanensis* all affected the hairy root growth, especially obviously stimulated saponin formation of the hairy roots.

Introduction

Panax japonicus var. *major* is a wild dicotyledonous herb of Araliaceae which is mainly distributed in southwest China and Himalayas areas[1]. Its root has been used as an expectorant, antitussive and hemostatic in Chinese herbal medicine for a long time. And the main active constituents were thought as a wide range of saponins and sapogenins[2]. Biotechnological alternative, i.e. cullus cultures, cell suspension cultures and hairy root cultures, has been adopted for *in vitro* saponin production. But, no study has been made for the tissue culture and secondary metabolism of this plant. This paper describes the hairy root culture and its saponin formation.

Material and Methods

1. *Culture of Agrobacterium rhizogenes. A. rhizogenes* strain pRi 15834 is a wild agropine type bacteria which is maintained at 28℃ on YEB agar medium[3].

2. *Induction and cultivation of the hairy roots.* Root pieces of a 3-year-old *P. japonicus* var. *major* plant is collected from Yunnan, China. They were surface sterilized with 70% ethanol for 30 sec followed by treatment with 0.1% (w/v) $HgCl_2$ for 30 min. and then were cut to 5～10 mm square pieces explants. The root explants were used as the materials for *A. rhizogenes* infection. The inoculated explants were cultured on MS medium[4] containing 50 μm of acetosyringone and 5 mM of $MgSO_4$. After two days' coculture in the dark, the explants were transferred on MS agar medium containing 200 mg/l of cefotaxime in order to stop bacteria multiplication. After the bacteria were removed thoroughly, the hairy roots were cultured on hormone free MS agar medium, and maintained at 21±1℃and 60% of relative humidity in darkness by subculturing at 30～40 days intervals. They were also cultured in hormone free MS liquid medium, and maintained at 21±1℃ in darkness by changing medium at 14 days intervals. A rotated shaker, with about 2.0cm displacement and a rotative velocity of 110±5 rpm was used for hairy root suspension culture.

3. *Dry weight of the hairy roots, total saponin content and saponin yield estimation.* The cultured hairy roots were harvested by filtration, and then dried up to a constant weight in a

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freezing drier. The total saponin content of the hairy roots was determined according to a modified procedure of Furuya et al[5] and our previous papers[6-8]. The yield of saponin was made by the saponin content times the yield of the hairy roots.

4. *Preparation and addition of oligosaccharins.* Oligosaccharins (oligosaccharides with regulatory activities) with different degree of polymerization (DP) were isolated from acid-hydrolyzed young cell wall polymers of both *Dendrobium candidum* (Orchidaceae) and *Paris polyphylla* var. *yunnanensis* (Trilliaceae). Thery were heptasaccharide, octasaccharide and nonasaccharide from *D. candidum*, and hexasaccharide, heptasaccharide and octasaccharide and from *P. polyphylla* var. *yunnanensis*. As DP of *D. candidum* oligosaccharins were 7, 8 and 9, and DP of *P. polyphylla* var. *yunnanensis* oligosaccharins were 6, 7 and 8 separately, so the oligosaccharins from *D. candidum* were named as DC-DP7, DC-DP8 and DC-DP9, and oligosaccharins from *P. polyphylla* var. *yunnanensis* were named as PP-DP6, PP-DP7 and PP-DP8. All the oligosaccharins consist of glucose and mannose, and were obtained with help of column chromatographic methods such as Bio-Gel P-2 column and semi-separative HPLC column. The purity of the oligosaccharins was above 99%. All the oligosaccharins were dissolved in water, then the solutions were passed through filters (0.45 μ m), and were added into the media after the hairy roots had been cultured for a period of time. In normal condition, the oligosaccharins were added into medium as the hairy roots had been cultured for 7 days.

Results

1. Induction of the hairy roots

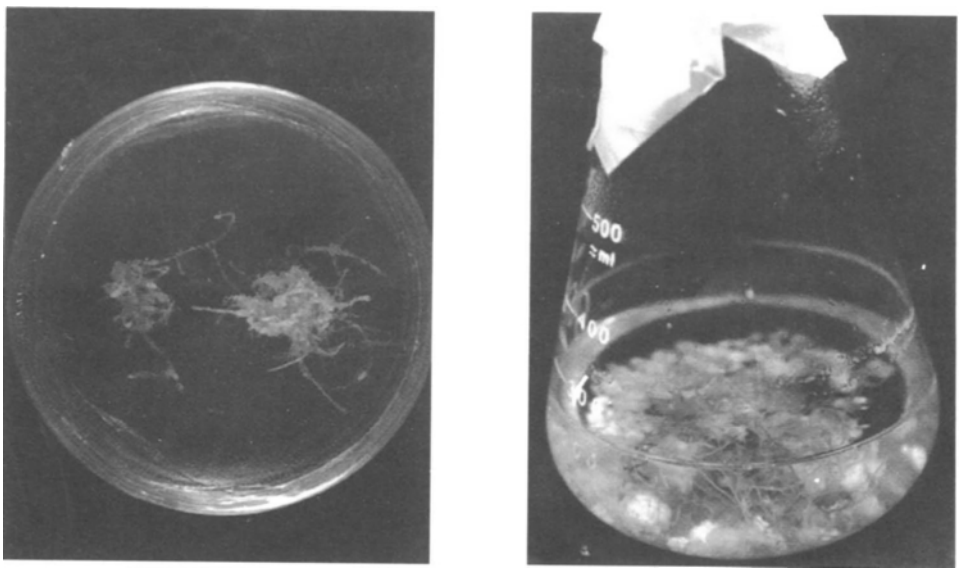
About three weeks after inoculation, some roots emerged from the infected areas. No roots emerged from the controls which were infected using only fresh medium or sterilized water. The induced roots were isolated and subcultivated in darkness at 21±1℃. The hormone free MS agar medium was used including cefotaxime in order to remove bacteria. These roots showed the typical characteristics of the "transformed root" which are hormone autotrophy, non-geotropic growth, high lateral branching, and dense cover of root hairs. In comparison with the cultures of normal roots, they grew very slowly and finally died when they were cultivated on hormone free medium.

2. Identification of the hairy roots

The hairy roots of *P. japonicus* var. *major*, which bacteria were removed thoroughly, could be used for the identification of genetic transformation. Using paper electrophoresis, mannopine was determined to exist in the hot water extracts of roots induced by *A. rhizogenes*. Other silver nitrate positive substances also existed in the extracts which should be other opines. Nevertheless, controls using normal plant roots were always negative for the silver nitrate positive substances. This indicated that T-DNA of Ri plasmid was inserted into plant DNA. So, a hairy root system of *P. japonicus* var. *major* has been established from the root explants by using a wild strain pRi 15834 of *A. rhizogenes*. The hairy roots grew well both on MS hormone free agar medium (Fig. 1, A) and in liquid medium (Fig. 1, B).

3. Time course of the hairy root suspension culture

Studies on time course of the hairy root culture could help us better to know the hairy root growth kinetics and accumulation of saponin. The results are given in Fig. 2. The dry weight of the hairy roots reached its top (5.36g/L) on the 30th day, and saponin yield (70.20mg/L) approached its maximum value after being cultured for 30 days. The pH value was 5.48 at the beginning of the culture and slowly dropped to 5.18 on the 15th day, then rose to 5.58 on the 20th day, and grudually dropped to 4.26 at the end. The results metioned above suggested that an appropriate harvesting time for *P. japonicus* var. *major* hairy roots to produce saponin was 30 days. The experimental data indicated that saponin formation proceeds almost in parallel with hairy root growth.



A B

Fig. 1 Cultivation of the hairy roots of *P. japonicus* var. *major*
(A) The hairy roots were subcultivated on hormone free MS agar medium;
(B) The hairy roots were subcultivated in hormone free MS liquid medium.

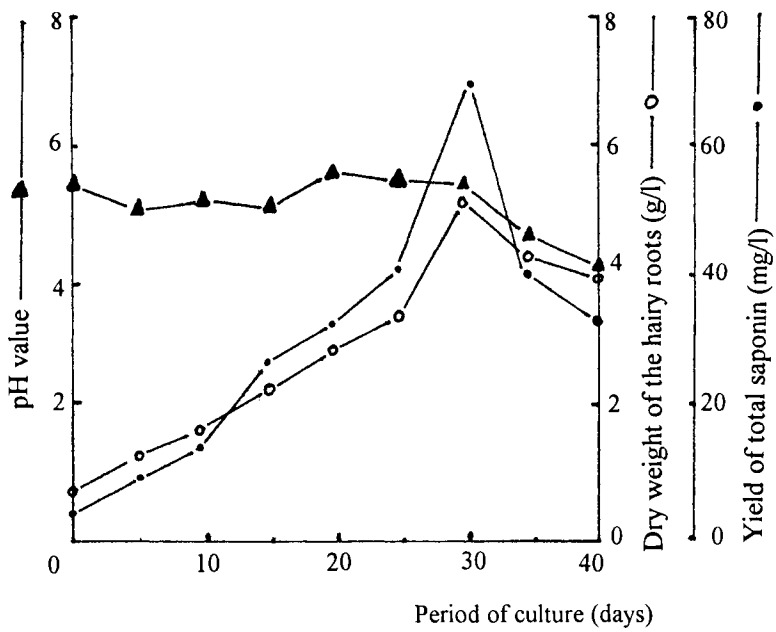


Fig. 2 Time course of the hairy root culture of *P. japonicus* var. *major* in liquid medium

4. Effects of oligosaccharins DC-DP7, DC-DP8 and DC-DP9 on hairy root culture

Effects of oligosaccharins DC-DP7, DC-DP8 and DC-DP9 on hairy root culture of *P. japonicus* var. *major* are shown in figure 3. Yield of total saponin was synthesized by the hairy root growth and saponin content. The appropriate concentrations for DC-DP7, DC-DP8 and DC-DP9 affecting the hairy roots to produce saponin were 20, 20 and 30mg/L separately, and their yield of saponin were 360mg/L, 227mg/L and 426mg/L, respectively, while, the yield of

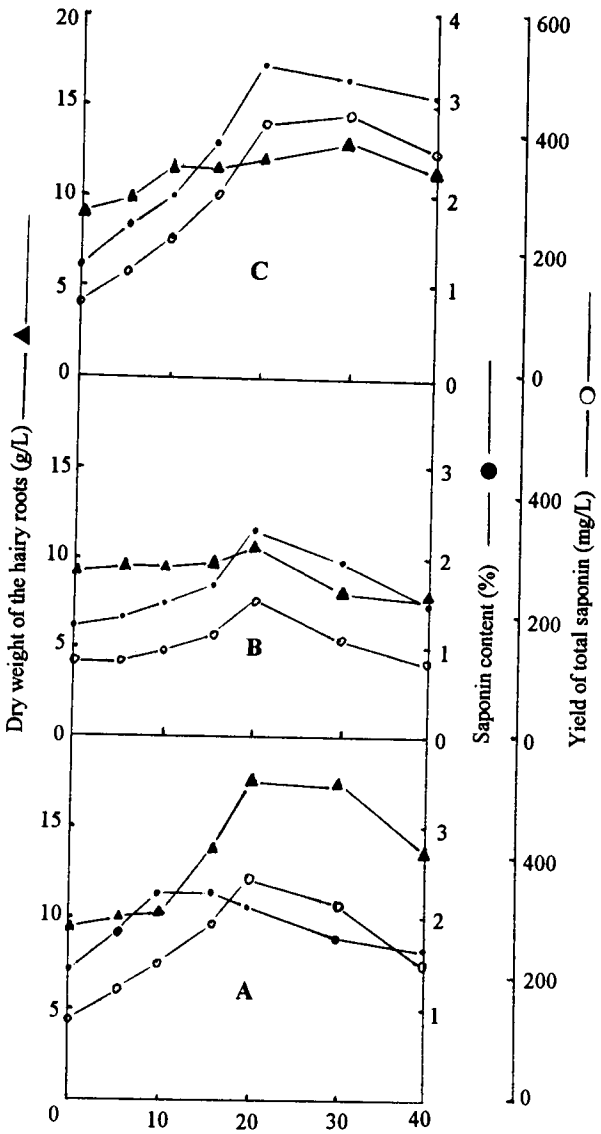


Fig. 3 Effects of oligosaccharins DC-DP7, DC-DP8 and DC-DP9 on the hairy root growth and saponin formation of *P. japonicus* var. *major*
A: DC-DP7; B: DC-DP8; C: DC-DP9

control which no oligosaccharin was added into medium was only 122.59mg/L. Among the three oligosaccharins, DC-DP9 was the most effective one to increase saponin content at concentration of 30mg/L, and DC-DP7 was the most effective one to stimulate the hairy root growth at concentration of 20mg/L.

Effects of DC-DP9 on hairy root suspension culture of *P. japonicus* var. *major* in a different addtion time are shown in table 1. Results show that it was suitable for DC-DP9 was added into medium as the hairy roots had been cultured for 7 or 14 days. The yield of saponin was more than 400mg/L, and that of control was only 132.28mg/L. It was less effective for DC-DP9 to affect saponin formation when DC-DP9 was added into medium at beginning of the hairy root suspension culture or the suspension culture lasted more than 21 days.

DC-DP9 was added into medium at the concentration of 30mg/L as the hairy roots had been cultured for 7 days. Results of the time course of hairy root supension culture were shown in figure 4. As the period of suspension culture was lasted to 25th day, the yield of hairy roots reached its top value (12.23g dw/L). At this time, the yield of control was only 7.83 g dw/L. As the period was lasted to 30th day, both the saponin content and yield reached their top values which were 3.564% and 442.29 mg/L separately, and those of control were 1.441% and 131.71mg/L separately. The results suggest that an appropriate harvesting time for the hairy root suspension culture to produce saponin was 30 days the same as that of the control. It was not obvious for pH value changing between addition of DC-DP9 and control.

Table 1 Effects of DC-DP9 on hairy root suspension culture of *P. japonicus* var. *major* in a different addition time

Cultured time before DC-DP9 was added into medium (d)	Hairy root yield (g dw/L)	Saponin content (%)	Saponin yield (mg/L)
Control	9.09	1.46	132.28
0	9.48	3.21	304.31
7	12.10	3.56	430.76
14	12.26	3.28	402.13
21	9.28	2.01	186.53

*Concentration of DC-DP9 was 30mg/L;"Control" means no DC-DP9 was added into medium.

5. Effects of oligosaccharins PP-DP6, PP-DP7 and PP-DP8 on hairy root culture

Effects of oligosaccharins PP-DP6, PP-DP7 and PP-DP8 on hairy root culture of *P. japonicus* var. *major* are shown in figure 5. By comparing saponin yield, the appropriate concentrations for PP-DP6, PP-DP7 and PP-DP8 to affect the hairy roots to produce saponin were 20, 30 and 20mg/L separately. At the appropriate concentrations of PP-DP7, PP-DP8 and PP-DP9, the saponin yield were 465mg/L, 521mg/L and 403mg/L. The saponin yield of control which no oligosaccharin was added into medium was only 127mg/L. Of them, PP-DP7 was the most effective one to increase saponin yield. At the concentration of 10mg/L, PP-DP6 was most effective to increase saponin content which was 3.90%, and saponin content of control was only 1.33%.

Discussion

A hairy root strain has been established from the root explants of *P. japonicus* var. *major* by using a wild strain pRi 15834 of *A. Rhizogenes* and grew well both on hormone free MS agar medium and in liquid medium. Mannopine was characterized in the hairy roots by paper electrophoresis. These roots showed the typical characteristics of transformed roots which are non-geotropic growth, high lateral branching, and dense cover of root hairs. The experimental

data on the time-course of product formation indicate that production-growth patterns can be classiified into three major types[9]. Results on the time course of the hairy roots of *P. japonicus* var. *major* in liquid culture showed that both the dry weight of hairy roots and total saponin yield reached their maximum values on the 30th day which might be suggested as the

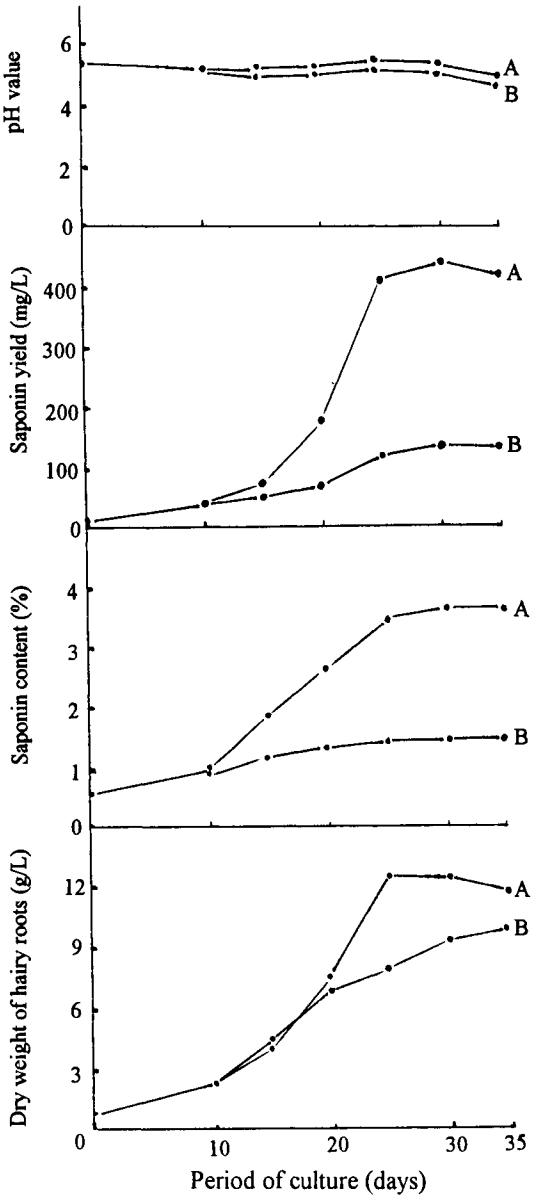


Fig. 4 Effects of oligosaccharin DC-DP9 on the time course of the hairy root suspension culture of *P. japonicus* var. *major*
A: addition of DC-DP9; B: control

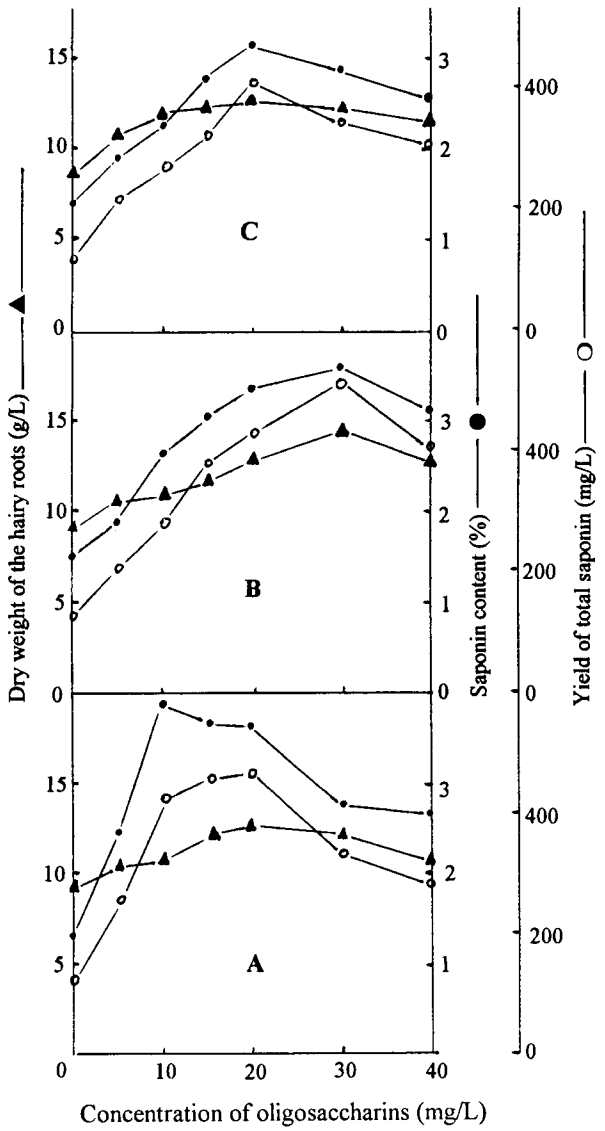


Fig. 5 Effects of oligosaccharins PP-DP6, PP-DP7 and PP-DP8 on the hairy root growth and saponin formation of *P. japonicus* var. *major*
A: PP-DP6; B: PP-DP7; C: PP-DP8

appropriate harvesting time to produce saponins. The experimental data indicated that saponin formation proceeds almost in parallel with hairy root growth. It belonged to the first production-growth pattern, and was very similar to that of cell suspension culture of *P. notoginseng*[8].

Oligosaccharins, which are oligosacchrides with regulatory activity on plant cells, have devoted to much attention in recent years[10,11], and been discovered to help plants to control its functions, such as growth, development, reproduction and defense against disease. It has

been in great demand for the accumulation of secondary metabolites from cultures, such as hairy roots and suspension cells by adding oligosaccharins, mycelia of fungi, and other elicitors. Oligosaccharins affect not only growth of plant cells but also secondary metabolism[12]. Their effects on secondary metabolism vary greatly depending upon the kinds of secondary metabolites. All the oligosaccharins, DC-DP7, DC-DP8, DC-DP9, PP-DP6, PP-DP7, PP-DP8, affected the growth of hairy root, especially obviously stimulated saponin formation. The experimental data indicated that addition of oligosaccharins in the medium could increase the saponin formation in hairy roots of *P. japonicus* var. *major*. Funk et al reported that the growth of *Glycine max* cells was inhibited but the amounts of glyceollin was increased rapidly when a kind of carbohydrate from yeast was added into the cell culture broth[13]. Our previous results also showed that the oligosaccharins from both *P. gingeng* and *D. candidum* young cell wall polymers obviously stimulated saponin formation of *P. notoginseng* and *P. quinquefolium* culutred cells[6-8] and shikonin formation of *Onosma paniculatum* cultured cells[14]

All investigations so far have used oligosaccharins prepared *in vitro*. Natural occurrence of the oligosaccharins, which is a prerequisite for studying natural regulatory role, has been reported only in a few cases[12]. The main difficulty facing studies of the natural biologically active oligosaccharins is their low concentration. We agreed with the working hypothesis originally put forward by Fry[12] that the walls of plant living cells contain built-in oligosaccharin units which, *in vivo*, can be enzymically released from their immobile state into a freely diffusible state. To our knowledge, this is the first report on the hairy root induction and culture of *P. japonicus* var. *major*. The hairy root strain should be useful for basic studies such as metabolism regulation, biosynthesis and biotransformation, and also for practical application such as large scale production of saponin.

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SOME PROGRESS ON CHEMICAL STUDIES OF SAPONINS FROM CHINESE MEDICINAL PLANTS

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Saponins occur widely in plant kingdom and possess various bioactivities such as sedative, antipyretic, analgesic, anticough, antiinflammatory, immunopromoting etc. Many commonly-used traditional Chinese medicines like ginseng, glycyrrhiza, *Bupleurm*, *Platycodon* are noted as their rich saponins, which have been studied since long time ago. By using reverse-phase chromatography and polyporous resins for separation and purification, and various advanced 2D-NMR techniques for structural determination, saponin chemistry have been developed rapidly in last two decades. This paper deals with some progress on chemical studies of saponins isolated from Chinese medicinal plants.

***Clematis chinesis* Osbeck (Ranunculaceae)**

This plant is a liana distributed widely in south China. The plant roots are used as a source of the Chinese crude drug "Wei-Ling-Xian", which drug has commonly been used as an analgesic, diuretic and antiinflammatory agent for ages, and therefore recorded in Chinese Pharmacopoeia (1990 ed.). Although a number of chemical constituents have been isolated from this plants, it is still an unsolved problem to elucidate its activer principles corresponding above indications. From past work two lactones, anemonin and protoanemonin were obtained and considered as its antiinflammatory compounds. But recent pharmacological studies showed that such effects were very weak. Due to rich saponins contained in the plant roots, the polar fraction of root extracts has been systematically studied by Japanese chemists since 1979. As the results of their studies more than twenty prosapogenins were isolated from alkaline hydrolysate of its crude saponins and among them six less polar prosapogenins were proved to be present in the root as genuine saponins by HPLC detection. However, these obtained saponins and prosapogenins are all monodesmosides. To promote fundamental research on this Chinese crude drug, we have made our efforts to elucidate its genuine saponin constituents .

From ethanol extracts of the plant roots, we obtained thirteen compounds, (**1-13**) (see Fig 1), which were elucidated as oleanolic acid **1** and its glycosides **2-6**, and hederagenin **7** and its glycoside **8-13** respectively [1,2]. Among them Compound **3**, **6** and **12** were proved to be new triterpenoid saponins. The proton system of each sugar unit in these new saponins was analyzed by a combined use of DQF-COSY and TOCSY experiments on Bruker AMX 600 spectrometer. Starting from signals of anomeric protons and methyls of rhamnose units, the chain of coupled protons could be pursued by a sequential "walk" via observed cross-peaks in ^{1}H-^{1}H correlation spectrum. The TOCSY spectrum was further studied to discover the delayed correlations among protons more than four bonds away. All correlation signals and sequence of protons in each corresponding residue were then deduced. A ^{1}H-^{13}C one-bond chemical shift correlation experiment (HMQC) correlated all proton resonances with those of the corresponding carbons in each sugar unit. Thus all proton and carbon signals of each sugar unit were assigned unambiguously. The sequence of oligosaccharide chain and the linkage sites to the aglycone were obtained by NOESY and HMBC.

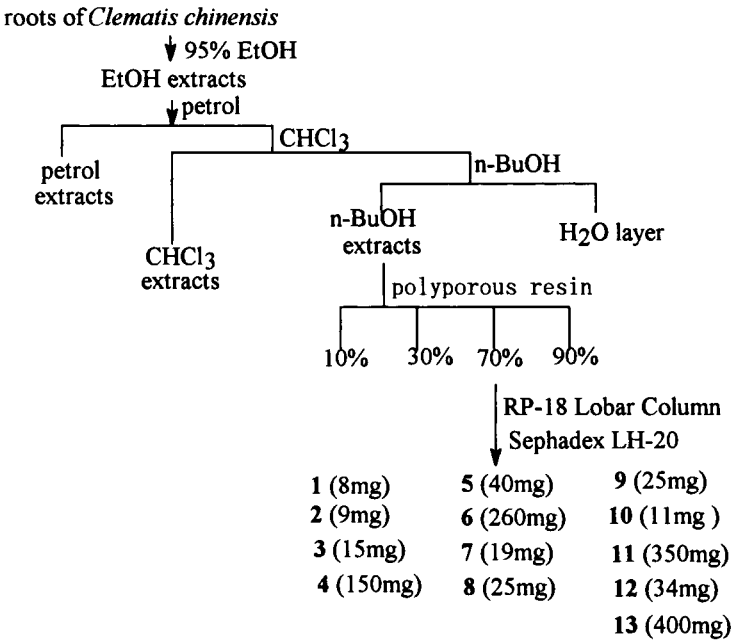
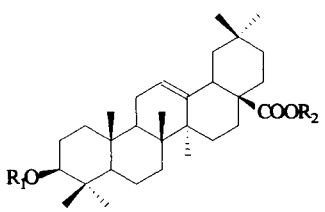
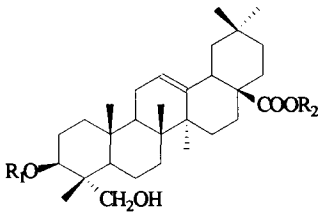


Fig 1: Isolation and separation of EtOH extracts from *C. chinesis*



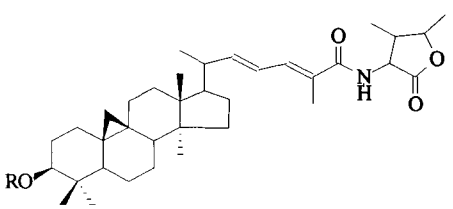
	R_1	R_2
1	H	H
2	-ara $\underline{2\ 1}$ rha $\underline{3\ 1}$ rib	H
3	-ara $\underline{2\ 1}$ rha $\underline{3\ 1}$ rib	-glu
4	-ara $\underline{2\ 1}$ rha $\underline{3\ 1}$ rib	-glu $\underline{6\ 1}$ glu $\underline{4\ 1}$ rha
5	-ara $\underline{2\ 1}$ rha $\underline{3\ 1}$ rib $\underline{4\ 1}$ glu	H
6	-ara $\underline{2\ 1}$ rha $\underline{3\ 1}$ rib $\underline{4\ 1}$ glu	-glu $\underline{6\ 1}$ glu $\underline{4\ 1}$ rha



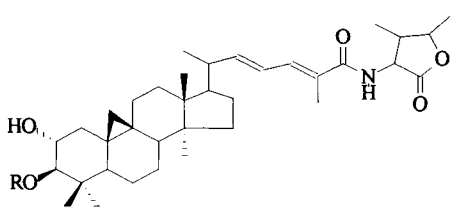
	R_1	R_2
7	H	H
8	H	-glu 6 1 glu 4 1 rha
9	-ara 2 1 rha 3 1 rib	H
10	-ara 2 1 rha 3 1 rib	-glu 6 1 glu 4 1 rha
11	-ara 2 1 rha 3 1 rib	H
12	-ara 2 1 rha 3 1 rib 4 1 glu	-glu 6 1 glu 4 1 rha
13	-ara 2 1 rha 3 1 rib 4 1 glu	H

***Mussaenda pubescens* Ait. f. (Rubiaceae)**

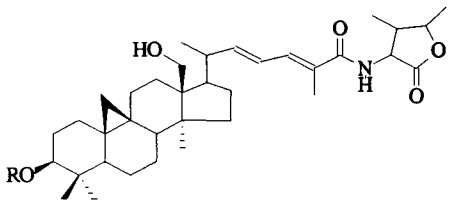
It is a Chinese medicinal plant widely distributed in south China and used commonly for treatment of the common cold, laryngopharyngitis, acute gastroenteritis and diarrhea. It is also used as a contraceptive in some districts of Fujian Province. The aqueous extracts of this plant and its precipitate obtained by additing 95% ethanol showed significant effects on terminating pregnancy in rats. In order to elucidate its active principles, we systematically studied the hydrophilic components of the whole plant.



mussaendosides		R
D	14	glu 1 2 glu–
E	15	glu 1 6 glu 1 2 glu–
O	16	rha 1 2 glu 1 2 glu– (4 1 rha)
F	17	glu 1 2 glu 1 2 glu– (4 1 rha)
G	18	rha 1 2 glu 1 2 glu– (6 1 glu; 4 1 rha)



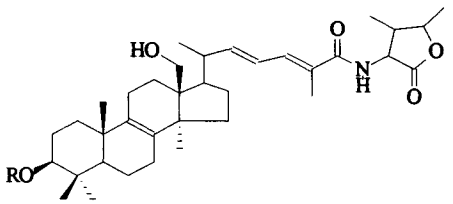
mussaendoside		R
P	19	rha 1 2 glu 1 2 glu– (4 1 rha)



mussaendoside H **20**

R

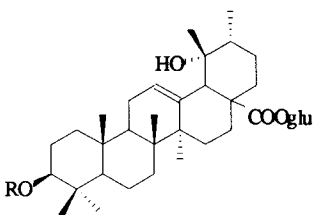
rha 1 2 glu 1 2 glu—
4
1
rha



mussaendoside Q **21**

R

rha 1 2 glu 1 2 glu—
4
1
rha



mussaendosides

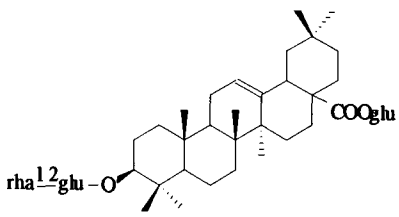
R **22**

I **23**

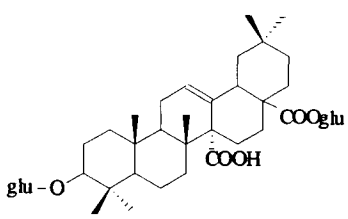
R

glu-

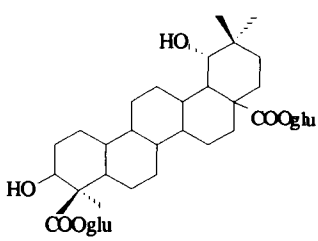
glu 1 2 glu—



mussaendoside J **24**



mussaendoside S **25**



mussaendoside K **26**

The dried and powdered plant materials (6 kg) were extracted with 95% ethanol. The EtOH solution was evaporated in vacuo to give concentrates (450 g), which were diluted with H_2O and then extracted with ether, ethyl acetate and butanol respectively. The butanol-soluble fraction (20.5 g) was chromatographed on a highly porous polymer (DA-201), eluted stepwise with H_2O containing increasing concentration of EtOH (20, 40, 60-90%). The fractions (60-90% EtOH) were evaporated to dryness to yield crude saponins. These crude saponins were subjected to column chromatography on silica gel, Sephadex and RP-18 Lobar column to give thirteen new saponins **14-26**, which structures were elucidated by chemical and spectral method, especially various 2D NMR techniques, and named as mussaendosides D **14**, E **15**, H **20** [3] , O **16**, P **19**, Q **21** [4], F **17** [5] , G **18**, K **26** [6] , I **23**, J **24** [7], R **22** and S **25**

[8]. The saponins isolated in the plant possess aglycones of three skeletons: cycloartane, ursane and oleanane, with some variation in each structure--type. It's very interesting to discover such cycloartane-type saponins, that its side-chain has been modified naturally to conjugated diene-carboxylic acid, which formed amide with 2'-amino-3', 4'-dimethyl-γ-lactone moiety.

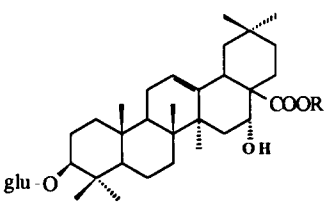
In our study the butanol-soluble fraction was proved to be effective for terminating pregnancy in rats. The results agreed with literature reports and suggested the active principles should be hydrophilic components. However, some soponins we subjected to test showed not effective and the water eluent on a highly porous polymer chromatography seems much better than individual saponin in animal test. The experiments should be repeated and confirmed to make a conclusion carefully.

***Aster lingulatus* (Compositae)**

The genus *Aster* contains hundreds of species in the world. Member of this genus are mainly distributed in the north temperate zone. Most species represent herbs and shrubs which are largely perennial and to a lesser extent annual. Taxonomically *Aster* is a complex genus due to its variable nature and polyploidy. There are more than 100 species of the genus *Aster* found in China. 15 species of *Aster* plants have been used in traditional medicine for treatment of fever, cold, tonsillitis, bronchitis, snake bites and bee stings. In the pharmacological experiments, some *Aster* plants have ben reported to have antitumor activity. As a part of our basic research to investigate chemical constituents of Chinese medicinal plants, we have now undertaken a phytochemical examination of *Aster lingulatus*, which has not been chemically studied before.

The whole plants of *A. lingalatus* were collected from Li-Jiang county, Yunnan Province. The butanol-soluble fraction of a 70% ethanol extract was chromatographed over Diaion HP-20, silica gel, Sephadex LH-20 and C8 reversed-phase silica gel to yield four new saponines **27-30**, The structures of the saponins we isolated were determined by acidic and alkaline hydrolyses, esterification and various spectral analyses. The common aglycone to all new saponins was proved to be 3β, 16α-dihydroxy-olean-12-en-28-oic acid, which connected a commone glucose unit at C_3 and different sugar units at C_{28}.

Compounds **27-30** were evaluated for *in vitro* antitumour activity by means of their inhibitory effect on [^{3}H] thymidine incorporation into DNA in HL-60 cells. Their inhibition was showed in Table 1.



	R
27	-ara
28	-ara$\overset{2\ 1}{__}$rha
29	-ara$\overset{2\ 1}{__}$rha$\overset{4\ 1}{__}$xyl
30	-ara$\overset{2\ 1}{__}$rha$\overset{4\ 1}{__}$xyl$\overset{3\ 1}{__}$xy

Table 1. Inhibition of DNA synthesis in HL-60 cells

compounds	concentration (μM)	inhibition(%)	IC_{50}(μM)
27	5	20.1	22.1
	25	54.4	
	100	87.2	
28	5	18.2	34.3
	25	43.3	
	100	81.9	
29	5	39.0	8.8
	25	70.4	
	100	94.9	
30	5	44.8	6.1
	25	79.1	
	100	97.8	

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SAPONINS PRODUCED DURING THE LIFE CYCLE OF MUNGBEANS AND THEIR ROLE AS ALLELOCHEMICALS

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Key Word Index -- allelochemical activity, bioassay, continuous cropping, epicotyl, extraction, inhibitory, lettuce, life cycle, mass spectrometry, mono- and bi-desmoside saponins, mungbeans, purification, radicle, soyasaponins, soil, stimulation, thin layer chromatography, *Vigna radiata* L., *Lactuca sativa* L.

Abstract Continuous cropping of mungbean (*Vigna radiata* L.) can lead to plant growth inhibition. We have found that allelopathy may be the cause of as much as 10-25% of the inhibition in mungbean plants grown following mungbean plants. Mungbean plants are allelopathic, and the surrounding soil is often toxic. The phytotoxic activity is found primarily in the stems and the aerial parts (excluding the stems). We partitioned stem extracts with water and with organic solvents: the water extracts produced the greatest inhibition of mungbean and lettuce; the organic solvents caused both inhibition and stimulation. The discovery of enhancement of mungbean growth by crude mungbean saponins was serendipitous; the plants showed quicker germination and enhanced growth, but such treatment did not increase the yield.

Leaves, stem, and roots of mungbeans (*Vigna radiata* L.) were harvested at 2, 4, 7, and 10 weeks during the spring growth period of the crop in Taiwan. Crude saponins were isolated from defatted plant residue with 80% EtOH, partially purified with 1-BuOH extraction, and analyzed by using TLC, HPLC, LC/MS, FAB/MS, and FAB/MS/MS. Soyasaponin I [(SSI) the main saponin], soyasaponin III, 3-O-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl] sophradiol, were identified and several mono- and bi-desmosides saponins were not identified. The BuOH extracts and SSI were stimulatory at concentration's of 1, 10, and 100 ppm when tested using seeds of mungbeans and lettuce (*Lactuca sativa* L.) in a 72-h bioassay on filter paper with distilled water as control. At 1,000 ppm the growth of both plants were inhibited. The bioassay results were significant at the 95% level of confidence. A mechanism of action is proposed.

Introduction

The variation of secondary metabolites produced by plants is enormous and differs greatly with the stages of growth and development (1-5). Of these natural products, saponins are being carefully examined because of their biological effects on humans, plants, and animals.

Saponins exhibit allelopathy in mungbean plants and in the soil around them in both inhibitory and growth-promoting activity. Saponins are localized in plant organelles that have a high turnover rate, which implies that they are metabolically active as well as sequestered from the remaining parts of the cell. Allelochemical activity is found in agriculture, forestry, natural, and developed ecological systems and may provide, in part, an explanation of the reason for plant survival in a hostile world.

Allelopathic interactions between plants, soil, and organisms have been recognized by scientists worldwide because a) they offer an understanding of the growth and development process and b) when recognized they may find new uses in agriculture such as decreasing our reliance on synthetic herbicides, insecticides, and nematocides for disease and insect control. The recognition of allelochemicals that have a part in influencing the growth of plants and microorganisms is not yet understood. Crop yields are of fundamental importance; and to improve them we must incorporate the ecological changes brought about through the appropriate use of allelopathy in each country. Despite much optimism and some progress in unraveling the complexities of chemical interactions between biological species, a firm foundation for the existence and function of allelopathic interaction has not been forthcoming. It is my hope that this International Symposium on Plant Glycosides will markedly assist in developing knowledge needed that will carry mankind and womankind into the 21st century.

I would like to give my perception of the conceptual setting for production of food and fiber in the world today. In the history of the Earth there has not been a species as successful as *Homo sapiens* in establishing and maintaining itself! Meeting the essential dietary requirements of the increasing numbers of human beings is a global, persistent, and arduous challenge for those people involved in agricultural and biological research, development, extension, and production. Most of us are familiar with how well the challenge has been met: fewer people on the land are feeding more of the world's population than ever before. It could not have been done without advances in knowledge through research and development. Ultimately the practical application by the farmers of the world made the difference in the successful production.

Mungbeans were not known to have allelopathic activity until recently (6-11). Mungbeans (*Vigna radiata* L.) are a crop plant of economic significance in Taiwan, China, and many other countries around the world. Multiple cropping is common in subtropical and tropical Taiwan; in the southern part of the island three crops (two crops of rice and one crop of mungbeans) can be raised on the same field in a year. Temperatures that are higher in the subtropical and tropical regions increase the phytotoxicity of decomposing residues (12-14). In 1980, the Asian Vegetable Research and Development Center (AVRDC), Taiwan (15-17) determined that five continuous mungbean croppings showed lack of uniformity of growth patterns; the later plants were smaller and produced fewer pods per plant, fewer and lighter seed and poor grain yields (only 25 kg/ha). By comparison, where mungbeans had not been grown for at least three cropping seasons, grain yield was 440 kg/ha. This led to the recommendation that a mungbean crop should not be followed by another such crop for at least three cropping seasons (15-17). Among the different preceding crops (mungbean, soybean, tomato, Chinese cabbage, sweet potato, corn, crotalaria, sorghum and buckwheat), mungbean was the most detrimental to a succeeding mungbean crop. In 1984, a mungbean root disease in the Philippines was described and it was reported that the primary cause was not fungi; however, no mention was made of

involvement of allelochemicals from the mungbean plant (18).

A series of experiments using a plant culture system designed to determine whether an allelopathic agent existed in the mungbean plant was performed by Young (National Chung-Hsing University, Taichung, Taiwan, personal communication). The results strongly indicated that the mungbean plant produced phytotoxic substance(s) in its aerial parts and its root system. They were found to produce mono- and bi-desmosidic saponins (8-11, 19), with soyasaponin I being dominant. Little is known about the biological activity of saponins, although some reports on allelopathic activity (both inhibitory and stimulatory) have been published for mungbeans (8-11). Research showing the presence of steroid saponins in the plant kingdom and their significance to plant growth was reported by Balansard et al. in 1946 (20). This was followed by Helmkamp and Bonner (21), who studied the growth rate of wheat embryos and found a doubling by the optimum concentration of saponins. The treatment of tomato seed and cereals with dilute solutions of saponins accelerated germination and increased the growth rate. Seeds of pea or corn absorbed water more rapidly in the presence of saponins with a corresponding increase of growth rate. They speculated as to why steroidal saponins increase growth of pea embryos by 40%, and postulated that these effects may be due to surface-active activity, which modulates the relation of water to embryos and seedlings in cultivars. A decade later Vendrig (22) determined that steroidal saponins exhibited strong auxin activity at very low concentration which placed them into the area of plant growth regulators. Grunwald (23) found plant sterols influenced the cell wall permeability of plants; the review of Price et al. (24) states that the primary action of saponins upon cells is to cause a general increase in the permeability of the plasma membrane. Bisdesmosides appear to be primarily a transport form, and when the plant is damaged they could rapidly be converted by enzymes into monodesmosides which tend to be more active; however, Bissett (25) did not provide any evidence to support this theory.

Allelopathy can be stimulatory as well as inhibitory toward plant growth; these biochemical reactions occur in all types of plants and microorganisms which are dependent on the soil and little is known about how their interaction with insects or organisms causing diseases might be brought about. The focus of this paper is to review the research from mungbean plants grown in the laboratory as well as those grown in the field and sampled at 2-week intervals. This work was done using soils at the Asian Vegetable Research Development Center (AVRDC), Tainan, Taiwan. Higher concentrations of saponins cause inhibitory reactions in the mungbean plant which is the most common cause of allelopathy. The phenomenon of growth promotion at lower concentrations of saponins and inhibition of growth in higher concentrations calls for careful studies of the plants in the future. Such studies should focus on plant adaptability, stages of growth, concentration and type of allelochemical measurement of biological activity, etc. to fully explore the allelochemical response.

Materials And Methods

Materials and methods have been described previously (8-11).

Allelopathic Inhibition in the Field. Fig. 1a, b and c shows the effect of planting mungbeans following the earlier crop of mungbeans with data being statistically significant. Fig. 1a shows replanting the second time; Fig. 1b the third time, and Fig. 1c shows the fourth replanting. Differences in height were around 75-80% between the second and third crop, and about 40-

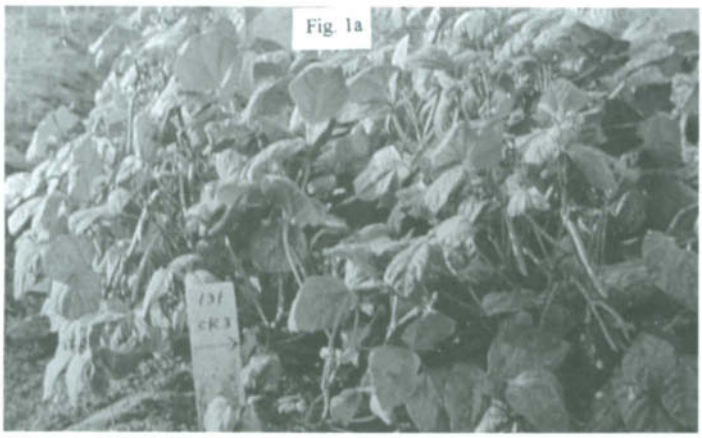

Fig. 1a, b, c. Mungbeans planted following mungbeans in the same field of Asian Vegetable Research and Development Center, Tainan, Taiwan during 1993, a) Second planting, b) Third planting, and c) Fourth planting

50% for the third and fourth crop. The yields of mungbeans were not measured but they were significantly lower each time the replanting occurred which agreed with the results reported by AVRDC (15-17). It was our objective to demonstrate what role, if any, the saponins have in protection of the mungbean plants.

Distribution Of Saponins In Mungbean Plants

Mungbeans Grown in the Field. The results of harvesting three replicates of mungbeans with respect to the dry weight increase are shown in Fig. 2. The greatest increase at 90 days of age occurs with 18 g/plant; the roots were 1.1 g/plant and the stem weights were relatively constant from 50-90 days at 200 mg/plant.

Method Used for Isolating Saponins. Extraction and partial purification using different methods showed that the best procedure was to extract mungbean plant parts with $CHCl_3$ in a Soxhlet apparatus for 24 h, extract the defatted residue with 80% ethanol three times, take the residue to dryness, extract with 1-butanol saturated with distilled water for three times, evaporate to dryness *in vacuo*, and filter through a 0.45-mm filter. This produces a partially purified mungbean fraction of saponins which was 70-80% pure saponins. In some experiments the partially purified saponins were put through dialysis using a 1,000 M.W. cut-off tubing; the results showed that soyasaponin I and III were present in both the dialysate and inside the tubing; however, this technique was abandoned.

Saponins Isolated from Plants Grown in the Field. The crude saponins (Fig. 3) were isolated from each part of the plant. For all of the data report on TLC, HPLC, MS, and bioassay the saponins were processed through the first stage of purification, which was extracted with 1-butanol. Leaf biosynthesis of crude saponins (Fig. 3) occurred in 3 bursts at approximately 30-50, 55-65, and 75-90 days, which corresponded to the initiation of flowers, leaf development, and maturity. The total saponins accumulated corresponds to 1.8 g saponins/plant; however, in the purification with 1-butanol about 40% of the crude saponins were lost leaving about 1 g saponins/plant being produced during the life cycle. The stems were relatively constant at around 20-30 mg saponins/plant after 30-40 days of age; the roots were similar in content to the leaves in that at 30-40 days of age they increased from 20 mg/plant to 70-80 mg/plant which corresponded with the second and third trifoliate leaf development. This burst of biosynthetic activity of saponins coincides with the onset of fungal root disease which was identified earlier as due to *Rhizoctonia* spp, *Pythium* spp, and *Fusarium* spp (8,9, 15-17).

The rates of biosynthesis and catabolism are reflected in the pool size of the saponins, which is considerable in the whole plant (i.e., nearly 2 g/plant); however, the bulk of the metabolic activity was found in the leaves, particularly the young ones. We believe that the rates of biosynthesis and catabolism vary with respect to physiological states of development, diurnal variation, time of year that the crop is grown, types of soil, fertilizers, herbicides, insecticides and cultivars, as well as the functionally different parts of the plant. Different patterns of saponins may be formed. Saponins undergo biotransformation within the plant as is shown in the section on bioassay in this paper. Biotransformation also occurs in the soil surrounding the plant; often these reactions occur at appreciable rates; however, little is known about their effect either in the plant or in the soil.

Occurrence of Soyasaponin I and Mono- and Bidesmosides Studied by Thin Layer Chromatography (TLC). Preparative TLC was used to fractionate the crude saponins from

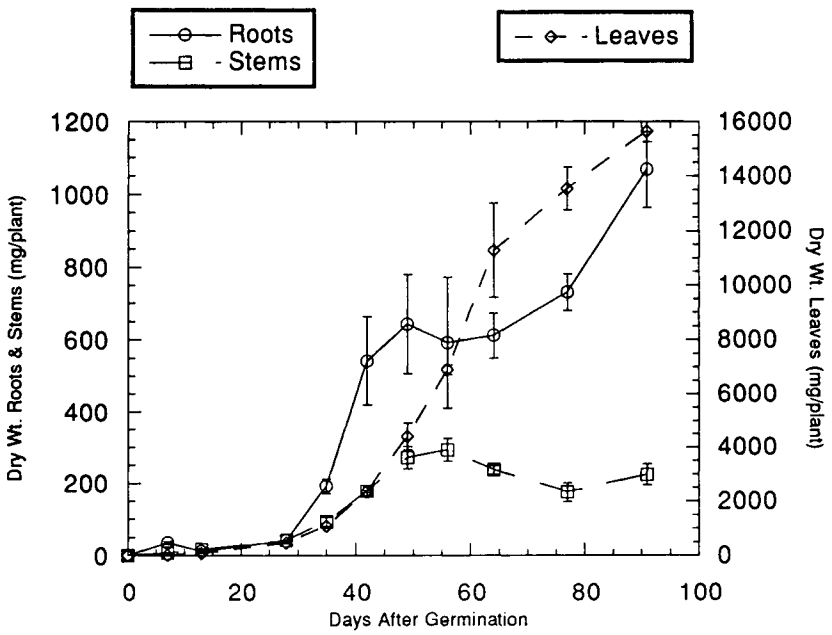

Fig. 2. Increase in dry weight of mungbeans throughout germination. Average of 3 replications: No. of plants collected for each age; 150 for 7 days; 100 for 13 days; 75 for 28 days; 50 for 35 days; 25 for each of the later collection periods.

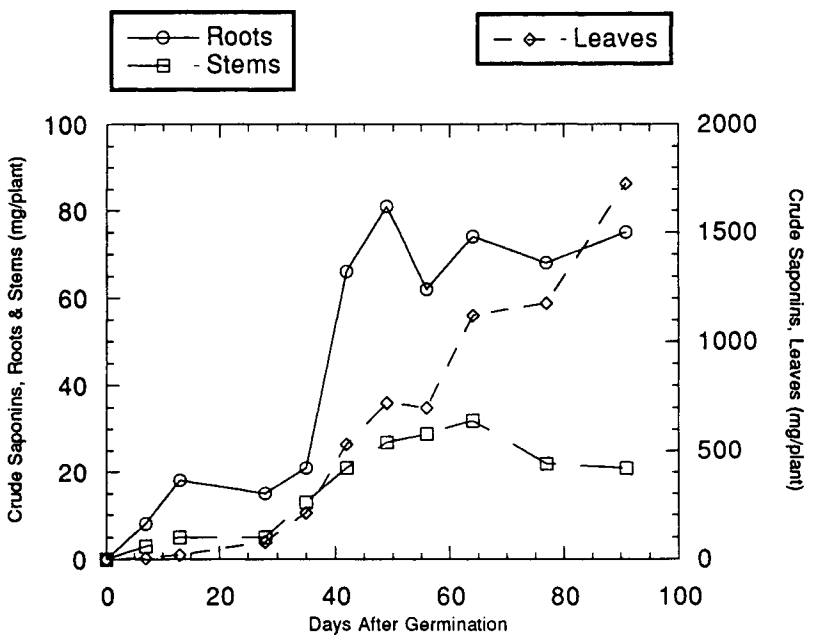

Fig. 3. Crude saponins isolated from roots, stems, and leaves of mungbean plants throughout the life cycle.

mungbeans. Soyasaponin I (Fig. 4a), which has a molecular weight (MW) of 942 and an Rf value of 0.48-0.53, was found to be the main saponin in all leaf, roots, and stem samples isolated throughout the life cycle of mungbeans. The bidesmosides [R_f=0.10-0.48] and the other monodesmosides (R_f=0.54-0.68) were also found in each fraction. The monodesmosides contained a new saponin (Fig. 4b) of MW 780, and Fig. 4c shows soyasaponin III (MW 796). Kitagawa et al. (26) first identified soyasaponins I and III from soybeans. The upper part of the plate (R_f=0.70-1.00) contained soyasapogenol B as well as other aglycones, some not yet identified. Preparative plates were run and the fractions were collected that had R_f=0-0.21, 0.21-0.35, 0.35-0.48, 0.48-0.53, 0.53-0.65, and 0.65-1.00; information on this is summarized in Table 1. Table 1 shows the distribution of the saponins at 2, 4, 7, and 10 weeks of age, with more saponins being formed as the mungbean plants reach maturity. It also indicates that the saponins are being biosynthesized at rates corresponding to increase in dry weight. The disappearance of saponins (biodegradation) occurs much more slowly. Both are important metabolic processes that occur throughout the life cycle. More bidesmosides and monodesmosides are formed during the last 5-10 week period, which corresponds to the pre-flowering, flowering, and pod development.

HPLC. The preparative TLC samples were used for both the analytical and preparative modes for analysis, with collections being made for the mass spectrometer.

MS. The JEOL JMS SX/SX 102A high resolution double-focusing sector tandem mass spectrometer was used to determine the structures of soyasaponin I (MW *m/z* 942), soyasaponin III (MW *m/z* 796), and 3-O-[β-D-galactopyranosyl-(1→2)-b-D-glucurono-pyranosyl] sophradiol (MW *m/z* 780) as shown in Fig. 4 (9, 27-29). Several other mono- and bidesmoside saponins were detected; however, the samples were not pure enough to yield parent (M.W.), daughter or granddaughter ions high enough in intensity to permit tentative identification. Soyasaponin I was predominant (60-70%); it was present in leaves, stems, and roots until 20-30 days of growth; however, the roots contained no mono- and bidesmosides throughout the life cycle of the mungbean plant (Table 1). The majority of the saponins were found to occur in 60-90 days (Table 1, Fig. 3) where the soyasaponin I made up only about 50% of the mixture with more mono- and bidesmoside saponins being present in the leaf and stem tissue.

Growth of Mungbean Plants in Soil Taken at Different Intervals After a Previous Mungbean Harvest. Soils were used without adding fertilizers, insecticides, fumigants, or other agents except the mungbean plant parts that were mixed with soil. The mungbeans were allowed to grow naturally, and the pH varied from 6.6-8.0 (10). Fig. 5 provides evidence of allelopathy for plants grown for 40 and 81 days. The numbers on the pots refer to one of the twelve plants grown in a particular plot of soil. The pH of plot number 33 was 7.8, and 65-70% inhibition occurred (Fig. 5). Fig. 6 indicates about 60-65% inhibitory activity in plot 33, whereas plot number 34 (pH 6.7) showed 90% of the normal growth. This provides evidence that alkaline soil promotes allelopathic activity, whereas only a negligible amount of allelopathic activity was observed in plot number 34. There was no apparent fungal attack on the plants grown in plot 34 (acidic soil), which indicates that the phytotoxic activity in the acidic soil was considerably less than in the alkaline soil. This effect of pH on the allelopathic factors in the growth of mungbean was not previously recognized.

Control Soil Mixed with Mungbean Plant Parts. Table 2 shows the overall result of

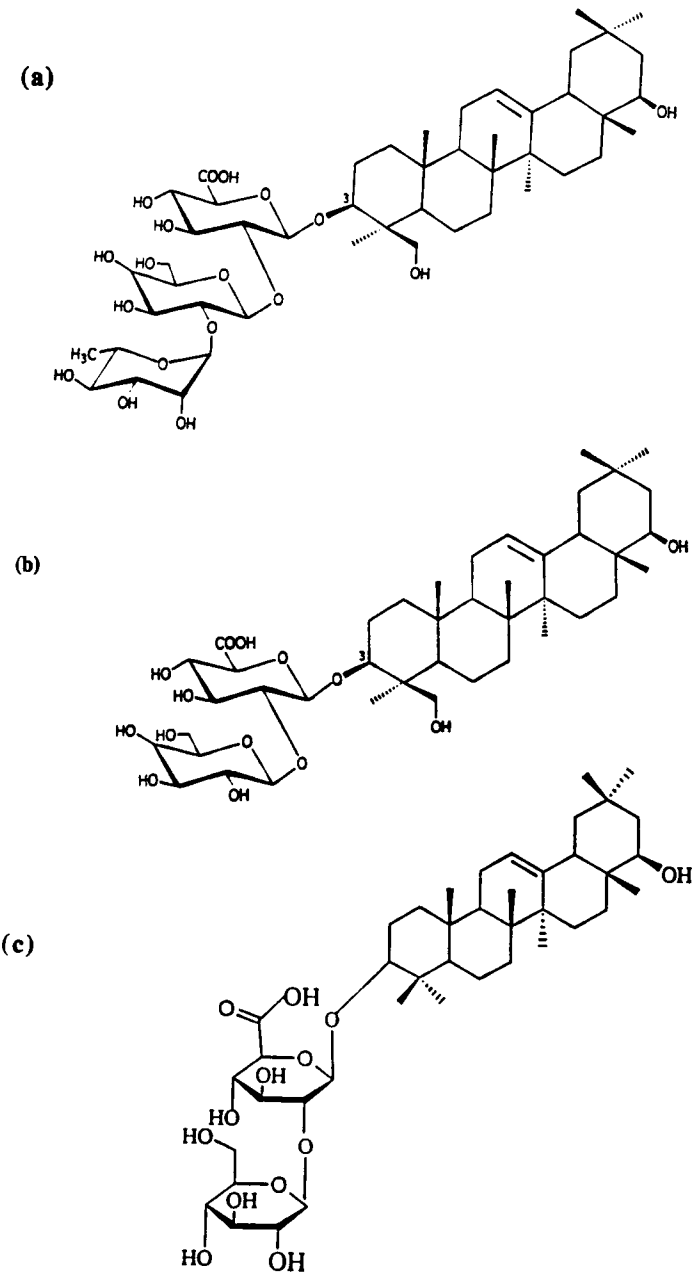

Fig. 4. Structures of saponins identified in mungbeans (Vigna radiata L.): a) soyasaponin I, 3-O-[α-L-rhamnopyranosyl-(1→2)- β-D-galactopyranosyl-(1→2)- β-D-glucoronopyranosyl] soyasapogenol B; b) newly identified saponin [3-O-[β-D-galactopyranosyl-(1→2)- β-D-glucuronopyranosyl] sophradiol; and c) soyasaponin III, 3-O-[β-D-galactopyranosyl-(1→2)- β-D-glucuronopyranosyl] soyasapogenol B.

Table 1. Quantitative comparison of the TLC of saponins formed throughout the mungbean plant life cycle.[1,2]

	Weeks			
Saponin Spots Corresponding with	2	4	7	10
Bidesmosides				
Leaf	+	+	++	++
Root	-	-	-	-
Stems	++	++	++	++
Soyasaponin I				
Leaf	++	+++	++++[3]	++++[3]
Root	++	++	++	++
Stems	+	+	+++	+++
Other Monodesmosides				
Leaf	+	+	++	++
Root	-	-	-	-
Stems	++	+	++	++

[1]Quantitatively the amount of each series of compounds spotted was similar.
[2]Relative quantities: - (absence); + (low presence); ++ (medium more than 250 mg/g sample); +++ (high about 500 g/g sample); ++++ (very high about 1 mg/g sample); all samples on a dry weight basis.
[3]Many compounds clustered around soyasaponin I.

Table 2. The effect of mixing mungbean residue powder with control soil on the growth of mungbean at 81 days.

Treatment	Growth and weight, % of control	
mixing rate (g/g)	Height	Dry weight
Root powder/soil (0.2%)	98ab	76
Stem powder/soil (0.5%)	87b	33
Leaf/aerial powder/soil (0.9%)	115a	124

Values are the means of 12 replicates. The letters in a column not followed by the same letter are different, P = 0.05, ANOVA with Duncan's Multiple-Range Test.

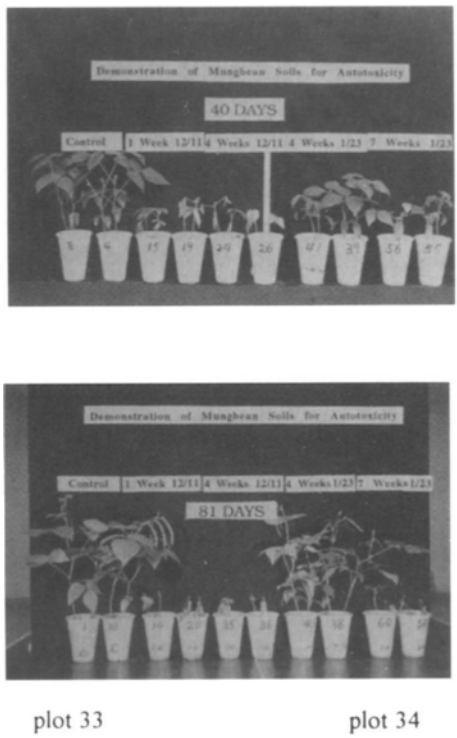

Fig. 5. Allelopathy of mungbeans growing in AVRDC soil for 40 and 81 days.

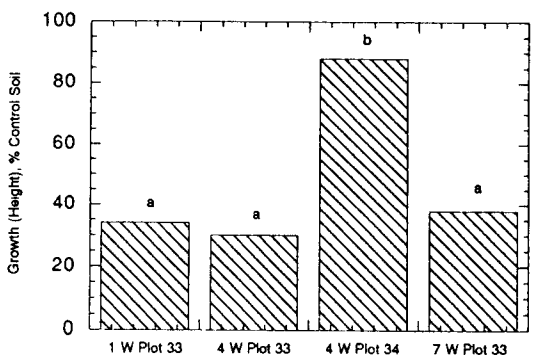

Fig. 6. Allelopathy of mungbean plants grown in AVRDC soil following mungbeans. Legend: Duration of experiment (81 days); W = weeks following harvest of mungbeans; bars having different letters are significantly different, P = 0.05, ANOVA with Duncan's Multiple-Range Test.

comparing the height and weight of plants grown for 81 days in the modified soil to those grown in the control soil. The stems decreased the height at a 0.5% concentration and the roots caused almost the same degree of inhibition at a concentration of 0.2%, but leaf or other aerial parts at a concentration of 0.9% caused about a 15% increase in plant height. The stems caused the greatest inhibition of dry weight, the roots showed somewhat less, but the leaf/aerial soil mixture showed there was a 24% increase. It is clear that stems of the mungbean plant contain the predominant chemicals that exert their allelopathy activity toward mungbean plants. The plant material added was estimated, based on weights obtained from plants grown in the field just prior to harvest, to be about the normal amount plowed under in the field. These data suggest that the complete mungbean plant when plowed under might have a negligible allelopathic effect on the new crop of mungbeans, since the effect of secondary chemical compounds that can inhibit plant growth can be overcome by those which have stimulatory activity, i.e., saponins. As the plant material decays in the soil, residual compounds, sometimes in high concentrations can occur. Such compounds if allelopathic, could also stimulate or inhibit the growth of a crop of mungbeans planted soon after the harvest.

Bioassay of Mungbean Plant Parts. Aqueous extracts of roots (Fig. 7) showed a significant inhibition of lettuce and wheat seedlings and a small but typical inhibition of mungbean seedlings. The assay was run at the same pH (7.0±0.3) as that of the plant parts extract. The osmotic concentration ranged from 16 to 82 milliosmol kg-1 in the 1-5% concentrations. A bioassay was run on mungbean and lettuce at 25 milliosmol kg-1 (30); it showed 12% and 27% inhibition, respectively (30). The bioassay of extract of mungbeans leaves (Fig. 8) demonstrated a significant inhibition of mungbean, lettuce, and wheat. The pH of the extracts were 6.0±0.2 and remained constant throughout the assays. The osmotic concentration ranged from 20 to 87 milliosmol kg-1, which was nearly the same value as for the roots. The inhibition by stem extracts (not shown) was similar to that of the leaf extracts. The stem extracts had pH values of 6.3±0.3 and an osmotic concentration that was almost identical to that of the leaf fraction. The results of the root extract bioassay agree with the findings on the whole plants grown for 81 days (Fig. 5).

Bioassay of Compounds from Mungbean Stems Extracted into Water and Partitioned into Organic Solvents. The effect of extracts made with distilled water and then partitioned with organic solvents is shown in Fig. 9 (top and bottom) at 1% and 15% of the original plant weight. There was mixed inhibition and stimulation of mungbean and lettuce. Chloroform and hexane extracts showed slight stimulation of growth, whereas the water, ether, 1-butanol, and ethyl acetate extracts inhibited mungbean growth. At a concentration of 1% the extracts of lettuce stems were somewhat less inhibitory than were those of mungbeans, but showed greater inhibitory activity at 15%.

It appears that this partitioning system provides information on the compounds responsible for the effects described. The water extracts were the most inhibitory at both concentrations; it contains phenolic acids and other soluble chemical compounds from the partitioning by organic solvents which are known allelopathic compounds.

Bioassay of Soils Collected After Mungbean Harvest. The bioassay of mungbean soils (Fig. 10) supports the experiments with mungbean plants grown in maturity (Figs. 5 and 6) with the exception of the lack of significant allelopathic activity shown by the 1-week soil. The control soil (plot 40, 41) showed no inhibitory or stimulatory activity. The soil samples taken 4

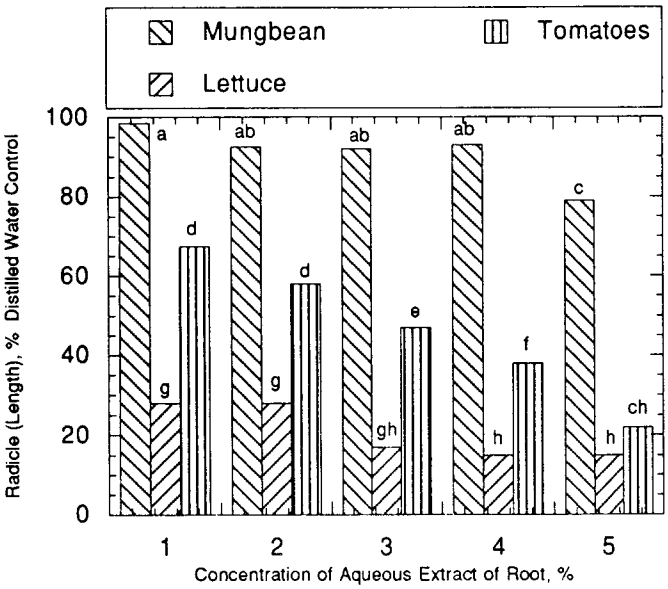

Fig. 7. Inhibition of mungbeans, lettuce, and tomato by application of aqueous extracts of mungbean roots for 72 h. Legend: Bars having different letters are significantly different, P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

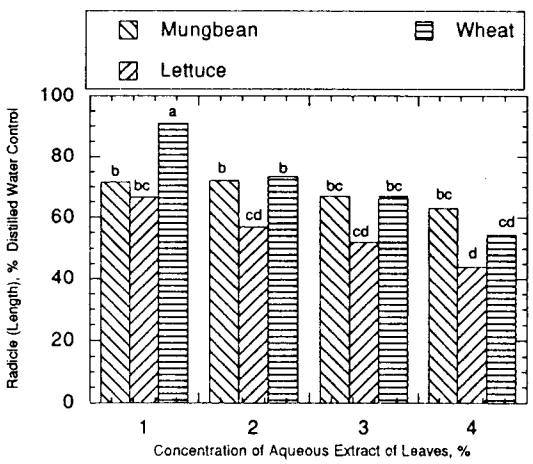

Fig. 8. Inhibition of mungbeans, lettuce, and wheat by application of aqueous extractions of mungbean leaves for 72 h. Legend: Bars having different letters are significantly different. P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

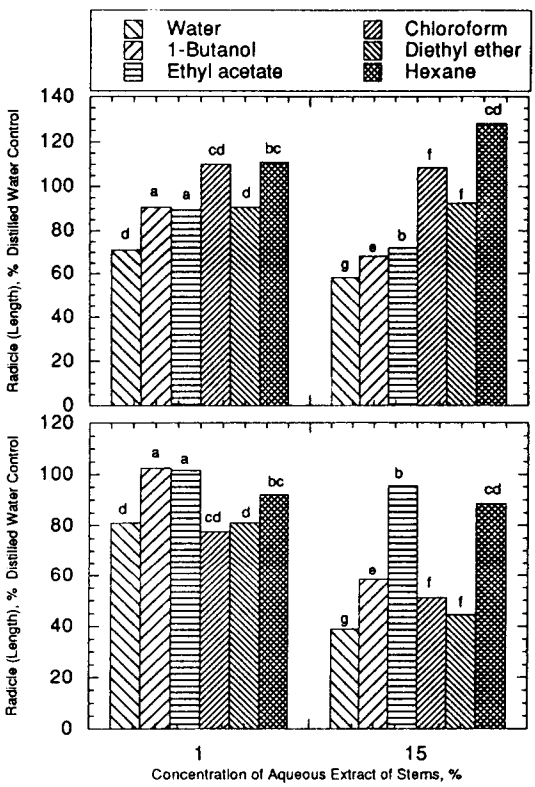

Fig. 9. Allelopathic effect of application of water- and organic solvent- extracts of mungbean stems on mungbeans (top) and lettuce (bottom) for 72 h. Legend: Bars having different letters are significantly different, P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

weeks after growing mungbeans from plots 33 (pH 7.8) and 34 (pH 6.7) showed approximately 8% and 18% inhibition respectively, where as at 7 weeks the inhibition of mungbean was negligible.

The effects on lettuce in 1- and 4-weeks alkaline soil were most inhibitory, but the 4- and 7-weeks alkaline and acidic soils showed no inhibition or stimulation. The compounds extracted from each soil differed markedly in amount and type; however, this experiment was only preliminary.

Enhancement of Mungbean Growth by Crude Mungbean Saponins Added to Soil. Crude mungbean saponins (15, 17, 18, 31) were applied to the soil at concentrations of 15, 150, and 450 ppm. Mungbean seeds were germinated in pots and allowed to grow until maturity. The plots were 40 and 41 the control pots. The height of the plants was recorded for several experiments, but only the pertinent results are included in Table 3. The mixture of mungbean saponins was around 50% soyasaponin I. The experimental plants clearly showed elongation of stems and other growth-enhancement effects when the 1-butanol-extracted and 5-h-dialysis-treated saponins were added, but we cannot be certain that all the active compounds were saponins. Mungbean plants showed quicker germination and other enhancement effects throughout their growth. The plants had larger, darker green leaves, which is an indication of enhanced photosynthesis, but the number of seed pods was about the same; the increased growth of the plant was not accompanied by an increased yield. After 40 days, all concentrations caused about 10% enhancement of growth, but after 67 days the two higher concentrations showed 10% and 15% enhancement respectively, while the effect of 15 ppm of

Table 3. Enhancement of the growth of mungbeans by soyasaponin I and other saponins produced by mungbean plants added to AVRDC soil

Date (1992)	Day	Observation of pots [a,b,c,d] (Compared to control without saponis)
Feb. 13	0	Experiment started
Feb. 21	8	Primary leaves, no trifoliate, growth enhancement with 88% germination as compared with 58% of the control
Feb. 25	12	First trifoliate, growth enhancement
Feb. 28	15	Second trifoliate, growth enhancement
Mar. 4	20	Third trifoliate, growth enhancement
Mar. 7	23	27% higher than control; more third trifoliate
Mar. 14	30	Less growth enhancement
Mar. 24	40	Plants average 10% higher than control; some flowering
Apr. 20	67	Plants average 13% higher than control; pods appear; maturity attained in a shorter time than for control
May 6	83	Plants 20-25% higher than control

[a]Saponins where extracted with 1-butanol and purified by 5 h dialysis with 3000 MW tubing

[b]concentrations of added saponins were 15, 150, and 450 ppm

[c]Soils from Asian Vegetable Research and Development Center where no mungbeans were grown during the previous three years; plots 41 and 42

[d]Small differences of enhancement may be seen at: flowering, pod maturity, not pod number.

saponin continued to drop to nearly zero. At the end of the experiment (84 days) the lowest concentration had no effect, and 150 and 450 ppm saponins showed 20-25% enhancement (measured by height of the plant). The preliminary indications are that 150 ppm of crude saponins (5-h dialysis) is required to enhance mungbean growth.

The crude saponins added to the soil may have provided a source of nutrients. Little effort was made to remove inorganic elements, other than dialysis for 5 h (a common technique to reduce the inorganic nutrients and small organic molecules by a factor of 30-50). Our results should be considered preliminary.

Attempts to recover the saponins immediately following their addition to the soil were unsuccessful by using several extraction techniques, but no humic or fulvic acid was isolated. This was interpreted to mean that the saponins added might become bound to the humus fraction of the soil

Roots and their rhizosphere have been the subject of research for more than a hundred years, but their action on biological membranes and sites of enzyme action have not been thoroughly treated, although Tang et al. (7) alluded to the reactions that occur at the molecular level. Soil moisture, temperature, gases, humus, and inorganic (mineral) and organic compounds have important effects on the development of the root system, and interact with each other in establishing the pattern of root development and function. Mungbean roots can take part in symbiotic associations with mycorrhizae and bacterial nodules when fungi and bacteria bring in mineral constituents in exchange for some of the plant vitamins, carbohydrates, etc. We suggest that saponins, such as soyasaponin I (Fig. 11) which is the predominant saponin present in 7-day-old mungbeans (8, 9) can become attached to the root hair and facilitate the transport of water and nutrients. The sugar portion of the saponin is responsible for the primary enhancement of mungbean growth; but it could be hydrolyzed off by enzymatic cleavage and produce another saponin molecule.

The saponins occurring in the mungbean rhizosphere prior to plant maturity may undergo several changes in the microstructure of cell membranes (32, 33). The rhamnose-galactose-glucuronic acid-soyasapogenol B (soyasaponin I) (Fig. 11) is the dominant structure during the early germination period. We suggest that as the plant develops, hydrolysis catalyzed by enzymes produced by microorganisms in the soil cleaves the sugars from the saponin in the same manner as for medicagenic acid glycosides (9, 11, 18, 31, 33) to give the aglycone (soyasapogenol B). Although the structural integrity of soyasapogenol B is not understood, this compound can be further broken down by microorganisms to serve as a carbon source. Hydrolysis may progress through the sequential cleavage of sugars (which we think most likely) (Fig. 11), or cleavage could release the rhamnose-galactose-glucuronic acid as 2- or 3-piece fragments, leaving soyasapogenol B. Because plant root hairs have a short lifetime, the process would be repeated many times, continually exposing the root hairs to saponin molecules.

Effect on the Growth of Mungbeans and Lettuce in a 72-H Bioassay. Partially purified saponins at concentrations of 1, 10, 100, and 1,000 ppm were dissolved in distilled water, adjusted to pH 7.0±0.1, and used in bioassays for 72 h. After that time, the lengths of mungbean and lettuce seedlings were measured in millimeters and compared to those grown in distilled water. Results of the complete study are reported in documents filed in the Oklahoma State University Library. Some typical results are presented for mungbean radicles in Fig. 12a, b, c and for lettuce radicals in Fig. 13a, b, c; however, most of the results are not shown in this

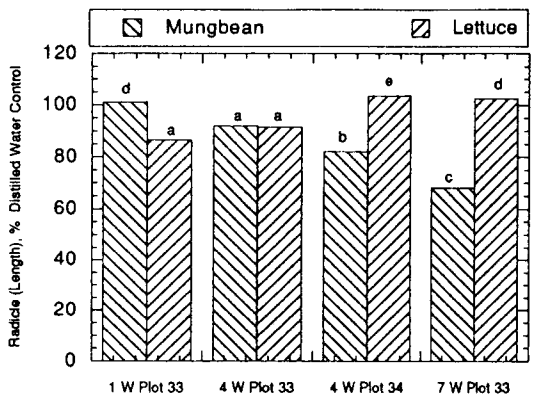

Fig. 10. Allelopathic effect of soil on mungbeans and lettuce seedlings after 72 h. Legend: Soil (5 g) was placed on filter paper, distilled water and seeds were added, and the culture was set to incubate. Bars having different letters are significantly different, P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

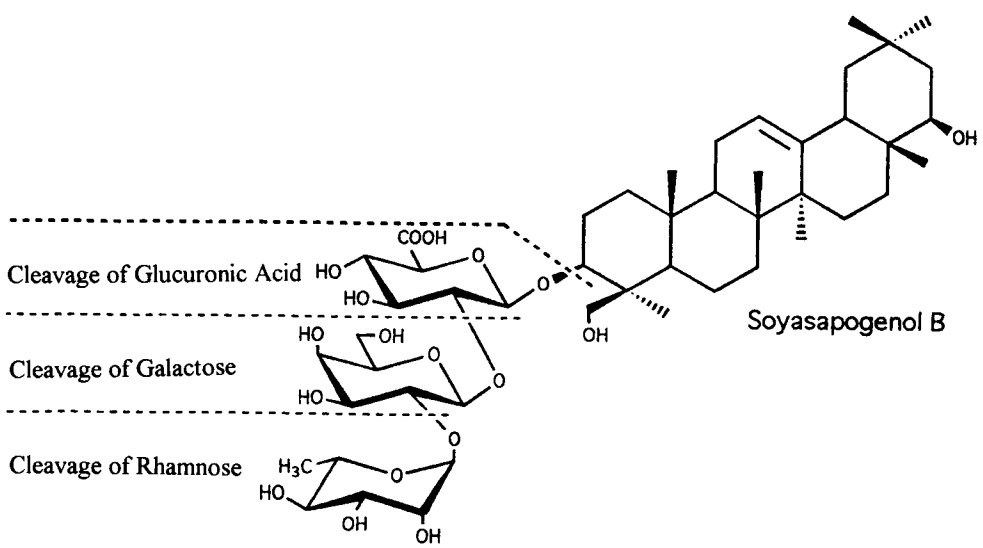

Fig. 11. Suggested structural changes in soyasaponin I (3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl] soyasapogenol B) that occur on the mungbean root surface and in the surrounding rhizosphere. Legend: Stimulation occurs when soil is added; inhibition occurs when no soil is present (in the 72 h bioassay). Cleavage or loss of sugars can occur sequentially or by 2- or 3-piece fragments.

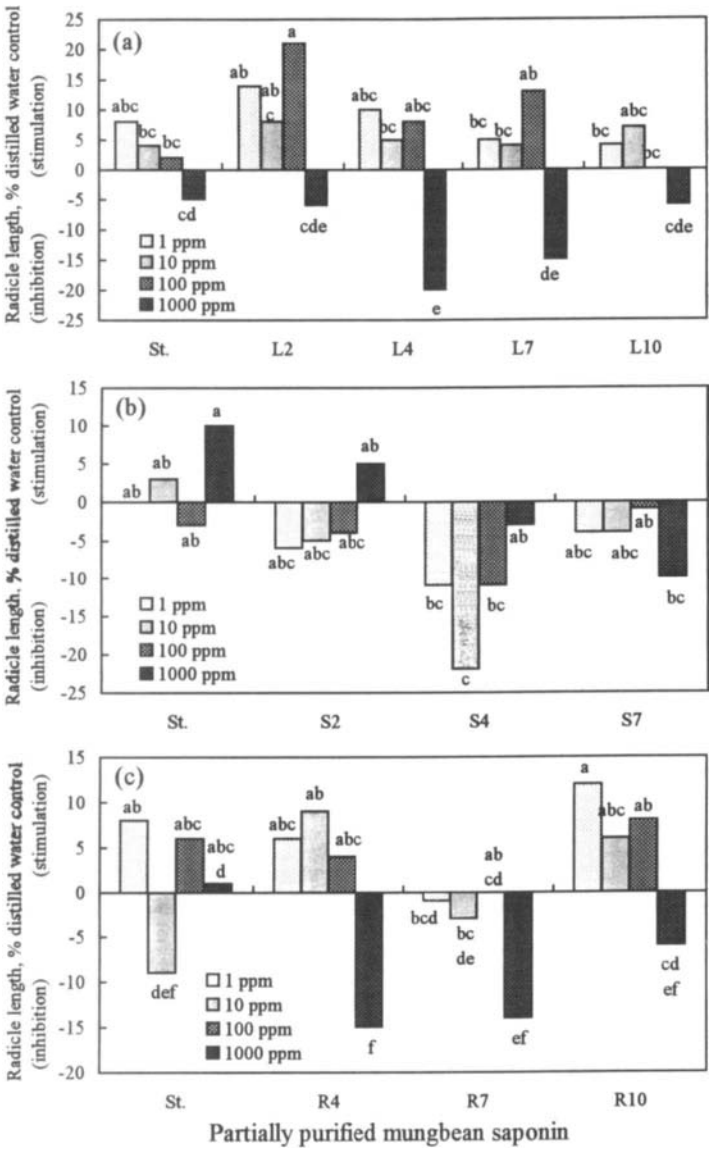

Fig. 12a, b, c. The effects of partially purified saponins extracted from the (a) leaves, (b) stems, and (c) roots of mungbean at 2, 4, 7, and 10 weeks of growth on the growth of mungbean radicle at 72-h bioassay. Bars having different letters are significantly different, P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

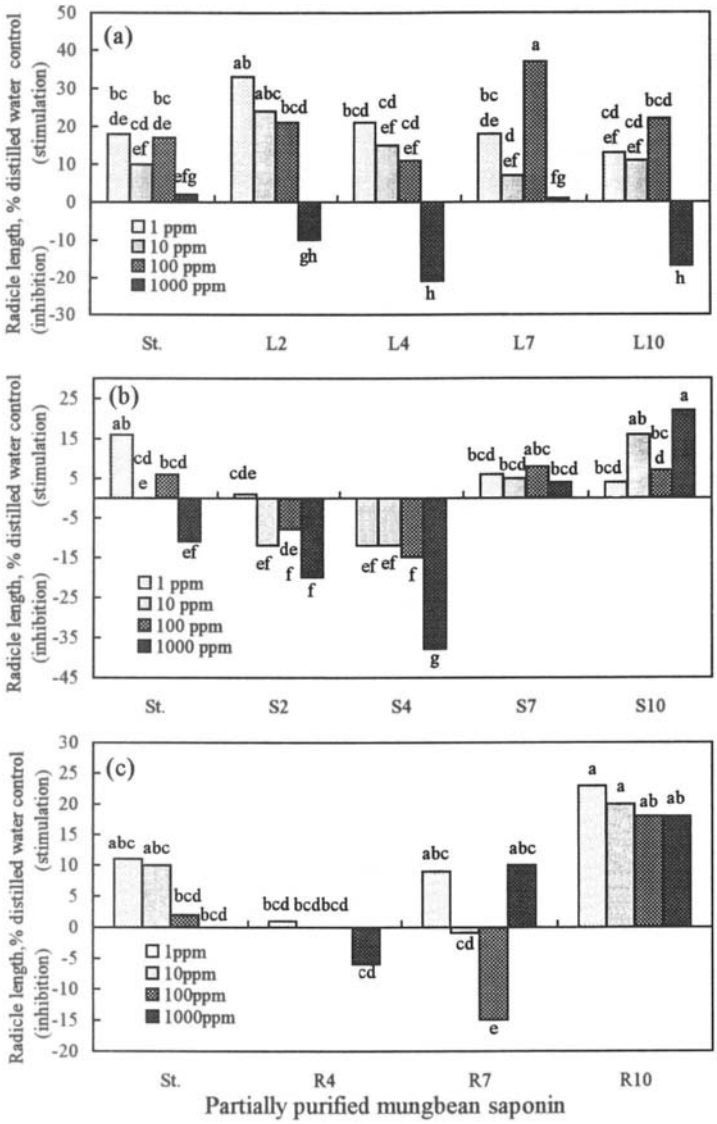

Fig. 13a, b, c. The effects of partially purified saponins extracted from the (a) leaves, (b) stems, and (c) roots of mungbean at 2, 4, 7, and 10 weeks of growth on the growth of lettuce radicle for 72-h bioassay. Bars having different letters are significantly different, P = 0.05, ANOVA, with Duncan's Multiple-Range Test.

paper. The results with mungbeans were mixed, with the leaves showing the most enhancement of growth, and the stem showing inhibition, whereas the root growth was slightly promoted. The most inhibition of growth was found at higher concentrations (i.e., 1000 ppm). The results with lettuce show (Fig. 13a, b, c) that mungbean stem saponins were inhibitory at 2 and 4 weeks, but slightly stimulatory at 7 and 10 weeks; the stem saponin produced stimulatory action at all times during the growth season whereas the roots were slightly inhibitory at all times.

Fig. 14a and b show the effect of pure soyasaponin I on the growth of mungbean and lettuce epicotyl and the radicle, and the difference is striking. In the presence of soyasaponin I most of the growth for lettuce radicle occurs at 1 ppm, less so at 10 and 100, and there is inhibition at 1,000 ppm; for the epicotyl an increase in concentration of soyasaponin I causes an increase in growth. The effect of soyasaponin I on the mungbean is slight enhancement of growth of the radical and slightly inhibitory for the epicotyl.

The evidence is quite clear for lettuce and mungbean that the presence of partially purified saponins is stimulatory. The leaves showed the greatest enhancement of growth, possibly because they contain more bidesmosides. This evidence suggests that there is stimulation of growth and inhibition of growth in the mungbean and lettuce occurring independently. A summary of the results for 2, 4, 7, and 10 weeks is shown in Tables 4 and 5. The biosynthetic pathway leading to the formation of saponins is dependent in part on the growing parts of the plant; monodesmosides probably being formed first and the bidesmosides being derived from the monodesmosides. Just exactly how and when the bidesmosides can serve in a transport form to be present in the mungbean plant at a site of damage, and be enzymatically converted to mondesmoside, as Bissett (25) suggests remains unclear.

A Proposed Mechanism Of Action For Saponins Added Without Soil. Low concentrations of mungbean saponins generally promote the growth of mungbeans and lettuce whereas higher concentrations are inhibitory. Oakenfull (34) suggested that saponins exist in aggregates up to 1x106 molecular weight when in aqueous solution. We would assume that the concentrations being used that are growth enhancing lead to such an aggregated state. A suggested series of events (Fig. 14) which might occur in the seed-plantlet (root hairs)-water interface is proposed for mungbeans: a) some saponin molecules may be bound to the outer coating of the seeds but most molecules will be in a uncombined form, b) as the seed begins to imbibe water some of the saponin molecules become active in transport of water molecules into the plant, i.e., by b processes where there is a loose binding to the surface of the seed throughout an unknown mechanism but which I think will be mediated in part by an enzyme-lipid bilayer-saponin interface, c) as the seed develops the plant hairs become available to additional unreacted molecules of saponins, which react with the lipid bilayer of the seedling and become attached to an enzyme receptor on the surface, thus promoting water going into the plant. Fig. 14d-g shows that the saponin loses sugar molecules by cleavage by enzymes until what remains on the enzyme surface is the aglycone, which is loosely attached and eventually comes off the root hair leaving the enzyme available for another reaction. When inhibitory action is manifested, the saponin molecules are in such a high concentration that they are assembled around the rootlet as clusters or matrixes as high in molecular weight as one million. This prevents water or nutrients from the surrounding soil to be taken up by the root. Although the 72-h bioassay was not sterile, the growth of any microorganism will be minimal;

Table 4. Summary of effects of partly purified mungbean saponins on growth of mungbeans.[1,2]

Field Plant Part	Bioassay Plant Part	Weeks SS-I[3]	2	4	7	10	Growth in 72 h
Roots	Radicle	++		NC	NC	+++	Low inhibition
	Epicotyl	NC		NC	NC	——	Low inhibition
Stems	Radicle	+	—	—	+	++	Low stimulation
	Epicotyl	++	++	++	NC	NC	Medium stimulation
Leaves	Radicle	+	+++	+++	+++	+++	High stimulation
	Epicotyl	++	++	+	+	+	Low stimulation

[1]Relative quantities: No detectable change -NC; Low (+), Medium (++), High (+++) Stimulatory; Low (-), Medium(—), High(——) Inhibitory
[2]In most test there was a significant amount of inhibitory action at 1,000 ppm
[3]Soyasaponin I

Table 5. Summary of effects of partly purified mungbean saponins on growth of lettuce[1,2]

Field Plant Part	Bioassay Plant Part	Weeks SS-I[3]	2	4	7	10	Growth in 72 h
Roots	Radicle	++		NC	NC	+++	Low stimulation
	Epicotyl	NC		NC	NC	——	No change
Stems	Radicle	+	—	—	+	++	Low stimulation
	Epicotyl	++	++	++	NC	NC	Low stimulation
Leaves	Radicle	+	+++	+++	+++	+++	High stimulation
	Epicotyl	++	++	+	+	+	Low stimulation

[1]Relative quantities: No detectable change -NC; Low (+), Medium (++), High (+++) Stimulatory; Low (-), Medium(—), High(——) Inhibitory
[2]In most test there was a significant amount of inhibitory action at 1,000 ppm
[3]Soyasaponin I

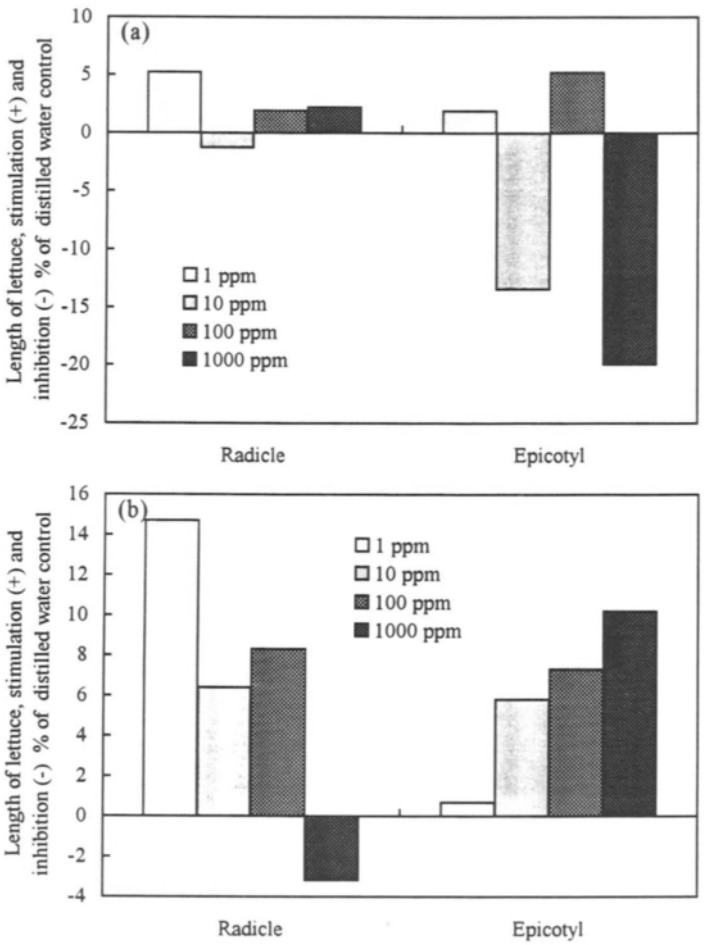

Fig. 14a, b. The effects of soyasaponin I on the radicle and epicotyl growth of (a) mungbean and (b) lettuce in a 72-h bioassay.

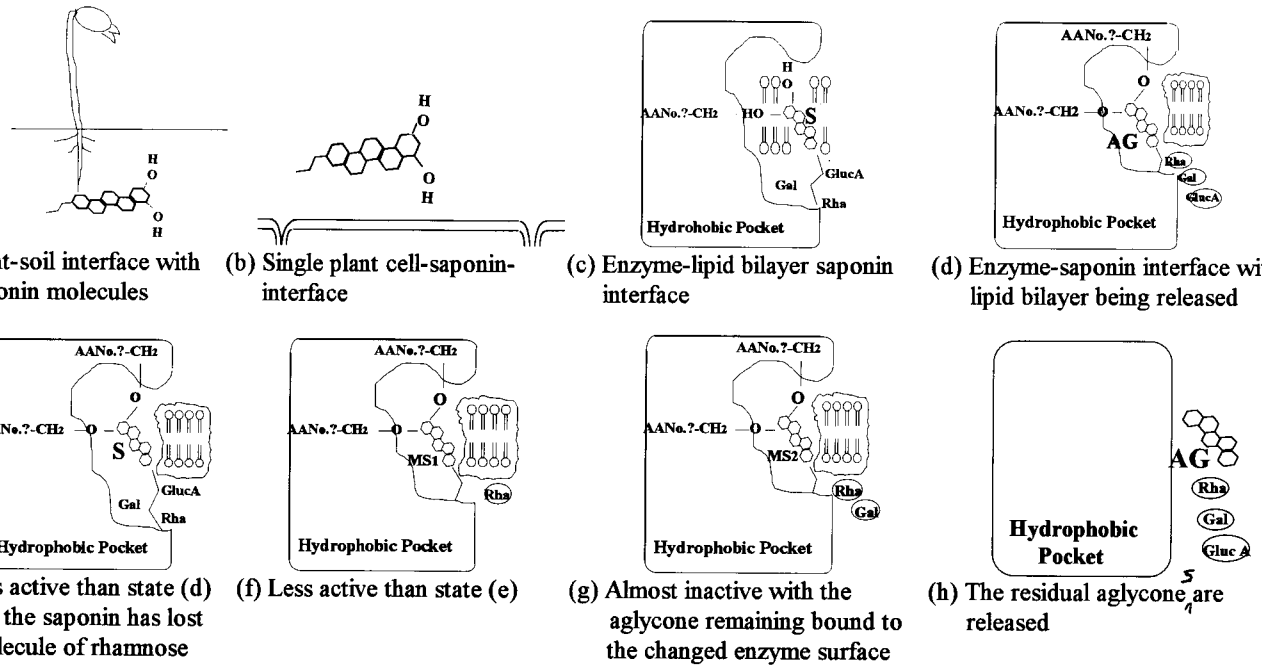

Figure 14. Suggested mechanism of action of mono- and bidesmoside saponins that occur in mungbeans used as an addition to water in the growth of mungbeans and lettuce in a 72-h bioassay. Note: Sugars from soyasaponin I were used as an example; which amino acid number is not known for co-valent binding; S = saponin; MS1 = saponin without one molecule of sugar; MS2 = saponin with loss of two molecules of sugar; AG = aglycone with rhamnose, galactose and glucuronic acid free in the medium.

however, saponins can arrest the growth of bacteria or fungi. What we do not know is whether the external cell concentration of the saponins in the medium may be high enough to cause the aglycone to remain on the active site; however, it seems that too high a concentration would produce inhibition in plant growth because of the blockage of the enzymatic sites by the aglycone; the concentration of saponin that would be required for this passive diffusion to occur; however, remains an open question. We might ask, is the root hair cell resistant to this series of events? The cell is in constant search of water and nutrients. In this scenario it has no source of nutrients other than what was present in the seed itself. As the molecules of saponins are synthesized during the 72-h bioassay they may be present as clusters that migrate freely within the plantlet and some are released to the medium. The saponins may be positioned so that the plant cell-saponin may react with concomitant passage of water (and nutrients). As the root grows it may contact more molecules of saponins, and the same cycle will be repeated so that a growth enhancement may be assured as long as saponins are present in the environment.

Conclusion

Inhibition. The inhibition of growth of mungbeans in pots was found to be 20-25%, and was often less, depending upon temperature, water, and soil characteristics. As the plants developed for 30-45 days, the effect of phytotoxins from the mungbean plant almost disappeared, and the root pathogens grew with pronounced deleterious effect on the mature plants. There is evidence in the literature that Taiwan, the Philippines, and Kenya all have a problem with continuous cropping of mungbeans (Poehlman, 35) that is associated with root pathogens. The present paper recognizes for the first time the role that allelopathy or allelochemicals have in causing damage when mungbeans are planted in the soil in which mungbeans were previously grown.

The inhibitory effect of partitioned extracts of mungbean stems show that the naturally occurring allelopathic phytotoxins were distributed throughout the water, 1-butanol, ethyl ether, and ethyl acetate fractions, which indicates that several types of compounds are represented.

We find that under certain conditions, mungbean plants grown under continuos cropping conditions in subtropical or tropical regions suffer reduction in height and yield from allelochemicals. There are allelopathic phytotoxins from the mungbean plants that are present at all stages of growth. Soil acidity is an important factor; the more alkaline the soil, the more active the metabolism of the microorganism population. Each of these factors, and perhaps others, should be considered when growing mungbeans.

Enhancement. The substantial increase in saponins by the individual mungbean plant through the life cycle is due in part to their allelochemical response. This is the first report of saponins produced during a life cycle. In a 72-h bioassay the mungbean saponins obtained around 5-10% throughout the life cycle showed growth enhancement with the higher activity being shown as the plants matured. These results confirm and extend the results obtained previously on pot growth experiments using mungbean saponins mixed with soil which indicated a 15-25% increase using mungbeans (6-10) Early reference (Heftman, 36 and reference cited therein; Rosenthal and Berenbaum, 37) to the role of various saponins produced by plants indicate that low concentrations promote germination, high concentrations inhibit the growth, and treating the seed has a lasting growth-promoting effect. Bisset (25) states that one reason saponins are advantageous to the plant producing them is that they function as growth

regulators. This research provides the first definitive evidence that when saponins produced by mungbean plants are added to the soil, they enhance the growth of new mungbean plants as an allelochemical plant growth regulator.

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LEGUMINOUS GLYCOSIDES EFFECTIVE FOR HEPATITIS

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From the reason that leguminous plants so widely distribute and are used as foodstuffs and folk medicines, we have focused on these plants and are trying to develop natural medicines after proving the effectiveness of these crude drugs and to find the lead compounds among these natural source. As presented in the preceding ACS Saponin Symposium in Chicago 1995 [1], we reported the oleanene glucuronides in Abri Herba and Puerariae Flos. Here, we descrive the outline of these plant studies and the recent results of our studies on the oleanene glucuronides of Puerariae Radix and Edible beans, and their hepatoprotective activities.

1. Abri Herba, the whole plant of *Abrus cantoniensis* HANCE, has been used as a folk medicine for infectious hepatitis in Hong Kong [2]. Recently, the efficacy of this herb was clinically confirmed [3, 4]. Two crude saponin fractions were prepared by separation. One is less polar than the other. The efficacy of these fractions and the methanolic extract were confirmed towards liver injury induced by CCl_4 (the effect on mice treated with CCl_4). By comparison with CCl_4 control, these saponins were clearly effective for liver injury. Especially, the crude saponin Fr I showed over 70% inhibition [5]. Since the saponin fr. showed activity, we tried to examine their chemical ingredient. At first, we investigated their sapogenols. We found fifteen sapogenols named abrisapogenols along with the known sapogenols [6, 7]. The common features of these sapogenols are that they possess a methyl group at C-17 and some oxygen functions on the E-ring. Furthermore, we have isolated 23 oleanene glycosides (OGs) named abrisaponins [8, 9] as shown in Fig. 1. It was very difficult to isolate these OGs because of a wide variety of its sapogenols. These OGs have glucuronic acid in the *endo*-sugar chain. In the study on the hepatic injury induced by CCl_4 on primary cultured rat hepacytes, a representative OG in the crude saponin Fr I from Abri Herba, that is, kaikasaponin III was much effective than soyasaponin I and glycyrrhizin and showed the antihepatic activity even at 50 μg/mL as shown in Fig. 2 [10]. The sapogenol of kaikasaponin III is sophoradiol, being non-polar sapogenol.

2. Puerariae Flos, the flower of *Puerariae lobata* Ohwi, which is used as a counteract drinking. The methanolic extract of Puerariae Flos was separated into the isoflavonoid, and the crude saponin fraction. The isoflavonoid fr. was composed of kakkalide as major ingredient. The saponin fraction was separated into soyasaponin I, kaikasaponin III and kakkasaponin I [11]. Since Puerariae Flos is used to counteract drinking, we confirmed its effect to the alcoholic metabolism.

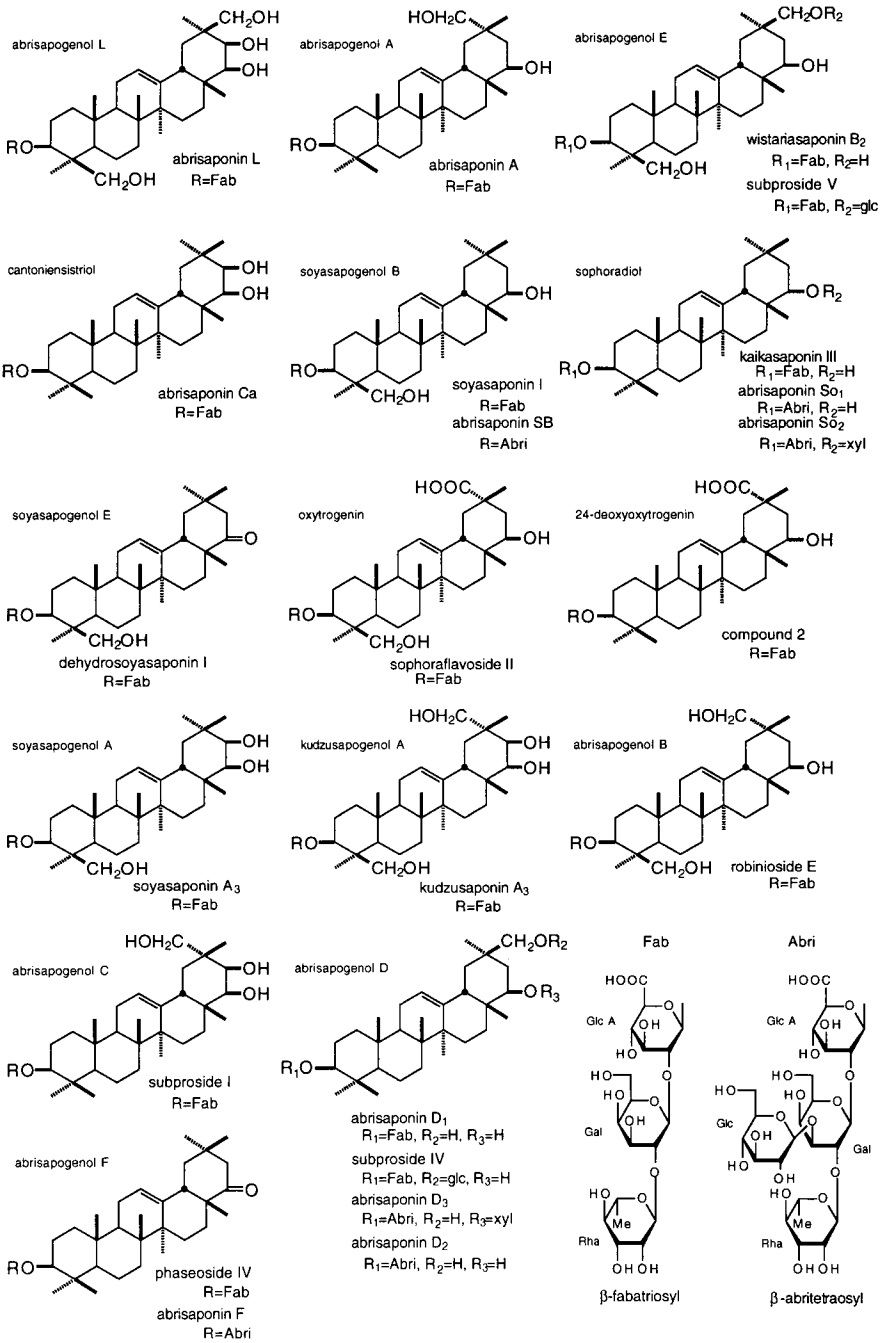


Fig. 1. Oleanene Glycosides obtained from Abri Herba

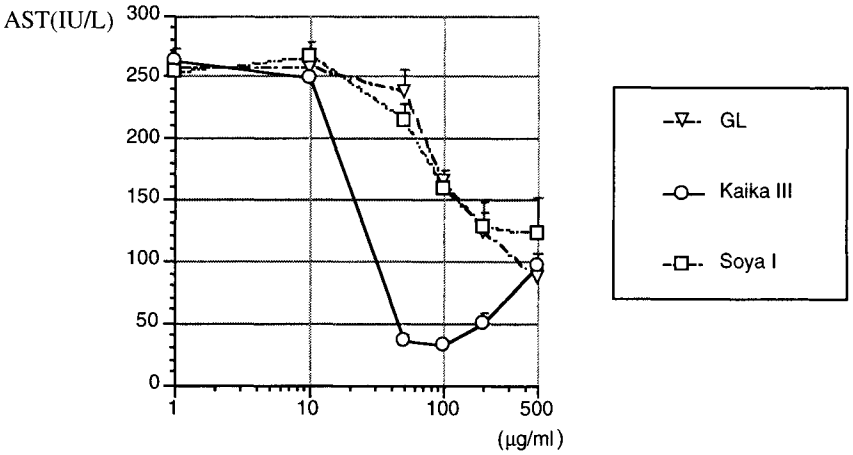


Fig.2. Effects of Glycyrrhizin (GL), Kaikasaponin III (Kaika III) and Soyasaponin I (Soya I) on CCl_4 (5mM)-Induced Cytotoxicity In Primary Cultured Rat Hepatocytes. The data are the mean ± S.D. for 3 independent cell preparations.

Chart 1 illustrates our experimental results of "Pharmacological Effect of Puerariae Flos and Its Constituents" [12, 13]. The MeOH extract of this crude drug depresses various alcohol-induced unusual metabolisms, a decrease in ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase), suppression of spontaneous movement, decrease in BG and TG, and a significant decrease in blood alcohol and acetaldehyde level. After separation of the MeOH extract, the obtained triterpenoidal saponin fr. (PF-SP) shows a decrease in ALT and AST, and TG and

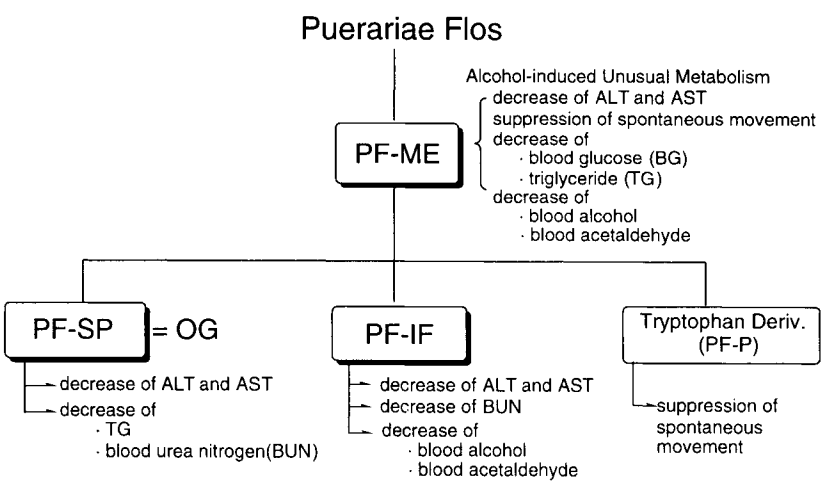


Chart 1. Pharmacological Effects of Puerariae Flos on Alcohol-Induced Unusual Metabolism

BUN. The isoflavonoid fr. (PF-IF) shows a decrease in ALT, AST and BUN, and a significant decrease in the blood alcohol and acetaldehyde level.

3. Pueraiae Radix, the root of *Pueraria lobata* OHWI, is widely used as a specific for antispasmodic, antipyretic and perspiration in the Chinese orthodox medicine, Kampo. Daidzein covers antispasmodic action. However, the substances responsible for the other actions have never been characterized from the crude drug. Now, we can guess that the triterpenoid saponin might be responsible for antipyretic and perspiration actions. We have firstly found the presence of 13 triterpenoidal saponins in Japanese Puerariae lobatae Radix [14, 15]. On the other hand, from Chinese Puerariae Thomsonii Radix, 5 saponins have been obtained [16] as shown in Fig. 3

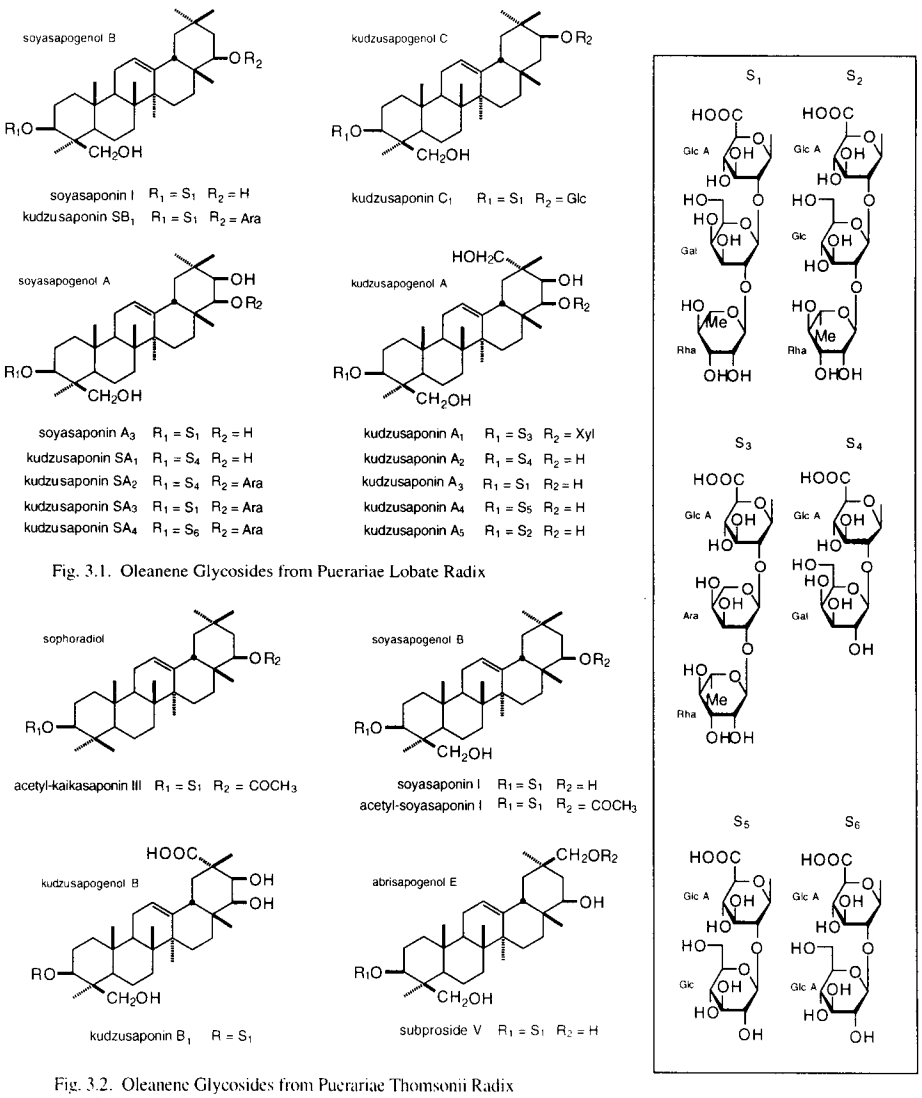


Fig. 3.1. Oleanene Glycosides from Puerariae Lobate Radix

Fig. 3.2. Oleanene Glycosides from Puerariae Thomsonii Radix

In the way of studies on the hepatoprotective drugs, from the reason that the antipyretic and perspiration of Puerariae Radix is related to an inflammatory reaction, we have investigated the

conditions for an *in vitro* assay method using immunological liver injury of rat primary hepatocytes cultures [17]. This method is regarded as one of the most analogous method to the hepatitis. After the preparations of antiserum against the rat liver plasma membranes and the primary cultured rat hepatites, the hepatoprotective activities were quantified by measuring their preventive effects on the release of ALT into culture media of primary cultures of hepatocytes injured with antiserum against the liver plasma membranes.

By using this method, we have measured hepatoprotective activities of crude saponins from Puerariae Lobatae Radix and Puerariae Thomsonii Radix, various soyasapogenol glycosides and kudzusapogenol glycosides isolated from these crude drugs. As results, the crude saponin of Japanese Puerariae Lobatae Radix was much effective than that of Chinese Puerariae Thomsonii Radix and glycyrrhizin of control. Moreover, soyasapogenol glycosides were much stronger than kudzusapogenol glycosides. For example, in case of soyasaponin A_3, it completely depressed the ALT release at 500 μM. Concerning the hepatoprotective activities for saponins obtained from Puerariae Lobatae Radix, some of them are listed in Table 1-6 [18].

Table 1. Hepatoprotective Activity of Crude Saponin from Puerariae Lobatae Radix

Dose (μg / mL)	ALT (IU / L)	Protection (%)
Referecce	155.50 ± 5.9	
10	148.50 ± 3.3 *	5
30	147.75 ± 8.0	5
90	140.00 ± 3.4 *	11
200	63.25 ± 14.6***	65
500	37.25 ± 7.6 ***	84

Reference value was challenged with the antiserum and not treated with Crude Saponin.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective ***$p < 0.001$.

Table 2. Hepatoprotective Activity of Crude Saponin from Puerariae Thomsonii Radix

Dose (μg / mL)	ALT (IU / L)	Protection (%)
Referecce	156.25 ± 2.2	
10	148.25 ± 1.0	6
30	151.25 ± 4.1	4
90	150.00 ± 1.4 *	4
200	138.50 ± 2.4 ***	13
500	107.50 ± 5.6 ***	34

Reference value was challenged with the antiserum and not treated with Crude Saponin.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective *$p < 0.05$, ***$p < 0.001$.

Table 3. Hepatoprotective Activity of Kudzusaponin SA_2

Dose (μM)	ALT (IU / L)	Protection (%)
Referecce	139.75 ± 4.6	
10	130.50 ± 12.4	7
30	138.25 ± 3.8	1
90	118.25 ± 5.4	17
200	37.00 ± 4.8 ***	82
500	22.25 ± 1.9 ***	94

Reference value was challenged with the antiserum and not treated with Kudzusaponin SA_2.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective *** $p < 0.001$.

Table 4. Hepatoprotective Activity of Soyasaponin A_3

Dose (μM)	ALT (IU / L)	Protection (%)
Reference	129.00 ± 2.8	
10	120.75 ± 4.3	7
30	125.75 ± 2.6	3
90	83.00 ± 6.9 ***	40
200	29.00 ± 3.5 ***	87
500	14.50 ± 1.3 ***	100

Reference value was challenged with the antiserum and not treated with Soyasaponin A_3.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective *** $p < 0.001$.

Table 5. Hepatoprotective Activity of Kudzusaponin A_2

Dose (μM)	ALT (IU / L)	Protection (%)
Referecce	132.25 ± 1.3	
10	126.75 ± 3.8	5
30	126.75 ± 5.0	5
90	129.00 ± 7.1	3
200	124.25 ± 3.3	7
500	105.50 ± 1.0 ***	23

Reference value was challenged with the antiserum and not treated with Kudzusaponin A_2.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective ***$p < 0.001$.

Table 6. Hepatoprotective Activity of Kudzusaponin A_3

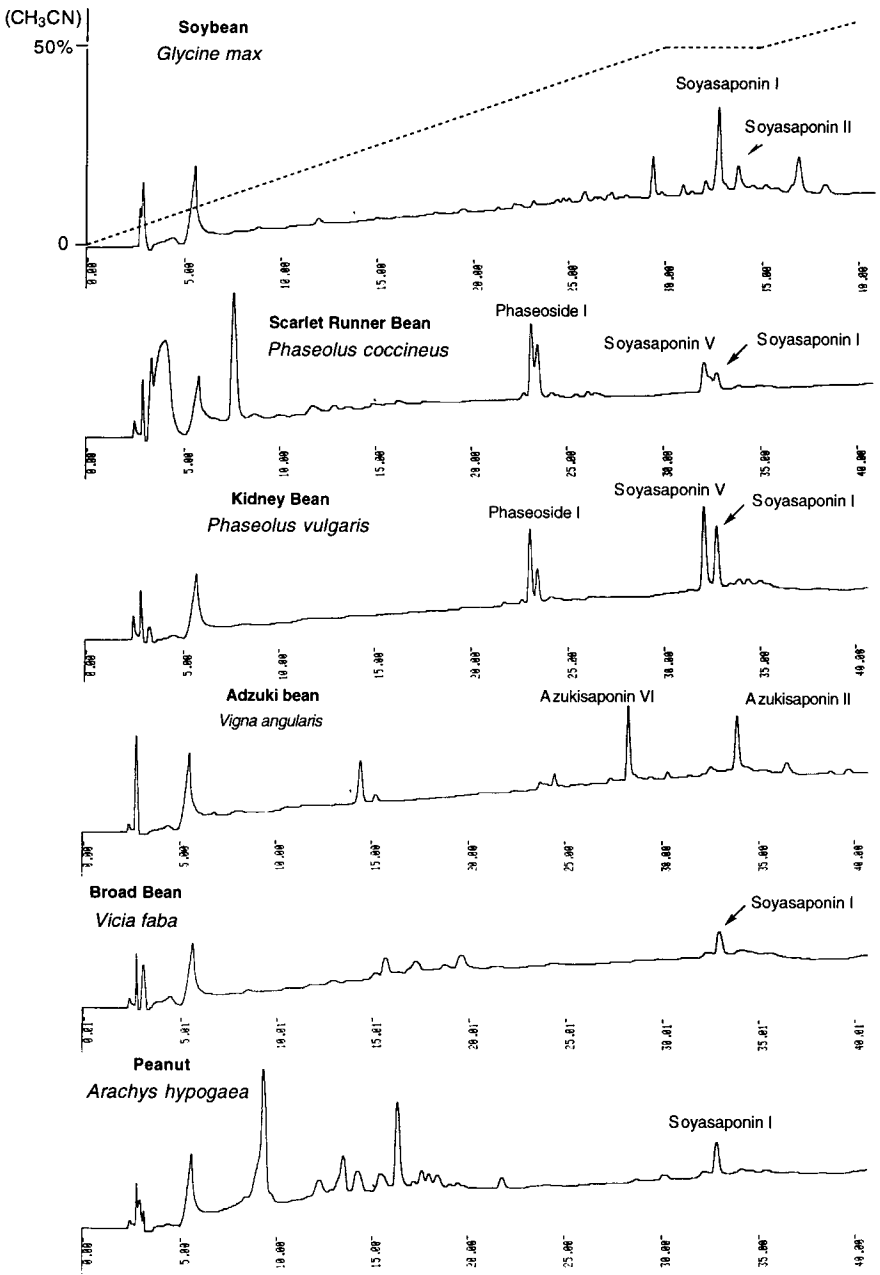
Dose (μM)	ALT (IU / L)	Protection (%)
Referecce	129.00 ± 2.4	
10	124.00 ± 2.0	4
30	123.50 ± 2.1	5
90	123.75 ± 2.5	5
200	115.75 ± 1.0 **	12
500	101.50 ± 5.8 ***	24

Reference value was challenged with the antiserum and not treated with Kudzusaponin A_3.
The percent of protection is calculated as {1-(Sample-Control) / (Reference-Control)} × 100.
Significantly different from Reference, effective $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Edible Beans With regard to saponins of the most important soybean, Kitagawa's chemical studies are well known [19]. In addition, various pharmacological actions were reported about soybean [20-24]. We have examined several beans as listed Table 7, that is, they are kidney bean, haricot bean, cowpea, azuki bean and so on and listed yields of crude oleanene triterpene glucuronides [25]. The HPLC profiles of oleanene glucuronides in some edible beans are shown in Fig. 4. Almost of beans include soyasaponin I.

Table 7. Yields of MeOH Extracts and Crude Oleanene Glucuronides (OG) in Some Edible Beans

Bean's Name	Japanese Name	English Name	Botanical Name	Amounts (g)		
				Seeds	MeOH ext.	Crude OG
Torosuku	Ingenmame	Kidney Bean Haricot Bean	*Phaseolus vulgaris cv. Torosuku*	100	3.3	0.20
Toramame			*P. vulgaris cv. Toramame*	100	3.9	0.17
Taishokintoki			*P. vulgaris cv. Kintokimame*	100	3.9	0.15
Ooshirobanamame	Benibanaingen	Scarlet Runner Bean	*P. coccineus cv. Ooshirobanamame*	100	5.1	0.17
Murasakihanamame			*P. coccineus cv. Murasakihanamame*	100	4.6	0.19
Kuromame	Daizu	Soybean	*Glycine max cv. Kuromame*	100	6.5	0.28
Daizu			*G. max*	100	5.8	0.20
Chuguro	Sasage	Cowpea	*Vigna unguiculata cv. Chuguro*	100	3.0	0.12
Dainagon	Azuki	Azuki Bean	*Vigna angularis cv. Dainagon*	100	2.1	0.22
Green-Peace	Endou	Garden Pea	*Pisum sativum*	71	2.5	0.04
Rakkasei	Rakkasei	Peanut	*Arachis hipogaea*	100	4.5	0.10
Soramame	Soramame	Broad Bean	*Vicia taba*	100	3.4	0.05



Column: Nova-Pak C_{18} (4μm, 8 x 100mm) with Radial-Pak RCM 8 x 100 module
Solvent A : H_2O/TFA=100:0.05 (v/v); Solvent B : CH_3CN/H_2O/TFA=60:40:0.05 (v/v)
Flow Rate : 1mL/min, Detection : UV (205nm)

Fig. 4. HPLC Profiles of OGs in Some Edible Beans

The chemical structures of oleanene glucuronides included in several edible beans is shown in Fig 5.

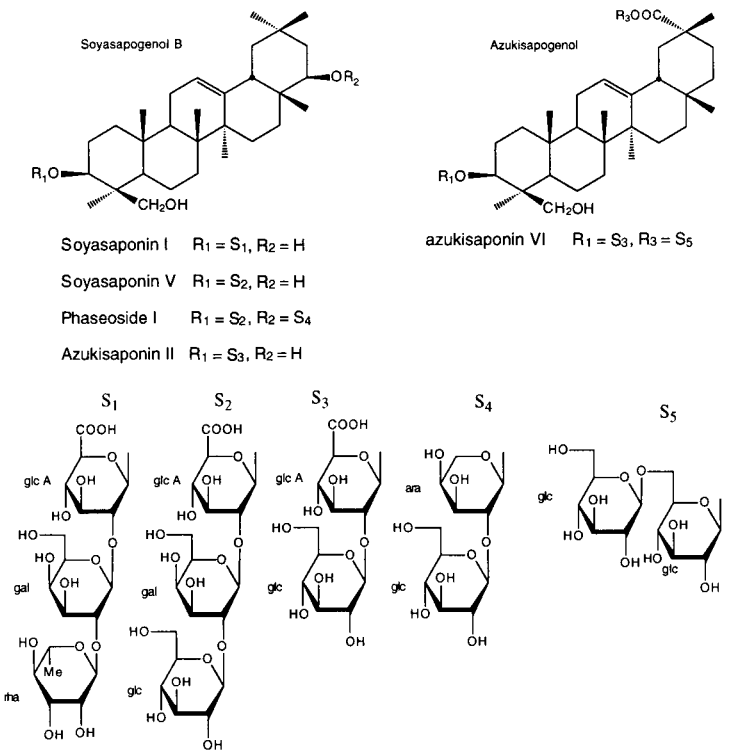


Fig. 5. Structures of OGs from Several Edible Beans

Next, the anti-hepatotoxic activity of crude oleanene glucuronides from some edible beans are listed in this Table 8. In soyasapogenol glycosides, biosyl glycosides are prominent at activity

Table 8. Hepatoprotective Activity of Crude OGs and Pure OGs from Some Edible Beans

	Concentration	*n*	**Protection (%)**
GL (Glycyrrhizin)	5×10^{-4} M	4	**37%**
Crude OG (Soybean)	5×10^{-4} M	4	**24%**
Crude OG (Adzuki bean)	5×10^{-4} M	4	**34%**
Crude OG (Kidney bean)	5×10^{-4} M	4	**27%**
Crude OG (Scarlet Runner Bean)	5×10^{-4} M	4	**10%**
Soyasaponin I	5×10^{-4} M	4	**40%**
Soyasaponin V	5×10^{-4} M	4	**22%**
Phaseoside I	5×10^{-4} M	4	**8%**
Azukisaponin IV	5×10^{-4} M	4	**16%**

among these saponins [26].

5. Other Plants As stated in Puerariae Radix, we have examined the hepatoprotective activity of soyasaponins I-IV obtained from wild soya, namely, *Glycine soya* [27].

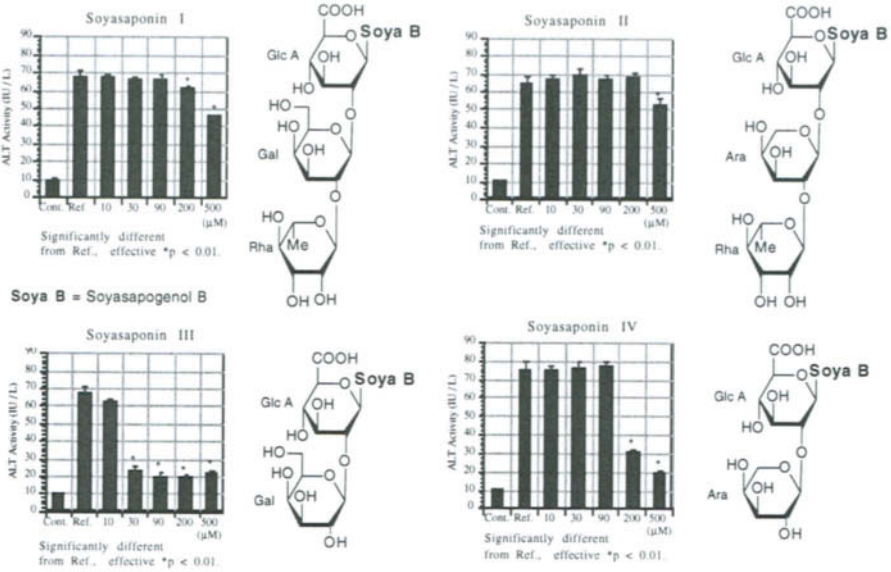


Fig. 6. Hepatoprotective Activity of Soyasaponins I ~ IV from *Glycine soya*

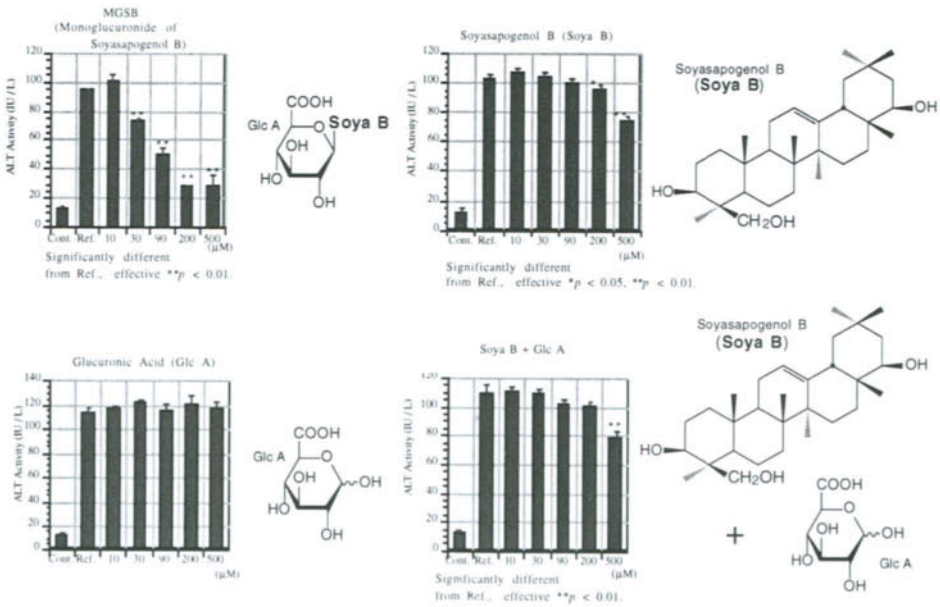


Fig. 7. Hepatoprotective Activity of Soyasapogenol B Analogs

Fig. 6 and 7 show the activities correlated to soyasapogenol B, its glycosidic derivatives and

glucuronic acid. Only with soyasapogenol B or glucuronic acid or a mixture of both were no effective, while soyasapogenol B monoglucuronic acid was very effctive.

6. Trans-Glycosylation Through the above-mentioned hepatoprotective activity of leguminous oleanene glucuronides, we have recognized that the sugar moiety apparently plays an important role for activity, so that, we tried to transport a functional sugar moiety to the other aglycone. Julibrosides [28, 29], which has been isolated from *Albizzia juribrissin* belonging Caesalpinioideae, a subfamily of Leguminosae possessed cytotoxic activity, however, when the ester sugar linkage at C-28 was taken off, it turned to be no activity. Therefore, it was supposed that this sugar moiety possessed any interaction for activity. So that, we cut off this sugar moiety which was derived into the imidate [30]. The imidate was subjected to the Schmidt method [31] to give a diosgenin glycosides in β-form (Chart 2), which showed a cytotoxic activity although it was not so strong. On the other hand, the fabatriosyl moiety regarded as a functional sugar linkage having the antihepatotoxic activity was taken up with aid of the glycyrrhinic acid hydrolase and transported into diosgenin to yield a new spirostanol fabatrioside (Chart 3). This diosgenin fabatrioside showed a hepatoprotective activity (Fig. 8), therefore, the functions of the respective sugar moieties were verified.

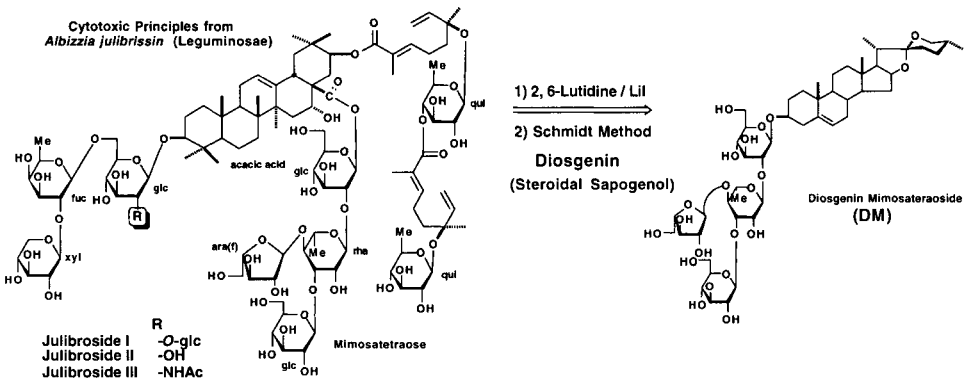


Chart 2. Trans-Glycosylation of Mimosatetraosyl Moiety

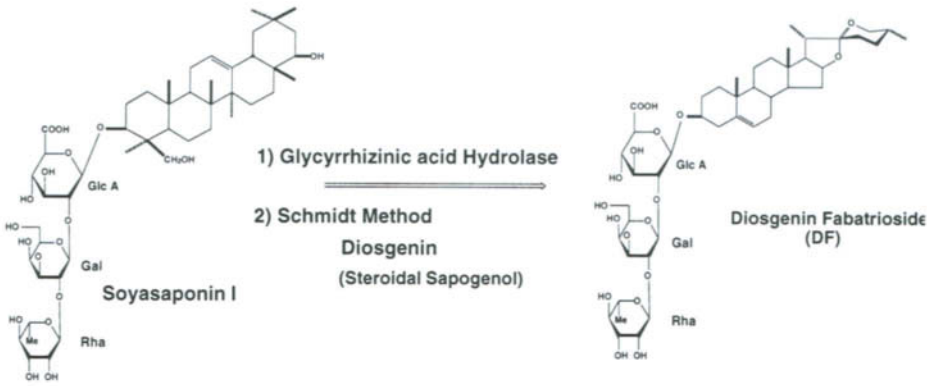


Chart 3. Trans-Glycosylation of Fabatriosyl Moiety

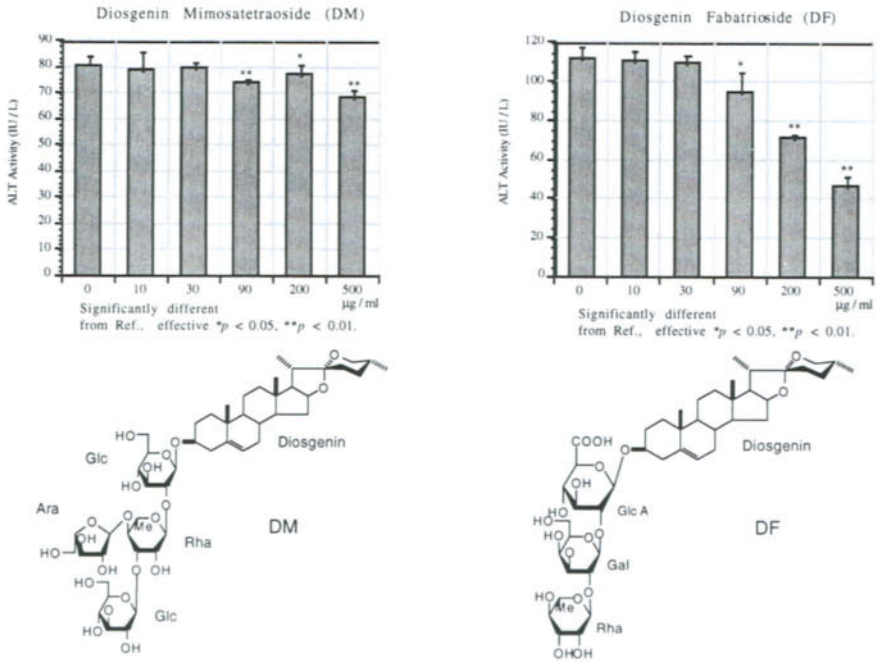


Fig. 8. Hepatoprotective Activity of Trans-Glycosylation Products

Summary

We have so far studied on the oleanene glucuronides in 24 species of Leguminous plants, isolated total 150 glycosides, and found new 21 sapogenols (Table 9). In conclusion, we would like to emphasize that the typical oleanene glycosides with methyl group at C-28 and having the fabatriosyl moiety are occurred in the Faboideae of Leguminous plants and they showed strong anti-hepatitis (Fig. 9).

Table 9. Isolated Oleanene Glycosides in Our Lab.

Plants	Parts	Total OGs	New Aglycones	New OGs
Abrus cantoniensis	whole	23	10	11
Abrus precatorius	seed	1	1	0
Aeschynomene indica	whole	1	0	0
Astragalus complanatus	seed	6	1	4
Astragalus sinicus	seed	6	0	1
Campylotropis hirtella	root	2	0	0
Canavalia gladiata	root	4	0	2
Crotalaria albida	whole	6	0	2
Dalbergia hupeana	bark	3	2	2
Desmodium styracifolium	whole	2	0	0
Melilotus officinalis	root	4	0	1
Medicago polymorpha	aerial	1	0	0
Mucuna sempervirens	leaf	2	0	1
Lathyrus palustris	aerial	5	0	2
Lupinus polyphyllus hybrid	root	7	0	5
〃	seed	4	0	0
Pachyrhizus erosus	tuber	2	0	0
Phaseolus coccineus	seed	1	0	0
〃	aerial	2	0	0
Phaseolus vulgaris (Uzura)	seed	2	0	0
Phaseolus vulgaris (Kintoki)	seed	1	0	1
Phaseolus vulgaris (Tora)	aerial	2	0	0
〃	root	3	0	1
Pueraria lobata	flower	3	0	1
〃	root	13	3	10
Pueraria thomsonii	root	5	0	2
Robinia pseudo-acacia	bark	13	0	10
Sophora subprostrata	root	11	4	7
Sophora flavescens	root	4	0	3
Vigna unguiculata	seed	2	0	0
Wisteria brachybotrys	bark	9	0	5
Total **24**	**31**	**150**	**21**	**71**

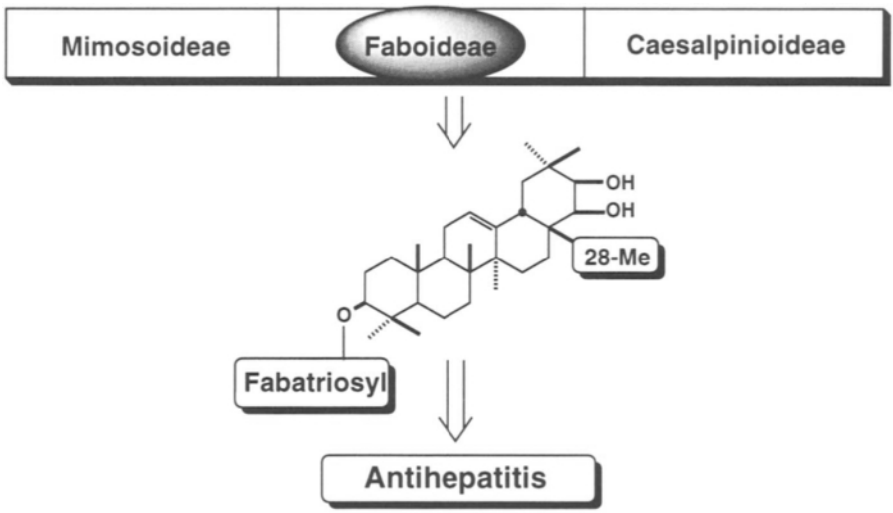


Fig. 9. Oleanene Glucuronides (OGs) in Leguminous Plants

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GLYCOSIDES FROM KU-DING-CHA, A KIND OF CHINESE TRADITIONAL TEA

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Introduction

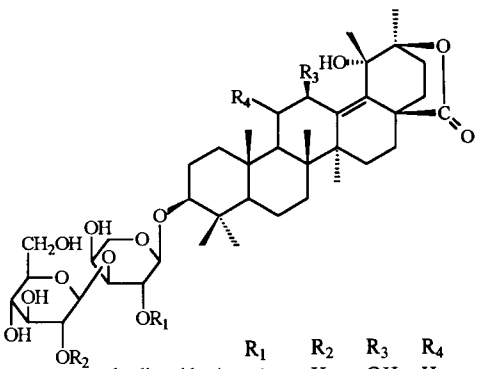
As a kind of special tea, Ku-Ding-Cha has been used in the southern China from many hundred years ago. Instead of tea, local people always drink Ku-Ding-Cha in the summer for clearing away heat and toxic materials in body, and satisfy one's thisrt. It is also used in folk medicine as a diuretic and slimming agent, and for the treatment of hypertension, sore throat as well as inflammation. As for its original materials, there are more than ten plants from different families and genera being used (Table.1) in different places of China. In Yunnan, Sichuan and Guizhou provinces, the leaves of *Ligustrum* (Oleaceae) species are mainly used as Ku-Ding-Cha, while in Guangxi, Guangdong, Hunan and Jiangxi provinces, the leaves of *Ilex* (Aquifoliaceae) species are commonly used[1]. As a part of studies on plant glycosides and Chinese tea plants in our research group, we have paid attention to these interesting plants from several years ago. This is a brief review for our chemical and pharmaceutical works on these plants.

Table 1 The original plants of Ku-Ding-Cha in different areas of China

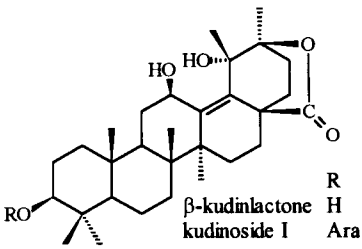
Original plants	Family	Place
Ligustrum pedunculare Rehd.	Oleaceae	Sichuan
L. purpurasceas Y.C.Yang	Oleaceae	Yunnan
L. japonicum var. *pubescens* Koidz.	Oleaceae	Guizhou
L. robustum (Roxb.) Bl.	Oleaceae	Guizhou
Ilex cornuta Lindl. ex Paxt.	Aquifoliaceae	Zhejiang
I. kudincha C.J.Tseng	Aquifoliaceae	Guangxi, Guangdong
I. macrophylla	Aquifoliaceae	Guangdong
I. latifolia Thunb.	Aquifoliaceae	Zhejiang, Hunan
Cratoxylum prunifolium (Kurz) Dyer	Guttiferae	Guangxi
Ehretia thyrsiflora (S.et Z.) Nakai	Boraginaceae	Guangxi
Photinia serrulata Lindl.	Rosaceae	Zhejiang

Glycosides from *I. kudincha*

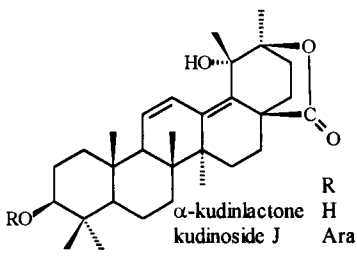
I. kudincha C.J.Tseng is a new species endemically distributed in Guangxi province and commonly used as a material of Ku-Ding-Cha in the locals. After suspended in water, the 70% EtOH extracts of the dried leaves were extracted with $CHCl_3$, EtOAc and n-BuOH, respectively. Further repeatedly separation by column chromatography and HPLC led to the isolation and purification of a lot of triterpenoidal saponins and sapogenins from each fractions. Among them, sixteen new saponins which named as kudinosides A-G, K-Q, S and T, together with zigu-glucoside I were isolated from n-BuOH fraction; two new saponins, kudinosides I and J, were obtained from EtOAc fraction; and from $CHCl_3$ fraction, α- and β-kudinlactones as two new free lactonic aglycones were afforded[3,4]. Kudinosides A-F, I and J are monodesmosides, which aglycones contain a six-member lactone ring between C-20 and C-28. Kudinosides G, K, L-Q, S and T, as well as zigu-glucoside I are bisdesmosides, which have two sugar chains attached at the hydroxyl group of C-3 positions and C-28 carboxyl group of the aglycones, respectively.



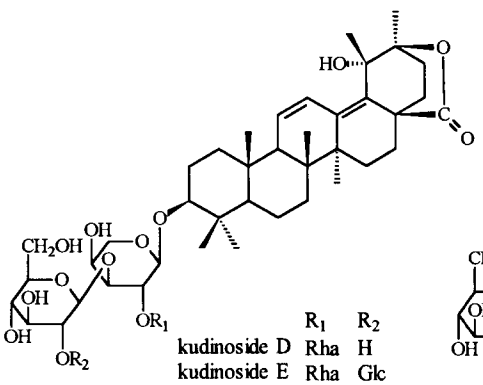
	R_1	R_2	R_3	R_4
kudinoside A	Rha	H	OH	H
kudinoside B	H	Glc	OH	H
kudinoside C	Rha	Glc	OH	H
kudinoside F	Rha	H	H	OH



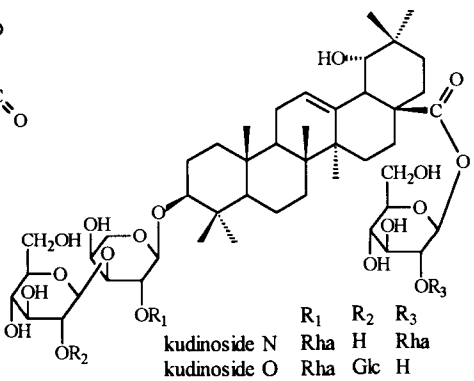
	R
β-kudinlactone	H
kudinoside I	Ara



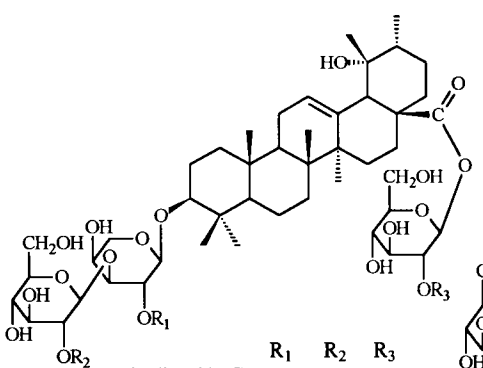
	R
α-kudinlactone	H
kudinoside J	Ara



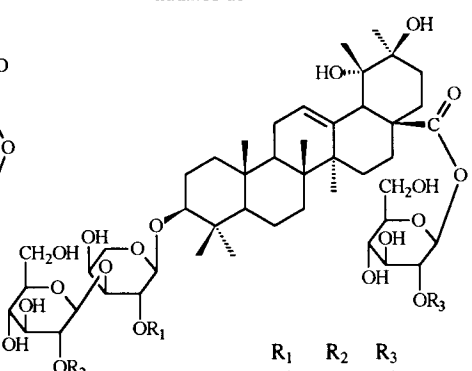
	R_1	R_2
kudinoside D	Rha	H
kudinoside E	Rha	Glc



	R_1	R_2	R_3
kudinoside N	Rha	H	Rha
kudinoside O	Rha	Glc	H
kudinoside P	Rha	Glc	Rha



	R_1	R_2	R_3
kudinoside G	Rha	H	H
kudinoside K	Rha	Glc	H
kudinoside L	Rha	H	Rha
kudinoside M	Rha	Glc	Rha



	R_1	R_2	R_3
kudinoside Q	Rha	H	Rha
kudinoside S	Rha	Glc	H
kudinoside T	Rha	Glc	Rha

The structures of all saponins are elucidated by chemical and spectral methods. For determine the interglycosyl positions, 2D NMR experiments were used. For example, the

NOESY experiment of kudinoside C showed characteristic cross peaks between the anomeric proton (δ 4.76) of internal α-L-arabinopyranosyl unit and both of the aglycone C-3 proton (δ 3.30) and anomeric proton (δ 6.40) of α-L-rhamnopyranosyl unit, between the anomeric proton of β-D-glucopyranosyl unit (δ 5.13) and both of the α-L-arabinopyraosyl H-3 (δ 4.20) and the anomeric proton (δ 5.24) of terminal β-D-glucopyranosyl unit. These data provided an effective evidence to determine the structure of sugar chain of kudinoside C. The sugar moiety of this saponin to be elucidated as -O-β-D-glucopyranosyl(1-2)-β-D-glucopyranosyl(1-3)-[α-L-rhamnopyranosyl (1-2)]-α-L-arabinopyranosyl.

The formation of the triterpenoidal lactonic cycle may be caused by the oxidation of α-amyrin skeleton(Fig.1). It was interesting that each biosynthetic intermediate aglycone has been provided in a glycosidic form from this plant by our isolation. The key intermediate aglycone might have a β-hydroxyl group on C-20 position and belonging to kudinoside Q, S and T. It might be significant that each oxidative step of α-amyrin skeleton can be represented by these aglycones, and possess an important role on the biosynthesis of triterpenoidal skeleton.

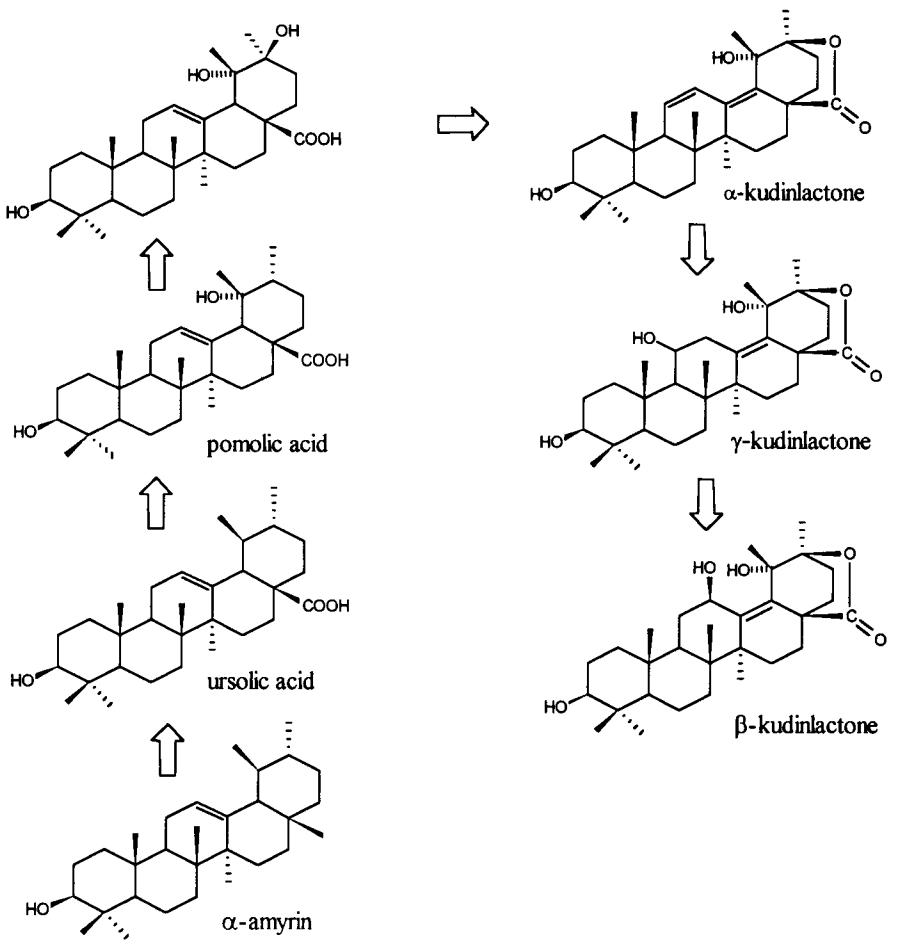
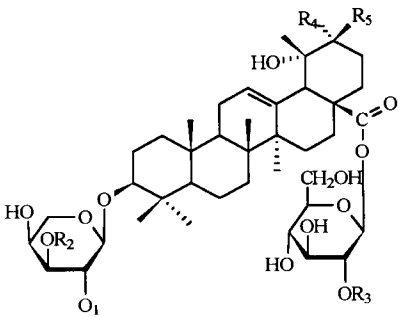


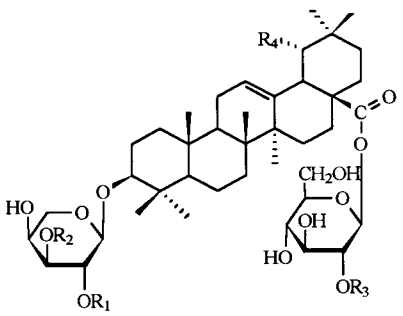
Fig. 1 Biosynthesis pathway of triterpenoid aglycone

Glycosides from *I. latifolia*

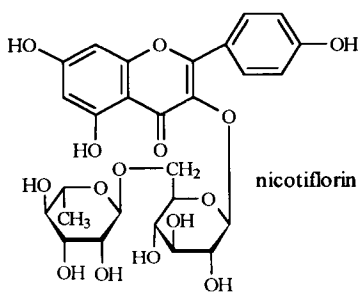
In Hunan and Zhejiang Provinces, the leaves of *I. latifolia* Thunb. are commonly used as the materials of Ku-Ding-Cha. The butanol soluble fraction of the methanol extract of its leaves was repeatedly chromatographed on silica gel column and preparative HPLC to afford sixteen new triterpenoidal saponins, latifolosides A-P, together with two known saponins, kudinosides A and G, and an ionone glycoside, cis-roseoside, as well as a flavonoid glycoside, nicotiflorin[5].



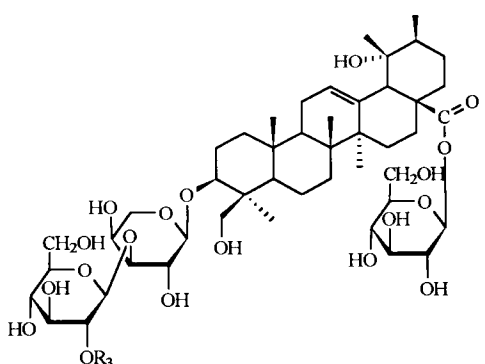
	R_1	R_2	R_3	R_4	R_5
latifoloside A	Rha	H	H	CH_3	H
latifoloside D	Rha	H	H	H	CH_3
latifoloside E	Rha	Glc	H	H	CH_3
latifoloside F	Rha	Glc	Rha	H	CH_3
latifoloside L	H	Glc	H	H	CH_3
latifoloside G	Rha	Glc	Rha	CH_3	H
latifoloside I	H	Glc	H	CH_3	H
latifoloside J	H	Glc	Rha	H	CH_3



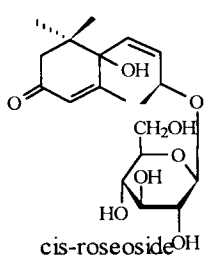
	R_1	R_2	R_3	R_4
latifoloside B	Rha	H	H	OH
latifoloside C	Rha	Glc	H	OH
latifoloside H	Rha	Glc	Rha	OH
latifoloside N	Rha	Glc	H	H
latifoloside O	Rha	H	Rha	H



nicotiflorin



latifoloside K



cis-roseoside

Except kudinoside A, all of these saponins are bisdesmosides and the sugar moieties attached at the both positions of C-3 and C-28 of aglycones, which assignated by glycosylation shift effects of ^{13}C NMR spectra. It is noticed that latifoloside A, D and B; kudinoside G, latifoloside E and C; latifoloside G, F and H are three isomer groups respectively, which aglycones are pomolic acid, ilexgenin B and siaresinolic acid, respectively, and different only on the location of a methyl group in E ring.

The negative ion FAB-MS spectrum of latifoloside F give a quasi molecular ion peak at m/z 1219[M($C_{59}H_{96}O_{26}$)-H]$^-$ and typical fragment ions at m/z 1073[M-146]$^-$, 911[M-146-162]$^-$ and 765[M-2x146-162]$^-$. On acid hydrolysis with 5% H_2SO_4, it afforded glucose, rhamnose and arabinose on the ratio of 2:2:1, and the aglycone which was determined as ilexgenin B. The sugar sequence and interglycosyl linkage positions were achieved by HMBC experiment (Fig.2). The correlative signals between aglycone C-28 and a β-D-glucopyranosyl anomeric proton, between this β-D-glucopyranosyl unit C-2 and an α-L-rhamnopyranosyl anomeric proton indicated that a terminal rhamnosyl unit is linked at the C-2 position of an inner glucosyl unit which is linked at the carboxyl group of C-28 of ilexgenin B. Moreover, correlative signals between aglycone H-3 and an α-L-arabinopyranosyl anomeric carbon, between this arabinosyl C-2 and an α-L-rhamnopyranosyl anomeric proton, between a β-D-glucopyranosyl anomeric proton and the same arabinosyl C-3 indicated that a rhamnosyl and a glucosyl unit located on the terminal position and linked at the inner arabinosyl C-2 and C-3 position, respectively.

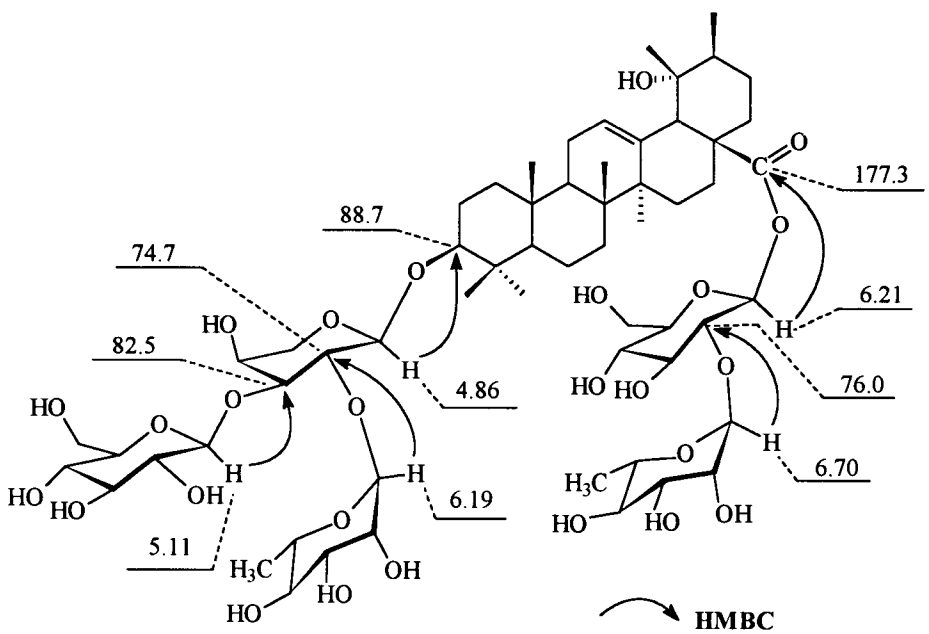


Fig. 2. HMBC of latifoloside F

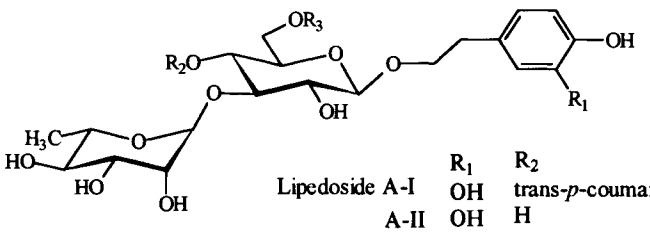
Glycosides from *L. pedunculare*

In Sichuan and Hubei province, *L. pedunculare* Rehd., a oleaceous plant, is commonly used as the material of Ku-Ding-Cha. The water soluble fraction of the methanol extract of its

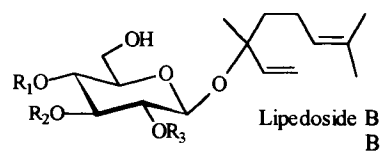
dried leaves was fractionated and purified by Diaion HP-20, silica gel and Rp-18 column chromatographies. Two new phenylethanoid glycosides, lipedosides A-I and A-II as well as six new monoterpenoid glycosides, lipedosides B-I to B-VI together with known osmanthuside B, anatolioside and linalool were isolated[2].

Among these glycosides, lipedosides A-I, A-II, B-II, B-III, B-V and B-VI contain a *p*-coumaroyl group linked at the sugar moiety. Usually, the *p*-coumaroyl group is present as a mixture of *cis*- and *trans*-isomers in plants, which led to the difficulty of purifying each isomer. By using a specific separate procedure which keep solvent in low temperature, the pure forms of each isomer were obtained. Lipedosides A-I, A-II, B-III and B-V are *trans*-forms, while, B-II and B-VI are *cis*-forms.

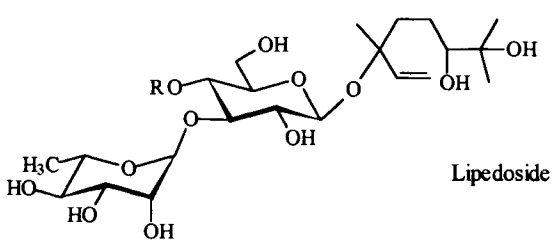
The aglycones of all the monoterpenoid glycosides isolated from this plant are the common constituents of essential oil in plant kingdom. All of sugar moiety of these glycosides linked at an asymmetric carbon atom of aglycone's C-3 hydroxyl group. Through a enzymatic hydrolysis, lipedoside B-I gave (R) and (S) mixture of linalool (R:S=13:87, detected by GC). Moreover, both of B-II and B-III were transformed to B-I after alkaline hydrolysis(Fig.3). Therefore, these monoterpenoid glycosides could be characterized as a mixture of 3(R) and 3(S) forms.



	R_1	R_2	R_3
Lipedoside A-I	OH	trans-*p*-coumaroyl	H
A-II	OH	H	trans-*p*-coumaroyl
Osmanthuside B	H	trans-*p*-coumaroyl	H



	R_1	R_2	R_3
Lipedoside B-I	H	Rha	H
B-II	cis-*p*-coumaroyl	Rha	H
B-III	trans-*p*-coumaroyl	Rha	H
Anatolioside	H	H	Rha



	R
Lipedoside B-IV	H
B-V	cis-*p*-coumaroyl
B-VI	trans-*p*-coumaroyl

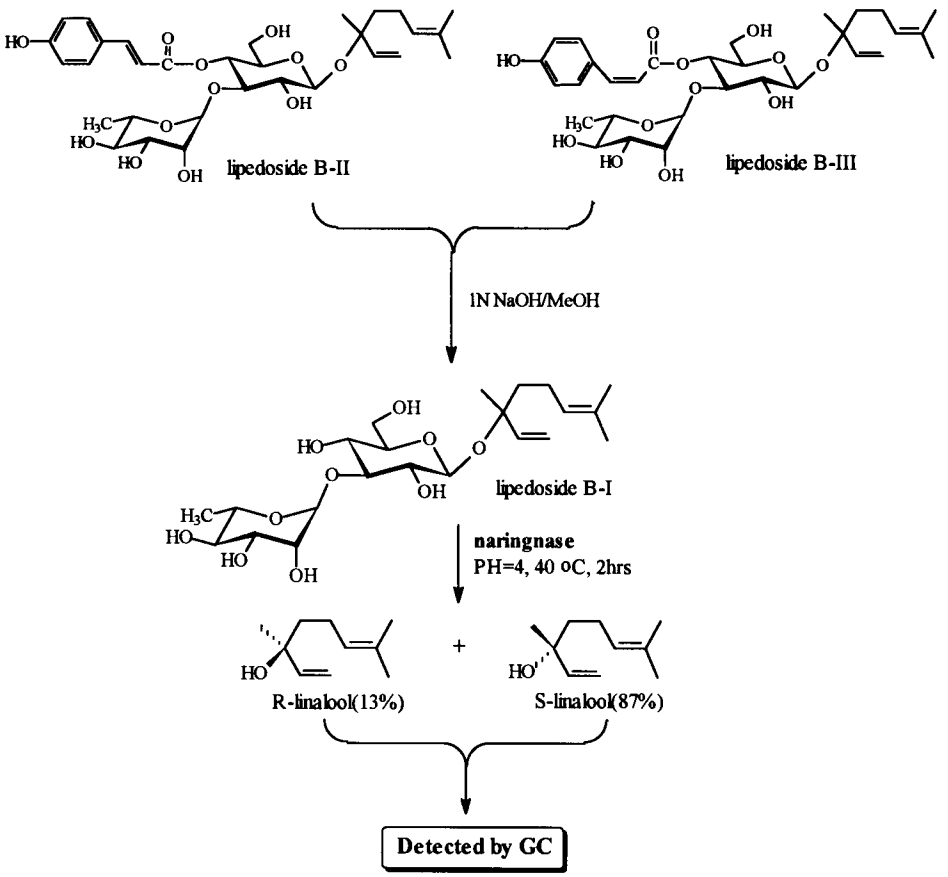
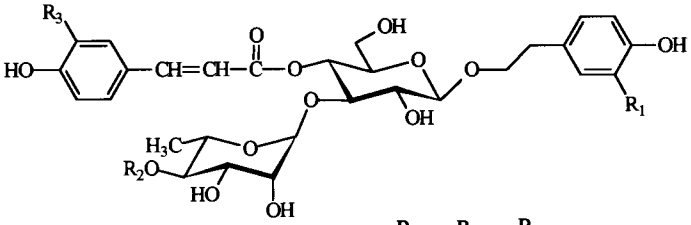


Fig. 3 Hydrolysis of isomers and determining (R,S) linalool

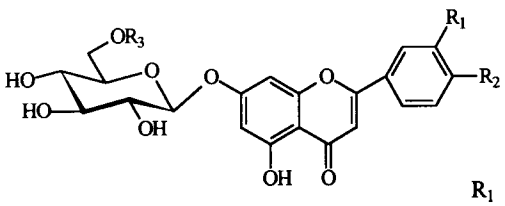
Glycosides from *L. purpurescens*

The leaves of *L. purpurescens* Y.C.Yang as the materials of Ku-Ding-Cha are only used in the northeast of Yunnan province. Two new phenylpropanoid glycosides, ligupurpuroside A and B together with the Z/E isomer mixture of 2-(3,4-hydroxyphenyl) ethyl (3-O-α-L-rhamnopyranosyl)(4-O-coumaroyl)-O-β-D-glucopyranoside(glycoside A), the mixture of osmanthuside B and its *cis* isomer (glycoside B), and acteoside were isolated from the methanol extract of its dried leaves. Three known flavonoid glycosides, luteolin-7-O-glucoside, cosmosiin and rhoifolin were also obtained from this plant[1].

It is noticed that the content of acteoside in this plant is very high by HPLC analysis and all of the flavonoid glycosides are isolated from the genus *Ligustrum* for the first time.



	R_1	R_2	R_3
Acteoside (E)	OH	H	OH
Ligupurpuroside A (E)	OH	Rha	OH
Ligupurpuroside B (E)	H	Rha	H
glycoside A (Z/E)	OH	H	H
glycoside B (Z/E)	H	H	H



	R_1	R_2	R_3
Luteolin-7-glucoside	OH	OH	H
Cosmosiin	H	OH	H
Rhoifolin	H	OH	Rha

Biological Activties

The diversity of the original materials of Ku-Ding-Cha and the structures of their glycosides provided an opportunity for search new bioactive lead compounds from natural resources. It is well known that free radical induced lipid peroxidation damage as a critical initiating event leading to cell injures[6]. The lipid peroxidative reaction may disturb the fine structure of the cells and may thus effect the permeability and function of the membranes. These processes may cause irreversible damage of the cells and may initiate and/or promote the pathogenesis of certain disease[7-8]. Therefore, it has important significance to find effective antioxidants and free radical scavengers for preventing and treating diseases.

According to a primary screening test, the anti-PAF activity of some triterpenoid saponins from *Ilex* and the antioxidant activity of phenylethanoid glycosides from *Ligustrum* had been found. For further evaluate against lipid peroxidative activity of acteoside, ligupurpuroside A and B, we successfully induced the lipid peroxidation of the rat liver microsomes with Fe^{2+}-cysteine and the generating system of oxygen free radicals, as well as LDL oxidation by the electron supporter Cu^{2+}.

The effect of phenylethanoid glycosides on the rat liver microsomal lipid peroxidation

The results indicated that acteoside, ligupurpuroside A and B dose-dependently inhibit MDA formation induced by Fe^{2+}-cysteine. The inhibitory rate(%) of acteoside and ligupurpuroside B is close to Vitamin E, and better than ligupurpuroside A.(Table 2)

Table 2. The inhibitory effect of three phenylethanoid glycosides on rat liver microsome lipid peroxidation

	Inhibitory Rate(%)			
ug/ml	acteoside	ligupurpuroside B	ligupurpuroside A	Vit E
52	92.1	90.4	---	---
26	74.2	72.4	67.7	75.4
13	60.3	62.1	52.5	---
7	50.1	49.4	40.0	---

The inhibitory effect of glycosides on LDL oxidation

These three glycosides can increase the lag time before the onset of LDL oxidation remarkably as compared with the control. The effect is concentration-dependant, and better than the same concentration of Vitamin C, a positive control. Among them, acteoside and ligupurpuroside B showed a better antioxidant activity (Table 3).

Table 3. The effect of three phenylethanoid glycosides on the lag time of LDL oxidation modification

	Lag Time (min)		
	acteoside	ligupurpurosideA	ligupurpuroside B
control	84.9	56.6	74.6
Vit C	105.4[a]	66.9[a]	131.1[b]
400 ng/ml	complete	126.0	153.0
200	153.0	90.0	144.0
100	136.3	73.0	101.0
50	128.6	64.0	86.0

[a] Final concentrtion = 140ng/ml [b] Final concentrtion = 280ng/ml

Though, there have been many researches about the antioxidative effect of polyphenols[9,10], phenylethanoid glycosides as a kind of stronger antioxidative agent had not been much reported. The antioxidative properties of acteoside, ligupurpuroside A and B may be related to their multiple phenolic hydroxyl groups. The action of acteoside and ligupurpuroside B are more potent than that of ligupurpuroside A. That might not only due to the amount of the phenolic hydroxyl groups, but also for the structure of sugar moiety. The function of antioxidative action might be through the interruption of the free radical chain reaction by accepting electrons.

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TRITERPENOID SAPONINS FROM *ASTER AURICULATUS*

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Key Word Index---*Aster auriculatus*; Compositae; triterpenoid saponins; 2D NMR.

Abstract---Two new triterpenoid saponins were isolated from the alcoholic extract of roots of *Aster auriculatus*. Their structures have been determined on the basis of spectral and chemical evidence as 3-O-β-D-glucuronyl-16α-hydroxyolean-12-en-28-oic acid-28-O-α-L-arabinopyranosyl(1-4)-α-L-rhamnopyranosyl(1-2)-β-D-xylopyranosyl-(1-3)-β-D-xylopyranosyl(1-3)-α-L-arabinopyranoside and methyl ester of 3-O-β-D-glucuronyl-16α-hydroxyolean-12-en-28-oic acid-28-O-β-D-xylopyranosyl(1-3)-α-L-arabinopyranosyl(1-4)-β-D-apiofuranosyl(1-3)-α-L-rhamnopyranosyl(1-2)-β-D-xylopyranoside.

Introduction

Members of the genus *Aster* are used in Chinese folk medicine. For example, *A. tataricus* is used as dissolving phlegm and stopping cough [1]. The result of pharmacological test showed that the crude saponin mixture from roots of *A. auriculatus* [2, 3] have strong anti-inflammatory and anti-ulcer activities. In our studies on the glycosidic constituents of this plant, this paper describes the isolation and structural elucidation two new saponins **1** and **2** from roots of *A. auriculatus*.

Results and Discussion

Compound **1** was obtained as colourless needles from MeOH, m.p 232-235°C, $[\alpha]_D^{21}$ -56.1 (MeOH, c 0.027). It showed the ions of [M+Na] at m/z 1345.6129 and [M+2Na-1]+ at m/z 1367.5881 in the ESI mass spectrum (HRMS), suggesting a molecular formula of $C_{62}H_{98}O_{30}$. The IR spectrum indicated the presence of hydroxy at 3431 cm^{-1}, ester group at 1730 cm^{-1}, double bond at 1634 cm^{-1} and glycosidic linkage at 1076 ~1043 cm^{-1}. Its ^{1}HNMR spectrum showed the signals of seven tertiary methyl groups at δ 0.82, 0.98, 0.99, 1.70, 1.15, 1.27 and 1.82 (each 3H, s), one trisubstituted olefinic proton at δ 5.58 (1H, brs) and six anomeric protons at δ 4.98 (d, J=7.5Hz), 5.03 (d, J=7.5Hz), 5,15 (d, J=7.2Hz), 5.46 (d, J=7.8Hz), 5.64 (brs) and 6.55 (d, J=1.6Hz). Its ^{13}CNMR spectrum revealed the presence of six sp^3 quaternary carbon atoms at δ 39.6, 40.1, 37.0, 42.1, 49.6 and 31.0, a pair of olefinic carbon atoms at δ 122.9 and 144.4, an ester carbonyl at δ 176.0 and six anomeric carbon atoms at δ 93.4, 100.9, 104.4, 106.0, 106.1 and 107.2. The NMR spectral data and molecular formula suggested that compound **1** was an oleanane-type triterpene hexoglycoside. The significant downfield shift of the anomeric proton at δ 6.55, and the upfield shift of anomeric carbon at δ 93.4 indicated that this sugar was attached to the aglycone through an ester linkage [4].

Acid hydrolysis of **1** yielded a sapogenin **1a**, and glucuronic acid, rhamnose, arabinose and xylose as sugar components. The EI mass spectrum of **1a** showed a molecular ions at m/z 472, corresponding to the molecular formula of $C_{30}H_{48}O_4$. The EI-MS of **1a** revealed fragment ions at m/z 264, 246, 201 derived from the D/E ring, and m/z 208, 190 from the A/B ring, which were formed through the characteristic retro-Diels-Alder fragmentation at the C ring in olean-12-en-28-oic acid skeleton with a hydroxy group on each of A/B and D/E ring. The ^{1}HNMR

spectrum of **1a** showed signals of seven tertiary methyl groups, two hydroxy-bearing ax-eq methine protons at δ 3.22 (1H, dd, J = 4.5, 11.2 Hz, H-3a) and δ 4.50 (1H, dd, J = 3.7, 3.7 Hz, H-16e), together with the signal of trisubstituted olefinic proton at δ 5.41 (1H, t, J =3.0 Hz, H-12). These spectral data suggested that **1a** was 3β, 16α-dihydroxyolean-12-en-28-oic acid, *i. e.* echinocystic acid. The sugars were identified by PC and HRTLC through direct comparison with authentic samples.

Upon alkaline of compound **1**, the ester-linked sugar chain was selectively cleaved and a prosapogenin **1b** was obtained. The FAB-MS of **1b** showed $[M+Na]^+$ ion at 671, corresponding to the molecular formula of $C_{36}H_{56}O_{10}$. Acid hydrolysis of **1b** afforded aglycone **1a** and glucuronic acid, which were identified by direct comparison with authentic samples. The NMR spectra of **1b** showed signals at δ 89.1 (CH-3) and 3.35 (1H, dd, J = 10.5, 3.0 Hz, H-3), suggesting the glucuronic acid linked at C-3 in a β position. From its FAB-MS, ^1H and ^{13}CNMR data, **1b** was established as 3-O-β-D-glucuronyl-16a-hydroxyolean-12-en-28-oic acid.

The above results indicated that remaining 5 mol of sugar units must be bound to the C-28 of the aglycone with an ester linkage. Elucidation of the structure of the 28-O-saccharide was performed by the following procedures.

The first step was to assign unambiguously the proton and carbon resonances of every monosaccharide unit by 2D NMR experiments including ^1H-^1H COSY, ^{13}C-^1H COSY, NOESY and HMBC spectra. In detail:

Beginning from the anomeric proton of rhamnose (δ 5.46, brs), 2- and 3-position protons of Rha. were easily assigned according to ^1H-^1H COSY (R_1-R_2-R_3), the remaining proton signals of Rha. residue (H_4 through H_6) were also assigned in the ^1H-^1H COSY spectrum from the distinct methyl proton doublet of Rha H-6 (δ 1.69, d, J=6.1Hz) to H-5 to H-4. With protons of Rha. assigned, ^{13}C-^1H COSY spectrum then allowed assignments of the carbon resonances of Rha. unit.

As for full assignments of the remaining 4 mol pentoses, beginning from the anomeric protons, all 2-position protons of the sugar moieties were easily located in the ^1H-^1H COSY spectrum, and the corresponding signals of 1- and 2-position carbons were then assigned by the ^{13}C-^1H COSY spectrum. In addition, beginning from their well resolved resonances of methene-5 protons, which had strong cross peaks with their anomeric carbons in the HMBC spectrum, the 4-position protons of these sugars were also easily assigned, and correspondingly the 4- and 5-position carbons were also assigned from the ^{13}C-^1H COSY spectrum. With the 2- and 4-position protons in the four sugars assigned, the remaining 3-position proton signals could be also delineated by careful analysis of the correlations between H-2 with H-3, H-4 with H-3 in the ^1H-^1H COSY spectrum, as well as by careful examination of the cross peaks between C-2 with H-3, C-4 with H-3 in the HMBC spectrum. Thus, the assignments of NMR data due to the sugar units were completed, the carbon and proton chemical shifts were listed in Table 2.

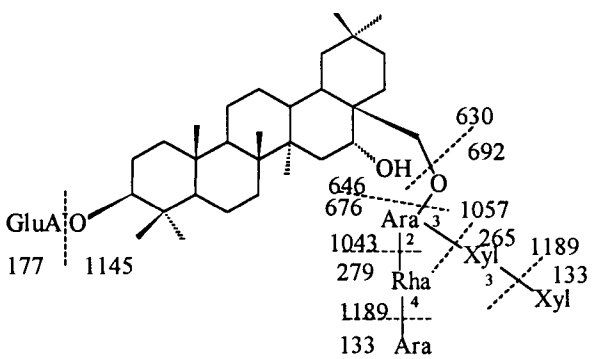
The next step was the determination of the sugar sequences and linkage sites through analysis of the carbon chemical shifts, observation of inter-correlation signals in the HMBC and NOESY spectra, and analysis of fragmentation patterns in the FAB mass spectrum.

Comparison of ^{13}CNMR data of the sugar part with those of methyl-α-L-rhamnopyranoside, methyl-α-L-arabinopyranoside and methyl-β-D-xylopyranoside [5, 6] indicated that 28-O-sugar moiety consisted of one rhamnose unit, two arabinose and two xylose units, and that one xylose unit and one arabinose unit were in terminal locations. C-3 of the inner xylose unit in the 28-O-sugar moiety resonated at δ 87.9, C-4 of the inner rhamnose at δ 82.3, C-2 and C-3 of the inner arabinose at δ 75.6 and 78.2, respectively, which were more downfield shifted than the corresponding carbon signals in individual methyl glycoside, suggested that glycosylation positions were at C-3 of the inner xylose, C-4 of the inner rhamnose, C-2 and C-3

of the inner arabinose. The HMBC spectrum of compound **1** showed strong cross peaks between the outer xylose H-1 and the inner xylose C-3, the inner xylose H-1 and the inner arabinose C-3, the outer arabinose H-1 and the inner rhamnose C-4, the inner rhamnose H-1 and the inner arabinose C-2, the inner arabinose H-1 and the ester carbonyl C-28. In addition, the NOESY spectrum of 1 showed correlations between the outer Xyl.H-1 and the inner Xyl.H-3, the inner Xyl.H-1 and the inner Ara.H-3, the outer Ara.H-1 and the inner Rha.H-4, the inner Rha.H-1 and the inner Ara.H-2. These results allowed the establishment of the linkage of 28-O-sugar chain as follows:

Xyl'-(1-3)-Xyl-(1-3)-Ara-(1---28)- Aglycone
| (1-2)
Ara'-(1-4)-Rha

Moreover, the presence of ions at 1213 $[M+Na-133+H]^+$, 1169 $[M+Na-177+H]^+$, 1081 $[M+Na-265+H]^+$, 1067 $[M+Na-279+H]^+$, 1037 $[M+Na-132-176]^+$ and 631 $[M-692+H]^+$ in the FAB mass spectrum also supported the sugar sequence above (Fig. 1).



1

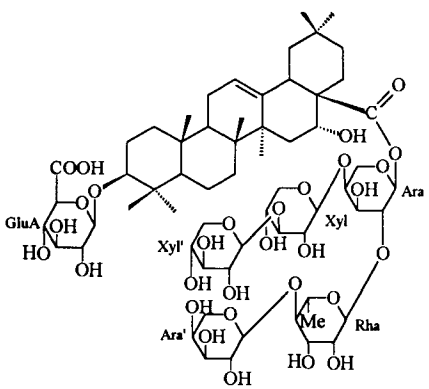
Fig 1

The $J_{1,2}$ couplings of the anomeric protons and the chemical shifts of protons and carbons of the sugar residues suggested that the anomeric configurations of xyloses and glucuronic acid were in β, and rhamnose and arabinose in α [7, 8]. From the above results, Compound **1** was established as 3-O-β-D-glucuronyl-16α-hydroxyolean-12-en-28-oic acid-28-O-α-L-arabino-pyranosyl(1-4)-α-L-rhamnopyranosyl(1-2)-β-D-xylopyranosyl-(1-3)-β-D-xylopyranosyl(1-3)-α-L-arabinopyranoside.

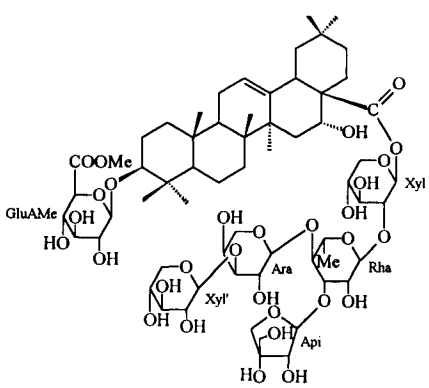
Compound **2** was obtained as white amorphous powder, m.p. 280-284°C, $[\alpha]_D^{31}$ -23.68 (MeOH, c 0.046). Positive ion FAB-MS provided a molecular ion m/z 1336, suggesting a molecular formula of $C_{63}H_{100}O_{30}$, which was consistent with the result of elemental analysis (Found: C, 53.33%; H, 7.72%; Cald: C, 53.35%, H, 7.75% for $C_{63}H_{100}O_{30}.9/2H_2O$). Its IR, ^1H and ^{13}CNMR spectra due to aglycone part were almost identical with those of **1**, which suggested that compound **2** was also an echinocystic acid 3, 28-bidesmoside.

Acid hydrolysis of compound **2** afforded echinocystic acid **1a**, as well as glucuronic acid, arabinose, xylose, rhamnose and apiose. While on alkaline hydrolysis of **2**, the prosapogenin **1b** was obtained. They were identified by direct comparison (PC and HRTLC) with authentic samples.

The NMR data of **2** showed the presence of six sugars from the six anomeric proton signals at δ 5.17 (d, J= 7.5 Hz), 5.24 (d, J=7.6Hz), 5.27 (brs), 5.62 (brs), 5.99 (d, J=4.2 Hz) and 6.56 (d, J=6.3 Hz) and from the six anomeric carbon signals at δ 112.0, 107.2, 105.9, 104.8, 101.2 and 93.4, together with a methoxyl group at δ H: 3.59 (3H, s) and δ C: 49.6 (CH_3). One of these sugars was rhamnose (δ 101.2), suggested by the distinct methyl proton doublet (Rha.H-6: δ 1.69, d, J=6.0Hz), and the significant downfield signal of the anomeric carbon (δ 112.0) was assigned as apiose, suggested by the quaternary carbon at δ 79.9. The above results indicated that the 3-O-sugar unit was glucuronic acid methyl ester, and the 28-O-sugar moiety consisted of one unit of rhamnose, one unit of apiose, three units of xylose and arabinose.



1



2

The unambiguously assignment of ^{1}H and ^{13}C resonances of each monosaccharide was made (Table 2) by using 2D NMR spectra.. Comparison of the ^{13}CNMR data of 28-O-sugar moiety in compound **2** with those of the corresponding individual methyl glycosides indicated that one apiose unit and one xylose unit were at terminal positions, and that the glycosylation positions were at C-3 of inner arabinose (+12.2 ppm), C-2 of inner xylose (+5.8 ppm), C-3 and C-4 of inner rhamnose (+11.3 and +5.0 ppm, respectively) [5, 6]. In the HMBC spectrum of

Table 1. NMR chemical shift assignments of aglycone moieties for **1**, **2** and **1b** (500MHz for ^{1}H and 125 MHz for ^{13}C, δ, ppm, in C_5D_5N)

C	**1**		**2**		**1b**		DEPT
	δ H	δ C	δ H	δ C	δ H	δ C	
1	0.88, 1.40	38.83	0.89, 1.39	38.85		38.81	CH_2
2	1.83	26.93	1.84	26.95		26.56	CH_2
3	3.38dd, (10.5, 3.0)	89.08	3.32 dd (10.2, 3.5)	89.10	3.35 dd	89.17	CH
4		39.57		39.52		39.56	C
5	0.80 brd (11.6)	55.91	0.76 brd (11.2)	56.00		55.98	CH
6	1.31-1.35	18.50	1.31-1.35	18.51		18.60	CH_2
7	1.33-1.40	33.56	1.31-1.44	33.60		33.58	CH_2
8		40.09		40.09		39.99	C
9	1.69	47.18	1.69	47.17		47.24	CH
10		37.03		37.02		37.08	C
11	1.89	23.85	1.89	23.84		23.88	CH_2
12	5.58	122.89	5.58	123.10	5.62	122.44	CH
13		144.43		144.45		145.19	C
14		42.11		42.09		42.19	C
15	2.15-2.45	36.20	2.15-2.47	36.21		36.22	CH_2
16	5.25 brs	74.13	5.25 brs	74.10	5.24	74.80	CH
17		49.64		49.64		49.01	C
18	3.58 dd (11.3, 3.0)	41.29	3.57dd (11.3, 3.0)	41.29	3.61 dd (13.8, 3.0)	41.54	CH
19	1.34, 2.77	47.11	1.35, 2.78	47.10		47.37	CH_2
20		30.99		30.97		31.10	C
21	1.28	36.02	1.26	36.01		36.22	CH_2
22		32.15		32.14		32.43	CH_2
23	1.270	28.25	1.250	28.29	1.269	28.31	CH_3
24	0.977	17.06	0.972	17.07	0.959	17.08	CH_3
25	0.819	15.72	0.819	15.71	0.827	15.67	CH_3
26	1.070	17.66	1.060	17.66	1.058	17.57	CH_3
27	1.816	27.27	1.806	27.26	1.854	27.33	CH_3
28		175.99		175.97		180.14	C
29	1.007	33.34	1.016	33.33	1.007	33.43	CH_3
30	1.150	24.87	1.170	24.90	1.176	24.88	CH_3

Table 2. NMR chemical shift assignments of sugar moieties for 1, 2 and 1b (500MHz for ^{1}H and 125 MHz for ^{13}C, δ, ppm, in C_5D_5N)

	1		2		1b	
	GluA		GluAMe		GluA	
1	107.2	4.98 d 7.5	107.2	5.17 d 7.5	106.9	4.88 d 7.8
2	75.5	4.12	74.7		75.5	4.00
3	77.8	4.49	77.8		76.8	4.23
4	70.9	4.14	71.0		73.6	4.38
5	78.2	4.50	78.5		78.2	4.46
6	176.7		---		177.2	
OMe			49.6	3.59 s		
	Ara		Xyl			
1	93.4	6.55 d 1.6	93.4	6.56 d 6.3		
2	75.6	4.52 brd 5.8	79.8	4.56		
3	78.2	4.34	75.3	4.15		
4	65.5	4.45	70.9	4.12		
5	62.4	3.94 dd 8.3, 12.0	65.5	3.49 dd 11, 11		
		4.56		4.10		
	Rha		Rha			
1	100.9	5.64 brs	101.2	5.62 brs		
2	71.6	4.79 dd 8.7, 2.8	71.5	4.84 brd 4.2		
3	72.9	4.73	82.4	4.60 dd 4.2, 8.0		
4	82.3	4.60 dd 3.1, 9.6	78.3	4.52		
5	68.7	4.43 dd 9.6, 6.1	68.7	4.46		
6	18.6	1.76 d 6.1	18.6	1.69 d 6.0		
	Xyl		Ara			
1	104.4	5.46 d 7.8	104.8	5.27 brs		
2	74.7	3.97 dd 7.8, 9	71.0	4.63		
3	87.9	4.05 dd 9, 9	86.6	4.01		
4	69.4	4.00	66.5	4.50		
5	66.4	3.31 dd 11.0, 11.0	67.1	3.99 , 4.60		
		4.10				
	Xyl'		Xyl'			
1	106.1	5.15 d 7.2	105.9	5.24 d 7.6		
2	75.4	4.01	75.4	4.04		
3	77.9	4.08	77.9	4.09		
4	69.6	4.17	69.4	4.18		
5	67.1	4.09 dd 11.3, 11.3	67.2	3.42 dd 11, 11		
		4.11		4.10		
	Ara'		Api			
1	106.0	5.03 d 7.5	112.0	5.99 d 4.2		
2	72.9	4.47	77.7	4.76 d 4.2		
3	74.5	4.02	79.9	---		
4	68.9	4.57	74.6	4.22 d 9, 4.52		
5	67.2	3.43 dd 11, 11	64.5	4.02		
		4.10				

compound **2**, the interactions between Api.H-1 with Rha.C-3, Xyl'.H-1 with Ara.C-3, Ara.H-1 with Rha.C-4, Rha.H-1 with Xyl.C-2 and Xyl.H-1 with the aglycone C-28 were observed, suggesting that 28-O-saccharide chain had the structure:

Xyl'-(1-3)-Ara-(1-4)-Rha-(1-2)-Xyl- (1---28)-Aglycone
| (1-3)
Api

The HMBC spectrum also displayed the interaction between GluAMe.H-1 with the aglycone C-3, connecting this glucuronic acid methyl ester moiety to the aglycone 3-position.

The coupling constants of H-1 and H-2 and the carbon chemical shifts of each monosaccharide showed that GluAMe, Xyl. and Api were in β, while Rha. and Ara. were in α configurations [7, 8]. Therefore, the structure of compound **2** was determined as methyl ester of 3-O-β-D-glucuronyl-16α-hydroxyolean-12-en-28-oic acid-28-O-β-D-xylopyranosyl(1-3)-α-L-arabinopyranosyl(1-4)-β-D-apiofuranosyl(1-3)-α-L-rhamnopyranosyl(1-2)-β-D-xylopyranoside.

Experimental

General. Mps were determined on a XT4-100X micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin Elmer 241 automatic digital polarimeter. The IR spectra were recorded on a Perkin Elmer 683 IR spectrometer. FAB-MS were taken on a ZAB SpecE instrument, ESI MS on an IonSpec HiResMALDI instrument and EI-MS on a VG ZAB-2f instrument. NMR spectra were taken on a Bruker AM-500 (500MHz for ^{1}H and 125MHz for ^{13}C) spectrometer in C_5D_5N with TMS as int. standard. TLC was on silica gel GF254 and HRTLC on silica gel H (5-7 μ). Separation and purification were performed by CC on silica gel (300-400 mesh and 180-200 mesh).

Plant material. The roots of *Aster auriculatus* were collected in Sichuan Province, P. R. China. A voucher specimen (880801) was identified by Prof. W. Z. Song and deposited in the Herbarium of the authors' Institute.

Extraction and Purification. Air dried roots of plants (2.5kg) were extracted with 70% ethanol (5 x 5 l) under reflux. The combined extracts were evapd under reduced pressure to obtain a crude syrup (500g), which was chromatographed over a highly porous polymer column (9.5 x 50 cm, 2 x 750g) eluting successively with H_2O, 30%, 60% and 95% ethanol.

The 95% ethanol part (54g) was then chromatographed over silica gel CC (5.5 x 85 cm, 180-200mesh, 1000g) eluting with $CHCl_3$-MeOH (10:1) followed by gradually increasing the polarity with MeOH, seven fractions were collected. Fr.5 ($CHCl_3$- MeOH: 2:1, 158-165, 500ml each eluent) was chromatographed repeatly over silica gel CC, eluting with $CHCl_3$-MeOH-H_2O (1:1:0.1) to obtain pure **2** (80mg).

The 60% ethanol part (250g) was subjected to CC over silica gel (8.0 x 64 cm, 180-200 mesh, 2000g) eluting with $CHCl_3$-MeOH-H_2O (9:1:0.01---1:1:0.2) gradient (500 ml each eluent) to separate into eight crude fractions monitoring by HRTLC (silica gel 5-7 μ). Fr.8 ($CHCl_3$-MeOH-H_2O, 1:1:0.2, 120-180, 500 ml each eluent) was recrystallized with MeOH to obtain pure **1** (5g) after silica gel CC eluting with $CHCl_3$-MeOH-H_2O (26:14:3).

Compound 1, white needles, m. p. 232-235 °C, $[\alpha]_D^{21}$ -56.1° (MeOH, c 0.027). $C_{62}H_{98}O_{30}$. FAB-MS *m/z*: 1345 $[M+Na]^+$, 1213, 1169, 1081, 1037, 631, 585, 543, 455, 133, 115. ESI-MS (HR): 1367.5881 (4.7) $[M+2Na-H]^+$, 1345.6129 (4.7) $[M+Na]^+$, 1171.5769 (4.1), 1170.5753 (7.6), 1169.5788 (16.0), 1037.5260 (4.6), 727.2260 (18.5), 698.2193 (34.1), 697.2150 (100.0), 493.3577 (16.3). IR υ max cm^{-1}: 3431, 2930, 1730, 1634, 1387, 1076-1043.

^1H and ^{13}CNMR: see Tables 1 and 2.

Compound 2, an amorphous powder, m. p. 280-284°C, $[\alpha]_D^{31}$ -23.68° (MeOH, c 0.046). $C_{63}H_{100}O_{30}$. FAB-MS *m/z*: 1359 $[M+Na]^+$, 1227 $[M+Na-132]^+$, 1169 $[M+Na-GluAMe]^+$, 1095 $[M+Na-Xyl'-Ara]^+$, 631, 585, 483, 455, 437, 391, 330, 303, 207, 133, 115. Elemental analysis: C 53.33%, H 7.72% for $C_{63}H_{100}O_{30}.9/2H_2O$. IR υ max cm^{-1}: 3400, 1735, 1640, 1100-1000. ^1H and ^{13}CNMR: see Table 1 and 2.

Acid hydrolysis of 1 and 2. A soln of sample (20mg) in 4 ml 2N HCl-MeOH (1:1) was refluxed for 2 hr. After cooling to room temp., the reaction mixture was neutralized with 0.05 N NaOH, and then extracted with $CHCl_3$. The $CHCl_3$ soln was evapd to dryness and chromatographed over silica gel CC eluting with petrol-CH_3COCH_3 (4:1) to obtain pure aglycone **1a** for both compounds **1** and **2**. **1a**, needles, m. p. 310-314°C, $[\alpha]_D^{31}$ +13.6° ($CHCl_3$, c 0.055). $C_{30}H_{48}O_4$. EI-MS *m/z*: 472 $[M]^+$, 454, 427, 410, 392, 264, 246, 230, 219, 208, 201, 190, 175. ^{1}HNMR ($CDCl_3$, δ, ppm): 0.77, 0.88, 0.91, 0.92, 0.98, 0.99 and 1.35 (each 3H, s), 3.02 (1H, dd, J = 4.2, 14.0 Hz, H-18), 3.22 (1H, dd, J = 4.5, 11.2 Hz, H-3), 4.50 (1H, dd, J = 3.7, 3.7 Hz, H-16), 5.41 (1H, t, J = 3.0 Hz, H-12).

The aq. layer was evapd *in vacuo* and subjected to HPTLC analysis on Kieselgel 60 F_{254} [using $CHCl_3$-MeOH-H_2O (30:12:4) 9 ml and HOAc 1 ml] and PC [using n-BuOH-benzene-C_5H_5N-H_2O (5:1:3:3) and n-BuOH-HOAc-H_2O (4:1:5)] with comparison with authentic samples, indicating GluA., Xyl., Ara. and Rha. in **1**, GluA., Xyl., Ara., Rha. and Api. in **2.**

Alkaline hydrolysis of 1 and 2. A soln of sample (20 mg) in 2% NaOH-EtOH (1:1, 10ml) was refluxed for 6 hr. The reaction mixt. was cooled to room temp. and carefully neutralized with 0.1N HCl, and then extracted with n-BuOH for three times. The n-BuOH soln was evapd under red. pres. and the residue was chromatographed over silica gel CC, eluting with $CHCl_3$-MeOH-H_2O (3:1:0.1), followed by recrystallization in 95% EtOH to obtain prosapogenin **1b** for both **1** and **2**. **1b**, amorphous powder, m. p. 182-186°C, $[\alpha]_D^{31}$ +23.6° (MeOH, c 0.051). $C_{36}H_{56}O_{10}$. FAB-MS *m/z*: 671 $[M+Na]^+$, 455,391,207,115. ^1H and ^{13}CNMR: see Table 1 and 2.

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CONSTITUENTS OF THE FRUIT OF *RANDIA SIAMENSIS*, BIOLOGICAL ACTIVITY OF SAPONIN PSEUDOGINSENOSIDE-RT_1

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Key word index -- *Cardiovascular activity, pseudoginsenoside-RT_1, Randia siamensis, saponins, uterine contractility, ichthyotoxic activity.*

Abstract- The following components were isolated from the fruit of *Randia siamensis* Craib (Rubiaceae); ursolic acid (**1**), pseudoginsenoside-RP_1 (3-O-β-GlcUA-(2-1)-β-Xyl of Oleanolic acid) (**2**), pseudoginsenoside-RT_1 (3-O-β-GlcUA-(2-1)-β-Xyl of Glucosyl oleanolate) (**3**) and siamenoside (3-O-β-GlcUA-(2-1)-β-Xyl-(2-1)-α-Rha of Glucosyl oleanolate) (**4**). ^{13}C NMR spectroscopy was particularly useful in confirming the structures of these components. *In vivo* studies with rats showed that the saponin **3** caused a decrease in blood pressure, an increase in heart rates and an increase in spontaneous contractility of uterus. Alcoholic extract of the fruit exhibited acute ichthyotoxic activity in low dose.

Introduction

Randia Linn. is a large genus of erect of climbing shrubs and trees of the subfamily Gardennieae, family Rubiaceae. One hundred and fifty species of *Randia* are distributed widely in the tropical and sub-tropical regions, especially in Asia and Africa (Rendle, 1953). In Thailand there are at least 37 valid species of *Randia* which are commonly found in evergreen forests (Craib, 1939). Craib (1939) reported that *Randia siamensis* Craib is synonymous with *R. longiflora* Hook.f., *R. uncata* Ridl., *Griffithia siamensis* Miq. and *Webera siamensis* Kurz. It can be found in all parts of Thailand and Burma, especially in the humid regions.

This plant is known by various local names in Thailand : Khat khao, Khet khao, Khat khao naam and Khat khao khuea. All parts of *R. siamensis* have been used in folkloric medicine; its fruits have been used for inducing abortion and as an emmenagogue or hematinic, the leaves are claimed to control blood pressure, the root is used for its antipyretic and antiscurvy activity, the flowers have been used to stop nosebleeds and its stems have been used as a hematinic (Pongboonrod, 1959). There have been no previous reports in the literature on pharmacological investigations of this plant.

Chemical studies of many other species of *Randia* have shown the presence of triterpenoid acids such as oleanolic acid and randialic acid. Previous work on the roots of *R. siamensis* yielded mesembryanthemoidigenic acid, 3-β-acetyloleanolic acid, ursolic acid and 3-O-(α-L-arabinosyl)-oleanolic acid (Lapikanom *et al.*, 1983; Woo *et al.*, 1984). However, there have been no previous reports of compounds isolated from the fruit of this plant.

Accordingly, a number of components have been isolated from the fruit of *Randia siamensis* and their structures established. A preliminary study found that an alcohol extract of the fruit of *R. siamensis* caused a significant uterine contraction *in vivo*. Thus the major component isolated in this study was examined for its *in vivo* activity on blood pressure, heart rates and spontaneous uterine contractility.

Results and Discussion

The ethanol extract from the fruit of *Randia siamensis* after separation by column chromatography yielded four components: ursolic acid (**1**), pseudoginsenoside-RP_1 (**2**), pseudoginsenoside-RT_1 (**3**) and siamenoside (**4**). **1** is a commonly occurring triterpenoid acid while **2** and **3** are saponins which were first isolated from *Panax* (Araliaceae) species and were identified by Tanaka *et al.* (1985). The major component isolated in our study, oleanolic acid glycoside **3**, has since been reported to be present in additional species (Morita *et al.*, 1986; Shukla and Thakur, 1988; Shukla, 1989; Ida *et al.*, 1994; Sakai *et al.*, 1994). In the structural elucidation of the four components from *R. siamensis*, ^{13}C NMR spectroscopy was found to be particularly useful for comparison with published spectra.

Although saponins are known to exhibit a variety of biological effects (Rouhi, 1995), the activity of component **3** has not been reported. Thus the effect in rats of **3** on blood pressure, heart rate and spontaneous uterine contractility *in vivo* was examined. As shown in table 2 and Figure 1, intravenous injection of **3** caused a dose-dependent decrease in the mean arterial blood pressure (MAP), an increase in the heart rate and a small increase in the spontaneous uterine contractility. However, when a solution of **3** was injected directly into the uterine lumen, no effects on blood pressure or heart rate were detected, while all doses of **3** caused strong increases in both amplitude and frequency of the spontaneous uterine contractions. There are no signs of acute toxicity such as internal bleeding of liver, lung, gastrointestinal tract or urinary bladder, at any doses of **3** studies, either injected the **3** through the jugular vein or the intrauterine. Traditional medicine of Thailand has indicated that extracts from *Randia siamensis* are effective in inducing abortions and in treating cardiovascular problems. The *in vivo* biological activity exhibited by saponin **3** suggests that these traditional uses have, in fact, some scientific support. Additionally, it is found that crude ethanolic extract of the fruit exhibits acute ichthyotoxic activity in low dose by testing method of Sprague (Sprague, 1969, 1971). The various concentration and time of entirely lethal dose are shown in Table 3.

Experimental

Materials and Methods

Plant material The fruit of *Randia siamensis* was collected from Nakornpathom Province, Thailand, in July, 1984. Authentication was achieved by comparison with herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. A voucher specimen of plant material has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and Isolation The dried, powdered fruit (250 g) of *R. siamensis* was macerated twice with 95% ethanol (1 L x 2) for three-day periods and filtered. The combined ethanolic extract was evaporate under reduced pressure to dryness. The residue was washed with petroleum ether (300 ml) and filtered. The pet. ether wash, which showed the presence of traces of terpenoids by TLC, was not investigated further. The pet. ether insoluble portion (19.6 g) was divided into four equal portions. Each portion was chromatographed on a silica gel column (4 x 16 cm). Elution with chloroform:methanol (95:5) afforded 20 fractions of 25 ml each, which were examined by TLC. Those fractions of similar composition were combined. Fractions 8-11 were combined and the solvent removed under reduced pressure to give a white amorphous solid. Recrystallization of the solid from methanol gave ursolic acid (**1**) as white needles (62 mg, 0.025%).

The columns were then washed with methanol until no trace of triterpenoids were detected. The methanol eluant was evaporated under reduced pressure to give 8.19 g of a residue which was divided in 8 equal portions. Each portion was chromatographed on a silica gel column (2.5 x 16 cm) and eluted with chloroform:methanol:water (65:35:10). Fractions of 25ml each were collected and examined by TLC. Homogeneous fractions 8-10, 25-30 and 41-45 were combined, evaporated and lyophilized to dryness. The resultant three fractions consisted of pseudoginsenoside-RP_1 **(2)** (21 mg, 0.0084%), the major component pseudoginsenoside-RT_1 **(3)** (312 mg, 0.125%) and siamenoside **(4)** (67 mg, 0.027%), respectively.

Ursolic Acid (1) White needles from methanol, m.p. 285°C; EIMS *m/z* 456 (M^+,8), 411(2), 248(11); ^1H and ^{13}C NMR spectra (pyridine-d_5, 270 and 67.8 MHz, resp.) are in agreement with previously published values for this triterpenoid acid (Ogura *et al.*, 1977; Seo *et al.*, 1975).

Pseudoginsenoside-RP_1 {Oleanolic acid-3-β-O-[β-glucuronopyranosyl (1-2)-β-xylo pyranoside]} (2) Colorless needles from methanol, m.p. 230-232°C; ^{13}C NMR spectrum (pyridine-d_5, 67.8 MHz) was in agreement with that previously published for this saponin (Tanaka *et al.*, 1985).

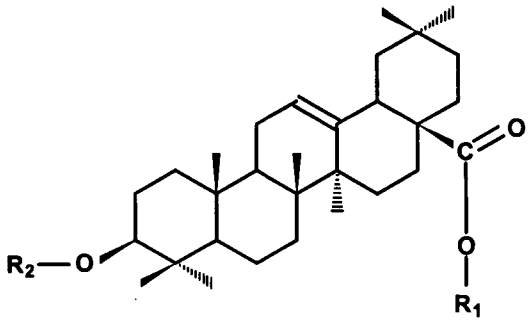
Pseudoginsenoside-RT_1 {Oleanolic acid -28-O-β-glucopyranoside-3-β-O-[β-glucuronopyranosyl (1-2)-β-xylopyranoside]} (3) White amorphous solid from methanol, m.p. 246-249°C (decomp.); FAB MS (negative ion mode) *m/z* 925 $(M-H)^-$ (100), 793 $(M-H-Xyl)^-$ (14), 763 $(M-H-glc)^-$ (60); ^{13}C NMR spectrum (pyridine-d_5, 100 MHz) was in agreement with that previously published (Tanaka *et al.*, 1985).

Siamenoside (4) White amorphous powder from methanol, m.p. 215-220°C (decomp.); ^{13}C NMR spectrum (pyridine-d_5, 100 MHz) (see Table 1)

Table 1 ^{13}C-NMR chemical shifts (δ) data of **4** in pyridine-d_5

Carbon	δ	Carbon	δ
C-1	39.0	C-28	176.3
C-2	26.5	C-29	33.1
C-3	89.9	C-30	23.7
C-4	39.7	3-GlcUa 1	105.3
C-5	56.1	2	79.1*
C-6	18.8	3	78.1*
C-7	33.3	4	73.3
C-8	40.1	5	78.5*
C-9	48.2	6	173.0
C-10	37.1	Xyl 1	102.8
C-11	23.7	2	78.9*
C-12	123.0	3	77.2
C-13	144.1	4	71.5
C-14	42.3	5	66.5
C-15	28.3	Rha 1	101.9
C-16	23.7	2	72.2
C-17	47.1	3	72.6
C-18	41.9	4	74.2
C-19	46.4	5	69.6
C-20	30.8	6	18.8
C-21	34.2	28-Glc 1	95.7
C-22	32.6	2	74.1
C-23	28.3	3	78.5*
C-24	16.7	4	71.1
C-25	15.5	5	78.9*
C-26	17.5	6	62.2
C-27	26.1		

* Assignment may be reversed



(2) $R_1 = H$, $R_2 = \beta$-GlcUA-(2-1)-β-Xyl
(3) $R_1 = \beta$-Glc, $R_2 = \beta$-GlcUA-(2-1)-β-Xyl
(4) $R_1 = \beta$-Glc, $R_2 = \beta$-GlcUA-(2-1)-β-Xyl-(2-1)-α-Rha

Biological Activity Studies of 3 on Rats: Blood Pressure, Heart Rate and Uterine Contractility Measurements The adult female Wistar rats in estrus, body weight 220-270 g were anesthetized with Nembutal (60 mg/kg, i.p.). A tracheal tube was inserted in the trachea. A polyethylene catheter was cannulated through the right common carotid artery and connected to a pressure transducer and polygraph for monitoring blood pressure and heart rate. The right abdomen was opened and a small balloon catheter (filled with 0.9% normal saline) was introduced through a small incision into the uterus at the ovarian end and connected to a pressure transducer and a polygraph for uterine contractility recording. A second polyethylene catheter was introduced through a small incision at the vaginal end of the same uterus for intrauterine injection of drug. The animal was then equilibrated for 1 hr. The response relationships to **3** (4-32 mg/kg, dissolved in saline) were studied by injection of the drug through the jugular vein or directly into the intrauterine lumen.

Table 2 Effects of **3** on blood pressure (MAP) and heart rates (H.R.) in anesthetized rats. Data are expressed as means±s.e.mean of 5-6 experiments.

Doses (mg/kg)	Decrease in MAP (mmHg)	Increase in H.R. (beats/min)
4	11.6±2.0	9.2±4.3
8	14.6±2.0	27.5±12.0
16	27.3±5.5	43.0±13.6
32	44.4±7.9	60.5±12.5

Ichthyotoxic Activity of Alcohol Extract The fish used in this experiment are *Puntius gonionotus* Smith, *Poecilia reticulata* Peters, *Cyprinus carpio* Linn. and *Tilapia nilotica* Linn. The ichthyotoxic activity of the alcoholic extract was performed by following the method of Sprague (Sprague, 1969, 1971).

Table 3 Time (hour) of entirely lethal concentration of alcoholic extract of the fruit of *R. siamensis*

Fish used	Conc.				
	Control	0.0312 %	0.0675 %	0.9375 %	0.1250 %
Puntius gonionotus Smith	36	12	5.51	3.25	1.08
Poecilia reticulata Peters	72	36	12	8	3.4
Cyprinus carpio Linn.	18	6	5.5	3.25	1.33
Tilapia nilotica Linn.	20	7	5.66	3.3	1.25

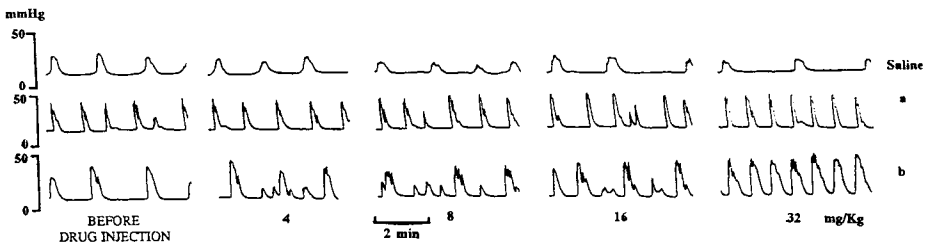


Figure 1. Typical traces of the effects of intravenous injection (a) or intrauterine injection (b) of **3** on spontaneous uterine contractility of anesthetized rats *in vivo*. Each episode shows only a few minutes of the maximal activity of each dose of **3**.

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A NEW TRITERPENOID GLYCOSIDES FROM *DECAIANEA FARGESII*

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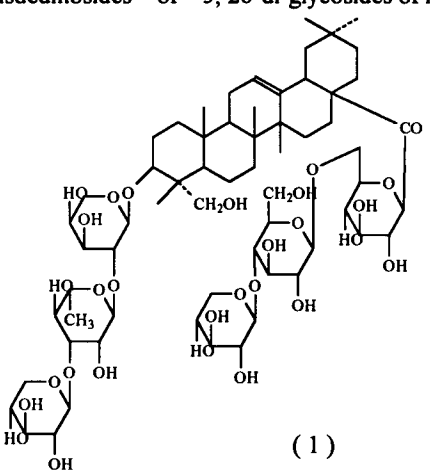
Abstract--A new triterpenoid glycosides, named decaisoside F, has been isolated from *Decaisnea fargesii* and its structure was elucidated on basis of spectral and chemical methods to be 3-O-β-D-xylopyranosyl (1→3)-α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-xylopyranosyl(1→4)-β-D-glucopyanosyl (1→6)-β-D-glucopyranoside.

Decaisnea fargesii Franch. is a traditional Chinese medicinal plant widely distributed in china, which has been used as an anti-rheumatic and antitussive drug for a long time in Chinese folk medicine [1]. We found recently that its methanol extract showed antitumour activity in vivo against S180, Hepa and Ehrlich cells. In the search for active principles from traditional Chinese medicinal plants, we have investigated the chemical constituents of the plant *Decaisnea* fargesii Franch. from Gansu province, and ten triterpenoids have been obtained[2]. In this paper we wish to report the isolation and structural elucidation of a triterpenoid Glycosides named as decaisoside F, from the n-butyl alcohol extract of *Decaisnea fargesii* Franch.

Result and Discussion

The new glycoside (**1**) designated decaisoside F, has a molecular formula $C_{63}H_{102}O_{29}$ deduced from the negative FABMS. The ^{13}C and $^{1}HNMR$ signals due to aglycone moieties of (**1**), showed that it was bisdedmosides of 3, 28-di-glycosides of hederagenin [3]. On acid



(1)

hydrolysis on TLC [5], (**1**) yielded rhamnose, arabinose, glucose and xylose. The sugar sequence and interglycosidic linkage positions were established as follows. In the negative FABMS of (**1**), besides the quasimolecular ion peak, fragment ion peaks at *m/z* 1337 $[M-H]^-$,

1205[M-Xyl-H]$^-$, 881[M-2Glc-Xyl-H]$^-$, 749[M-2Glc-2Xyl-H]$^-$ were observed. On acid hydrolysis with 0.2N HCl in 60% EtOH, (**1**) afforded hederagenin and prosapogenin (**2**). Compound (**2**) was determined to be hederagenin 3-O-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranoside based on the comparison of ^{1}H, ^{13}CMNR and FABMS as well as the result of monosaccharide analysis, the structure of (**2**) is identical with Saponin PG from *Akebia quinata*. The ^{13}CNMR spectrum disclosed that it was 28-O-triglycoside of hederagenin and the terminal xylose was deduced to be attached to the C-4 of inner glucose in terms of glycosylation shifts. Thus, the structure of (**1**) was established to be 3-O-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-xylopyranosyl(1→4)-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.

Table 1. ^{13}CMNR chemical shifts of compounds **1** in pyridine d_5 (ppm)

C	1	C	1
Aglycone		Sugar	
1	39.0	3-O-Are-1	104.3
2	26.2	2	75.4[a]
3	81.0	3	74.7
4	43.5	4	69.4
5	48.2	5	65.7
6	18.1	Rha-1	101.1
7	32.5	2	72.8
8	39.9	3	82.7
9	47.6	4	71.8
10	36.9	5	69.5
11	23.4[a]	6	18.3
12	122.9	Xyl-1	107.1
13	144.1	2	75.2[a]
14	42.1	3	78.1[b]
15	28.3	4	70.6[c]
16	23.7[a]	5	67.1
17	47.0	28-O-Glc-1	95.6
18	41.7	2	73.8
19	46.2	3	78.5[b]
20	30.7	4	70.9[c]
21	34.6	5	77.7
22	32.8	6	69.3
23	64.1	Glc-1	104.7
24	14.0	2	74.4
25	16.2	3	76.4
26	17.5	4	80.8
27	26.0	5	76.1
28	176.5	6	61.6
29	33.1	Xyl-1	105.3
30	23.6[a]	2	74.7
		3	77.9
		4	70.6[c]
		5	67.1

[a-c]Signals may be interchanged within each column

Experimental

Mps: uncorr. NMR spectra were recorded in pyridine-d5 at 400 MHz for 13C NMR, using TMR as int. Standard. EIMS were measured at 20ev accelerating voltage after micro-scale acetylation[5].

Plant material. The stems of *D. fargesii* Franch were collected in Kang county, Gansu province, China, and identified by Mr. Guo-liang Zhang. A voucher specimen is deposited in the Herbarium of the Botany Institute, Northwest Noumal University.

Extraction and isolation. The dried stems (2.23 kg) were percolated with 95% EtOH. The percolates were concd in vacuo and the residue (380 g) was subjected to macroporous absorption resin D-101 eluting with aq. MeOH gradiently. The 80% MeOH eluate was conce to dryness to give a crude glycoside fr. This fr. was chromatographed on silica gel with $CHCl_3$-MeOH-H_2O (40:10:1-10:10:1) and finally with MeOH to yield 7 frs, which of 2 were separated by repeated CC on RP-8(60-70% MeOH) to afford glycoside 1.

Dacaisoside G (1). White powder from MeOH, mp. 227-230° (dec.), $[\alpha]_D^{23}$-18.896° (MeOH c 0.09); FAB-MS (neg.) *m/z*: 1337 $[M(C_{63}H_{102}O_{29})-H]^-$, 1205 [M-Xyl-H]$^-$, 881 [M-2GLc-Xyl-H]$^-$,749[M-2Glc-2Xyl-H]$^-$, 603 [M-2Glc-2Xyl-Rha-H]$^-$, 471 [M-2Glc-2Xyl-Rha-Ara-H]$^-$. Herein the signals of ^{13}C NMR chemical shifts of the compounds (Table 1). ^{1}HNMR: δ 0.86, 0.88, 0.98, 1.07,1.08, 1.19 (each 3H, s, CH_3), 1.62 (3H, d, J=5.85Hz, Glc-H), 4.80 (1H, d, J=4.52Hz), 5.21 (1H, d, J=6.92Hz, Glc H-1), 5.40 (1H, s, 12-H), 5,72 (1H, s, Rha H-1), 6.14 (1H, d, J=7.80Hz, Glc H-1).

Acid hydrolysis of glycosides and prosapogenins on TLC plate and identification of the resulting monosccharides. See previous paper[4].

Partial acid hydrolysis of 1. Glycoside 1 (200 g) was heated on a boiling bath with 0.2N HCl in 60% EtOH for 40 min. after cooling, the reaction mixt. was neutralized with KHCO3, and then extracted with n-BuOH layers were concd to dryness to afford a residue, The residue was chromatographed on silica gel with $CHCl_3$-MeOH-H_2O (8:2:1, lower layer) to give hederagenin and prosapogenin (2). Hederagenin was identified by comparson of TLC behaviour and 1h and 13C NMR spectra with those of an authentic sample[6]. Compound 2: white powder, mp 234-236° (dec.), $[\alpha]_D^{23}$-7° (pyridine; c 0.12); FAB-MS (neg.) *m/z*: 881 [M-H]$^-$, 749[M-Xyl-H]$^-$, 603[M-Xyl-Rha-H]$^-$, 471 [M-Xyl-Rha-Ara-H]$^-$; ^{1}HNMR: δ 5.02(1H, d, J=5.8Hz, Ara H-1), 5.16 (1H, d, J=7.4Hz, Xyl H-1), 6.05 (1H, s, Rha H-1).

Acknowledgment -- We are hereby to express special thanks to National Phytochemistry laboratory of Yunnan Botany Institute, Chinese academy of Sciences for its kindly financial assistant and for the cordial helps of Dr. Xing-Cong Li and the staffs of the laboratory.

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27-NOR-TRITERPENOID GLYCOSIDES FROM ADINA RUBELLA HANCE

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Key Word Index -- *Adina rubella*; Rubiaceae; saponins; 27-nor-olean-13-en-3β-hydroxy-28-oic acid; pyrocincholic acid glycosides

Abstract Three new 27-nor-triterpenoid saponins were isolated from the roots of *Adina rubella*. They were characterized as pyrocincholic acid 3β-O-α-L-rhamnopyranosyl-(28→1)-β-D-glucopyranosyl ester, pyrocincholic acid 3β-O-β-D-glucopyranosyl(1 → 2)-β-D-fucopyranosyl-(28→1)-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl ester, pyro-cincholic acid 3β-O -β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(28→1)-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl ester. Their structures were elucidated by spectral methods, especially with the aid of 2D NMR experiment.

Introduction

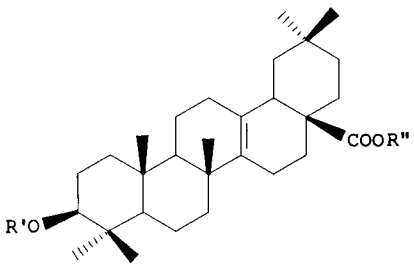
Adina rubella Hance, a Chinese folk plant, has been reported to contain several new pyrocincholic acid glycosides[1][2] and other triterpenoid saponins[3]. In continution of our work, three new 27-nor-triterpenoid saponins, pyrocincholic acid 3β-O-α-L-rhamnopyranosyl-(28→1)-β-D-glucopyranosyl ester, pyrocincholic acid 3β-O-β-D-glucopyranosyl(1→2)-β-D-fucopyranosyl-(28 → 1)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl ester, pyrocincholic acid 3β-O -β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(28→1)-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl ester were isolated from the butanol extract of the roots. This paper describes their isolation and structure elucidation.

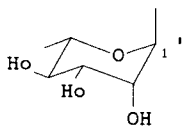
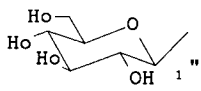
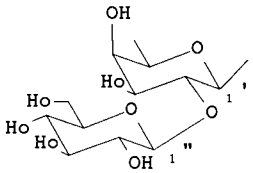
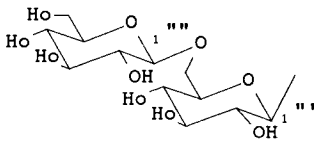
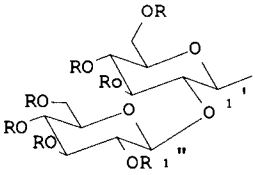
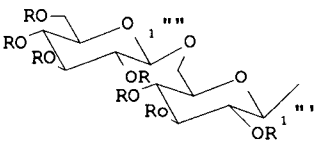
Results and Discussion

Compound **1** was obtained as white powder. The molecular formula was determined as $C_{41}H_{66}O_{12}$ by ^{13}C NMR DEPT spectrum and FAB MS spectral data(m/z 773 $[M+Na]^+$). The IR spectrum indicated the presence of hydroxyl (ν_{max} 3400, 1070cm^{-1}) and carboxylic ester(ν_{max} 1733cm^{-1}) groups. Acid hydrolysis of **1** yielded L-rhamnose and D-glucose identified by PC comparison with authentic samples.

The ^{13}C NMR spectrum of **1** (Table 1) contained 41 carbon signals, among which 29 were assigned to the aglycone part and 12 to the disaccharide moiety. In the aglycone carbon signals, two quaternary olefinic carbon signals (δ130.3, 136.9) and one carboxyl carbon (δ176.8) were displayed. Comparison of the ^{13}C NMR data of **1** with those of compound **4**, pyrocincholic acid 3β-O-α-L-rhamnopyranoside [1], revealed that the aglycone was pyrocincholic acid and the glycosidation sites were C-3 and C-28. The anomeric signals of one glucose unit appearing at δ6.30(d, J=8.1) in its ^1H NMR and δ95.6 in the ^{13}C NMR spectrum indicated that the glucose moiety gave a β-configuration and attached to the 28-carboxyl group of aglycone. The presence of a α-rhamnose unit linked to the 3β-hydroxy of the aglycone with an ether bond was suggested by the ^1H NMR(δ5.35, 1H, s, H-1') and the ^{13}C NMR (δ104.3) anomeric signals, that was confirmed by the alkaline hydrolysis of **1** to yield **4** (TLC). Therefore, the structure of

1 was deduced to be pyrocincholic acid 3β-O-α-L-rhamnopyranosyl-(28 → 1)-β-D-glucopyranosyl ester.



compound	R'	R"
1		
2		
3 R=H 3a R=Ac		

Compound **2**, obtained as white powder, showed the $[M+Na]^+$ peak at m/z 1097 in the FAB mass spectrum. Acid hydrolysis of **2** afforded D-fucose and D-glucose. The ^1H and ^{13}C NMR spectrum of **2** indicated that the aglycone was pyrocincholic acid with the glycosidation sites at C-3 and C-28. Four anomeric signals were displayed at δ 4.73(d, 7.6), 5.19(d, 7.6), 6.19(d, 8.1), 4.97(d, 7.8) in the ^1H NMR spectrum and at δ 105.0, 105.9, 95.5, 105.1in the ^{13}C NMR spectrum respectively. The J values of those anomeric protons indicated that those sugar moietes gave a β-configuration. The assignment of the corresponding protoned carbon shifts was completed with the aid of 2D experiments. The lowfield shift of one of the glucose anomeric signals(H-1'''at δ6.19, C-1'''at δ95.5) suggested the esterifying unit of C-28 aglycone. The position of the attachment of the sugar chains to the aglycone were revealed by the glycosidation shift of the sugar carbon signals, that were confirmed by the NOESY experiment(Table 3). From these, the structure pyrocincholic acid 3β-O-β-D-glucopyranosyl(1 → 2)-β-D-fucopyranosyl-(28 → 1)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl ester was assigned to **2**.

Table 1. ^{13}C NMR spectral data for 1, 2 and 3a

No.	1	2	3a	4*	DEPT	No.	1	2	3a
1	38.3	38.4	38.0	38.5	CH2	***3-o-***	rha	fuc	glc
2	26.0	26.7	26.4	26.8	CH2	1'	104.3	105.0	103.5
3	88.8	88.9	90.5	88.9	CH	2'	72.4	81.6	77.9
4	39.2	39.5	39.4	39.6	C	3'	72.8	73.8	75.3
5	55.4	55.7	55.5	55.8	CH	4'	74.0	72.3	69.4
6	18.8	18.6	18.6	18.7	CH2	5'	69.8	70.7	72.1
7	39.5	39.5	39.3	39.6	CH2	6'	18.4	17.2	62.5
8	38.0	37.9	37.8	37.9	C	***3/28-***	glc	glc	glc
9	56.4	56.4	56.1	56.5	CH	1"	95.6	105.9	100.9
10	37.1	37.0	37.0	37.2	C	2"	74.1	76.8	71.5
11	18.0	17.9	17.9	18.1	CH2	3"	79.1	77.8	73.5
12	32.0	31.9	31.8	32.2	CH2	4"	71.2	71.3	69.0
13	130.3	130.1	130.6	130.7	C	5"	78.7	77.9	71.8
14	136.9	136.8	136.2	136.9	C	6"	62.3	62.5	62.2
15	20.9	20.8	20.5	21.2	CH2	***28-o-***		glc	glc
16	24.0	24.1	23.5	24.1	CH2	1'''		95.5	92.0
17	45.7	45.5	45.6	45.2	C	2'''		75.2	70.7
18	39.4	39.3	38.9	39.8	CH	3'''		78.5	73.3
19	41.5	41.3	41.2	41.7	CH2	4'''		70.8	68.9
20	30.5	30.4	30.4	30.8	C	5'''		77.8	74.0
21	34.3	34.2	34.0	34.6	CH2	6'''		69.3	67.6
22	31.2	31.0	31.0	31.2	CH2	*28-o-*		glc	glc
23	28.1	28.0	27.6	28.2	Me	1''''		105.1	100.9
24	16.5	16.6	16.6	16.6	Me	2''''		75.0	71.6
25	16.4	16.4	16.1	16.6	Me	3''''		78.2	73.3
26	20.8	20.7	20.9	20.8	Me	4''''		71.5	69.1
27	-	-	-	-	-	5''''		78.2	72.1
28	176.8	176.6	175.6	180.2	C	6''''		62.5	62.4
29	32.4	32.2	32.4	32.5	Me				
30	24.9	24.9	24.5	25.1	Me				

Table 2. ^{1}H NMR spectral data for 1, 2 and 3a

No.	1	2	3a
H-3	3.19 dd (3.6,11.3)	3.28 dd (4.1,11.5)	3.24 dd (3.6,11.3)
Me-23	1.13 s	1.28 s	1.24 s
Me-24	0.78 s	0.79 s	0.93 s
Me-25	0.91 s	1.04 s	1.09 s
Me-26	0.90 s	1.10 s	1.08 s
Me-29	0.89 s	0.86 s	0.91 s
Me-30	0.78 s	0.85 s	0.96 s
H-1'	5.35 s	4.73 d (7.6)	4.84 d (7.7)
H-1"	6.30 d (8.1)	5.19 d (7.6)	5.23 d (7.4)
H-1"'		6.19 d (8.1)	6.28 d (8.1)
H-1""		4.97 d (7.8)	5.00 d (7.4)
Rha/fuc-Me	1.70 d (5.6)	1.51 d (6.3)	

Table 3. Cross-peaks in the NOESY spectra of 2 and 3a

compound	H-3/H-1'	H-2'/H-1"	H-6"'/H-1""
2	3.28/4.73	4.50/5.19	4.30/4.97
3a	3.24/4.84	4.14/5.23	3.89/5.00

Acid hydrolysis of **3** produced D-glucose only. On acetylation of **3** gave the peracetyl derivative **3a**. The genin of **3a** was identified as pyrocincholic acid with the glycosidation sites at C-3 and C-28 by comparison of the ^{1}H and ^{13}C NMR data with those of **1** and **2**. In the ^{1}H NMR spectrum of **3a**, three proton anomeric signals were observed at δ4.84(d, J=7.7), 5.23(d, 7.4), 6.28(d, J=8.1), 5.00(d, J=7.4). The complete assignment of the ^{1}H and ^{13}C NMR signals of four glucose units for **3a** were carried out by 2D NMR techniques. Comparison of the ^{13}C NMRsignals of four glucose units showed an upfield shift of C-1"'(δ92.0), suggesting to the ester-linked anomeric carbon ; and two downfield shifts of C-2'(δ77.9) and C-6"'(δ67.7), suggesting the glycosidation site of its corresponding glucose units. Those sugar attachments were confirmed by the NOESY correlations(Table 3). All these considations about **3a** led us to assign to **3** the structure of pyrocincholic acid 3β-O -β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(28→1)-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl ester.

Experimental

General. Melting points are uncorrected. The NMR spectra of **1, 2** and **3a** were recorded on a Bruker AM-400 spectrometers, all with TMS as internal standard and pyridine-d_5 as solvent.

Extraction and isolation. The roots of *A. rubella* were collected in Jiang-su, China and authenticated by vice professor Huang xu-lan. A voucher specimen is deposited at Shanghai Institute of Materia Medica. The air-dried roots(5.0 kg) were extracted with EtOH and 228 g extract was obtained, which was partitioned with petrol ether, Et_2O, $CHCl_3$, EtOAc and n-BuOH successively from a MeOH-H_2O soln. The butanol fraction (92 g) was chromatographed on a silica gel column using EtoAc-MeOH as eluent. The fractions, eluted with EtoAc-MeOH 9:1(fraction A) and 3:1(fraction B), were further chromatographed on a silica gel column with $CHCl_3$:MeOH:H_2O and ODS(RP-18) column with MeOH:H_2O to obtain from fraction A compound **1**(13mg), and from fraction B compound **2** (280mg), **3**(90mg).

Compound **3** was acetylated as usual with Ac_2O/pyridine to give **3a**.

Compound **1.** White power; Mp 165~170^{0}C; [α]$^{27}_D$ -39.55°(MeOH; *c* 0.002690); IR ν max (KBr) cm^{-1} 3400(br), 2940, 1730, 1645, 1072; FABMS m/z 773[M+Na]$^+$; ^{1}H and ^{13}C nmr see table 1 and 2.

Compound **2**. Mp 217~220^{0}C; white powder; [α]$^{20}_D$ - 24.18° (MeOH; *c* 0.009511); IR ν max (KBr) cm^{-1} 3406, 2941, 1734, 1645, 1072; FAB MS m/z 1097[M+Na]$^+$; ^{1}H and ^{13}C nmr see table 1 and 2.

Compound **3a**. Mp 125~130^{0}C ; white powder; [α]$^{20}_D$ - 23.08° (MeOH; *c* 0.004767); IR ν max (KBr) cm^{-1} 2945, 1755, 1367, 1221, 1040; ^{1}H and ^{13}C nmr see table 1 and 2.

Acid hydrolysis of 1, 2 and 3. Compound **1, 2** and **3** (5 mg respectively) were submitted to acid hydrolysis in the usual manner. The sugars were identified by comparison with authentic samples of L-rhamnose, D-glucose and D-fucose by PC.

Basic hydrolysis of 1. Compound **1** (5mg) were submitted to alkaline hydrolysis in the usual manner to yielded 4 identified by TLC.

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NEW NEOHOPANE TRITERPENOIDAL SAPONIN GLYCOSIDES FROM *GLIMUS LOTOIDS* VAR. *DECTAMNOIDS*

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Key Word index--*Glinus lotoids var. dectaminoids*, Molluginaceae; neohopane, triterpenoidal saponins; glinisides D and E.

Abstract--Two neohopane triterpenoidal saponins(glinusides D and E) were isolated from the n-butanol fraction of *Glinus lotoids var. dectaminoids*, their structures elucidated as 3-O- β -L-arabinopyranosyl -22-O- β -D-glucopyranosyl (4←1)-α-L-rhamnopyranosyl-16- β -hydroxy-5- β -neohoane and 3-O- β -larabinopyranosyl -22-O- β -D-glucopyransyl(4←1)- β -D-glucopyranosyl-16- β -5- β -neohopane respectively by means of spectral data, especially NMR including COSY, HMQC, HMBC, NOESY techniques.

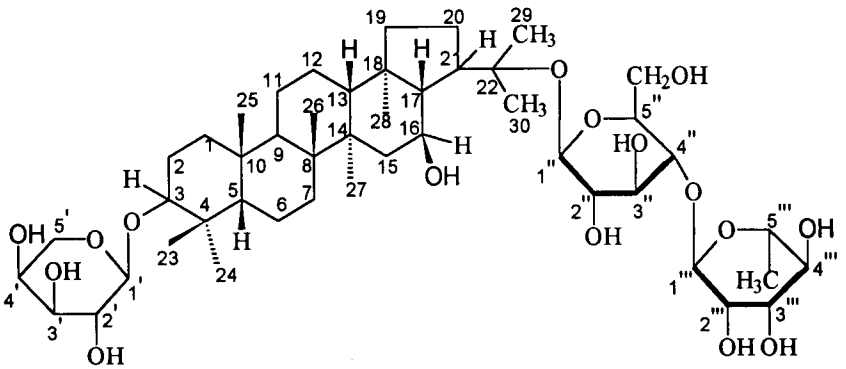
Introduction

In previous paper we have reported the isolation and structure elucidation of three new saponins[1] from the plant *Glinus lotoides* L. =*dictamnoides Burm*, (=*Mollugo glinus* A. Rich)[2]. All of these have been shown to be hopane glycosides carrying a tetrasaccharide unit and disaccharide unit. The present paper deals with the structure elucidation of two saponins of neohopane. The plant is widely distributed in the Allaqiarea, southof Aswan[3]. Various species of the genus Glinus are used in the folk medicine for treatment of an antiseptic, anthelmintic, diarrhoea, bilious attacks, purgaeve, curing boils, wound, pains and strengthen weak children[4, 5].

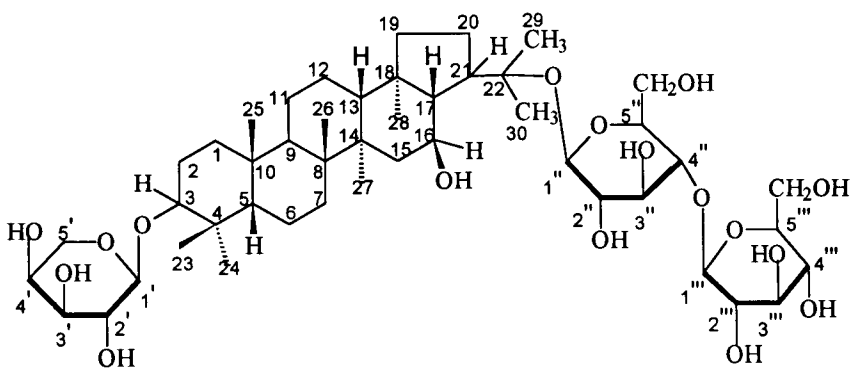
Results and Discussion

Successive extraction of a methanolic extract of *Glinus lotoides* var. *dectamnoides* with n-buanol led to isolation of five glycosidic compounds, three known[1] and two new. the first new compound(**1**) showed its $[M+2Na]^+$ ion peak at m/z 944 in the **FAB** mass spectrum, which was consistent with $C_{47}H_{78}O_{16}$.The 1HNMR suggested the presence of six methyl singlets at δ 0.71, 0.93, 0.98, 1.06, 1.55, 1.61 and two methyl singlets at δ 1.40 [6-9]; there was also a broad singlet integrated for one proton at δ 2.03 for H-5 which showed a cross peak with carbon at δ 88.99 of proton signal at δ 3.43(1H, dd, J=4.09, 9.14Hz, H-3)[6-15]. the dawn field shift of H-5 and its carbon(δ 31.19)indicated that its configuration as B with C-23[19]. there was a signal for one proton overlapping with sugar signals at δ 4.25 for H-16 which showed cross peak(HMBC) with C-13 and C-21.The HMNR spectrum contained signals for three anomeric protons at δ 6.56(1H, br s), δ 5.17(1H, d, J=7.6Hz) and δ 4.89(1H, d J=7.4Hz). A three proton doublet at δ 1.70(J=6.13Hz)was typical for the methyl group of a 6-deoxy sugar. The 13CNMR spectrum data revealed the presence of six C-saturated quaternary carbons(δ 38.78, 40.34, 42.81, 43.50, 45.41, 83.11) and three anomeric carbons (δ 98.43, 101.85, 106.40). The numbers and chemicals shifts of 1 with the tertiary methyl functions. Quaternary carbons and shift of C-5 (which resonated at δ 31.19) suggested that **1** was neohopan triterpene triglycoside. Upon acid hydrolysis of **1** with 5%-Methanol[15, 16], gave arabinose glucose and rhamnose as sugar components which were identified by TLC with direst comparison with authentic samples. The anomeric centers of the glucosyl moieties were each determined to have B-configuration based on large $J_{1,2}$values

(7.4-7.6Hz). The C-5 of the rhamnosyl unit resonated δ 69.70 and thus had B-configuration[18]. The identify of the 22-O-disacchride and 3-O-monosccharid was deduced by means of the 2 COSY, HMBC and NOESY, where there were cross peaks between H-1''(δ 5.17)and C-22(88.11), H-1'''(δ 6.56)and C-4''(δ) and H-1'(δ 4.89) and C-3(δ 88.99). The H and ^{13}CNMR spectra revealed that the aglycone was a triterpenoid with neohopane skeleton [6, 7, 13, 9] and contained three hydroxyl groups (two secondary at C-3, C-16 and one tertiary at C-22). All above data suggested that compound 1 was a glycoside with a new aglycone, 3 β , 16 β , 22 β -hydroxy-5- β -neohopane, which is isolated from a natural source for the first time. Hence, the isolated compound has the structure 3-O- β -L-arabinopyranosyl-22-O- β -D-glucopyranosyl (4-1)-A-L-rhamnopyranosyl-16- β hydroxy-5- β - neohopane (**1**); this compound has been given the name glinusid **D**.



1



2

The second new compound (**2**) exhibited its $[M+K+H_2O]^+$ ion peak at m/z 971 in the FAB mass spectrum and was consistent with $C_{47}H_{78}O_{17}$. The ^{1}HNMR suggested the presence of six methyl singlets at δ 0.70, 0.95,1.00,1.17,1.52, 1.58 and two methyl singlets at δ 1.40[7-10]; there was also a broad singlt integrated for one proton at 2.03 for H-5 that simillar

Table 1. ^{1}H NMR spectral data for the isolated saponin glycosides (400MHz, pyridine-d_5)

No.	Ginuside D aglycone	Glinuside E aglycone	No.	Glinuside D aglycone	Glinuside E aglycone
Aglycone moieties					
C-1	1.65, 1.00(each 1H, m)	1.65, 1.00(each1H, m)	C-27	1.61(3H, s)	1.58(3H, s)
C-2	2.24, 1.93(eachn 1H, m)	2.45, 1.93(each 1H, m)	C-28	1.55(3H, s)	1.52(3H, s)
C-3	3.43(1H, dd, J=4.09, 9.14Hz)	3.45(1H, dd, J=5.16, 10.84Hz)	C-29	1.40(3H, s)	1.40(3H, s)
C-4	----	----	C-30	1.40(3h, s)	1.40(3H, s)
C-5	2.03(1H, S)	2.03(1H, s)	Sugar moieties		
C-6	1.07, 1.38(each 1H, m)	1.07, 1.38(each 1H, m)		Arabinose	Arabinose
C-7	1.95(2h, m)	1.95(2H, m)	1'	4.98(1H, d, J=7.4Hz)	4.92(1H) *
C-8	------	-------	2'	4.02(1H)*	4.05(1H)*
C-9	1.27(1H)*	1.18(1H)*	3'	4.00(1H)*	4.01(1H)*
C-10	-------	----------	4'	4.17(1H)*	4.17(1H)*
C-11	1.53, 1.29(each 1H, m)	1.53, 1.29(each 1H, m)	5'	3.72, 4.16(each 1H)*	3.72, 4.16(each 1H)8
C-12	1.34, 1.29(each 1H, m)	1.34, 1.29(each 1H, m)		Glucose	glucose
C-13	1.53(1H, m)	1.53(1H, m)	1''	5.17(1H, d, 7.6Hz)	5.16(1H, d, J=7.65Hz)
C-14	-------	-------	2''	3.99(1H, d, J=8.4Hz)	3.99(1H, d, J=8.4Hz)
C-15	1.60, 1.15(each 1H, m)	1.60, 1.15(each 1H, m)	3''	4.13(1H, d, J=8.4Hz)	4.15(1H, d, J=8.4Hz)
C-16	4.25(1H)*	4.25(1H)*	4''	4.86(1H, m)	4.92(1H, m)
C-17	1.89(1H)*	1.90(1H)*	5''	3.70(1H, m)	3.78(1H, m)
C-18	-----	-------	6''	4.30, 4.40(each 1H)*	4.30, 4.40(each 1H)*
C-19	1.40, 0.90(each 1H, m)	1.40, 0.90(each 1H, m)		Rhamnose	Glucose
C-20	1.60, 1.35(each 1H, m)	1.70, 1.40(each 1H, m)	1'''	6.56(1H, br s)	4.99(1H, d, J=7.20Hz)
C-21	2.73(1H, m)	2.72(1H, m)	2'''	4.87(1H)*	4.92(1H)*
C-22	-------	---------	3'''	4.37(1H)*	4.14(1H)*
C-23	0.98(3H, s)	1.00(3H, s)	4'''	4.27(1H)*	4.86(1H)*
C-24	1.06(3H, s)	1.17(3H, s)	5'''	4.23 (1H, m)	3.80(1H), t, J=4.89Hz)
C-25	0.71(3H, s)	0.70(3H, s)	6'''	1.72(3H, d, j+6.13Hz)	4.32(1H, m),
C-26	0.93(3H, s)	0.95(3H, s)			4.56(1H, dd, J=2.40, 11.68Hz)

Multiplicity was detected by DEPT experiment.
* Overlapping with other signals.

configuration with compound(**1**). The ^{1}HNMR spectrum contained signals for three anometic protons at δ 5.15 (1H, d J=7.65Hz), δ 4.99(1H, d, J=7, 15Hz) and δ 4.92 overlapping with water signal. Acid hydrolysis of compound (**2**) afforded glucose and arabinose which were identified by TLC(direct comparison with authentic samples. Comparison of the H and CNMR data (table 1 and table 2)of **2** with those of 1 it contain the same sugar moieties as 1 except rhamnose unit which showed dawn shift of its carbon(δ 107.69). From above evidence, the structure of compound **2** was proved to be 3-O- β -L arabinopyranosyl -22-O- β -D-glucopyranosyl(4←1) - β -D-glucopyranosyl -16- β hydroxy-5 β -neohopane which is isolated from a natural source for the first time and this compound has been given the name glinusid E.

Table 2. ^{13}C NMR spectral data for the isolated saponin glycosides (100MHz, pyridine-d5)

No.	Ginuside D aglycone	Glinuside E aglycone	No.	Glinuside D aglycone	Glinuside E aglycone
Aglycone moieties					
C-1	27.5(t)	27.2(t)	C-27	18.7(q)	18.7(q)
C-2	26.9(t)	26.5(t)	C-28	18.4(q)	18.4(q)
C-3	88.9(d)	88.8(d)	C-29	23.8(q)	23.8(q)
C-4	45.4(s)	46.0(s)	C-30	24.9(q)	25.0(q)
C-5	31.1(d)	31.1(d)	Sugar moieties		
C-6	39.3(t)	39.2(t)		Arabinose	Arabinose
C-7	39.2(t)	39.2(t)	1'	106.4(d)	106.4(d)
C-8	42.8(s)	42.8(s)	2'	74.1(d)	74.1(d)
C-9	48.9(d)	48.9(d)	3'	78.6(d)	78.4(d)
C-10	38.8(s)	38.8(s)	4'	71.7(d)	71.0(d)
C-11	21.2(t)	21.1(t)	5'	66.9(t)	66.8(t)
C-12	23.9(t)	23.8(t)		Glucose	Glucose
C-13	49.7(d)	98.4(d)	1''	98.4(d)	98.4(d)
C-14	43.5(s)	43.5(s)	2''	79.0(d)	79.0(d)
C-15	44.1(t)	44.1(t)	3''	75.3(d)	75.6(d)
C-16	77.9(d)	77.8(d)	4''	66.9(d)	66.9(d)
C-17	50.6(d)	50.5(s)	5''	78.6(d)	78.6(d)
C-18	40.3(s)	40.3(s)	6''	62.8(t)	62.8(t)
C-19	43.7(t)	43.7(t)		Rhamnose	Glucose
C-20	45.0(t)	45.0(t)	1'''	101.8(d)	107.8(d)
C-21	67.6(d)	67.6(d)	2'''	72.5(d)	79.5(d)
C-22	83.1(s)	83.0(s)	3'''	71.5(d)	75.0(d)
C-23	17.3(q)	17.3(q)	4'''	77.9(d)	66.8(d)
C-24	17.3(q)	17.3(q)	5'''	69.6(d)	78.4(d)
C-25	17.1(q)	17.1(q)	6'''	18.4(q)	62.9(t)
C-26	17.1(q)	17.1(q)			

Multiplicity was detected by DEPT experiment.

Experimental

Melting points were taking on Yamazawa micro-melting point apparatus; optical rotations were measured on a JASCO-360 digital polarimeter; UV spectra were obtained on a Hitachi 200-10 spectrophotometer; IR spectra were taken on a JASCO IR-A-2 spectrometer; HNMR, !3CNMR, 1H-H COSY, NOESY, HMQC and HMBC spectra were taken on a Bruker AM-400, Bruker AM-500; MS were obtained on Hitachi Rmu -7M spectrometer.

Extraction and isolation of the compounds. As mentioned earlier ref. [1].

Acid hydrolysis of the saponin. The saponin (5mg)was refluxed with 5ml 5% HCl-MeOH on a steam bath for 6 hr. The product was diluted with H_2O and extracted with $CHCl_3$ in a separatory funnel, and therespective aglycones were sepd. The aq. Filtrate was neutralized

with $AgCO_3$, filtered and evapd. The resulting syrup was subjected to TLC on silica gal using EtOAc -MeOH -HoAc -H_2O (13:3:4:3) as developing solvent against ref. Sugars, and the dried chromatograms was sprayed with p-anisadehyde-H_2SO_4 reagent.

Glinuside D(1). Powder, [α]$_D$ -9.73(c=0.26 MeOH); IR v $^{Kbr}_{max}$cm-1:3245.Negative FAB-MS m/z (rel. int):944 $[M+2Na]^+$ (25) ($C_{47}H78O_{16}$), 687 $[M\text{-}Glu\text{-}Rhan\text{-}H]^+$ (25), 517 [M-Glu Rham -Ara-CH3+Na]$^+$ (20), 325(100), 183(58); ^{1}HNMR(400MHz, pyrine-d5) and ^{13}CNMR(100MHz, pyridine-d5): Table 1 and 2.

Glinuside E(2). Powder, [α]$_D$ -4.34(c=0.05 MeOH); IR V^{KBr}_{max} cm-1 1:3245.. Negative FAB -MS m/z (rel. int.): 971 $[M+K+H_2O]^+$ (45) ($C_{47}H_{80}O_{17}$), 451 $[M\text{-}Glu]^+$ (70), 555 $[M\text{-}Glu\text{-}Glu\text{-}3H]^+$ (70), 455 $[M\text{-}Glu\text{-}Glu\text{-}Ara]^+$ (40); ^{1}HNMR(400MHz, pyridine-d5) and ^{13}CNMR(100 MHz, pyridine -d5): Table 1 and 2.

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TRITERPENOID SAPONINS FROM *BERNEUXIA THEBETICA* DECNE

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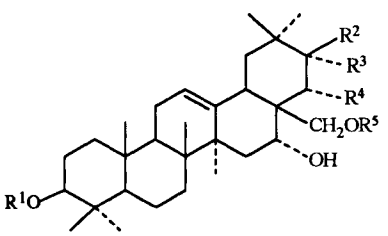
Key Word Index — *Berneuxia thibetica* Decne; triterpenoid saponins; berneuxia saponins A, B, C; desacyl jegosaponin.

Abstract — Four triterpenoid saponins were isolated from *Berneuxia thibetica* Decne. On the basis of chemical and spectroscopic evidence, three new saponins, berneuxia saponins A, B and C were elucidated as 21-tigloylbarringtogenol C-3β-O-α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside], 28-tigloyl-barringtogenol C-3β-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside] and 16α-28-dihydroxyolean-12-en-12-one-3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside]. The known saponin was desacyl jegosaponin.

Introduction

Berneuxia thibetica Decne, which is spread in the Southwest of China, is used as Chinese folk medicine for curing cough due to pathogenic wind-cold factors, overstrain, asthma and dyspea, and wound[1].We have reported sterols, triterpenes and flavones from the EtOAc part of EtOH extracts[2,3,4].We now report the triterpenoid saponins isolated from the leaves of this plant.



	R^1	R^2	R^3	R^4	R^5
1	rha(1→2)gal(1→3)[glc(1→2)glu]	tigloyl	H	OH	H
1a	H	tigloyl	H	OH	H
1b	glc(1→2)glu	tigloyl	H	OH	H
2	rha(1→2)gal(1→3)[glc(1→2)glu]	OH	H	OH	tigloyl
2a	H	OH	H	OH	tigloyl
3	rha(1→2)gal(1→3)[glc(1→2)glu]	$R^2, R^3 = O$		H	H
3a	H	$R^2, R^3 = O$		H	H
4	rha(1→2)gal(1→3)[glc(1→2)glu]	OH	H	OH	H

Results and Discussion

The crude saponin fractions were subjected to repeated CC on silica gel and silanised silica gel, affording saponins **1**, **2**, **3** and **4**.The yields were 0.2% , 0.02%, 0.1% and 0.004% of the dry leaves, respectively.

On mineral acid hydrolysis, saponin **1** yielded the aglycone **1a**, which was identified as

21-tigloylbarringtogenol C by comparison with an authentic sample. This aglycone was also isolated from the EtOAc of this plant. Four kinds of sugars, D-glucuronose, D-glucose, D-galactose and L-rhamnose, were detected by PC in the water after the remove of the aglycone. The EI-mass spectrum of its acetate showed fragment ions at *m/z* 273[terminal rhamnose$(Ac)_3]^+$, 331[terminal glucose $(Ac)_4]^+$ and 561[rhamnose$(Ac)_3$ galactose$(Ac)_3]^+$. The FAB-mass spectrum showed the molecular ion at *m/z* 1219 $\{[M+H]^+\}$. The negative FAB-mass spectrum showed the fragment ions at *m/z* 1217 $[M-H]^-$, 1071[M-rhamnose]$^-$, 1055[M-glucose]$^-$ and 909[M-rhamnose-galactose]$^-$. The ^{13}CNMR spectrum due to sugar moieties indicated the presence of four monosaccharide units. On 1M HCl hydrolysis, **1** gave prosapogenin **1b**. The hydrolysis of **1b** gave the 21-tigloylbarringtogenol C as the aglycone, and D-glucuronose and D-glucose as the sugar components. The EI-mass spectrum of the acetate of **1b** showed fragment ions at 331[terminal glucose$(Ac)_4]^+$, 605[glucose$(Ac)_4$ glucuronose-OMe $(Ac)_2]^+$. The ^{13}CNMR spectrum due to sugar moieties indicated the presence of two monosaccharide units. The glycosylation shift (82.4ppm) indicated that the β-D-glucuronopyranose was 2-O-glycosylated[5]. Therefore, the structure of **1b** was 21-tigloylbarringtogenol C-3-O-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside. Comparison of the ^{13}CNMR signals due to sugar moieties of **1** with those reported by I. Calis et al [6], the sugar moieties were the same. The downfield shift of **1b** (δ78.6 of **1** to δ82.4 of **1b**) may be caused by 3-O-glycosylation of β-D-glucuronopyranose. On the basis of above, saponin **1** was elucidated as 21-tigloylbarringtogenol C-3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside], named berneuxia saponin A.

Saponin **2** yielded the aglycone **2a** by mineral acid hydrolysis. **2a** was identified as 28-tigloylbarringtogenol C by comparison of the ^{1}HNMR signals with those reported[7]. Four kinds of sugars, D-glucuronose, D-glucose, D-galactose and L-rhamnose, were detected by PC in the water after the remove of the aglycone. The FAB-mass spectrum showed the molecular ion at *m/z* 1219 $\{[M+H]^+\}$. The negative FAB-mass spectrum showed the fragment ions at *m/z* 1217 $[M-H]^-$, 1071[M-rhamnose]$^-$, 1055 [M-glucose]$^-$ and 909 [M-rhamnose-galactose]$^-$. The ^{13}CNMR spectrum of **2** resembled that of **1**, indicating they were only different in the substitution position of tigloyl group. By comparison of ^{13}CNMR signals due to aglycone of **1** with those of **2**, the obvious difference of chemical shifts occurred at C-16, 21, 22, 28, 29, indicating the C-28 was substituted by tigloyl group. Therefore, saponin **2** were identified as 28-tigloylbarringtogenol C-3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside], named berneuxia saponin B.

On mineral acid hydrolysis, saponin **3** yielded the aglycone **3a**. There are 30 signals in ^{13}CNMR spectrum of **3a**. EI-mass spectrum showed molecular ion at *m/z* 472$[M]^+$. According to EI-mass spectrum and ^{13}CNMR spectrum, **3a** was identified as 3β, 16α, 28-trihydroxylolean-12-en-21-one, whose EI-mass spectrum and ^{1}HNMR were identical to those of armillarigenin from *Jacquinia armillaris* [8]. Four kinds of sugars, D-glucuronose, D-glucose, D-galactose and L-rhamnose, were detected by PC in the water after the remove of the aglycone. The negative FAB-mass spectrum showed the fragment ions at *m/z* 1117 $[M-H]^-$, 971[M-rhamnose]$^-$, 955[M-glucose]$^-$ and 809[M-rhamnose-galactose]$^-$. By comparison of the ^{13}CNMR signals due to sugar moieties of **3** with those of **1**, the sugar moieties were the same. On the basis of above, saponin **3** was elucidated as armillarigenin-3-O-α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl (1→3)[β-D-glucopyranosyl (1→2)-β-D-glucuronopyranoside], named berneuxia saponin C.

Hydrolysis of **4** yielded the **4a** as the aglycone and D-glucuronose, D-glucose, D-galactose and L-rhamnose as sugars. The EI-mass spectrum of its acetate showed fragment ions at *m/z* 273[terminal rhamnose$(Ac)_3$]$^+$, 331[terminal glucose $(Ac)_4$]$^+$ and 561[rhamnose$(Ac)_3$ galactose$(Ac)_3$]$^+$. The negative FAB-mass spectrum showed the fragment ions at *m/z* 1135 [M-H]$^-$, 989 [M-rhamnose]$^-$, 973 [M-glucose]$^-$ and 827 [M-rhamnose-galactose]$^-$. **4a** has the same R*f* as the alkaline hydrolysate of **1a** on silica HPTLC with different solvent systems. ^{13}CNMR signals due to aglycone were accord with barringtogenol C except for the position of C-3[9]. By comparison of the ^{13}CNMR signals due to sugar moieties of **4** with those of **1**, the sugar moieties were the same. On the basis of above, **4** was elucidated as barringtogenol C-3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranoside], which was the same as desacyl jegosaponin from *Styrax japonica* [10].

Experimental

^{1}H and ^{13}CNMR spectra were recorded at 300MHz in pyridine-d_5 and $CDCl_3$ using TMS as int. standard. EI-MS was measured at 40eV accelerating voltage after acetylation. FAB-MS was measured with VG ZAB mass spectrometer. Optical rotations were measured with PE241 automatic recording spectropolarimeter.

Plant material. Leaves of *Berneuxia thibetica* Decne. were collected in Xichang of Sichuan Province, China, and identified by Prof. R. N. Zhao. A specimen is deposited in the Herbarium of the Chengdu Institute of Biology, Chinese Academy of Sciences.

Extraction and isolation of saponins. Dry leaves (1.8kg) were extracted with 95% EtOH. After removal of solvent by evapn, the combined extracts (180g) were suspended in H_2O, extracted with petroleum ether, EtOAc and *n*-BuOH successively. The *n*-BuOH part (45g) were dissolved with MeOH, precipitated with ether to obtain crude saponins (40g). The crude saponin fr. was chromatographed on a silica gel column, eluting with $CHCl_3$-MeOH-H_2O to yield frs **1** - **46**. Fr. **26**(1.12g) was purified by silanised silica gel 60 to give saponins **1** (150.2mg) and **2** (106mg). Fr. **43** was purified by silanised silica gel 60 to give saponin **3** (202mg). Fr. **46** was recrystallized to give saponin **4** (80mg). Compound **1**, $C_{59}H_{94}O_{26}$, $[\alpha]_D^{13}$ −12.2°(MeOH, *c* 1.1). EI-MS *m/z*: 273 [terminal rhamnose$(Ac)_3$]$^+$, 561[rhamnose$(Ac)_3$ galactose$(Ac)_3$]$^+$, 331[terminal glucose$(Ac)_3$]$^+$. FAB-MS *m/z*: 1219{[M+1]$^+$ ($C_{59}H_{94}O_{26}$+H)}. Negative FAB-MS *m/z*: 1217{[M-1]$^-$ ($C_{59}H_{94}O_{26}$ −H)}, 1071[M-rhamnose]$^-$, 1055[M-glucose]$^-$, 909[M-rhamnose-galactose]$^-$. Compound **1a** was identified as 21-tigloylbarringtogenol C by comparison with an authentic sample. Compound **2**, $C_{59}H_{94}O_{26}$, $[\alpha]_D^{26}$ −15.6°(MeOH, *c* 1.0). Negative FAB-MS *m/z*: 1217 {[M-1]$^-$ ($C_{59}H_{94}O_{26}$ - H)}, 1071 [M-rhamnose]$^-$, 1055[M-glucose]$^-$, 909 [M-rhamnose-galactose]$^-$. **2a**, white powder, ^{1}HNMR($CDCl_3$) δ(ppm): 6.89(1H, q, J=6.4Hz, 3'-H), 5.35(1H, br s, 12-H), 4.31(1H, br s, 16-H), 4.09(1H, d, J=12.3Hz, H-28), 3.84(1H, d, J=10.5Hz, 21α-H), 3.75(1H, d, J=12.7Hz, H-28), 3.70(1H, d, J=10.5Hz, 22β-H), 3.23(1H, m, 3α-H). Compound **3**, $C_{54}H_{86}O_{24}$, $[\alpha]_D$ −6.8°(MeOH, *c* 0.75). FAB-MS *m/z*:1141 {[M + Na]$^+$ ($C_{54}H_{86}O_{24}$+H)}. Negative FAB-MS *m/z*: 1117 {[M-1]$^-$ ($C_{54}H_{86}O_{24}$ - H)}, 971 [M-rhamnose]$^-$, 955[M-glucose]$^-$, 809 [M-rhamnose-galactose]$^-$. **3a**, colorless needles, mp 296~298 °C{ Ref. 299~301°C [9]}(MeOH). EI-MS *m/z*: 472{[M]$^+$}, 454, 436, 424, 264(a), 233, 215, 208(b), 190. ^{1}HNMR ($CDCl_3$) δ (ppm) : 5.44(1H, t, J=3.3Hz, 12-H), 3.85(1H, m, 16-H), 3.28(1H, m, 3-H), 3.25(1H, d, J=5.0Hz, 28-H), 3.21(1H, d, J=5Hz, 28-H), 2.52(2H, d, J=6Hz, 22-H), 1.32, 1.25, 1.14, 1.07, 1.00, 0.93,

0.79(each 3H, s). Compound **4**, $C_{54}H_{88}O_{25}$, white powder. EI-MS *m/z*: 273 [terminal rhamnose$(Ac)_3]^+$, 561[rhamnose$(Ac)_3$ galactose$(Ac)_3]^+$, 331[terminal glucose$(Ac)_4]^+$. FAB-MS *m/z*:1159 {$[M+Na]^+$ ($C_{54}H_{88}O_{25}$+Na)}. Negative FAB-MS *m/z*: 1135 {$[M-1]^-$ ($C_{54}H_{88}O_{25}$ - H)}, 989 $[M\text{-rhamnose}]^-$, 973[M-glucose]$^-$, 827 $[M\text{-rhamnose-galactose}]^-$.

Acid hydrolysis of the saponins and identification of the resulting monosaccharide. Saponin **1** (100mg) was dissolved in MeOH and heated with 1M HCl/MeOH for 50min. The solution was evapn below 30°C. The residue was chromatographed on silica gel to obtain **1b** (13mg). Other frs were further hydrolysed with 5%H_2SO_4. The reaction mixture was diluted in H_2O and extracted with $CHCl_3$. **1a** was isolated from the $CHCl_3$ layer. The aq. Layer was neutralized with $Ba(OH)_2$ and concentrated, then subjected to PC analysis with authentic samples, Developing solvent BuOH–AcOH–H_2O (4:1:5) (upper layer), detection reagent: aniline-phthalate. Each saponin of **2–4** (20mg) was heated with 5%H_2SO_4. The sugars were detected as above. The aglycones were obtained from $CHCl_3$ layer.

Acetylation of saponins. To each saponin (5mg) was added Ac_2O-pyridine (1:1) (0.5ml) in a microtube. After standing at room temperature for 48 hrs, the soln was evapd to dryness and then subjected to EIMS analysis.

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Table 1 ^{13}CNMR shifts of compounds **1 – 4** and their prosapogenins(δ, ppm)

Carbon	1^1	$1a^2$	$1b^1$	2^1	$2a^2$	3^1	$3a^2$	4^1
1	38.6	38.4	38.8	39.2	38.6	38.7	38.8	38.4
2	26.0	26.5	26.5	26.7	26.4	27.5	27.2	25.9
3	90.2	78.5	89.2	90.8	79.2	90.2	78.9	90.1
4	39.4	38.4	38.8	40.1	38.8	39.5	38.7	39.2
5	55.4	54.9	55.7	56.1	55.2	55.7	55.3	55.4
6	18.1	18.0	18.3	18.9	18.2	18.1	18.3	18.1
7	32.8	32.4	33.0	33.5	32.8	33.0	32.5	32.7
8	39.7	39.4	39.5	40.4	39.7	39.5	40.3	39.6
9	46.6	46.3	46.9	47.3	47.0	48.1	47.1	46.5
10	36.4	36.6	36.7	37.1	37.9	36.7	36.9	36.3
11	23.5	23.2	23.8	24.3	23.7	23.8	23.5	23.4
12	123.7	123.3	122.7	123.8	124.4	123.9	124.1	122.8
13	143.1	141.5	143.5	143.8	141.1	143.1	140.8	143.4
14	41.4	41.0	41.8	42.2	41.3	42.0	41.5	41.5
15	33.8	32.9	34.2	35.1	33.7	34.3	34.4	33.8
16	67.4	68.8	67.8	68.5	68.3	75.6	74.0	67.8
17	47.7	46.3	47.7	47.2	46.7	46.7	46.5	46.8
18	40.1	40.3	40.3	41.3	40.5	42.0	42.5	40.6
19	47.3	46.7	48.0	48.6	47.1	46.9	47.6	47.7
20	36.0	35.4	36.3	36.8	36.9	44.7	44.6	35.9
21	81.4	80.8	81.8	78.6	78.9	216.4	209.8	77.7
22	75.2	75.8	75.3	77.4	75.7	40.5	40.3	75.8
23	27.4	28.7	28.0	28.4	28.9	27.8	28.0	27.5
24	16.2	15.2	16.6	17.1	15.6	16.6	15.8	16.2
25	15.4	15.2	15.6	16.0	15.2	15.6	15.6	15.2
26	16.6	16.2	16.9	17.5	16.9	17.0	17.3	16.5
27	26.9	26.5	27.3	28.2	27.2	26.1	26.4	27.0
28	65.9	66.6	65.9	67.3	68.1	65.2	69.5	67.3
29	29.6	27.6	29.8	31.0	28.0	27.8	27.8	30.1

30	20.1	19.3	20.3	19.8	20.7	25.9	23.8	19.0
1'	168.4	169.4	168.5	168.4	167.9			
2'	129.4	128.4	129.8	129.5	128.7			
3'	135.9	137.5	136.1	137.7	138.0			
4'	12.0	11.7	12.4	12.7	12.2			
5'	14.1	14.0	14.1	14.7	14.2			
glu-1	104.9		105.7	105.5		104.9		104.6
2	78.6		82.4	79.3		78.9		78.6
3	81.1		77.8	82.1		81.7		81.3
4	71.9		73.0	71.9		72.5		72.1
5	75.2		76.8	76.2		75.7		75.3
6	172.2		170.3	176.9		175.3		175.1
			52.0					
glc-1	101.4		101.6	102.4		101.7		101.5
2	73.1		72.6	74.1		72.9		73.2
3	77.6		78.1	78.6		78.1		77.7
4	70.8		71.5	71.8		71.2		71.3
5	76.5		77.4	76.6		76.8		75.8
6	62.0		62.6	62.8		62.0		61.7
gal-1	101.9			102.8		102.0		101.9
2	77.8			79.0		78.1		78.1
3	75.6			76.2		76.1		75.3
4	70.8			71.8		71.2		71.3
5	76.5			77.4		76.8		76.7
6	63.1			64.0		63.5		63.1
rha-1	100.1			101.2		100.7		100.4
2	72.1			72.9		72.5		72.1
3	71.8			72.9		72.5		72.1
4	73.2			74.2		73.6		73.2
5	69.2			70.1		69.6		69.3
6	17.6			18.6		18.1		17.7

[1]: pyridine-d_5; [2]: $CDCl_3$

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EUPHANE TRITERPENE BISDESMOSIDE AND TRIDESMOSIDES FROM *RHOIPTELEA CHILIANTHA*

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Abstract--Four triterpene tridesmosides rhoiptelesides A~D (**1~4**) and a triterpene bisdesmoside rhoipteleside E (**5**) were isolated from *Rhoiptelea chiliantha* Diels *et* Hand.-Mazz. (Rhoipteleaceae). Their structures were elucidated on the basis of extensive analyses of 1D and 2D-NMR spectroscopic data and chemical reactions. Rhoiptelesides represent the first example of the glycoside of euphane-type triterpene.

Introduction

Rhoiptelea chiliantha Diels *et* Hand.-Mazz. which is distributed in Yunnan, Guizhou, Guangxi of China and northern Vietnam, is the sole species of the family Rhoipteleaceae. Since 1930's, the systematic position of this family has been widely studied from morphological, anatomical and palynological aspects, but still remains obscure [1]. From the viewpoint of chemotaxonomy, we have investigated the chemical constituents of this plant, and reported the structures of triterpenes, triterpene caffeates and triterpene-lignan esters from barks [2], diarylheptanoids [3] and dimeric ellagitannins formed by intermolecular oxidative C-C coupling [4] from fruits and leaves. Further studies on the chemical constituents of leaves and fruits led to the isolation of five triterpene glycosides named rhoiptelesides A (**1**)~ E (**5**). We described herein the isolation and structure determination of these novel compounds.

Results and Dicussion

The MeOH extract of the air-dried leaves (510 g) was extracted with Et_2O and EtOAc, successively. The H_2O layer was separated by a combination of column chromatographies over Sephadex LH-20, MCI-gel CHP 20P, ODS and silica gel to afford rhoiptelesides A (**1**)~ E (**5**). In a similar isolation procedure, **1** and **5** were obtained from the fruits.

Rhoipteleside A (**1**), a white amorphous powder, gave an $[M+Na]^+$ peak at *m/z* 919 in the positive FAB-MS. Careful analyses of ^{1}H-NMR, ^{1}H-^{1}H COSY and ^{13}C-NMR spectral data revealed that **1** is a triterpene glycoside with a fucose and two rhamnose residues in the molecule. The plane structure of the aglycone was unequivocally determined on the basis of ^{1}H-^{1}H COSY, HSQC and HMBC correlations. The locations of two rhamnoses and a fucose at C-3, C-23 and C-11, respectively, were also established by the observation of the HMBC correlations between the respective anomeric protons and the oxygen bearing carbon signals of the aglycone. Thus, **1** is a triterpene tridesmoside which is extremely rare in the nature [5].

The relative configuration of the aglycone was determined as follows. First, the small coupling constant of H-3 indicated that it is in an *equatorial* position, and the large coupling constant (11 Hz) between H-9 and H-11 revealed *trans* di-*axial* relationship between these protons. Next, the relative configurations of the methyl groups and the other protons in the rings A-D were determined on the basis of the ROESY correlations. Thus, the aglycone of **1** was deduced to be an euphane or tirucallane-type triterpene with different configuration at C-20. Hydrolysis of **1** with 10 % H_2SO_4 yielded an artificial aglycone **1a** along with L-rhamnose and D-fucose. A careful spin decoupling ^{1}H-NMR and a differential NOE experiment of **1a** showed the large coupling constant (10 Hz) between H-17 and H-20, and the NOE correlation between 18-Me and H-20, respectively, indicating that H-20 and 18-Me maintain a *pseudo* 1, 3-*diaxial* conformation. This is consistent with the X-ray crystallographic studies reported on tirucallane

and euphane-type triterpenes[6]. Furthermore, since the differential NOE spectrum of **1a** showed NOE correlation between 18-Me and H-23, without the correlation between 18-Me and 21-Me, the configuration of C-20 of **1a** was confirmed to be *S**, *i.e.*, euphane-type.

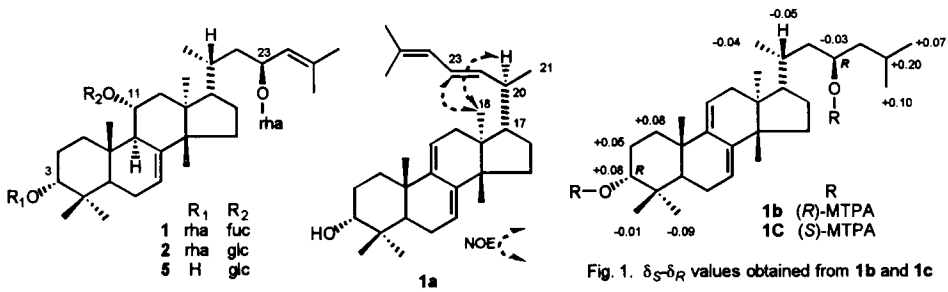


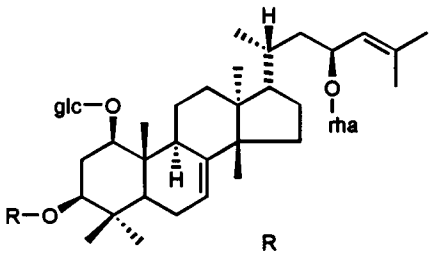
Fig. 1. δ_S-δ_R values obtained from **1b** and **1c**

To determine the absolute configuration of **1**, modified Mosher's method [7] was applied to MTPA esters (**1b** and **1c**), which were derived from **1** by successive hydrogenation, hydrolysis, and acylation (Fig. 1). The distribution of the positive and negative δ (δ_S-δ_R) values around the MTPA ester groups unequivocally confirmed that the configurations of C-3 and C-23 of **1** are *R* and *S*, respectively. Thus the absolute structure of rhoipteleside A was established to be as shown by the formula **1**.

Rhoipteleside B (**2**) was isolated as a white amorphous powder. Its positive FAB-MS exhibited an $[M+Na]^+$ ion peak at *m/z* 935, which is 16 mass unit more than that of **1**. The ^1H and ^{13}C-NMR spectra of **2** resembled those of **1**, especially the ^{13}C-NMR spectral data attributable to the aglycone are almost superimposable to those of **1**. The only difference is the presence of a glucose residue in **2** instead of a fucose in **1**. The locations of two rhamnoses and a glucose were determined to be at C-3, C-23 and C-11, respectively, by the NOE observations between the anomeric protons and oxygen-bearing methine protons. Consequently, the structure of **2** was assigned to rhoipteleside B.

Rhoipteleside E (**5**) was indicated to be a bisdesmoside because only two anomeric proton signals were observed in its ^1H-NMR spectrum. These two sugars were elucidated to be rhamnose and glucose by analyzing the ^1H and ^{13}C-NMR data of **5** and its acetate. The ^{13}C-NMR signals derived from the aglycone are almost identical to those of **1**, except for the downfield shift of C-2 and upfield shift of C-3. These indicated that **5** possesses the same aglycone as that of **1** and **2**, the C-3 hydroxyl group of which is free. A glucose and a rhamnose were established to be located at C-11 and C-23, respectively, by the NOE correlations of their anomeric protons with H-11 and H-23, respectively. Accordingly, the structure of rhoipteleside E was represented by formula **5**.

Rhoipteleside C (**3**), $C_{42}H_{70}O_{12}$, was indicated to be also a triterpene tridesmoside from its FAB-MS, ^1H and ^{13}C-NMR spectral data, the sugar components of which were elucidated to be glucose, 2-*O*-acetyl-glucose and rhamnose. The ^1H and ^{13}C-NMR signals due to aglycone are similar to those of **1**, suggesting that the aglycone is also a trioxygenated euphane-type triterpene. However, some differences in the spectra of **3** and **1** were observed: a methine signal at δ 3.82 (dd, *J*=4, 12 Hz) in **3** showed correlation with the methylene signals (H_2-2) at δ 2.45, 1.67 which in turn correlated with H-3 signal (δ 3.21, dd, *J*=5, 12 Hz) in the ^1H-^1H COSY spectrum. This observation indicated the presence of oxygen atom at C-1, and this was supported by HMBC correlations. On the basis of the above evidence, the structure of rhoipteleside C was elucidated to be as shown by the formula **3**.



R

3 2-O-Ac-glc

4 glc

Rhoipteleside D (**4**) was deduced to be a deacetyl derivative of **3** from FAB-MS (*m/z* 951 $[M+Na]^+$), ^1H and ^{13}C-NMR spectral data, which was further confirmed by alkaline hydrolysis (5 % NaOH) of **3** yielding **4**.

To the best of our knowledge, rhoiptelesides A-E represent the first example of the glycoside of euphane-type triterpene. From the chemotaxonomical viewpoint, the presence of these unique constituents in Rhoipteleaceae supports the establishment of the order Rhoipteleales [1].

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SOME EDIBLE VEGETABLES OF SAPONIN CONTAINING INDIGENOUS TO CHINA

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In the past, people have always considered the toxicity of saponin, but many kinds of vegetables, melons and fruits containing saponins have been demonstrated by modern scientific research. Those saponins belong to pentacyclic triterpene, tetracyclic triterpene and steroidal structures respectively. In the present report saponins from some vegetables, melons and fruits used to be eaten in china were disscused as examples in order to explain that physiological activities of saponin may always give helps to the health ofpeople and cure some diseases without toxicity foradminitration in longer period. Of course,in those vegetables, melons and fiuits always contain components other than saponin which, in fact, were one kind of the main activecomponents. They possessed effects of anti-bacteria, antiphlogistic; sedative and analgesic; antagonizing of senility, anticarcinogen, antimutaion; anticoagulation of blood, protecting myocardium from anoxia and ischemic, heart tonicantagonizing of arrhythmia and dilatation of coronary artery; lowering the level of blood sugar and lipids, provention of atherosclerosis; antagonizing of excitability; promotingimmunity and protection of liver; tonic and nourishing etc. Those saponins were good healthy foods and, when mixed in cosmotics, protector of skin to antago nize senility.

(I) chenopodiaceae

Spinacia oleracea L.

The tender whole plant was edible and contained spinosap-ponin A and B, with oleanolic acid and hederagenin astheir genins respectively.as well as steroidal glycosides. The physiological effects were hemostatic, nourishing blood, treatment of diabetes, moisturizing dryness and moving bowels. etc.

(II) Cucurbitaceae

Citrullus vulgaris schrad:

The edible fruits contained cucurbocitrin which demonstrated effects of clearing away summer heat, easing restlessness and quenching thirst.

Luffa cylindrica (L) Roem:

The whole plant contained saponins. From the edible fruits many saponins have been isolated. Sapogenins obtained after hydrolysis of lucyosides were pentacyclic triterpene genins, such as oleanolic acid, hederagenin, gypsogenin, moslinic acidand arjunolic acid etc. The isolated tetracyclic triterpens were dammarane saponin such as gensinoside-Re, -Rg_1 and saponon I. It was used as durgs for clearing away heat to relieve cough, dissipating phlegm and detoxication in traditional medicine. Achievements from research in recent years demonstrated its anti-inflamatory, sedative, analgesic, promoting immunity, anti-infection, anticoagulant, abatment of obesity and cure beauty of skin effects, thus it was used for treatment of chronic bronchitis and to manufacture healthy foods and cosmetics for cure beauty.

Momordica chranlia L.

The unriped fruits were edible. Fruits and seeds all possessed potent physiological effects. The main active components were glycoside derivated from tetra cyclic triterpene, cucurbitacenc. It contained glycosides of stigmastanol also.It was used as a drug for clearing away summer-heat, treatment of extreme thirst and desire for drinking, improving eyesight and detoxication, relieving carbuncle and sore in traditional medicine. Studies in recent years

discoverd more physiological effects for it, such as decreasing of blood-sugar content, anticarcinogen, spermatozocide, criminalobortion, improving appetite, relieving rheumatalgia and anti-mutaton.

(III) Leguminsoae

Arachia hypogaea L

The seeds were edible and used to nourish the lung, regulate stomach-energy and improve agalactia of parturient. It contained soyasaponin I, large amount of oil, sterols and leucocyanidin. Tonic and sedative effects of the seeds were discovered in recent years as well as the hemostatic effect of testa and hypotensive effect of the shells.

Glycine max Merr.

From the edible seeds more than 18 soyasaponins have been isolated which were composed from genins of oleanane compounds, they were soyasapogenol A, B, C, D, E, F, Gand B'. It was used to promote blood circulation and diuresis, expel wind evil and detoxicate. Studies in recent years demonstrated that no hemolytic effect have been induced by those saponins and nontoxic; protective effecton liver damage induced by peroxides of fat; preventing and healing thromboembolism; promating the tolerance to anoxia; better healing effect on hyperlipemia, hypentersion, atherosclerosis, diabetes; relieving obesity; controlling theformation of lipidic peroxides; anticarcinogenic and virus static activities etc. It was used to manufature healthy foods and cosmetics.

Gleditsia sinensis Lam.

The seeds were edible and contained triterpenoid saponins and used as expectorant, insecticide and antiseptic etc.

Phaseolus valgaris L.

Fruits and seeds were edible and contained phaseoluside A, B, C, D and E etc. With genins of oleanane type, such as soya-sapogenol B, etc. They demonstrated nourishing, antipyretic, diuretic and detumescence effects. Effects of owering hyperlipemia and antifertility were discovered recently also.

Pisum sativum L.

From the edible seeds, soyasapoinin I, a bad taste saponin. and an oleanene saponin, chromo-saponin I were isolated. It was used as drug for regulating of middle warmer and keepingthe adverse energy downward, diuretic and toxicide of sore inchinese medicine. The antibacterial and hypolipemia effects were discovered recently.

Vigna angularis (willd) Ohw, et ohashi

From the edible seeds six oligosaccharidic glycosides of oleanane type were isolated and designated as azunisaponinI-VI. The total saponin contained three known petancyclic triterpene sapogenins, which were sophoradiol, soyasapogenol B and gypsogenic acid, and an additional new sapogenin, azukisopogenol. It was used as diuretic and detoxicant, to eliminatewetness and treat beriberi in chinese medicine. It demonstrated a static effect to the synthesis of lipid peroxides, also can be used to manufacture cosmotic to keep skin beauty.

(IV) Liliaceae

Allium cepa L.

The edible bulb contained furostane and spirostane typesaponins, from which three new saponins, alloiospiroside A, B and allofuroside A have been isolated. Oleanolic acid α-amyrin and β-sitosterol were obtained from the hydrolysateof total saponin. It possessed effects of congragation staticofplatelets, decreasing lipid in serum, hypotensive, decreasing the content of blood sugar, antiasthma antiallergy and bacteriostatic.

Allium fistuosuni L.

The whole plant was edible from which fistuloside A, Band C were isolated with

yuccagenin as their genin. It possessed effects of detoxication relieving rheumatic and bacteriostatic.The juice demonstrated aphrodisiac effect onmice. The decoctum may be used to cure urticaria.

Allium sativum L.

From the edible bulb contained many saponins of furost-ane type,sativoside-B_1 , a new furostane glycoside, and proto-desgalactotigonin were isolated from the scales of the bulb.Two new steroid saponins, sativoside-R1 and -R2 were isolated from the roots. Another new and bacteriostatic furostane saponin, protocruboside B was isolated also. It was used to treatment of retention of undigested food, abdominal cold and pain, edema and dysentery in Chinese medicine. Studies in recent years demonstrated that it possessed the effects of bacteriostatic; antagonizing atherosclerosis, decreasing the level of blood sugar and increasing the level of plasmainsuline; anticoagulation of blood, clearance of free radicals; blood vascullar dilatation; excidation of immunity anti carcinogen; protection of liver and antagonizing of senility etc. It was a good healthy food.

Allium tuberosum Rottler.

The whole plant was edible and contained steroid saponins. It possessed effects of antitussive, antidiptic, prothotinglactation, warm and tonic; anti-mutation and healed injury ofgristle by external use.

(V) Rhamnaceae

Hovenia duleis thunb.

From the fruits with pedicel some hovenosides and hovenidulcioside A1, A2 were isolated, which contained tetracyclictriterpene genins of dammarane type. chelinlactone and hovenolactone were produced in acidic hydlrolysis of jujubogenin. Hodulein is a triterpene saponin with sweet taste, containing a genin which was samilar in structure with gymnemic acid. It was used to relieve drunkenness, fever with irritability and fidget, fidgetand thirstiness; to strengthen the middle-warmer and benefit vital eneryg. Tincture of Hovenia duleispossessed an effect to relax and activate the tendons. Modern scientific studies demonstrated the effects of antagonizingto peroxidation of lipids and to senility. It was practically non-toxic and can be administered for a longer duration.

Zizphus jujube Mill

The riped fruits were edible and mainly containedsaponins of dammarane type, zizyphus sapcnin I , II , III andjujuboside B_1, with jujubogenin as theri genins.It also containing additional pentacyclic triterpene saponins with betulic acid cleanolic acid and cratogolic acid as genins.It was used as tonic, nourisnment and tranquilizer. Modern scientific studies demonstrated the effects of central nervous system sedative; protection of liver, promotion of the muscular strength; carcinostatic; lowering the cholesterol level in serum, hypotensiye, protection of cultured myocardia, stomachic tonic, nourishing; dilatationof chronchus; antimicrobial and antisenility.

(VI)Rosaceae

Chaenomeles logenovia (Loisel) Koidz

The riped fruits were edible and contained triterpenoidsaponin. It demonstrated effects to subdue hyperactivity ofthe liver and regulate the stomach energy; elimminated wetness and relax the tendons.It was used in clinicaltreatment for acute dysentery and jaundice hepatities. Extractfrom it was used to manufacture cosmetic, clearing milk forskin.

Crataegus pinnatifida Bge.

From the edible riped fruits, saponins, rucoside A. B and C with ruscogenin as their genins, were isolated. It was used in Chinese medicine to promote digestion and dissipate bloodstasis, as anthelmintic for tape warm. Studies in recent years revealed good effects in

provention and treatment of cardiovascular disseases, as well as effects of hypotension, promotion of blood circulation in coronary artery, cardio-tonic, antiatherosclerosis. lowering cholesterol level in serum, antagonizing of myocardium ischemic and anticongragation of platelet. It was a good healthy food for its promotion of digestive function.

(Ⅶ) Theaceae

Camellia sinensis Linn O, Ktze

Buds of tender leaves contained triterpene saponins from which genins were obtained after hydrolysis, they were barringtogenol C and R1-barrigenol, along with cinnamic acidangelic acid, tiglinic acid, arbinose, xylose, galactose andglucuronic acid etc.The tender leaves have the effects toimprove eyesight, relieve excessive thirst, dissipate phlegm, promote digestion; of diuretic and detoxication. The saponinshave the effects of antifungus, microrganismcide, anti-inflamation; antigonizing of atherosclerosis, provention and treatment of hypertension; antagonizing of gastric ulcer and anticarcinogen. Saponins from fermented wulun tea was used as toniz, as well as it's extract has the effect of litholysisin urethra and non-toxic for long time administration.

REVIEW OF STUDIES ON BIOLOGICAL ACTIVITIES OF SOYASAPONINS

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Key Word Index—Soyasaponin; Biological Activities; Review

Abstract—This review is about the studies on biological activities of soyasaponins(ss).These studies show that ss have extensive biological activities and pharmocological values.Thus, ss may become new drug resources for treating angiocardiopathy and inhibiting cancer.

Introduction

Soyasaponins(ss) are contained in leguminous plants.The well-known soybean is a source of ss. But until recently people have regarded it as impurity and tried to remove it from soybean because of its pungent, bitter taste. Even in academic field, the study on ss has been conducted for only twenty years. Nowadays,many studies show that ss has extentive pharmocological functions, and is a natu-ral material with biological activities worthy of be-ing exploited. It may becom therapeutic and pre-ventive drug and functional food for angiocardio-pathy and cancer.

Biological Activities and Pharmoceutical Values of Ss

1.Its function of elevating blood pressure and accelerating heart rate

Since 1994, Li Jianhua injected a little ss into posterior hypothalamic nucleus(PHN) of wister rats of normal,clipped common carotid artery and anesthetic models, with the result of elevating artery blood pressure(MAP) and accelerating heart rate. Meanwhile, with the method of electrophysiology, it was verified that ss could increase discharge activitis of PHN and elevate blood pressure (1.2.3.4.). It is thus evident that PHN is the main target area for ss to elevate central blood pressure. The me-chanism of ss's elevating blood pressure is that ss elevates blood pressure and accelerate heart rate by strengthening sympathetic nerve activities and by pro-moting the change of hypophysis—eneocrine system. It is also retated to NPY and sympathetic nerve activities.

2.The function of ss on blood platelet

Certain amount of blood platelet is the basic material for artery smc hyperplasia and fibric plaque formation. Blood platelet is attached to artery endothelium,which plays an

could resist thrombus.Kubo's fur-ther study showed that under extracorporeal condition, different kinds of ss (Ⅰ , Ⅱ , Ⅲ , A, A_2) and total ss all prevented fibrinogen from turning to fibrin and that ss Ⅰ and Ⅱ could activate fibrin lysosome.(6) In the treatment of experimental diabetes (DM) of rats, it was observed that ss could decrease blood platelet aggregation rate and TXB_2/6-keto-PGF_1L (T/P) ratio,and increase insulin.(8) This result shows that ss has the same function with that of saponin of ginseng stem (GS). So it is inferred that they have the same functional mechanism. That is, they all play a role in the later period of TXA_2 composition.

3. Soyasaponin's antilipid peroxidation

Ss obiously decreases blood-lipid. Kitagawa et al gave 80 patients with hypertension and hyperlipemia 100 mg ss per day for 4, 8, 12 weeks to find that their blood serum lipid was improved greatly. It also decreased Dm rats,total cholesterol content.(7) Gao Guiqing et al added ss into high-lipid for age to feed rabbits in order to prevent their blood serum cholesterol and triglyceride from increasing.(8)

In vitro test, ss has antiperoxidation.(9) Oxygen was infused into the compound of ss and salad oil. After heating the compound for 40 min,the compound was measured to see the amound of peroxide lipid, with result that the compound with ss produced less peroside lipid than that without ss. In addition,the content of SOD in serum of experimental diabetes rats(DM) fed on forage contained ss increased,while the peroside lipid and free radical amount decreased. Thus the damage of peroxide lipid to endothelial cells was decreased, and the deterioration of DM rats' angiopathy was postponed or prevented. It is thus inferred that ss inhibits lipid peroxidation by inhibiting the onset period of peroxidation.

Ohminami affirmed the above results through his test. He also pointed out that ss could decrease cholesterol in serum and inhibit the damage of peroxide lipid to the liver cells.(6)

4.Zhang Wenjie et al's test showed that immediately after ss was added to the community, the spontaneous pulsation cardiac cells in it contract-ed and action potential was inhibited. If ss was washed off with routine medium and calcium concentration outside cells was increased with calcium passage excitant—adrenalin, contraction of cardiac cells and inhibition of action potential all reversed. Thus ss could block calcium passage.(10) The studies showed that calcium antagonist could postpone or inhibit artery atheromatous sclerosis(11), thus it could treat this disease.Because calcium passages distribute all the tissue of the body and relate to nervous excitement, muscle contraction, glandular secretion etc., ss may have other utilization value.

5. Ss's inhibition of cancer and regulation of organic immunologic function

Yu Liping et al studies ss's inhibition of cancer. Test in vivo showed that ss could indirectly inhibit S180 cancer cells in mice with S180 cancer.Test in vitro showed that ss could directly kill cancer cells. Its mechanism may be that ss can inhibit the DNA synthesis in S180 and YAC-1 cells and have obvious cytotoxic reaction to K562 and YAC-1 cells.

In the test of immunologic function of mouse cells, it was indicated that ss could promote CONA and CPS to reproduce spenocyte of the mouse, and could strengthen the cytotoxic activity of NK , LAK cells.Obviously, ss has remarkable immuno-regulation.(14)

6. Ss's other functions

Gao Guiqing et al found that ss could prolong the life of anoxic mice, which showed that ss could improve anoxic cardiac muscle.(8) Hu Jisheng et al conducted a clinic experiment to find that ss paste could cure herpes zoster with high cure rate and analgesic effect.(15) Through

clinic observation, Japaness scholars found that ss could treat adiposis and inhibit AIDS virus from infecting human beings.

Ss's Side Effect and Toxicity

1. Ss's Hemolyzation

Ss isolated by Kitagawa had hemolytic index below 100, almost without hemolyzation.(9)

2. Acute Toxicity Test

Toxicity test of rats and mice all showed that no death, increase of serum GOT and abnormal symptoms happened, in the animals (3.2g/Kg).(9) After three rabbits had taken ss (5mg/Kg/day) for four weeks, it was found that their appetite reduced, their weight increased slowly and their hematological, liver and kidney function examinations and vital organ microscopy had no abnormity.(8)

3. Ss's Goitrogenic Effect

The mice fed on soybean for a long time may have thyroid enlargement. By comparison with other forage, it was found that ss was a factor that caused thyroid enlargement. The above toxicity tests showed that ss had little side effect,which did not affect ss's utilization.

In one word, ss has extentive pharmocological effect,little side effct.It may be used as new drugs for angiocardiopathy and cancer.Therefore studies on its clinical application should be made more and its application should be expanded.

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SYNTHESIS OF STEROID SAPONINS FROM CHINESE HERBS

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Abstract Several bioactive monodesmosidic steroid saponins from traditional Chinese herbs were synthesized by glycosylating a steroid either directly with a fabricated oligosaccharide donor or stepwisely with monosaccharide donors.

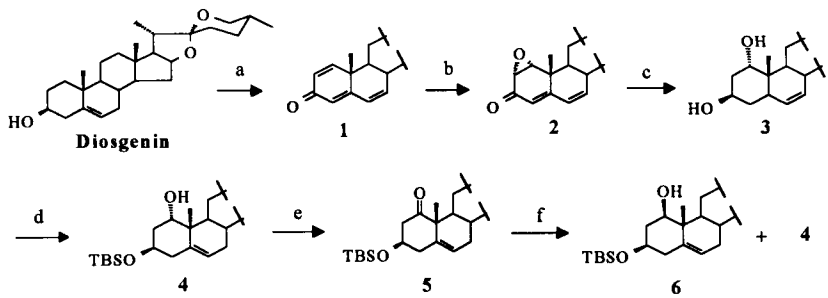
Saponins constitute a structurally and biologically diverse class of molecule that are widely distributed in terrestrial plant and some marine organisms.[1] The extreme difficulties associated with the purification of closely related saponins from a natural source provides synthesis with a realistic opportunity to contribute to the availability of homogeneous saponins. One of our current effort was being directed toward the total synthesis of bioactive steroid saponins from Chinese herbs.

A key step to build a steroid saponin is the attachment of sugar moieties to a steroid. Focusing on this key, there are two basic strategies for assembling a monodesmosidic saponin. The first is to fabricate a suitably protected and activated oligosaccharide first, then attach it to the steroid; The second is to connect the first monosaccharide to the aglycone first, then manipulate the protecting groups on the monosaccharide saponin and extend the sugar chain sequentially. Employing the first strategy, it is easier in handling the protecting groups but more risky on the stereospecificity and the yield of the final attachment of the sugar to aglycone. On the contrary, using the second strategy, the formation of the glycosidic bond between the sugar and the steroid is easier to be stereospecific and in high yield but the manipulation of the protecting pattern becomes more complicated. On the other hand, it is convenient to produce a family of saponins with a same sugar moiety but different steroids by employing the first strategy, and a family of saponins with a same steroid but different sugar moieties by using the second strategy. Using either of the strategies, the present presentation described the total syntheses of three structurally typical steroid saponins (**17**, **29**, **34**) with promising pharmacological activities from Chinese herbs.

25(R)-ruscogenin 1-*O*-β-D-glucopyranosyl (1→2) [β-D-xylopyranosyl (1→3)]-β-D-fucopyranoside (**17**) was one of the saponins isolated from the tuber of *Liriope muscari (Decne.) Bailey* (Maidong), [2] an Ophiopogon plant whose tuber was commonly used in Chinese herb medicines to promote the production of body fluid.[3] Pharmacological studies showed that **17** possessed strong antiinflammatory activities without gaining the body weight for tested mice.[4] Employing the first strategy, **17** was synthesized as shown in scheme 1-3.

Firstly, steroid acceptor **6** was prepared by introduction of a 1α hydroxyl group to diosgenin followed by epimerization under an oxidation-reduction sequence.(Scheme 1)

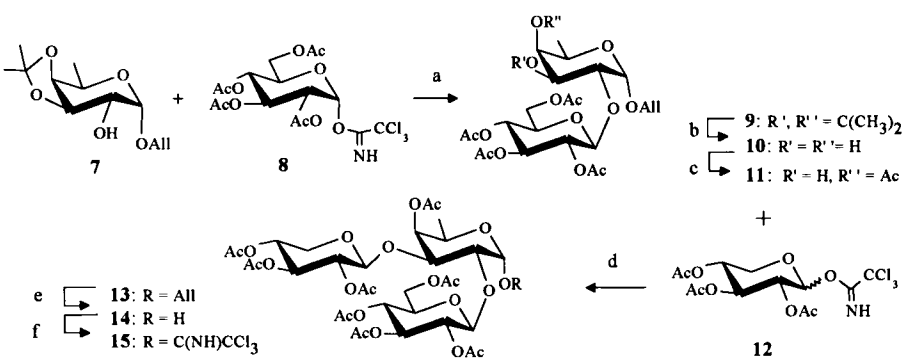
Scheme 1



Reagents and Conditions: a) DDQ, dioxane, reflux, 8 h, 69%; b) H_2O_2, NaOMe , MeOH, rt, overnight, 68%; c) Li/NH_3, THF, then NH_4Cl, 53%; d) TBDMSCl, imidazol, rt, overnight, 50%; e) PDC, CH_2Cl_2, rt, 92%; f) $NaBH_4$, THF, 70% for **6**, 25% for **4**.

Secondly, trisaccharide donor **15** was fabricated taking advantage of Schmidt glycosylation. (Scheme 2)

Scheme 2

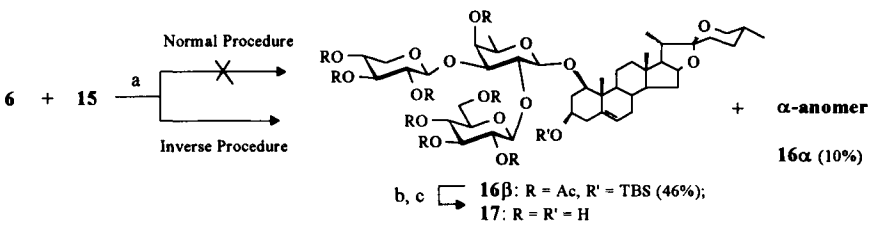


Reagents and Conditions: a) BF_3OEt_2 (0.7 equiv.), CH_2Cl_2, 4ÅMS, -20°C, 73.1%; b) 50% HOAc, 60°C, 1 h, 92.5□; c) $CH_3C(OCH_2CH_3)_3$, TsOH (0.1 equiv.), rt, 20min; then 20% HOAc, rt, overnight, 87.3%; d) BF_3OEt_2 (0.7 equiv.), CH_2Cl_2, 4ÅMS, -20 °C, 90%; e) $PdCl_2$, NaOAc, 50% HOAc, 60 °C, 87%; f) Cl_3CCN, DBU,CH_2Cl_2 , 49.2%.

Finally, with donor **15** and acceptor **6** at hand, we sought to effect the final glycosylation to construct the target saponin. (Scheme 3) However, all attempts to glycosylate the steroid acceptor **6** with imidate donor **15** failed to provide the resulting glycoside under the normal procedure for imidate glycosidation. It is reasonable regarding the steric hindrance of 1β-OH of steroid **6**. Fortunately, under Schmidt's "Inverse procedure" [5] that acceptor was

activated firstly by a catalytic amount of TMSOTf before adding donor, the desired glycosylation products **16** were obtained, but in a mixture of α- and β-anomer, which were separated by silica gel column chromatography. This synthesis provided an entry to a series of saponins with the same trisaccharide but different steroids.

Scheme 3

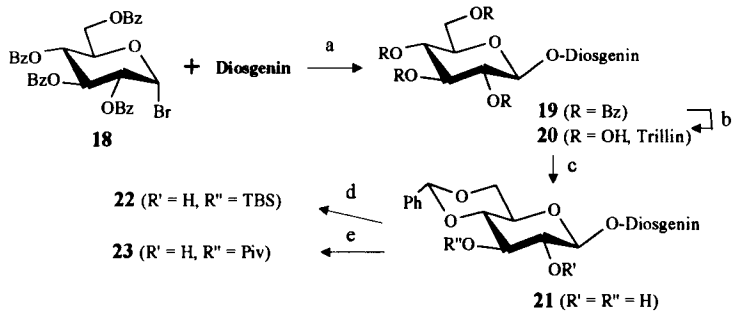


Reagents and Conditions: a) TMSOTf (0.2 equiv.), -5~0 °C, CH_2Cl_2, 4ÅMS, 2 h; b) TMSOTf (2.0 equiv.), CH_2Cl_2, -78 °C, 5min; c) MeONa, MeOH, rt, 3-4h, 85%.

Diosgenin α-L-rhamnopyranosyl- (1→2)-[β- D- glucopyranosyl-(1→3)]- β- D-glucopyranoside (**29**,Gracillin) and Diosgenin α-L-rhamnopyranosyl-(1→2)-[α-L-arabinofuranosyl-(1→4)]-β-D-glucopyranoside (**34**) were two structurally typical diosgenyl glycosides isolated from *paris* [6,7] and other species which have multiple pharmacological background in traditional Chinese herb medicines (Zaoxiu, Qiyeyizhihua, and Yunnan paiyao, etc.).[3] **29** and **34** themselves have been evaluated to have promising cardiovascular [6] and antitumour activities [7]. Employing the second strategy, **29** and **34** were synthesized as shown in scheme 4-5.

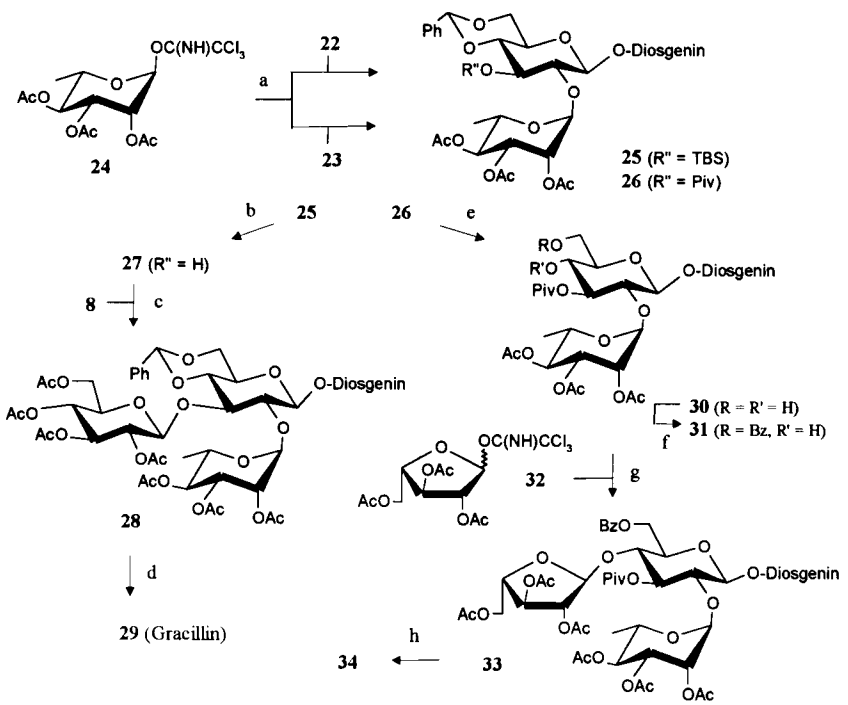
The first glycosylation of a steroid with a monosaccharide donor can be sought to be stereospecific and in high yield. (Scheme 4) In this glycosylation, the protecting groups of the glycosyl donor and the promoter used are two crucial factors. For example, using peracetyl protected glucosyl bromide instead as a glycosyl donor resulted in a very low yield of glycosylation product; using AgOTf/collidine as a promoter led to 80% of the orthorester. Multi-step protecting group manipulation and glycosylation on the resulting monosaccharide saponin furnished the target molecules. (Scheme 5) It is worthy noting that many derivatives with a same steroid but different sugar moieties could easily be produced on the way to the final molecule.

Scheme 4



Reagents and Conditions: a) AgOTf (1.1 equiv.), 4ÅMS, -20 °C-rt, 2 h, 73%; b) MeONa, MeOH, CH_2Cl_2, overnight, 88%; c) $PhCH(OMe)_2$, CSA, DMF, 50 °C, 2 h, 89%; d) TBDMSCl (2.9 equiv.), imidazole (4.0 equiv.), 0°C-rt, 1 day, 52.2%; e) PivCl, Py, 0 °C, 2 h, 64%.

Scheme 5



Reagents and Conditions: a) BF_3OEt_2 (1.9 equiv.), 4Å MS, CH_2Cl_2, -78°C-rt, 100%; b) Bu_4NF (0.5 M), THF, rt, 5 h, 84%; c) BF_3OEt_2, 4Å MS, CH_2Cl_2, -78°C-rt, 84%; d) 80% AcOH, 70 °C, 2 h; then MeONa, MeOH, CH_2Cl_2, rt, overnight, 79%; e) TsOH, CH_2Cl_2/MeOH, rt, 2 h, 53%; f) BzCl, Py, 0

°C-rt, 2 h, 75%; g) BF_3OEt_2, 4Å MS, CH_2Cl_2, 0 °C, 95%; h) MeONa, MeOH, CH_2Cl_2, rt, overnight, 90%.

Practically, after the synthesis of several pharmacological promising saponins from Chinese herbs, both two synthetic strategies were used orthogonally to build two groups of saponins, either with a same sugar moiety or with a same steroid. The pharmacological evaluation of these two groups of saponins is going to be directed toward the revealing of the pharmacological roles of either sugar or steroid in saponins.

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STEROIDAL GLYCOSIDES FROM LILIACEAE PLANTS AND THEIR BIOLOGICAL ACTIVITIES

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Abstract--Liliaceae is a rich source of steroidal glycosides. About 400 of new glycosides including triterpene glycosides and several staroidal alkaloids have been isolated and their structures were elucidated by our systematic studies on chemical constituents of Liliaceae plants. Some of these glycosides have unique structures.

The isolated glycosides were examined their physiological activities and many of theglycosides exhibited remarkable activities. Especially the glycoside that isolated from *Ornithogalum saundersiae* showed prominently strong cytostatic activity against human malignant tumor cells.

Our studies indicated that steroidal glycosides are very important as the source of medicine.

Introduction

Liliaceae is one of the largest families of flowering plants and about 220-240 genera with 3500-4000 species belong to this family. According to the A. Engler's classification(1964)[1], it is divided into 13 subfamilies as shown bellow:

Subfamilies and main genera of Liliaceae by Engler(1964)

Alstromerioideae: *Alstromeria*
Allioideae: *Agapanthus, Allium, Brodiaea*
Asparagoideae: *Asparagus, Convallaria, Paris, Polygonatum, Ruscus, Trillium*
Asphodelideae: *Aloe, Chlorophytum, Hemerocallis, Hosta*
Aletroideae: *Aletris*
Lilioideae: *Erithronium, Fritillaria, Lilium, Nomocaris, Tulipa*
Herrerioideae: *Herreria*
Luzuriagoideae: *Luzuriaga*
Melanthioideae: *Narthecium, Tricyrtis, Veratrum, Zygadenum*
Ophiopogonioideae: *Liriope, Ophiopogon*
Scilloideae: *Camassia, Chionodoxa, Eucomis, Hyacinthus, Ornithogalum, Scilla, Urginea*
Smilacoideae: *Smilax*
Wurmbaeoideae: *Colchicum*

Some plants of Liliaceae are well known as important origins of crude drugs, for example, Rhizoma Anemarrhenae, Radix Ophiopogonis and Rhizoma Smilacis Glabrae are listed in both Chinese and Japanese Pharmacopoea.

Liliaceae is a rich source of steroidal glycosides. In the course of our continuous studies on chemical constituents of Liliaceae plants, a number of new steroidal glycosides including cholestane glycosides and steroidal alkaloids have been isolated.

The biological activities of theseglycosides were examined with the following tests:

1. cyclicAMP phosphodiesterase inhibition test
2. Na^+/K^+ATPase inhibition test

3. *in vitro* Antitumor promotor activity test
4. Antitumor activity test:cytotoxity towards HL-60 and MOLT-4 leukemia cells

Result and Discussion

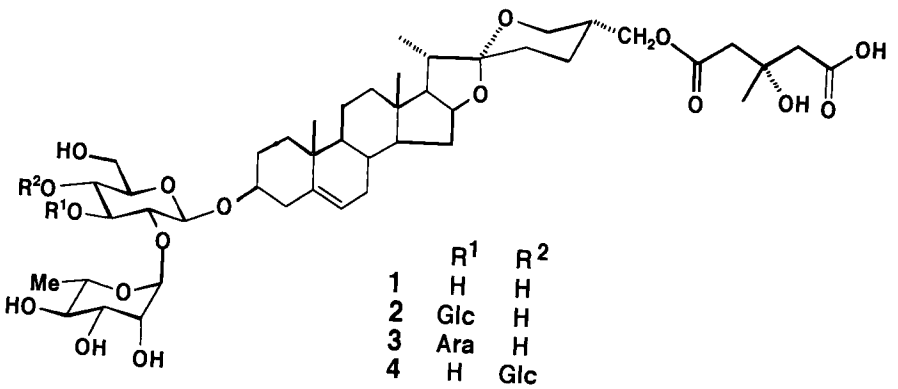
Steroidal Glycosides from Liliaceae

1. Lilioideae Plants

Lilium, the typical genus of this subfamily, distributed from the subtropical zone to the subarctic zone of the North Hemisphere.

In the first stage of our studies on chemical constituents of Liliaceae, we researched *Lilium* plants.

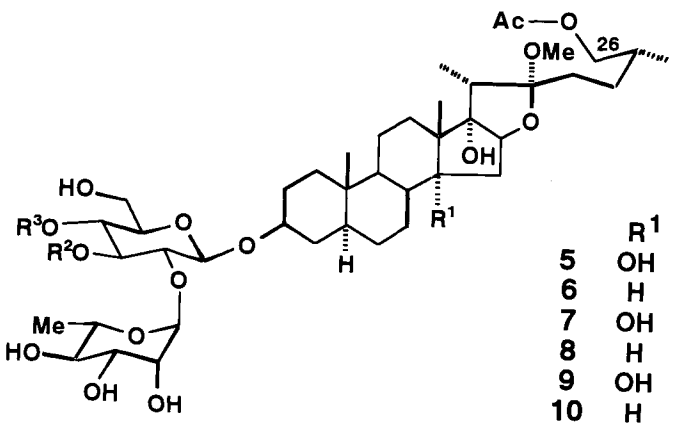
From *L. brownii*(Hongkong lily), *L. brownii* var. *cholchesteri*, *L. speciosum* x *L. nobilissimum* and *L. candidum*(Madonna lily), novel steroidal glycosides(**1-4**) bearing 3-hydroxy-3-methylglutaryl (HMG) moiety at the C-27 hydroxyl position have been isolated[2].



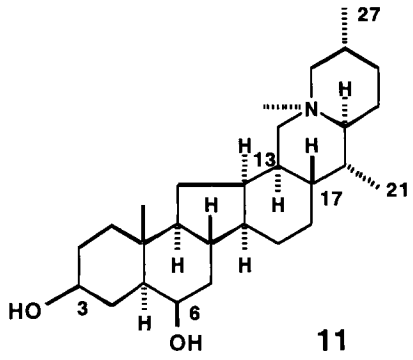
	R^1	R^2
1	H	H
2	Glc	H
3	Ara	H
4	H	Glc

From *L. pardarinum* of North America, novel frostanol glycosides(**5-10**) were obtained[3]. These glycosides were the first instance of the frostanol glycosides bearing acetoxyl group instead of glucose at C-26 hydroxyl position.

Fritillaria is closely related to Lilium taxonomically. Three steroidal alkaloids were isolated from *F. percica*. Compound **11** was the first example of cis quinolitizine alkaloid[4]. 22,26-Imino and 23,26-imino steroidal alkaloids such as compound **12** and **13** [5]were very rare in nature.



	R^1	R^2	R^3
5	OH	H	H
6	H	H	H
7	OH	Ara	H
8	H	Ara	H
9	OH	H	Glc
10	H	H	Glc



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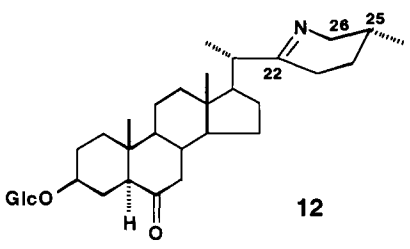

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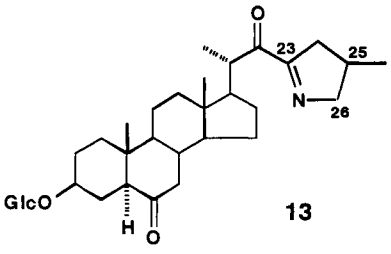

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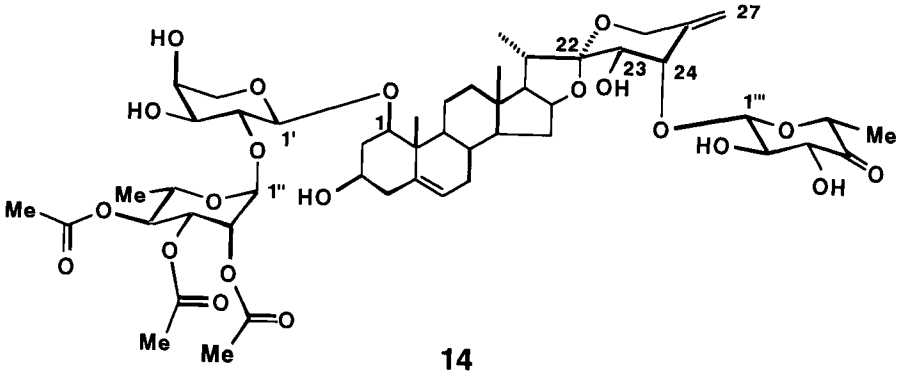
2. Asparagoideae Plants

The plants of this subfamily have rhizomes, not bulbs. Some genera such as *Convallaria, Rhodea* contain cardiac glycosides.

Ruscus aculeatus is a small shrub growing around the Mediteranean. Novel polyhydroxylated steroidal glycoside (**14**) possessing a deoxyaldoketose, 6-deoxy-D-glycero-L-threo-4-hexosulose at the C-24 hydroxyl position was isolated from this plant[6]. This is the first representative of a steroidal glycosides bearing an aldketose as one of the sugars.



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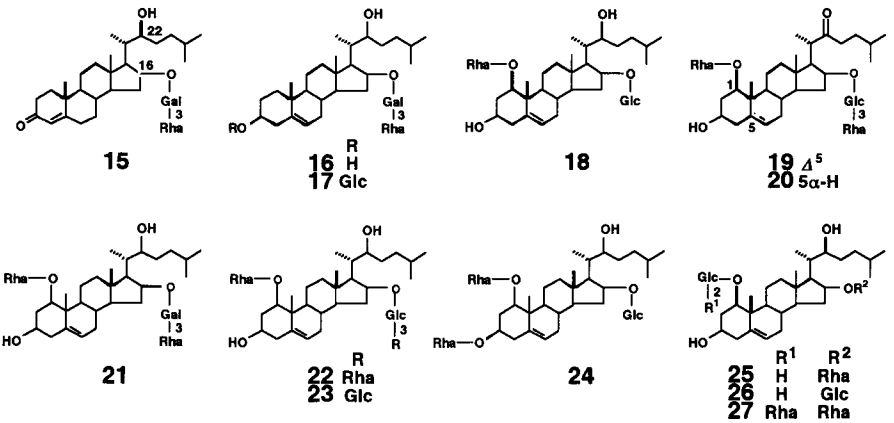
3. Allioideae Plants

Alloideae is a big group of Liliaceae. Some botanists transfer it to Amaryllidaceae because of the umbellata inflorescence.

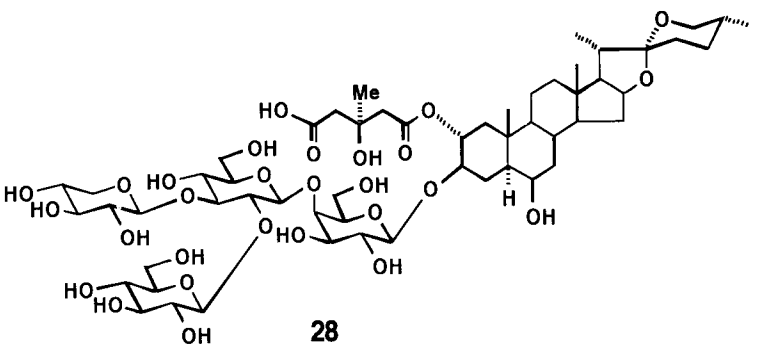
Though the plants of Allioideae are known to contain sulphur compounds, we have found out from our studies that the plants of this subfamily are also a rich source of steroidal glycosides.

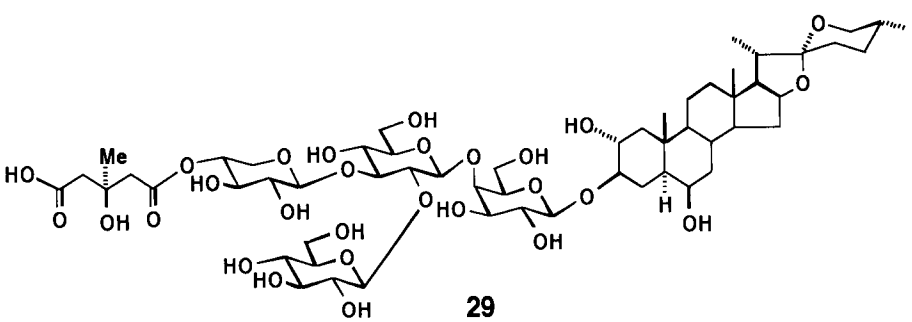
Analysis of 24 species of *Allium* led to the isolation of many novel steroidal glycosides. From *A. schubertii*, *A. albopilosum*, *A. ostrowskianum* and *A. elatum*, cholestane glycosides (**15-27**) have been isolated[7]. These glycosides bear 1β-, 3β-, 16β- and 22- hydroxyl groups and some of hydroxyl groups except of 22 position were combined with sugars.

The abusolute configuration of the C-22 hydroxyl group was confirmed by the advanced Moscher's method.

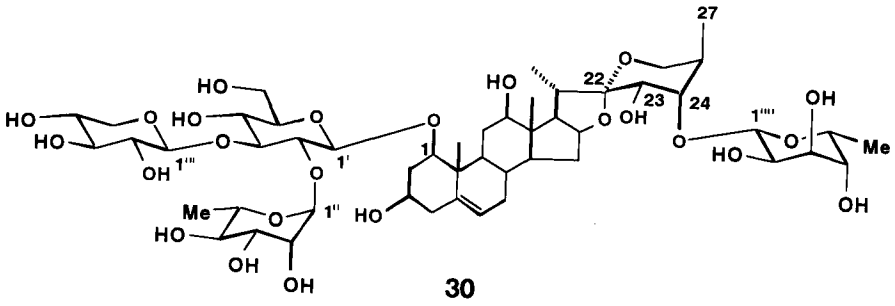


Steroids(**28**,**29**) possessing HMG moieties were obtained from A. albopilosum7-b) and *A. schubertii*[8].





Brodiaea is a small group of Allioideae occurred in North America. From *B. californica*, a novel steroidal glycoside (**30**) bearing 6-deoxygulose at C-24 hydroxyl position was isolated[9]. 6-Deoxygulose is the first example of the sugar which make up the steroidal glycoside.

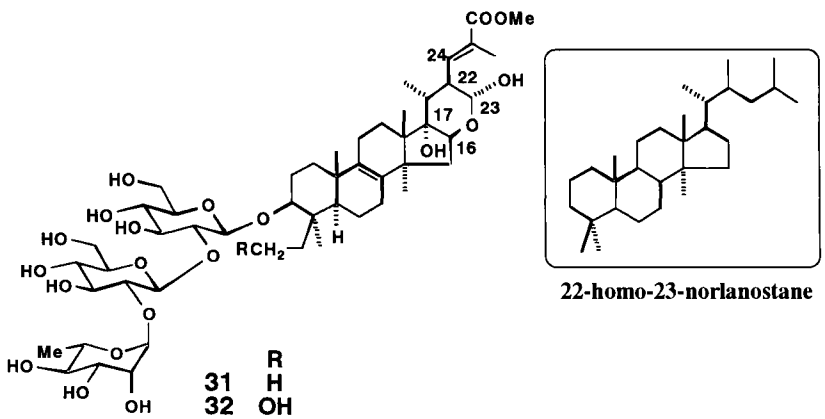


30

4. Schilloideae Plants

Some Schilloideae plants, such as *Urginea scilla* are well known to contain cardiac glycosides. We have studied 21 species of this subfamily and isolated many kinds of steroidal glycosides, but have not found any cardiac glycoside.

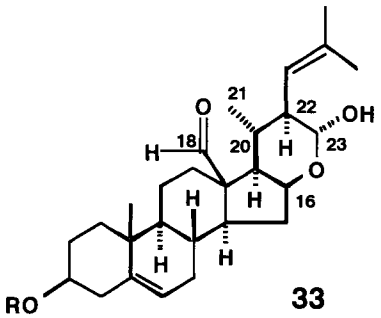
Scilla peruviana occrred in Portugal and North Africa contained novel triterpene glycosides (**31-32**)with a rearranged carbon skeleton in which C-24 to C-27 side chain is transfered to C-22position[10].



22-homo-23-norlanostane

Ornithogalum is distributed in a temperate region in Europe, Asia and Africa. Some plants of this genus are known to be poisonous and sometimes, poisoning of stocks which ate the bulbs of *Ornithogalum* were reported.

O. saundersiae is a large bulbous plant and native to South Africa. Compounds (**33-36**) isolated from the bulbs of this species have a rearranged carbon skeleton; C-24 to C-27 side chain was rearranged to the C-22 position with a six membered hemiacetal ring between C-16 and C-23 (**33**)[11] and a more five membered acetal ring between C-18 and C-20(**34-36**) in their structures[12].



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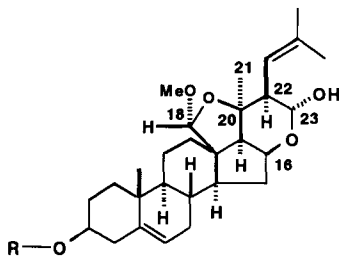
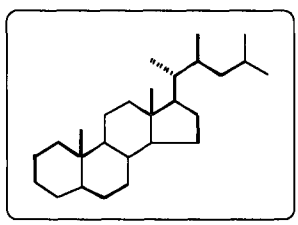


R

34 Rha-(1→2)-Glc
35 4-*O*-*p*-methoxybenzoyl-Rha-(1→2)-Glc
36 Rha-(1→2)-Glc-(1→2)-Glc



22-homo-23-norcholestane

Biological Activities of Steroidal Glycosides

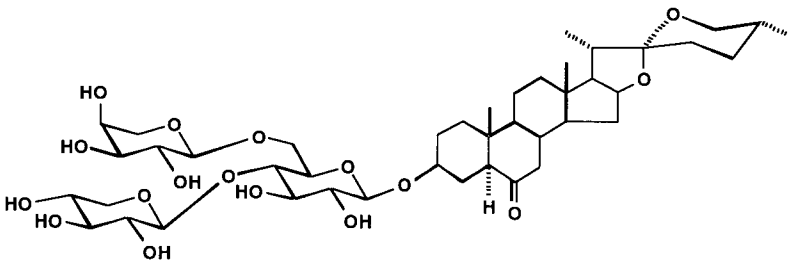
1. cAMP phosphodiasterase Inhibition

Cyclic adenosine 3',5'-monophosphate(cAMP) plays an important role as a second messenger in animal cells. cAMP is synthesized by adenylate cyclase and decomposed by cAMP phosphodiesterase(PDE) in the cells. Inhibition of cAMP PDE causes increase of cAMP in the cells and as a result physiological changes such as rise of blood sugar level, increase of contraction of cardiac muscle, inhibition of aggregation of platelets and/or enlarging of bronchus are induced. Therefore, cAMP PDE inhibition test is useful for the first screening of biological active compounds[13].

We have evaluated about 400 steroidal and triterpene glycosides for their activities on cAMP PDE. Many glycosides showed strong inhibitory activities. The activity generally increases with number of sugars. In addition, the type of sugar attached also affects the activity. Linkage of acyl moiety to the sugar of the glycoside increases the activity more

The activities of frostanol glycosides is generally very weak compared to those of spirostanol glycosodes.

Compounds which possess inhibitory activity for cAMP PDE are expected to work as cardiac agents. Therefore, several steroidal glycosides possessing the activity were subjected to *in vivo* screening test for cardiotonic effects using dogs and compound **37** was discovered as a cardiotonic compound. It is interesting to note that **37** is a main steroidal glycoside of *Allium chinensis* bulb[14] which is used to cure some heart disease as one of Chinese traditional medicines.

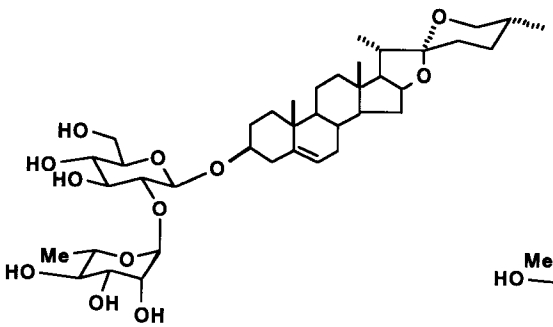


37

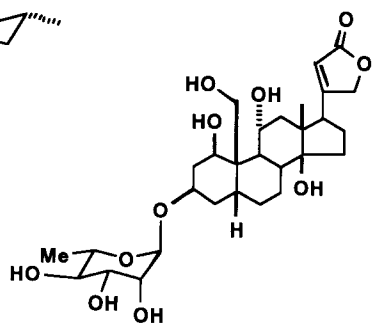
2. Na^+/K^+ ATPase Inhibition

Na^+/K^+ ATPase occurs in cells of higher animals and act as a sodium pump. It is reported that Na^+/K^+ ATPase was concerned with differeciation -induction of leukemia

The Na^+/K^+ ATPase inhibition test of glycosides was carried out and ihhibitory activities were compared with that of a positive control, ouabain. A simple spirostanol glycoside(**38**) showed relatively strong (1/10 of that of ouabin) activity.



38 IC_{50} 1.5 x 10^{-5} M

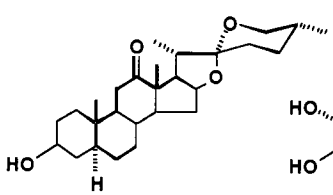


ouabain: IC_{50} 1.0 x 10^{-6} M

3. In vitro Antitumor Promotor Activity

Some foods originated from Liliaceae plants, such as *Allium sativum*(garlic) and *A. wakegi* are effective in the prevention of a malignant tumor and a sterol , laxogenin was identified as the active compound[16]. *in vitro* Antitumor promotor test of the steroidal glycosides isolated by our studies have been carried out and the activity was estimated from the rate of incorporation of phospholipid into HeLa cells stimulated with TPA(12-O-tetradecanoylphorbol 13-acetate).

The steroids (**39-41**) having carbonyl group like the laxogenin showed relatively strong activities.



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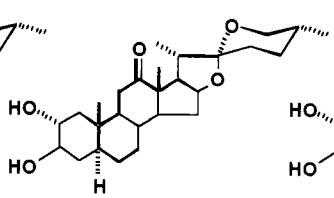

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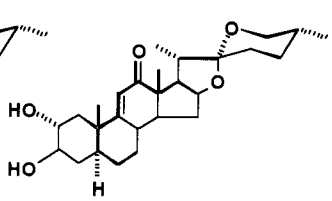

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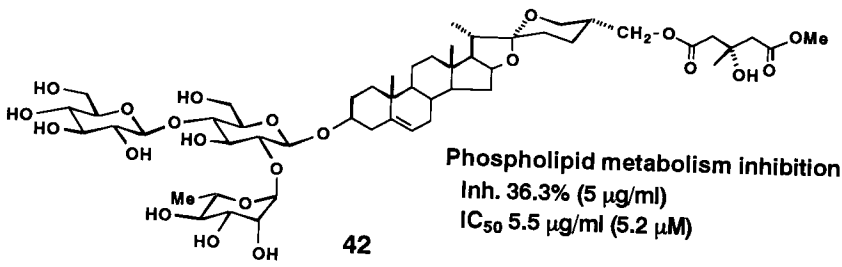
A steroidal glycoside(**42**) bearing HMG methyl ester at C-27 hydroxyl position showed prominent activity which was ten times higher than that of the laxogenin. It also showed comparatively strong inhibition to the proliferation of several human malignant tumor cells[17] (Table I).



42

Phospholipid metabolism inhibition
Inh. 36.3% (5 μg/ml)
IC_{50} 5.5 μg/ml (5.2 μM)

Table I
Inhibition of human malignant tumor cell proliferation with 42

Cells	Inhibition (%)
Pancreatic cancer (PANC-1)	95.5
Osteosarcoma (OST)	72.2
Human gastric cancer (HGC-27)	70.8
Pheochromocytoma (PC-12)	24.8

42: IC_{50} 4 μg/ml (3.8 μM) **Sample concentration: 5μg/ml**

4. Antitumor Activity

The series of cholestane glycosides isolated from Ornithogalum saundersiae exhibited very strong cytostatic activity towards several malignant tumor cells. The activities of two rearranged cholestane glycosides(**33**,**35**) against human leukemia cells, HL-60 and MOLT-4 were almost equal or more potent than those of the clinically applied anticancer agants, etoposide, methotrexate, adriamycin(ADM) and vincristine[18](Table II).

Table II Cytostatic activities of 33 and 35 towards HL-60 andMOLT-4 cells

	HL-60	MOLT-4	
33	0.0092	0.0024	
35	0.025	0.018	
etoposide	0.025	0.054	
adriamycin	0.0072	0.035	
vincristine	0.0029	0.00059	
methotrexate	0.012	0.048	IC_{50} (μM)

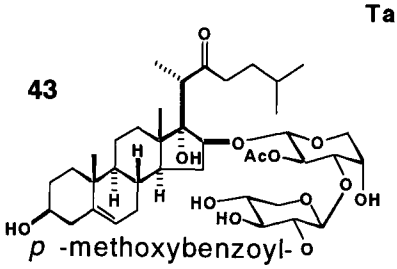
The cytostatic activities of the cholestane glycosides partly resulted from the induction of apoptosis. The apoptosis is caused by the activation of calcium dependent endonuclease. An analysis of flow cytometry pointed out that glycosides stopped the cell cycle of HL-60 at S2/M phase and induced apoptosis at G0/G1 phase[19].

The cholestane glycoside (**43**),the main constituent of the bulbs of *O. saundersiae*, showed a remarkably strong cytostatic activity which is about 10-100times stronger than those of mitomycin C(MMC), ADM, cisplatin(CDPP), camptpthecin(CPT) and taxol(TAX)[20](Table III).

Table III

Cytostatic activities of 43 and some anticancer agents towards HL-60 and MOLT-4 cells

43



	HL-60	MOLT-4	
43	0.00025	0.00049	
etoposide	0.025	0.054	
adriamycin	0.0072	0.035	
vincristine	0.0029	0.00059	
methotrexate	0.012	0.048	IC_{50} (μM)

It is worthy to note that the avtivity of **43** towards tumor cells including carcinostatic-resistant cells is about 10,000times more potent than that towards normal cells(CCD-19Lu)[20](Table IV).

Table IV

Cytotoxic activities of 43 and clinically applied anticancer agents on various malignant tumor cells

Malignant cells	IC_{50} (mg/ml)					
	43	MMC	ADM	CDDP	CPT	TAX
CCD-19Lu	1.5	2.0	2.0	10	2	2
P388	0.00013	0.01	0.003	0.05	0.005	0.01
P388/ADM	0.00077					
P388/CPT	0.00010					
FM3A	0.00016					
A-549	0.00068					
Lu-65	0.00020					
Lu-99	0.00020	0.01	0.002	0.001	0.001	0.002
RERF-LC-AI	0.00026					
CCRF-CEM	0.00016	0.02	0.01	0.005	0.005	0.001

CCD-19Lu (human normal pulmonary cell)
P388 (mouse leukemia)
P388/ADM (adriamycin-resistant P388)
P388/CPT (camptothecin-resistant P388)
FM3A (mouse mastrocarcinoma)
A-549 (human pulmonary adenocarcinoma)
Lu-65 (human pulmonary large cell carcinoma)
Lu-99 (human pulmonary large cell carcinoma)
RERF-LC-AI (human pulmonary squamous cell carcinoma)
CCRF-CEM (human leukemia)

The data of the 60Cell Line Assay by National Cancer Institute(NCI) in America indicated that **43** has a wide antitumor spectrum and is especially effective in proliferation of melanoma cells.

In *in vivo* test, **43** increased the life span of mice bearing the P388 leukemia by 59% with one-time administration of 0.01mg/kg[20]. **43** also inhibited the proliferation of human hepatoma 134 transplanted in nude mice at 0.06mg/kg (iv)[21] (Fig. 1).

The compounds isolated from plants have attracted special attention as the source for medicines. Our studies clarify that the steroidal glycosides are important compounds for resorces of new medicines.

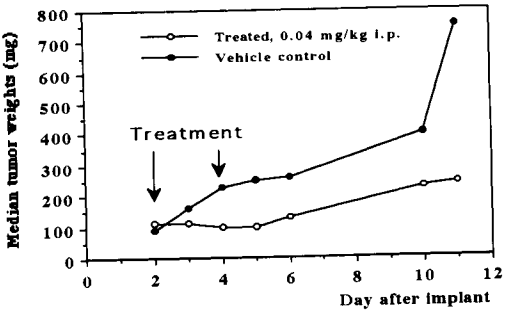


Fig.1 Response of HEP 134 xenografts to 43

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STEROIDAL SAPONINS FROM THE BULBS OF *ALLIUM CHINENSE*

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Key Word Index-- *Allium chinense*; Liliaceae; steroidal saponins

Abstract Two new steroidal saponins, chinenoside VI (**1**) and neomacrostemonoside D (**8b**), together with ten known saponins were isolated from the bulbs of *Allium chinense*. On the basis of chemical evidence and spectral analysis (IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC, HMBC and SI-MS), chinenoside VI was established as (25R)-24-O-β-D-glucopyranosyl-3β,24β-dihydroxy-5α-spirost 3-O-α-arabinopyranosyl-(1→6)-β-D-glucopyranoside, and neomacrostemonoside D was elucidated as neotigogenin 3-O-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-(6-acetyl-β-D-glucopyranosyl)-(1→4)-β-D-galactopyranoside, respectively.

Introduction

The dried bulbs of both *Allium macrostemon* Bunge (Liliaceae) and *Allium chinense* G. Don are main sources of traditional Chinese medicine "Xiebai", which is used for treatment of thoracic pain, stenocardia, so-called stagnant blood and diarrhea [1].The former specimen is mainly distributed in Northern and Northeast China and was collected in the Pharmacopoeia of People's Republic of China (1990 version). While the latter is cultivated in Southern China, which is widely used as the substituent of *A. macrostemon*. The components of *A. macrosemon* have been reported by our group in the previous papers [2-4]. In the continuation, we made a further study on the chemical components of *A. chinense*. From the bulbs of it, twenty compounds were isolated and twelve of which were steroidal saponins. Among the saponins, chinenoside VI (**1**) and neomacrostemonoside D (**8b**) are two new compounds. Timosaponin AIII (**6**) and a laxogenin trisaccharide (**3**) are two saponins isolated from this genus for the first time, and macrostmonoside D (**8a**) is a saponin first isolated from the title plant. In this paper, we reported the structure elucidation of these saponins especially by two-dimensional NMR (2D-NMR) techniques.

Results and Disscusion

The 60% ethanol extract of the dried bulbs of *A. chinense* was fractionated by a combination of silica gel, octadecylsilanized (ODS) silica gel column chromatographies and reversed-phase preparative HPLC procedures to yield compounds **1-9b** (Fig.1).

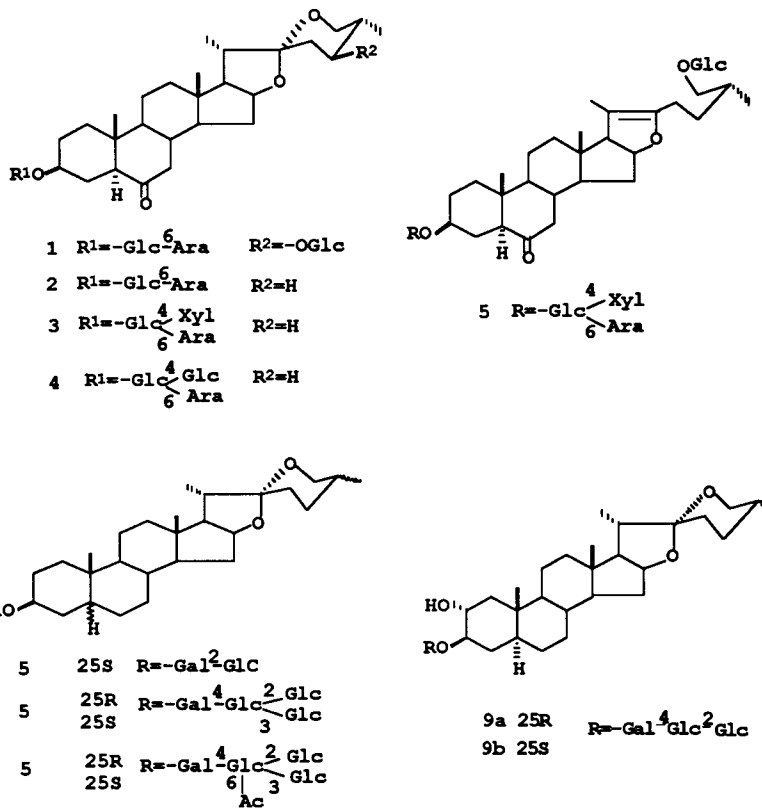

Fig. 1 The structures of compounds **1-9b**

Compound **1** was obtained as amorphous power and was positive to both Liebermann-Burchard and Molish tests. On acid hydrolysis, compound **1** give arabinose and glucose. By Comparing ^{13}C-NMR of **1** with that of glycoside E [5], the signals almost appeared at the same positions except those of E and F rings. In HMBC spectrum, 27-H_3 (δ 1.16) showed correlations with C-24 (δ81.5), C-26 (δ65.2) and C-25 (δ38.2), respectively. In ^{1}H-^{1}H COSY spectrum, signal at δ4.05 (H-24) was also observed to correlate with signals at δ1.98, 2.67 (H-23) and 1.92 (H-25), respectively. Furthermore, one anomeric proton signal of glucose at δ 4.94 (Glc-1′) exhibited correlation with δ 81.5 (C-24) in HMBC spectrum. All these data suggested that one glucose attached to C-24 of the aglycone. Also, the signals of two other sugars anomeric protons at δ5.01 (2H, Ara-1 and Glc-1) showed correlations with signals at δ 69.7 (Glc-6′) and 76.7 (C-3 of the aglycone), respectively. This suggested that compound **1** was a bisdesmosidic saponin, with the sugar chains attaching to C-3 and C-24 of the aglycone, respectively. β-Configuration of C-24 was determined on the basis of *J* values of H-23 (2.67, 1H, *dd*, *J* = 4.77, 12.46 Hz, 23-He and 1.98, 1H, *t*, *J* = 12.46 Hz, 23-Ha). R-Configuration of C-25 was elucidated by means of *J* values of H-26 (3.65 , 1H, *dd*, *J* = 4.77, 11.36 Hz, 26-He

and 3.58 , 1H, *t*, *J* = 11.36 Hz, 26-Ha). On comparison of ^{13}C-NMR data of F-ring with those of anzuronin I [6], the configuration of C-24 and C-25 were confirmed furtherly. Based on all the data mentioned above, compound **1** was established as (25R)-24-O-β-D-glucopyranosyl-3β, 24β-dihydroxy-5α-spirost 3-O-α-arabinopyranosyl (1→6)-β-D-glucopyranoside, which was a new spirostanol saponin, named chinenoside VI.

The ^{1}H-NMR and ^{13}C-NMR spectra of compounds **2-5** gave the typical signals of steroidal saponins. On acid hydrolysis, they gave the common aglycone of laxogenin and respective sugars. Both **2** and **3** gave arabinoses and glucoses, while **4** and **5** gave arabinoses, xyloses and glucoses. The locations of glycosidic linkages were elucidated by analysis of the 2D-NMR spectra, especially by HMBC spectra. On comparison of the ^{13}C-NMR spectra with the literatures [5, 7, 8] furtherly, the structures of compounds **2-5** were elucidated as laxogenin 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (**2**), laxogenin 3-O-β-D-glucopyranosyl-(1→4)-[α-L-arabinopyranosyl-(1→6)]-β-D-glucopyranoside (**3**), laxogenin 3-O-β-D-xylopyranosyl-(1→4)-[α-L-arabinopyranosyl- (1→6)]-β-D-glucopyranoside (**4**) and (25R)-26-O-β-D-glucopyranosyl-3β, 26-dihydroxy-5α-furost-20(22)-en-6-one 3-O-β-D-xylopyranosyl-(1→4)-[α-L-arabinopyranosyl-(1→6)]-β-D-glucopyranoside (**5**), respectively. The wrong assignments of compound **4** in the literature (glycoside B [5]) were corrected (Table 1).

Compound **6** was obtained as amorphous power and was positive to both Liebermann-Burchard and Molish tests. On acid hydrolysis, compound **6** gave galactose and glucose. Its formula was elucidated as $C_{39}H_{64}O_{13}$ by analysis of ^{13}C-NMR and SI-MS [763 $(M + Na)^+$, 741 $(M + H)^+$]. The ^{1}H-NMR and ^{13}C-NMR spectra of compound **6** exhibited the typical signals of 25R, 5β spirostanol saponin [δ 1.08 (27-H_3), 3.38 (26-He), 4.09 (26-Ha), 37.0 (C-5) and 24.0 (C-19)], so the aglycone was identified as sarsasapogenin [9]. The carbon and proton signals of the sugars were assigned by ^{1}H-^{1}H COSY and HMQC spectra. By comparing those literatural data of authentic methyl glycoside [10], one terminal glucose substituted at the position of C-2 of inner galactose was indicated. In HMBC spectrum, the anomeric proton signals at δ 5.30 (G-1) and 4.90 (Ga-1) showed cross-peaks with the carbon signals at δ 81.9 (Gal-2) and 75.5 (C-3 of the aglycone), respectively. These signals were as the ample evidence to determine the linkages by which the sugars were connected. Thus, the structure of compound **6** was determined to be sarsasapogenin 3-O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside, namely timosaponin A III. The wrong assignments of the sugar moiety in literature [9] were also corrected (Table 2).

Compounds **7a** and **7b** showed a series of ion peak at m/z 1087 $[M + Na]^+$, 925 $[M + Na - Glc]^+$, 579 $[M + H - Glc \times 3]^+$ and 417 $[aglycone + H]^+$ in the secondary ion mass spectrum (SI-MS). In the ^{13}C-NMR spectra, the carbon signals of E and F rings existed in pairs at C-16, C-17 and from C-20 to C-27. Correspondingly, the H signals of 27-H_3, 18-H_3 and 26-H_2 were existed also in pairs (intensity ratio 7a:7b = 4:3). Thus, the 25R and 25S spirostanol saponins were elucidated to coexist, and their aglycone was identified as tigogenin (25R) and neotigogenin (25S), respectively [10]. On acid hydrolysis, **7a** and **7b** gave glucose and galactose. The locations of glycosidic linkages were elucidated by analysis of two-dimensional NMR spectra, especially by the HMBC spectra. The anomeric protons at δ 5.59, 5.31 (two terminal glucoses), 5.16 (inner glucose) and 4.89 (galactose) exhibited correlations with carbon signals at δ 81.5 (C-2 of inner glucose), 88.6 (C-3 of inner glucose), 80.3 (C-4 of

galatose) and 77.6 (C-3 of the aglycone). Based on all the data described above, **7a** was determined to be tigogenin 3-O-β-D-glucopyranosyl-(1→ 2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, namely macrostemonoside A [2]. **7b** was determined to be neotigogenin 3-O-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside.

Table 1. ^{13}C-NMR shifts[a] of compounds **1-5** (δ ppm, in C_5D_5N)

C	1[b]	2	3[b]	4[b]	5	sugar	1[b]	2	3[b]	4[b]	glycoside B[5]	5
1	36.8	36.5	36.7	36.7	36.7	3-O-sugar						
2	26.9	29.3	29.5	29.5	29.5	Glc- 1	102.1	101.9	102.0	102.1	102.1	102.1
3	76.7	76.7	77.0	77.0	77.0	2	75.2	74.9	74.8	74.9	74.9[c]	74.9
4	27.0	26.9	27.0	27.0	27.0	3	78.6	78.3	76.6	76.3	78.5[e]	76.3
5	56.4	56.1	56.4	56.5	56.5	4	71.9	72.1	81.1	79.8	79.9	79.8
6	209.6	209.3	209.6	209.5	209.6	5	77.1	76.8	74.9	75.0	75.0[c]	78.6
7	46.7	46.7	46.8	46.8	46.9	6	69.7	69.5	68.4	68.1	68.1	68.1
8	37.3	37.0	37.4	37.4	37.2	Ara- 1	105.5	105.2	105.7	105.1	105.1[d]	105.7
9	53.6	53.3	53.7	53.7	53.6	2	72.4	71.6	72.6	72.6	72.6	72.5
10	40.9	40.8	40.9	41.1	40.9	3	74.5	74.2	74.6	74.6	74.6[c]	78.6
11	21.5	21.5	21.5	21.6	21.7	4	69.2	68.9	69.7	69.9	69.8	69.8
12	39.5	39.3	39.6	39.6	39.4	5	66.7	66.6	67.2	67.3	67.3	67.3
13	40.9	40.8	41.1	40.9	44.0	Xyl- 1				105.8	105.7[d]	105.1
14	56.4	56.1	56.5	56.5	54.8	2				74.9	74.9[c]	75.2
15	31.7	31.5	31.8	31.8	34.1	3				78.5	76.3[e]	78.6
16	81.3	80.5	80.9	80.9	84.2	4				71.1	71.1	71.1
17	62.4	62.5	62.8	62.8	64.4	5				67.3	67.3	67.3
18	16.4	16.1	16.5	16.5	14.3	24-O-Glc						26-O-Glc
19	13.1	12.7	13.1	13.1	13.1	Glc- 1′	106.5		104.9			104.9
20	42.1	41.6	42.0	42.0	103.5	2′	75.7		75.2			75.0
21	13.5	14.7	15.0	15.0	11.8	3′	78.6		78.5			78.7
22	111.6	108.9	109.3	109.3	152.6	4′	71.8		71.9			71.8
23	41.0	31.5	31.8	31.8	23.7	5′	78.1		78.2			78.5
24	81.5	28.9	29.2	29.2	31.4	6′	62.8		62.6			62.9
25	38.2	30.3	30.6	30.6	33.4							
26	65.2	66.2	66.9	66.9	75.0							
27	14.9	17.0	17.3	17.3	17.4							

a) All compounds were measured on JEOL JNM-GX 400 spectrometer except 2 on Bruker ARX-330 spectrometer.

b) Signals were assigned by ^{1}H-^{1}H COSY, HMQC and HMBC spectra.

c-d) Signals within the same column may be interchangeable

e) Literature assignments underlined were improved

Table 2. ^{13}C-NMR shifts[a] of Compounds **1**, **7a**, **7b**, **8a**, **8b**, **9a** and **9b** (δ ppm, in C_5D_5N)

C	**6**	**7a**	**7b**	**8a**	**8b**	**9a**	**9**	sugar	**6** Timosaponin AIII[9]		**7a,7b**	**8a,8b**	**9a,9b**
1	31.0	37.2	37.2	37.2	37.2	45.7	45.7	Gal- 1	102.6	102.4	102.4	102.7	105.2
2	26.4	29.9	29.9	30.0	30.0	70.5	70.5	2	81.9	81.6	73.2	73.4	72.7
3	75.5	77.6	77.6	77.5	77.5	84.7	84.7	3	75.3	76.7[b]	75.6	75.4	75.5
4	30.9	34.8	34.8	34.7	34.7	34.2	34.2	4	69.9	69.7	80.3	80.6	80.9
5	37.0	44.7	44.7	44.7	44.7	44.7	44.7	5	76.7	76.4	76.2	76.2	75.5
6	26.8	28.9	28.9	28.9	28.9	28.1	28.1	6	62.2	62.1	60.6	60.5	60.4
7	26.8	32.1	32.1	32.4	32.4	32.1	32.1	Glc- 1′	106.2	105.9	105.1	105.4	103.4
8	35.6	35.3	35.3	35.3	35.3	34.6	34.6	2′	77.0	75.4[b]	81.5	81.0	85.9
9	40.3	54.4	54.4	54.4	54.4	54.4	54.4	3′	78.1	77.9	88.6	88.2	77.8
10	35.3	35.8	35.8	35.8	35.8	36.9	36.9	4′	71.8	71.6	70.9	70.7	70.5
11	21.2	21.3	21.3	21.3	21.3	21.4	21.4	5′	78.5	78.2	77.9	75.7	79.0
12	40.3	40.1	40.1	40.2	40.2	40.1	40.1	6′	63.0	62.7	62.3	64.2	61.8
13	40.9	40.8	40.8	40.8	40.8	40.8	40.8	Glc-1′′			104.9	104.6	106.9
14	56.5	56.5	56.5	56.5	56.5	56.3	56.3	2′′			75.3	75.2	76.7
15	32.2	32.1	32.1	32.1	32.1	32.2	32.2	3′′			78.7	78.5	78.5
16	81.4	81.1	81.2	81.1	81.2	81.1	81.2	4′′			70.9	70.6	71.8
17	63.0	62.9	63.0	62.9	63.1	62.8	63.0	5′′			77.4	77.3	78.3
18	16.6	16.7	16.7	16.6	16.6	16.6	16.6	6′′			62.9	62.2	63.2
19	24.0	12.3	12.3	12.3	12.3	13.4	13.4	Glc-1′′′			104.6	104.5	
20	42.5	42.0	42.5	42.0	42.5	42.0	42.5	2′′′			75.3	75.2	
21	4.3	15.1	14.9	15.1	14.9	15.0	14.9	3′′′			78.7	78.6	
22	109.7	109.2	109.7	109.2	109.7	109.2	109.7	4′′′			71.6	71.6	
23	26.2	31.8	26.4	31.8	26.4	31.8	26.2	5′′′			77.6	77.5	
24	26.2	29.9	26.2	29.3	26.2	29.3	26.4	6′′′			63.1	62.9	
25	27.6	30.6	27.6	30.6	27.6	30.6	27.6	AC-C=O			171.2		
26	65.1	66.9	65.1	66.9	65.1	66.9	65.1	AC-CH_3			21.1		
27	16.3	17.4	16.3	17.4	16.3	17.3	16.3						

a) Signals were assigned by ^{1}H-^{1}H COSY, HMBC and HMQC spectra
b) Literature assignments underlined were corrected

The ^{1}H-NMR and ^{13}C-NMR spectra of compounds **8a** and **8b** were similar to those of compounds **7a** and **7b**. The presence of an acetyl group in the molecular was showed by IR (1733 cm^{-1}), ^{1}H-NMR (δ 2.12, 3H, *s*) and ^{13}C-NMR (δ 171.2 and 21.2) spectra. When **8a** and **8b** were treated with 4% NaOH in methanol, they were hydrolyzed to yield **7a** and **7b**. Therefore, compounds **8a** and **8b** should be monoacetate of **7a** and **7b.** By comparing NMR spectra of compounds **8a** and **8b** with compounds **7a** and **7b**, the signal of H-6 of the inner glucose shifted to 5.18 and 4.85, along with C-6 and C-5 of **8a** and **8b** shifting to lower field by 1.9, and to upper field by 2.2, respectively. Furthermore, the H-6 signal (δ 5.18) was observed to correlat with the carbonyl signal (δ 171.2). All these suggested the acetyl group link to the C-6 position of the inner glucose. Thus, the structure of **8a** was elucidated as tigogenin 3-O-β-

D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-(6-acetyl-β-D-glucopyranosyl)-(1→4)-β-D-galactopyranoside, namely macrostemonoside D [2]. **8b** was a new compound, which was determined as neotigogenin 3-O-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-(6-acetyl-β-D-glucopyranosyl)-(1→4)-β-D-galactopyranoside, named neomacrostemonoside D. Similarly, the epimers of **9a** and **9b** were identified as gitogenin 3-O-β-D-gluopyrnosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside. **9b** was determined as neogito-genin 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside[11]

Experimental

General. Dianguang brand micro-melting point apparatus (Beijing Instrument Factory), uncorr. C. C. silica gel (200-300 mesh, Qingdao Haiyang Chemical Factory). IR: Bruker IFS-55 (KBr). ^1H and ^{13}CNMR: JEOL JNM-GX 400 spectrometer (^1H 400 MHz, ^{13}C 100 MHz) and Bruker ARX-330 (^1H 330 MHz, ^{13}C 82.5 MHz), with TMS as int. standard in C_5D_5N. SI-MS: JM-DX 302. Prep. HPLC: Liquid Chromatograph LC-10 (Japan Analytical Industry Co. Ltd.) equipped with RI detector, using a $5C_{18}$ column (Waters, 10×250 mm, 5 μm) with MeOH:H_2O as mobile phase, in a flow rate of 2.0 ml min^{-1}. TLC spots were visualized by spraying with 10% H_2SO_4 ethanol solution followed by heating.

Plant material. The bulbs of *Allium chinense* G. Don (Liliaceae) were collected in Huaihua Region in Hunan Province (China) and were identified by Prof. Zheyong Jiang (Division of Pharmacognosy, Shenyang Pharmaceutical University). A voucher speciman is deposited at herbarium of Shenyang Pharmaceutical University.

*Extract and isolation.*The dried bulbs of *A. chinense* (30 kg) were extracted with 60% EtOH (300 l, 2 h and 240 l, 1 h).The combined EtOH solns were concentrated *in vacuo* to gave 5.8 Kg ethanol extract. A suspension of the extract in H_2O was partitioned successively with $CHCl_3$ and EtOAc to afford fr. CJ-1 (142.4 g), fr. CJ-2 (21.5 g) and the residue of fr. CJ-3 (5.7 kg), respectively.

Fr. CJ-1 was subjected to slica gel C. C. (1.2 kg) with a gradient mixture of petroleum: EtOAc: MeOH (10:1:0, 9:1:0, 8:2:0, 7:3:0, 6:4:0, 0:1:0, 0:15:1, 0:10:1) to gave eight fractions (CJ-1.1 - CJ-1.8) . Fr. CJ-1.7 eluted by EtOAc:MeOH (15:1) was rechromatographed on silica gel C. C. (300 g) eluting with $CHCl_3$:MeOH (9:1) and ODS silica gel (80 g) with MeOH: H_2O (7:3) to gave **2** (55.7 mg) and **6** (9.1 mg). Fr. CJ-1.8 was separated furtherly by silica gel C. C. with $CHCl_3$:MeOH:H_2O (70:30:1) to gave five fractions (CJ-1.8.1 - CJ-1.8.5). CJ-1.8.2 was chromatographed on lobar column (75% aq. MeOH) to yield **4** (133.5 mg). By lobar column separation (80% aq. MeOH), CJ-1.8.3 gave two saponin fractions (frs. OS_1 and OS_2). Fr.OS_1 was further separated by prep. TLC on silica gel to give **3** (22.4 mg) and **4** (10.1 mg). While fr. OS_2 was further isolated by prep. HPLC (ODS column, 5 μm, solvent, 78% aq. MeOH, flow rate, 2.0 ml min^{-1}) to gave **9a** and **9b** (29.9 mg). Fr. CJ-1.8.4 was purified by prep. HPLC, eluted with MeOH:H_2O (8:2) to gave **8a** and **8b** (23.1 mg). Fr. CJ-1.8.5 were recrystallized with $CHCl_3$:MeOH to give **7a** and **7b** (357.3 mg).

A Part of fr. CJ-3 (3.7 kg) was subjected to a column of D-101 macrophous resin column (1.5 kg), with stepwise elution of water (10 l), 40% aq. EtOH (3 l) and 95% aq. EtOH (1 l). The elution of 40% and 95% combined was refluxed with MeOH (850 ml×7). The MeOH sol. was fractionated on a silica gel column (400 g) using $CHCl_3$:MeOH:H_2O gradiently to give six

fractions (CJ-3.1-CJ-3.6). Fr. CJ-3.6 eluted with $CHCl_3$: MeOH:H_2O (70:30:5, lower phase) was separated repeatedly by silica gel C. C. and purified by HPLC with 50% aq. MeOH to give **1** (9.2 mg) and **5** (102.3 mg).

Compound 1. Amorphous powder, m.p 219-221℃. IR ν_{max} cm^{-1}: 3423 (OH), 2933, 1702 (C=O), 1380, 1049 (C-O), 950, 899, 863. ^{1}H-NMR: δ0.64 (3H, *s*, 19-H_3), 0.72 (3H, *s*, 18-H_3), 1.08 (3H, *d*, *J* = 6.6 Hz, 21-H_3), 1.16 (3H, *d*, *J* = 7.6 Hz, 27-H_3), 1.72 (1H, *t*, *J* = 12.45 Hz, 4-Ha), 1.98 (1H, *t*, *J* = 12.46 Hz, 23-Ha), 2.40 (1H, *br.d*, *J* = 12.45 Hz, 4-He), 2.67 (1H, *dd*, *J* = 4.77, 12.46 Hz, 23-He), 3.58 (1H, *t*, *J* = 11.36 Hz, 26-Ha), 3.65 (1H, *dd*, *J* = 4.77, 11.36 Hz, 26-He), 5.01 (2H, *d*, *J* = 6.6 Hz, Ara-1 and Glc-1). ^{13}C-NMR: Table 1.

Compound 2. Amorphous powder, m.p 235-238℃. IR ν_{max} cm^{-1}: 3450 (OH), 2950, 1705 (C=O), 980, 915, 895, 860 (915< 895, 25R). ^{1}H-NMR (300 MHz): δ0.72 (3H, *s*), 0.77 (3H, *d*, *J* = 5.7 Hz), 0.86 (3H, *s*), 1.22 (1H, *d*, *J* = 6.9 Hz), 4.92 (1H, *d*, *J* = 9.8 Hz), 5.06 (1H, *d*, *J* = 6.6 Hz). ^{13}C-NMR: Table 1.

Compound 3. Amorphous powder, m.p 238-240℃. IR ν_{max} cm^{-1}: 3409 (OH), 2947, 1708 (C=O), 1054, 1053 (C-O), 958, 921, 899, 864 (921< 899, 25R). ^{1}H-NMR: δ0.64 (3H, *s*, 18-H_3), 0.70 (3H, *d*, *J* = 5.86 Hz, 27-H_3), 0.79 (3H, *s*, 19-H_3), 1.15 (3H, *d*, *J* = 6.96 Hz, 21-H_3), 4.94 (1H, *d*, *J* = 7.69 Hz, inner Glc-1), 5.11 (1H, *d*, *J* = 7.32 Hz, Ara-1), 5.55 (1H, *d*, *J* = 8.06 Hz, Glc-1′). ^{13}C-NMR: Table 1.

Compound 4. Amorphous powder, m.p 252-254℃. SI-MS m/z: 879 $[M + Na]^+$, 431 $[aglycone + H]^+$, 413, 395, 379, 361, 341, 313. ^{1}H-NMR: δ0.64 (3H, *s*, 19-H_3), 0.70 (3H, *d*, *J* = 5.13Hz, 27-H_3), 0.79 (3H, *s*, 18-H_3), 1.15 (3H, *d*, *J* = 7.34 Hz, 21-H_3), 4.97 (1H, *d*, *J* = 7.33 Hz, Glc-1), 5.08 (1H, *d*, *J* = 7.34, Ara-1), 5.51 (1H, *d*, *J* = 8.07 Hz, Xyl-1). ^{13}C-NMR: Table 1.

Compound 5. Amorphous powder, mp > 300 ℃. SI-MS m/z 763 $[M + Na]^+$, 741 $[M + H]^+$, 579 $[M + H - Glc)^+$, 417 $[aglycone + H]^+$, 399, 363, 327. ^{1}H-NMR: δ0.83 (3H, *s*, 18-H_3), 0.98 (3H, *s*, 19-H_3), 1.08 (3H, *d*, *J* = 6.96 Hz, 27-H_3), 1.16 (3H, *d*, *J* = 6.96 Hz, 21-H_3), 4.94 (1H, *d*, *J* = 7.69 Hz, Gal-1), 5.30 (1H, *d*, *J* = 7.69 Hz, Glc-1). ^{13}C-NMR: Table 1.

Compound 6. Amorphous powder, m.p 257-258 ℃. IR ν_{max} cm^{-1}: 3400 (OH), 2920, 2850, 1700 (C=O), 1000-1100 (C-O). ^{1}H-NMR: δ0.65 (3H, *s*, 19-H_3), 0.67 (3H, *s*, 18-H_3), 1.04 (3H, *d*, *J* = 6.96 Hz, 27-H_3), 1.65 (3H, *s*, 21-H_3), 4.94 (1H, *d*, *J* = 7.69 Hz), 5.11 (1H, *d*, *J* = 7.32 Hz), 5.55 (1H, *d*, *J* = 8.06 Hz). ^{13}C-NMR: Table 2.

Compounds 7a and 7b. Amorphous powder, m.p 290-293℃。SI-MS m/z: 1087 $[M + Na]^+$, 925 $[M + Na - Glc]^+$, 579 $[M + H - Glc \times 3]^+$, 417 $[aglycone + H]^+$, 389 $[aglycone + H - H_2O]^+$, 325, 307. IR ν_{max} cm^{-1}: 3422 (OH), 2931, 1376, 1073, 919, 896 (919 < 896). ^{1}H-NMR: δ4.89 (1H, *d*, *J* = 7.34 Hz, Gal-1), 5.16 (1H, *d*, *J* = 8.07 Hz, inner Glc-1), 5.31 (1H, *d*, *J* = 7.33 Hz, Glc-1'), 5.59 (1H, *d*, J = 7.34 Hz, Glc-1'). 7a (25R): (0.66 (3H, s, 19-H3), 0.70 (3H, d, J = 5.86 Hz, 27-H3), 0.83 (3H, s, 18-H3), 1.15 (3H, d, J = 6.61 Hz, 21-H3), 3.52 (1H, t, J = 11.27 Hz, 26-Ha), 3.60 (1H, br.d, J = 11.74 Hz, 26-He). 7b (25S): (0.66 (3H, s, 19-H3), 1.08 (3H, d, J = 5.86 Hz, 27-H3), 0.82 (3H, s, 18-H3), 1.15 (3H, d, J = 6.61 Hz, 21-H3), 3.37 (1H, br. d, J = 11.01 Hz, 26-He). 13C-NMR: Table 2.

Compounds 8a and 8b. Amophous powder, m.p 272-274℃. SI-MS m/z: 1129 [M + Na]+, 417 [aglycone + H]+, 399 [aglycone + H - H2O]+, 385, 367, 343, 321. IR (max cm-1: 3427 (OH), 2932, 1733 (C=O), 1376, 1073 (C-O), 956, 919, 896, 852 (919 (896). 1H-NMR: (2.14

(3H, s, acetyl-H3), 4.81 (1H, d, J = 7.3 Hz, Gal-1), 5.10 (1H, d, J = 8.1 Hz, inner Glc-1), 5.28 (1H, d, J = 7.3 Hz, Glc-1(), 5.65 (1H, d, J = 7.3 Hz, Glc-1(). 8a (25R): (0.66 (3H, s, 19-H3), 0.70 (3H, d, J = 5.86 Hz, 27-H3), 0.83 (3H, s, 18-H3), 1.15 (3H, d, J = 6.61 Hz, 21-H3), 3.54 (1H, t, J = 11.27 Hz, 26-Ha), 3.60 (1H, br.d, J = 11.74 Hz, 26-He). 8b (25S): (0.66 (3H, s, 19-H3), 1.08 (3H, d, J = 5.86 Hz, 27-H3), 0.82 (3H, s, 18-H3), 1.15 (3H, d, J = 6.61 Hz, 21-H3), 3.37 (1H, br.d, J = 11.01 Hz, 26-He). 13C-NMR: Table 2.

Alkaline hydrolysis of 8a and 8b. 8a and 8b (2 mg) were refluxed with 1 M NaOH-50(aq. MeOH (1:1, 2 ml) in a sealed tube at 80℃ for 2 hr. 7a and 7b were detected in the hydrolysate by means of TLC with developing solvent CHCl3:MeOH:H2O (65:35:10, lower phase).

Compounds 9a and 9b. Amorphous powder, m.p 266-269℃. IR (max cm-1: 3423 (OH), 2930, 1382, 1073 (C-O), 921, 896, 850 (921 (896). 1H-NMR: (4.95 (1H, d, J = 7.34 Hz, Gal-1), 5.17 (1H, d, J = 7.34 Hz, Glc-1), 5.30 (1H, d, J = 7.34 Hz, Glc-1'). 9a (25R): (0.69 (3H, d, J = 5.1 Hz, 27-H3), 0.71 (3H, s, 19-H3), 0.81 (3H, s, 18-H3), 1.13 (H, d, J = 7.0 Hz, 21-H3), 3.54 (1H, t, J = 11.01, 10.27 Hz, 26-Ha), 3.60 (1H, br. d, J = 11.01 Hz, 26-He). 9b (25S): (0.71 (3H, s, 19-H3), 0.80 (3H, s, 18-H3), 1.08 (3H, d, J = 7.33 Hz, 27-H3), 1.14 (1H, d, J = 6.6 Hz, 21-H3), 3.37 (1H, br. d, J = 10.27 Hz, 26-He). 13C-NMR: Table 2.

Acid hydrolysis. Compounds 1-9b (a few mg) were refluxed with 2 M HCl-dioxane (1:1, 2 ml) in a sealed tube at 100℃ for 4hr. The reaction mixtures were concentrated to dryness in vacuuo and the residues were examined on TLC with the authentic samples of glucose, galactose, xylose and arabinose. The developing solvent was CHCl3:MeOH:H2O (160:92:20), and the visualizing reagent was o-phthalic acid-aniline.

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ANTINEOPLASTIC STEROIDAL SAPONINS FROM RHIZOMES OF *DIOSCOREA COLLETTII* VAR. *HYPOGLAUCA*

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Key Word Index--*Dioscorea collettii* var. *hypoglauca*; Dioscoreaceae; steroidal saponins; antineoplastic agents; *Pyricularia oryzae*.

Abstract By activity-guided fractionation, two new and twelve known steroidal saponins were isolated from the total saponin fraction of *Dioscorea collettii* var. *hypoglauca* as bioactive agents causing morphological abnormality of *Pyricularia oryzae* mycelia. The compounds were also evaluated with cytotoxic activities against the cancer cell line of K562 *in vitro*. All the structures of the compounds were elucidated on the basis of chemical evidence and IR, FAB-MS, ^{1}H-NMR, ^{13}C-NMR, and two-dimentional NMR (2D-NMR) analysis.

Introduction

Morphological deformations of mycelia or cornidia of microorganisms, such as curling, swelling, hyper-divergency, bead formation along with inhibition of germination are often induced in the presence of bioactive substances [1, 2]. A bioassay detecting deformation of mycelia germinated from conidia of *Pyricularia oryzae* (a phytopathogenic fungus) was modified for quantitative application. This bioassay method could be efficiently applied for the primary screening of antineoplastic and antifungal agents such as griseofulvin, rhizoxin [3], fusarielin A [4] from fungus metabolites. We applied the method to traditional Chinese medicines (TCM) for the first time. So far, 92 kinds of TCM used in the treatment of cancer have been collected and bioassayed. The ethanol extract of *Dioscorea collettii* var. *hypoglauca* showed the strongest activity among the seven active TCM.

The rhizomes of *Dioscorea collettii* var. *hypoglauca* (Dioscoreaceae) are used in the traditional Chinese medicine "Fen Bei Bi Xie" for the treatment of cervical carcinoma, carcinoma of urinary bladder and renal tumor in China. It is widely distributed in Southeast China and was included in the Pharmacopoeia of People's Republic of China (1990 version). Tang and co-workers reported the isolation of four steroidal saponins [5, 6]. Ten steroidal sapogenins, such as diosgenin, yamogenin and isonarthogenin, have been isolated from the acid-treated rhizomes of the plant [7].

In this paper, bioactivity-guided fractionation of the ethanol extract of *D. collettii* var. *hypoglauca* yielded prosapogenin A of dioscin (**1**), dioscin (**2**), gracillin (**3**), protoneodioscin (**4**), protodioscin (**5**), protoneogracillin (**6**), protogracillin (**7**), methyl protoneodioscin (**8**), methyl protodioscin (**9**), methyl protoneogracillin (**10**), methyl protogracillin (**11**), hypoglaucin F (**12**), hypoglaucin G (**13**) and hypoglaucin H (**14**) (Fig. 1). Among them, **12** and **13** are two new steroidal saponins. Compounds **1**, **3**, **4**, **5**, **6** and **14** are first reported from the title plant. Compounds **4** and **5**, **6** and **7**, **8** and **9**, **10** and **11** are four pairs of stereoisomers at carbon-25, respectively. These fourteen steroidal glycosides all caused morphological abnormality of *Pyricularia oryzae* mycelia. Furthermore, compounds **1-11** also showed cytotoxic activities against the cancer cell line of K562 *in vitro* as antineoplastic agents. The structures of **1-14**

were elucidated on the basis of chemical evidence and IR, FAB-MS, ^{1}H-NMR, ^{13}C-NMR, and two-dimensional NMR (2D-NMR) analysis.

Results and Discussion

The crude bioactive saponin fraction of *D. collettii* var. *hypoglauca* was fractionated by a combination of silica gel chromatography and HPLC on silica gel RP-18 to afford compounds **1-14**. Compounds **1-11** and **14** were identified as the known prosapogenin A of dioscin (**1**), dioscin (**2**), gracillin (**3**), protoneodioscin (**4**), protodioscin (**5**), protoneogracillin (**6**), protogracillin (**7**), methyl protoneodioscin (**8**), methyl protodioscin (**9**), methyl protoneogracillin (**10**), methyl protogracillin (**11**) and pregna-5, 16-dien-20-one 3-O-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-O-β-D-glucopyranoside, respectively, based on their spectral data and by a comparison of their physical properties with those reported in the literature. It seems that the 22-hydroxyl compounds **4-7** existed in nature and that they were methylated to form the corresponding 22-methoxyfurostanol glycosides **8-11** when treated with methanol during the process of isolation, respectively [8].

Hypoglaucin F (**12**) was obtained as a white amorphous powder and showed a purple coloration with Ehrlich reagent. On acid hydrolysis, GC analysis of the pertrimethylsilyated sugars in the hydrolysate of **12** showed rhamnose and glucose to be present in a ratio of 1:1. The aglycone of **12** was identified as isonarthogenin (25S) with the authentic sample. On enzymic hydrolysis with β-glucosidase, compound **12** gave glucose and the corresponding spirostanol saponin **12a**, which was identified as isonarthogenin 3-O-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside [9].

The loactions of the glycosidic linkages were elucidated by analysis of the two-dimentional NMR (2D-NMR) spectra, especially the HMBC spectrum. Complete assignments of the ^{1}H and ^{13}C NMR signals of four sugars and the aglycone were achieved with the aid of the ^{1}H-^{1}H COSY and HMQC spectra (Table 1).

In Table 1, the ^{1}H and ^{13}C NMR spectral data of **12** are listed for comparison with those of **5**. The ^{1}H NMR spectrum of **12** showed C-18, C-19 and C-21 methyl groups at δ 0.81 (*s*), 1.04 (*s*) and 1.09 (*d*), respectively. However, the C-27 methyl doublet observed in the spectrum of **5** at δ 0.98 (*d*) was absent in that of **12**, and was replaced by a two-proton signal at δ 3.71 and 4.01, which revealed a typical CH_2OH group next to a chiral centre. The substituent hydroxyl group attached to C-27 led to the chemical shift down-field at C-25 (β-C) and up-field at C-24 and C-26 (γ-C) in the ^{13}C NMR spectrum of **12** when compared with the signals in the spectrum of **5**. Signals for four anomeric protons at δ 6.34 (*d*, J = 0.9 Hz), 5.80 (*d*, J = 0.9 Hz), 4.92 (*d*, J = 7.2 Hz) and 4.74 (*d*, J = 7.8 Hz), and an olefinic proton at δ 5.32 (*br d*) were also observed in the ^{1}H NMR spectrum of **12**, which were the same as those of **5**. Similarly, the ^{13}C NMR spectral data for the four sugars in **12** were also in agreement with those for **5**. Furthermore, in the HMBC spectrum of **12**, the anomeric proton signals at δ 6.34 (H-1'' of the terminal rhamnose attached to C-2' of the inner glucose), 5.80 (H-1''' of the terminal rhamnose attached to C-4' of the inner glucose), 4.92 (H-1' of the 2', 4'-substituted inner glucose attached to C-3 of the aglycone) and 4.74 (H-1'''' of the terminal glucose attached to C-26 of the aglycone) showed cross-peaks with the carbon signals at δ 78.0 (C-2' of the 2', 4'-substituted inner glucose), 78.9 (C-4' of the 2', 4'-substituted inner glucose), 78.2 (C-3 of the aglycone) and 72.0 (C-26 of the aglycone), respectively. These signals were sufficient to determine the linkages by which the sugars were connected. Based on the data mentioned above, **12** was determined to be (25S)-26-O-β-D-glucopyranosyl 22-hydroxy-5-en-furostane-3β, 26, 27-triol 3-O-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-

glucopyranoside.

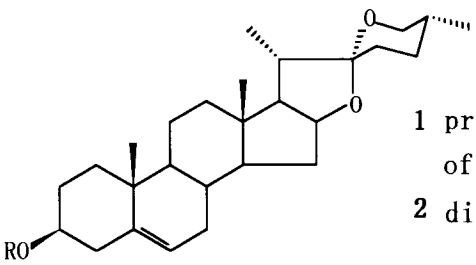
Compound **13** was obtained as white amorphous powder and responsed positively to the Libermann-Burchardt and Molish test, indicating it to be a glycoside. The HR-MS showed a molecular ion peak at m/z 1085.5186 corresponding to $C_{51}H_{82}O_{23}Na$. On acid hydrolysis, GC analysis of the pertrimethylsilated sugars in the hydrolysate of **13** showed rhamnose and glucose to be present in a ratio of 1:1. The precipitates was identified as pregna-5, 16-dien-3β-ol-one with the authentic compound.

Fully decoupled ^{13}C-NMR and DEPT NMR spectra of **13** exhibited 51 carbon signals, consisting six methyls, twelve methylenes, and five quaternary carbons. The ^{1}H-NMR spectrum of **13** confirmed the presence of six methyl groups at δ 0.90 (*d*, J = 6.5 Hz, 27-H_3), 1.03 (*s*, 18-H_3), 1.21 (*s*, 19-H_3), 1.62 (*d*, J = 6.0 Hz, Rha 6'''-H_3), 1.74 (*d*, J = 6.0 Hz, Rha 6''-H_3) and 2.11 (*s*, 21-H_3), and revealed in addition four anomeric protons at δ 6.40 (*d*, J = 1.0 Hz, Rha-1''), 5.85 (*d*, J = 1.0 Hz, Rha-1'''), 4.93 (*d*, J = 7.8 Hz, Glc-1') and 4.78 (*d*, J = 7.8 Hz, Glc-1''''), together with an olefinic protonat δ 5.30 (*br d*, H-6).

The ^{13}C-NMR assignments of the aglycone moiety of **13** were based largely on HMQC and HMBC spectra. Many unambiguous assignments were made on the basis of long-range correlations of the methyl protons. Such experiments may be exemplified by 21-H_3 at δ 2.11 showing correlations with C-17 and C-20 at δ 66.6 and 205.4, respectively. Furthermore, H-16 at δ 5.66 (*m*) exhibited correlation with C-22 at δ 173.3. The J value of 8.0 Hz between H-16 and H-17 suggested the β-configuration of the side chain of γ-methyl valeroxy group attached to C-16.

The ^{13}C-NMR assignments of the sugar moiety of **13** were achieved by referring to those literature data of the authentic methyl glycosides [10, 11], and were confirmed by ^{1}H-^{1}H COSY, HMQC and HMBC spectra. The data also indicated the presence of two rhamnose unit and two glucose unit. The β-configuration of the anomeric carbon of the glucose in **13** was proved by the large $J_{H1\text{-}H2}$ value (> 7.0 Hz). The α-configuration of the anomeric carbon of the rhamnose was assured by comparison of the chemical shift values of carbons 3'', 5'', 3''' and 5''' with those of the corresponding carbons of methyl α- and β-rhamnopyronoside. In HMBC spectrum, the anomeric proton signals at δ 6.40 (Rha-1''), 5.85 (Rha-1'''), 4.93 (Glc-1') and 4.78 (Glc-1'''') showed cross-peaks with the carbon signals at δ 77.7 (Glc-2'), 78.6 (Glc-4'), 78.1 (C-3) and 74.7 (C-26), respectively.These signals were complete to determine the linkages by which the sugars were connected. From an analysis of all the above data, the structure of **13** was established as 26-O-β-glucopyranosyl 16β-(γ-methyl valeroxy)-pregn-5-en-3β, 26-diol-20-one 3-O-α-L-rhamnopyranosyl -(1→2)-[α-L-rhamnopyranosyl-(1→4)-]-β-D-glucopyranoside.

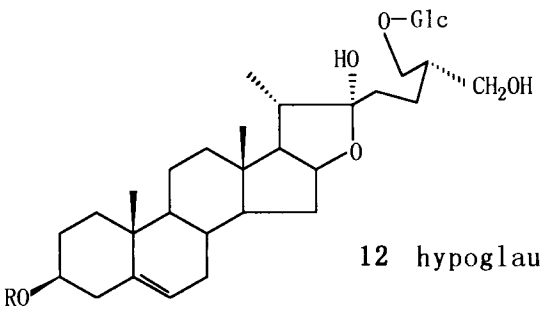
As listed in Table 2, fourteen active steroidal saponins **1-14** isolated under guidence of the *P. oryzae* model were further bioassayed with cytotoxic activities against the cancer cell line of K562 *in vitro*. As to causing morphological abnormality of *P. oryzae* mycelia, the active intensities of spirostanol saponins **1-3** are much stronger than those of furostanol saponins **4-12** and pregnane glycosides **13** and **14**. The 22-hydroxy furostanol saponins **4-7** showed weaker activities against *P. oryzae* than those of the 22-methoxyl artifacts **8-11**. But for inhibition of the cancer cell line of K562, furostanol saponins **4-11** possessed similar activities to those of spirostanol saponins **1-3**, whereas **12-14** showed no activities. The 22-hydroxy furostanol saponins **4-7** also showed activities compared with 22-methoxy artifacts **8-11** at about the same degree. 25R-Furostanol saponins **5**, **7**, **9** and **11** exhibited no significant difference in bioactivities with their corresponding 25S steroisomers **4**, **6**, **8**, and **10** in the bioassays of *P. oryzae* and K562.



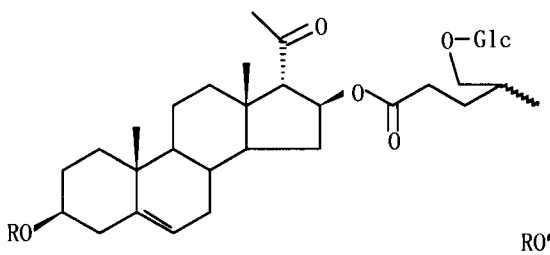
1 prosapogenin A of dioscin R = -Glc(2)-Rha

2 dioscin R = -Glc(4)-Rha, Glc(2)-Rha

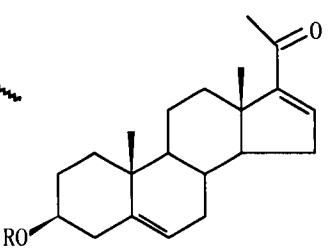
3 gracillin R = -Glc(3)-Glc, Glc(2)-Rha



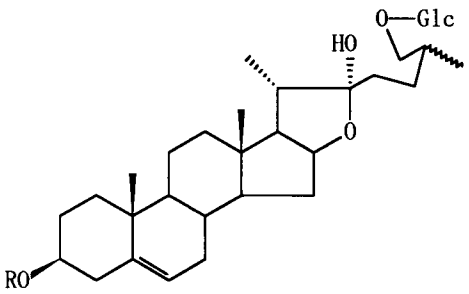
12 hypoglaucin F R = -Glc(4)-Rha, Glc(2)-Rha



13 hypoglaucin G R = -Glc(4)-Rha, Glc(2)-Rha



14 hypoglaucin H R = -Glc(4)-Rha, Glc(2)-Rha

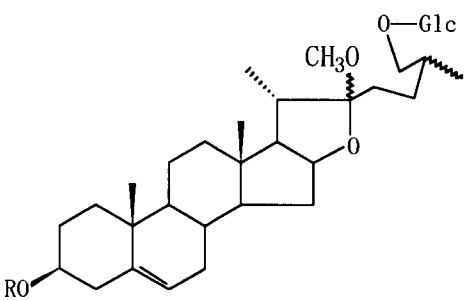


4 protoneodioscin R = −Glc(4→1)Rha, Glc(2→1)Rha — 25R

5 protodioscin R = −Glc(4→1)Rha, Glc(2→1)Rha — 25S

6 protoneogracillin R = −Glc(3→1)Glc, Glc(2→1)Rha — 25R

7 protogracillin R = −Glc(3→1)Glc, Glc(2→1)Rha — 25S



8 methyl protoneodioscin R = −Glc(4→1)Rha, Glc(2→1)Rha — 25R

9 methyl protodioscin R = −Glc(4→1)Rha, Glc(2→1)Rha — 25S

10 methyl protoneogracillin R = −Glc(3→1)Glc, Glc(2→1)Rha — 25R

11 methyl protogracillin R = −Glc(3→1)Glc, Glc(2→1)Rha — 25S

Table 1. ^{1}H and ^{13}C NMR data* for compounds **12** and **5** in pyridine-d_5 (δ values)†

position	**12**		**5**	
	H	C	H	C
1	0.98, 1.04 (o)‡	37.5	0.96, 1.74 (o)	37.5
2	1.86, 2.07 (o)	30.2	1.82, 2.07 (o)	30.2
3	3.86 (m)	78.2	3.86 (m)	78.2
4	2.70, 2.79 (m)	39.0	2.71, 2.78 (m)	39.0
5		140.9		140.9
6	5.32 (*br d*)	121.8	5.31 (*br d*)	121.8
7	1.50, 1.87 (o)	32.3	1.48, 1.87 (o)	32.4
8	1.55 (o)	31.7	1.56 (o)	31.7
9	0.89 (o)	50.4	0.88 (o)	50.4
10		37.2		37.2
11	1.41 (o)	21.1	1.45 (o)	21.1
12	1.06, 1.66 (o)	39.9	1.10, 1.74 (o)	40.0
13		40.5		40.8
14	1.07 (o)	56.7	1.07 (o)	56.6
15	1.43, 2.02 (o)	32.2	1.44, 2.02 (o)	32.5
16	4.49 (o)	81.5	4.92 (o)	81.1
17	1.76 (o)	62.9	1.92 (o)	63.9
18	0.81 (s)	16.3	0.89 (s)	16.5
19	1.04 (s)	19.4	1.04 (s)	19.4
20	1.92 (o)	42.0	2.23 (o)	40.7
21	1.09 (*d*, 6.9)§	14.9	1.32 (*d*, 6.5)	16.5
22		109.5		110.7
23	1.65 (o)	31.3	2.01, 2.03 (o)	37.2
24	1.65 (o)	24.0	1.66, 2.03 (o)	28.3
25	2.04 (o)	36.7	1.91 (o)	34.3
26	3.45, 3.92 (o)	72.0	3.62, 3.92 (o)	75.2
27	3.71, 4.01 (o)	63.7	0.98 (*d*, 6.8)	17.5
C-3 sugar part				
Glc	4.92 (*d*, 7.2)	100.3	4.92 (*d*, 7.2)	100.3
(inner)	4.19 (o)	78.0	4.19 (o)	77.9
	4.19 (o)	77.9	4.19 (o)	78.0
	4.34 (o)	78.9	4.34 (o)	78.8
	3.63 (m)	76.9	3.62 (m)	76.9
	4.07, 4.19 (o)	61.4	4.07, 4.19 (o)	61.4
Rha	6.34 (*d*, 0.9)	102.0	6.34 (*d*, 0.9)	102.0
(1→2)	4.79 (*dd*, 0.9, 3.5)	72.5	4.80 (*dd*, 0.9, 3.5)	72.5
	4.59 (*dd*, 3.5, 9.0)	72.8	4.59 (*dd*, 3.5, 9.0)	72.8
	4.31 (o)	74.2	4.32 (o)	74.2
	4.92 (o)	69.5	4.92 (o)	69.5
	1.74 (*d*, 6.5)	18.6	1.74 (*d*, 6.5)	18.5

Rha	5.80 (*d*, 0.9)	103.0	5.80 (*d*, 0.9)	102.9
(1→4)	4.64 (*dd*, 0.9, 3.5)	72.5	4.64 (*dd*, 0.9, 3.5)	72.5
	4.50 (o)	72.7	4.50 (o)	72.7
	4.29 (o)	73.9	4.29 (o)	73.9
	4.85 (o)	70.5	4.86 (o)	70.4
	1.60 (*d*, 6.5)	18.5	1.60 (*d*, 6.5)	18.6
C-26 sugar				
part	4.74 (d, 7.8)	105.0	4.75 (*d*, 7.8)	104.9
Glc	4.00 (o)	75.2	3.99 (o)	75.2
	4.19 (o)	78.6	4.19 (o)	78.4
	4.20 (o)	71.8	4.20 (o)	71.8
	3.92 (o)	78.6	3.91 (o)	78.6
	4.37, 4.54 (o)	62.9	4.34, 4.50 (o)	62.9

* Recorded on a JNM Alpha-500 (^{1}H 500 MHz, ^{13}C 125 MHz) spectrometer in C_5D_5N.
† All of the signals were assigned by ^{1}H-^{1}H COSY, HMQC and HMBC spectra.
‡ Overlapped signals are indicated by "(o)".
§ *J* values (in parentheses) are reported in Hz.

Table 2. The bioactivities of **1-14** against *P. oryzae* and K562 in comparison to rhizoxin.

Compounds		P. oryzae (MMDC* μM)	K562 (IC_{50} μM)
1	prosapogenin A of dioscin	5.5	7.0
2	dioscin	2.3	1.0
3	gracillin	9.0	1.2
4	protoneodioscin	95.4	2.7
5	protodiscin	95.4	1.6
6	protoneogracillin	94.0	6.6
7	protogracillin	94.0	3.3
8	methyl protoneodiscin	15.1	2.7
9	methyl protodioscin	15.1	1.6
10	methly protoneogracillin	14.8	6.6
11	methyl protogracillin	14.8	3.3
12	hypoglaucin F	324	-
13	hypoglaucin G	135	-
14	hypoglaucin H	236	-
	rhizoxin	0.008	0.003

* MMDC (minimum morphological deformation concentration).
† No activity agaist the cancer cell line K562.

Experimental

General. Mps: Yanaco MP-S3 micro-melting point apparatus, uncorr. Optical rotations: Perkin-Elmer 241 polarimeter at 13°. IR: Jasco A-102 (KBr). ^1H and ^{13}C-NMR: JNM Alpha-500 (^1H 500 MHz, ^{13}C 125 MHz) spectrometer with TMS as int. standard in C_5D_5N. Positive-ion FAB-MS: JEOL JMS-DX302. GC: HP-5890 SERIEIS II. Prep. HPLC: Liquid Chromatograph LC-10 (Japan Analytical Industry Co., Ltd.) using an ODS column (Waters, 20 × 250 mm, 5 μm) with flow rate of mobile phase 3.0 ml min^{-1}. C. C. silica gel H (10-40 μm, Qindao Haiyang Chemical Factory). TLC and VLC: silica gel G (10-40 μm, Qindao Haiyang Chemical Factory). Spots were visualized by spraying with 10 % H_2SO_4 followed by heating.

Plant material. Rhizomes of *Dioscorea collettii* var. *hypoglauca* (Dioscoreaceae) were collected in 1994 from Zhejiang Province (China) and were identified by Prof. Zherong Jiang (Division of Pharmacognosy, Shenyang Pharmaceutical University). A voucher specimen is deposited at the herbarium of Shenyang Pharmaceutical University (No. 104), Liaoning Province.

Bioassay. K562 cells were provided by Prof. T. Tsuruo (Institute of Molecular & Celluar Biosciences, The University of Tokyo) and maintained in PRMI1640 medium supplemented with 5% fetal bovine serum. The cultures were incubated at 37°C in a 5% CO_2 humidified incubator and subcultured every 2d to maintain them in a state of logarithmic growth (12). Test compounds **1-14** were dissolved in DMSO and diluted with ethanol. Addition of the samples to cell cultures was performed in such a way that the final concentration of DMSO/ethanol did not exceed 0.5% (v/v). K562 cells (1×10^5 cells/ml) were incubated for 2d in the presence of samples. The results are presented in Table 2.

Extraction and isolation. Air-dried powdered rhizomes (1500 g) of *D. collettii* var. *hypoglauca* were refluxed with 75% EtOH (15 l). The combined EtOH solns were concd *in vacuuo* to give 133.5 g extract. A suspension of the resulting extract in H_2O was patitioned successively with $CHCl_3$, EtOAc, and n-BuOH to afford 4 frs, i.e., DC (4.5 g), DE (4.2 g), DB (13.5 g) and DH (102.1 g) residues.

Fr. DC (4.5) was subjected to VLC [13] on silica gel G (250 g) and eluted with stepwise with $CHCl_3$ (2700 ml), $CHCl_3$-EtOAc (19:1, 1820 ml; 9:1, 900 ml; 17:3, 1920 ml;7:3, 1450 ml; 13:7, 2120 ml; 2:3, 1800 ml), EtOAc (1 l) and Me_2CO (1 l) to afford 9 frs (DC-1 - DC-9). Fr. DC-9 was further chrommatographed on silica gel H (30 g) with $CHCl_3$-MeOH (6:1) as eluent to give **1** (18.2 mg) and **2** (20.3 mg).

Fr. DE (4.2 g) was subjected to GC on silica gel H (330 g) and was eluted with $CHCl_3$-MeOH (5:1) to afford **3** (46.8 mg).

Fr. DB (13.5 g) was subjected to CC on silica gel H (300 g) and eluted stepwise by $CHCl_3$-MeOH-H_2O (80:5:0.1, 80:20:1, 80:25:1 and 14:6:1; each 3 l) to give 4 corresponding frs, i.e., fr. DB-1-DB-4). Fr. DB-4 (4718 mg) was further sepd by prep. HPLC (column, ODS, 5 μM , 20 × 250 μm; solvent, 50 % aq. MeOH; flow rate, 3.0 ml min^{-1}) to give **8** (366 mg), **9** (2042 mg), **10** (76.7 mg), **11** (441 mg), **12** (22.1 mg), **13** (60.7 mg) and **14** (7.4 mg). Compounds **8-11** were refluxed with 50% aq. Me_2CO at 90° for 24 hr to give **4** (340 mg), **5** (1810 mg), **6** (60.6 mg) and **7** (394 mg), respectively. Compounds **4-7** were easily converted into the corresponding compounds **8-11** when refluxed with dry MeOH at 95° for 36 hr. This suggested that **4-7** were 22-hydroxyfurostanol saponins, whereas **8-11** were their corresponding artifacts, i.e., 22-methoxyl derivatives [14].

Hypoglaucine F (**12**). Amorphous powder, m.p > 300° (dec.); $[\alpha]_D$: - 94.3° (pyridine; *c* 0.01). IR ν_{max} cm^{-1}: 3400 (OH), 1000-1100 (glycosyl C-O). Anal. calc. for $C_{51}H_{84}O_{23}$. $2H_2O$; C, 55.64; H, 8.00; found , C, 55.54; H, 8.05%. FAB-MS (positive) m/z: 1047 $[M + H - H_2O]^+$, 885 $[M + H - H_2O - Glc]^+$, 739 $[M + H - H_2O - Glc - Rha]^+$, 721 $[M + H - H_2O \times 2 - Glc -$

Rha]$^+$, 575 [M + H - H_2O - Glc - Rha × 2]$^+$, 413 [M + H - H_2O - Glc × 2 - Rha × 2]$^+$. ^{1}H-NMR: δ 0.81 (3H, *s*, 18-H_3), 1.04 (3H, *s*, 19-H_3), 1.09 (3H, *d*, *J* = 6.5 Hz, 21-H_3), 1.60 (3H, *d*, *J* = 6.5 Hz, Rha 6'''-H_3), 1.74 (3H, *d*, *J* = 6.5 Hz, Rha 6''-H_3), 4.74 (1H, *d*, *J* = 7.8 Hz, Glc-1''''), 4.92 (1H, *d*, *J* = 7.2 Hz, Glc-1'), 5.30 (1H, *br d*, 6-H), 5.80 (1H, *d*, *J* = 0.9 Hz, Rha-1'''), 6.34 (1H, *d*, *J* = 0.9 Hz, Rha-1''). ^{13}C-NMR: Table 1.

Hypoglaucine G (**13**). Amorphous powder, m.p 154-156°; $[\alpha]_D$: - 48.6° (MeOH; *c* 0.21). IR ν_{max} cm^{-1}: 3400 (OH), 1705 (C=O), 1040 (glycosyl C-O). HRMS m/z: [M+Na]$^+$ 1085.5186 (calcd for $C_{51}H_{82}O_{23}Na$, 1085.5145). FAB-MS (positive) m/z: 1087, 923, 791, 787, 613, 495, 333, 297, 185, 115, 93, 43. ^{1}H-NMR: δ 0.90 (3H, *d*, *J* = 6.5 Hz, 27-H_3), 1.03 (3H, *s*, 19-H_3), 1.21 (3H, *s*, 18-H_3), 1.62 (3H, *d*, *J* = 6.5 Hz, Rha 6'''-H_3), 1.74 (3H, *d*, *J* = 6.0 Hz, Rha 6''-H_3), 2.11 (3H, *s*, 21-H_3), 2.47 (1H, *d*, *J* = 8.0 Hz, 17-H), 4.78 (1H, *d*, *J* = 8.0 Hz, Glc-1''''), 4.93 (1H, *d*, *J* = 7.8 Hz, Glc-1'), 5.30 (1H, *br d*, 6-H), 5.85 (1H, *d*, *J* = 1.0 Hz, Rha-1'''), 6.40 (1H, *d*, *J* = 1.0 Hz, Rha-1''). ^{13}C-NMR: δ aglycone 37.4 (C-1), 30.1 (C-2), 78.1 (C-3), 39.0 (C-4), 140.9 (C-5), 121.6 (C-6), 31.9 (C-7), 31.0 (C-8), 50.4 (C-9), 37.0 (C-10), 20.6 (C-11), 38.1 (C-12), 42.3 (C-13), 54.1 (C-14), 35.5 (C-15), 74.7 (C-16), 66.6 (C-17), 13.8 (C-18), 19.4 (C-19), 205.4 (C-20), 30.4 (C-21), 173.3 (C-22), 32.2 (C-23), 29.0 (C-24), 33.4 (C-25), 74.7 (C-26), 16.9 (C-27), 3-O-Glc (inner) 100.3 (C-1'), 77.7 (C-2'), 78.0 (C-3'), 78.6 (C-4'), 77.0 (C-5'), 61.3 (C-6'), Rha (1→2) 102.0 (C-1''), 72.5 (C-2''), 72.8 (C-3''), 74.1 (C-4''), 69.5 (C-5''), 18.6 (C-6''), Rha (1→4) 102.9 (C-1'''), 72.6 (C-2'''), 72.7 (C-3'''), 73.9 (C-4'''), 70.4 (C-5'''), 18.5 (C-6'''), 26-O-Glc 104.9 (C-1''''), 75.2 (C-2''''), 78.5 (C-3''''), 71.7 (C-4''''), 78.5 (C-5''''), 62.8 (C-5'''').

Acid hydrolisis of **12**. Compound **12** (a few mg) was heated with 2N HCl-dioxane (1:1, 2ml) in a sealed tube at 100°C for 4hr. The aglycone was identified as isonarthogenin when compared with the corresponding authentic sample. The reaction mixt. was concd to dryness under N_2 at room temp. For GC analysis, the residue was trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane (2:1) [15] at room temp. GC: SE30 capillary column (12 m × 0.22 mm i.d.); detector: FID (270°); column temp. 170-210°, rate 5° min^{-1}; carrier gas: N_2 (30 ml min^{-1}); R_t: rhamnose (3.72 min) and glucose (7.12 min).

Acid hydrolisis of **13**. Compound **13** (a few mg) was refluxed with 1N H_2SO_4 in 50% aq. Me_2CO for 5 hr. The precipitates **13a** formed on cooling was confirmed as pregna-5, 16-dien-3β-ol-20-one. The filtrate was evapd *in vaccuo* to dryness and then the residue was examined on TLC with the authentic compounds of glucose and rhamnose.

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SCREENING FOR MOLLUSCICIDAL ACTIVITY IN MEDICINAL PLANT

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Abstract Thirty-two extracts of medicinal plants were screened for activity against *Oncomelania hupensis*, the intermediate host of schistosomiasis due to *Schistosoma japonicum*. Strong molluscicidal activities were found in the n-Butyl alcohol extracts of rhizomae of *Dioscorea zingiberensis* and *Anemarrhena asphodeloides*, flower of *Liriope gramnifolia*. Further studies showed that the major molluscicidal constituents of *Dioscorea zingiberensis* were gracillin and zingiberenin A, which had a snail mortality of 98% and 96% at 5mg/l in 72 hours respectively.

Introduction

Schistosomiasis is a parasitic disease which may afflict people's health seriously, and it is widely distributed in South America, Africa and Asia. In China, this disease is mainly endemic in Yangtze river area. To control schistosomiasis, one of the effective ways is to kill its transmitter hosts—aquatic snails or amphibious snails. Synthetic molluscicides are expensive and may lead to environmental problems, so the use of plant molluscicides becomes a proper approach to the control of the snail host in the developing countries.

The control of the aquatic snails such as *Biomphalaria glabrata* and *Bulinus trancatus* have been frequently studied in European, African and South-American countries. But in China, this endemic disease is caused by the amphibious snail *Oncomelania hupensis*. To kill this kind of snail, the molluscicides should not only toxic to it, but also have the abilities to prevent it from up-crawling out of the water while the molluscicides take effect.

In connection with our systematic isolation and structure studies on molluscicidal constituents from medicinal plants, we noticed that the crude n-Butyl alcohol extracts of rhizomae of *Dioscorea zingiberensis* and *Anemarrhena asphodeloides*, flower of *Liriope gramnifolia* possessed strong molluscicidal activities. In this paper, we report the results of preliminary molluscicidal screening of medicinal plants using *Oncomelania hupensis* snails, and describe the isolation of the active constituents of *D. zingiberensis*.

Results and Discussion

Thirty-two extracts of medicinal plants have been screened for molluscicidal activities against *O. hupensis*, the intermediate host of the chinese strain of *Schistosoma japonicum*. Of these samples tested, 7 samples showed strong molluscicidal activity, they are: *Liriope gramnifolia* (flower), *Dioscorea zingiberensis* (rhizoma), *Anemarrhena asphodeloides* (rhizoma), *Anemone raddeana* (rhizoma), *Angelica taiwaniana* (rhizoma), *Sapindus mukorosii*(fruit), *Melia azedarach* (fruit). However, only *D. zingiberensis* and *L. gramnifolia* can effectively prevent the snails from up-crawling out of the water in the up-crawling tests. And though *A. asphodeloides* has a significant molluscicidal activity, it can not inhibit the snail's up-crawling effectively.

Among the active extracts, *D. zingiberensis* was selected for further study. Bioassay-guided fractionation has led to the isolation and structure determination of two steroidal saponins: DZ_1 (gracillin), Diosgenin-3-O- [α-L-Rhamnopyranosyl-(1→2)] -O- [β-D-glucopyranolyl-(1→

3)] -O-β-D-pyranoglucoside;DZ_3 (zingiberenin A), Diosgenin-3-0- [β-D-glucopyranosyl-(1 → 2)] -O-[α-L-rhamnopyranosyl-(1 → 3)] -O-β-D-pyranoglucoside. DZ_1 has a snail mortality of 98% at 5 mg/l in 72 hours, while DZ_3 has a snail mortality of 96% at the same condition.

Besides gracillin and zingiberenin A, two protosaponins (protogracillin and protozingiberenin A) has also been isolated from the rhizoma of *D. zingiberensis*, but they have no molluscicidal activity.

Table 1 Results of Preliminary Molluscicidal Screening of Plant Extracts

Plant name	Parts	After 48h in test solv.	After 120h in test solv.
Aspidistra lurida	rhizoma	2/50	4/50
Aspidistra elatior	rhizoma,leaf	1/50	4/50
Liriope gramnifolia	rhizoma,flower	48/50	50/50
Ophiopogon japonicus	rhizoma,fruit	6/50	18/50
Pterocarya stenoptera	leaf	0/50	2/50
Celastrus angulatus	root	1/50	10/50
Platycodon grandiflorum	rhizoma	0/50	3/50
Polygala tenuifolia	rhizoma	2/50	10/50
Dioscorea zingiberensis	rhizoma	49/50	50/50
Clematic chinensis	rhizoma	0/50	0/50
Cenfella asiatica	whole plant	2/50	3/50
Achyranthes bidentata	rhizoma	0/50	3/50
Melia azedarach	bark, fruit	50/50	50/50
Sapindus mukorossi	fruit	18/50	43/50
Sophora flavescens	rhizoma	08/50	12/50
Anemone raddeana	rhizoma	46/50	50/50
Angelica taiwaniana	rhizoma	19/50	39/50
Belamcanda chinensis	rhizoma	3/50	8/50
Rumex japonicus	rhizoma	6/50	10/50
Phytolacca acinosa	rhizoma	8/50	15/50
Anemarrhena asphodeloides	rhizoma	43/50	50/50

a) Concentration 50mg/l b) number of dead snails/number of tested snails

Experimental

Extraction and isolation. The air-dried powdered rhizoma of *D. zingiberensis* (5kg) was extracted 4 times with 90% EtOH at room temperature. The extract was evaporated under reduced pressure and the residue (480g) was suspended in water. Centrifugation of this solution gived two parts, precipitate and mother liquor. The precipitate was treated with charcoal and recrystallized by 80% EtOH gived 40 g mixed crystal. Chromatography of 6g this crystal on silica gel gived 1.5g gracillin and 2.0g zingiberenin A.

The mother liquor was extracted with n-Butyl alcohol,the butanol fraction (80g) was rechromatographed on silica gel using $CHCl_3$-MeOH-H_2O as eluent. Two isospirostanol saponins (gracillin and zingiberenin A) and two furostanol saponins (protogracillin and protozingiberein A) were also obtained.

Materials and methods. Bioassay was done with snails of the species O. hupensis in a glass tank(35cm × 20cm) in the laboratory of the Department of Parasitology, Hubei Institute

of Preventive Medicine. Snails used in this experiment were mature and were newly collected from the field.

The test were carrried out by exposing the snails to a solution of a known concentration of test sample in distilled water at a temperature of 20 ± 2 ℃ for 24~120h .Following the exposure period, the snails were placed in distilled water, and the activities of the snails were observed. Whether the snails were living or dead based on observation after smashing the snails with a glass plate.

Plant materials were collected in Wuhan Botanical Garden. Rhizoma of *D. zingiberensis* was purchased from Yunxi County, Hubei Province.

A sample of each material was extracted with MeOH and the extract were concentrated, then dissolved in water, partitioned with n-Butyl alcolol-water. The Butanol part was concentrated, frozen and dried.

The samples were tested first at concentrations of 1000、100、50mg/l, then,the active samples were repeatedly tested at concentrations of 50、30、10mg/l, while the isolated saponins were tested at concentrations of 20、10、5mg/l at first, then tested at concentrations of 10、5、3mg/l.

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RAPID DETERMINATIONOF PLANT GLYCOISDES BY LC/MS AND LC/NMR

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1. INTRODUCTION

Efficient detection and rapid characterisation of natural products play an important role as an analytical support in the word of phytochemists. The identification of a metabolite at the earliest stage of separation is a strategic element for guiding an efficient and selective isolation procedure. In this respect, the role of hyphenated techniques such as high performance liquid chromatography coupled to mass spectrometry (LC/MS) or to nuclear magnetic resonance (LC/NMR) has became of great interest.

The plant kingdom represents an extraordinary reservoir of novel molecules. Of the estimated 400,000-500,000 plant species around the globe, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even lower. Since plants may contain hundreds, or even thousands, of metabolites, there is currently a growing interest in the vegetable kingdom as a possible source of new lead compounds for introduction into therapeutical screening programmes. The rapid disappearance of tropical forests and other important areas of vegetation has meant that it is essential to have access to methods which lead to the rapid isolation and identification of bioactive natural products.

The approach adopted to obtain an exploitable pure plant constituent involves interdisciplinary work in botany, pharmacognosy, pharmacology, chemistry, toxicology. The plant material is extracted by solvents of increasing polarity, the extracts are screened with different bioassays and submitted to fractionation with chromatographic techniques. This process is repeated until the isolation of a pure active constituent which is finally identified by spectroscopic methods (bioactivity-guided isolation)(Fig. 1).

By following this approach alone, there is a risk of unnecessarily isolating known plant constituents. Furthermore, interesting lead compounds which do not exhibit the tested activity will simply be missed. In order to avoid the time-consuming isolation of known constituent, hyphenated technique such as LC/MS, LC/UV and recently even

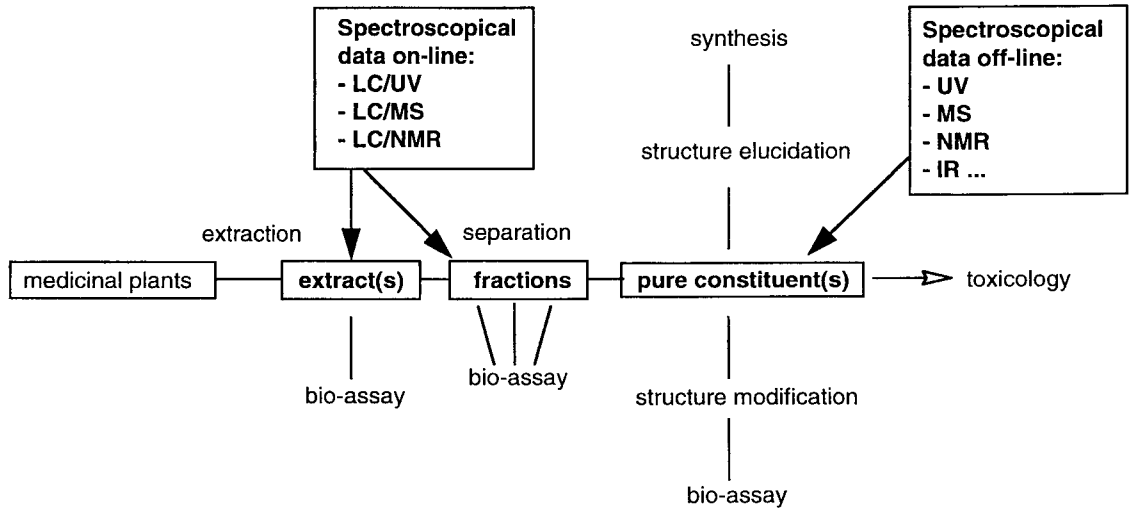


Fig. 1. Procedure for obtaining the active principles from plants and use of LC hyphenated techniques as strategic analytical screening tools during the isolation of constituents from a plant extract

LC/NMR can be used at the earliest stage of separation to screen chemically the crude extracts (Fig. 1)[1].

In this paper, the role of the LC chemical screening of crude extracts used as a complement to the biological screening will be particularly emphasised and mainly examples of analysis of polyphenol and triterpene glycosides will be discussed.

2. HYPHENATION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography (HPLC) is used routinely in phytochemistry to "pilot" the preparative isolation of natural products (optimisation of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds [2]. HPLC is the most well fitted technique for an efficient separation of crude plant extracts and can be coupled with different spectroscopic detection methods.

In our laboratory, for the screening of crude plant extracts, HPLC coupled with UV photodiode array detection (LC/UV-DAD) and mass spectrometry (LC/MS) has been mainly used [1,3,4]. Recently several trials have also shown that HPLC coupled with nuclear magnetic resonance (LC/NMR) is a very powerful complementary technique for the on-line plant metabolite identification [5,6]. This new tool will now be also implemented to the existing LC/UV/MS configuration [7].

2.1. Liquid chromatography / UV photodiode array (LC/DAD-UV)

HPLC coupled with UV photodiode array detection (LC/UV) has been used since more than a decade by phytochemists for screening extracts [8] and is now widely spread in many laboratories. The UV spectra of natural products give useful information on the type of constituents and also, as it is the case for polyphenols, information on the oxidation pattern. New instruments allow the recording of UV spectra of reference

compounds in databases and computer matching can be realised automatically when screening for known constituents.

2.2. Liquid chromatography / mass spectrometry (LC/MS)

HPLC coupled to mass spectrometry (LC/MS) has been introduced recently and is still not widely spread in the phytochemical community [1]. At present, MS is one of the most sensitive method of molecular analysis. Moreover, it has the potential to yield information on the molecular weight as well as on the structure of the analytes. Due to its high power of mass separation, very good selectivities can be obtained. The coupling between LC and MS has not been straightforward since the normal operating conditions of a mass spectrometer are diametrically opposed to those used in high performance liquid chromatography (HPLC) [9]. To cope with these different problems, many LC/MS interfaces have been built up. Each of them has its own characteristics and range of applications [10]. In our approach to LC/MS, mainly used for the HPLC screening of crude plant extracts, three interfaces, thermospray (TSP) [11], continuous flow FAB (CF-FAB) [12] and electrospray (ES) [13] have been investigated [10]. They cover the ionisation of relatively small non-polar products (aglycones, 200 u) to highly polar molecules (glycosides, 2000 u). LC/TSP-MS allowed a satisfactory ionisation of moderately polar constituents such as polyphenols or terpenoids in the mass range of 200-800 u. For larger polar molecules such as saponins (MW > 800 u), CF-FAB or ES are the methods of choice [10]. In our laboratory, thermospray is the most widely used interface.

2.3. Liquid chromatography / nuclear magnetic resonance (LC/NMR)

HPLC coupled with nuclear magnetic resonance (LC/NMR), despite being known for over fifteen years [14], has not been yet a widely accepted technique, mainly because of its lack of sensitivity. However, the recent progress in pulse field gradients and solvent suppression, the improvement in probe technology and the construction of high field magnets have given a new impulse to this technique [15], which has an

important potential for on-line structure identification of natural products. While the LC coupling itself was rather straightforward compared to LC/MS [16], the samples are flowing in a non-rotating glass tube (60-180 µl) connected at both ends with HPLC tubing, the main problem of LC/NMR was the difficulty of observing analyte resonances in the presence of the much larger resonances of the mobile phase. This problem was even worsened in the case of typical LC reversed phase operating conditions, where more than one protonated solvent was used and where the resonances changed frequencies during the analysis in gradient mode. Furthermore, the continuous flow of sample in the detector coil complicated solvent suppression. These problems have now been overcome thanks to the development of fast reliable and powerful solvent suppression techniques such as WET [17], which produced in both on-flow and stop-flow modes high quality spectra. These techniques consist of a combination of pulsed field gradients, shaped rf pulses, shifted laminar pulses and selective ^{13}C decoupling and are much faster than classical presaturation techniques previously used in this field [17]. Thus, in reversed HPLC conditions, non-deuterated solvents such as MeOH or MeCN can be used, while water is replaced by D_2O.

A general setup of the experimental configuration used for performing LC/UV/MS and LC/NMR analysis is presented in figure 2.

3. LC/MS ANALYSIS OF GLYCOSIDES

Natural products often exist in the form of glycosides. These conjugates may or may not occur together with their respective aglycones in the plants. Glycosides are thermally labile, polar and non-volatile compounds. Off-line mass spectral investigation requires soft ionisation techniques such as desorption chemical ionisation (D/CI) or fast atom bombardment (FAB) [18,19], if information on molecular weights or sugar sequences are desired. LC/MS with interfaces such as TSP, ESP or CF-FAB provides usually a soft ionisation of the glycosides but as this will be shown, the ionisation is mostly dependant on the number of sugar substituents on a given natural product.

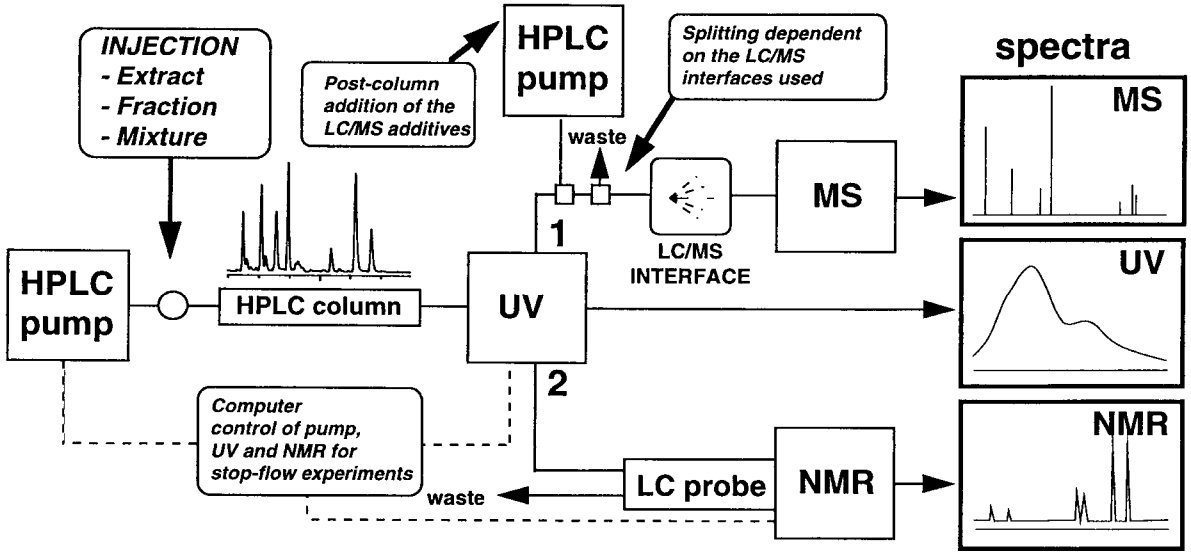


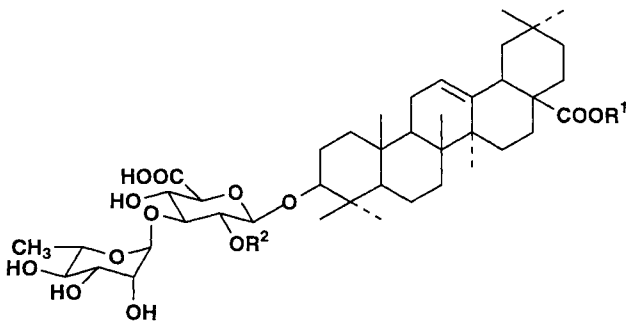
Fig. 2. Schematic representation of the experimental setup used for LC/UV/MS (1) and LC/UV/NMR (2) analyses

3.1. Comparison of different ionisation techniques for the analysis of saponins

In order to have a rapid idea about the saponin content of a plant extract, LC/ES-MS was compared to LC/TSP-MS and LC/CF-FAB-MS for the analysis of saponins in the crude MeOH extracts of the fruits of *Swartzia madagascariensis* [20]. In order to discuss the LC/MS analysis of *S. madagascariensis* extracts, three oleanolic acid saponins **1-3**, bearing respectively 4, 3 and 2 sugar units were selected (Fig. 3).

Swartzia madagascariensis Devaux (Leguminosae) is one of the most promising plants for the potential control of schistosomiasis-transmitting snails in Africa. Aqueous extracts of the seed pods contain large amounts of saponins with high molluscicidal activity. These saponins have been characterised and field trials with extracts of the fruits have been performed in Tanzania [21]. The most active saponins are glycosides of oleanolic acid.

The LC/TSP-MS (P.I., NH_4OAc buffer) analysis of the extract exhibited the presence of triterpene glycosides derived from oleanolic acid (MW: 456). Indeed, the TSP trace recorded at *m/z* 439 was characteristic for dehydrated oleanolic acid moieties $[A+H-H_2O]^+$ (trace, Fig. 4a). For **3**, a distinctive ion at *m/z* 796 and a fragment ion at *m/z* 650 were characteristic for a saponin bearing a diglycosidic moiety consisting of a terminal deoxyhexosyl unit (-146 u) and a glucuronic acid (-176 u) moiety. As rhamnose is the most frequent deoxyhexose occurring in saponins, it can be assumed from these on-line MS data that **3** was a saponin of oleanolic acid, substituted by a glucuronic acid unit and a rhamnosyl unit in the terminal position. The TSP spectra of saponins **1** and **2** were less clear than those of **3**. In both cases, characteristic signals for the oleanolic acid moiety were present, and fragment ions at *m/z* 795 were indicative of the presence of at least a glucuronic acid with a hexosyl unit. On the traces at *m/z* 941 and *m/z* 1103, no clear molecular ions for tri- or higher glycosylation were visible (trace, Fig. 4a). For these two metabolites, the LC/TSP-MS analysis alone could not give enough structural information on-line.



	R1	R2	MW	Sug.
3	H	H	778	2
2	H	Glc	940	3
1	Glc	Glc	1102	4

Fig. 3. Structures of selected saponins isolated from *Swartzia madagascariensis* (Leguminosae)

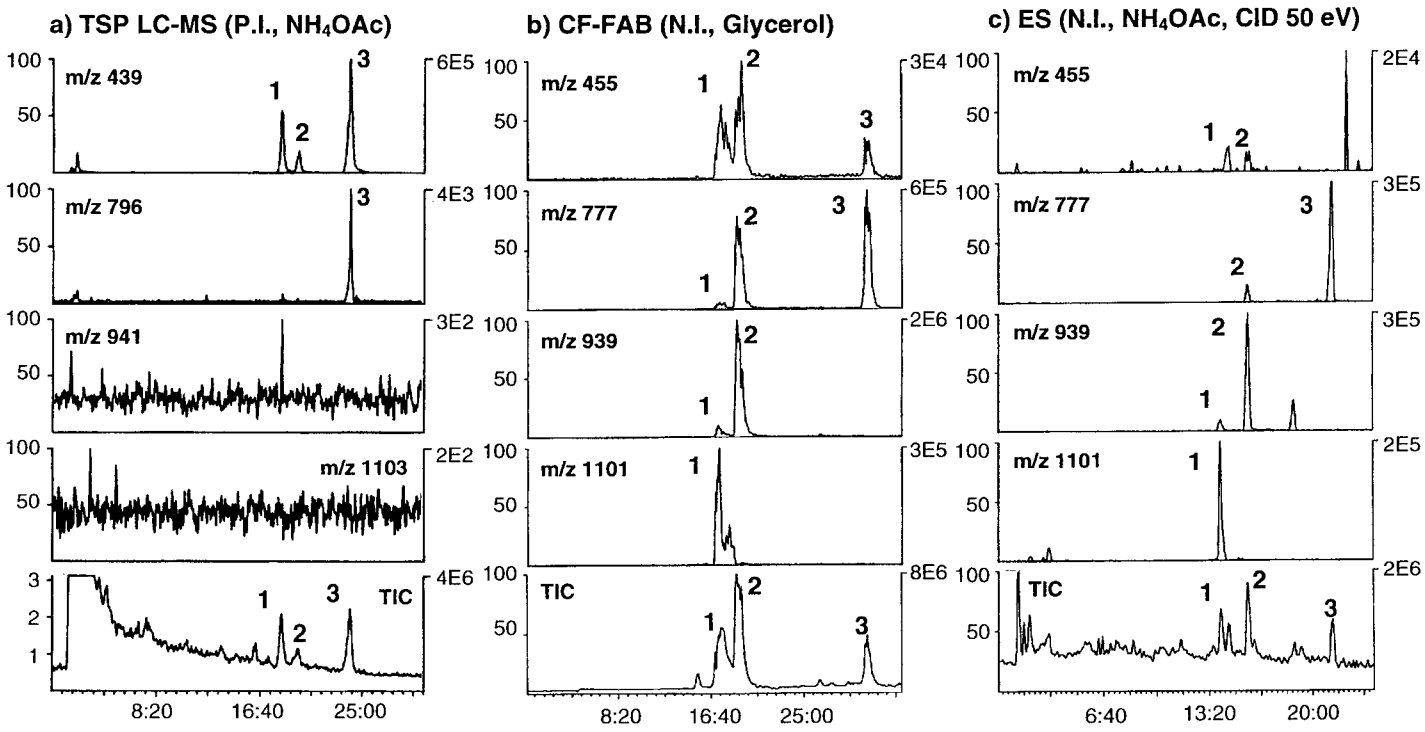


Fig. 4. Combined LC/TSP-MS, LC/CF-FAB-MS and LC/ES-MS of the MeOH extract of *Swartzia madagascariensis* (Leguminosae). HPLC: C18 Nova-Pak (4µm, 150 x 3.9 mm i.d.); gradient, CH_3CN-H_2O (0.05% TFA) 30:70 ->50:50 in 30 min (0.9 ml/min)

In the LC/CF-FAB-MS (N.I., glycerol matrix) analysis of the same extract (Fig. 4b), in contrast to the TSP results, all the saponins found in the extract exhibited intense deprotonated molecular ions $[M-H]^-$ and very weak ions characteristic for the aglycone moiety $[A-H]^-$ (*m/z* 455) and $[A-H-H_2O]^-$ (*m/z* 437). Furthermore, different characteristic cleavages were distinguishable. For **3**, ions at *m/z* 777 $[M-H]^-$, *m/z* 631 $[M-H-146]^-$ and *m/z* 455 $[A-H]^-$ confirmed the results obtained with TSP. For **2**, an intense $[M-H]^-$ ion at *m/z* 939 was observed in the CF-FAB spectrum, showing that it was a triglycosylated saponin (see trace *m/z* 939, Fig. 4 b). The different fragment ions recorded in the CF-FAB spectrum of **2** (*m/z* 777 $[M-H-162]^-$ and *m/z* 793 $[M-H-146]^-$) confirmed that **2** was probably similar to **3** with one more hexosyl unit in position C-28 or branched at the diglycosidic moiety. The CF-FAB spectra of **1** exhibited an intense molecular ion at *m/z* 1101 $[M-H]^-$ (see trace *m/z* 1101, Fig. 4b and spectrum, Fig. 5b). This indicated that **1** has one hexosyl unit (164 u) more than **2**. Saponin **1** was thus a tetraglycosylated triterpene.

The LC/ES-MS analysis of the same extract (N.I., NH_4OAc buffer, CID 50V) gave intense and clearly discernible $[M-H]^-$ ions and weak acetate anion adducts $[M+CH_3COO^-]$ for saponins **1-3** (trace, Fig. 4c, spectrum of **1**, Fig. 5c). With the aid of up front CID in the entrance octapole of the ES source, ions due to the loss of different sugar moieties were also observable. Aglycone ions at *m/z* 455 appeared only for **3** and **2** but not for **1**. In ES, almost no peak broadening was observed producing clearly defined peaks in the selected ion traces (Fig. 4c). In CF-FAB, on the contrary, large diminution of the chromatographic resolution was due to the important splitting and the post column addition of the glycerol matrix (Fig. 4b).

This example clearly shows the importance of the choice of the right interface for the ionisation of a given type of molecule. Conditions have to be chosen carefully according to the type of compounds that have to be screened by LC/MS. LC/ES-MS gave mainly molecular ions but structural information can be efficiently obtained by the subsequent fragmentation of these ions in MS/MS.

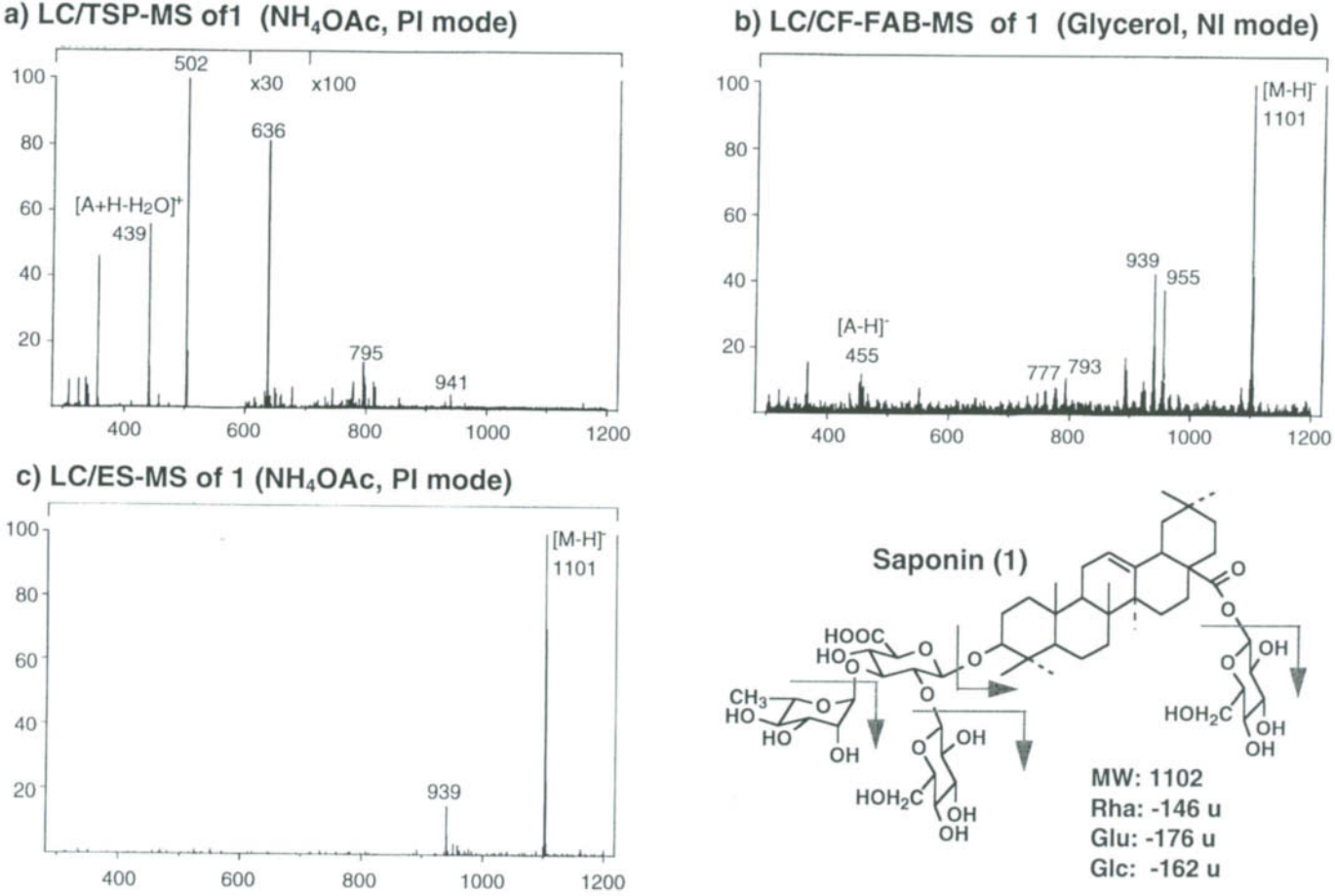


Fig. 5. Comparison of the LC/TSP-MS, LC/CF-FAB-MS and LC/ES-MS spectra of the tetraglycosylated saponin **1** in the MeOH extract of *Swartzia madagascariensis* (Leguminosae). Same conditions as in Fig. 4

3.2. Multiple stage LC/MS (MS^n) analysis for the sugar sequence determination

As shown, LC/ES-MS of saponins gives few fragments and complementary techniques such as MS/MS are needed to obtain more structural information on the sugar sequence. In order to illustrate this approach, the sugar sequence of some saponins of *Phytolacca dodecandra* L'Hérit (Phytolaccaceae) were tentatively identified on-line by LC/MS/MS. The dried berries of this plant, are used in Ethiopia as a soap substitute. The molluscicidal properties of their constituents were discovered by Lemma in 1965 and this plant became rapidly of great importance for the local control of schistosomiasis.

The LC/MS analysis of the methanolic extract of the berries was performed by LC/ES-MS in the negative ion mode with TFA as buffer. Under these conditions, intense TFA molecular ion adducts $[M+CF_3COO^-]$ for the different saponins of the extract were observed (See spectrum of **4**, Fig. 6a).

For the determination of the sugar sequence of these saponins, an early prototype of a new ion trap mass spectrometer was tested. With this instrument, it has been possible to perform not only an MS/MS experiment, but a multiple stage MS/MS experiment (MS^n). In MS^n, it was possible to isolate and excite only one ion of interest and thus, to decrease the amount of consecutive reactions [10]. For example on the pentaglycosylated saponin **4**, six stages of MS/MS were performed (MS^6). The sugar sequence information was obtained by successive decomposition of the main ion, as shown in Fig. 6.

The first step was the fragmentation of the strong TFA adduct at *m/z* 1363 (Fig. 6a), giving a deprotonated molecular ion $[M-H]^-$ (Fig. 6b). This latter ion yielded a first fragment at *m/z* 1087 (Fig. 6c). This first loss of 162 u corresponded to the departure of the glucosyl moiety at position C-28. This sugar was particularly sensitive due to the ester linkage. Then, the $[(M-Glc)-H]^-$ ion cleaved into two fragments at *m/z* 941 and *m/z* 925 (Fig. 6d), showing the simultaneous loss of a rhamnosyl or a glucosyl unit, respectively. These losses were characteristic for a branched sugar chain. In figure 6e., the ion at *m/z* 779 issuing from the fragmentation of *m/z* 941 (-Rha) or *m/z* 925 (-Glc)

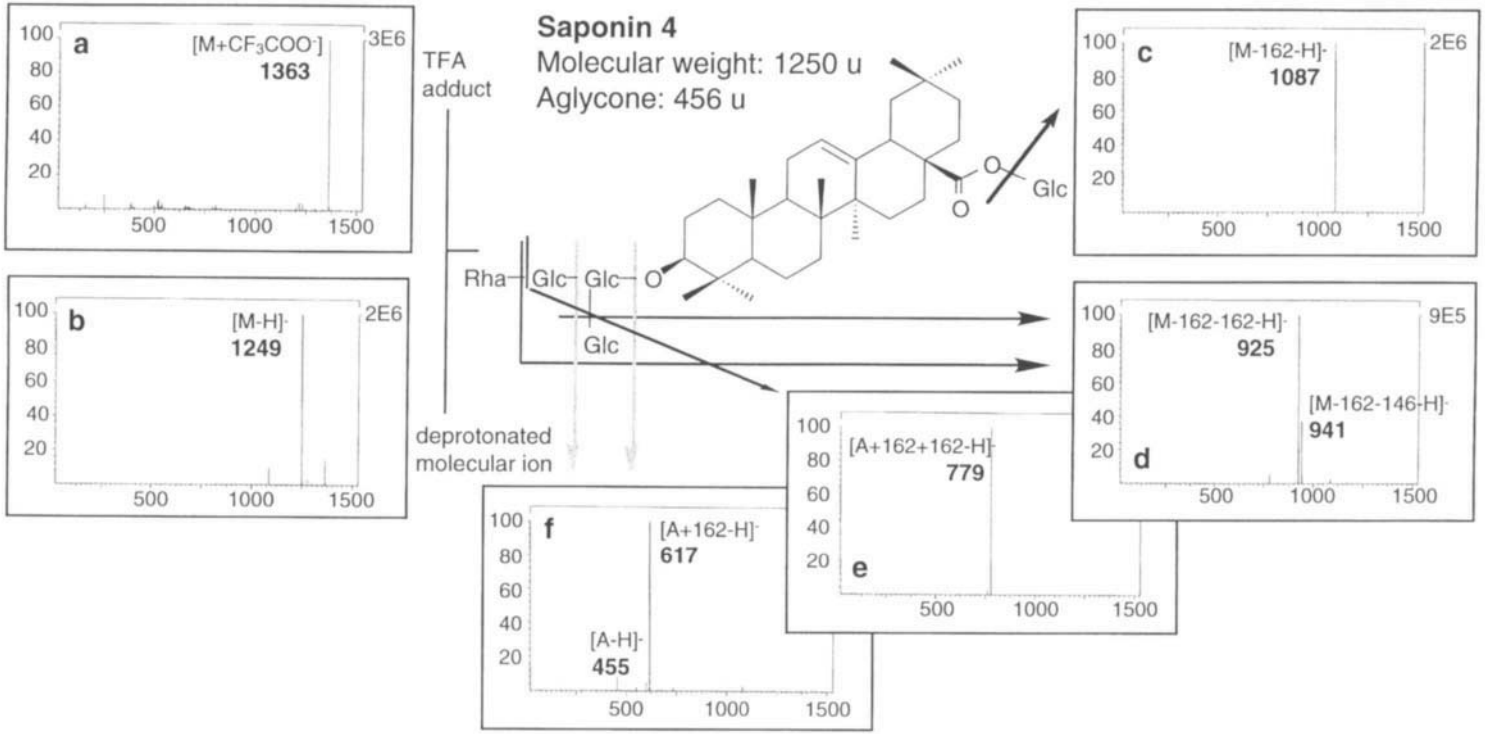


Fig. 6. ES MS^n spectra of the pentaglycosylated saponin **4** from the fruit methanolic extract of *P. dodecandra* (Phytolaccaceae). Sample (1 mg/ml) injected by a syringe pump (5µl/min) (infusion experiment). For LC/MS conditions, see experimental. This experiment allowed a sequential fragmentation of the saponin sugar chain (cleavage of only one sugar at each MS/MS step), clarifying the structural determination.

was observed. This ion corresponded to the diglucoside moiety, which gave finally the monoglucoside $[(A+Glc)-H]^-$ at *m/z* 617 (Fig. 6f) and the aglycone ion characteristic of oleanolic acid at *m/z* 455.

Thus, this MS^n analysis showed that it was possible to cleave only one sugar at a time by adjusting the collision energy, making the interpretation simpler. This type of experiment was found to be very useful for clarifying the sugar sequence of saponins.

3.3. Combined LC/MS analyses for the detection of secoiridoid glycosides

Secoiridoid glycosides are monoterpenes that are derived from secologanin, their biosynthetic precursor. They represent the bitter principles of many plant families, especially in the Cornales, Dipsacales and Gentianales orders. They may occur as esters with different acids, and especially with biphenylcarbonic acids, which enhance strongly their bitter taste. Plant containing these compounds are used in many herbal preparations for their stomachic properties.

Monoterpenes, like triterpene glycosides, have been analysed in LC/MS by both TSP of CF-FAB ionisation. As secoiridoid glycosides occur mainly as monoglucosides, TSP is often the method of choice for their detection and identification. Thus, the bitter principles of various Gentianaceae species have efficiently been screened by this mode [22,23]. However, in certain cases LC/CF-FAB-MS was found to be essential for the screening of unknown larger secoiridoids.

For example, the methanolic extract of *Gentiana rhodantha* Fr., a Chinese Gentianaceae species, was screened by LC/TSP-MS and LC/UV using our routine procedure for the Gentianaceae [24] in order to obtain rapid and precise information on its composition.

LC/UV analysis exhibited different peaks (**5-8**) with retention times less then 10 min (Fig. 7). Their UV spectra showed only one maximum around 240 nm that was characteristic for a chromophore containing an α,β-unsaturated ketone function, attributable to a secoiridoid glycoside(swertiamarin (**5**) (MW:374), kingiside (**6**) (MW:404), epi-kingiside (**7**) (MW:404) and sweroside (**8**) (MW:358)). A very

predominant component **9** (rt. 9'40") (MW: 422) was identified as the xanthone *C*-glycoside mangiferin by comparison with an authentic sample. The slower running compounds (**10-13**) (rt. 20-25 min) (Fig. 10b) also exhibited the same characteristic UV spectra of secoiridoids (one band at around 240 nm) (see **10** in Fig. 7b). These compounds, which were less polar than the common secoiridoids, were studied in more detail. A LC/TSP-MS analysis with ammonium acetate as buffer (positive ion mode) was carried out (Fig. 7a). Under these conditions, the technique usually gives intense pseudomolecular $[M+H]^+$ or $[M+NH_4]^+$ ions for the compounds under investigation. The analysis revealed the presence of sweroside **8** (MW: 358) in trace amounts (tr. 9'50" min) (Fig. 7a). This compound exhibited a characteristic TSP-MS spectrum with an intense pseudomolecular ion $[M+H]^+$ at *m/z* 359. The display of the single ion trace *m/z* 359 allowed the specific assignment of peak **8** , but it also showed important signals corresponding to the less polar "secoiridoid-like" compounds (**10-13**) (Fig. 7a). Indeed the TSP spectra recorded for **10-13** were identical and all exhibited an intense ion at 359 amu and no ion at higher masses. These first results obtained on-line with the crude extract of *G. rhodentha*, showed that all the compounds exhibited the same UV and TSP-MS spectra as sweroside **8.** However their chromatographic behaviour was quite different. In order to obtain complementary information on the constituents, a second LC/MS analysis with CF-FAB was achieved, using the same HPLC conditions (see experimental setup, Fig. 2). The total ion current recorded for the whole chromatogram showed a very important MS response for compounds **10-13**, while the more polar metabolites were only weakly ionised (Fig 10 B). The CF-FAB spectrum of **10** recorded on-line exhibited a very intense pseudomolecular ion $[M-H]^-$ at *m/z* 913 together with a weak ion at *m/z* 555 corresponding to the loss of a "sweroside like" unit $[M+H-358]^+$ (CF-FAB spectrum of **10**, Fig. 7b). This complementary information indicated clearly that the molecular weight of **10** was 914 amu. For the same compound, only a fragment corresponding to a "sweroside like" unit *m/z* 359 was recorded during the LC/TSP-MS analysis (TSP spectrum of **10**, Fig. 7a). According to the different results obtained on-line for **10** in the HPLC screening of the extract of *G rhodentha,* it was concluded that

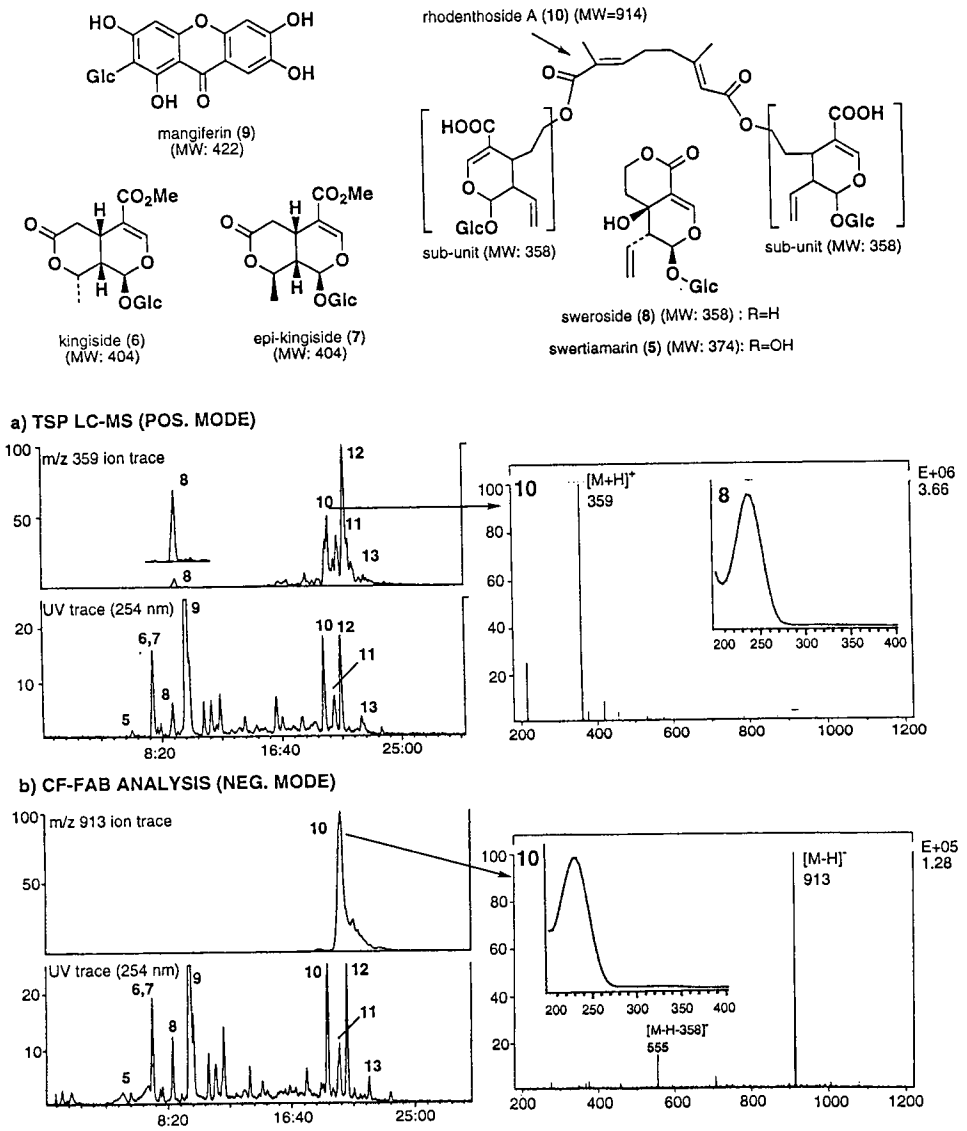


Fig. 7. Combined TSP (a) and CF-FAB (b) LC/MS of the enriched BuOH fraction of the methanolic extract of *Gentiana rhodentha* (Gentianaceae). HPLC: Column, RP-18 NovaPak (4μm, 150 x 3.9 mm i.d.); gradient, CH_3CN-H_2O (0.05% TFA) 5:95 ->50:50 in 30 min (0.9 ml/min). TSP (a): Positive ion mode; filament off; vaporiser, 90°C; source, 230°C; $AcONH_4$ 0.5 M (0.2 ml/min post-column). CF-FAB (b): Negative ion mode; FAB tip 50°C; source 100°C; glycerol 50% (v/v) (0.15 ml/min post-column); LC flow post column split 1:100.

10 was probably a type of moderately polar large secoiridoid containing at least one unit very similar to sweroside (**8**).

The CF-FAB spectra of compounds **11-13** exhibited pseudomolecular ions $[M-H]^-$ at *m/z* 1271, 1629 and 1643 respectively. Thus, these compounds have even higher molecular weights than **13** and they all should have at least a common sweroside-type unit.

Following the LC/MS screening results, a targeted isolation of **10-13** was undertaken and full structure determination of **10** showed it to consist of two secoiridoid units linked together with a monoterpene unit through two ester groups (Fig. 7) [25]. Compounds **11** and **12** have proved to be of the same type but with two supplementary monoterpene units [26]. All these compounds are a new natural products.

This example shows well the use of both LC/MS ionisation techniques for targeting unknowns. LC/TSP-MS indicated that compounds **8** and **10-13** had common sub-units (identical fragments (MW 358)), while CF-FAB allow the on-line molecular weight determination of all of these types of oligomers. The combination of both types of information has thus allowed the early recognition of this type of large secoiridoid glycosides.

4. LC/NMR ANALYSIS OF GLYCOSIDES

As shown, the LC/UV/MS and LC/MS/MS analyses of plant extracts provides numerous useful structural information but often this information is not sufficient enough for a full on-line structural identification of plant metabolites. Other hyphenated techniques such as LC/NMR are thus needed for deeper structural investigations.

4.1. Combined LC/UV/MS and LC/NMR analysis of Gentianaceae glycosides

The LC/UV/MS analysis of Gentianaceae extracts gives useful structural information on-line for a partial identification of their metabolites. Indeed Gentianaceae plants contains UV active polyphenolic constituents such as xanthones and flavone

aglycones and glycosides which possess characteristic UV spectra. A comparison of these UV spectra with those of standards as well as molecular weight information obtained thank to LC/TSP-MS analysis gives usually a rather good idea on the nature of these constituents. However for a full identification on-line complementary information are needed. In order to obtain more detailed structural information, the extracts of different Gentianaceae plants have been analysed also by LC/NMR and LC/MS/MS. In order to illustrate this approach, the analysis of the root methanolic extract of another Gentianaceae from Chile *Gentiana ottonis* Phil. are discussed.

The LC/UV analysis of this extract (Fig. 8) presented peaks with UV spectra characteristic for secoiridoids (**5**), flavones (**14** and **16**) and xanthones (**15**, **17-19**). The LC/TSP-MS analysis of this extract allowed the attribution of molecular weights of all these compounds. Secoiridoid **5** exhibited $[M+H^+]$ and $[M+NH_4^+]$ ions at *m/z* 375 and 392 together with a fragment at 213 characteristic for the loss of an hexosyl moiety. These MS information suggested that **5** could be swertiamarin, a widespread secoiridoid of the Gentianaceae family. Compounds **9**, **14** and **16** presented all fragments characteristic for C-glycosides (losses of 90 and 120 u). According to their UV spectra, **14** and **16** (MW 448 and 446) were respectively tri- and tetraoxygenated C-glycoside flavones. Compound **9** (MW 422) was readily identified as the widespread 1,3,6,7-tetrahydroxy-2-C-glucosylxanthone, mangiferin [27]. Xanthones **15**, **17-19** presented all similar UV spectra, suggesting an identical oxidation pattern. Compounds **15** and **18** presented both intense ions at *m/z* 261 characteristic for tetrahydroxylated xanthones. Substance **15** exhibited also a protonated molecular ion $[M+H]^+$ at *m/z* 423. This additional hexosyl moiety (loss of 162 u) suggested that **15** was the corresponding glucoside of aglycone **18**. The LC/TSP-MS spectra of **17** and **19** indicated the presence of another couple of related xanthones with a common aglycone ion at *m/z* 275, characteristic for substitution by one methoxyl and three hydroxyl groups.

In order to confirm these attributions and to obtain more structure information on-line, the same extract of *G. ottonis was* submitted to an on-line LC/^{1}H-NMR analysis. The same LC conditions as for the LC/UV/MS analysis were used except that the water

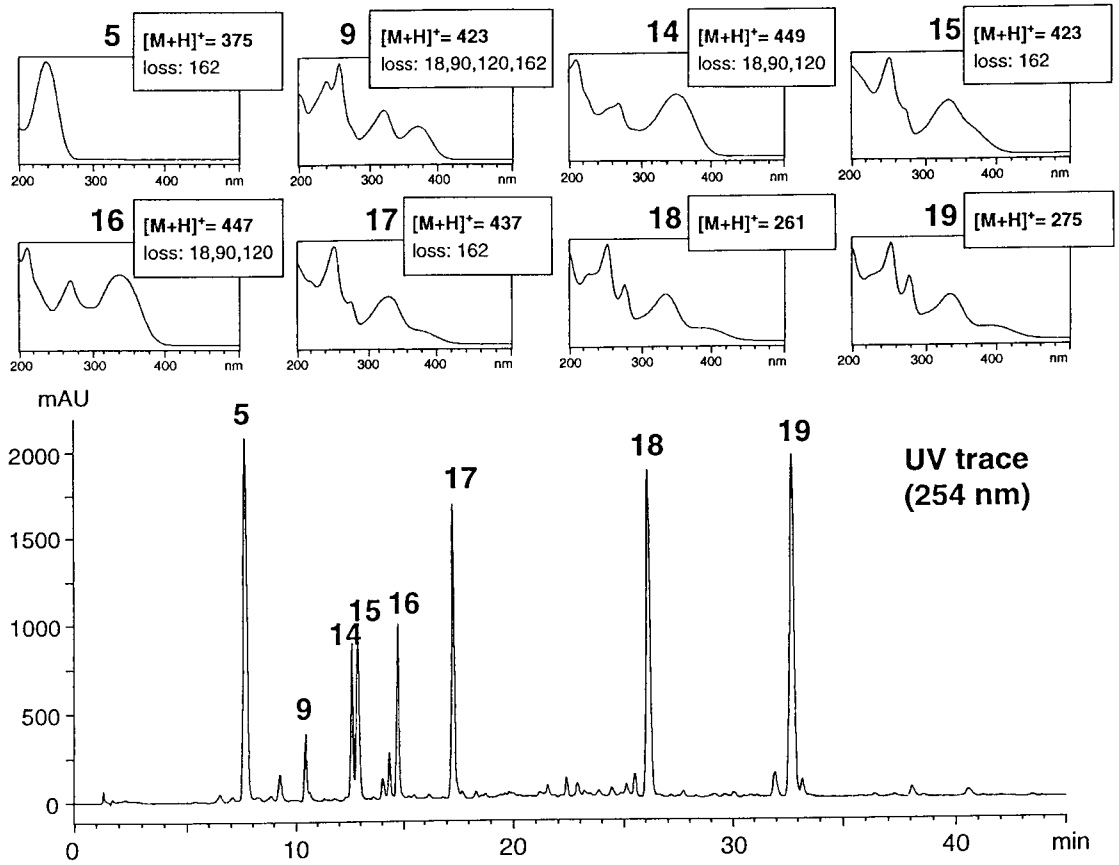


Fig. 8. LC/UV chromatogram of *G. ottonis* with UV spectra and main FSP/LC-MS ions of the major constituents. HPLC: Column, RP-18 NovaPak (4µm, 150 x 3.9 mm i.d.); gradient, CH_3CN-H_2O (0.05% TFA) 5:95 ->65:35 in 50 min (1.0 ml/min). UV spectra of **14** and **16** were characteristic for flavones, while those of **15** and **17-19** were indicative of xanthones and those of **5** of a secoiridoid glycoside.

of the LC gradient system was replaced by D_2O. However the quantity of extract injected onto the column was increased because of the relative insensitivity of LC/NMR compared to LC/UV and LC/MS detection. As the extract of *G. ottonis* was rather complex, only 0.4 mg could be injected onto the column without a loss of resolution. For this reason, the LC/NMR analysis was performed in the stopped-flow mode using the same gradient conditions as for the LC/UV/MS analysis but H_2O was replaced by D_2O. In this mode, the HPLC flow was stopped as soon as a UV active constituent of the extract reached the LC/NMR cell. For simpler Gentianaceae extracts, such as those of *Swertia calycina* N.E.BR. which allow more material to be loaded on the column, on-flow LC/NMR runs are also possible [7]. In this case, the measurement of the LC/^{1}H-NMR spectra of the main constituents can be performed without stopping the flow rate. The acquisition of the LC/^{1}H-NMR spectra with solvent suppression was then carried out for each constituent until a satisfactory signal-to-noise ratio was obtained for the peak of interest (ca 512 transients, acquisition time ca 10 min). In order to illustrate the type of results obtained, the LC/^{1}H-NMR of four types of metabolites (**5**, **16**, **17** and **19**) are displayed (Fig. 9). Secoiridoid glycoside **4** presented signals characteristic for a glucosyl moiety between 3 and 4 ppm, together with an anomeric proton at 4.45 ppm. A singlet at δ 7.75 was characteristic for the H-3 methine proton of most secoiridoids. Signals between 5 and 6 ppm signals were attributable to the proton of an exocyclic double bound and to H-1. A careful comparison of the LC/^{1}H-NMR spectra of these secoiridoids demonstrated clearly the absence of the H-5 signal in the case of **5**, proving an hydroxylation at C-5. A comparison of these LC/^{1}H-NMR data with those of literature allows an unambiguous identification of **5** as swertiamarin, confirming thus the attribution made according to the LC/UV/MS results.

Xanthone O-glycoside **17** (MW 436) presented signals characteristic for a O-glucosyl moiety (δ 3.3-5.1) and one methoxyl group (δ 3.96). A pair of *meta* coupled aromatic protons (1H, δ 6.70, *d*, *J*=2.0, H-4 and 1H, δ 6.47, *d*, *J*=2.0, H-2) was indicative for a 1,3-disubstituted A-ring. The B-ring protons exhibited a pair of *ortho* coupled protons (1H, δ 7.34, *d*, *J*=8.8, H-6 and 1H, 7.17, *d*, *J*=8.8, H-7). This

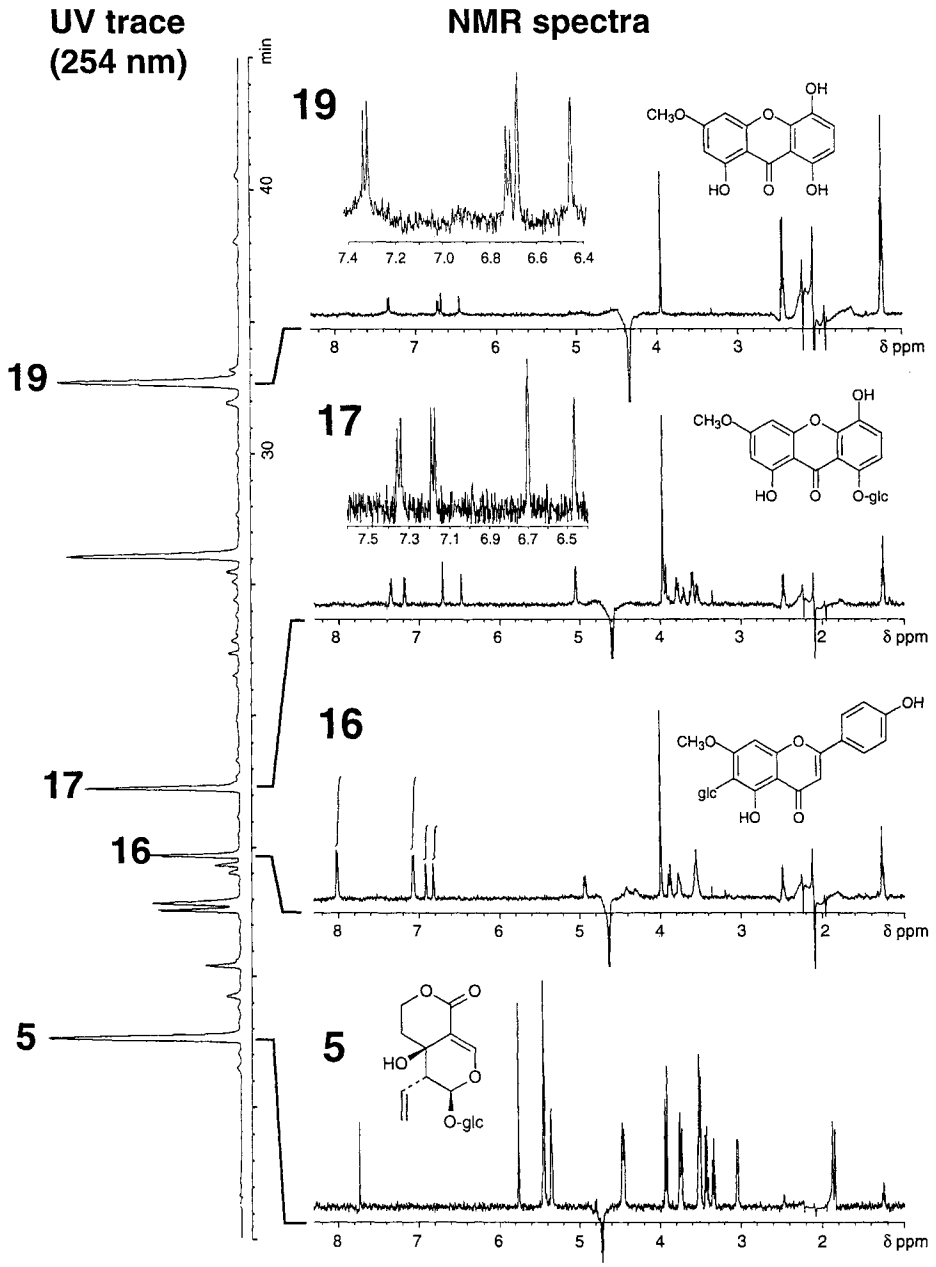


Fig. 9. LC/UV chromatogram of *G. ottonis* (vertical display) with the stop-flow LC/^{1}H-NMR spectra of **5**, **16**, **17** and **19**. On these LC/^{1}H-NMR spectra, the coupling constants and the integration of the aromatic signals were easily measured.

information, together with UV data, confirmed a 1,3,5,8 substitution pattern for **17**. The LC/^{1}H-NMR of **19**, aglycone of **17**, also exhibited a pair of *meta* and *ortho* coupled protons, as well as a methoxyl group. However, with respect to **17**, one of the *ortho* protons was shifted upfield indicating a glycosylation on the B-ring (at C-5 or C-8). A careful comparison of the LC/UV data with authentic samples allowed finally the identification of **19** and **17** respectively as 1,5,8-trihydroxy-3-methoxyxanthone (bellidifolin) and 1,5-dihydroxy-3-methoxy-8-O-glucosylxanthone (8-O-glucosylbellidifolin) [28]. Similar deductions allowed the identification of **18** and **15** as 1,3,5,8-tetrahydroxyxanthone (desmethylbellidifolin) and 1,3,5-trihydroxy-8-O-glucosylxanthone (8-O-glucosyldesmethybellidifolin) [28].

Flavone C-glycoside **16** (MW 446) presented signals of 6 aromatic protons (δ 6.8-8.1) and one methoxyl group (δ 4.0), together with those of the C-glycoside moiety (δ 3,5-5.0). A pair of symmetric *ortho* coupled protons (2H, δ 7.06, *d*, *J*=8.3, H-3',5' and 2H, δ 8.00, *d*, *J*=8.3, H-2',6') was characteristic for a B-ring substituted in C-4'. The singlet at δ 6.8 was attributable to H-3. The other singlet at δ 6.9 was due to proton either at position C-6 or C-8 on the A-ring. At this stage the LC/UV/MS, together with the LC/^{1}H-NMR data, did not allow a full identification of **16**. In order to ascertain the position of C-glycosylation, a LC/MS/MS experiment was performed. As shown in Fig. 10, the LC/TSP-MS/MS spectrum of **16** obtained by choosing the $[M+H-120]^+$ as parent ion exhibited fragments at *m/z* 191 and 163 characteristic for 6-C-glycosylated flavones. It is known that TSP-MS/MS spectra of the $[M+H-120]^+$ fragments of isomeric C-glycosylflavones show different specific daughter ions [29]. Indeed, the A-C-ring fragments issued from a retro Diels-Alder cleavage are only observable for the C-6 position isomers, as it was the case for **16**. Furthermore the fragment observed at *m/z* 121 was indicative for a monohydroxylated B-ring, confirming the position of the methoxyl group on the A-ring.

These UV, MS, ^{1}H-NMR and MS/MS information allowed the identification of **16** as the known 5,4'-dihydroxy-7-methoxy-6-C-glucosylflavone (swertisin). Similarly, the

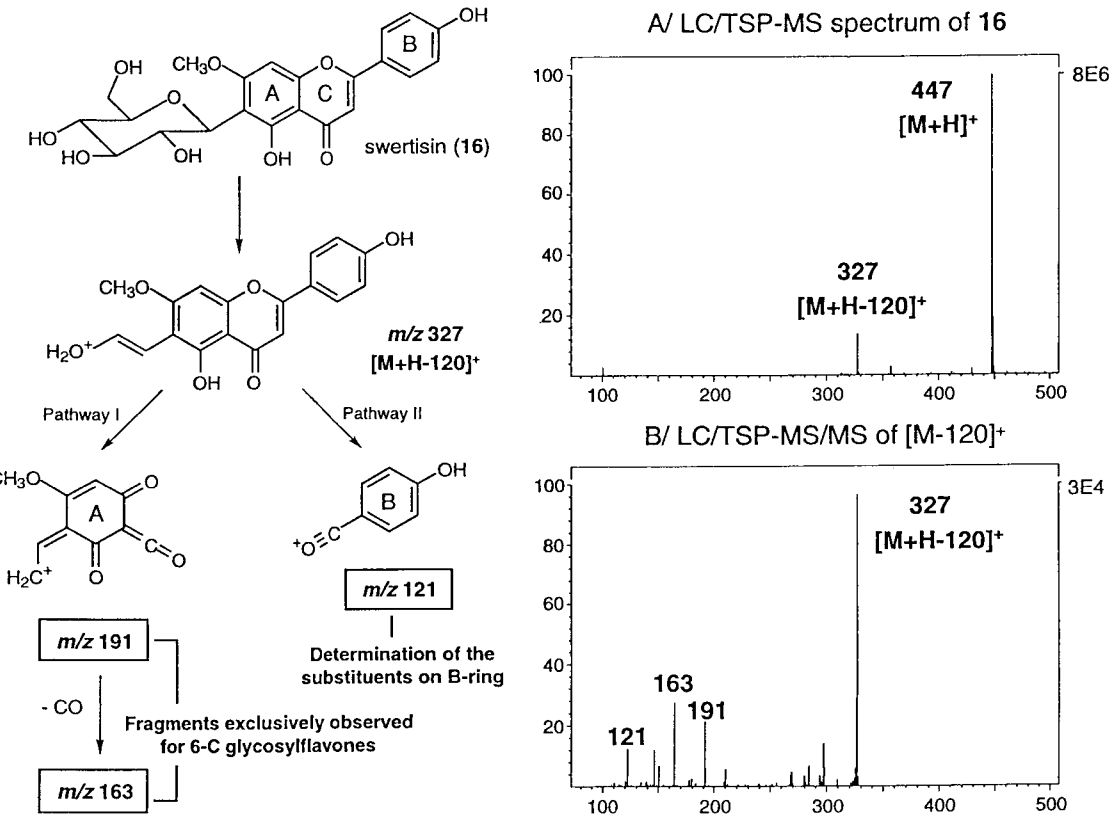


Fig. 10. LC/TSP-MS (a) and LC/TSP-MS/MS (b) spectra of swertisin (**16**) in the crude extract of *G. ottonis*. The LC/MS/MS analysis was performed using the fragment [M+H-120]+ (spectrum a) as parent ion. Characteristic daughter ions at m/z 121, 163 and 191 were observed, indicating the substitution on the A- and B-ring of the C-glycosylflavone **16**.

flavone C-glycoside **14** (MW 448) was identified as the common 5,7,3',4'-tetrahydroxy-6-C-glucosylflavone (isoorientin).

This example showed that LC/NMR and LC/MS/MS represent an excellent complement to LC/UV/MS. The combined use of all these hyphenated techniques allow an efficient structure identification of the main constituents of an extract on-line.

5. CONCLUSIONS

Today the work of phytochemists lies mainly on bioassay-guided fractionation schemes of crude plant extracts. This type of approach has led to the isolation of numerous compounds with interesting activities. Since plants contain thousands of constituents with considerable different biological activities. Obviously for any successful investigation of plant material with such a wide range of properties, the future lies in the ability to have as large a number of biological screens as possible.

Chemical screening of crude plant extracts which allows the localisation and the efficient targeted isolation of new types of constituents with potential activities, can be considered as an efficient complementary approach to the biological screening. As shown, the use of hyphenated techniques such as LC/UV, LC/MS and LC/NMR enables rapid initial screening of crude plant extracts, providing a great deal of preliminary information about the content and nature of constituents of these extracts. According to these structural information, once the novelty or utility of a given constituent is established. It is then important to process the plant extracts in the usual manner, to obtain samples for full structure elucidation and biological or pharmacological testing. In such a way, the unnecessary isolation of common compounds of minor interest is avoided.

The examples discussed have shown that the LC/UV/MS or LC/MS/MS approach alone permits the identification of small metabolites such as xanthones or flavones, provided that some information about the chemotaxonomy of the plant families of

interest is already available. For screening more complicated constituent or unknowns, LC/NMR data are of crucial importance for a deeper structural investigation. The recent introduction of LC/NMR for the crude plant extract screening will probably make another breakthrough in the on-line structural determination of natural products.

The chemical screening of extracts with such complex hyphenated techniques generates a huge amount of information. In order to rationalise this approach and use it efficiently with a high sample throughput, the next challenge will be to find a way to centralise all these data for rapid pattern recognition by reference to standard compounds. With such an analytical system, natural product chemists will then be able to concentrate their efforts on finding new biological targets. This aspect still remains the more difficult problem to solve when searching for lead compounds.

6. ACKNOWLEDGEMENTS

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BIOACTIVE GLYCOSIDES IN CITRUS FRUIT PEELS

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Key Word Index -- glycoside; Citrus fruit; biological activity; flavonoid glycoside; phenylpropanoid glycoside.

Abstract Since 1980's we have been investigating bioactive compounds in citrus fruit peels. We have successfully isolated seventy-three glycosides including thirty-four new compounds. They were flavonoid glycosides, phenylpropanoid glycosides, terpenoid glycosides, limonoid glycosides, and alkyl glycosides. The biological activities of the compounds were studied for the utilization as hypotensive and hypertensive drugs. In this manuscript, recent works are briefly reviewed focussing on the isolation and a mutual relationship between the structures of the compounds and their biological activities.

Introduction

The citrus fruit peels in general have been used as food with or without processing so far, and also extremely small amount of essential oils of the peels have been utilized as flavors, fragrances, or synthetic starting materials such as pharmaceutical and agricultural chemicals and other chemical products. However, a large parts of them have been wasted even now. Therefore, it is very important to find an efficient way of utilization of citrus fruit peels as one of the valuable organic chemical resources.

Citrus fruits is used for aromatic stomachic, diaphoresis, expectorans, a bath charges as crude drug and folk medicine from ancient times in China and Japan. Agents with biological activities such as inhibitory action of intestine exercise and antiallergic action may be involved.

Since 1980's we have been investigating bioactive compounds in citrus fruit peels [1-27]. We have chosen eleven kinds of citrus fruit peels: namely lemon (*Citrus limon*), grapefruit (*Citrus paradishi*) and orange (*Citrus sinensis*) which are imported from the United States, unshiu (*Citrus unshiu*), hassaku (*Citrus hassaku*), zabon (*Citrus gradis*), iyo (*Citrus iyo*) and amanatsu (*Citrus natsudaidai*) produced abundantly in Japan, and kinkan (*Fortunella japonica*), sudachi (*Citrus sudachi*) and yuzu (*Citrus junos*), part of the peels of which are edible in Japan. We have successfully isolated seventy-three glycosides including thirty-four new compounds. They were flavonoid glycosides, phenylpropanoid glycosides, terpenoid glycosides, limonoid glycosides, and alkyl glycosides. The biological activities of the compounds were studied for the utilization as hypotensive and hypertensive drugs.

In this manuscript, recent works are briefly reviewed focussing on the isolation and a mutual relationship between the structures of the compounds and their biological activities.

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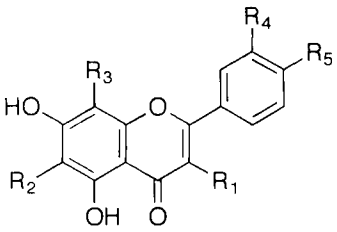
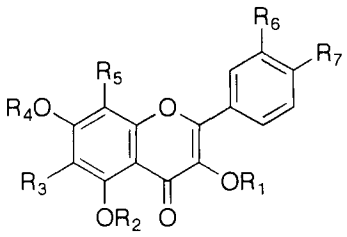
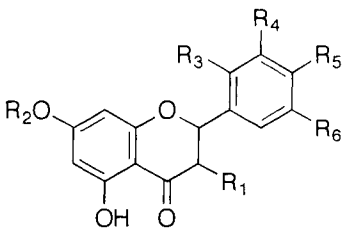


di-*C*-glucosyl flavones (**1 - 6**)
mono-*C*-glucosyl flavones (**7 - 12**)
C-xyloglucosyl flavone (**13**)
C-rhamnoglucosyl flavones (**14 -18**)
flavone glycosides (**33 - 38**)

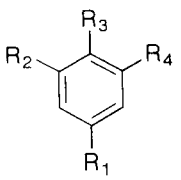


flavonol glycosides (**19 - 32**)

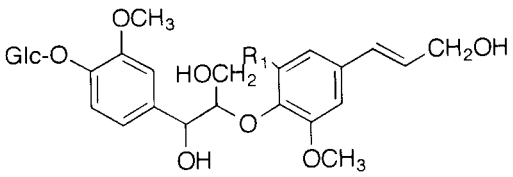


flavanone glycosides (**39 - 51**)

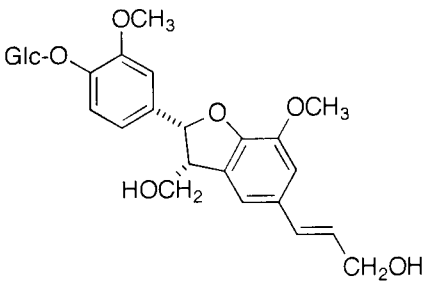
Fig. 1. Structures of flavonoid glycosides isolated from citrus fruit peels.



(**52 - 57**)

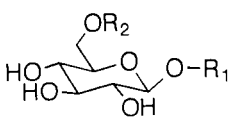


(**58, 59**)



(**60**)

Fig. 2. Structures of phenylpropanoid glycosides isolated from citrus fruit peels.



(**70 - 73**)

Fig. 3. Structures of alkyl glycosides isolated from citrus fruit peels.

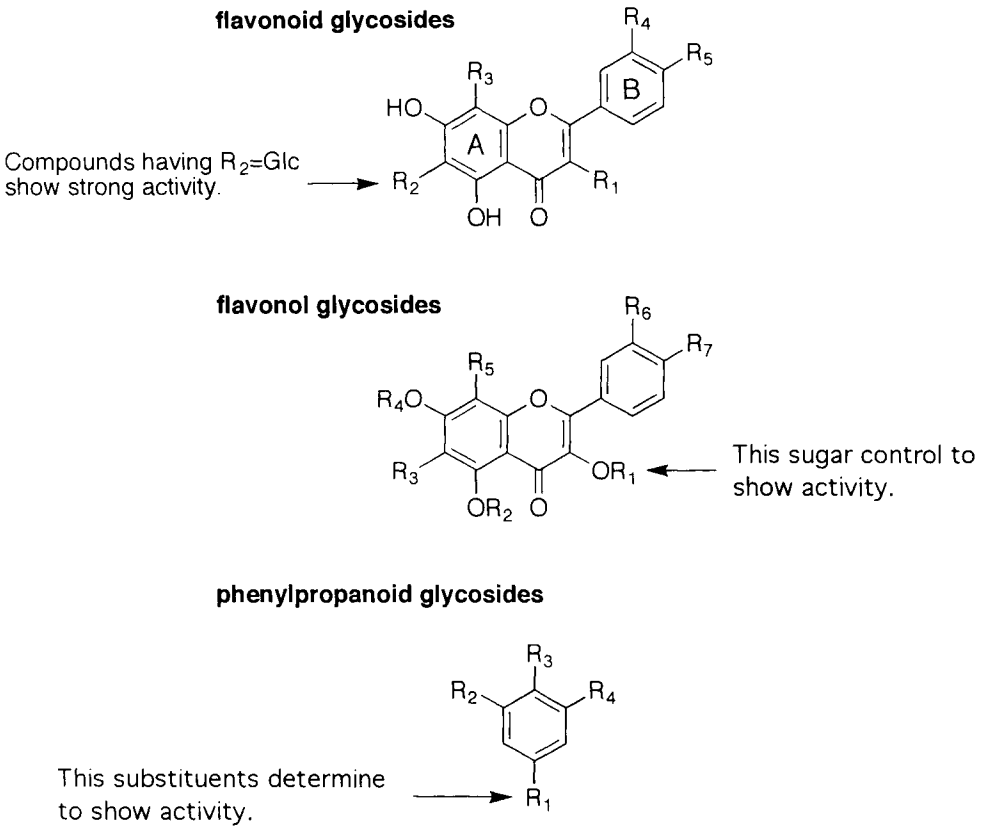


Fig. 4. A mutual relationship between the structures of the compounds and their biological activities.

Isolation and Purification

Method A The fresh peel of each citrus fruit was kept in hot H_2O at 96° for 20 min, chopped using a commercial blender, and homogenized after making up the total volume to 3 l with hot H_2O. Cold EtOH (7 l) was added to the hot H_2O solution, and the mixture was allowed to stand overnight in the dark. Then the hot H_2O extract was treated with n-hexane and n-BuOH, successively. A saturated aqueous solution of lead subacetate was added to the n-BuOH extract, and the resulting precipitates were removed by filtration.

Flavonoid glycosides [3-13] were obtained by the following procedures. The precipitates were treated with a saturated aqueous solution of sodium carbonate. After stirring for 1 hr, a white precipitate of lead carbonate was removed by filtration, and the filtrate adjusted to pH 5.3 with 6M HCl was extracted with n-BuOH to afford a mixture of crude flavonoid glycosides from the n-BuOH solution. Flavonoid glycosides (**1-51**, Fig.1 and Table 1) were purified by gel filtration on HW-40F TSK gel and by column chromatography on silica gel.

Phenylpropanoid glycosides [14, 23], terpenoid glycosides[15-19], and alkyl glycosides [21] were obtained by the following procedures. The filtrate was adjusted to pH 9.0 and then extracted with n-BuOH to afford a mixture of crude phenylpropanoid glycosides, terpenoid glycosides, and alkyl glycosides from the n-BuOH solution. Phenylpropanoid glycosides (**52**, **53**, **54**, **58**, **59**, **60**, Fig. 2 and Table 2), terpenoid glycosides (**61-67**, Table 3), and alkyl glycosides (**70-73**, Fig. 3 and Table 4) were purified by gel filtration on HW-40F TSK gel and by column chromatography on silica gel.

Method B The peel obtained from each citrus fruit was air-dried, chopped by a commercial blender, and homogenized after adding hot H_2O. The mixture was kept at 96° for 30 min and then filtered. The filtrate was deposited on an Amberlite XAD-2 column, washed with H_2O, and fractionated by successive elution with 20% MeOH-H_2O, 50% MeOH-H_2O, and MeOH. The 50% MeOH-H_2O eluate was concentrated in vacuo, dissolved in MeOH, charged on a neutral Al_2O_3 column, and chromatographed with 50% MeOH-H_2O. The eluate was concentrated in vacuo, giving a mixture of crude phenylpropanoid glycosides and limonoid glycosides. Phenylpropanoid glycosides (**52**, **53**, **55**, Fig. 2 and Table 2)[23] and limonoid glycosides (**68**, **69**, Table 3)[24] were purified by column chromatography on silica gel and by gel filtration on HW-60F TSK gel.

Method C The peel obtained from each citrus was chopped and extracted with cold MeOH in darkness for three days. The MeOH extract was successively treated with n-hexane, EtOAc and n-BuOH. The n-BuOH extract was chromatographed over a Diaion HP 20 column. The column was washed with H_2O, and successively eluted with 20% MeOH-H_2O, 60% MeOH-H_2O and MeOH. The 20% MeOH-H_2O eluate was concentrated in vacuo, giving a mixture of crude phenylpropanoid glycosides. Phenylpropanoid glycosides (**52**, **53**, **54**, **56**, **57**, Fig. 2 and Table 2)[25] were purified by column chromatography on silica gel and by gel filtration on HW-40F TSK gel.

Table 1. Hypotentive effects of flavonoid glycosides isolated from citrus fruit peels

Compounds	Maximal decrase of blood pressure (mmHg)	citrus fruit peels
di-C-glucosyl flavones		
6,8-di-C-glucosylapigenin (**1**)	-86 (0.5mg/b.w., i.v.) -30 (1.0mg/b.w., i.p.)	lemon, kinkan**, zabon**, grapefruit**, unripe unshiu**
6,8-di-C-glucosyldiosmetin (**2**)	-53 (1.0mg/b.w., i.v.)	lemon
3,6-di-C-glucosylapigenin (**3**)*	-50 (1.0mg/b.w., i.v.) -20 (1.5mg/b.w., i.p.)	unshiu, sudachi
3,6-di-C-glucosylacacetin (**4**)*	-35 (0.5mg/b.w., i.v.)	kinkan
3,8-di-C-glucosylapigenin (**5**)*	0 (1.0mg/b.w., i.v.)	sudachi, yuzu, zabon, hassaku, orange, unripe unshiu
3,8-di-C-glucosyldiosmetin (**6**)*	0 (1.0mg/b.w., i.v.)	sudachi, orange
mono-C-glucosyl flavones		
6-C-glucosyldiosmetin (**7**)	-33 (1.0mg/b.w., i.v.)	lemon
8-C-glucosyldiosmetin (**8**)	-30 (1.0mg/b.w., i.v.)	lemon
isovitexin (6-C-glucosylapigenin) (**9**)	-12 (1.0mg/b.w., i.v.)	lemon
vitexin (8-C-glucosylapigenin) (**10**)		kinkan
8-C-glucosylacacetin (**11**)		kinkan
6-C-glucosylacacetin (**12**)		kinkan
C-rhamnoglucosyl flavones (C-xyloglucosyl flavone)		
2"-*O*-xylosylvitexin (8-C-xyloglucosylapigenin) (**13**)	-18 (1.0mg/b.w., i.v.)	orange
2"-*O*-α-rhamnosylvitexin (8-C-rhamnoglucosylapigenin) (**14**)	-28 (0.5mg/b.w., i.v.)	kinkan**
2"-*O*-α-rhamnosylorientin (8-C-rhamnoglucosylluteorin) (**15**)*	-25 (0.5mg/b.w., i.v.)	kinkan
2"-*O*-α-rhamnosyl-4'-*O*-methylvitexin (8-C-rhamnoglucosylacacetin) (**16**)*	-4 (0.5mg/b.w., i.v.)	kinkan
2"-*O*-α-rhamnosyl-4'-*O*-methylorientin (8-C-rhamnoglucosyldiosmetin) (**17**)*	0 (0.5mg/b.w., i.v.)	kinkan
2"-*O*-α-rhamnosyl-4'-*O*-methylisovitexin (6-C-rhamnoglucosylacacetin) (**18**)*	0 (0.5mg/b.w., i.v.)	kinkan
flavonol glycosides		
limocitrol 3-β-D-glucopyranoside (**19**)	-63 (1.0mg/b.w., i.v.)	lemon
3,7,4'-trihydroxy-5,6,8,3'-tetramethoxyflavone 3-β-D-glucopyranoside (**20**)*	-28 (1.0mg/b.w., i.v.)	unripe unshiu
3-hydroxy-5,6,7,8,3',4'-hexamethoxyflavone 3-β-D-glucopyranoside (**21**)*	-12 (0.3mg/b.w., i.v.)	unshiu, orange, unripe unshiu
limocitrin 3-β-D-glucopyranoside (**22**)	-12 (1.0mg/b.w., i.v.)	lemon, unshiu**, unripe unshiu
isolimocitrin 3-β-D-glucopyranoside (**23**)	-11 (0.5mg/b.w., i.v.)	unshiu**
limocitrin 3-*O*-rhamnopyranoside (**24**)*	0 (1.0mg/b.w., i.v.)	unshiu
rutin (**25**)	-45 (0.015mg/b.w., i.v.)	unshiu, unripe unshiu
limocitrin 3-*O*-rutinose (**26**)*	-10 (1.0mg/b.w., i.v.)	lemon
limocitrin 3-*O*-{[3-hydroxy-3-methylglutaryl(1—>2)]-β-D-glucopyranoside} (**27**)*		lemon

Continued

Table 1. (*continued*)

limocitrin 3-*O*-{[3-hydroxy-3-methylglutaryl(1—>6)]-β-D-glucopyranoside} (**28**)*	-20 (1.0mg/b.w., i.v.)	unripe unshiu
3,7,4'-trihydroxy-5,6,8,3'-tetramethoxyflavone 3-*O*-{[3-hydroxy-3-methylglutaryl (1—>6)]-β-D-glucopyranoside} (**29**)*	-15 (1.0mg/b.w., i.v.)	unripe unshiu
limocitrol 3-*O*-{[5-a-glucopyranosyl-3-hydroxy-3-methylglutaryl (1—>2)]-β-D-glucopyranoside} (**30**)*	-15 (1.0mg/b.w., i.v.)	lemon
limocitrin 3-*O*-{[5-α-glucopyranosyl-3-hydroxy-3-methylglutaryl (1—>2)]-β-D-glucopyranoside} (**31**)*	-13 (1.0mg/b.w., i.v.)	lemon, unshiu, hassaku
narcissin (**32**)	0 (1.0mg/b.w., i.v.)	unshiu**, unripe unshiu
flavone glycosides		
acacetin 7-*O*-neohesperidose (**33**)	-35 (1.0mg/b.w., i.v.)	kinkan
apigenin 7-*O*-neohesperidose (**34**)	-25 (1.0mg/b.w., i.v.)	lemon**, hassaku
apigenin 7-*O*-rutinose (**35**)	-22 (1.0mg/b.w., i.v.)	lemon**
luteorin 7-*O*-rutinose (**36**)	0 (1.0mg/b.w., i.v.)	lemon**
sudachiin A (**37**)	-12 (1.0mg/b.w., i.v.)	sudachi
sudachiin B (**38**)*	-13 (1.0mg/b.w., i.v.)	sudachi
flavanone glycosides		
naringenin 4'-*O*-glucopyranosyl-7-*O*-rutinose (**39**)	-47 (1.0mg/b.w., i.v.)	unshiu, sudachi
naringenin 7-*O*-{[α-rhamnopyranosyl(1—>2)]-[α-rhamnopyranosyl (1—>6)]-β-glucopyranoside} (**40**)*	-12 (1.0mg/b.w., i.v.)	yuzu
5,7,2',3',5'-pentahydroxyflavanone 7-*O*-rutinose (**41**)*	-18 (1.0mg/b.w., i.v.)	lemon
poncirin (**42**)	-7 (0.5mg/b.w., i.v.)	grapefruit, kinkan
eriocitrin (**43**)	-5 (1.0mg/b.w., i.v.)	lemon
hesperetin 7-*O*-{[α-rhamnopyranosyl(1—>2)]-[α-rhamnopyranosyl (1—>6)]-β-glucopyranoside} (**44**)*	0 (1.0mg/b.w., i.v.)	sudachi, yuzu
3,5,7-trihydroxy-3'-methoxyflavanone 7-*O*-neohesperidose (**45**)*	0 (1.0mg/b.w., i.v.)	yuzu
naringin (**46**)	0 (1.0mg/b.w., i.v.)	grapefruit, yuzu, hassaku, zabon
narirutin (**47**)	0 (1.0mg/b.w., i.v.)	unshiu, yuzu, orange, grapefruit, unripe unshiu
prunin (**48**)		zabon
hesperidin (**49**)		lemon, unshiu, yuzu, sudachi, hassaku, unripe unshiu
neohesperidin (**50**)		sudachi
homoeriodictiol 7-*O*-neohesperidose (**51**)		yuzu**

* : new compounds

** : The compounds have not been previously found in those citrus fruits.

Table 2. Hypertentive and hypotensive effects of phenyl propanoid glycosides isolated from citrus fruit peels

Compounds	Maximal incrase and decrease of blood pressure (mmHg)	citrus fruit peels
phenylpropanoid glycosides		
coniferin (**52**)	+10 (1.0mg/b.w., i.v.)	lemon, unshiu, kinkan, yuzu, sudachi, hassaku, zabon, iyo, orange, amanatsu
syringin (**53**)	+16 (1.0mg/b.w., i.v.)	lemon, unshiu, kinkan, yuzu, sudachi, hassaku, zabon, iyo, orange, amanatsu
citrusin C* (**54**, 1-(4-β-D-glucopyranosyl-3-methoxyphenyl)propane-2-en)	-20 (1.0mg/b.w., i.v.)	kinkan, hassaku, zabon, orange, amanatsu
citrusin D* (**55**, 3-(4-hydroxy-3-methoxyphenyl)-1-β-D-glucopyranosyl-2-propene)	-30 (1.0mg/b.w., i.v.)	unshiu
citrusin E* (**56**, methyl 3-(4-β-D-glucopyranosyl-3-methoxyphenyl)propionate)	-25 (0.5mg/b.w., i.v.)	lemon
citrusin F* (**57**, methyl 3-{4-(6-O-α-glucopyranosyl-β-glucopyranosyl)-3-hydroxyphenyl}propionate)		lemon
citrusin A* (**58**, 1-(4-β-D-glucopyranosyl-3-methoxyphenyl)-2-{2-methoxy-4-[1-(*E*)-propane-3-ol]phenoxy}propane-1,3-diol)	-11 (1.0mg/b.w., i.v.)	lemon, unshiu, kinkan, yuzu, sudachi, hassaku, zabon, iyo, orange, amanatsu
citrusin B* (**59**, 1-(4-β-D-glucopyranosyl-3-methoxyphenyl)-2-{2,6-dimethoxy-4-[1-(*E*)-propane-3-ol]phenoxy}propane-1,3-diol)		lemon, unshiu, kinkan, yuzu, sudachi, hassaku, zabon, iyo, orange, amanatsu
dehydrodiconiferyl alcohol 4-β-D-glucopyranoside (**60**)	-14 (1.0mg/b.w., i.v.)	lemon, kinkan, yuzu, hassaku, orange, amanatsu

* : new compounds

Table 3. Terpenoid glycosides isolated from citrus fruit peels

Compounds	Citrus fruit peels
terpenoid glycosides	
trans-carveol 6-β-D--glucopyranoside (**61**)*	unshiu, unripe unshiu, yuzu, hassaku, orange
α-terpineol 8-β-D-glucopyranoside (**62**)*	orange
(2*E*,6*R*)-2,6-dimethyl-2,7-octadien-6-ol-1-*O*-β-D-glucopyranoside (**63**)	unshiu, unripe unshiu, yuzu, hassaku, orange
(1*S*,4*R*,6*S*)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-*O*-β-D-glucopyranoside (**64**)*	unripe unshiu
(4*S*,6*S*)-6-*O*-β-D-glucopyranosyl-p-menth-1-en-3-one (**65**)*	sudachi
vomifoliol 9-*O*-β-D-glucopyranoside (**66**)	unshiu, unripe unshiu, yuzu, hassaku, orange
(6*R*,7*E*,9*R*)-9-hydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-glucopyranoside (**67**)	unshiu, unripe unshiu, hassaku, orange
limonoid glycosides	
ichangin 4-β-D-glucopyranoside (**68**)*	lemon, unshiu
nomilinic acid 4-β-D-glucopyranoside (**69**)*	lemon, unshiu

* : new compounds

Table 4. Hypertentive and hypotensive effects of alkyl glycosides isolated from citrus fruit peels

Compounds	Maximal incrase and decrease of blood pressure (mmHg)	citrus fruit peels
alkyl glycosides		
ethyl-1-*O*-β-D-glucopyranoside (**70**)	-25 (1.0mg/b.w., i.v.)	yuzu
propyl-1-*O*-β-D-glucopyranoside (**71**)		yuzu
hexyl-1-*O*-β-D-glucopyranoside (**72**)	+25 (1.0mg/b.w., i.v.)	iyo
hexyl-1-*O*-(6-*O*-apiosyl-β-D-glucopyranoside (**73**)	+28 (1.0mg/b.w., i.v.)	hassaku

Biological Activity

Flavonoid Glycosides

Flavonoids have already been well documented for numerous biological activities such as effect which increase hip systole and decrease heart rate [28, 29], and catharsis effect [30, 31], effect for the core which coronary vessel is widened and improve recycle in myocardium [32], and a component, vitamin P (hesperidin) [33, 34] exhibits complete response in scorbutus with vitamin C or capillary bursting prevention effect declining capillary permeability. Recently, antioxidative activity was reported on flavonoids. For example, it is known that flavonols such as quercetin restrains decoloration of pigmentary carotenoids caused by 1O2 [35], and also exhibited radical scavenging ability on non-enzymatic generation of O_2-•. Although rutin (25) which is a glycoside of quercetin exhibited such an antioxidative activity, it is weak compared with quercetin [36].

We have been investigating, hypotensive substances in food which have no need of concern about drug side reactions, contrary to the usual hypotensive agent. The hot H_2O extract of citrus fruits was examined with intravenous administration for SHR (spontaneously hypertensive rats) and SHR-SP (stroke-prone spontaneously hypertensive rats). As a result, we confirmed that flavonoid glycosides have conspicuous hypotensive effect [26, 27]. We also found that the many components among the flavonoid glycosides in citrus fruits exhibits strong hypotensive effect. Thus, we confirmed one of major components of hypotensive effect in citrus fruit peels to be a flavonoid glycoside. In the study of the peels of nine citrus fruit peels, we have successfully isolated fifty-one flavonoid glycosides (**1-51**) including twenty-two new compounds. Then, we have studied the mutual relationship between their structures and hypotensive activity by injecting those flavonoid glycosides intravenously in SHR-SP.

Di-C-glucosyl flavones Strong depressive effects were found for these four compounds: namely apigenin (**1**) and diosmetin (**2**) having C-C bound glucose at C-6 and C-8 of the flavonoid nucleus, apigenin (**3**) and acacetin (**4**) having C-C bound glucose at C-3 and C-6. The strongest hypotensive effects are found by 6,8-di-C-glucosylapigenin (**1**) and 3,6-di-C-glucosylapigenin (**3**) after stomachic administration. In such case C-glucosyl flavonoids are considered to be not hydrolyzed by the digestion enzyme and hydrochloric acid in the stomach. Apigenin (**5**) and diosmetin (**6**) having C-C bound glucose at C-3 and C-8 do not exhibit any hypotensive effect.

Mono-C-glucosyl flavones Diosmetin (7 or 8) having the glucosyl group at C-6 or C-8 exhibits a moderate hypotensive effect, whereas isovitexin (**9**) shows only a weak hypotensive effect.

C-Rhamnoglucosyl flavones (C-xyloglucosyl flavones) Apigenin (**14**) and luteorin (**15**) having C-C bound neohesperidose at C-8 exhibit moderate hypotensive effects, whereas 2"-O-xylosylvitexin (**13**) exhibited a weak hypotensive effect. However, acacetin (**18**) and diosmetin (**17**) having C-C bound neohesperidose at C-6 and C-8, respectively, exhibit virtually no hypotensive effect.

Flavonol glycosides Compounds **19** and **21** having fully substituted A-ring exhibit strong hypotensive effects. Compound **20** exhibits a moderate hypotensive effect. Also limocitrin (**22**) and isolimocitrin (**23**) containing C-O bound glucose at C-3 exhibit weak hypotensive effects, but limocitrin (24) having C-O bound rhamnose does not exhibit any hypotensive effect. Rutin (**25**) exhibits a strong hypotensive effect, whereas limocitrins having

C-O bound rutinose (**26**) and 3-hydroxy-3-methylglutaric acid (**28**), respectively, at C-3 exhibit weak hypotensive effects. Also compounds (**29**, **30**, and **31**) having 3-hydroxy-3-methylglutaric acid at C-3 exhibit weak hypotensive effects. Narcissin (**32**), however, does not exhibit any hypotensive effect.

Flavone glycosides Apigenins (**34**, **35**) containing C-O bound neohesperidose and rutinose at C-7 exhibit moderate hypotensive effects, whereas luteorin 7-O-rutinose (**36**) does not exhibit any hypotensive effect. The compound (**38**) having C-O bound glucose at C-4' and the flavonoid glycoside (**37**) having the fully substituted A-ring exhibit weak hypotensive effects.

Flavanone glycosides Naringenin (**39**) containing C-O bound glucose at C-4' and rutinose at C-7 exhibits strong hypotensive effect. Also compounds **40** and **41** exhibit weak hypotensive effects, but poncirin (**42**) and eriocitrin (**43**) exhibit almost no hypotensive effect, other components (**44-47**) do not exhibit hypotensive effect at all. The hypotensive effects of **48-51** have not been determined, because of their poor solubility in water.

A mutual relationship between the structures of flavonoid glycosides and their physiological activity As we have shown so far, fifty-one kinds of flavonoid and flavanoid glycosides have now been separated, and their structures and physiological activity have been investigated. As a result, we have found that the kinds and the position of the substituents and the sugar moieties strongly effect the physiological activity, as shown in Table 1. In particular, components having fully substituted A-ring or components having C-C bound glucose at C-6 of the flavonoid nucleus tend to show strong activity, as shown in Fig. 4. We have also found that the kind of the sugar moieties at C-3 of the flavonol nucleus strongly effects the physiological activity [1, 2, 13].

Phenylpropanoid Glycosides

Phenylpropanoids have been known for numerous biological activities such as alleviation of fever, relief, antioxidative activity, growth inhibitory activity for spore of plant, or higher plant, insect attraction activity, and so on.

Nine phenylpropanoid glycosides (**52-60**) including six new compounds have been successfully isolated from ten kinds of citrus fruit peels. The effect of these phenylpropanoid glycosides on the blood pressure was examined by using SHR-SP. Compounds **52** and **53** exhibited hypertensive effects, whereas compounds **54**, **55**, **56**, **58** and **60** exhibited hypotensive effects, as shown in Table 2. The finding of the presence of components having a hypertensive effect is of particular importance in view of the other components with the same fundamental skeleton showing the opposite physiological effect. In particular, by comparing **52** with **55**, it was clear that 52 showed a hypertensive effect, while **55** showed a hypotensive effect by only a difference in the position of sugar moiety. Comparisons between **52** and **54**, and between **52** and **56** showed the opposite physiological effect induced by a difference in the substituents of C-1. Therefore, we found that the substituents of C-1 (R1) were the critical in the development of the physiological activity of the phenylpropanoid glycosides [1, 2], as shown in Fig. 4.

Terpenoid Glycosides

Since terpenes are well known to be one of the most widely distributed components in food and are utilized as industrial intermediates for perfumes, agricultural chemicals, and medicine, numerous physiological activities of terpenes have already been well documented. However, very little is known so far about the activity of the glycosides of terpenoids.

Therefore, the studies on their physiological activities are highly anticipated.

Seven terpenoid glycosides (**61-67**) including four new compounds have been successfully isolated from five kinds of citrus fruit peels. The effect of these terpenoid glycosides on the germination was examined by using lettuce seeds. However, these terpenoid glycosides were found to have no effect on the germination of lettuce.

Limonoid Glycosides

Limonoids [37-41] are known as bitter taste components of Rutaceae and Meliaceae. Recently, Lam et al. [42-44] reported that limonoids have an antitumor activity.

Two new limonoid glycosides (**68**, **69**) have been successfully isolated from lemon and unshiu peels. The antitumor activity of these limonoid glycosides was examined by using mouse P-388 leukemia. However, these limonoid glycosides were found to have no activity.

Alkyl Glycosides

Four alkyl glycosides (**70-73**) were obtained from the hot water extract of citrus fruit peels. The effect of these alkyl glycosides on the blood pressure was examined by using SHR-SP. Compound **70** exhibited hypotensive effect, whereas compounds **72** and **73** exhibited hypertensive effects, as shown in Table 4. The finding of the components having a hypertensive effect (**72**, **73**) is of particular importance in view of the presence of the other components having the different carbon number of alkyl group which show the opposite physiological effect (**70**). Thus, we found that the carbon number of alkyl group was the most critical for a mutual relationship between the structure of the alkyl glycosides and their physiological activity.

During the course of our studies on physiologically active substances in citrus fruit peels for exploiting the most effective utilization of the peels, we have investigated eleven kinds of citrus fruits. As a consequence, we have successfully isolated seventy-three glycosides including thirty-four new compounds. Among these compounds, we found a series of flavonoid glycosides having a hypotensive effect, and phenylpropanoid glycosides and alkyl glycosides having an interesting effect on the blood pressure of SHR-SP.

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THE 2-(β-GLUCOPYRANOSYLOXY)-5-HYDROXYBENZYL ALCOHOL DERIVATIVES OF THE FLACOURTIACEAE

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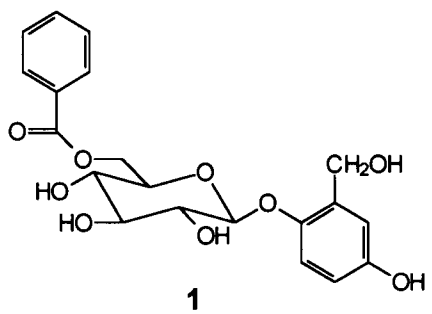
The first isolation of a glucoside of 2,5--hydroxybenzyl alcohol from the Flacourtiaceae appears to have been in 1969 when Plouvier[1] reported the presence of poliothrysoside (**1**) in the Chinese *species Poliothrysis sinensis.* Xylosmacin (**2**) from *Xylosma velutina,* the second example to be isolated from the family,[2] is very unusual in that it is based on a 5-*O*-glucosyl-2,5-hydroxybenzyl alcohol rather than the normal 2-*O*-glucosyl skeleton.



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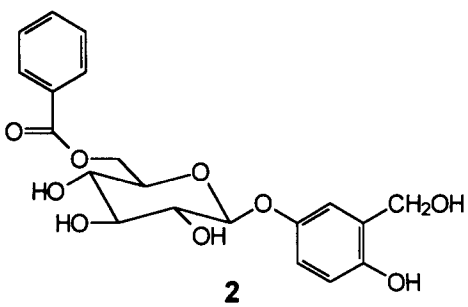


2

In the course of an investigation of the Flacourtiaceae for novel metabolites we have isolated further glycosides of this type from four species; *Poliothrysis sinensis,*[3] *Xylosma flexuosa,*[4] *Homalium longifolium,*[5,6] and *Scolopia spinosa.*[7] In this paper I am going to discuss the isolation and structure elucidation of some of these compounds.

Most of the glycosides proved to be soluble in ethyl acetate and initial separation was performed by vacuum liquid chromatography, eluting with solvent mixtures of increasing polarity. Subsequent purification to individual compounds was achieved by preparative TLC, centrifugal TLC or semi-preparative HPLC. Structure elucidation was undertaken using 400 MHz NMR, usually using pyridine-d_6 as solvent. FAB MS gave excellent M +1 and M + Na ions.

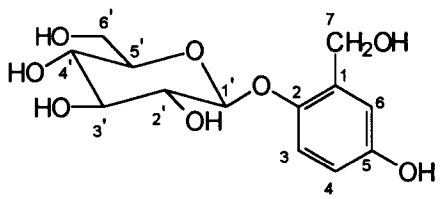
Of the new compounds obtained most proved to possess a central core of 2-(β-glucopyranosyloxy)-5-hydroxybenzyl alcohol (**3**), the position of glycosylation being confirmed by an HMBC experiment[8] which showed both the anomeric H-l' of the sugar and H-7 of the benzyi alcohol coupling to C-2. The sugar moiety, which was easily resolved in the ^{1}H NMR spectrum in all cases (see Table 1), was generally partially esterified. The esterifying groups were usually benzoic acid and occasionally *trans-para*-coumaric acid in *Homalium, Poliothrysis and Xylosma,* but in *Scolopia* these were replaced on the sugar by (*S*)-aleprolic

acid (**4**), which had previously been isolated in free form from the oil of *Hydnocarpus wightiana* (also Flacourtiaceae).[9]

Table 1. ^{1}H NMR chemical shift values for esterified and free positions in the glucose moiety of the glycosides of *Homalium longifolium*[5]

Position	δ esterified	δ free
H-1'		5.24-5.54
H-2'	6.06	4.22-4.44
H-34'		4.29-4.60
H-4'	5.90-5.96	3.88-4.32
H-5,		3.88-4.40
H2-6'	4.80-5.30	4.39-4.54

all spectra run in pyridine-d_6. 8 compounds included.



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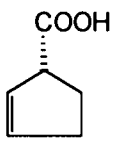

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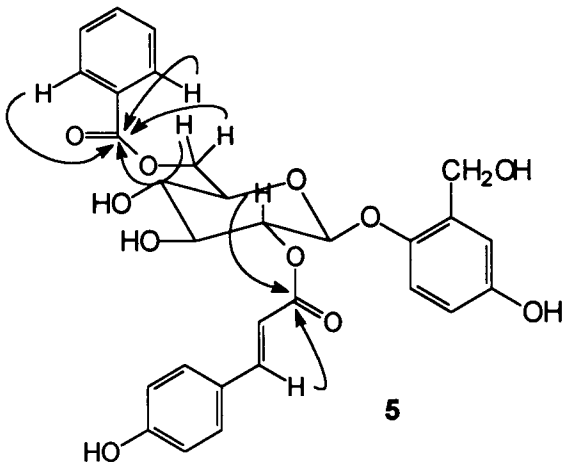
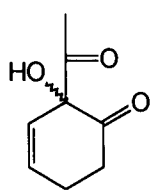
As is clear from Table 1 the presence of substituents at either C-2' or C-4' was immediately obvious from the deshielding of H-2' or H-4' (about 1.7 ppm). For the methyleneoxy C-6' the deshielding was less pronounced (about 0.5 ppm), but was still unambiguous.

In rare cases where more than one esterifying acid was involved (e.g. **5**) each could be located by detecting long-range H-C coupling through the HMBC experiment. Thus, in **5** the observation of ^3J H-C coupling from both H-2' and the β-olefinic proton of the *trans para*-coumaric acid with the ester carbonyl at 167.5 ppm placed the cinnamoyl group at C-2'. Likewise, the coupling of the equivalent H-2 and H-6 protons of the benzoyl group and the two H-6' protons of the glucose with the ester carbonyl at 167.0 ppm placed the benzoyl group at C-6"



5

Much variability in the structures obtained was caused by a further series of esterifications to C-7 of the benzyi alcohol. The first of these to be reported was 1-hydroxy-6-oxocyciohex-2-en-1-carboxyiic acid (**6**) which was independently identified by Ekabo *et al.*[10] from *Homalium ceylanicum* and by our group *from Poliothrysis sinensis*.[3] Neither group resolved the stereochemistry of **6** at C-1. We later re-isolated this acid from *Scolopia spinosa*[7] where it occurred in scoloposide-D. Another isolate from this species, scoloposide-E, contained 1,2,3-trihydroxy-6-oxocyclo-hexane-1-carboxyiic acid (**7**) which is presumed to derive from **6** by oxidation across the double bond. The two additional hydroxyls in **7** were found to be *trans*-diaxial but their relationship to the 1-hydroxyl was again unresolved. In a further modification the 2-hydroxyl was esterified with the aleprolic acid esterifying group that is so prolific in this species.



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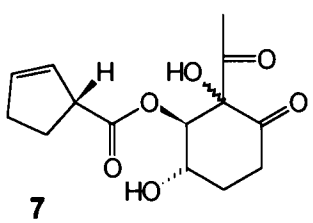
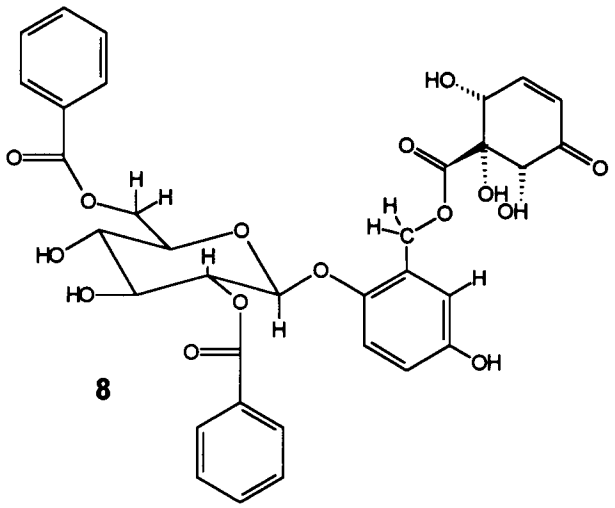


7

Our later studies of *Homalium longifolium*[5] and *Xylosma flexuosa* revealed yet another variant on the C-7 esterifying acids, 1,2,6-trihydroxy-5-oxocyclohex-3-en-1-carboxylic add, which also occurred in the former species with an esterifying benzoic acid group at C-6. We were fortunate to obtain one of the glycosides, xylosmin (**8**), in crystalline form. Despite the poor quality of those crystals White and his colleagues at the University of Western Australia were able to perform an X-ray study and this resolved that the three hydroxyls were all on the same face of the cyclohexenoic acid with the 2 and 6 hydroxyls in a pseudoequatorial configuration and the 1-hydroxyl pseudoaxial.

One further C-7 esterifying group found in two of the glycosides from *Homalium longifolium* was identified as **9**. This compound was also found free, together with the simple isocoumarin (**10**).[6]



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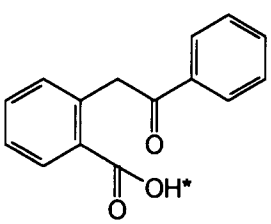
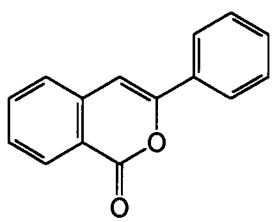


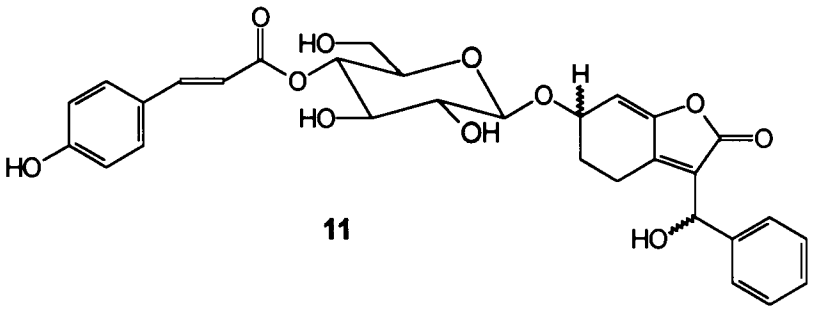
9 * point of linkage to C-7 of benzyl alcohol



10

Finally, *Homalium longifolium*[6] also yielded two aberrant glycosides, one of which has been identified as **11**. The origin of the C_{15} unit which is linked to the glucose at C-1 in this compound is not known.

A complete list of all of the glycosides isolated to date by our group is given in an Appendix to this paper. This reveals that each species has a unique set of compounds but that each contains poliothrysoside.



11

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Appendix. Glycosides isolated from Flacourtiaceae isolated by the Strathclyde group.

Esterification on sugar			Esterification of C-7		Species/
2	4	6	of benzyl alcohol		Ref.
-	-	bz	-		Ps[3] Xf[4] Hl[5], Ss[7]
-	-	-	A		Ps[3]
-	-	bz	A		Ps[3]
bz	-	bz	-		Xf[4]
-	-	-	bz		Xf[4]
bz	-	bz	C		Xf[4]
-	-	-	D		Hl[5]
bz	-	-	D		Hl[5]
pc	-	bz	-		Hl[5]
-	bz	bz	-		Hl[5]
-	-	bz	C		Hl[5]
-	-	bz	C-6-bz	Hl[5]	
-	bz	bz	C		Hl[5]
-	bz	bz	C-6-bz	Hl[5]	
-	pc	-	C15		Hl[6]
al	-	-	-		Ss[7]
al	-	al	-		Ss[7]
al	-	al	A		Ss[7]
al	-	al	B-2-al		Ss[7]

bz = benzoic acid, pc = *para*-coumaric acid, al = aleprolic acid.
A = 1-hydroxy-6-oxocyclohex-2-en-1-carboxylic acid
B = 1,2,3-trihydroxy-6-oxocyclohexan-1-carboxylic acid
C = 1,2,6-trihydroxy-5-oxocyclohex-3-en-1-carboxylic acid
D = 2-(2-oxo-2-phenyl)ethylbenzoic acid
C15 = 3-(1-hydroxybenzyl)-6-hydroxy-2-oxo-2,4,5,6-tetrahydrobenzofuran
Ps = *Poliothrysis sinensis*; Xf = *Xylosma flexuosa*; Hl = *Homalium longifolium*; Ss = *Scolopia spinosa*.

ELUCIDATION OF THE BIOACTIVE CONSTITUENTS IN TRADITIONAL CHINESE MEDICINE "MORI CORTEX"

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Key word Index--*Morus alba*, Moraceae, mulberroside A, oxyresveratrol, metabolites

Abstract--In search of the bioactive substances of the bioactive substances in Mori Cortex, a Mori Cortex extract (MCE) was orally administered to rats, and the characteristic constituents in plasma, urine and bile were analyzed. mulberroside A and cis-mulberroside A were identified in plasma, oxyresveratrol, oxyresveratrol 2,3'-O- β -D-diglucuronide and oxyresveratrol and oxyresveratrol 2-O- β -D-glucuronide-3'-O-sulfate in urine and mulberroside A, oxyresveratrol, oxyresveratrol 2,3'-O- β -D-diglucuronide and oxyresveratrol and oxyresveratrol 2-O- β -D-glucuronide-3'-O-sulfate in bile. The origins of these metabolites were considered to be mainly derived from mulberroside A in Mori Cortex.

Oxyresveratrol showed a relaxant effect on contractions of Guinea pig tracheal smooth muscle induced by histamine and its IC50 was 25 μg/mg.

Introduction

Sangbaipi(桑白皮), Mori Cortex or toot bark of *Morus alba* L.(Moraceae), is mainly used as an antiasthmatic component in traditional Chinese medicinal prescriptions such as Wuhutang(五虎汤)[1]. Although many compounds such as flavonoids[2], coumarins [3] and stilbenes [4] had been identified in Mori Cortex, few pharmacological reports on the traditional usage of Mori Cortex have been published. Since traditional medicines are prepared by extraction with hot water, we make a search for the active components of Mori Cortex from a water extract and try to establish an efficient way for finding the bioactive constituents from the traditional Chinese medicines. In this paper, we report the identification and tracheal smooth muscle relaxing effect of the major metabolites in blood, urine and bile of rats after oral administration of a water extract of the crude drug and mulberroside A.

Results

Identification of M-1, M-2, M-3, M-4 and M-5

After oral administration of water extraction of Mori Cortex to rats, M-1 and M-2 were detected in blood, M-3, M-4 and M-5 in urine, and M-1, M-2, M-4 and M-5 in bile [5].

M-1 and M-2 were identified as mulberroside A and cis-mulberroside A by chemical and spectral evidences[6].

The structures of M-3, M-4 and M-5 were elucidated as elucidated as follows. M-3, M-4 and M-5 showed the UV spectrum similar to that of Mulberroside A, suggesting that these metabolites retained the skeleton structure (oxyresveratrol) of mulberroside A. This suggestion was further supported by their H-NMR as C-NMR data. On enzymatic hydrolysis with β -glucuronidase and arylsulfatase, M-3 and M-5 gave the common aglycone,

oxyresveratrol which was identified by comparing the retention time and UV spectrum with those of an authentic sample on 3D-HPLC. M4 was identified as oxyresveratrol by comparing the MS, UV, H-NMR and C-NMR spectral data with those in a literature [6](Fig. 1).

M-3 was obtained as yellow powder. The molecular formula was determined to be C26H27O16 by HR-SIMS. The two α - anomeric proton signals at δ 4.63 (d, J=7.0Hz) and 4.76(d, J=7.6Hz) in the H-NMR, a pseudomolecular ion peak at m/z 595(M-H) and fragment ion peak at m/z 419 (M-H-2*C6H8O6) in negative SIMS, and the C_NMR signals of two glucuronic acid molecules showed that M-3 contained two β -D-glucuronic acid moieties (Fig. 1).

The location of the glucuronide moieties in M-3 were determined by comparing the C-NMR spectrum of M-3 with that of oxyresveratrol and by the observation of the N<OE between the anomeric proton δ 4.63 (d, J=7.0Hz) and the aromatic proton at δ 6.60, and also between the anomeric proton at δ 6.63 and 6.92. Thus, M3 was confirmed to be oxyresveratrol 2,3'-O- β -diglucuronide (Fig. 1).

M-5 was obtained as yellow powder. The molecular formula was determined to be $C_{20}H_{19}O_{13}S$ by HR-SIMS. Presence of a $-OSO_3^-$ group [7] was suggested by and intense absorption band a5 1050cm-1 in the IR spectrum. The C-NMR spectrum of M-5 showed one glucuronic acid signals (Table 2) and the H-NMR spectrum gave one a – anomeric proton signal at 4.77 (d, 7.6Hz). The negative SIMS gave the pseudomolecular ion peak at m/z 499 (M-1) and the fragment ion peak at m/z 419 (M-H-SO_3), 323 (M-H-$C_6H_8O_6$), 243(M-H-SO_3-$C_6H_8O_6$). M-5 was considered to consist of one glucuronide, one sulfate and oxyresveratrol, which was proved by the enzymatic hydrolysis of M-5 with β -glucuronidase and arylsulfatase. The glucuronide group was showed to be at C-2 by the NOE of M-5.

The hydrolysis of M-5 with β -glucuronidase did not give oxyresveratrol. A comparison of the DC-NMR spectrum of M-5 with that of M-4 showed that the C-3' downfield shifts of C2'(5.7 ppm), C-4' showed that the C-3' signal of M-5 shifted upfield by 3.8, accompanied by downfield shifts of C-2'(5.7ppm), C-4'(5.5ppm) and C-6'(3.8ppm_). These shifts concealed the presence of an intense electron-withdrawing group at C-3', indicating the presence of A –OSO3- group at C-3'. M-5 was thus concluded to be oxyresveratrol 2-O- β — D-glucuronide-3'-O-sulfate.

Relaxant effect of stilbene constituents on tracheal smooth muscles

Both mulberroside A and cis-mulberroside A showed no relaxant effect on tracheal muscle of guinea pig in concentrations of 5×10^{-5}, 1×10^{-4}, 2×10^{-4}, 4×10^{-4} g/ml(Fig. 2). However, in the case of oxyresveratrol 3'-O- β -glucopyranoside and oxyresveratrol, significant relaxant effects were discovered at a concentration of 5×10^{-5} g/ml, especially oxyresveratrol seemed to be stronger than oxyresveratrol 3'-O- β -glucopyranoside.

Relaxant effect of oxyresveratrol on contractions of tracheal muscles induced by histamine

Fig.3 shows a concentration-dependent relaxation of oxyresveratrol on the spontaneous contraction of the guinea pig tracheal muscle induced by histamine. The IC50 of oxyresveratrol was 2.5×10^{-5} g/ml and its concentration at complete inhibition was 5*10-5 g/ml. Although the shapes for oxyresveratrol and diphenhydramine concentration-response curves are different and maybe oxyresveratrol and diphenhydramine have the different

Table 1. Chemical Shifts of ^{1}H-NMR of Mulberroside A, *cis*-Mulberroside A, M-3, M-4 and M-5

	Mublerroside A δ_H (CD_3OD)	*cis*-Mublerroside A δ_H (CD_3OD)	M-3 δ_H(DMSO-d_6)	M-4 δ_H(DMSO-d_6)	M-5 δ_H(DMSO-d_6)
3	6.59 d (2.0)	6.59 d (2.0)	6.60 s	6.32 d (2.0)	6.62 d (2.0)
5	6.62 dd (8.6,2.0)	6.45 dd (8.6,2.0)	6.44 dd (8.0,2.0)	6.24 dd (8.0,2.0)	6.45 dd (8.6,2.0)
6	7.42 d (8.6)	7.02 d (8.6)	7.43 d (8.0)	7.33 d (8.4)	7.47 d (8.6)
α	7.32 d (16.5)	6.56 d (12.0)	7.45 d (16.5)	7.15 d (16.0)	7.39 d (16.5)
β	6.94 d (16.5)	6.40 d (12.0)	6.83 d (16.5)	6.75 d (16.0)	6.85 d (16.5)
2'	6.61 s	6.52 s[a)]	6.92 s	6.34 d (2.0)	6.66 s[a)]
4'	6.46 t (2.0)	6.34 t (2.0)	6.33 s	6.07 t (2.0)	6.63 t (2.0)
6'	6.77 s	6.38 s[a)]	6.58 s	6.34 d (2.0)	6.73 s[a)]
Anomeric proton					
	4.88 d (7.3)	4.56 d (7.3)	4.63 d (7.0)	-	4.77 d (7.6)
	4.90 d (7.3)	4.87 d (7.3)	4.76 d (7.6)	-	-
Anomeric proton					
	3.30-3.95 (12H)	3.30-3.95 (12H)	3.20-3.70 (8H)	-	3.10-3.50 (4H)

δ Values in ppm. Values in parentheses are coupling constants (Hz)

a) Assignments may be interchanged in each column.

Table 2. Chemical Shifts of ^{13}C-NMR of Mulberroside A, *cis*-Mulberroside A, M-3, M-4 and M-5

	Mublerroside A (CD_3OD)		*cis*-Mublerroside A (CD_3OD)		M-3 (DMSO-d_6)		M-4 (DMSO-d_6)	M-5 (DMSO-d_6)
1	120.3		120.2		117.7		115.1	117.8
2	157.1[a)]		157.1[a)]		155.9[a)]		155.9[a)]	156.0[a)]
3	104.9		104.9		103.1		102.5	103.4
4	159.5[a)]		159.1[a)]		158.1[a)]		158.0[a)]	158.3[a)]
5	109.4		108.5		109.4		107.5	109.8
6	128.4		132.0		126.5		126.9	126.6
α	125.0		127.0		123.7		123.1	123.1
β	127.6		130.1		125.6		124.5	125.4
1'	141.9		140.7		135.8		139.8	139.2
2'	107.2		108.8[b)]		105.0		103.9	109.6[b)]
3'	160.3[a)]		159.8[a)]		159.0[a)]		158.3	154.5[a)]
4'	104.0		104.1		103.2		101.2	106.7
5'	159.5[a)]		159.4[a)]		158.3[a)]		158.3	157.8[a)]
6'	108.2		111.4[b)]		108.2		103.9	107.7[b)]
	glucose		glucose		Glucuronic acid			Glucuronic acid
1	102.4	102.1	102.3	102.1	102.5	100.7	-	101.0
2	74.9	74.8	74.8	74.7	73.2	72.8	-	73.3
3	78.1[c)]	78.1[c)]	78.0[c)]	77.9[c)]	73.9	73.9	-	74.1
4	71.3	71.3	71.3	70.9	71.9	71.8	-	72.0
5	78.0[c)]	78.0[c)]	77.9[c)]	77.5[c)]	76.8	76.6	-	76.7
6	62.5	62.3	62.5	62.0	173.1	172.4	-	172.4

δ Values in ppm. Values in parentheses are coupling constants (Hz)

[a), b), c)] Assignments may be interchanged in each column.

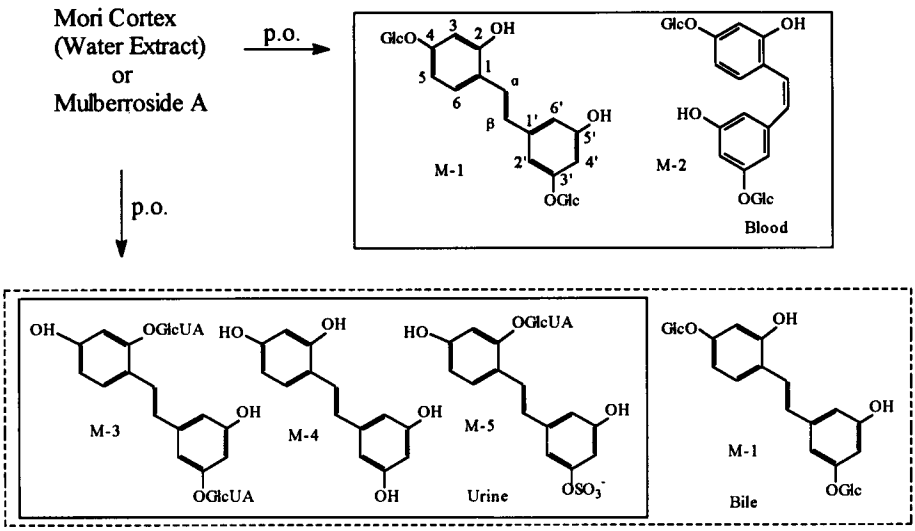

Fig. 1. Absorption and Metabolism of Mulberroside A
M-1: mulberroside A, M-2: cis-mulberroside A,
M-3: oxyresveratrol 2,3'-di-o- β-D-glucuronide
M-4: oxyresveratrol ,
M-5: oxyresveratrol 2-O- β-glucuronide-3'-O-sulfate

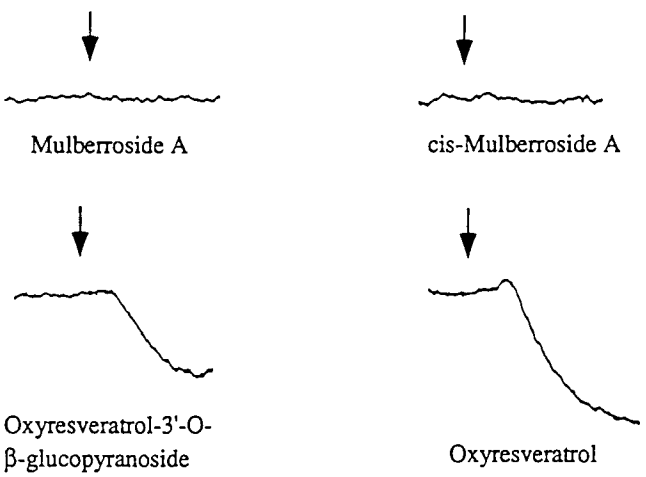

Fig. 2. Response of Stilbene Constituents on the Guinea Pig Tracheal Smooth Muscle

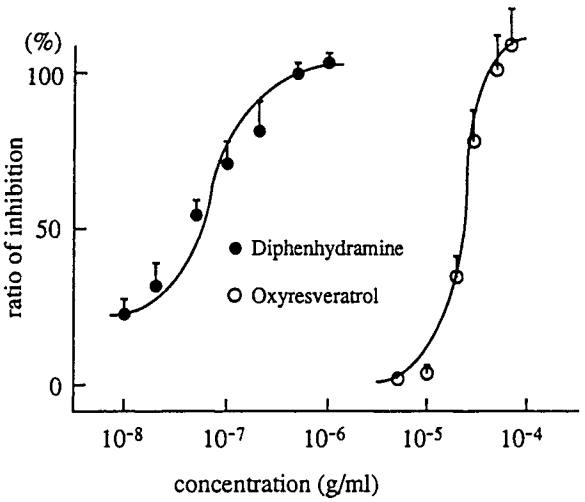

Fig. 3. Concentration-Response Curves for the Inhibitory Effect of Oxyresveratrol and Diphenhydramine on the Spontaneous Contractions of Guinea Pig Tracheal Muscle.

Values are expressed as the percentages of inhibition of contractile response calculated as the mean of five data ± SD.

mechanism of action, oxyresveratrol appeared approximately 1/500 of the strength of diphenhydramine by comparison of IC_{50}.

Discussion

A new method was applied in studies of bioactive constituents of Mori Cortex. This method is established based on two points: One, traditional Chinese medicine is taken as a decoction in most cases, and two, the material base for traditional Chinese medicine to show its effective chemical constituents *in vivo*. In order to elucidate bioactive component of Chinese traditional medicine "Mori Cortex", a Mori Cortex extract (MCE) and mulberroside A were orally administered to rats, as presented in Fig. 1, mulberroside A and cis-mulserroside A were identified in plasma, oxyresveratrol, oxyresveratrol 2,3'-O- β -diglucuronide and oxyresveratrol 2-O- β -D-glucuronide-3'-O-sulfate in urine and mulberroside A, oxyresveratrol 2,3'-O- β -diglucuronide and oxyresveratrol 2-O- β -D-glucuronide-3'-O-sulfate in bile. Since mulberroside A is a characteristic constituent in Mori Cortex. To clarify the pharmacokinetics of mulberroside A and its metabolites, biopharmaceutical studies were also carried out [8]. The distribution volume of mulberroside A was about 8L per kg, and its extent of bioavilibility was less than 1%. However, the percentage of dose absorbed for mulberroside A and its de4rivatives was about 50%, oxyresveratrol is a main existing form in the body. The above results suggested that oxyresveratrol is an important objective constituent in investigation of bioactivity. Mori Cortex was reported to have an antiasthmatic effect, and relaxant activity of oxyresveratrol on Guinea pig tracheal smooth muscle can just explain the effectiveness of Mori Cortex. Therefore, the metabolite oxyresveratrol is a bioactive substance and mulberroside A in Mori Cortex is just a prodrug. The approach used in this study most closely correlates to the theoretical system of traditional Chinese medicine and will provide practicable reference for elucidating effectiveness of tradition Chinese medicine.

Experiment

Crude drug. Mori cortex, dried root bark of *Morus alba* L, of the Japanese Pharmacopoeia XII quality was purchased form to Tochimoto Co., Ltd. (Japan) in 1994 and identified by Dr. N. Ishizaka, Professor of the department of Biology in Hokkaido College of Pharmacy, Japan.

Chemicals. Mulberroside A and cis-mulberroside A were isolate form Morus Alba L. and identified by chemical and spectral evidences [6]. Oxyresveratrol was obtained by hydrolysis of mulberroside A with β -D-glucosidase. Hitamine, diphenhydramine were obtained commercially form Sigma (St. Louis. MO, U.S.A.).

Melting points were determined on a Yanako MP micro melting point apparatus and not corrected. IR spectra were measured on Jasco FT-IR 7000 spectrophotometer, UV spectra on Shimadzu UV-3000 spectrometer. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{13}C COSY and ^{1}H-^{1}H COSY spectra were measured with a JNMGX270 spectrometer. Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard. MS was taken on a Hitachi M-6000 spectrometer.

Preparation of MCE. Mori Cortex(100g) was added to 2l of distilled water, and the whole was boiled until the volume decreased to 1/2 of the original volume. Then, the extract was filtered through 5-layer gauze to give a filtrate (MCE), which was freeze-dried. Two hundred milligrams of the freeze-dried MCE corresponded to 1g of the crude drug. This freeze-dried MCE was stored at 4 ^{0}C until use.

Animals. Male Wistar rats (6-week-old, 150-180) and Guinea pigs (male, 400-600g, 10-week-old) were purchased from Shizuoka Laboratory Animal Center Co., Ltd.(Hamanatsu, Japan). The animal were kept in a breeding room (temperature: 24+1 ^{0}C, humidity: 50+5%, 12h dark-light cycle) for 7 days before the start of the experiments. Tap water and normal foods were given *ad libitum*. They were fasted for about 24 h before start of the experiments. The freeze dried MCE and mulberroside A were orally administrated to rats at doses of 10g/kg (as aqueous suspension) and 100 mg/kg (as aqueous solution). Eight rats were used in one assay.

Isolation of metabolites in urine and bile. 1) Metabolites in urine: About 1.5l of 24 h urine was obtained from 50 rats. Three liters of methanol was added to the urine. The mixture was centrifuged at 3000 rpm for 10 min and the4 supernatant was evaporated to dryness at 40 ^{0}C *in vacuo*. The residue was chromatographed on Sephadex LH-20 with methanol as an eluant. Then, the fractions containing urinary metabolites (M-3, M-4 and M-5) were evaporated to dryness at 40 ^{0}C *in vacuo*, and the residue was subjected to preparative HPLC. The conditions were as follows: column; Inertsil ODS-2 (20 × 250 mm, Gl Science Inc.), mobile phase; acetonitrile / water=4/96, flow rate; 10ml/min. Each eluate containing M-3(4mg), M-4(26mg) or M-5(1mg) was evaporated to dryness at 40 ^{0}C *in vacuo*.

2) Metabolite in bile: About 600 ml of pooled rat bile was treated in the same manner as described above. Mulberroside A (4mg), M-3(180mg), M-4(2mg) and M-5(7mg) was obtained.

Enzymatic hydrolysis. M-3(0.5mg) was incubated with β -glucuronidase (0.4μl) in 0.1M acetate buffer (pH 5.0) for 3h at 37 ^{0}C. M-5 (0.5mg) was treated with β -glucuronidase(0.2μl) and arylsulfatase(0.2mg) in the same way. The aglycone (oxyresveratrol) in each reacted mixture was identified by comparing with an authentic sample on 3D-HPLC.

M-3. oxyresveratrol 2,3'-O- β -diglucuronide (184mg), yellow powder. High-resolution SIMS: Foun:595.0374; Calcd for $C_{26}H_{27}O_{16}$(M-H):592.1299. Negative SIMS m/z: 595(M-H), 419(M-H-$C_6H_8O_6$), 243 (M-H-2×$C_6H_8O_6$). IR ν KBr max cm^{-1}: 3450,1600,1410,1280. ^{1}H-NMR: shown in Table 1; ^{13}C-NMR in Table 2.

M-5. oxyresveratrol 2-O- β -D-glucuronide-3'-O-sulfate (8mg), yellow powder. High-resolution SIMS: Found: 499.1435; Calcd for $C_{29}H_{19}O_{13}S$ (M-H): 499.0546. Negative SIMS m/z : 499(M-H), 419(M-H- $C_6H_8O_6$). IR ν KBr max cm^{-1}: 3550, 1600, 1280, 1050, 770. ^{1}H-NMR: shown in Table 1; ^{13}C-NMR in Table 2.

The guinea pig tracheal muscles assay. Male guinea pigs were killed with a blow to the head and exsanguination. The whole tracheal tube was excised and cut open longitudinally along the anterior side of the trachea. The opened trachea strips, were tied to a UL-2GR transducer (Minebea) connected with a C-4A recorder (shimadzu), and they were suspended in a 50 ml organ bath containing Tyrode solution of the following composition (g/l): NaCl 80, $NaHCO_3$ 10 KCl_2, NaH_2PO_4 0.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 bubbled with air. The bath temperature was maintained at 37 ^{0}C. After a stabilization time of 60 min, the test was started.

Relaxant effect of stilbene constituents on tracheal smooth muscles. Mulberroside A, cis-mulberroside A, oxyresveratrol-3'-O- β -glucopyranoside were dissolved in water to concentrations of 2.5, 5, 10,20mg/ml for mulberroside A and cis-mulberroside A and 2.5mg/ml for oxyreveratrol-3'- O- β -glucopyranoside. Oxyresveratrol was dissolved in 2% DMSO (dimethyl sulfoxide) aqueous solution to make a concentrantion of 2.5mg/ml. One milliliter of each solution was added to the organ bath and the reactions of guinea pig tracheal muscle were observed for 10 minutes.

Relaxant effect of oxyresveratrol on contractions of tracheal muscles induced by histamine. Histamine solution (10^{-6} g/ml) was added to the organ bath. After the contraction of tracheal muscle reached the maximum, oxyresveratrol was administered at the following concentrations:5×10^{-5}, 1×10^{-4}, 2×10^{-5}, 3×10^{-5}, 5×10^{-5}, 7×10^{-5} g/ml. The contractile changes were recorded.

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INVESTIGATION OF FUNCTIONAL MOLECULES IN AFRICAN *CELOSIA ARGENTEA* L.

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Key Word Index-*Celosia argentea* L.; glycosides; constituents; bioassay; lettuce.

Abstract Five glycosides were isolated from the leaves of *Celosia argentea* L. and their structures were determined based on UV, MS, ^{1}H NMR and ^{13}C NMR spectroscopic data as 1-(4-*O*-β-glucopyranosyl-3-methoxyphenyl) propane-2-ene (citrusin C, **1**), 3-*O*-β-glucopyranosyl-1*H*-indole (indican, **2**), (3*Z*)-hexenyl-(1-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**3**), (3*Z*)-hexenyl-1-*O*-β-glucopyranoside (**4**), and (7*E*)-6,9-dihydromegastigma-7-ene-3-one-9-*O*-β-glucopyranoside (**5**). On bioassay on the germination of lettuce, compound **1**, the aglycone of **1** (**1a**), and the aglycone of **3** (**3a**) exhibited growth inhibitory activity, whereas compound **2** and **3** exhibited growth promotive activity. Compounds **1-5** were detected in the leaves of *Celosia argentea* L. for the first time in this study.

Introduction

Sierra Leone is a country located in the Middle West of Africa. In this country, people are longevity, and it is interesting to know what may be the cause. We thought that their food may be related to the longevity, and started to investigate the constituents in the leaves of *Celosia argentea* L., which is used for diet in the country. *Celosia argentea* L. grows in the torrid zone of Africa by origin. It is found also in a warm place of the westward of Japan. For constituents in *Celosia argentea* L., it is estimated to contain some terpenoids and saponins in the roots, and flavonoids in the leaves and stems [1]. Recently, Shah *et al.* reported that the alcohol extracts of the leaves have an antibacterial activity and also the seeds have a diuretic activity for albino rats and human volunteers [1].

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For the effect of the germination of plants, Pandya reported that aqueous extracts of fresh leaves, stem, and roots of *Celosia argentea* L. inhibited growth of shoot and root of bajra (*Pennisetum americanum*) [2, 3]. Also Ashraf and Sen presented supporting evidence for the inhibition effects of aqueous extracts of *Celosia argentea* L. on bajra and reported that the similar inhibitory effects on til (*Sesamun indicum*) [4].

This paper describes an investigation of glycosides in the leaves of African *Celosia argentea* L., and their effect on the germination of lettuce.

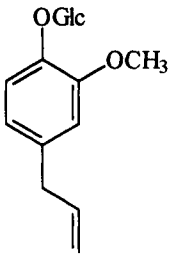
Results and Discussion

A mixture of crude glycosides were obtained from a 70% EtOH-H_2O extract of the leaves of *Celosia argentea* L. (dry wt., 789.9 g). Four glycosides (Fig.1) were purified by gel filtration on HW-40F TSK gel and column chromatography on silica gel. The yields of isolated compounds are **1:** 9.6 mg, **2:** 6.3 mg, **3:** 5.5 mg, **4:** 9.2 mg, and **5:** 9.2 mg. The melting point (mp), specific rotation, and spectral data (UV, FAB MS, ^{1}H NMR and ^{13}C NMR) of **1** agreed with citrusin C, which had been isolated from hassaku (*Citrus hassaku*) and orange (*Citrus sinensis*) peels in our earlier study [5].

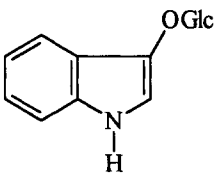
Compound **2**, a colorless crystal, had mp 58°, and showed a specific rotation of -24.49° (*c* 0.075, MeOH). The molecular weight of **2** was found to be 295 by FAB MS; positive FAB MS showed peaks at *m/z* 296 $[M+H]^+$, 133 $[MH\text{-}Glc]^+$ and 115 $[M\text{-}GlcOH]^+$, and negative FAB MS showed a peak at *m/z* 294 $[M-H]^-$. The ^{1}H NMR spectrum of **2** indicated the presence of five olefinic protons of indole ring [H-2 at δ 6.98 (1H, *s*), H-4 at δ 7.58 (1H, *dd*, *J*=1.0, 7.5 Hz), H-5 at δ 6.87 (1H, *ddd*, *J*=1.0, 7.5, 8.0 Hz), H-6 at δ 6.98 (1H, *ddd*, *J*=1.0, 7.5, 8.0 Hz), and H-7 at δ 7.17 (1H, *dd*, *J*=1.0, 7.5 Hz)] and an anomeric proton of β-glucopyranosyl moiety at δ 4.62 (1H, *d*, *J*=7.8 Hz). The structure of this indole glycoside was supported by the appearance of fourteen signals in the ^{13}C NMR spectrum, including six carbon signals of a glucopyranosyl moiety and eight carbon signals of an indole moiety. Based on the evidence, the structure of **2** was identified to be 3-*O*-β-glucopyranosyl-1*H*-indole (indican).

Compound **3** was colorless oil, and showed a specific rotation of -48.26° (*c* 0.5, MeOH). The molecular weight of **3** was found to be 408 by FAB MS; positive FAB MS showed peaks at *m/z* 409 $[M+H]^+$, 263 $[MH\text{-}Rham]^+$ and 501 $[MH+Glycerol]^+$, and negative FAB MS showed peaks at *m/z* 407 $[M-H]^-$, 261 $[M\text{-}H\text{-}Rham]^-$. The ^{1}H NMR spectrum of **3** indicated the presence of a methyl group at δ 0.87 (3H, *t*, *J*=7.5 Hz), methylene protons attached to an unsaturated bond at δ 1.98 (2H, *qt*, *J*=7.5 Hz) and 2.28 (2H, *q*, *J*=7.5 Hz), *Z*-olefinic protons at δ 5.32 (2H, *m*), and two anomeric protons of a α-rhamnopyranosyl moiety at δ 4.64 (1H, *d*, *J*=1.5 Hz) and a β-glucopyranosyl moiety at 4.16 (1H, *d*, *J*=7.8 Hz). The structure of an alkyl glycoside was supported by ^{13}C NMR spectrum which exhibited eighteen signals including twelve carbon signals of a glucopyranosyl moiety and a rhamnopyranosyl moiety, and two olefinic carbon signals. In the ^{13}C NMR spectrum of **3**, the signal due to C-6 of glucose shifted downfield by *ca.*

5 ppm and that of C-5 of glucose shifted upfield by *ca.* 1 ppm in comparison with those of usual glucopyranoside [6]. Therefore, a rhamnopyranosyl moiety of **3** proved to be attached to C-6 of glucose. Based on the evidence, the structure of compound **3** was determined to be (3*Z*)-hexenyl-1-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyanoside).



1



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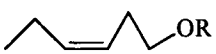
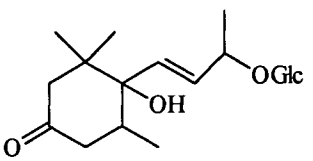


3 :R=Glc^{6}—Rham
4 :R=Glc



5

Compound **4**, a colorless oil showing a specific rotation of -35.5° (*c* 0.2, MeOH), was identified to be (3*Z*)-hexenyl-1-*O*-β-glucopyranoside by comparison with its ^{1}H and ^{13}C NMR data with those of an authentic sample isolated from *Epimedium grandiflorum* MORR. var. *thunbergianum* (MIQ) NAKAI [6, 7].

Compound **5** was a colorless oil and showed a specific rotation of -35.49° (*c* 0.245, MeOH). The molecular weight of **5** was found to be 388 by FAB MS; positive FAB MS showed a peak at *m/z* 389 $[M+H]^{+}$, and negative FAB MS showed a peak at *m/z* 387 $[M-H]^{-}$. The ^{1}H NMR spectrum of **5** indicated the presence of geminal methyl groups at δ 0.84 and 1.02 (both, 3H, *s*), other two methyl groups δ 1.11 (3H, *d*, *J*=7.0 Hz) and 1.13 (3H, *d*, *J*=7.0 Hz), *E*-olefinic protons at δ 5.57 (1H, *dd*, *J*=15.5, 5.5 Hz) and 5.82 (1H, *d*, *J*=15.5 Hz), and an anomeric proton of a β-glucopyranosyl moiety at δ 4.24 (1H, *dd*, *J*=7.8 Hz). The structure of terpenoid glycoside was confirmed by the ^{13}C NMR spectrum which showed nineteen signals including six carbon signals of a glucopyranosyl moiety, two olefinic carbon signals, two carbon signals attached to an oxygen atom and one carboxyl carbon of terpenoid moiety. Based on the foregoing evidence, the structure of **4** was assumed to be (7*E*)-6,9-dihydromegastigma-7-ene-3-one-9-*O*-β-

glucopyranoside, which has a saturated bond at C-4 instead of an unsaturated bond at C-4 of vomiforiol-9-*O*-β-glucopyranoside [8, 9].

Thus, five kinds of glycosides have now been successfully isolated from the leaves of *Celosia argentea* L.

Although a mixture of crude glycosides (neutral components) was found to have no antioxidative activity in the analysis by DPPH method, the effect of these glycosides on the germination was examined by using lettuce seeds. As a result, compound **1**, the aglycone of **1** (**1a**), and the aglycone of **3** (**3a**) exhibited growth inhibitory activity, whereas compound **2** and **3** exhibited growth promotive activity, as shown in Table 1. The difference between **1** and **1a** or **3** and **3a** which showed the opposite biological effect is dependent only to whether the compound having sugar moiety or not. Therefore, it may be suggested that if the compounds have sugar moiety (glucose), they tend to have growth promotive activity, and if they do not have sugar moiety, they may have growth inhibitory activity.

All compounds isolated in this study, **1-5**, were found in the leaves of *Celosia argentea* L. for the first time.

Table. 1 Effect of **1-3**, **1a** and **3a** on the Germination of the Lettuce.

Compound	length of root (%)	Length of hypocotyl (%)	Length of cotyledon (%)	Weight (%)
citrusin C (**1**)				
1000ppm	71.4	80.9	81.6	85.2
500ppm	66.5	85.2	74.1	75.1
100ppm	68.8	88.6	86.8	96.0
indican (**2**)				
1000ppm	75.5	170.4	70.6	77.7
500ppm	88.8	135.2	77.4	79.0
100ppm	105.5	111.1	84.7	85.1
3*Z*-hxenyl-1-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**3**)				
500ppm	87.0	138.9	98.1	93.3
100ppm	113.4	128.9	106.5	107.6
eugenol (**1a**)				
1000ppm	0	0	0	0
500ppm	0	0	0	0
100ppm	65.1	40.0	86.8	96.0
3*Z*-hexenol (**3a**)				
1000ppm	0	0	0	0
500ppm	77.3	69.6	92.5	89.3
100ppm	92.2	77.2	98.3	102.8
Control	100.0	100.0	100.0	100.0

Experimental

General. Melting point (mp) was collected on Yanagimoto micro melting point apparatus. Optical rotation was measured with a JASCO DIP-140. UV and IR spectra were recorded on Shimadzu UV-160A spectrometer and a Perkin-Elmer 1760-X infrared fourier transform spectrometer, respectively. MS spectra were obtained with JEOL JMX-HX 100 and JMA-DA 5000 spectrometers under Xe bombardment (6.0 keV). NMR spectra were measured with a JOEL EX-270 instrument in CD_3OD with TMS as an internal standard (270 MHz for ^1H and 67.5 MHz for ^{13}C).

Materials. Commercially available dry powder of the leaves of African *Celosia argentea* L. were used.

Separation of the crude glycoside. The dry powder of leaves of *Celosia argentea* L. (789.9 g) were extracted with 70% EtOH-H_2O (3 l) in the dark for one day. The 70% EtOH-H_2O extract (100.8 g) was concentrated to 700 ml *in vacuo*, and then extracted with hexane and *n*-BuOH, successively. A saturated aqueous solution of lead subacetate was added to the *n*-BuOH extract, and the precipitates were removed by filtration. The filtrate was adjusted to pH 9.0 and then extracted with *n*-BuOH to afford a mixture of crude glycoside (neutral components, 3.75 g) from the *n*-BuOH solution.

Gel filtration. Gel filtration was carried out in prepacked column (5 cm X 1 m) of HW-40F TSK gel (Tosho Co., Ltd) under medium pressure, and absorptions of the elutes were measured at 280 nm with an UVICON 750 instrument (Advantec Toyo Co., Ltd.). The neutral components was eluted with 20 l of distilled H_2O and 10 l of 50% MeOH-H_2O, successively. The eluate was collected in every min., and each fraction was concentrated *in vacuo* and freeze-dried.

Column chromatography on silica gel. Each fraction obtained by gel filtration was chromatographed on silica gel (C-300 Wako gel, Wako Pure Chemical Industries Ltd.) with $CHCl_3$-MeOH (5:1 or 8:1) as the eluent.

Antioxidative assay. Antioxidative activity was measured by DPPH method [10, 11].

Bioassay with lettuce seeds. 2 ml of each compounds at the requisite concentration in pure H_2O or in an aqueous solution containing tween 80 (100 ppm) was absorbed on filter paper in Petri dish. Ten germinated seeds of lettuce were placed on each filter paper in Petri dish and kept at 25°. The relative germination ratio was calculated based on ten germinated seeds in Petri dish which contained only H_2O or aqueous tween 80 without any other additives (control).

Enzymatic hydrolysis. A solution of the sample (3 mg) in a Na_2HPO_4-citric acid buffer (pH 4.0, 2 ml) was treated with a β-glucosidase or a hesperidinase (Wako Pure Chemical Industries Ltd; 2 mg) and the whole mixture was stirred for 3 days at 37°. The reaction mixtures was extracted with Et_2O, and the Et_2O extract was analyzed by the EI MS and GLC FID; column, PEG-20M, 0.25 mm X 20 m, temp., 80-180° (2° min^{-1}); carrier gas, N_2, 1.0 $kg \cdot cm^{2-1}$.

3-O-β-glucopyranosyl-1H-indole (indican) 2, colorless; mp 58°; $[\alpha]_D^{20}$ -27.49° (*c* 0.075,

MeOH); FAB MS data (positive and negative ion modes, glycerol) *m/z* 296 $[M+H]^+$, 294 $[M-H]^-$, 133 $[MH-Glc]^+$; UV λ_{max}(MeOH): 282 nm; IR (NaCl) ν_{max}: 3402, 2360, 2342, 2122, 1648, 1108, 1016 cm^-; ^1H NMR (270 MHz, CD_3OD) δ: 3.24-3.47 (4H, *m*, Glc-H2,3,4,5), 3.63 (1H, *dd*, *J*=12, 5 Hz, Glc-H6B), 3.82 (1H, *dd*, *J*=12, 2 Hz, Glc-H6A), 4.62 (1H, *d*, *J*=7.8 Hz, Glc-H1), 6.87 (1H, *ddt*, *J*=8.0, 7.5, 1.0 Hz, H-5), 6.98(1H, s, H-2), 6.98 (1H, *ddt*, *J*=8.0, 7.5, 1.0 Hz, H-6), 7.17 (1H, *dd*, *J*=7.5, 1.0 Hz, H-7), 7.58 (1H, *d*, *J*=7.5, 1.0 Hz, H-4); ^{13}C NMR (67.5 MHz, CD_3OD) δ: Sugar 62.6 (Glc-C6), 71.5 (Glc-C4), 75.1 (Glc-C2), 78.0 (Glc-C5), 78.2 (Glc-C3), 105.8 (Glc-C1), Aglycone 112.2 (C-3), 112.4 (C-7), 118.6 (C-4), 119.5 (C-6), 121.4 (C-2), 122.8 (C-5), 135.3 (C-8), 138.1 (C-9).

(3Z)-hexenyl-1-O-(6-O-α-rhamnopyranosyl-β-glucopyranoside) ***3***, colorless oil; $[\alpha]_D^{20}$ -48.26° (*c* 0.5, MeOH); FAB MS data (positive and negative ion modes, glycerol) *m/z* 409 $[M+H]^+$, 407 $[M-H]^-$, 263 $[MH-Rham]^+$, 261 $[M-H-Rham]^-$; UV λ_{max} (MeOH): 259 nm; IR (NaCl) ν_{max}: 3436, 1641, 1073 cm^{-1}; ^1H NMR (270 MHz, CD_3OD) δ: 0.87 (3H, *t*, *J*=7.5 Hz, H-6), 1.16 (3H, *d*, *J*=6.5 Hz, Rham-H6), 1.98 (2H, *qt*, *J*=7.5 Hz, H-2), 2.28 (2H, *q*, *J*=7.5 Hz, H-5), 3.44 (4H, *m*, Glc-H4,3 and Rham-H3,4), 3.56 (3H, *m*, Glc-H5,2 and Rham-H5), 3.73 (2H, *m*, Glc-H6B and Rham-H2), 3.87 (1H, *dd*, Glc-H6A), 4.16 (1H, *d*, *J*=7.8 Hz, Glc-H1), 4.64 (1H, *d*, *J*=1.5 Hz, Rham-H1), 5.32 (2H, *m*, H-3,4); ^{13}C NMR δ: (67.5 MHz, CD_3OD) Sugar 17.9 (Rham-C6), 68.0 (Glc-C6), 70.6 (Rham-C5), 71.6 (Glc-C4), 72.2 (Rham-C3), 72.3 (Rham-C2), 74.0 (Rham-C4), 75.1 (Glc-C2), 76.8 (Glc-C5), 78.0 (Glc-C3), 102.1 (Glc-C1), 104.5 (Glc-C1), Aglycone 14.6 (C-6), 21.5 (C-5), 28.8 (C-2), 69.7 (C-1), 125.8 (C-3), 134.5 (C-4).

(7E)-6,9-dihydromegastigma-7-en-3-one-9-O-β-glucopyranoside ***5***, colorless oil; $[\alpha]_D^{20}$ -35.49° (*c* 0.245, MeOH); FAB MS data (positive and negative ion modes, glycerol) *m/z* 389 $[M+H]^+$, 387 $[M-H]^-$, 209 $[MH-GlcOH]^+$; UV λ_{max} (MeOH): 261 nm; IR (NaCl) ν_{max}: 3343, 2360, 1670, 1654, 1077, 1044 cm^{-1}; ^1H NMR (270 MHz, CD_3OD) δ: 0.84 ppm (3H, *s*, H-11), 1.02 (3H, *s*, H-12), 1.11 (3H, *d*, *J*=7.0 Hz, H-13), 1.13 (3H, *d*, *J*=7.0 Hz, H-10), 1.21 (2H, *m*, H-4), 1.62 (1H, *m*, H-2A), 2.28 (1H, *m*, H-2B), 3.20-3.38 (4H, *m*, Glc-H2,3,4,5), 3.55 (1H, *m*, Glc-H6B), 3.75 (1H, *dd*, *m*, Glc-H6A), 4.20 (1H, *m*, H-9), 4.24 (1H, *d*, *J*=7.8 Hz, Glc-H1), 5.57 (1H, *dd*, *J*=15.5, 5.5 Hz, H-8), 5.82 (1H, *d*, *J*=15.5 Hz, H-7); ^{13}C NMR (67.5 MHz, CD_3OD) δ: Sugar 62.7 (Glc-C6), 71.6 (Glc-C4), 75.2 (Glc-C2), 77.8 (Glc-C5), 78.0 (Glc-C3), 102.8 (Glc-C1), Aglycone 20.2 (C-13), 23.8 (C-12), 25.2 (C-11), 29.8 (C-10), 35.8 (C-4), 37.6 (C-1), 38.6 (C-5), 45.7 (C-2), 68.6 (C-6), 72.8 (C-9), 135.5 (C-8), 201.3 (C-3).

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PHENYLPROPANOID GLYCOSIDES FROM *ILLICIUM* PLANTS

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Abstract--Two glycerol phenylpropanoid glucosides **1**, **2** and a glycoside of 2-hydroxyphenylpropanoid **3** were isolated from the barks of *Illicium difengpi* K. I. B. et K. I. M. (Illiciaceae). Compounds **4** and **5**, the analogous compounds of **3**, were obtained from the fresh fruits of *I. anisatum* L.. The structures of these compounds were elucidated on the basis of spectral and chemical evidence.

Introduction

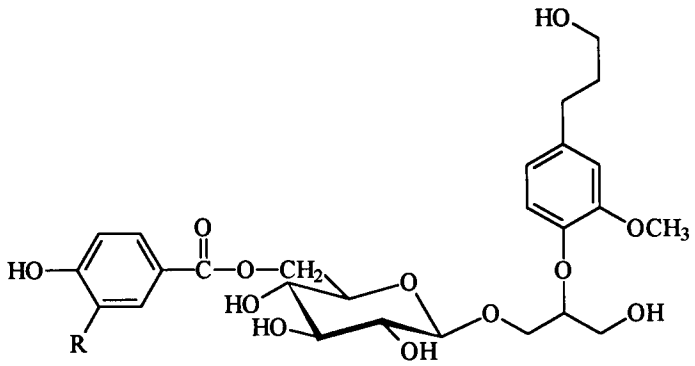
We have been interested in the toxic constituents of *Illicium* plants (Illiciaceae), and have reported on the isolation and structural elucidation of a series of sesquiterpenes possessing unique framework from these plants in Japan and China [1]. As a continuous work on the chemical studies on the *Illicium* plants, we investigated the constituents of the bark of non-toxic *Illicium difengpi* K. I. B. et K. I. M., which was used as antiarthritic agent in China, and those of the fresh fruits of toxic *Illicium anisatum* L.. This paper deals with the isolation and structural elucidation of five new phenylpropanoid glycosides from the above plants.

Results and Discussion

The MeOH extract of the bark of *I. difengpi* was extracted with *n*-hexane, EtOAc and *n*-BuOH, successively. The *n*-BuOH layer was subjected to column chromatographies over Toyopearl HW-40, silica gel, ODS and preparative HPLC (ODS) to furnish compounds **1** [2], **2** [3] and **3** [2].

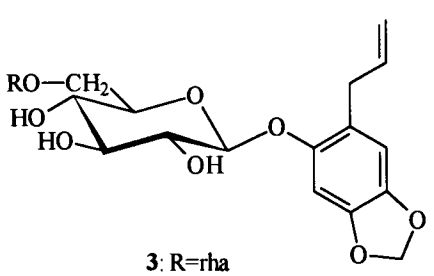
Compound **1** was isolated as colorless syrup whose molecular formula was determined to be $C_{26}H_{34}O_{12}$ by its positive FAB-MS [*m/z* 561 $(M+Na)^+$] and ^{13}C-NMR spectral data. The presence of dihydroconiferyl alcohol and *p*-hydroxybenzoic acid moieties was inferred from its ^1H and ^{13}C-NMR spectral data. A phenolic and five alcoholic acetoxy groups were suggested from the ^1H-NMR spectrum of the acetate of **1**. Acid hydrolysis of **1** with 2N HCl afforded *p*-hydroxybenzoic acid, glucose and 4-*O*-(2-hydroxy-1-hydroxymethylethyl)-dihydroconiferyl alcohol. The linkage of these three segments in **1** was established on the basis of the comparison of ^1H and ^{13}C-NMR spectral data. Thus, the structure of **1** was assigned as in the figure.

Compound **2** was assigned the molecular formula $C_{27}H_{36}O_{13}$ from the results of its positive FAB-MS and ^{13}C-NMR data. Its ^1H and ^{13}C-NMR spectral data strongly resembled those of **1**, except for the acyl unit. Detailed analyses of the NMR data revealed that **2** should have a vanniloyl group in the molecule instead of the *p*-hydroxybenzoyl group in **1**. The presence of glycerol, dihydroconiferyl alcohol and glucose was also confirmed by the NMR data. The stereochemistry of the asymmetric centre of C-2' in the glycerol moiety of **1** and **2** was not clarified.



1: R=H
2: R=OCH_3

Compound **3**, $C_{22}H_{30}O_{12}$, was isolated as a white amorphous powder. It showed $[M+H]^+$ ion peak at *m/z* 487 in the positive FAB-MS. The ^{1}H-NMR spectrum showed the signals at δ_H 5.03 (dd, 1H, *J*=17.2, 1.8 Hz), 4.98 (1H, dd, 1H, *J*=9.3, 1.8 Hz), 4.02 (ddd, 1H, *J*=17.2, 9.3, 6.4 Hz) and 3.40 (d, 2H, *J*=6.4 Hz), indicating the presence of a 2-propenyl side-chain group. The signals of aromatic protons were observed at δ_H 6.59 and 6.79 (each 1H, s) due to 1, 2, 4, 5-tetrasubstituted benzene ring, together with δ_H 5.88 and 5.87 (each 1H, d, *J*=1.1 Hz) derived from a methylene dioxy group. Thus, **3** should have a phenylpropanoid moiety. The ^{13}C-NMR spectrum of **3** also exihibited the signals due to a glucose and a rhamnose moieties, as was confirmed by the acid hydrolysis of **3** with 2N HCl. Accordingly, **3** is suggested to be a diglycosyl phenylpropanoid. When **3** was hydroloyzed with crude hesperidase for 24 h at 37 °C, it gave colorless needles **3a**, mp 77-78 °C, which was identified to be 2-allyl-4, 5-methylenedioxyphenol by its MS, ^{1}H and ^{13}C-NMR data. The aglycone **3a** was synthesized and reported by Alexander *et. al.*[4], the melting point of which is identical to that of the reported value. Moreover, the rhamnose was determined to link to C-6' of the glucose moiety because the signal of C-6' appeared at δ_C 68.1. As a result of the above evidence, **3** was determined to be 2-allyl-4, 5-methylenedioxyphenol-1-*O*-α-L-rhamnopyranosyl-(16)-*O*-β-D-glucopyranoside.



3: R=rha
4: R=ara
5: R=api

Compounds **4** and **5**, together with three known sesquiterpenes, *i.e.*, pseudoanisatin, pseudomajucin and illicinolide A, were isolated from the acetone extract (50 g) of the fresh fruits by column chromatographies over Chromatorex ODS, Bondapak ODS and silica gel. Both **4** and **5** are white amorphous powders, showed $[M+Na]^+$ ion peaks at *m/z* 495 in the

positive FAB-MS. Their ^{1}H and ^{13}C-NMR spectra closely resembled those of **3**, especially the signals derived from the aglycone and glucopyranose residue are almost superimposable with those of **3**. This suggested that both **4** and **5** are 6'-glycosidated 2-allyl-4, 5-methylenedioxyphenol-1-*O*-β-D-glucopyranosides. Further detailed analyses of their ^{1}H and ^{13}C-NMR data revealed that an arabinofuranose and an apiofuranose moieties exist in the molecules of **4** and **5**, respectively. From the above results, **4** and **5** were established to be 2-allyl-4, 5-methylenedioxyphenol-1-*O*-α-L-arabinofuranosyl-(16)-*O*-β-D-glucopyranoside and 2-allyl-4, 5-methylenedioxyphenol-1-*O*-β-D-apiofuranosyl-(16)-*O*-β-D-glucopyranoside, respectively.

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GLYCOSIDES FROM *ERIGERON MULTIRADIATUS*

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Erigeron multiradiatus (Wall.) *Benth.*(Compositae),which grows in the southwest of China, is used in traditional Chinese medicine for promoting blood circulation. We report here the isolation and structure elucidation of five glycosides from *Erigeron multiradiatus.*Their structures were identified as 6'-O-caffeylerigeroside (I), apigenin-7-O-β-D-glucuronide (II), isoquercitrin (III), scutellarein-7-O-β-D-glucuronide (IV), plantagin (V) on the basis of physical and chemical properties and spectroscopic analysis.Compound III were found in *Genus Erigeron* for the first time.Compound I was novel.

Experimental

m.p.:uncorr.; NMR:200MHz, DMSO-d_6; IR:KBr; MS:70ev; UV:MeOH; C.C.:polyamide (100-120 mesh) and silica gel (14-40μ).

Plant material:Erigeron multiradiatus(Wall.) *Benth.*was collected at Ganzi, Sichuan province, China and identified by Prof. Zhang Hao, School of Pharmacy, West China University of Medical Sciences,where a voucher specimen is deposited.

Extraction and isolation: The air dried powdered whole plant (1680 g) was extracted (× 6) with 95% EtOH. The extract was evaporated to dryness under reduced pressure and dissolved in water. The aqueous solution was partitioned with $CHCl_3$, Et_2O, EtOAc and n-BuOH to give fractions A (6.8 g), B (5.6 g), C (11 g) and D (16.8 g). The fraction C was column-chromatographed on polyamide eluting with a gradient of H_2O-EtOH with increasing EtOH and on silica gel eluting with $CHCl_3$-MeOH-H_2O (7:2:1, lower layer) and gave I (30 mg), II (60 mg), III (50mg). The fraction D was column-chromatographed as described above to give I (20mg), IV (40mg),V(15mg).

Compound I: $C_{20}H_{20}O_{11}$ (M^+, Found: 436.0984, require: 436.1005), amorphous powder, m.p.: 154-156℃. UV_{max}^{MeOH}nm: 213, 252,331; +$AlCl_3$: 213, 261, 360; + $AlCl_3$/HCl: 213,251,330; $IR_{max}^{KBr}cm^{-1}$: 3500-3100, 1674, 1628, 1596, 1550, 1442; EIMS m/z(rel. int): 436 [M^+] (3), 163 (25), 162 (10), 112 (100); ^{1}HNMR δ(ppm): 8.17 (1H,s,H-2), 8.05 (1H,d,J=5.6Hz,H-6), 7.46 (1H,d,J=15.8Hz,H-7"), 6.97-7.04 (2H, m, H-2",6"), 6.77 (1H, d, J=7.8Hz, H-5"), 6.19(1H, d, J=5.6Hz, H-5), 6.24 (1H, d, J=15.8Hz, H-8"), 4.88 (1H, d, J=6.3Hz, H-1'); ^{13}CNMR δ(ppm):145.7(C-2), 148.8(C-3), 172.8(C-4), 116.4(C-5), 156.2 (C-6), 125.8(C-1"), 115.0(C-2"), 145.9(C-3"), 146.1(C-4"), 114.1(C-5"), 121.8(C-6"), 144.3(C-7"), 116.1(C-8"), 166.7(C-9"), 100.4 (C-1'), 74.3(C-2'), 76.5 (C-3'), 70.0(C-4'),73.4(C-5'),63.5(C-6'). On the basis of above data, the compound was identified as 6'-O-caffeylerigeroside.[1]

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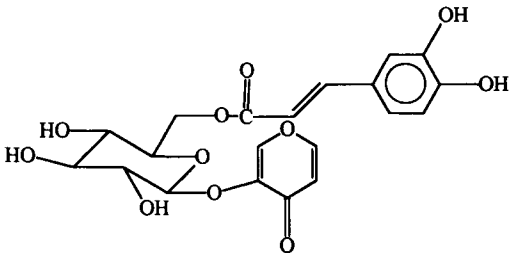

(I)

Compound II: pale yellow powder, m.p.>300℃. UV_{max}^{MeOH}nm: 268, 331; $IR_{max}^{KBr}cm^{-1}$: 3391, 1736, 1664, 1508, 1490; ^{1}HNMR δ(ppm): 12.98 (1H, s, C_5-OH), 10.43(1H, s, C_4'-OH), 7.94(2H, d, J=8.6Hz, H-2',6'), 6.94(2H, d, J=8.6Hz, H-3',5'), 6.84(1H, d, J=1.6Hz, H-8), 6.80(1H, s, H-3), 6.45(1H, d, J=1.6Hz, H-6), 5.31(1H, d, J=6.4Hz, H-1''); ^{13}CNMR δ(ppm):165.7(C-2), 102.8(C-3),183.6(C-4), 161.8(C-5), 99.8 (C-6), 162.3(C-7), 95.5(C-8), 157.6(C-9), 103.7(C-10), 121.7(C-1'), 129.3(C-2'), 116.7 (C-3'), 161.4(C-4'), 116.7(C-5'), 129.3(C-6'), 100.0(C-1''), 73.2(C-2''), 75.8(C-3''), 71.8(C-4''), 75.8(C-5''), 169.4(C-6''). The compound was identified as apigenin-7-O-β-D-glucuronide.[2]

Compound III: yellow powder, m.p.225-226℃. UV_{max}^{MeOH}nm: 257, 266sh, 303sh, 358; $IR_{max}^{KBr}cm^{-1}$: 3263, 1656, 1604, 1559, 1496; ^{1}HNMR δ(ppm): 12.64 (1H, s, C_5-OH), 7.59(2H, m, H-2',6'), 6.83(1H, d, J=8.6Hz, H-5'), 6.40(1H, s, H-8), 6.20(1H, s, H-6), 5.48(1H,d, J=6.4Hz, H-1''); ^{13}CNMR δ(ppm):156.5(C-2), 133.7(C-3), 177.7(C-4), 161.3(C-5), 98.9(C-6), 164.2(C-7), 93.9(C-8), 156.7(C-9), 104.3(C-10), 121.5(C-1'), 115.2(C-2'), 145.0(C-3'), 148.6(C-4'), 116.5(C-5'), 122.0(C-6'), 101.2(C-1''), 74.3(C-2''), 77.8(C-3''), 70.1(C-4''), 76.6(C-5''), 61.2(C-6''). The compound was identified as isoquercitrin.[3]

Compound IV: yellow powder, m.p.>300℃. UV_{max}^{MeOH}nm: 279, 331; $IR_{max}^{KBr}cm^{-1}$: 3379, 1734, 1661, 1606, 1494, 1445; ^{1}HNMR δ(ppm): 12.75 (1H,s,C_5-OH), 10.39(1H, s, C_6-OH), 8.62(1H, s, C_4'-OH), 7.94(2H, d, J=8.6Hz, H-2',6'), 6.97(2H, d, J=8.6Hz, H-3',5'), 6.91(1H, s, H-8), 6.83(1H, s, H-3), 5.52(1H, d, J=6.0Hz, H-1''); ^{13}CNMR δ(ppm):162.3(C-2), 102.2(C-3), 181.8(C-4), 146.5(C-5), 132.4 (C-6), 164.7(C-7), 94.7(C-8), 150.9(C-9), 105.6(C-10), 121.0(C-1'), 128.5(C-2'), 116.0(C-3'), 161.0(C-4'), 116.0(C-5'), 128.5(C-6'), 99.8(C-1''), 72.6(C-2''), 75.3(C-3''), 71.8(C-4''), 75.0 (C-5''), 170.6(C-6''). The compound was identified as scutellarein-7-O-β-D-glucuronide.[2]

Compound V: yellow powder, m.p.264-265℃. UV_{max}^{MeOH}nm: 282, 335; $IR_{max}^{KBr}cm^{-1}$: 3400, 1666, 1606, 1444; ^{1}HNMR δ(ppm): 12.72(1H,s,C_5-OH), 10.40(1H, s, C_6-OH), 8.56(1H, s, C_4'-OH), 7.94(2H, d, J=8.6Hz, H-2',6'), 6.97(2H, d, J=8.6Hz, H-3',5'), 6.91(1H, s, H-8), 6.83(1H, s, H-3), 5.00(1H, d, J=7.5Hz, H-1'') . Sugar was identified as glucose by acid hydrolysis. On the basis of above data, the compound was identified as plantagin.[2]

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STUDIES OF FLAVONE GLYCOSIDES OF *ERYSIMUM CHEIRANTHOIDES* L

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Key Word Index -- Flavone; *Erysimum cheiranthoides* L; Glycoside.

Abstract --Five flavone were isolated first from the seeds of *Erysinum cheiranthoides* L. Those compounds were characteried by spectral methods as kaempferol-3- rhamnosyl glucoside (Nicotiflorin), Quercetin 3 rutinoside (rutin), Quercetin 3-O-α-L-arabinopyranoside (Guaijaverin), isorhamnetin 3-O-α-L-arabinopyranoside (Distichin), Quercetin.

Erysimum cheiranthoides L is a two-year herb belonging to Erysimum genus, Cruciterae Family, mainly distributed over the areas of Northeast, China, Cardiac glycosides have been studied deeply, but we have not seen the study's report about flavone ingredient so far. When studied the cardiac glycoside, we found rich yield of Flavones in this plant, in yields of total flavones of 0.52%. On order to develop rich in natural resources, we utilize fully these chemical compositions . Flavones compounds have been extracted and isolated from the seed of E. cheiranthoides and then MeOH extract was chromatographed on silica gel and Polyclar column. We have obtained five flavone monomers. After idetificated by the chemical methods and spectral means of FAB-MS, ^{1}H-NMR, ^{13}C-NMR, IR, UV, the structure of five flavones is identified respectively, Kaemperol-3-O-rutinose(Nicotiflorin), Quercetin-3-O-rutinose(rutin), Quercetin-3-O-arabinose (Guaijaverin), Isorhamnetin-3-O-arabinose (Distichin), Quercetin. These compounds have been found first in E. cheiranthoides.

Experimental

All melting points were determined on a Yanagimoto mictomelting point appatatus and were uncorrected. Opitical totations were measured with a JASCD DIP-360 digital polarimeter (L=0.5). IR spectra were recorded on a Beckman-1300 spectrometer. Mass spectra were obtained with JEOL JMS DX-303 HF mass spectrometer. ^{1}H and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in DMSO-d_6 using TMS as an internal standard. TLC was performed on precoated silica gel (Qing dao) and detection was achieved by spraying 10% $AlCl_3$/EtOH following under ultraviolet.

Extraction and separation The seeds (2.5kg) of *E. cheiranthoides* were extracted with MeOH and the estract (125g) was suspended in H_2O, filtered and then extracted with ethyl acetate; the solvent was evaporated under reduced pressure. The ethyl acetate extract (38g) was chromatographed on a column of silica-gel with CHC_{13}-MeOH (10:1, 4:1) to give 1 (120mg), 2(80mg), 3(65mg), 4(32mg), 5 (60mg).

Table 1. ^{13}C-NMR spectral Data for Compounds 1, 2, 3 and 4 in DMSO-d_6

	1	2	3	4
C- 2	155.92	156.62	156.3	156.2
3	133.42	134.17	133.5	133.7
4	177.34	177.86	177.4	177.5
5	161.21	161.69	161.2	161.2
6	99.07	99.1	98.7	98.6
7	165.39	164.61	164.2	164.1
8	93.89	93.95	93.7	93.5
9	156.43	156.70	156.3	156.2
10	103.61	104.39	104.0	103.9
1'	122.17	122.65	120.9	120.9
2'	130.91	116.77	113.2	115.3
3'	115.37	145.48	149.5	144.9
4'	160.16	149.01	147.1	148.5
5'	115.37	115.77	115.2	115.7
6'	130.91	121.33	122.3	122.0
glc-1	100.15	100.45		
2	70.61	70.98		
3	70.72	72.36		
4	69.85	69.07		
5	71.90	75.01		
6	63.89	64.10		
rha-1	99.29	99.88		
2	68.69	69.07		
3	70.39	66.80		
4	70.66	70.98		
5	66.99	66.64		
6	17.38	17.97		
ara-1			101.4	101.6
2			71.6	71.8
3			70.7	71.0
4			66.0	66.6
5			64.2	65.0
				55.8 (OMe)

Nicotiflorin (1) A yellow powder ($CHCl_3$-CH_3OH), mp 180-182℃, IR cm^{-1}: 3400 (OH), 1690 (C=O), FD-MS m/z 594 (M^+), $C_{27}H_{30}O_{15}$. ^{1}H-NMR(DMSO-d_6): δ:0.96 (3H, d, J=6.23 Hz, rha-6-Me), 4.92 (1H, S, rha-1), 5.45 (1H, d, J=5.76 Hz, glc-1-H), 6.39 (1H, d, J=1.83 Hz, 6-H), 6.64 (1H, d, J=6.06 Hz, 8-H), 6.88 (2H, d, J=8.8 Hz, 3',5'-H), 8.05 (2H, d, J=8.8 Hz, 2',6'-H), 10.15 (1H,S,C_4'-OH), 10.87 (1H, S, C_7-OH), 12.57 (1H, S, C_5-OH). ^{13}C-NMR spectrum: see Table 1. spectrum datas identify with the datas of Nicotoflorin by report of references [1].

Rutin (2) A yellow powder (MeOH) , mp 188-190℃, $[\alpha]_D$-30℃IR cm^{-1}: 3400 (OH), 1690 (C=O). FD-MS m/z 610 (M^+) $C_{27}H_{30}O_{16}$. ^{1}H-NMR (DMSO-d_6) δ:0.98 (3H, d, J=5.86 Hz rha-6-Me), 4.93 (1H, S, rha-1), 5.41 (1H, J=4.76 Hz, glc-1), 6.19 (1H, d, J=1.83 Hz, 6-H), 6.40 (1H, d, J=2.2Hz, 8-H), 6.83 (1H, d, J=8.42 Hz, 5'-H), 7.47 (1H, J=1.83 Hz , 6'-H), 7.68 (1H,

d, d, J=2, 2, 8.4 Hz 2'-H), 9.25 (1H, S, $C_{3'}$-Oh), 9.75 (1H,S, $C_{4'}$-OH) 10.91 (1H, S, C_7-OH), 12.67 (1H,S, C_5-OH). ^{13}C-NMR spectrum: see Table 1. spectrum datas identify with the datas rutin by report of references[2].

Guaijaverin (3) A yellow powder (MeOH) , mp 239-256℃, $[a]_D$-120℃ (C=1.41, DMSO), IR cm-1: 3400 (OH), 1690 (C=O), Negative FAB-MS: m/z 433 $(M-H)^-$, 301-(433-ara)$^-$, $C_{20}H_{18}O_{11}$ ^{1}H-NMR (DMSO-d_6) δ: 3.25-3.78 (5H, m, ara H- 2~5), 4.57 (1H, brs,S, OH), 4.69 (1H, br, S, OH), 5.23 (1H, br, S, OH), 5.29 (1H, d, J=4.9 Hz, ara-1), 6.21 (1H, d, J=1.8 Hz, H-6), 6.41 (1H, d, J=1.8 Hz, H-8), 6.85 (1H, d, J=8.5 Hz, H-5'), 7.52 (1H, d, J=2.2 Hz, H-2'), 7.67 (1H, dd, J=2.2, 8.5 Hz, H-6'), 12.65 (1H, S, C_5-OH). ^{13}C-NMR spectrum: see Table 1. spectrum datas identify with the datas of Guaijaverin by report of references [3].

Distichin (4) A yellow powder (MeOH), mp 262-264℃ $[a]_D$-17℃ (C=0.54, MeOH), Negarive FAB-MS: m/z 447$[M-H]^-$, 315 $[447\text{-ara}]^-$ $C_{20}H_{20}O_{11}$, ^{1}H-NMR (DMSO-d_6) δ:3.25-3.75 (5H, m, ara H-2~5), 3.87 (3H, S, OMe), 4.58 (1H, d, J=3.6 Hz, OH), 4.80 (1H, d, J=4.9 Hz, OH), 5.27 (1H, d, J=4.3 Hz, OH), 5.34 (1H, d, J=5.5 Hz, araH-1), 6.21 (1H, d, J=1.8 Hz, H-6), 6.44 (1H, d, J=1.8 Hz, H-8), 6.91 (1H, d, J=8.0 Hz, H-5'), 7.58 (1H, dd, J=1.3, 8.0 Hz, H-6'), 7.91 (1H, br, S, H-2'), 12.61 (1H, S, C_5-OH). ^{13}C-NMR spectrum: see Table 1. spectrum datas identify with the datas of Guaijaverin by report of references[4].

Quercetin (5) A yellow crystalline, mp 312-316℃. The characteristic patterns of TLC chromatograms identify with Rf of Quercrtin.

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NEW FLAVONOID GLYCOSIDES FROM *SCUTELLARIA AMOENA*

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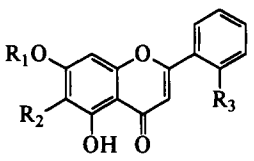
Key Word Index -- Labiatae; *Scutellaria amoena*; flavonoids.

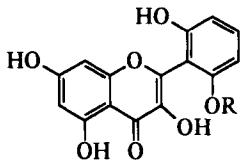
Abstract -- Three new flavonoid glycosides, 5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucuronide methyl ester, 5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucoside and 5,7,2',6'-tetrahyroxyflavonol 2'-O-β-D-glucoside, along with eleven known flavonoids and two known phenyl ethanoid glycosides, were isolated from the roots of *Scutellaria amoena*. Their structures were determined by spectral and chemical methods.

Introduction

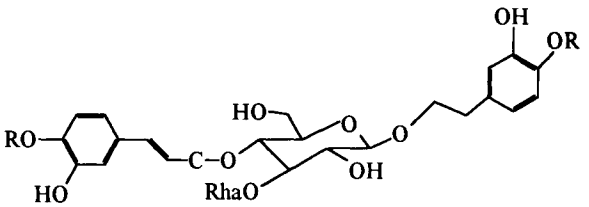
Scutellaria amoena C. H. Wright as a labiataeous herb is natived in the southwest of China. Its roots as one of the original material of "Huang-qin" has been used for the treatment of suppurative dermatitis, diarrhea and inflammatory diseases in traditional chinese medicine. Some chemical studies have been reported on this plant and several flavonoids were obtained[1-3]. As a part of our research work on important traditional medicinal herbs in Yunnan, we re-investigated the chemical constitutents of this plant, and sixteen phenolic compounds were obtained. Among them, compounds **1-3** are new flavonoid glycosides.



	R_1	R_2	R_3
1	glcUA^{6}—Me	OMe	OH
2	glc	OMe	OH
4	glcUA	OMe	OH
5	glcUA	OMe	H
6	glcU^{6}—Me	OMe	H
7	glcUA	H	H
8	glc	OH	H
9	glcUA	OH	H
10	H	OH	H
11	H	OMe	H
12	H	H	H
13	H	OMe	OH



	R
3	glc
14	H



	R
15	Me
16	H

* Author to whom correspondence should be addressed.

Table 1. ^{13}C NMR data of compound **1-7**

	1	**2**	**3**	**4**	**5**	**6**	**7**
2	161.96	161.85	146.63	161.74	163.54	163.73	163.56
3	108.77	108.75	130.03	108.57	104.95	104.95	105.37
4	182.39	182.39	177.20	182.32	182.32	182.39	181.94
5	152.25	152.25	161.01	152.25	152.31	152.53	161.50
6	132.46	132.46	97.60	132.62	132.78	132.66	99.65
7	155.98	156.52	163.42	156.40	156.64	156.09	163.15
8	93.89	94.30	93.35	94.18	94.24	94.05	94.77
9	152.43	152.25	158.13	152.25	152.31	152.19	157.02
10	105.87	105.69	103.95	105.76	106.50	106.12	105.37
1'	117.16	117.11	109.32	116.69	130.61	130.58	130.51
2'	156.67	156.67	156.48	157.40	126.34	126.35	126.35
3'	117.05	117.04	110.48	117.30	129.10	129.05	129.02
4'	132.88	132.84	131.16	132.62	132.05	132.07	132.00
5'	119.33	119.36	104.71	118.82	129.10	129.05	129.02
6'	128.43	128.43	157.20	128.11	126.34	126.35	126.35
Sugar-1	99.41	100.15	100.46	99.87	99.98	99.46	99.65
2	72.77	73.15	73.30	73.02	73.02	72.77	72.88
3	75.20	76.66	76.63	74.40	74.49	75.25	74.29
4	71.18	69.60	69.74	71.81	71.50	71.21	71.74
5	75.60	77.18	77.00	76.45	76.57	75.61	76.25
6	168.98	60.62	60.78	172.89	172.66	169.00	172.15
OMe	60.21	60.20		60.15	60.23	60.23	
OMe	51.81					51.85	

Table 2. ^{13}C NMR data of compound **8-14**

	8	**9**	**10**	**11**	**12**	**13**	**14**
2	163.61	163.54	162.85	163.15	163.00	161.38	146.62
3	104.76	104.65	104.40	104.57	104.96	108.53	130.95
4	182.61	182.50	182.01	182.13	181.56	182.27	177.21
5	146.61	146.71	146.95	152.45	157.32	152.62	160.82
6	130.71	130.79	130.91	131.43	104.96	131.29	97.65
7	149.30	149.17	149.79	157.44	164.22	157.39	163.37
8	94.38	94.17	93.93	99.30	93.77	94.19	93.19
9	151.68	151.57	153.55	152.63	161.44	152.62	157.09
10	106.16	106.12	104.23	104.29	103.94	104.12	103.64
1'	130.88	130.79	129.25	130.68	130.72	119.47	106.66
2'	126.43	126.33	126.19	126.26	125.91	156.64	157.23
3'	129.21	129.11	128.99	129.11	128.67	117.03	106.66
4'	132.11	131.96	131.68	131.84	130.72	132.76	130.95
5'	129.21	129.11	128.99	129.11	128.67	117.31	106.66
6'	126.43	126.33	126.19	126.26	125.91	128.50	157.23
Sugar-1	101.07	100.59					
2	73.25	72.91					
3	75.98	74.79					
4	69.84	71.81					
5	77.39	75.64					
6	60.78	171.74					
OMe						59.93	

Results and Discussion

MeOH extract of the roots of *S. amoena* was repeatedly chromatographed on Silica gel , Sephadex LH-20, MCI gel CHP 20P, RP-18 and TSK gel columns to yield sixteen compounds. Compounds **4-16** were identified by comparing their spectral data with those of reported values and authentic sample as eleven known flavonoids and two known phenyl ethanoid glycosides, i.e. 5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucuronide (**4**)[3], oroxylin A 7-O-β-D-glucuronide(**5**)[4] , oroxylin A 7-O-β-D-glucuronide methyl ester (**6**)[5], chrysin 7-O-β-D-glucuronide(**7**)[6], baicalein 7-O-β-D-glucoside (**8**)[7], baicalin (**9**)[4,6], baicalein (**10**)[4], oroxylin A (**11**)[2], chrysin (**12**)[6], 5,7,2'-trihydroxy-6-methoxyflavone (**13**)[3], 5,7,2',6'-tetrahydroxyflavonol (**14**)[3], 3-hydroxy-4-methoxyphenyl ethyl 1-O-α-L-rhamnosyl-(1→3)-β-D-(4-feruloyl) glucoside(**15**)[8], acteoside(**16**).

Compound **1** was obtained as pale yellow powder. Its molecular formula was analysed as $C_{23}H_{22}O_{12}$ from its negative FAB mass spectrum, in which there appeared a quasi-molecular ion peak at *m/z* 489[M-H]$^-$, in conjunction with its ^{13}C NMR spectrum. Combining with its chemical shifts of ^1H and ^{13}C NMR spectra, it could be considered as a flavone skeleton. Its ^1H NMR spectrum showed that there were six aromatic protons at δ 7.88(d, J=8.0Hz), 7.42(t, J=7.6Hz) and 7.00-7.10 (4H, m), two hydroxyl proton at δ 12.88 and 10.88, and two methyl groups at δ 3.75 (3H, s) and 3.65 (3H, s). The ^1H and ^{13}C NMR signals of **1** due to sugar moiety indicated the presence of a β-D-glucuronopyranosyl unit [δ_C 168.98 (COO), 99.41(anomeric C) and δ_H 5.51(d, J=5.6Hz, anomeric H)], and the signals for the aglycone were very similar to those of 2',5,7-trihydroxy-6-methoxyflavone[3], but **1** has one more methoxy group at δ_C 51.81 and δ_H 3.65(s). The HMBC spectrum of **1** showed that there are correlation signals between the protons of methoxyl (δ 3.75) and C-6 (δ 132.46), between the protons of methyl (δ 3.65) and the carbonyl carbon (δ 168.98) of glucuronide, and between anometic proton (δ 5.51) and C-7 (δ 155.98). This indicated that the carboxyl group of β-D-glucuronopyranosyl unit which was linked at C-7 position of aglycone was esterified by the methyl group at δ 3.65, and the another methyl group at δ 3.75 was attached at C-6 position of aglycone, as a methoxyl form. Therefore, compound **1** was assigned as 5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucuronide methyl ester.

Compound **2** was obtained as pale yellow powder. Its molecular formula was analysed by negative FAB mass spectrum, in which there appeared a quasi-molecular ion peak at *m/z* 461[M-H]$^-$. Comparing its ^1H and ^{13}C NMR spectra with those of **1** indicated that both compounds were very similar, but instead of a β-D-glucuronopyranosyl methyl ester attached at C-7 position in **1**, **2** has a β-D-glucopyranosyl unit attached at C-7 position of aglycone. This assignment was also comletely supported by the COLOC spectrum of **2** that there are correlaiton signals between the anomeric proton of β-D-glucopyranosyl unit at δ 5.12 and C-7 (δ 156.32) position of aglycone and between the methoxyl group at δ 3.77 and C-6 (δ 132.46) position of aglycon. Thus, **2** was determined as 5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucoside.

Compound **3** was obtained as brown powder. Its molecular formula was analysed as $C_{21}H_{20}O_{12}$ from its negative FAB mass spectrum, in which there appeared a quasi-molecular ion peak at *m/z* 463[M-H]$^-$, in conjunction with its ^{13}C NMR spectrum. Combining with its chemical shifts of ^1H and ^{13}C NMR spectra, it could be considered as a flavonoid compound. Its ^1H NMR spectrum showed a hydroxyl proton signal at δ 12.79 (C_5-OH), and five aromatic protons at δ 7.21(t, J=8.4Hz), 6.63(d, J=8.4Hz), 6.54(d, J=8.4Hz), 6.28(d, J=1.2Hz) and 6.14(d, J=1.2Hz). The latter two aromatic proton signals which were coupled *via* a ^4J coupling should be assigned as H-6 and H-8. The former three aromatic proton signals exhibited as an ABC-type coupling system, which indicated that they were at B-ring. The ^1H and ^{13}C NMR

signals of **3** due to sugar moiety indicated the presence of a β-glucopyranosyl unit [δ_C 100.46 (anomeric C) and δ_H 4.86(d, J=7.6Hz, anomeric H)]. Acid hydrolysis of **3** yielded **14** as its aglycone, which is identified with authentic sample. Thus, **3** should be a glucoside of **14**. The β-D-glucopyranosyl group should be attached at C-2' position of B-ring in **3**, according to the glycosylation effect in B-ring, which the chemical shift of C-1', 3' were shifted to downfield from 106.66 in **14** to 109.32 and 110.48 in **3**, C-5' was shifted to upfield from 106.66 in **14** to 104.71 in **3**. This elucidation was also supported by the results of comparing the ^1H and ^{13}C NMR signals of **3** with those of 5,7,2',6'-tetrahydroxy-flavone 2'-O-β-D-glycopyranoside[9] that both compound had a same B-ring substituence. Therefore, compound **3** was determined as 5,7,2',6'-tetrahydroxyflavanol 2'-O-β-D-glucoside.

Experimental

Genera. Mps were determined on a Kofler hot stage apparatus and are corrected by authentic sample of caffeine(237 ^{0}C). UV and IR were recorded on Shimadzu UV-210A and IR-450 spectrophotometers, in MeOH and KBr pellets, respectively. ^1H, ^{13}C and COLOC NMR spectras were measured on a Bruker AM-400 NMR spectrometer and HMBC NMR spectras on a Bruker AM-500 NMR spectrometer in DMSO-d_6 using TMS as internal standards. FAB and EI Mass spectra were obtained using a VG Autospec mass spectrometer. CC was carried out on silica gel (200-300 mesh), Sephadex LH-20, MCI-gel CHP 20P and TSK-gel Toyopearl HW-40F, Lichroprep RP_8 (40-63 μ, merck). TLC was conducted on precoated silica gel plates. Spots were detected by spraying with $FeCl_3$.

Extraction and isolation. Dried roots (18.0 kg) of *Scutellaria amoena* were extracted with MeOH under reflux. After removal of the solvent in vacuo, the residue was suspended in H_2O and then successively extracted with petroleum ether, $CHCl_3$, EtOAc and n-BuOH. After concentrated, it gave petroleum ether extract (49 g), $CHCl_3$ extract (64 g), EtOAc extract (54.5 g), n-BuOH extract (328 g) and H_2O layer fraction (600 g). $CHCl_3$ extract (40g) was chromatographed on silica gel column, eluting with different proportional solution of $CHCl_3$-MeOH, to give **11**(80mg), **12** (200mg), **13**(25mg). The EtOAc extract (54.5g) was repeatedly chromatographed on silica gel column with different proportional solution system of $CHCl_3$-MeOH, Sephadex LH-20 and MCI-gel CHP 20P column with different concentration of aq. MeOH to afford **3**(50mg), **4**(100mg), **10**(85mg) and **14** (40mg). The n-BuOH extract (50g) was repeatedly chromatographed on MCI-gel CHP 20P, Sephadex LH-20 and reverse phase column of RP_8, eluting with different proportional solution system of MeOH-H_2O to give **2** (100mg), **7** (25mg, **9** (40mg), **15** (45mg) and **16** (50mg),. The water layer fraction (600g) was chromatographed on macroporous resin D101, eluting with H_2O and MeOH. After concentrated, it gave MeOH fraction (150g). The MeOH fraction (18g) was chromatographed on MCI-gel CHP 20P, TSK gel and reverse phase column of RP_8, eluting with different proportional solution system of MeOH-H_2O to give **1** (200mg), **5** (150mg), **6** (40mg), **8** (40mg).

5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucuronide methyl ester (**1**). Pale Yellow powder. mp: 279-281 ^{0}C; [α]$_D^{25.8}$ -85.18^0 (pyridine; c 0.0026). UVλ_{max}nm (MeOH): 304.5, 274.5, 245.5. IR ν_{max} cm^{-1}: 3500-3100, 2900, 1645, 1600, 1450, 1375, 1275, 760. FAB-MS: *m/z* 489[M-H]$^-$, 299[M-glcUAMe]$^-$. ^1H NMR(DMSO-d_6): δ 12.88(C5-OH), 10.88, 7.88(d, J=8.0Hz, H-3'), 7.42(t, J=7.6Hz, H-4'), 7.00-7.10(m, H-5',6', 3, 8), 5.51(d, J=5.6Hz, anomeric H), 3.75(s, OMe), 3.65(s, OMe). ^{13}C NMR: see Table 1.

5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucoside (**2**). Yellow powder.[α]$_D^{26.0}$ -12.33^0 (pyridine; c 0.00223). FAB-MS: *m/z* 461[M-H]$^-$ (100). ^1H NMR(DMSO-d_6): δ 12.86 (C5-OH), 7.88(d, J=7.6Hz, H-3'), 7.41(m, H-4'), 7.11-6.98(m, H-3, 8, 3', 5'), 5.12(d, J=7.2Hz, anomeric H) 3.77(s, OMe). ^{13}C NMR: see Table 1.

5,7,2',6'-tetrahydroxyflavanol 2'-O-β-D-glucoside (**3**). Brown powder. mp:199-201 ^{0}C; $[\alpha]_D^{25.9}$-79.68^0 (pyridine; c 0.00251). UVλ_{max}nm(MeOH): 297, 270, 239, 210.5. IR ν_{max} cm^{-1}: 3500-3100, 2900, 1725, 1600. FAB-MS: *m/z* 463 $[M-H]^-$ (100). ^{1}H NMR (DMSO-d_6): δ 12.79(C5-OH), 7.21(t, J=8.4Hz, H-4'), 6.63(d, H- 5'), 6.54(d, J=8.4, H-3'), 6.28(d, J=1.2Hz, H-6), 6.14(d, J=1.2Hz, H-8), 4.86(d, J=7.6Hz, anomeric H). ^{13}C NMR: see Table 1.

Acid hydrolysis of **3**. A solution of **3** (3 mg) in 2N HCl/H_2O (5 ml) was heated at 98 ^{0}C for 40 minutes. From the reaction mixture, its aglycone was identified as 2',6',5,7-tetrahydroxyflavanol by comparing with an authentic sample on TLC (silica gel plates; solvent system: benzene-ethyl formate-formic acid (2:7:1); detected by spraying with $FeCl_3$. Rf = 0.6).

5,7,2'-trihydroxy-6-methoxyflavone 7-O-β-D-glucuronide (**4**). Pale Yellow crystals. FAB-MS: *m/z* 475$[M-H]^-$ (100). UVλ_{max}nm(MeOH): 335, 271, 212. IR ν_{max} cm^{-1}: 3400, 1650, 1600, 1440, 1340, 1280, 1240, 1180, 1080. ^{1}H NMR(DMSO-d_6):12.85(C5-OH), 7.77(d, J=8.0Hz, H-3'), 7.31(t, J=8.0Hz, H-4'), 7.10(s, H-3), 7.05(d, J=8.0Hz, H-6'), 6.95(s, H-8), 6.87(t, J=7.8Hz, H-5'), 5.17(d, J=6.4Hz, anomeric H), 3.75(s, OMe). ^{13}C NMR see Table 1.

oroxylin A-7-O-β-D-glucuronide (**5**). Yellow powder. FAB-MS: *m/z* 459$[M-H]^-$ (100). UVλ_{max}nm(MeOH): 287, 273, 245.5, 214, 204. IR ν_{max} cm^{-1}: 3500-3200, 1650, 1600, 1450, 1400, 1350, 1290. ^{1}H NMR(DMSO-d_6): 8.06(d, J=7.2Hz, H-2', 6'), 7.54-7.62(m, H-3', 4', 5'), 7.04(s, H-3), 7.02(s, H-8), 5.17(d, J=6.4Hz, anomeric), 3.75(OMe). ^{13}C NMR see Table 1.

oroxylin A-7-O-β-D-glucuronide methyl ester (**6**). Yellow powder. FAB-MS: *m/z* 473$[M-H]^-$ (100). UVλ_{max}nm(MeOH): 213.5, 242, 272.5, 309. IR ν_{max} cm^{-1}: 3380, 1730, 1655, 1610, 1580, 1075. ^{1}H NMR(DMSO-d_6): 12.82(C5-OH), 8.07(d, J=7.2Hz, H-2', 6'), 7.56-7.62(m, H-3', 4', 5'), 7.12(s, H-3), 7.04(s, H-8), 5.53(d, J=5.6Hz, anomeric H), 3.76(s, OMe), 3.65(OMe).

chrysin 7-O-β-D-glucuronide (**7**). Yellow powder. FAB-MS: *m/z* 429$[M-H]^-$ (100). IR ν_{max} cm^{-1}: 3500-3100, 1650, 1600, 1475, 1450, 1400, 1175, 1050. ^{1}H NMR(DMSO-d_6): 8.08(d, J=8.0Hz, H-2', 6'), 7.6-7.50(m, H-3', 4', 5'), 7.04(s, H-3), 6.67(s, H-6), 6.46(s, H-8), 5.11(d, J=7.2Hz, anomeric H). ^{13}C NMR see Table 1.

Baicalein 7-O-β-D-glucoside (**8**). Yellow powder. FAB-MS: *m/z* 431$[M-H]^-$ (100). UVλ_{max}nm(MeOH): 314.5, 277.5, 242.5, 215. IR ν_{max} cm^{-1}: 3500-3100, 1650, 1600, 1475, 1445, 1400, 1350, 1300, 1230, 1175. ^{1}H NMR(DMSO-d_6): 8.04(d, J=8.0Hz, H-2', 6'), 7.55-7.60(m, H-3', 4', 5'), 7.04(s, H-3), 6.96(s, H-8). ^{13}C NMR see Table 2.

baicalin (**9**). Yellow and green powder. FAB-MS: *m/z* 445$[M-H]^-$ (100). UVλ_{max}nm (MeOH): 312.5, 278.5, 243.5, 215, 203. IR ν_{max} cm^{-1}: 3500-3100, 1700, 1600, 1475, 1350, 1075. ^{1}H NMR (DMSO-d_6): 8.04(d, J=8.0Hz, H-2', 6'), 7.59-7.50(m, H-3', 4', 5'), 7.04(s, H-3), 6.97(s, H-8). ^{13}C NMR see Table 2.

baicalein (**10**). Brown and green powder. EI-MS: *m/z* 270$[M]^+$ (100). UVλ_{max}nm (MeOH): 324.5, 270, 215, 204. IR ν_{max} cm^{-1}: 3360, 1640,1605, 1585, 1525, 1300, 1220. ^{1}H NMR (DMSO-d_6): 12.65(C5-OH), 8.03(d, J=7.8Hz, H-2', 6'), 7.570-7.52(m, H-3', 4', 5'), 6.90(s, H-3), 6.62(s, H-8). ^{13}C NMR see Table 2.

oroxylin A (**11**). Yellow powder. EI-MS: *m/z* 284$[M]^+$ (100). ^{1}H NMR(DMSO-d_6): 12.91(C5-OH), 8.04(d, J=7.2Hz, H-2', 6'), 7.60-5.52(m, H-3', 4', 5'), 6.94(s, H-3), 6.61(s, H-8), 3.71(s, OMe). ^{13}C NMR see Table 2.

chrysin (**12**). Yellow powder. EI-MS: *m/z* 254$[M]^+$ (100). ^{1}H NMR(DMSO-d_6): 13.57(C5-OH), 7.93(d, J=7.2Hz, H-2', 6'), 7.45(m, H-3', 4', 5'), 7.19(s, H-3), 6.82(d, J=2.4Hz, H-6), 6.74(d, J=2.4Hz, H-8). ^{13}C NMR see Table 2.

5,7,2'-trihydroxy-6-methoxyflavone (**13**). Yellow powder. EI-MS: *m/z* 300$[M]^+$ (100). UVλ_{max}nm(MeOH): 337, 270.5, 212. IR ν_{max} cm^{-1}: 3450, 3100-2975, 1650, 1600, 1550, 1425,

1350, 1235, 1075. ^{1}H NMR(DMSO-d_6): 12.99(C5-OH), 7.85(d, J=7.6Hz, H-3'), 7.38(m, H-4'), 7.04(m, H-3, 6'), 6.97(m, H-5'), 6.57(s, H-8), 3.69(s, OMe). ^{13}C NMR see Table 2.

5,7,2',6'-tetrahydroxyflavanol (**14**). Brown powder. FAB-MS: *m/z* 301[M-H]$^-$ (100). ^{1}H NMR(DMSO-d_6): 12.64(C5-OH), 7.06(t, J=8.0Hz, H-4'), 6.34(m, H-3', 5'), 6.27(s, H-6), 6.16(s, H-8). ^{13}C NMR see Table 2.

3-hydroxy-4-methoxyphenyl-ethyl 1-O-α-L-rhamnosyl-(1-3)-β-D-(4-feruloyl) glucoside (**15**). Yellow powder. FAB-MS: *m/z* 651[M-H]$^-$ (100). UVλ_{max}nm(MeOH): 328.5, 295, 285.5, 213, 204. IR ν_{max} cm^{-1}: 3500-3000, 1600. ^{13}C NMR: 131.11(C-1), 112.45(C-2), 144.98(C-3), 146.06(C-4), 116.27(C-5), 119.35(C-6), 69.97(C-α), 34.90(C-β), 125.52 (C-1'), 111.22(C-2'), 147.92(C-3'), 149.48(C-4'), 115.55(C-5'), 123.02(C-6'), 146.29 (C-α'), 114.08(C-β'), 55.75(OMe), 55.65(OMe), glu.: 101.12(C-1), 74.54(C-2), 79.09 (C-3), 69.17(C-4), 74.54(C-5), 60.74(C-6), rha.: 102.30(C-1), 7.49(C-1), 70.40(C-2), 71.70(C-3), 68.69(C-5), 17.98(C-6). ^{1}H NMR(DMSO-d_6): 7.52(d, J=15.84Hz, H-Ar-C=CH), 6.62-7.30(m, Ar-H), 6.43(d, J=15.84Hz, Ar-CH=C), 3.79(s, OMe), 3.76(OMe), 0.96(d, J=6.0Hz, Me).

acteoside (**16**). Pale yellow powder. FAB- MS: *m/z* 623[M-H]$^-$ (100). UVλ_{max}nm (MeOH): 333, 296.5, 289, 242, 219. IR ν_{max} cm^{-1}: 3500-3000, 1975, 1680, 1600, 1500.

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SEPARATION OF THREE MAJOR ISOFLAVONOIDS FROM THE ROOT OF *PUERARIA LOBATA* BY HIGH SPEED CENTRIFUGAL PARTITION CHROMATOGRAPHY

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Key Word Index-*Pueraria lobata*; isoflavonoids; HSCPC

Abstract--High Speed Centrifugal Partition Chromatography (HSCPC) was applied for the first time in the preparative scale separation of isoflavonoids from *Pueraria* lobata Ohwi. The three major isoflavonoids in the root of *Pueraria lobata*, puerarin, 3'-methoxypuerar in and daidzin were isolated in one step, using n-butanol-t-butylmethylether-acetonitrile-water solvent system.

Introduction

In recent years there has been a renaissance of liquid-liquid chromatographic (LLC) method, especially in the separation of natural products. These developments mainly involved the emergence of different counter-current chromatographic techniques. Further, the advent of High-Speed Centrifugal Partition Chromatography led to considerable improvements in separation efficiency, analysis time and handling of the apparatus compared with eg. droplet counter-current chromatography.

The root of *Pueraria lobata* is a very important Chinese traditional drug, It has been used as an antipyretic and spasmolytic agent [1]. The major bioactive constituent in *Pueraria lobata* is isoflavonoids. In this paper we report the preparative separation of isoflavonoids as pure compounds from the crude plant extract of *Pueraria Lobata*.

Material and Methods

Extraction

The dry roots of *Pueraria lobata* Ohwi (100g), collected in China, were extracted with water under heat. The extract was concentrated and partitioned between n-butanol and water. Evaporation of the organic layer gave a residue (15g).

Fractionation by HSCPC

The separation was carried out with a multi-layer coil planet centrifugal called the Ito multi-layer coil separator-extractor (obtained from CP. C. Inc., Potomac, MD USA), equipped with a 145m ×1.6mm I.D. coil and with total column capacity of ca. 290ml. The solvent system is n-Butanol-t-butyl-methylether-acetonitrile-water (2:2:1:5, stationary phase: upper phase, mobile phase: lower phase). The column was revolved at 800rpm and the volume of the mobile phase in column was around 130ml, corresponding to a retention of 55% stationary phase. Solvent was delivered by a pump. After filling with the stationary phase, the sample was injected, mobile phase was applied at 2ml/min, UV detection at 254nm was performed, using a Uvicord type S II UV detector. Eluates were collected in an LKB ultroral 7000 fraction collector.

Identification of isoflavonoids

Composition of the fractions was determined by HPLC on ODS column (Rainin) using a

mixture of 20% MeOH/water as the mobile phase. The pure isolated products were compared by HPLC. TLC, mp with authentic samples of puerarin, 3'-methoxypuerar in and daidzin. They were also analyzed by ^{1}HMNR and ^{13}CNMR spectroscopy in DMSO-d_6. Spectra were recorded at 250Mhz on Bruker ARX 250 and compared with data found in the literature [1].

Reagents

All chemicals were analytical grade and HPLC grade

Results and Discussion

Figure 1 shows the HSCPC chromatogram, recorded at 254nm obtained after injecting 500mg of the crude plant extract (in 13ml mobile phase and 7ml stationary phase). The 12ml fractions were collected and further analyzed by HPLC. Identical fraction 23~25 were combined and evaporated to dryness, which were recrystallized from MeOH and gave 10mg colorless needles, mp 214~216^{0}C (3'-methoxypuerarin 2% of the extract). Similarly, the residue of fraction 28~33 were recrystallized from 80% AcOH to give 40mg colorless prisms, mp 184~185^{0}C (puerarin 8% of the extract). The residue of fraction 41~48 were recrystallized from MeOH to give 12mg colorless needles, mp 215~2170C (daidzin, 2.4% of the extract). Thus we obtained 62mg of pure isoflavonoids, which corresponds to 12.4% of the starting material. The remains (438mg) was a mixture of minor flavonoids, sugars and tannins.

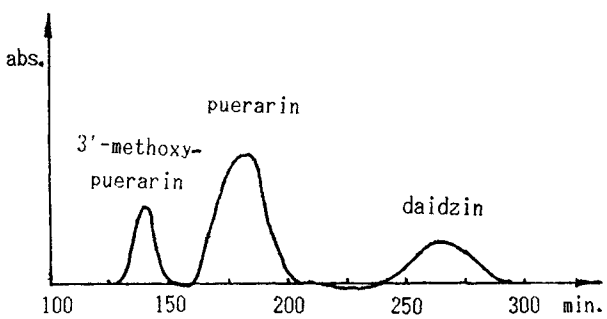


Fig. 1. preparative separation of isoflavonoids form crude plant extract of *P. labata*

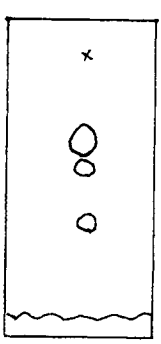
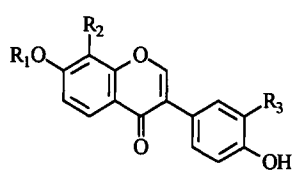


Fig. 2. Silica gel TLC
CH_2Cl_2-MeOH-H_2O 40:12:2



3'-methoxypuerarin R_2=-glc, R_3=OMe R_1=H

puerarin R_2=-glc R_1=R_3=H

daidzin R_1=-glc R_2=R_3=H

The observed elution sequence is in conformity with the reverse phase elution mode which has been used, 3'-Methoxy puerarin, which has a methoxy group in 3' position, is more polar than puerarin, and is eluted first. Daidzin, which does not have a hydroxy group in 7-position, is less polar than puerarin, and is eluted thirdly, But it is very interesting that Rf value of 3'-methoxypuerar in is larger than Rf value of puerarin on TLC plate of partition chromatography (Fig2). Due to the intramolecular hydrogen bond formed between 3'-methoxy and 4'-hydroxy which abated the ability of forming intermolecular hydrogen bond between 4'-hydroxy and water, the Rf value was enlarged.

Conclusion

Centrifugal partition chromatography is an effective tool for the separation and purification of polar and unstable bioactive molecules. It expends a lot of time when these kinds of compounds are isolated by conventional column chromatography because of strong adsorption. The three major isoflavonoids in the root of Pueraria lobata have been isolated in a baseline state and with preparative scale.

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NEW GLYCOSIDES FROM *HERNIARIA FONTANESII*

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Abstract-- *Herniaria fontanesii* Gay (caryophyllaceae) is widespread in the Mediterranean area. In the traditional pharmacopoeia of Morocco, the aerial parts of this plant are used for the treatment of lithiasis. We have studied the title plant and now we report the isolation and identification of tree flavonoids and four oleanane saponins. The mixture of glycosides was isolated from the methanol extract of the aerial parts of *H.fontanesii* and separated by multiple chromatographic steps. The structure of the pure glycosids was elucidated by means of mass spectrometry (FAB-MS), NMR technics (^1H, ^{13}C, 2D homo and heteronuclear COSY) and chemical analysis (methanolysis, methylation, alkaline hydrolysis). The flavonoids isolated are: (-) catechine : 1 ; 3-O-robinobioside Isorhamnetine: 2 ; and a new one : 3''' feruloyl-3-O-robinobioside Isorhamnetine: 3.

The four saponins are new oleanane saponins. Their structures were established as: Herniaria saponin A : 28-o-{[a-L-rhamnopyranosyl (1->2) [α-L-rhamnopyranosyl (1->3)]-β-D-xylopyranosyl (1->2)-β-D-fucopyranosyl} ester of 3-O-β-D-glucopyranosyluronic (1->4) α-L- rhamnopyranosyl] 2 β, 3 β-dihydroxy olean12-en 23, 28-dioic

Herniaria saponin B : 28-o-[α-L-rhamnopyranosyl (1->2)[α-L-rhamnopyranosyl(1->3)]-β-D-xylopyranosyl(1->2)-β-D-fucopyranosyl] ester of 3-O-[β-D-glucopyranosyluronic (1->4) α-L- rhamnopyranosyl] 2 β, 3 β, 16- α-trihydroxy olean-12-en 23, 28-dioic

Herniaria saponin C : 28-o-[α-L-rhamnopyranosyl (1->2) [α-L-rhamnopyranosyl (1->3)]-β-D-4-acetoxy fucopyranosyl] ester of 3-O-[α-L-rhamnopyranosyl(1->2)-β-D-glucopyranosyl uronic] 2 β, 3 β, 16- α-trihydroxy olean12-en 23, 28-dioic acid.

Herniaria saponin D : 28-o-{ a-L-rhamnopyranosyl (1->2)-[α-L-rhamnopyranosyl (1->3] β-D-fucopyranosyl} ester of 3-O-[α-L-rhamnopyranosyl(1->2)-β-D-glucopyranosyluronic] 2 β, 3 β, 16- α trihydroxy olean12-en 23, 28-dioic acid

Introduction

Stimulated by the use in moroccan folk medicine of the aerial parts of *Herniaria fontanesii* Gay (Caryophyllaceae) for the treatement of lithiasis or as a diuretic, we have undertaken the phytochemical study of this mediterranean endemic species. This led us to isolate three flavonoids [1] and four oleanane saponins [2-4] whose structures are described in this paper.

Results and Discussion

The dried aerial parts of *Herniaria fontanesii* were extracted with CH_2Cl_2 then with MeOH. The MeOH extract was suspended in water and extracted successively by CH_2Cl_2, AcOEt and n-BuOH. The AcOET extract was purified by a combination of flash

chromatography and preparative TLC on silica gel to give flavonoides 1-3. The n-BuOH extract was dissolved in MeOH and the saponins was precipitated with Et_20. The crude saponins was separated by a combination of gel filtration on Sephadex LH20 and HLPC to give Herniaria saponin A-D.

The structure elucidation was achieved using chemical methods. FAB-MS and NMR technics (^1H, ^{13}C, DEPT, homo and heteronuclear COSY). The carbohydrate composition of these glycosides are given in Table 1. They was identified by GC after methanolysis. The Alkaline hydrolysis under reductive conditions of crude saponins allowed the isolation of oligosaccharide alditol residue. Methanolysis of this later revealed the presence of fucitol and rhamnose from Herniaria saponin C and D. In Herniaria saponin A and B we identified an additional xylose residue. The inter glycosidic linkage was determined by methylation analysis. The molecular formula was deduced from the negatif mode FAB-MS this results are summerised in table 2. The ^1H and ^{13}C NMR data (Table 3 and 4) was determined using ^1H, ^{13}C, DEPT, 2D homo and heteronuclear COSY, HMQC, HMBC and HOHAHA.

Table 1 : Carbohydrate composition of coumpounds **1-7**

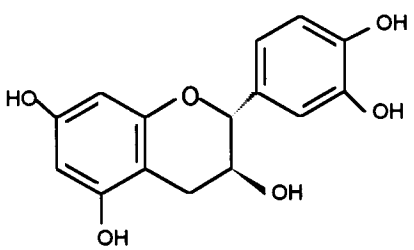
	Gal	Rha	GlcA	Fuc	Xyl
1	0	0	0	0	0
2	1	1	0	0	0
3	1	1	0	0	0
4	0	3	1	1	1
5	0	3	1	1	1
6	0	3	1	1	1
7	0	3	1	1	0

Table 2 : Molecular weight and Formula of **1-7**

	Molecular Weight	Formula
1	290	$C_{15}H_{14}O_6$
2	624	$C_{28}H_{32}O_{16}$
3	800	$C_{38}H_{40}O_{19}$
4	1394	$C_{65}H_{102}O_{32}$
5	1410	$C_{65}H_{102}O_{33}$
6	1319	$C_{62}H_{96}O_{30}$
7	1278	$C_{60}H_{94}O_{29}$

Table 3 : ^{1}H NMR data for **1-7**

	1	**2**	**3**		**4**	**5**	**6**	**7**
Aglycon				Aglycon				
2	4.61	-	-	2	4.27	4.51	4.50	4.28
3	4.02	-	-	3	4.06	4.28	4.50	4.10
4	2.55	-	-	12	5.26	5.49	5.55	5.12
	2.89	-	-	16	-	4.63	4.60	4.42
6	5.90	6.21	6.23	18	2.82	3.10	3.15	2.95
8	5.97	6.41	6.48	19	1.33	1.26	1.25	1.05
2'	6.89	8.03	8.10		1.73	2.50	2.48	2.31
5'	6.80	6.91	7.00	COCH3	-		2.35	
6'	6.80	7.60	7.65					
OCH3		3.97	3.98	3-O-sugar				
				GlcA	4.40	4.47	4.60	4.47
Gal-1		5.23	5.32	Rha	5.45	5.72	5.23	5.09
Rha-1		4.53	4.65					
Fer-2			7.29	28-O-sugar				
Fer-5			6.9	Fuc	5.34	5.46	5.60	5.38
Fer-6 Fer-7			7.18	Xyl	4.42	4.52	-	-
Fer-8			7.75	Rha	5.15	5.31	5.35	5.19
OCH3			6.45	Rha	5.16	5.40	5.35	5.19
			4.06					



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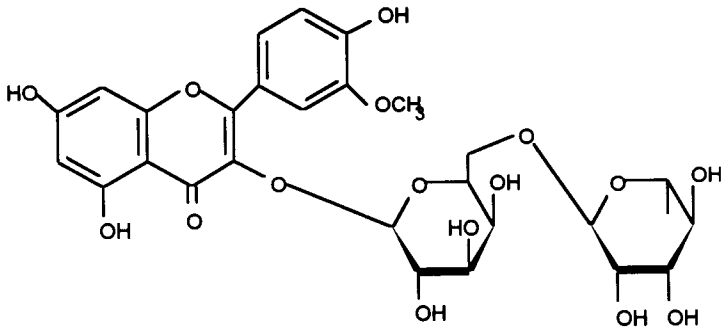

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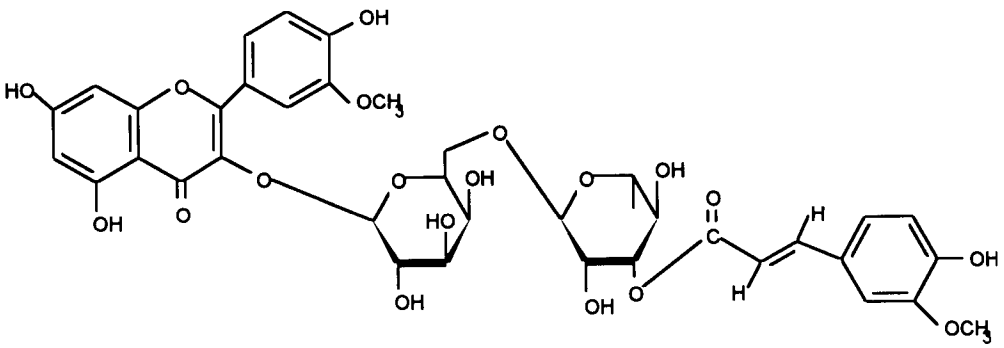

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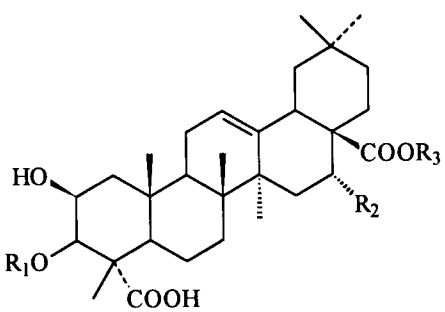

	R1	R2	R3
4 : Herniaria saponin A	GlcA (1-4)Rha	H	Rha(1-2)[Rha(1-3)]Xyl(1-2)Fuc
5 : Herniaria saponin B	GlcA (1-4)Rha	OH	Rha(1-2)[Rha(1-3)]Xyl(1-2)Fuc
6: Herniaria saponin C	Rha (1-2)GlcA	OH	Rha(1-2)[Rha(1-3)]-4-Ac-Fuc
7 : Herniaria saponin D	Rha (1-2)GlcA	OH	Rha(1-2)[Rha(1-3)]Fuc

Table 4 : ^{13}C NMR Data for **1-6** in CD_3OD

	1	**2**	**3**		**4**	**5**	**6**
Aglyco				Aglycon			
n	82.9	158.9	158.7	1	46.1	45.5	44.9
2	68.9	135.2	135.4	2	71.3	72.0	71.7
3	28.5	179.6	179.5	3	88.1	88.7	86.6
4	156.7	163.1	163.3	4	54.8	54.7	53.1
5	95.6	99.9	99.9	5	54.6	53.4	53.1
6	156.3	166.1	166.1	6	23.3	22.1	21.7
7	96.4	94.8	94.9	7	37.2	37.0	36.1
8	157.0	158.6	158.5	8	42.5	41.4	41.3
9	100.9	105.7	105.5	9	49.3	48.8	48.9
10	132.3	123.0	123.8	10	38.8	37.9	37.4
1'	115.4	114.6	114.6	11	25.2	25.0	24.7
2'	146.3	148.5	148.4	12	125.1	123.7	123.3
3'	146.3	150.9	150.8	13	146.1	144.9	144.7
4'	116.2	116.0	116.0	14	44.7	43.4	43.0
5'	120.1	123.8	124.0	15	30.8	31.6	31.3
6'		57.0	56.9	16	26.1	76.7	75.5
OCH3				17	49.6	49.2	49.8
		104.4	104.9	18	44.5	42.7	42.2
Gal-1		73.1	73.1	19	48.7	48.7	48.2
Gal-2		72.1	75.3	20	33.0	32.6	31.5
Gal-3		70.1	69.9	21	36.4	37.0	36.5
Gal-4		75.6	75.3	22	34.5	33.8	33.4
Gal-5		67.5	67.3	23	183.2	186.4	181.6
Gal-6		101.9	101.8	24	15.1	15.2	13.6
Rha-1		75.1	73.1	25	18.7	17.7	17.4
Rha-2		72.3	75.0	26	19.1	18.0	17.9
Rha-3		73.9	71.3	27	27.6	27.2	27.1
Rha-4		69.8	69.9	28	179.6	177.2	172.2
Rha-5		18.0	18.0	29	34.9	34.2	33.9
Rha-6			127.8	30	25.4	25.0	25.2
Fer-1			111.8	COCH3	-	-	172.6/20.
Fer-2			149.3				8
Fer-3			150.6	3-O-sugar			
Fer-4			116.5	GlcA-1	109.1	108.1	
Fer-5			122.7	GlcA-2	78.2	77.2	105.2
Fer-6			146.9	GlcA-3	75.5	74.4	80.9
Fer-7			115.7	GlcA-4	75.1	74.4	75.4
Fer-8			168.6	GlcA-5	75.5	74.0	74.9
Fer-9			56.3	GlcA-6	179.6	177.4	76.4
OCH3				Rha-1	102.7	100.9	177.4
				Rha-2	73.4	71.9	104.7
				Rha-3	73.7	72.5	73.6
				Rha-4	85.9	84.5	72.1
				Rha-5	70.0	68.8	73.6
				Rha-6	19.7	18.5	72.1
							17.3

28-O-sugar			
Fuc-1	96.6	95.3	
Fuc-2	73.8	72.6	95.3
Fuc-3	78.4	77.8	74.0
Fuc-4	73.2	72.6	82.4
Fuc-5	73.7	72.5	73.6
Fuc-6	17.9	16.8	16.8
Xyl-1	106.8	104.9	-
Xyl-2	78.2	76.7	-
Xyl-3	86.6	85.5	-
Xyl-4	71.1	70.6	-
Xyl-5	68.6	67.5	-
Rha-1	103.9	102.9	102.5
Rha-2	73.8	72.5	72.1
Rha-3	74.1	72.6	72.1
Rha-4	75.5	74.2	74.0
Rha-5	71.3	69.7	68.9
Rha-6	19.3	18.2	18.4
Rha-1	103.7	102.2	102.5
Rha-2	113.6	72.5	72.1
Rha-3	73.8	72.6	72.1
Rha-4	75.5	74.4	73.7
Rha-5	71.5	69.9	70.9
Rha-6	19.3	18.2	17.3

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XANTHONE GLYCOSIDES IN GENTIANCEAE OF QINGHAI-TIBET PLATEAU

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Key Word Index---Gentianaceae; Xanthone glycosides; Hepatitis; Cholecystitis; Tibetan medicine.

Abstract Most species of *Gentianaceae* in Qinghai-Tibet Plateau are traditional Tibetan medicines which were used for stabilizing liver functions and improving the gallbladder. They were used for curing hepatitis, cholecystitis and other diseases. We have studied the chemical composition of 12 species of Gentianaceae and extracted 17 xanthone glycosides. Nine of them are new natural products. Pharmacological studies showed that xanthone C-glycosides is beneficial to the gallbladder and that xanthone O-glycosides is the effective component in curing hepatitis.

Introduction

In 1801, Hentry et al. extracted the first natural 1,7-dihydroxy-3- methoxyxanthone from *Gentiana lutea* of Gentianaceae. Up to now the review on compounds of Xanthone glycosides is available only from Hosstettmann et al. (1977). At this period, only 24 xanthone O-glycosides and 5 xanthone C- glycosides were found. By now more than 120 xanthone glycosides have been found in various plants, including more than 20 xanthone C-glycosides. xanthone glycosides compounds are proved to have extensive physiological functions. They stimulate or suppress the central nervous system(CNS),improve urine secretion and strengthen heart function, and so on. In recent years, the discoveries showed that they are biologically active in suppressing platelet coagulation ATP release, activity of angiotensin and lowering blood sugar, resisting tumors enhancing immunization and so on. Most xanthone glycosides are distributed in the plants of Gentianaceae: *Gentiana, Swertia, Halenia*. xanthone glycosides have been concerned increasingly along with the studies of their chemical composition and the recognition of their importance in plant taxonomy.

Most Gentianaceae in Qinghai-Tibet Plateau (*Gentiana, Swertia, Halenia, Comastoma*) are traditional Tibetan medicines[1]. "Zangyinchen" or "Dida" is called by tibetan doctor (in Tibetan medicine).They growth mainly along riverside and in alpine meadow(at altitudes of 2,000 to 4,800 meters).The whole plant is used for medicines. It tastes bitter, has a cold nature. Being used for detoxification and fever relief, it is proved to be beneficial to the liver and gallbladder. Local Tibetan doctors have been used it to cure influenza, hepatitis, cholecystitis and other related diseases. In order to explore and utilize local herbal medicine resources, we have studied the chemical composition of 19 Gentianaceae plants. 17 xanthone glycosides were extracted from 12 Gentianaceae species. 9 of them are new natural compounds. The 12 species of Gentianaceae are *Swertia mussotii* Franch.(S.mus.)[2], *Swertia franchetiana* H. Smith(S.fra.)[3], *Swertia przewalskii* Pissiauk.(S.prz.) [4], *Swertia erythrosticta* Maxim.(S.ery.)[5], *Swertia verticillifolia* T. N. Ho et S. W. Liu(S.ver.)[6], *Comastoma pulmonarium* (Turcz.) Toyohuni (C.pul.) [7], *Comastoma falcatum* (Turcz.) Toyokuni (C.fal.), *Comastoma pedunculatum* (Royle ex D. Don) Holub (C.ped.)[8],*Gentianopsis paludosa* var. *ovato-deltoidea* (Burk.) Ma(G.pal.)[9], *Gentianopsis barbata* var. *stennocalyx* H. W. Li ex T.N. Ho(G.bar) [10], *Halenia ellipitica* D. Don(H.ell.)[11], *Lomatogonium rotayum* (L.) Fries ex Num(L.rot.)[12]. The xanthone glycosides in 12 species of Gentianaceae are showed in Table 1.

Table 1. Naturally occurring xanthone glycosides

xanthone glycosides No.	Natural sources S. mus.	S. fra.	S. prz	S. ery.	S. ver.	C. pul.	C. fal.	C. ped.	G. pal.	G. bar.	H. ell.	L. rot.	References
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-3,5-dimethoxyxanthone*		+						+					[3, 8]
1-O-β-D-glucopyranosyl-3,8-dihydroxy-7-methoxyxanthone*					+	+		+					[6, 7, 8]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-2,3,5,7-tetramethoxyxanthone*											+		[11]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-2,3,5-trimethoxyxanthone*											+		[11]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-2,3,4,5-tetramethoxyxanthone*											+		[11]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-7,8-dihydroxy-3-methoxyxanthone			+							+			[4, 10]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-8-hydroxy-3,7-dimethoxyxanthone			+				+	+					[4, 8]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-7-hydroxy-3,8-dimethoxyxanthone									+	+			[9, 10]
1-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-3,7,8-trimethoxyxanthone								+		+			[8, 10]
3-O-β-D-glucopyranosyl-1,8-dihydroxy-5-methoxyxanthone*	+												[2]
7-O-β-D-xylopyranosyl-1,8-dihydroxy-3-methoxyxanthone*	+												[2]
7-O-[α-L-rhamnopyrannosyl-(1-2)-β-D-xylopyranosyl]-1,8-dihydroxy-3-methoxyxanthone*	+												[2]
8-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-1,3,5-trimethoxyxanthone*												+	[12]
8-O-[β-D-xylopyranosyl-(1-6)-β-D-glucopyranosyl]-1,7-dihydroxy-3-methoxyxanthone	+		+										[2, 4]
8-O-β-D-glucopyranosyl-1,5-dihydroxy-3-methoxyxanthone				+	+								[5, 6]
8-O-β-D-glucopyranosyl-1,3,5-trihydroxyxanthone	+			+	+								[2, 5, 6]
2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone	+	+											[2, 3]

+ = occurring * = new natural products_

Results and Discussion

One kind of xanthone C-glycosides was separated from the 17 xanthone glycosides, the linking position of glycosyl and glycoproteins is 2. In the 16 xanthone O- glycosides, 1,8-followed by 3,7- are the most often glycosids position. By studying the chemical composition of Gentianaceae plants of Qinghai-Tibet Plateau, we have developed 4 new medicines: 1) Huanmao toblet(*Halenia ellipitica*) for curing hepatitis B; 2) Zangyinchen tablet (*Swertia mussotii, Swertia franchetiana*)for curing hepatitis;3) Zangyinchen injection(*Swertia mussotii*) for curing acute hepatitis; 4) Bianlai medicament (*Gentianopsis paludosa*) for curing children diarrhoea.

Pharmacological experiments showed that anti-hepatitis activity of xanthones in free state was not significant. Although oleanolic acid is an effective component of *Halenia ellipitica*, its content in water soluble part is minute. It can not be suggested that the main active component is in water soluble part. Pharmacological experiments indicated that the water soluble part was more effective in anti-hepatitis. It suggested that there must be other type(s) of active components in the water soluble part. For this reason we conducted further researches on the chemical composition of the water soluble part. Using chromatographed on silica gel column method, we obtained 3 new natural xanthone diglycosides:1-0-primeverosyl-2,3,5,7- tetramethoxyxanthone(I), 1-0- primeverosyl-2,3,5-trimethoxyxanthona(II) and 1-0-primeverosyl-2,3,4,5- tetrametho-xyxanthone. Pharmacological experiments showed that compounds I and II play markedly roles in protecting liver trauma. they increase the levels of ribonucleotide and hepatic glycogen, promote protein synthesis and liver cell re-generation, accelerate the repair of dead tissue. Injections made from *Swertia mussotii* have remarkable effects in anti-inflammation, they protect animals from experimental liver trauma and SGPT increase caused by CCl_4. Clinical results indicated that injections made from *Swertia mussotii* are effective in curing acute hepatitis. We separated and identified 8 glycoproteins and 3 Xanthone glycosides from the water soluble part. Major glycosids of *Swertia mussotii*. are mangiferin, amarogentin and 7-0-[α -L- rhamnopyanosyl- (1-2)- β -D-xylopyranosyl]- 1, 8-dihydroxy- 3-methoxy-xanthone. These pharmacological experiments demonstrated that xanthone glycosides has remarkable curative effects on hepatitis. Experiments about its biological activities in gallbladder protection are still in progress.

Acknowledgments The author wish to thank the all colleague who work in department of phytochemistry.

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BENZOFURAN GLYCOSIDES FROM THE SEEDS OF *PSORALEA PLICATA* DEL

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Key Word Index--*Psoralea plicata* Del., Leguminosae, Isocorylifonol glycoside, Corylifonol glycosid, Angelic acid glycoside, Isopsoralic acid glycoside.

Abstract--Three new benzofuran glycosides (1→4-O-β-D-glucopyranosid isocorylifonol, 1→6-O-β-D-glucopyranosid corylifonol and 1→4-O-β-D-glucopyranosid angeic acid) and anothr known (1→6-O-β-D-glucopyranosid isopsoralic acid) were isolated from seeds of *Psoralea plicata* Del., their structures being determined by means of spectroscopic methods.

Introduction

As a continuation of our studies on the bioactive compounds form the Lefuminosae plants, we have investigated the seeds of *Psoralea plicata* Del. (=*Cullen plicatum* Delile C. H. Stirt) that known in Arabic as Marmid [1] is a widely distributed wild herb in Allaqi area, South East of Aswan [2]. The plant has been used as folk medicine for the treatment of skin-photosensitizing activity, anthelmintic, antipyretic, analgesic, anti-inflammatory, diuretic, diaphoretic and useful in bilious infections, in liprosy and menstruation disorders [3,4]. The studies of the chemical constituents of this plant are extensive and interesting components including furocoumarings, chromenes, phenolic cinnamates, coumestans, a-tocopherolquinones, isoflavone, terpenoids, phenoilc cinnamates dimer were reported [5,6].

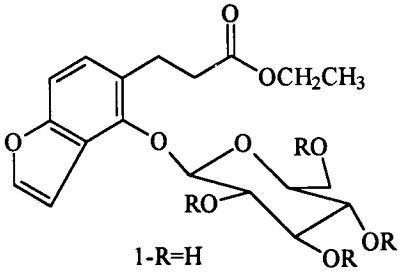
Results and Discussion

Successive extraction of methanolic extract of *Psoralea plicata* seeds with n-butanol led to isolation of four glycosidic compounds. The first compound (**1**) acetate showed its $[M-H]^+$ ion peak at m/z 627 in the FAB mass, consistent with $C_{27}H_{32}O_{17}$. The ^1H NMR spectrum (Table 1) suggested the presence of two furan protons at δ 7.55(1H, d, J=2.2 Hz) and δ 6.90(1H, dd, J=0.8, 2.2 Hz); also there was two ortho coupled aromatic protons at δ 7.14(1H, d, J=8.4 Hz) and δ 7.24(1H, d, J=8.4 Hz). The spectrum exhibited signals for an ethyl ester, represented by a methyl group signal at δ 0.89(3H, t, J=7.3 Hz); typical AB quartet for methylene group at δ 4.05(2H, q, J=7.2 Hz) and two methylene groups at δ 3.03(2H, t, J=6.6 Hz) and 2.61(2H, t, J=6.6 Hz). The ^1H NMR and ^{13}C NMR showed an anomeric proton signal at δ 5.13(1H, d, J=7.9 Hz) and its carbon at δ 99.68. The ^{13}C NMR showed also other five sugar carbons; ($1CH_2$) at δ 62.0, (4-CH) at δ 72.98, 72.01, 71.69 and 68.34. The ^{13}C spectrum of compound (**1**) acetate showed 4 acetate carbons; at δ 170.49, 170.31, 169.36 and 169.24. Acid hydrolysis of compound (**1**) with 5% HCl-Methanol gave D-Glucose and isocorylifonol [10]. The chemical shift and coupling constant of the anomeric proton and carbon indicated that the linkage of sugar with aglycon was β-linkage with carbon at δ 147.20.

From the above data we concluded that this compound was a glycoside with a known aglycone (isocorylifonol), which is isolated from a natural source for the fisrt time. Hence, the isolated compound has the sructure isocorylifonol 1→4-O-β-D-glucopyranosid.

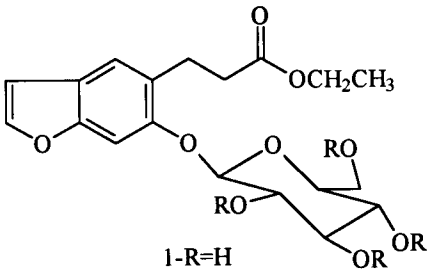
The second compound (**2**) acetate showed showed its $[M-H]^+$ ion peak at m/z 627 in the FAB mass, consistent with $C_{27}H_{32}O_{17}$. The ^1H NMR spectrum (Table 1) suggested the presence

1



1-R=H
2-R=Ac

2



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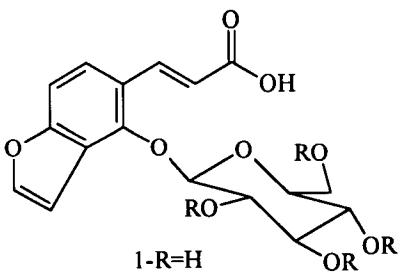
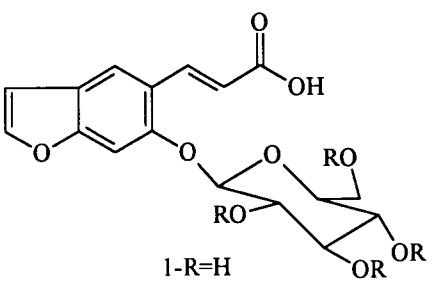
3



1-R=H
2-R=Ac

4



1-R=H
2-R=Ac

Table 1. ^{1}H NMR spectral data for the isolated benzofuran glycosides (400 MHz, $CDCl_3$)

No.	1	2	3	4
Aglycone moieties				
C-2	7.55(1H,d,J=2.2Hz)	7.54(1H,d,J=2.2Hz)	7.57(1H,d,J=2.2Hz)	7.60(1H,d,J=2.2Hz)
C-3	6.90(1H,dd,J=0.8,2.2Hz)	6.66(1H,dd,J=0.8,2.2Hz)	6.96(1H,dd,J=0.8,2.2Hz)	6.65(1H,dd,J=0.8,2.2Hz)
C-4	----	7.37(1H,s)	----	7.79(1H,s)
C-5	----	----	----	----
C-6	7.24(1H,d,J=8.4Hz)	----	7.56(1H,d,J=8.4Hz)	----
C-7	7.14(1H,d,J=8.4Hz)	7.26(1H,s)	7.31(1H,d,J=8.4Hz)	7.37(1H,s)
C-4a	----	----	----	----
C-7a	----	----	----	----
C-1'	3.03(2H,t,J=6.6Hz)	2.96(2H,t,J=6.6Hz)	8.21(1H,d,J=16.1Hz)	8.04(1H,d,J=16.1Hz)
C-2'	2.61(2H,t,J=6.6Hz)	2.63(2H,t,J=6.6Hz)	8.41(1H,d,J=16.1Hz)	6.40(1H,d,J=16.1Hz)
C-3'	----	----	----	----
C-4'	4.05(2H,q,J=7.2Hz)	4.05(2H,q,J=7.2Hz)		
C-5'	0.89(3H,t,J=7.3Hz)	0.89(3H,t,J=7.3Hz)		
CH_3CO	2.14,2.09,2.05,2.02(s)	2.13,2.09,2.04,2.03(s)	2.17,2.11,2.06,2.05(s)	2.17,2.13,2.05,2.03(s)
Glucosyl moieties				
1"	5.13(1H,d,J=7.9Hz)	5.10(1H,d,J=7.6Hz)	5.07(1H,d,J=7.8Hz)	5.08(1H,d,J=7.8Hz)
2"	5.37(1H,t,J=9.4Hz)	5.38(1H,t,J=9.4Hz)	5.44(1H,t,J=9.4Hz)	5.42(1H,t,J=9.4Hz)
3"	5.32(1H,t,J=9.4Hz)	5.28(1H,t,J=9.4Hz)	5.34(1H,t,J=9.4Hz)	5.30(1H,t,J=9.4Hz)
4"	5.16(1H,t,J=9.4Hz)	5.16(1H,t,J=9.4Hz)	5.20(1H,t,J=9.4Hz)	5.18(1H,t,J=9.4Hz)
5"	3.57(1H,m)	3.59(1H,m)	3.68(1H,m)	3.93(1H,m)
6"	4.12(1H,dd,J=2.4,12Hz)	4.13(1H,dd,J=2.4,12Hz)	4.06(1H,dd,J=1.1,12Hz)	4.26(2H,J=7.6,12Hz)
	4.21(1H,dd,J=2.4,12Hz)	4.30(1H,dd,J=2.4,12Hz)	4.25(1H,dd,J=1.1,12Hz)	

Multiplicity was detected by DEPT experiment

of two furan protons at δ 7.45(1H, d, J=2.2 Hz) and δ 6.66(1H, dd, J=0.8, 2.2 Hz); also there was two singlet aromatic protons at δ 7.37 and δ 7.26. The spectrum exhibited signals for ethyl ester, showing a methyl group triplet at δ 0.89(3H, t, J=7.3 Hz); an AB quartet for methylene group at δ 4.05(2H, q, J=7.2 Hz) and two methylene groups at δ 2.96(2H, t, J=6.6 Hz) and δ 2.63(2H, t, J=6.6 Hz). The ^{1}H NMR and ^{13}C NMR showed an anomeric proton signal at δ 5.10(1H, d, J=7.6 Hz) and its carbon at δ 99.04. The ^{13}C NMR showed also other five sugar carbons; ($1CH_2$) at δ 62.15, (4-CH) at δ 72.87, 72.20, 71.90 and 68.53. The carbon spectrum of compound (**2**) acetate showed 4 acetate carbons; at δ 170.51, 170.32, 169.45 and 169.31. Acid hydrolysis of compound (**2**) with 5% HCl-Methanol gave D-Glucose and corylifonol [10]. The chemical shift and coupling constant of the anomeric proton and carbon indicated that the linkage of sugar with aglycon was β-linkage with carbon at δ 155.36. From the above data we concluded that this compound was the isomer of compound (**1**) and its structure should be corylifonol 1→6-O-β-D-glucopyranosid, isolated for the fisrt time as a glycosid but its aglycone was isolated from *Psoralea corylifolia* [10].

The third compound (**3**) acetate showed showed its $[M-H]^+$ ion peak at m/z 534 in the FAB mass, which is consistent with $C_{25}H_{26}O_{13}$. The ^{1}H NMR spectrum (Table 1) suggested the presence of two furan protons at δ 7.57(1H, d, J=2.2 Hz) and δ 6.96(1H, dd, J=0.8, 2.2 Hz); also there was two ortho coupled aromatic protons at δ 7.56(1H, d, J=8.4 Hz) and 7.31(1H, d, J=8.4 Hz); in addition there are signals for cinnamic acid group (two trans-coupled olefinic protons at δ 8.21 and 6.41, J=16.10 Hz). The chemical shifts and spilitting pattern of furan, aromatic and olefinic protons are similar to angelic acid which was confirmed by C-H COSY spectrum that revealed a cross peak between proton at δ 5.07(1H,d, J=7.8 Hz) and carbon at δ 100.94(HMQC), a cross peak between proton at δ 6.96 and carbon at δ 158.65; a cross peak between proton at δ 7.56 and carbon at δ 158.65; and the latter exhibited a cross peak with carbons at δ 140.90 of cinnamic acid and 148.81 that is linkaged with sugar. The ^{1}H NMR and ^{13}C NMR (Table 1,2) exhibited one anomeric proton at δ 5.07(1H, d, J=7.8 Hz), its carbon at δ 100.94 showed a cross peak (C-H COSY) with carbon at δ 148.81 and this revealed that the linkage of sugar was β-linkage with C-4 of aglycone. The ^{1}H NMR of compound (**3**) acetate and ^{13}C NMR showed four signals for acetates and five methines, one methylene for sugar (DEPT expermint). Acid hydrolysis of **3** with 5% HCl-Methanol gave D-Glucose and angelic acid; there fore **3** was a new compound isolated from a natural source for the first time and its structure suggested as angelic acid 1→4-O-β-D-glucopyranosid.

The fourth compound (**4**) was an isomer of compound (**3**) and its structure was isopsoralic acid 1→6-O-β-D-glucopyranosid (Table 1,2) that was isolated previouslyform the same plant [6] and from *Coronilla glauca* [11].

Experimental

Optical rotation were measured on a JASCO-360 digital polarimeter; UV spectra were obtained on a Hitachi 200-10 specrophotometer; IR spectra were taken on JASCO IR-A-2 spectrophotometer; ^{1}H NMR and ^{13}C NMR spectra were taken on a Bruker AM-400 and AM-500; MS were obtained on Hitachi RMU-7M spectrometer.

Extraction and isolation of the consituents. The air-dried seeds of *Psoralea corylifolia* Del. (1 Kg) was powdered, defatted with hexane and extracted with methanol (75%) by meceration until exhaustion. The solbent free residue (25 g) was mixed with 100 ml water, 50 ml methanol, transferred to a separatory funnel and partitioned between chloroform and n-butanol. Each fraction was dried over anhydrous sodium sulphate and concentrated to a syrupy residue (10 g chloroform residue and 13 g n-butanol residue).

Table 2. ^{13}C NMR spectral data for the isolated benzofuran glycosides (100 MHz, $CDCl_3$)

No.	1	2	3	4
Aglycone moieties				
C-2	144.63(d)	144.95(d)	145.22(d)	146.14(d)
C-3	100.1 (d)	106.25(d)	105.23(d)	106.57(d)
C-4	147.20(s)	121.65(d)	148.81(s)	.20(d)
C-5	120.39(s)	121.65(s)	121.79(s)	121.40(s)
C-6	126.17(d)	155.36(s)	123.36(d)	153.51(s)
C-7	108.29(d)	106.26(d)	109.38(d)	101.83(d)
C-4a	120.30(s)	126.16(s)	121.17(s)	123.57(s)
C-7a	157.65(s)	157.65(s)	157.65(s)	156.54(s)
C-1'	35.17(t)	34.42(t)	140.90(d)	141.37(d)
C-2'	30.66(t)	29.70(t)	117.61(d)	117.52(d)
C-3'	173.12(s)	173.12(s)	171.54(s)	171.54(s)
C-4'	64.86(t)	64.86(t)		
C-5'	19.11(q)	21.96(q)		
$4CH_3CO$	20.59(q)	20.72(q)	20.56(q)	20.63(q)
C=O	169.24,169.36, 170.31,170.49(s)	169.31,169.45, 170.32,170.51(s)	169.33,169.49, 170.24,170.50(s)	169.40,169.59, 170.31,170.49(s)
Glucosyl moieties				
1"	99.68(d)	99.04(d)	100.94(d)	100.38(d)
2"	68.34(d)	68.53(d)	68.29(d)	68.4 (d)
3"	71.69(d)	71.90(d)	71.58(d)	71.58(d)
4"	72.01(d)	72.20(d)	72.88(d)	72.7 (d)
5"	72.98(d)	72.87(d)	72.08(d)	72.3 (d)
6"	62.0(t)	62.15(t)	62.85(t)	62.0 (t)

Multiplicity was detected by DEPT experiment

Separaton of n-butanol fraction. n-butanol fraction (13 g) was subjected to ODS column, eluted with methanol: water (8:2) to give three fraction **A**, **B** and **C** (9, 2.5 and 1g. respectively). Fraction **B** and **C** (100 mg) each were acetylated with acetic anhydride and then subjected to flash silica column.

Acetylation of fraction B and C. 100 mg of fractions **B** and **C** separately was added to pyridin (10 ml), acetic anhydride (5 ml) and DMP (traces), left for 4 hr. The mixture of each reaction was subjected to flash silica gel column eluted with Hexane:Acetone (3:2) to give compound **1**(20 mg) and **2** (15 mg) from fraction **B** and compound **3** (30 mg) and **4** (17 mg) from fraction **C**.

Acid hydrolysis of compounds 1, 2, 3 and 4. 3 mg of each compound was refluxed separately with 3 ml of 5% HCl-MeOH on a steam bath for 6 hr. The product was diluted with H_2O and extracted with $CHCl_3$ in a separatory funnel, and the respective aglycones were sepd. The aq. filtrate was neutralized with $AgCO_3$ filtrated and evpd. The resulting syrup was subjected to TLC on silica gel using EtOA-MeOH-HOAc-H2O (13:3:4:3) as developing solvent against ref. sugars, and the dried chromatogram was sprayed with *p*-anisaldehyde-H_2SO_4 reagent.

1→4-O-β-D-glucopyranosid isocorylifonol (1) acetate. Colourless gum [α]D-9.99 (c=0.006 $CHCl_3$); IR ν_{max}KBr cm^{-1}: 1730 (ester), 1725 (C=O), 2900 (CH stretching), 1680 (furan), 1620 (C=C); Positive FAB-MS *m/z* (rel.int.): 627$[M-H]^+$(27)($C_{27}H_{32}O_{17}$), 392[M-4 acetate]$^+$(60), 347[M-4 acetate-$OCH_2CH_3]^+$(25); UV λ $CHCl_3$ $_{max}$: 206, 244, 250, 290; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2.

1→6-O-β-D-glucopyranosid corylifonol (2) acetate. Colourless gum [α]D-3.33 (c=0.006 $CHCl_3$); IR ν_{max}KBr cm^{-1}: 1730 (ester), 1725 (C=O), 2900 (CH stretching), 1680 (furan), 1620 (C=C); Positive FAB-MS *m/z* (rel.int.): 627$[M-H]^+$(27)($C_{27}H_{32}O_{17}$), 392[M-4 acetate]$^+$(60), 347[M-4 acetate-$OCH_2CH_3]^+$(25); UV λ $CHCl_3$ $_{max}$: 206, 244, 250, 290; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2.

1→4-O-β-D-glucopyranosid angeic acid (3) acetate. Colourless gum [α]D-70 (c=0.02 $CHCl_3$); IR ν_{max}KBr cm^{-1}: 3460 (OH), 1725 (C=O), 2900 (CH stretching), 1680 (furan), 1620 (C=C); Positive FAB-MS *m/z* (rel.int.): 534$[M]^+$($C_{25}H_{26}O_{13}$), 368[M+2H-4 acetate]$^+$, 186[M-$C_{11}H_6O_3]^+$(15), 169[M-$C_{11}H_7O_4$]; UV λ $CHCl_3$ $_{max}$: 255, 300; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2.

1→6-O-β-D-glucopyranosid isopsoralic acid (4) acetate. Colourless gum [α]D-50 (c=0.02 $CHCl_3$); IR ν_{max}KBr cm^{-1}: 3460 (OH), 1725 (C=O), 2900 (CH stretching), 1680 (furan), 1620 (C=C); Positive FAB-MS *m/z* (rel.int.): 534$[M]^+$(27)($C_{25}H_{26}O_{13}$), 368[M+2H-4 acetate]$^+$, 186[M-$C_{11}H_6O_3]^+$(15), 169[M-$C_{11}H_7O_4$]; UV λ $CHCl_3$ $_{max}$: 255, 300; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2.

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A SIMPLIFIED SYNTHESIS OF PODOPHYLLOTOXIN GLUCOSIDE DERIVATIVES

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Key words Index--podohyllotoxin glucoside, catalytic agent, alcoholysic reaction, anticancer

Abstract Podophyllotoxin glucoside derivatives (Etoposide, Teniposide etc.) were synthesized from Podohyllotoxin by 5 steps of reactions. The method has many advantages, such as no need of protecting 4'-hydroxy, decrease of 2 reactions, and increase of more than 10% in total yield, in comparison with the conventional process. In this paper, alcoholysis reaction conditions were carefully studied and 5 metals as catalytic agent were selected. The new alcoholysis reaction is characterized by simple procedures, good quality and high yield.

Introduction (1～18)

Cancer remains a main enemy of mankind based on the observation that cancer causes several million deaths pere year in the world. For seeking new antitumor agents with antineoplastic activity against a variety of malignancies, numerous pharmacologists and doctors around the world are hard working. Now, one of more active fields is in the research and development of new antitumor agents from podophyllotoxin.

The American cancer institute first reported the structure of podophyllotoxin and 4'-demethylepipodophyllotoxin in 1951. Early researchers of American cancer institute took interest in podophyllotoxin for its antitumor activity. However, podophyllotoxin and its simple derivatives cannot be used clincal experiment due to their great toxicity. Then, Sandoz Pharmaceuticals Ltd. found that podophyllotoxin glucoside derivatives are more soluble in water and less poisonous than podophyllotoxin, therefor they synthesized the second period compound SP-1 and SP-G. Their clinical experiment proved that the latent element of SP-G in anti-leukaemia is 4'-demethyl epipodop- hyllotoxin- benzoyldiene-1- β -D-glucopyranosyl. Subsequently, some derivatives of 4'-demethylepipodophyllotoxin-dialkene-1- β -D-gluco-pyranosy were synthesized. For example two compounds VM-26 and VP-16, have anti-neopeastic activity against a varitty of malignancies and are used alone or in combination with other drugs, as an antitumor agent.

At present, podophyllotoxin glucoside derivatives are becoming the main body of podophyllotoxin derivatives as an antithmor agent. It has a bright applied perspective to sythesize podophyllotoxin glucoside derivatives 4'-demethylepipodophyllotoxin- β -D-glucopyranosy (Ⅰ) which is the necessary intermediate in sythesizing those compounds, so this

paper mainly relates to the preparation of (Ⅰ).

(Ⅰ) was sythesized from podophyllotoxin through 6 steps (including: halogenation, etc.) in the past. Pietro Allevi et al. improved the process for the synthesis of (Ⅰ) and synthesized (Ⅰ) for the first time from unprotected 4, -demethylepipodohyllotoxin〔20〕. The new process reduced 2 reactions, but there also exist some problems in the new process. First, the glucosidation which involves as the key step in that paper used a lot of solvent (0.4g 4'-demethylepipodohyllotoxin dissolved in 80ml dichloromethane), so the manipulation is difficult. Secondly, alcoholysic reaction of the process is performed simply according to known procedures. Alcoholysic product (unprotected 4'-hydroxy group) has more polar than old alcoholysic product (protected 4'-hydroxy group); Furthermore, the problem remains of removing the catalytic adgent [$ZnCL_2$ or $Zn(OOCH_3)_2$]. In this paper, we try to resolve these problems.

Experimental Section

1. chemicals

Podophyllotoxin was extracted from *Podophyllum emodi* Wall. var. *chinense* Sprague. 2, 3, 4, 6- tetra- o- acetyl- β - D- ghlcopyrunose was prepared in our laboratory Pichloromethane, BF3 • Et2O, Trichloromethane, methanol, zinc powder, copper powder, aluminium powder, magnesium powder and tin powder were purchased from chemical store.

2. Preparing 1-Bromo-4'-Demethylepipodohyllotoxin (Ⅲ) from Podophyllotoxin(Ⅱ)

Acording to known refrerence〔19〕, compound Ⅲ was synthesized. The yield is 51%. compound Ⅲ showed: mp 180-192 ℃ [α]20D=+6.2 ° (Trichloromethane); IR(KBr) 3375(OH), 1760(O=C), 1630, 1525, 1510, 1490(-C6H5); 1H-NMR(CDCl3): 6.83(1H, S)HC-8, 6.50(1H, S)HC-5, 6.24(2H, S)HC-2' and HC-6', 5.92(2H, S)O-CH2 -O, 5.58 (1H, d)HC-1, 4.67(1H, d)HC-4, 4.35(2H, d)-OCO-CH2, 3.75(6H, S)3'-OCH3 and 5'-OCH3, 3.45(1H, dd)HC-3, 2.60-3.20(1H, m) HC-2; Elemental analysis, calculated%(found%): C54.5(54.1), H4.1(4.2), Br17.2(17.1).

3. 4'-Demethylepipodohyllotoxin (Ⅳ) was prepare from (Ⅲ).

Seeing[19], compound(Ⅳ) was synthesized. The yield is 90% Compound (Ⅳ) showed as follow: mp 236-239℃ (CH3OH), 245-250℃(CH3COCH3); [α]20D=-60.4 ° (CH3Cl); IR(KBr): 3480, 3410(OH), 1755(C-O), 1620 1520 1500 1480 (-C6H5); Elemental analysis was as follow: colculated%(found%): C63.0(62.5), H5.0(5.0).

4. Preparing 2", 3", 4", 6"-tetra-O-acetyl-4'-Demethylepipodophyllotoxin β -D-glucopyranose(Ⅴ) from (Ⅳ)

Compound(Ⅴ) was synthesized by the following procedure. In a 100ml flask, a mixture of 2g (5mmol) of (Ⅳ). 40ml of CH2Cl2 and 4.1ml (15mmol BF3) of BF3. Et2O is stirred at -20℃, then is slowly added 2.6g (7.5mmol) of 2, 3, 4, 6-tetra-O-acetyl- β -D-glucopyrunose. The reaction was held at - 20 ℃ for 2～3 hours and checked the end point of reaction by thin layer

chromatography (TLC). Work-up of the reaction must be performed by simply pouring the reaction mixture into ice-cold water under sharp stirring. Under these conditions, a 90.4% of yield for V(3.7g) was obtained. Compound(V) showed: mp 223-225℃; [α]20D=-57° (CHCl3); IR(KBr) 3390(O-H), 1775, 1750(-OC-O-), 1600, 1510, 1500, 1475(-C6H5); 1H-NMR(CDCl3) 6.82(1H, S)HC-8, 6.50(1H, S)HC-5, 6.23(2H, S)HC-2' and HC-6', 5.92(2H, S) O-CH2-O, 5.18(1H, dd)HC-3", 5.02(1H, dd)HC-4", 4.97(1H, dd)HC-2", 4.92(1H, d)HC-4, 4.65(1H, d)HC-1", 4.56(1H, d)HC-1, 4.37(2H, d)OCO-CH2, 4.20(2H, d)HC-6", 3.74(6H, S)3'-OCH3 and 5'-OCH3, 3.66(1H, m)HC-5", 3.42(1H, dd)HC-3, 2.60-3.18(1H, m)HC-2, 2.12, 2.01, 1.99 and 1.85(12H, 4xS) 4xCH3COO; Elemental analysis; colculated%(found%): C57.3(56.5), H 5.2(5.3).

5. Preparing 4'-Demethylepipodophyllotoxin-β-D-glucopranose (Ⅰ)from (V)

A mixture of 3.3g of (V), 6.6g of metal powder and 132ml of CH3OH is refluxed for several hours under stirring. The end point of reaction is identified by thin layer chromatography (TLC). At the end of reaction, the reation mixture is, filtered for removing of metal powder, then the filtrate is concentrated and crystallized to yield 2.7g of (Ⅰ). The yield is 98%.

Compound (Ⅰ) showed: mp 225～227℃; [α]20D=-88.2° (CH3OH); IR(KBr) 3390(OH), 1765(-OC-O-); 1610, 1510, 1495, 1475(-C6H5); Elemental analysis, colculated%(found%) C: 57.6(58.2), H: 5.4(5.6); 1H-NMR(CDCl3): 6.83(1H, S)HC-8, 6.41(1H, S)H-C-5; 6.23(2H, s)HC-2' and HC-6', 5.91(2H, S)O-CH2-O, 5.19(1H, dd)HC-3", 5.02(1H, dd)HC-4", 4.97(1H, dd)HC-2", 4.92(1H, d)HC-4, 4.65(1H, d)HC-1", 4.55(1H, d)HC-1, 4.31(2H, d)HC-11, 4.20(2H, d)HC-6", 3.75(6H, S)3'-OCH3 and 5'-OCH3, 3.66(1H, dd)HC-5", 3.10(1H, dd)HC-2, 2.84(1H, m)HC-3, 2.13～1.85(12H,4×S)4×-CH3COO.

6. Synthesis of Podophyllotoxin glucoside derivatives(Ⅵ) from compound (Ⅰ)

Compound (Ⅵ) were prepared by reacting compound (Ⅰ) with aldehyde in the presence of lewis acids at 20℃ under protection and protecting in N2. A lot of podophyllotoxin glucoside derivatives such as Meoposide, Teniposide, Etoposide and SP-G ect. were prepared by this method.

Results and Discussions

With regard to alcoholysis reaction, five metal powders as catylytic agent (including: Copper powder, Tin powder, Zinc powder, Aluminium powder and Magnesium powder.) were used in which Zinc powder is the best one. Using Zinc powder as catalytic agent, the reation yield is 98%. which much better than 47.6% for old method and the problem of removing the catalytic agent [ZnCl2 or Zn(OOCCH3)2] was resolved, moreover, the prucedure is very simple.

Concerning glucosidation, solvent used in new method is only one tenth of former reaction, so the process is easy to enlarge.

Conclusion

Podophyllotoxin glucoside derivatives were synthesized the process reported in this paper. The total yield is higher than the other process. Furthemore, the mainpulation is simple.

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NON-CYANOGENIC CYANOGLUCOSIDES

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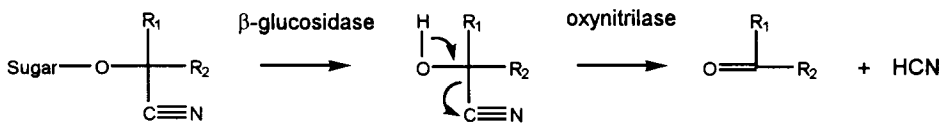
Key Word Index—non-cyanogenic;cyanoglucoside;nitrile glucoside; glycoside; simmondsin.

Abstract—Naturally occurring non-cyanogenic glucosides isolated and characterized from various plant sources are reviewed. Their occurrences, characteristic features, structure elucidations, biogenesis, biological activities and promising applications are discussed.

Introduction

Most of the cyanoglycosides in Nature are cyanogenic, since they have a nitrile group alpha to the glycosidic linkage. Hydrolysis of these cyanogenic glycosides by certain glycosidase or acid liberates aldehyde or ketone and hydrogen cyanide through the corresponding cyanohydrin. The liberated hydrogen cyanide is responsible for their physiological activities including toxicity.



However, there is a small group of cyanoglycosides possessing a nitrile group not adjacent to the glycosidic linkage, hence, are non-cyanogenic. In this review, several examples of these glycosides isolated from plants are introduced and their occurrences, structural features, plausible biogenesis, biological activities and promising applications are briefly discussed.

Occurrence

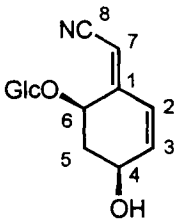
Non-cyanogenic cyanoglucosides of plant origin are listed in Table 1 and their structures are shown in Figure 1. Although only a few compounds of this type have been isolated so far, the taxonomical distribution is very wide, occurring in both classes of Angiospermae, the monocotyledoneae (Graminae) [28] and the Dicotyledoneae in the subclasses Sympetalae (Boraginaceae [7, 18]) and Archichlamydeae (Simmondsiaceae [1-3], Leguminosae [5,6, 16, 22], Menispermaceae [8, 13], Aquifoliaceae [10, 11], Euphorbiaceae [12], Ranunculaceae[14, 21], Ochnaceae [17], Rosaceae [20] and Crassulaceae [25, 27]). The non-cyanogenic cyanoglycosides seem to be ubiquitous and the limited examples make a discussion on their chemotaxonomy difficult.

Structural Features

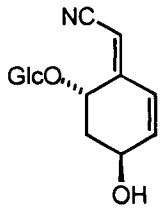
All of the non-cyanogenic cyanoglycosides isolated so far are monoglucosides, and they can be classified into two main groups, the cyanomethylenecyclohexyl glucosides (**1–13**), including phenylacetonitrile glucoside (**14**), and the acyclic isopentenyl cyanoglucosides (**15–21**). In this review, the former group is mainly discussed, while the latter group is only briefly mentioned.

Table 1. Structural Studies on Non-cyanogenic Cyanoglucosides

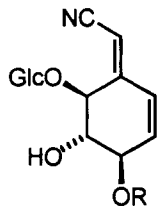
	Literature		Plant				Contents	Remarks
no.	Year	Senior author	*Latin name*	Family	Locality	Usage (Effect)	(Structure)	(Activity)
1	1973	Elliger	*Simmondsia californica*	Simmondiaceae	USA(jojoba)	weight loss	simmondsin (**6**)	LD_{50}>4g/kg
2	1974	Elliger	*Simmondsia californica*	Simmondiaceae	USA(jojoba)	weight loss	2'-feruloyl-**6**	no experimental
3	1978	Verbiscar	*Simmondsia californica*	Simmondiaceae	USA(jojoba)	replacement of sperm oil	HPLC,Quantitative analysis	
4	1992	Chida					synthesis of **6**	abs. configuration
5	1976	Badu	*Griffonia simplicifolia*	Leguminosae	Ghana	enema, wounds, kidney	griffonin (**3**), griffonilide(**3a**)	
6	1976	Gopalakrishna	*Griffonia simplicifolia*	Leguminosae	Ghana	enema, wounds, kidney	X-ray of **3a**	lactone only
7	1977	Sosa	*Lithospermum officinale*	Boraginaceae	France	contraceptive (Nevada)	lithospermoside (**3**)	
8	1978	Takahashi	*Menisperum dauricum*	Menispermaceae	Japan		menisdaurin (**1**), -lide (**1a**)	lactone (**1a**)
9	1980	Thomas	*Ilex aquifolium*	Aquifoliaceae	(holly)		**1a** (wrong structure)	lactone (-lide) only
10	1988	Willems	*Ilex aquifolium*	Aquifoliaceae	(holly)	poisonous fruits	**1** (wrong structure)	
11	1990	Nahrstedt	*Ilex aquifolium*	Aquifoliaceae	(holly)		**1** correction of lit.10	
12	1993	Bachmann	*Phyllanthus anisolobus*	Euphorbiaceae	Equador	fish poison	**1a, 2a** (only lactones)	
13	1993	Otsuka	*Sinomennium acutum*	Menispermaceae	Japan	anti-inflammation	**1, 1a, 2a**, etc.	
14	1979	Wu	*Thalictrum dasycarpum*	Ranunculaceae	USA		**3, 3a, 5, 5a** (absolute)	dibenzoate rule
15	1983	Ueda	*Ilex warburgii*	Aquifoliaceae	Ryukyu	fruits rarely eaten by birds	**1, 5** (epi ?)	
16	1985	Chen	*Bauhinia championii*	Leguminosae	Taiwan	piscicidal	bauhinin (**4**, X-ray), **4a**	300mg/kg: nontoxic
17	1993	Murakami	*Lophira alata*	Ochnaceae	Cameroon	anti-inflammation,analgesic	lophirosides (**7-10**)	bitter,abs config: CD
18	1994	Simpol	*Ehretia philippinensis*	Boraginaceae	Philippines	anti-inflammation	**6**, ehretiosides (**11-14**)	
19	1975	Ballester	*Erica scoparia*	Ericaceae	Spain		aglycone of **14**	no glusoside
20	1994	Nakanishi	*Purshia tridentata*	Rosaceae	USA	inhibit HIV-1 virus	**1, 2**	abs config: CD
21	1984	Guerriero	*Aquilegia atrata*	Ranunculaceae	Italy		**1a, 2a**	lactones only
22	1987	Swenson	*Acacia sutherlandii*	Leguminosae	Australia		sutherlandin (**15**)	
23	1993	Lechtenberg	*Osmaronia cerasiformis*	Rosaceae	USA		**15**, osmaronin (**16**)	epimeric epoxides
24	1994	Lechtenberg	*Osmaronia cerasiformis*	Rosaceae	USA		**15, 16**	
25	1996	Yoshikawa	*Rhodiola quadrifida*	Crassulaceae	China	anti-allergic	rhodiocyanosides (**17-18**)	inhibitor on PCA
26	1994	Nishida	*(Abraxas grossulariata)*	(Geometridae)	(moth)	defensive substance	sarmentosin (**19**)	Magpie moth
27	1982	Fang	*Sedum sarmentosum*	Crassulaceae	China		sarmentosin (**19**)	
28	1993	Pourmohseni	*Hordeum vulgare*	Graminae	(Barley)	resistence-susceptibility	epidermin (**20**), **21**	



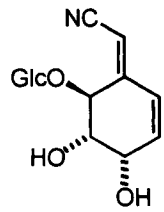
menisdaurin (1)
[lit. 8-13, 15, 20]



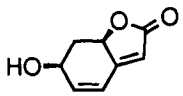
purshianin (2)
[lit. 20]



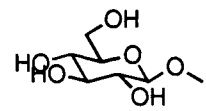
R = H:
lithospermoside (3)
(griffonin) [lit. 5, 7, 14]
R = Me:
bauhinin (4) [lit. 16]



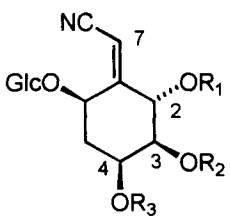
dasycarponin (5)
[lit.14, 15]



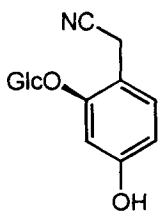
cf. menisdaurilide (1a)
[lit. 8, 9, 12, 13, 21]



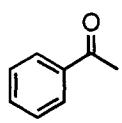
Glc (β-D-glucopyranosyl)



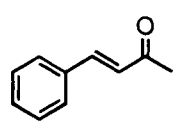
R_1	R_2	R_3	
H	Me	Me	simmondsin (6) [lit. 1-4, 18, 29-32]
H	Bz	Bz	lophiroside A1 (7) [lit. 17]
Bz	H	Bz	lophiroside A2 (8) [lit. 17]
H	Cin	Bz	lophiroside B1 (9) [lit. 17]
Cin	H	Bz	lophiroside B2 (10) [lit. 17]
H	Sen	H	ehretioside A1 (11) [lit. 18]
H	H	Sen	ehretioside A2 (12) [lit. 18]
Sen	H	H	ehretioside A3 (13) [lit. 18]



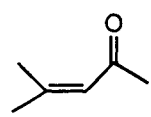
ehretioside B (14) [lit. 18]



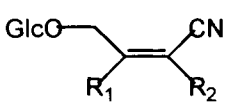
Bz (benzoyl)



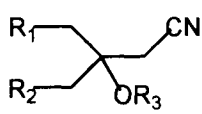
Cin (*trans*-cinnamoyl)



Sen (senecioyl)



R_1	R_2	
CH_2OH	H	sutherlandin (15) [lit. 22-24]
CH_3	H	osmaronin (16) [lit. 23-24]
H	CH_3	rhodiocyanoside A (17) [lit. 25
H	CH_2OH	sarmentosin (18) [lit. 26-27]
H	CH_2Ogalloyl	rhodiocyanoside B (19) [lit. 25



R_1	R_2	R_3	
H	H	Glc	epidermin (20) [lit. 28]
OGlc	OH	H	(no trivial name) (21) [lit. 28]

Figure 1. Noncyanogenic Cyanoglucosides

Table 2. Spectral Data for **1 - 3 and 5**

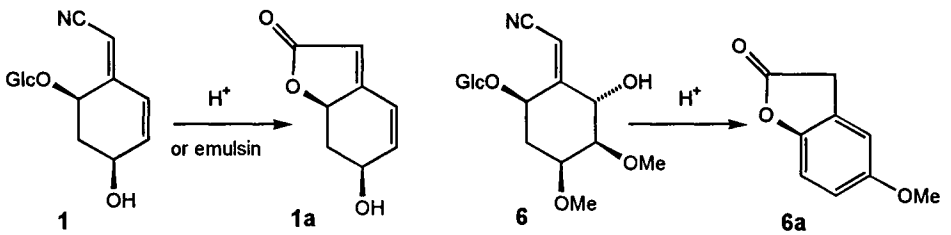
Compd	Menisdaurin		Purshiannin	Lithospermoside	Dasycarponin
Structure	**1**		**2**	**3**	**5**
Origin	*Menisperm*	*Menisperm*	*Purshia*	*Thalictrum*	*Thalictrum*
Fam.	Menispermaceae	Menispermaceae	Rosaceae	Ranunculaceae	Ranunculaceae
Author	Takahashi et al	Nakanishi et al	Nakanishi et al	Wu et al	Wu et al
Ref. (y)	8 (1978)	20 (1994)	20 (1994)	14 (1979)	14 (1979)
mp	175 -176°	170 -172°	167 -170°	272 -274°	253 -255°
$[\alpha]_D$	-185° (MeOH)	-215° (MeOH)	-90° (MeOH)	-138° (H_2O)	-11° (H_2O)
MS (m/z)	313 M^+		314 $(M+H)^+$	329 M^+	329 M^+
IR ν (KBr)	2220, 1625 ,1620		2220, 1620	2220	2230, 1633
UV λ (log ε)	260 (5.5)		271 (4.0)	259 (4.2)	261 (4.2)
^{13}C NMR					
Solvent	D_2O	CD_3OD	CD_3OD	D_2O	D_2O
C-1	156.4 s	157.8 s	157.1 s	157.6 s	155.4 s
C-2	127.6 d	128.4 d	127.5 d	129.2 d	128.5 d
C-3	139.3 d	141.3 d	144.3 d	138.7 d	139.8 d
C-4	64.5 d	66.0 d	65.3 d	76.2 d	68.1 d
C-5	35.2 t	36.7 t	38.4 t	78.5 d	79.4 d
C-6	73.8 d	73.2 d	75.2 d	72.3 d	72 d
C-7	96.9 d	97.6 d	98.7 d	99.4 d	102.5 d
CN	118.7 s	118.7 s	119.1 s	120.1 s	120.1 s
G-1	101.2 d	102.3 d	104.1 d	104.9 d	105.3 d
G-2	73.1 d	75.2 d	75.8 d	75.3 d	75.6 d
G-3	76.9 d	78.7 d	79.0 d	78.4 d	78.6 d
G-4	70.7 d	72.4 d	72.4 d	72.3 d	71.3 d
G-5	76.9 d	78.8 d	78.8 d	78.3 d	78.3 d
G-6	61.9 t	63.8 t	63.7 t	63.5 t	63.2 t
^1H NMR					
Solvent	CD_3OD	CD_3OD	CD_3OD	D_2O	D_2O
Freq.	100 MHz	600MHz	600MHz	90MHz	90MHz
H-2	6.27 d, 10	6.29 ddd	6.25 dddd	6.33 dd, 10,1	6.35 d, 10
H-3	6.20 dd, 10,3	6.21 ddd	6.22 dddd	6.12 dd, 10,3	6.05 d, 10
H-4	4.35 m	4.36 dddd	4.66 dddd	4.28 m	4.28 m
H-5a	1.87 m	2.25 ddd	2.57 dddd		
H-5b	2.39 m	2.04 ddd	1.60 ddd		
H-6	4.91 ddd, 2,4,8	4.93 ddd	4.93 dd		
H-7	5.48 d, 2	5.50 ddd	5.50 dd	5.61 d, 1	5.70 s
H-1′	4.54 d, 7	4.55 d, 8	4.54 d, 8	4.7 4.9	4.6 5.0
H-2′		3.34	3.16 dd		
H-3′		3.39	3.38 dd		
H-4′		3.29	3.29 dd	3.3 4.1	3.1 4.0
H-5′		3.34	3.31 ddd		
H-6′	3.68 dd, 4 12	3.67	3.68 dd		
H-6′	3.88 dd, 2,12	3.89	3.86 dd		

Table 3. Spectral Data for 6, 7, 11 and 14

Compd	Simmondsin	Lophiroside A1	Ehretioside A1	Ehretioside B
Structure	6	7	11	14
Origin	*Ehretia*	*Lophira*	*Ehretia*	*Ehretia*
Fam.	Boraginaceae	Ochnaceae	Boraginaceae	Boraginaceae
Auth.	Simpol et al	Murakami et al	Simpol et al	Simpol et al
Ref. (y)	18 (1994)	17 (1993)	18 (1994)	18 (1994)
mp	95-100°	amorphous	Amorphous	Amorphous
$[\alpha]_D$	-73° (MeOH)	-11.5° (MeOH)	+39° (pyridine)	-72° (MeOH)
MS (m/z)	374 $(M-H)^-$	556 $(M+H)^+$	428 $(M-H)^-$	310 $(M-H)^-$
IR ν(KBr)	2218, 1640[1]	2210, 1600	2230, 1645	
UVλ(logε)	217 (4.0)	274 (3.2)		
^{13}C NMR		pentaacetate		
Solvent	CD_3OD	Acetone-d_6	CD_3OD	CD_3OD
C-1	166.4 s	158.8 s	165.0 s	111.1 s
C-2	70.7 d	69.3 d	67.9 d	130.2 d
C-3	86.3 d	75.1 d	79.0 d	110.4 d
C-4	76.7 d	69.4 d	67.7 d	160.3 s
C-5	32.0 t	33.9 t	34.3 t	104.8 d
C-6	76.5 d	77.7 d	76.0 d	157.1 s
C-7	95.2 d	97.9 d	95.6 d	17.9 t
CN	117.6 s	116.2 s	116.9 s	119.5 s
G-1	104.1 d	102.1 d	102.7 d	103.3 d
G-2	74.7 d	72.3 d	75.0 d	74.8 d
G-3	78.1 d	73.7 d	79.4 d	78.8 d
G-4	71.4 d	69.1 d	71.4 d	71.0 d
G-5	78.1 d	73 d	78.5 d	78.4 d
G-6	62.7 t	62.5 t	62.7 t	62.2 t
OMe	58.5 q	3-Benzoyl		
OMe	58.2 q	CO, *o* 165.6, 130.3	Senecioyl	
		m, *p* 129.4, 134.0	166.0 s	
		4-Benzoyl	116.6 d	
		CO, *o* 166.4, 130.7	156.9 s	
		m, *p* 129.4, 134.0	26.9 q	
		Ac 20.5, 170.0 etc	20.0 q	
^1H NMR				
Solvent	CD_3OD	Acetone-d_6	CD_3OD	CD_3OD
Freq.	400 MHz	400 MHz	400 MHz	400 MHz
H-2	4.70 dd, 9,2	5.50 dd, 10.3,2.0	5.68 dd, 10,2	7.33 d, 8
H-3	3.15 dd, 9,3	5.16 dd, 10.3,3.3	5.18 dd, 10,3	6.83 dd, 8,2
H-4	3.91 ddd, 4,4,4	5.77 m	4.66 ddd, 3,3,3	
H-5a	1.79 dt, 15,4	2.34 ddd, 15.6,3.7,3.0	2.69 dt, 15,3	7.37 d, 2
H-5b	2.50 dt, 15,4	2.67 ddd, 15.6,3.2,2.9	1.82 dt, 15,3	
H-6	4.87 t, 4	5.09 ddd, 3.7,3.2,2.0	5.52 t, 3	
H-7	5.70 d, 2	5.96 d, 2.0	6.25 d, 2	4.00 d, 18
				3.90 d, 18
H-1'	4.38 d, 8	4.46 d, 7.8	5.03 d, 8	5.46 d, 7
H-2'	3.26 dd, 8,9		3.97 dd, 8,9	3.92 dd, 7,8
H-3'	3.30 m		4.21 dd, 9,8	4.26 m
H-4'	3.36 m	3.21-3.45 (4H)	4.21 dd, 9,8	4.28 m
H-5'	3.21 ddd, 8,2,5		3.91 ddd, 9,2,5	3.89 ddd, 2,5,9
H-6'a	3.65 dd, 5,12	3.66 dd, 11.7,5.9	4.44 dd, 12,2	4.38 dd, 12,2
H-6'b	3.83 dd, 12,2	3.89 dd, 11.7,1.8	4.28 dd, 12,5	4.31 dd, 12,5
etc	Me 3.44, s	Bz (*o*) 7.90 m, 8.14 m	Sen 5.56, sept, 1.3	
	Me 3.47, s	Bz (*m*) 7.40 m, 7.51 m	Sen 1.56, d, 1.3	
		Bz (*p*) 7.58 m, 7.65 m	Sen 2.07, d, 1.3	

Behaviour on hydrolysis

The cyanomethylenecyclohexyl glucosides (**1–13**) have a common skeleton, 2-β-D-glucopyranosyl cyclohexylidenemethyl cyanide, some of which have a double bond in the ring. Differing from the cyanogenic glucosides, menisdaurin (**1**) did not liberate hydrogen cyanide on acid hydrolysis, but cyclized to menisdaurilide (**1a**) [8]. Similar cyclization occurred for the compounds **2–6** on acid or enzymatic hydrolysis to afford the corresponding lactones [5, 14, 16]. In addition to the corresponding lactone, simmondsin (**6**) afforded the aromatized lactone (**6a**) on acid hydrolysis, while enzymatic hydrolysis with β-glucosidase (E. C. 3.2.1.21) gave mixtures of aromatic materials and glucose [1].

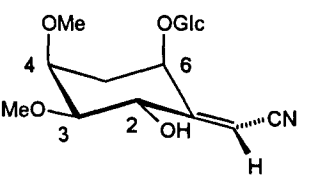


Spectral characteristics

The spectral data of the cyanomethylenecyclohexyl glucosides are tabulated in Tables 2 and 3. In the IR spectra, the most characteristic signal of these compounds is the CN stretching band. Strong absorption appearing at about 2220 cm^{-1} followed by absorption of the conjugated double bond around 1620 cm^{-1} are definite informations about this group. In addition, the ^{13}C NMR resonances of CN at δ 116–120 and the adjacent trisubstituted double bond at 155–166 (s) and 95–100 (d) are characteristic signals. Each compound showed six carbon signals attributable to a β-glucopyranosyl group with an anomeric proton signal around δ 5.0 having a coupling constant of *ca.* 7 Hz. In addition, an odd number molecular peak in the MS suggested the existence of sometimes unexpected nitrogen atom.

Simmondsin (6)

Historically, simmondsin (**6**) is the first reported compound of this series. It was isolated from *Simmondsia chinensis* [syn. *Simmondsia californica*] (Buxaceae [or Simmondsiaceae]) and characterized in 1973 by means of chemical degradation and proton NMR measured at 100 MHz [1]. The geometry of the double bond and the relative configuration was established on the basis of the coupling magnitude. However the absolute configuration was not decided. After two decades, simmondsin was stereoselectively synthesized from L-quebrachitol, confirming the geometry and the absolute configuration [4]. It is noteworthy that the conformations shown here is stable form in spite of the presence of 1,3-diaxial interaction of hydroxy functions [1, 4]. From the same plant, 2'-ferulate of simmondsin, 3-O-desmethyl simmondsin and 4-O-desmethyl simmondsin were also reported [2].



Stable conformation of **6** [1,4]

Griffonin (lithospermoside) (3) and dasycarponin (5)

Chronologically, griffonin (**3**) is probably the second compound isolated (1976) which belongs to this group. It was obtained from the roots of *Griffonia simplicifolia* Baill. (syn.

Bandeiraea simplisifolia Benth.) of the Leguminosae (Caesalpinaceae) family. The structure of **3**, with its corresponding lactone, griffonilide, was decided by ^{1}H NMR (250 MHz) and confirmed by X-ray analysis of the crystalline lactone, but the absolute stereochemistry was not solved [5,6]. In 1977, **3** was claimed to have been isolated from *Lithosperum purpureo-caeruleum* way back in 1955, and was named lithospermoside, although at that time the structure could not be fully elucidated. In the 1977 report, the structure and relative configuration were clarified using the sample isolated from *L. purpureo-caeruleum* and from *L. officinalis* [7]. In 1979, **3** and its diastereomer, dasycarponin (**5**) were isolated from the two plants, *Thalictrum rugosum* Ait. (syn. *T. glaucum* Desf.) and *T. dasycarpum* Fisch. and Lall. (Ranunculaceae). Their structures were elucidated and the absolute configurations were determined by utilization of dibenzoate chirality rule in CD spectra [14].

Menisdaurin (1) and purshianin (2)

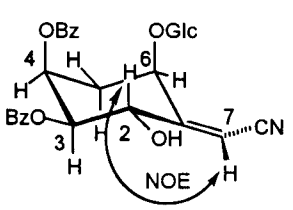
About the same time, in 1978, menisdaurin (**1**) was isolated from *Menisperm dauricum* DC (Menispermaceae) with the corresponding lactone, menisdaurilide (**1a**), and the structures were decided through ^{13}C and ^{1}H NMR. The geometry and relative configuration were also determined, but in spite of the CD data, the absolute configuration was tentative [8]. These compounds were later isolated from holly, *Ilex aquifolium* (Aquifolliaceae), but the wrong structures were proposed for the lactone [9] and the glucoside [10]. Later on, the structure in lit. 10 was corrected as **1** [11], and a new plant origin, *I. warburgii* Loesn. was reported [15]. The absolute structure of **1** was clarified using another plant origin, *Purshia tridentata* DC (Rosaceae), from which, a diastereomeric glucoside, purshianin (**2**) was also isolated [20]. From *Sinomennium acutum* Rehder et Wilson (Menispermaceae), we have isolated **1, 1a** and a lactone (**2a**) corresponding to **2** and two more similar lactones [13]. The absolute configuration of **1a** was determined by X-ray crystal analysis of *p*-bromobenzoate of **1a**. Compound **2a** was previously isolated from *Aquilegia atrata* var. *atroviolacea* (Ranunculaceae) and named aquilegiolide [21], and later from *Phyllanthus anisolobus* (Euphorbiaceae) along with **1a** [12].

Bauhinin (4)

Bauhinin (**4**) was isolated in 1985 from *Bauhinia championii* Benth. (Leguminosae) of Taiwan origin, and the structure was unambiguously decided by the results of single-crystal X-ray analysis of its dihydrate, and the absolute stereochemistry was based from the known absolute configuration of β-D-glucose [16].

Lophirosides (7-10)

Lophirosides (7–10) are bitter glucosides from the African Ocnaceae plant, *Lophira alata*. The geometry of the double bond was decided by the observation of a significant NOE between H-7 and H-2 after assigning all the proton signals, and the absolute stereochemistry was clarified by the application of dibenzoate chirality rule in the CD spectra of lophoriside A1 (**7**) itself, since it is one of the rare natural dibenzoate [17].



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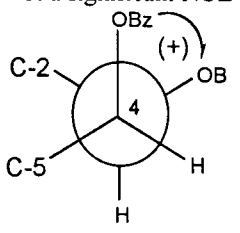
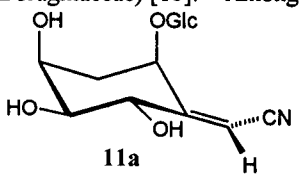


Ehretiosides (11-14)

Recently, we have isolated simmondsin (**6**) and four ehretiosides (**11-14**) from the Philippine endemic medicinal plant, *Ehretia philippinensis* (Boraginaceae) [18]. Among them, only ehretioside B (**14**) is an aromatic compound, suggesting the biogenesis of these compounds. Three alicyclic compounds, ehretiosides A1–A3 (**11–13**) had the same molecular formula, $C_{19}H_{27}O_{10}N$, and showed the characteristic CN band at 2230 cm^{-1} in the IR spectra. The



11a

^1H and ^{13}C NMR data suggested the presence of β- glucopyranosyl andsenecioyl moieties, and the structures of the aglycones were established through H-H COSY and HSQC NMR data. Methanolysis of **11** with KOH in MeOH afforded the deacylated compound (**11a**) which was identical with the compound obtained from the methanolysis of lophoriside A1 [17]. Comparison of the NMR spectra of **11** with those of **11a** led the position of the senecioyl group to be 3-O. In the same manner, structures of **12** and **13** were readily decided (Figure 1).

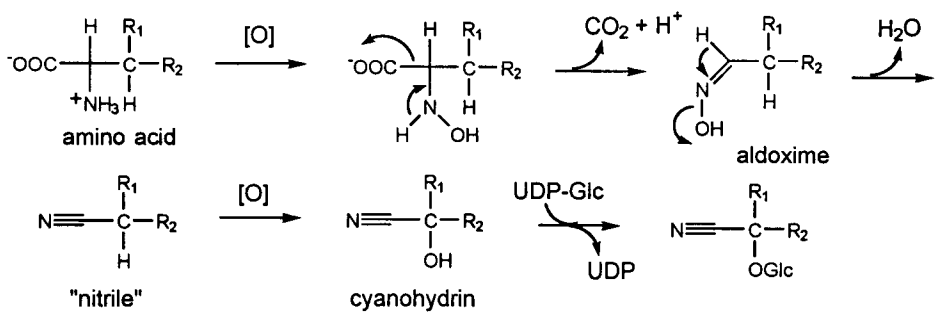
Ehretioside B (**14**) is the only aromatic compound, and the aglycone, 2,4-dihydroxyphenyl acetonitrile was previously isolated from *Erica scoparia* (Ericaceae) [19].

Isopentenyl glucosides (15-21)

Recently, sutherlandin (**15**), an unusual non-cyanogenic cyanoglucoside which has an isopentane skeleton was isolated from *Acacia sutherlandii* (Leguminosae) [22]. Following this, similar glucosides were reported [23–28] (Table 1 and Figure 1). Interestingly, all compounds have the same carbon skeleton.

Biogenesis

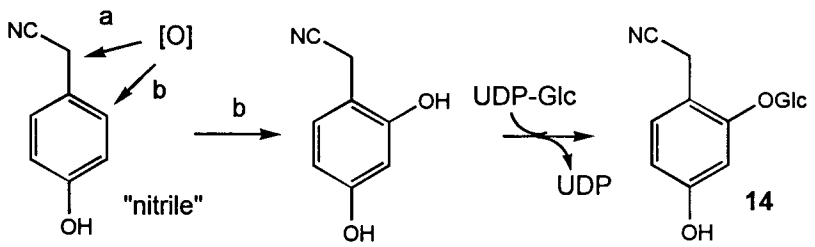
The biogenesis of cyanogenic glycosides, which have the cyano group alpha to the glycosyl bond, was established. It starts from α-amino acid as follows [29]:



$R_1 = R_2 = CH_3$: valine to linamarin

R_1=H, R_2 = phenyl: phenylalanine to prunasin

R_1 = H, R_2 = *p*-OH-phenyl : tyrosine to dhurrin



On the other hand, non-cyanogenic cyanoglycosides which possess cyano groups not adjacent to a glycosyl bond may be generated via a different pathway, but very probably from α-amino acid. Since **1–14** have the same main carbon skeleton, they might be generated from a common precursor, tyrosine. The co-existence of ehretioside B (**14**) with cyanomethylene-cyclohexyl glucosides in the same plant suggested this hypothesis. Instead of hydroxylation at the α-position (a) of the cyano group of "nitrile", if it occurs at the aromatic ortho position (b) followed by glucosylation, the target cyano glucoside (**14**) would be formed. Some of the isopentane glucosides would be derived from leucine.

Biological Activity

Most of the non-cyanogenic cyanoglucosides were isolated from plants used as medicinal, or known to have specific physiological activities. However, very few of the isolated pure compounds were reported to have biological activities (Table 1). Among these are the lophirosides (**7-10**), sarmentosin (**19**) and rhodiocyanoside A (**17**). Simmondsin (**6**) was also one of the exceptionally well studied compounds and will be discussed in detail.

Lophirosides (**7–10**) have weak anti-bacterial activity against *Micrococcus luteus* (minimum inhibitory amount = 100 μg each), but they may act as defensive substances against predatory mammals on account of their bitterness [17].

Sarmentosin (**19**), first isolated from *Sedum sarmentosum* [27], was accumulated in magpie moth. The high concentration of **19** in the imago (650 g per insect) suggests a defensive role for this substance [26].

Rhodiocyanoside A (**17**), isolated from *Rhodiola quadrifida*, was found to inhibit the histamine release by 10^{-5} to 10^{-4} in a concentration-dependent manner. It also significantly inhibited the PCA (passive cutaneous anaphylaxis) reaction, suggesting the anti-allergic activity of the plant [25].

Simmondsin (**6**) exhibited unique inhibitory activity on feeding. It was first isolated from jojoba (*Simmondsia californica*) which is a dioecious desert shrub that grows wild mainly in the south-western United States. Its seeds furnish high content of a liquid wax comprising mainly of C_{40}, C_{42} and C_{44} wax esters, as well as a meal containing 32% protein after removal of the oil which is a potential replacement for spermaceti. Incorporation of the jojoba meal into the diet of weanling rats caused extreme weight loss, and this reflected failure of the animals to consume food. From the active ethyl acetate fraction obtained from successive solvent extraction (heptane, benzene, ethyl acetate and methanol) of the jojoba seed, simmondsin (**6**) was obtained as an active substance and the chemical structure was determined. It exhibited inhibitory activity on feeding, although the oral acute toxicity (LD_{50} >4 g/kg) was extremely low [1].

Recently, the pharmacological effects of simmondsin was extensively studied by Cokelaere *et al.* using rats. The extracted and purified simmondsin from jojoba meal caused a food intake reduction in adult rats. Taste was apparently not involved because the same response was seen with intragastric intubation as oral administration.

The food intake reduction was probably due to an inhibition of hunger, rather than to an enhancement of satisfaction. Simmondsin treated with β-glucosidase and taken into gastrointestinal tract seemed to be more active than simmondsin itself [30]. Possible toxicological influences of simmondsin after subacute administration in the rat was studied, and a 5-day administration of 250 mg of simmondsin/kg of body weight did not have any toxicological influences on liver, pancreas and kidneys using several biological parameters. The non cyanogenecity of simmondsin in rats was also confirmed [31]. The influence of jojoba meal supplementation on growth and organ function in rats was also studied, and a lower food efficiency was due to an increase in T3 concentration, which can be explained by a relative protein shortage [32].

The application of simmondsin to regulate the feeding of livestock, especially domestic fowl, as well as the application to human diet food is in progress. So far, simmondsin seems to be safe, but antifertility effects in rats after long-term jojoba meal supplementation has been reported [33]. It may not be due to simmondsin, however, further study is needed to confirm the safety of this non-cyanogenic cyanoglucoside.

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STRUCTURAL ANALYSES OF GLYCOLIPIDS FROM EDIBLE MUSHROOM BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY

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Key Word Index -- glycolipid; mushroom; FAB MS; *Hypsizigus marmoreus* ; Pleurotus citrinopileatus .

Abstract The structures of glycolipids isolated from *Hypsizigus marmoreus* (Bunashimeji, a mushroom) and *Pleurotus citrinopileatus* (Nireouma, a mushroom) were determined to be (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucopyranosyl-9-methyl-C_{18}-sphinga-4,8-dienine (**1**), phosphodihexose N-(2-hydroxyoctadecanoyl)-4-hydroxy-C_{18}-sphinganine (**2**), phosphodihexose N-(2-hydroxyhexadecanoyl)-4-hydroxy-C_{18}-sphinganine (**3**), phosphodihexose N-(2-hydroxytetracosanoyl)-4-hydroxy-C_{18}-sphinganine (**4**), and phosphodihexose N-(2-hydroxydocosanoyl)-4-hydroxy-C_{18}-sphinganine (**5**). In particular, location of the double bonds in the long-chain base of **1** was clearly determined by the B/E constant linked scan method. The structure of cerebroside having a long-chain base of 9-methyl-C_{18}-sphinga-4,8-dienine could be determined in general by the presence of characteristic fragment ions of [C_{19}-sphingadienine + H-H_2O]+ at *m/z* 276 and [C_{19}-sphingadienine + H]$^+$ at *m/z* 294, and the fatty acid carbon number could be calculated from the characteristic fragment ion of [ceramide-180]$^+$ ([MH - GlcOH-180]$^+$) in positive ion mode FAB mass spectrometry. In the structural determination of 2-5, the ions of *m/z* 421 and 720 in the negative ion mode analyses are assigned to be characteristic of phosphodihexose and phytosphingosine containing phosphodihexose, respectively. This is a useful methodology for the structural determination of other unstable natural products such as lipids.

Introduction

As a part of our search for functional molecules in edible fungi, we report here the characterization and structural determination of glycolipids (**1-5**, Fig. 1) including new type of glycosyl phosphosphingolipids in *Hypsizigus marmoreus* (Bunashimeji, a mushroom) and *Pleurotus citrinopileatus* (Nireouma, a mushroom) by B/E constant linked scan fast atom bombardment (FAB) mass spectrometry [1-5].

Cerebrosides having a 9-methyl-C_{18}-sphingadienine unit were discovered independently by

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two groups almost at the same time in 1979 (Ballio et al. [6] and Karlsson et al. [7]) from an imperfect fungus, *Fusicoccum amygdali*, and from a sea anemone, *Metridium senile*, respectively. Recently, Kawai and Ikeda found a cerebroside having 9-methyl-C_{18}-sphinga-4,8-dienine in *Schizophyllum commune* (Suehirotake, a mushroom) and reported the complete structure. The long-chain base moiety with a 9-methyl branch of the cerebroside was suggested to constitute an essential part of the fruit-inducing activity in some fungi [8-11].

Recently, glycosphingolipids and sphingomyelin attract attention postulated as modulators of cell growth and cell differentiation [12,13].

Although structures of these glycolipids were analyzed in the past by gas chromatography-mass spectrometry (GC-MS) of derivatives such as trimethylsilyl (TMS) ethers [14-16], the characterization in this work, which involves determination of the fatty acid composition and the location of double bonds in the long-chain base (C_{19}-sphingadienine), is carried out by B/E linked scan FAB mass spectrometry of the glycolipids itself.

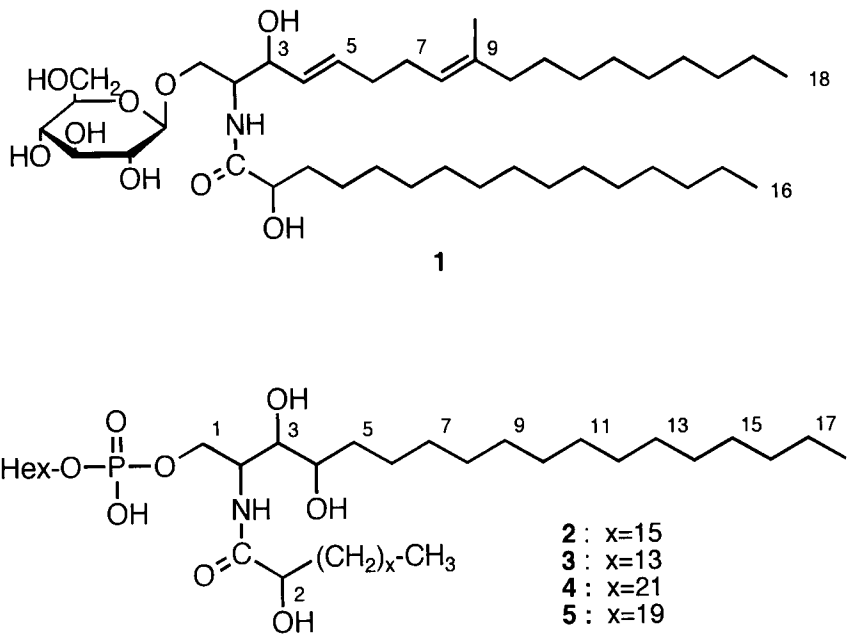

Fig. 1. Structures of glycolipids (1 - 5).

Results and Discussion

Structural elucidation of glycolipid 1

Positive and negative ion FAB mass spectra of cerebroside (**1**) are shown in Fig. 2. The positive ion mode FAB mass spectrum can be divided into three regions as reported already in Refs.17 and 18; (i) the molecular ion region, (ii) the ceramide ion region, and (iii) the long-chain base ion region. In region i, the molecular weight is successfully determined from m/z 750 $[M+Na]^+$ and *m/z* 710$[MH-H_2O]^+$ ions. In region ii, the ceramide ion peak was observed clearly at m/z 548$[MH-GlcOH]^+$. In region iii, the peaks derived from the long-chain base of C_{19}-sphingadienine were found at *m/z* 294 [C_{19}-sphingadienine+H]$^+$ and *m/z* 276 [C_{19}-sphingadienine +H-H_2O]$^+$.

The B/E constant linked scan spectra of the ceramide ion and the long-chain base ion in positive ion FAB mass spectra are shown in Figs. 3 and 4, respectively. The B/E constant linked scan spectrum of the ceramide ion indicated the fragmentation of ion *m/z* 548 at the doublly allylic position of two double bonds to give fragment A at *m/z* 368 and at the amide position to generate fragment B at *m/z* 294, and the following dehydration of fragment B to generate fragment C at *m/z* 276, as shown in Fig.3. High-resolution mass spectra of the peaks A and C at *m/z* 368 and 276, respectively, produced in the positive ion mode FAB mass spectrum of **1**, are summarized in Table 1. The possible elemental formulas calculated on the basis of the exact mass are also listed in Table 1. The B/E constant linked scan spectrum of the long-chain base ion in region iii gave the informative result shown in Fig. 4, which indicates the location of the double bonds in the long-chain. From the results of high resolution mass spectra, the fragment ions of high intensities in Fig. 3 at *m/z* 94[19] and 148 are assigned to $C_6H_8N^+$ produced by ring formation between C-6 and C-1, and $C_{10}H_{14}N^+$, respectively. On the basis of the ^{1}H-^{1}H COSY analysis of **1**, all the ^{1}H chemical shifts could be unambiguously determined, which indicated the presence of a vinylmethyl group at δ1.50(3H,s,C-19), two methylene groups of allylic positions at δ1.97(4H,m,C-6 and C-7), three olefinic protons at δ5.20(1H,br.s,C-8), 5.39(1H,dd,J=15.5, 7.1Hz,C-4), and 5.66(1H,br.d,J=15.5Hz,C-5), and an anomeric proton of a β-glucopyranoside moiety at δ 4.17(1H,d,J=7.6Hz,Glc-1). The location of the double bonds in the fragment ion at *m/z* 276 were then assigned at C2-C3, C4-C5, and C8-C9 on the basis of the above-mentioned results. The structure of the long-chain base was thus found to be 9-methyl-C_{18}-sphinga-4,8-dienine by B/E constant linked scan method. The characteristic fragment ions indicating the structure are determined to be [C_{19}-sphingadienine+H-H_2O]$^+$ at *m/z* 276 and [ceramide-180]$^+$ ([MH-GlcOH-180]$^+$) at *m/z* 368 in the positive ion mode FAB mass spectrum.

Table 1. Determination of the exact mass of molecular ion and fragment ions of compound **1**

Observed mass (error, m.m.u.)	Expected mass	Elemental formula	FAB mode	Fragment*
368.3165 (-0.5)	368.3160	$C_{22}H_{42}O_3N$	Positive ion	A
276.2697 (0.6)	276.2691	$C_{19}H_{34}N$	Positive ion	C
726.5521 (0.1)	726.5520	$C_{41}H_{76}O_9N$	Negative ion	--
312.2539 (2.1)	312.2560	$C_{18}H_{34}O_3N$	Negative ion	D
296.2589 (-0.4)	296.2585	$C_{18}H_{34}O_2N$	Negative ion	E
270.2433 (-3.4)	270.2399	$C_{16}H_{32}O_2N$	Negative ion	F
225.2218 (-0.9)	225.2209	$C_{15}H_{29}O$	Negative ion	G

*Assignments of fragment ions in Fig. 5.

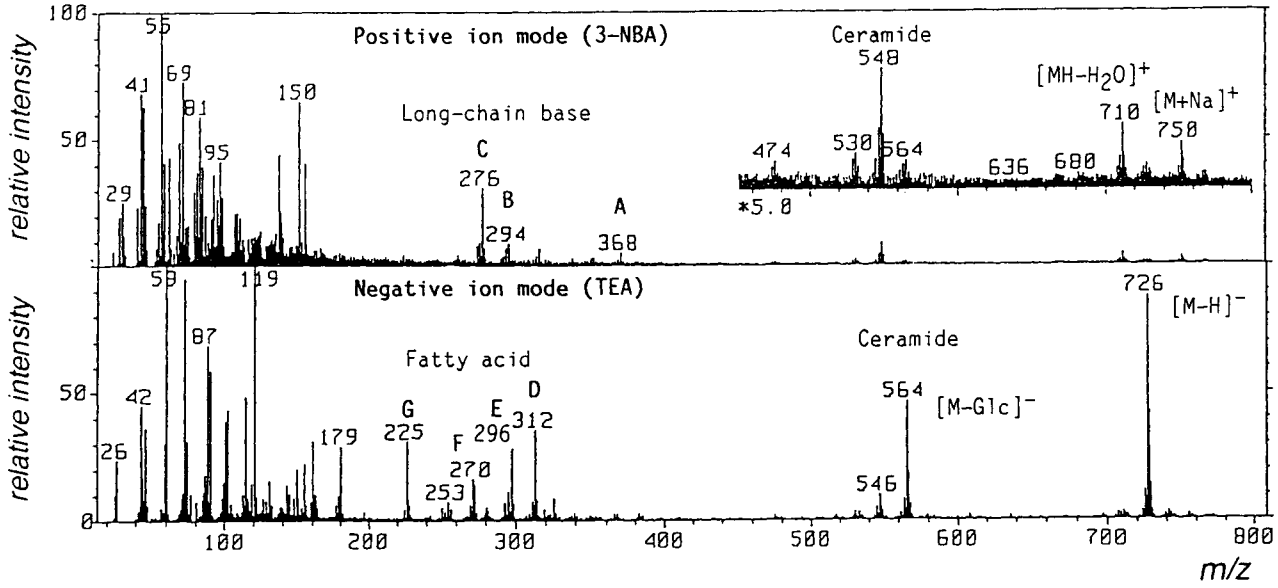

Fig. 2. FAB mass spectra of (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-glucopyranosyl-9-methyl-C_{18}-sphinga-4,8-dienine (1): (a) positive ion mode (matrix: 3-nitrobenzylalcohol); (b) negative ion mode (matrix : triethanolamine).

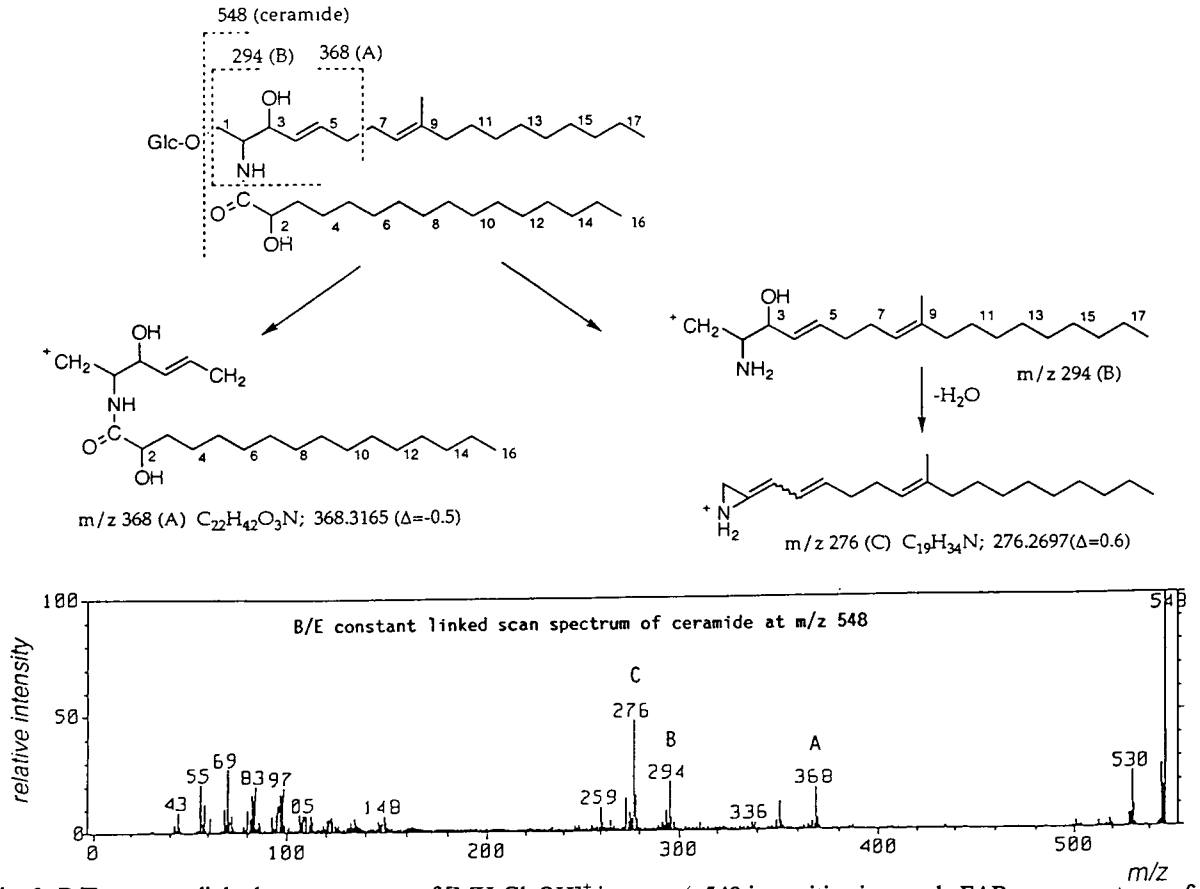

Fig. 3. B/E constant linked scan spectrum of $[MH\text{-}GlcOH]^+$ ion at *m/z* 548 in positive ion mode FAB mass spectrum of **1**.

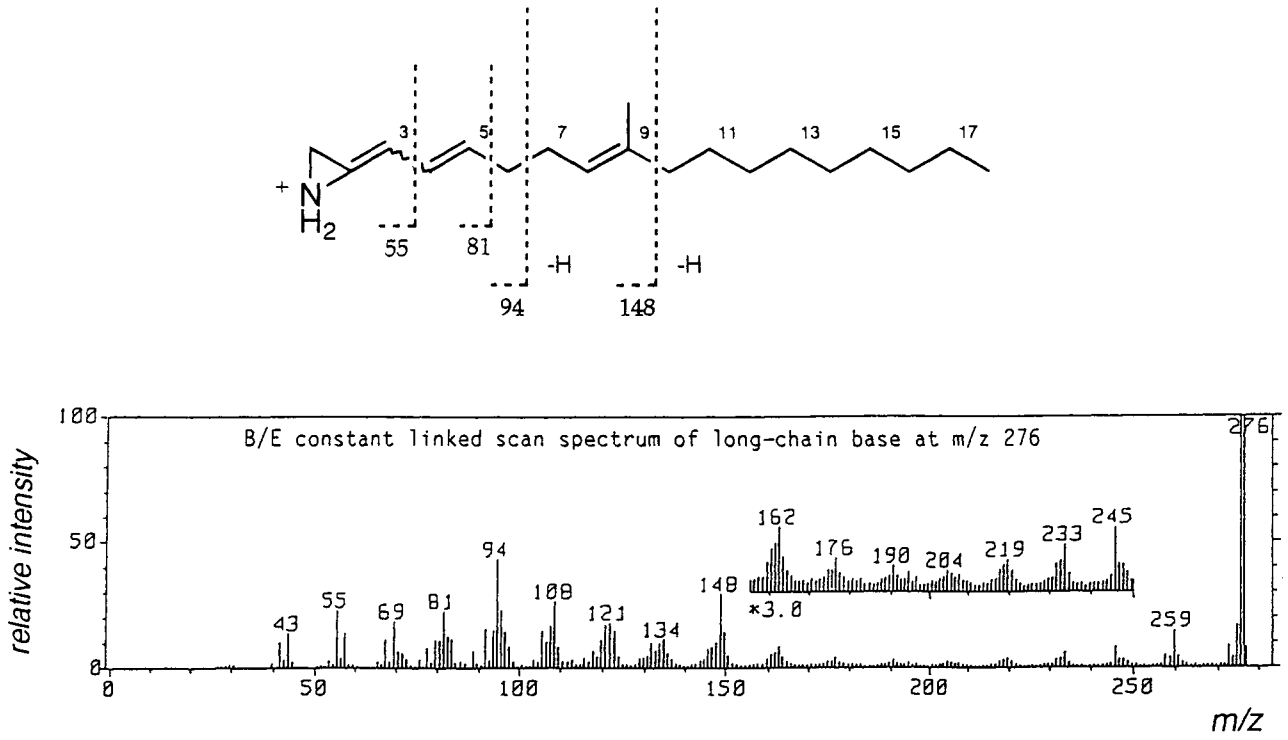

Fig. 4. B/E constant linked scan spectrum of $[C_{19}$-sphingadienine+H-H2O$]^+$ ion at *m/z* 276 in positive ion mode FAB mass spectrum of **1**.

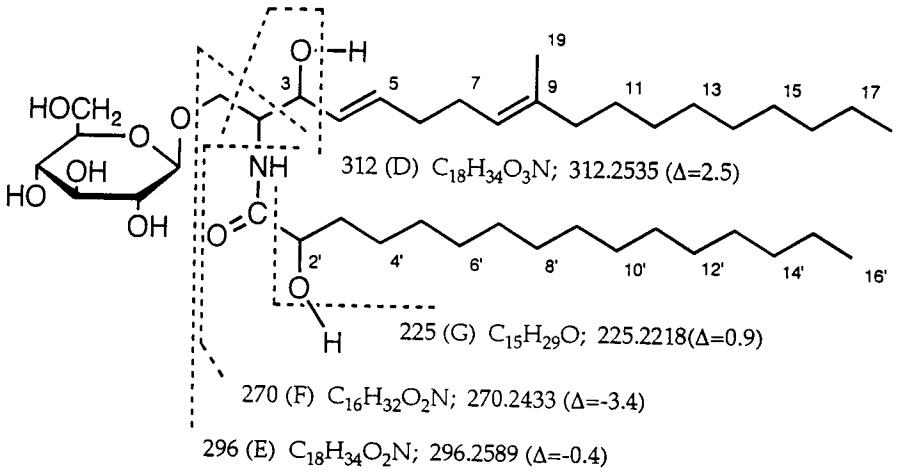

Fig. 5. Structure and fragmentations in negative ion mode FAB mass spectrum of **1**.

The molecular ion structure was not accessible from the positive ion mode FAB mass spectrum, but was more clearly elucidated by the negative ion mode experiments. The negative ion mode FAB mass spectrum was also divided into three regions [17,18]; (i) the molecular ion region, (ii) the ceramide ion region, and (iii) the fatty acid region. The molecular weight can be determined from the intense peak at *m/z* 726 $[M-H]^-$. The exact mass of the ion at *m/z* 726 in the negative ion mode is also included in Table 1, which clearly indicates the elemental formula in accordance with the proposed structure. The spectra in the ceramide ion region showed the presence of two ions at *m/z* 564 $[M-Glc]^-$ and *m/z* 546 $[M-H-GlcOH]^-$. The spectra in the fatty acid ion region showed fragment ions due to $[C_{14}H_{29}\text{-}CH(OH)\text{-}CONH\text{-}(CH\text{-}CH_2)O\text{-}H]^-$ at *m/z* 312, $[C_{14}H_{29}\text{-}CH(OH)\text{-}CONH\text{-}CH{=}CH_2\text{-}H]^-$ at *m/z* 296, $[C_{14}\text{-}H_{29}\text{-}CH(OH)\text{-}CONH_2\text{-}H]^-$ at *m/z* 270 and $[C_{13}H_{27}\text{-}(CH\text{-}CH_2)O\text{-}H]^-$ at *m/z* 225, which correspond to the fragmentations D, E, F, and G, respectively, in Figs. 2 and 5. The exact mass of fragments D, E, F, and G in the negative ion mode are also listed in Table 1. Consequently, the structure of fatty acid residue was found unequivocally to be 2-hydroxyhexadecanoic acid, which is supported by the result of positive ion mode analysis.

Based on the evidence of positive and negative ion mode FAB MS, the structure of cerebroside (1) was determined to be (4E,8E)-N-2-hydroxyhexadecanoyl-1-O-β-gluco-pyranosyl-9-methyl-C_{18}-sphinga-4,8-dienine (Fig. 1), which had been isolated from Schizophyllum commune by Kawai and Ikeda [8] as a fungal fruiting body inducer.

For glucosylceramides (cerebrosides) having the long-chain base of 9-methyl-C_{18}-sphinga-4,8-dienine, it might be concluded that the presence of characteristic fragment ion peaks at *m/z* 276 and 294 are accepted as the diagnosis of the long-chain base, and the fatty acid carbon number can be calculated from the characteristic fragment ion of $[ceramide\text{-}180]^+$ ($[MH - GlcOH\text{-}180]^+$) in positive ion mode FAB mass spectrum.

These data might contribute to the determination of structures of cerebrosides having the long-chain base of 9-methyl-C_{18}-sphinga-4,8-dienine, which are widely distributed among fungi.

Table 2. Determination of the exact mass of molecular ion and fragment ions of compounds **2-5**

Observed mass (error, m.m.u.)	Expected mass	Elemental formula	FAB mode
1002.6110 (-2.0)	1002.6130	$C_{48}H_{93}O_{18}NP$	Negative ion
974.5806 (-1.1)	974.5817	$C_{46}H_{89}O_{18}NP$	Negative ion
720.3572 (0.0)	720.3572	$C_{30}H_{59}O_{16}NP$	Negative ion
421.0724 (-2.3)	421.0747	$C_{12}H_{22}O_{14}P$	Negative ion
1086.7090 (2.0)	1086.7070	$C_{54}H_{105}O_{18}NP$	Negative ion
1058.6780 (2.3)	1058.6757	$C_{52}H_{101}O_{18}NP$	Negative ion

Structural elucidation of glycolipids 2-5

The molecular ion structures were not accessible from the positive ion mode FAB mass spectra, but were more clearly elucidated by the experiments of the negative ion mode. The negative ion FAB mass spectra of fraction II and fraction III are shown in Fig. 6. The molecular weights can be determined from intense peaks of $[M-H]^-$ in Figs. 6a and 6b, at *m/z* 1002 and 974, and 1086 and 1058, respectively. The difference of 28 a.m.u. suggests that these fractions are mixtures containing two molecular species. The exact mass of these molecular ions determined by the negative ion mode are listed in Table 2. The methanolysis of these fractions gave mixtures containing two FAMEs with the difference of carbon numbers as indicated above.

Structural elucidation of glycosyl phosphosphingolipids 2 and 3 in FAB mass spectrum of fraction II

The peaks at *m/z* 924 and 896 in Fig. 6a were assigned to fragment ions due to $[M-Hex]^-$ and peaks at *m/z* 678 and 650 to fragment ions due to $[M-(Hex)_2]^-$.

The B/E constant linked scan spectra of the molecular ions *m/z* 1002 and 974 indicated that fragmentations occurred at the amide position to generate fragment A at *m/z* 720, and at the polar groups to generate fragment B at *m/z* 421. Subsequent loss of a hexose from fragment B generated fragment C at *m/z* 259, and then loss of a phosphate group took place giving rise to ions of *m/z* 97 and 79, as shown in Fig.7. The results of high-resolution mass spectra of peaks A and B at m/z 720 and 421, respectively, produced in the negative ion mode FAB mass spectra (Fig. 6a), are summarized in Table 2. The possible elemental formula calculated on the basis of the exact masses are also listed in Table 2. From the results of high-resolution mass spectra, the fragment ions in Fig.7 at *m/z* 259, 97 and 79 are assigned to $C_6H_{12}O_9P^-$, $H_2O_4P^-$ and O_3P^-, respectively. The characteristic fragment ion at *m/z* 259 closely resembled the phosphoinositol part of phosphoinositol N-stearoyl-sphinganine which had been previously isolated from Leishmania by Singh et al.[10,11]. Moreover, the long-chain base can be elucidated as a phytosphingosine with a eighteen carbon chain (4-hydroxy-C_{18}-sphinganine) on the basis of a characteristic frag-ment ion at *m/z* 720. Consequently, the fatty acid carbon numbers can be calculated from the difference between the molecular ions and the characteristic ion at m/z 720, which indicated the presence of 2-hydroxyoctadecanoic acid for the compound of m/z 1002 and 2-hydroxyhexa-decanoic acid for the compound of *m/z* 974. The structures of these compounds having peaks at *m/z* 1002 and 974,respectively, in the negative ion spectrum(Fig. 6a) were thus found to be phosphodihexose N-(2-hydroxyoctadecanoyl)-4-hydroxy-C_{18}-sphinganine(**2**) and phospho-dihexose N-(2-hydroxyhexadecanoyl)-4-hydroxy-C_{18}-sphinganine(**3**) as shown in Fig.1

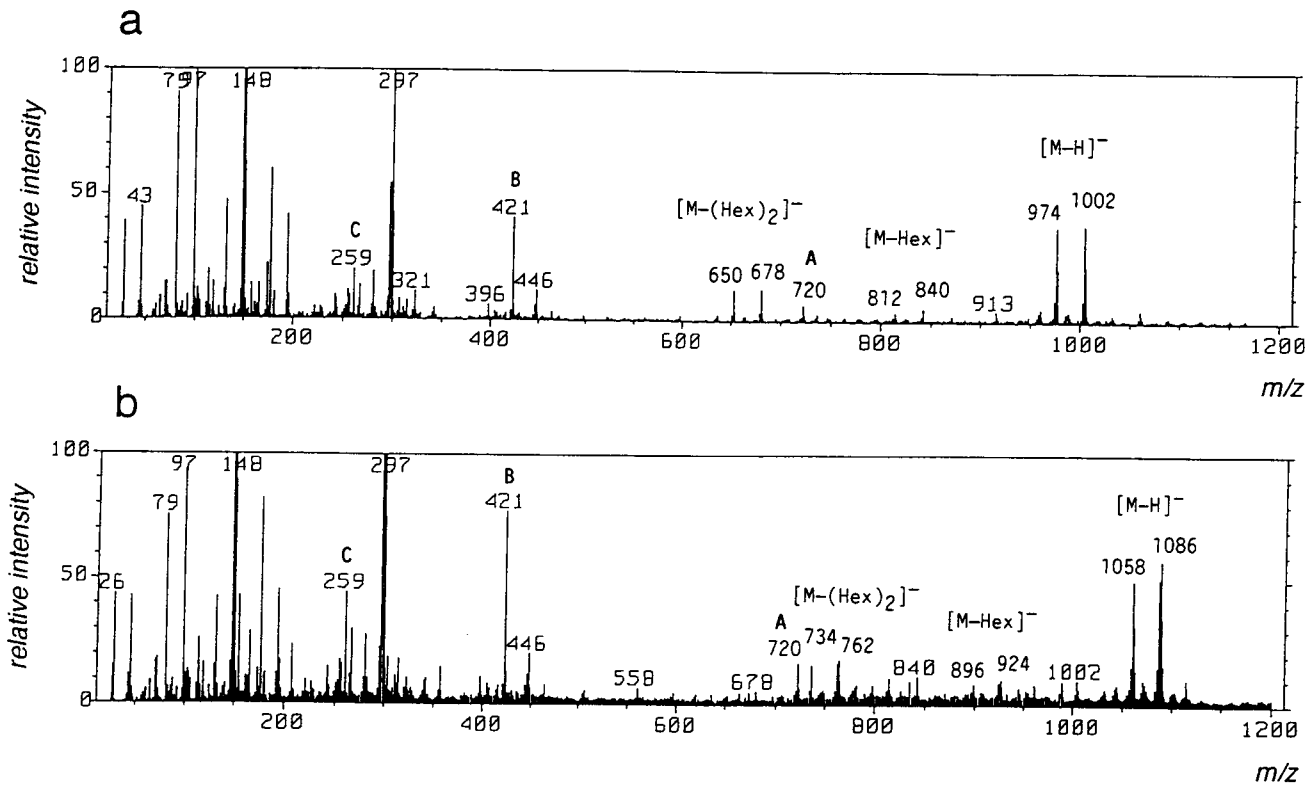

Fig. 6. FAB mass spectra of phosphodihexose-sphingolipids (2 - 5) : (a) negative ion mode spectrum of fraction II (matrix : triethanolamine) ; (b) negative ion mode spectrum of fraction III (matrix : triethanolamine).

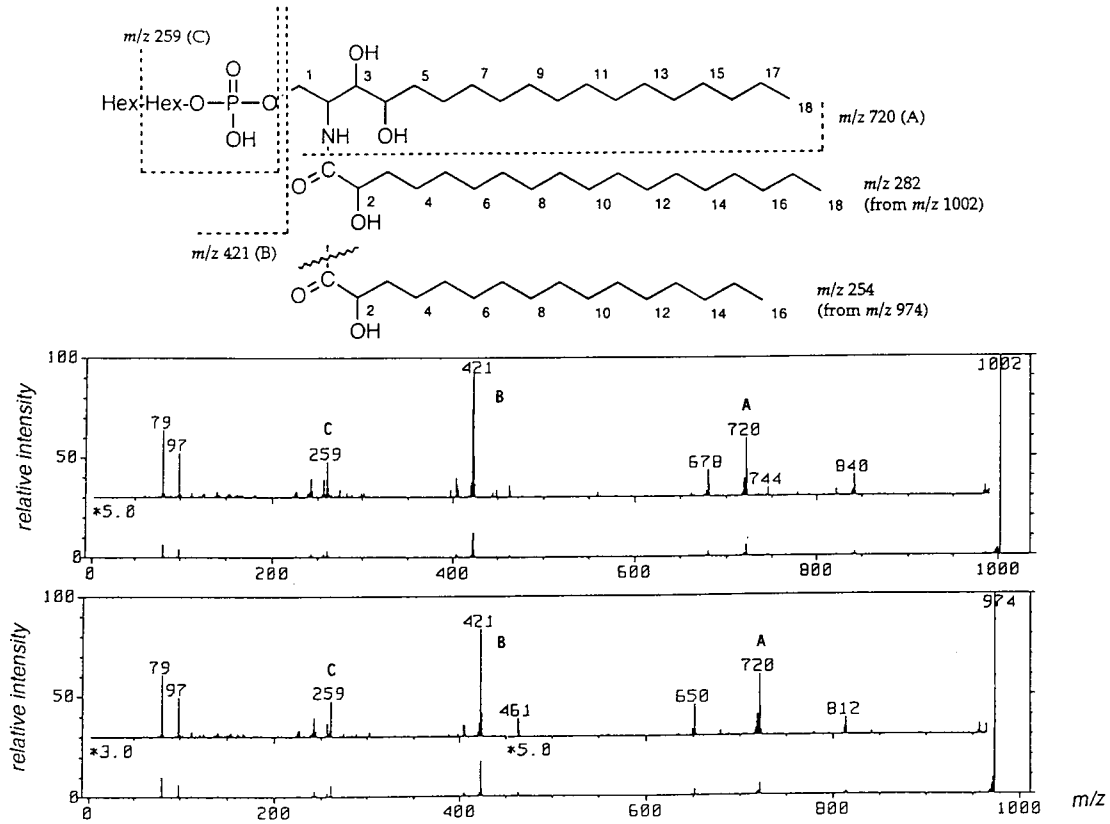

Fig. 7. B/E constant linked scan spectra of $[M-H]^-$ ions at *m/z* 1002 and 974 in negative ion mode FAB mass spectrum of fraction II.

Structural elucidation of glycosyl phosphosphingolipids 4 and 5 in FAB mass spectrum of fraction III

The structures of **4** and **5** were similarly determined as those of **2** and **3**, as follows. The two peaks at *m/z* 421 and 720 in the negative ion spectrum (Fig. 6b) indicated the presence of a phosphodihexose and a phytosphingosine containing phosphodihexose, respectively. The ions at *m/z* 259, 97, and 79 also support the structures of the phosphodihexoses. Consequently, the structures of fatty acids are determined to be 2-hydroxytetracosanoic acid for the compound of *m/z* 1086 and 2-hydroxydocosanoic acid for the compound of *m/z* 1058 from the characteristic fragment ion at m/z 720. The B/E constant linked scan spectra of the molecular ions *m/z* 1086 and 1058 (Fig. 8) supported the above-mentioned postulation. The structures of these compounds having peaks at m/z 1086 and 1058, respectively, in the negative ion spectrum (Fig. 6b) were thus found to be phosphodihexose N-(2-hydroxytetracosanoyl)-4-hydroxy-C_{18}-sphinganine (**4**) and phosphodihexose N-(2-hydroxydocosanoyl)-4-hydroxy-C_{18}-sphinganine (**5**) as shown in Fig. 1.

Thus, the ions of *m/z* 421 and 720 in the negative ion mode are found to be very useful for the structural determination of glycosyl phosphosphingolipids (phosphodihexose-sphingolipids) in the analyses by FAB mass spectrometry, because these ion peaks are characteristic of a phosphodihexose and a phytosphingosine (4-hydroxy-C_{18}-sphinganine) containing phosphodihexose, respectively.

FAB mass spectrometry is a powerful tool for analyzing the structure of glycolipids. Especially in combination with a linked scan at constant B/E, it provides unambiguous information on the fatty acid composition, the location of double bonds in the long-chain base (C_{19}-sphingadienine), or the presence of phosphodihexose.

Experimental

Isolation of Glycosyl Phosphosphingolipids. Fresh fruiting bodies (1.4 kg) of Hypsizigus marmoreus were kept in hot H_2O at 96° for 20 min, chopped by a commercial blender, and homogenized after making up the total volume to 1.5 l with hot H_2O. Cold EtOH (3.5 l) was added to the hot H_2O homogenate, and the mixture was allowed to stand overnight in the dark. The H_2O-EtOH extract (68.5 g) was extracted with n-hexane and n-BuOH, successively. The n-BuOH extract (4.7 g) was chromatographed over an Amberlite XAD-2 column (Japan Organo Co. Ltd., 2.3 x 24 cm). The loaded column was washed with 500 ml of H_2O, and successively eluted with 20% MeOH-H_2O, 50% MeOH-H_2O, and MeOH (500 ml each). The solvent-removed H_2O eluate (2.7 g) was chromatographed over silica gel (Wako gel C-300, Wako Pure Chemical Industries Ltd.) with $CHCl_3$-MeOH-H_2O (60:29:6) as an eluent to obtain fraction I (1335 mg), fraction II (22 mg), and fraction III (218 mg). The fraction I was rechromatographed over silica gel (Wako gel C-300) with $CHCl_3$-MeOH (5:1) as an eluent, and 85 mg of a pure cerebroside (1) was obtained. The fractions II and III were analyzed by FAB MS to have negative ion spectra shown in Figs. 6a and 6b, respectively.

Methanolysis of Fractions II and III. 3 mg of each fraction was heated at reflux with 0.9M HCl in 82% MeOH (1 ml) for 18 hr [21], respectively. The reaction mixture was extracted with n-hexane, the n-hexane layer was concentrated *in vacuo*, and the residue was analyzed by GC-MS to give the fatty acid methyl esters (FAMEs); methyl 2-hydroxy-

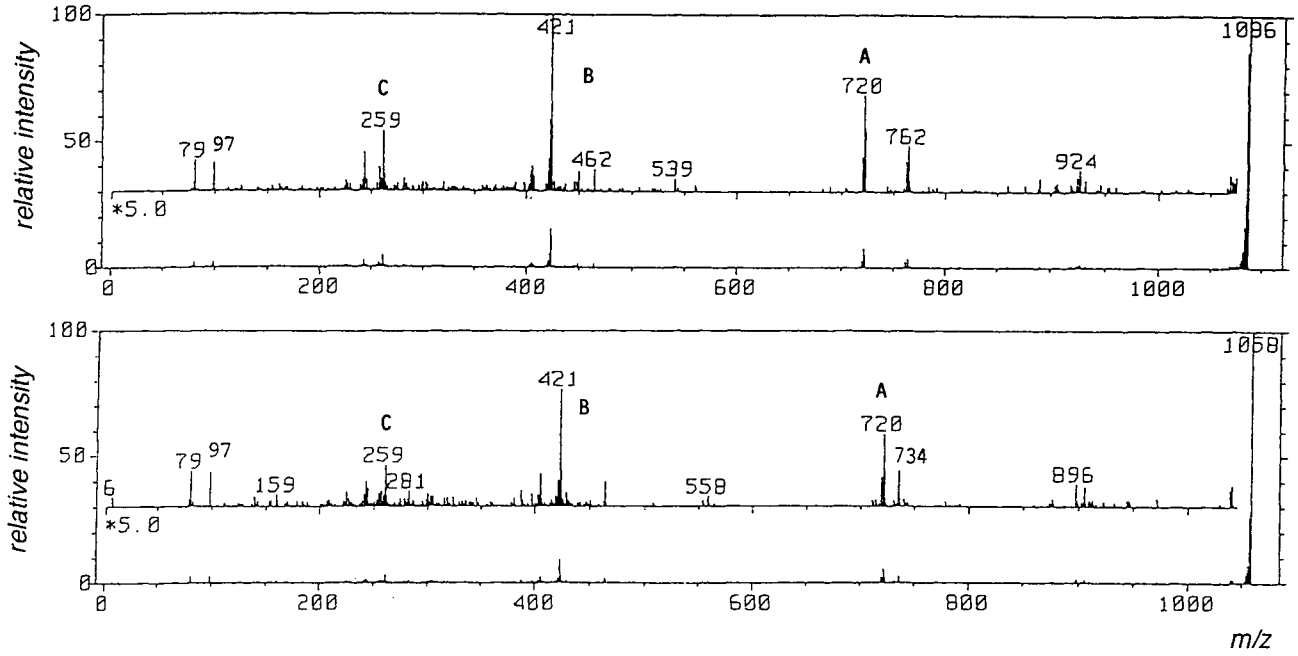

Fig. 8. B/E constant linked scan spectra of $[M-H]^-$ ions at *m/z* 1086 and 1058 in negative ion mode FAB mass spectrum of fraction III.

octadecanoate: *m/z* 314 $[M]^+$, 255 [M-59 ($COOCH_3$)] and methyl 2-hydroxyhexadecanoate: *m/z* 286 $[M]^+$, 227 [M-59] for fraction II; methyl 2-hydroxytetracosanoate: *m/z* 398 $[M]^+$, 339 [M-59] and methyl 2-hydroxydocosanoate: *m/z* 370 $[M]^+$, 311 [M-59] for fraction III, respectively.

Conditions of FAB mass spectrometry. Mass spectrometry was carried out with a JEOL JMS-HX 100 double-focusing mass spectrometer fitted with a high-field magnet and a FAB ion source. The mass spectrometer equipped with a post-accelerating detector was operated at an accelerating voltage of 5 KV. The sample was dissolved in chloroform-methanol (1:1 ; 1 mg/ml) for glycolipid **1** or water-methanol (5:1 ; 1 mg/ml) for glycolipids **2** - **5** and 1 ml of the solution was added to the matrix (triethanolamine for the negative ion mode) on the stainless-steel FAB probe target. The sample was bombarded with a 6 KeV xenon atom beam. The exact mass measurement by FAB mass spectrometry was carried out using a mixture of cesium iodide, sodium iodide, and glycerol (5:1:25) as a mass calibrant. The experiment of linked scan (B/E constant scan) method using a JEOL HX 100 mass spectrometer fitted with a DA 5000 data system was carried out with He gas introduced into a collision chamber placed in the first field-free region of the EB geometry to obtain spectra of the daughter ions.

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SYNTHETIC APPROACH TO INTENSELY SWEET GLYCOSIDES: BAIYUNOSIDE & OSLADIN

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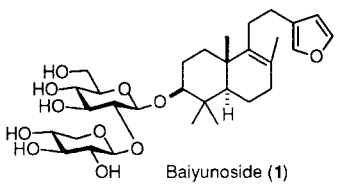
Abstract: Novel synthetic approaches into intensely sweet glycosides, baiyunoside and osladin, have been developed. Baiyunoside, a diterpene glycoside isolated from Chinese drug Bai-Yun-Shen, is 250 times sweeter than sucrose. The aglycone of baiyunoside, baiyunol, have been synthesized as racemate by means of the biomimetic olefin cyclization induced by mercury (II) triflate. Introduction of sugar residue afforded natural baiyunoside and stereoisomers or analogs with other sugar residues. One analog was sweeter than natural product, while some were very bitter. Well-known saponin osladin is the sweet principle of a fern, *Polypodium vulgare* (Polypodiaceae). Although we accomplished the total synthesis of the saponin corresponding to the reported structure for osladin, it was not sweet at all. Reextraction of the rhizomal sweet principle of the fern and a single crystal X-ray diffraction study revealed its real structure to be isomeric 26-*O*-α-L-rhamnopyranosyl-(22*R*,25*S*,26*R*)-22,26-epoxy-6-oxo-5α-cholestan-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside. The synthesis of the real osladin was achieved by using a newly developed β-selective and 2' hydroxyl group discriminating glucosylation procedure and an α-selective thermal rhamnosylation reaction. Synthetic osladin was very sweet, thus showing that osladin is proved to be the real sweet principle of the fern.

Introduction

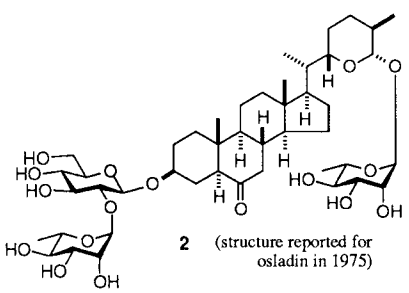
A large number of studies on artificial sweet tasting compounds have been carried out to develop additional noncaloric dietary sweeteners. Saccharin, dulcin, sodium cyclamate, and aspartame are well known. Intensely sweet tasting substances are also widely distributed in nature particularly in plant kingdom. These materials have been an exciting research area for natural product chemists. Among a variety kind of sweet tasting natural products, many of them are glycosides of terpenoids or steroids.[1,2,3] Since the sweet tasting natural glycosides have not been a subject of organic synthesis, we have been interested in developing synthetic approaches to these natural products.

Baiyunoside (**1**) is the sweet principle of a Chinese drug *Phlomis betonicoides* (Labiatae).[2] Through mercury (II) triflate induced biomimetic olefin cyclization followed by

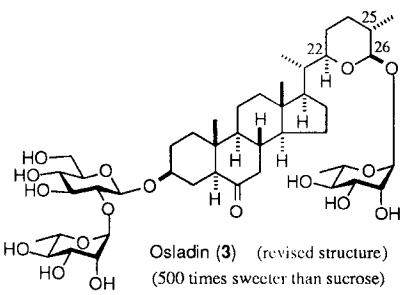
glycosylation, we have completed the total synthesis of **1** and a number of analogs. Among many synthetic analogs of **1**, a glycoside of (+)-baiyunol with α-glucose-β-glucose was sweeter than the natural product. Some analogs were bitter.[4] A fern metabolite osladin (**2**) is the intensely sweet glycoside of steroid isolated from the fern *Polypodium vulgare*.[3] Total synthesis of **2** showed that **2** is not sweet at all. Reinvestigation of the sweet principle of the fern by X-ray crystallography showed that the real structure of osladin is **3**, in which the stereochemistry at C-22, 25, and 26 is revised.[5] Total synthesis of **3** confirmed that osladin (**3**) is the real sweet principle of *P. vulgare*.[6]



Baiyunoside (**1**)
(250 times sweeter than sucrose)



2 (structure reported for osladin in 1975)

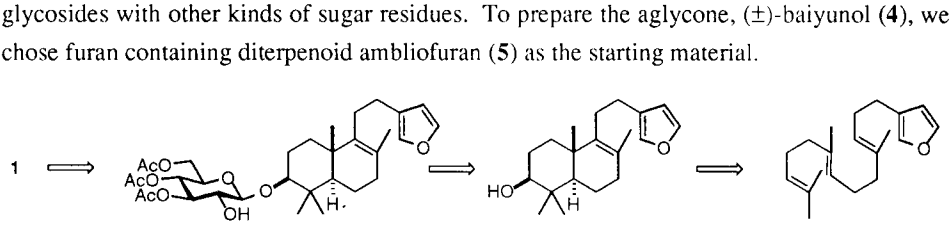


Osladin (**3**) (revised structure)
(500 times sweeter than sucrose)

Results and Discussion

1. Synthesis of Baiyunoside and Analogs

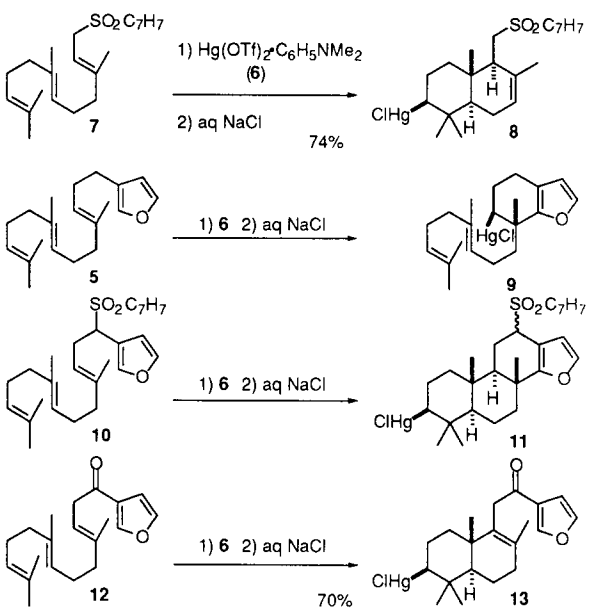
Baiyunoside (**1**) was isolated from the Labiatae plant, *Phlomis betonicoides* (Chinese name Bai-Yun-Shen) by Tanaka and coworkers, as the sweet principle that is 250 times sweeter than sucrose.[2] Since we have developed a very convenient synthetic tool to prepare the labdane skeleton, we decided to begin the synthetic study of this novel sweet compound. Our approach to construct **1** is based upon a stepwise introduction of sugar moieties into the racemic aglycone. This strategy allows us to prepare a large number of stereoisomers and glycosides with other kinds of sugar residues. To prepare the aglycone, (±)-baiyunol (**4**), we chose furan containing diterpenoid ambliofuran (**5**) as the starting material.

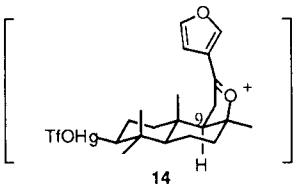


(±)-Baiyunol (**4**)

Ambliofuran (**5**)

Mercury triflate amine complex, $Hg(OSO_2CF_3)_2 \cdot C_6H_5NMe_2$ (**6**) is a powerful electrophile which is highly effective for cyclization of polyisoplenoids.[8] For example the cyclization of farnesyl sulfone **7** with **6** afforded bicyclic organomercuric compound **8** in 74% yield. This reagent will be useful to construct the framework of the aglycone of baiyunoside (**1**). In the cyclization of **5** with **6**, we however came across an abnormal product **9**. The product was generated by a cyclization from an internal double bond and this result suggests that the furan ring induces the cyclization at the adjacent olefin. In contrast, cyclization of sulfone **10** was initiated from the terminal double bond to give tetracyclic product **11** as the major product.[9] Thus the cyclization mode must be controlled not only the initiation but also the termination to prepare the labdane skeleton. Then we studied the conjugated ketone **12**. The furan ring of **12** should be deactivated by the electron withdrawing carbonyl group. As is the case, this carbonyl group controls the mode of cyclization dramatically. Cyclization was initiated only from the terminal double bond and terminated at the bicyclic stage. Moreover deprotonation to give the desired tetrasubstituted double bond became the major reaction course leading to **13** in 70% yield. Very stable cationic intermediate **14** might be generated in the reaction flask and the proton at C-9 is favor to eliminate due to stereoelectronic effects. Generally, it will be possible to control a biomimetic olefin cyclization by introducing a carbonyl group in the appropriate position of polyene chain.[13]

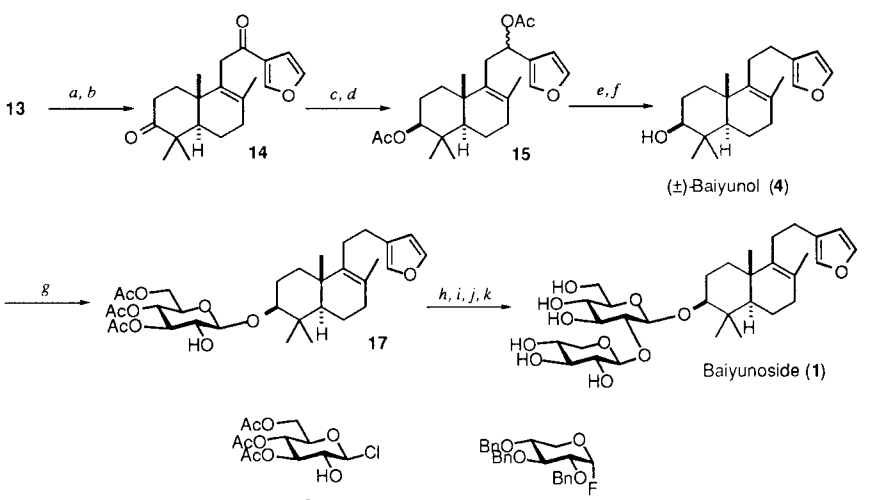




14

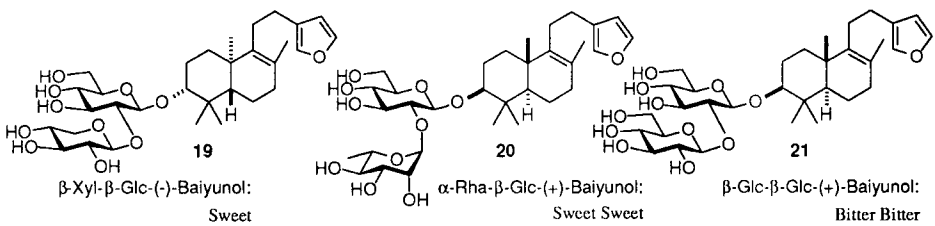
The organomercuric compound **13** was transformed into diketone **14** by hydroxylation with oxygen in the presence of $NaBH_4$ followed by Jones oxidation. LAH reduction and acetylation provided diacetate **15** as a mixture of diastereomers, and which was converted to (±)-baiyunol (**4**) by Li/NH_3 reduction followed by hydrolysis. Thus the aglycone **4** was obtained as a racemate by rather simple operation in good overall yield.[10, 11]

Next we focused on the glycosylation chemistry. Classic Koenigs-Knorr type glycosylation of (±)-**4** was carried out with 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride (**16**) in the presence of silver triflate and tetramethylurea (TMU) to give glycosides **17** as a diastereomeric and anomeric mixtures in a 2' hydroxyl group discriminated manner. The four stereoisomers were separated by HPLC and characterized by enzymatic hydrolysis leading to the optically active baiyunol. Since xylosylation of **17** by Koenigs-Knoll method was not successful, we employed a glycosylation based on the strong affinity between silicon and fluorine atoms developed by Noyori and coworkers.[12] The hydroxyl group of **17** was first converted into the corresponding silyl ether and then treated with xylosyl fluoride **18** in the presence of catalytic amount of TMS triflate. Disaccharides were obtained in reasonable yield with moderate β-selectivity. HPLC separation followed by deprotection afforded baiyunoside (**1**). It was very sweet.[13]



a: O_2, $NaBH_4$, DMF *b*: Jones oxidation *c*: LAH *d*: Ac_2O, pyridine *e*: Li, NH_3 *f*: LiOH *g*: **16**, AgOTf, TMU *h*: TMSCl *i*: **18**, TMSOTf *j*: Li, NH_3

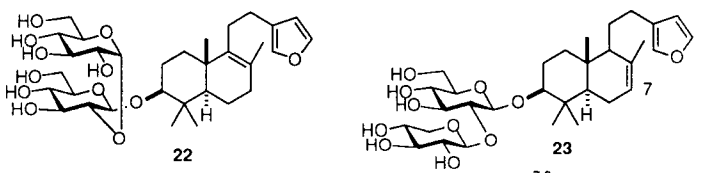
The three stereoisomers of **17** were also converted into the corresponding disaccharide by the same operation. Alternative sugar residues such as glucose and rhamnose were also introduced to **17** and its stereoisomers. Therefore, we have prepared 24 kinds of baiyunoside analogs and tested their sweetness. For example a diastereomer of baiyunoside, unnatural (-)-baiyunol with β-glucose and β-xylose (**19**), was moderately sweet. The disaccharide of (+)-baiyunol with β-glucose and α-rhamnose (**20**) was as sweet as baiyunoside, but (+)-baiyunol with β-glucose and β-glucose (**21**) was very bitter. These two glycosides, **20** and **21**, are minor components of Bai-Yun-Shen.[2] However, (+)-baiyunol with β-glucose and α-glucose (**22**) was sweeter than baiyunoside itself. Glycoside of $\Delta^{7,8}$-baiyunol with β-glucose and β-xylose (**23**) was as sweet as baiyunoside. Therefore throughout this investigation, we learned that taste changes dramatically upon very minor structural modifications.[14]



19
β-Xyl-β-Glc-(-)-Baiyunol:
Sweet

20
α-Rha-β-Glc-(+)-Baiyunol:
Sweet Sweet

21
β-Glc-β-Glc-(+)-Baiyunol:
Bitter Bitter



22
α-Glc-β-Glc-(+)-Baiyunol:
Sweet, Sweet, Sweet

23
$\Delta^{7,8}$-Baiyunoside:
Sweet Sweet

2. Osladin, A Historical Background and Summary

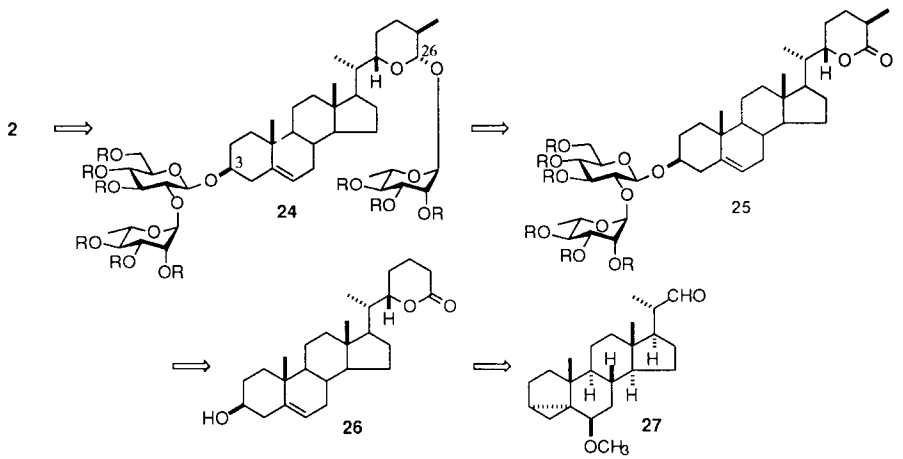
It is well known that the rhizome of the European fern *Polypodium vulgare* (Polypodiaceae) is intensely sweet. In 1967, Jizba and Herout reported the isolation of a structurally new saponin as the sweet tasting principle, and named it osladin based on the Czech name of this fern, osladic.[3] In 1971, they reported its structure.[15] Shortly thereafter, Havel and Cerny achieved a partial synthesis of the aglycone from solasodine, and established the absolute stereochemistry.[16] While the stereochemistry of the glucosidic bond was determined to be β based on an enzymatic hydrolysis using β-glucosidase, the stereochemistry of two rhamnosidic bonds as well as the stereochemistry at C-26 were not determined. Although the structure of **2** was not completely assigned, this compound became well known due to its intense sweetness. Farnsworth described that **2** is 3,000 times sweeter than sucrose in a review article.[17] We turned our interest towards a synthesis of the sweetest

glycoside, osladin. If the synthesis were realized, it would be the first synthesis of a saponin.[21]

Therefore, we found following thing throughout the synthetic study. 1) We achieved the total synthesis of saponin **2** that was found not to be sweet. This suggests that **2** is not the structure of the natural saponin osladin. 2) We isolated natural osladin from the rhizomes of the fern, *P. vulgare*. A single crystal X-ray diffraction study of natural osladin shows that it is the stereoisomeric compound **3**.[5] Furthermore, osladin is only 500 times sweeter than sucrose. 3) A total synthesis of **3** was achieved and thus the sweet principle of this fern was proved to be osladin.[6]

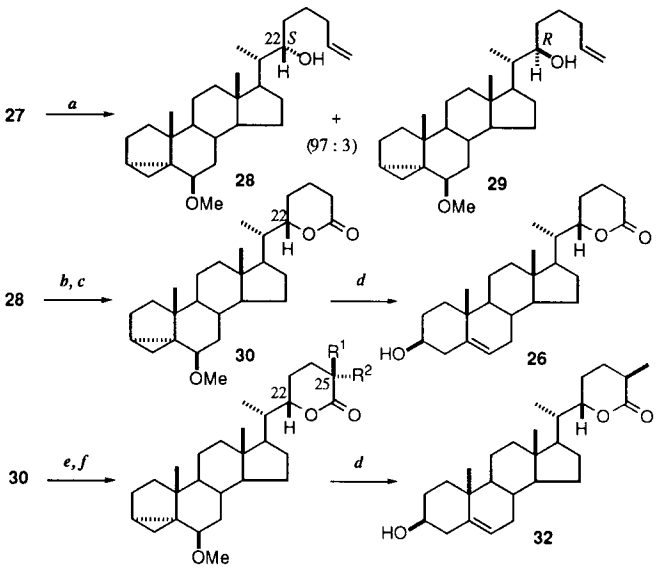
3. Synthesis of Compound 2

A retrosynthetic analysis suggests **24** as an advance intermediate toward the synthesis of **2**. Intermediate **24** will be accessible from disaccharide lactone **25**. Modification of steroidal aldehyde **27** into **26** and subsequent stereoselective glycosylation of **26** are anticipated to produce lactone **25**. The starting material **27** of this synthesis is easily accessible from commercially available (-)-stigmasterol. Sugar residues are used as protecting groups for the C-3 and 26 oxygen functionalities and are introduced in the early stages of the synthesis. Glycosylation of the hemiacetalic hydroxyl group at C-26 must be stereoselective.



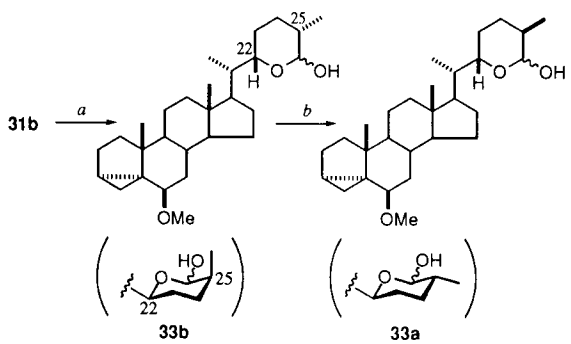
Grignard reaction of 4-pentenyl magnesium bromide with aldehyde **27** afforded alcohols **28** and **29** in 89% yield in a 97:3 ratio. At this point the stereochemistry of the C-22 center of **28** and **29** was not clear. Compound **28** was easily transformed into lactone **30** by ozonolysis followed by an oxidation of the resulting hemiacetal. Triflic acid catalyzed solvolysis of **30** afforded lactone **26** in 97% yield. The stereochemistry at C-22 of **28** was

determined to be *S* by an X-ray analysis of lactone **32** that was prepared by several steps from **28** through **30**. Methylation of **30** afforded two isomers **31a** and **31b**. Triflic acid catalyzed hydrolysis of **31a** afforded **32**. Thus the stereoselectivity of the Grignard reaction of **27** follows Cram's rule to provide C-22 *S* alcohol **28** as the major product.[19]



a: CH_2=CH(CH_2)$_3$MgBr, ether *b*: O_3, Sudan III, C_2H_5OH-H_2O, then $(CH_3)_2S$ *c*: PDC, CH_2Cl_2 *d*: TfOH (0.005 eq), dioxane-H_2O *e*: LDA, HMPA, THF, then CH_3I *f*: HPLC

Since compound **2** has C-25 *R* configuration, we attempted to isomerize the C-25 methyl group of **31ab**. Though neither under kinetic conditions nor thermodynamic conditions the 1:1 ratio of **31ab** changed, the DIBALH reduction product **33b** was isomerized into a 10:1 mixture of **33a** and **33b** under thermodynamic conditions.



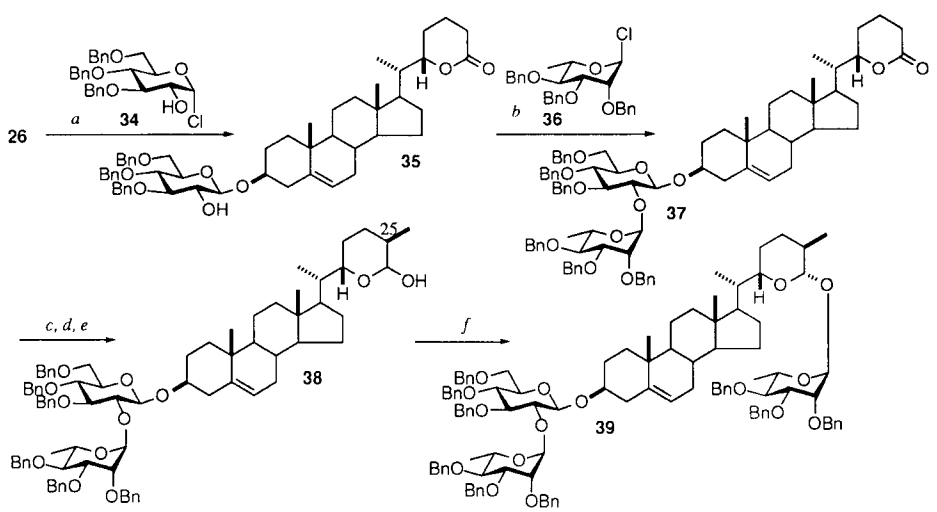
a: DIBALH, PhMe *b*: NaOMe, MeOH

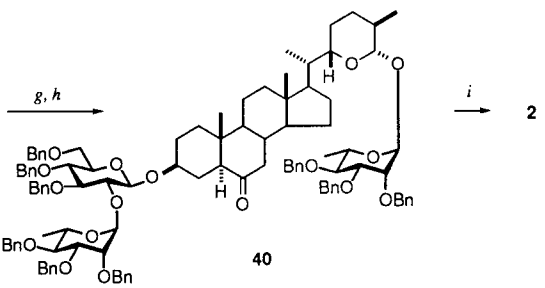
In this synthetic study we intended to use glycosyl moieties as protecting groups for the C-3 and C-26 hydroxyl functionalities. Glucosyl chloride **34** and rhamnosyl chloride **36** were employed as glycosylation donors. A 2'-discriminating and β-selective glucosylation of **26** was achieved by using glucosyl chloride **34**, a catalytic amount of triflic acid (0.05 equiv), and tetramethylurea as an acid scavenger to give β-glucoside **35** in 59% yield. The increased steric hindrance around C-2' limits the second glucosylation.

The α-rhamnoside linkage was introduced with the highly α-selective thermal glycosylation reaction that we recently developed.[20] A mixture of monoglycoside **35**, rhamnopyranosyl chloride **36** and α-methylstyrene (week acid scavenger) was heated at 80 °C for 60 h without solvent to give disaccharide **37** in 59% yield. The newly formed rhamnoside bond was found to be completely α. The stereochemistry of the original β-glucosidic linkage of **35** was retained during the thermal glycosylation.

A methyl group was introduced at C-25 of **37** by sequential treatment with LDA and methyl iodide to give 1:1 mixture of stereoisomers. After DIBALH reduction of lactone, the hemiacetal was smoothly isomerized into the C-25 *R* compound **38** by treatment with sodium methoxide.

Glycosylation at the C-26 hemiacetal of **38** was achieved by using the classical Koenigs-Knorr glycosylation. Treatment of **38** with rhamnosyl chloride **36** and silver triflate in the presence of TMU provided trisaccharide **39** in 55% yield. Hydroboration followed by PDC oxidation afforded desired ketone **40**. A palladium (II) hydroxide catalyzed debenzylation of **40** under hydrogen atmosphere gave **2**. This completes the first total synthesis of a saponin; however, aqueous solutions of **2** were not sweet at all.[6]

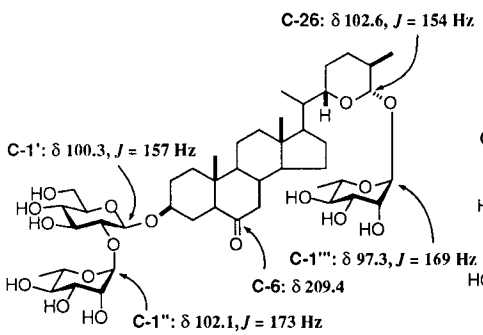




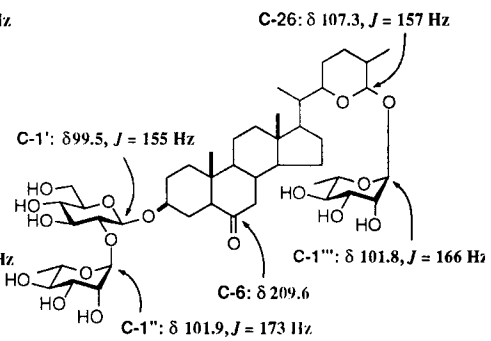
a: **34**, TMU, TfOH (0.05 equiv), CH_2Cl_2 *b*: **36**, α-methylstylene, neat, 80°C, 60 h *c*: LDA, HMPA, THF, then MeI *d*: DIBALH, ether *e*: MeONa, MeOH-THF *f*: **36**, AgOTf, TMU *g*: BH_3•THF,then H_2O_2, NaOH *h*: PDC, CH_2Cl_2 *i*: H_2, $Pd(OH)_2$

4. Isolation and Structure Determination of The Real Osladin

Since the original sample of osladin and spectral data were not available, we isolated the rhizomal sweet principle of *P. vulgare* from plants collected in the southern part of Germany by Professors Y. Asakawa and H. Becker. Sweet components of the fern were extracted by ethanol. Successive chromatography and recrystallizations afforded pure sweet compound as colorless crystals (0.02% isolated yield). Since the sweet compound has a mp of 202-204 °C (lit. 201-203 °C)[3] and a molecular formula $C_{45}H_{74}O_{17}$ suggested from HRMS (FAB), this is the same compound, osladin, which was isolated by Jizba and Herout. Both ^1H and ^{13}C NMR spectra of the natural osladin were not identical with those of synthetic **2**. Thus, the structure of real osladin differs from that of **2**.



^{13}C NMR Data of Synthetic Glycoside **2** in Py-d_5



^{13}C NMR Data of Natural Sweet Osladin (**3**) in Py-d_5

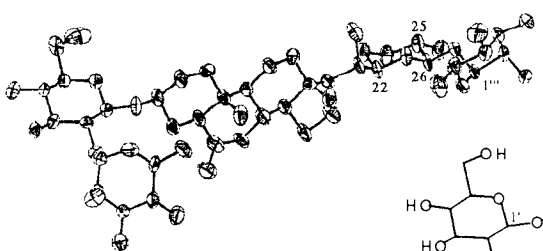
It is noteworthy that ^{13}C NMR chemical shift of C-26 of natural osladin (**3**) showed relatively low field (δ 107.3), while synthetic **2** showed δ 102.6. Glycosylation shift is a useful procedure to distinguish the absolute structure of chiral alcohol. Generally, a combination of an *R*-alcohol with *R*-sugar (β-glucose, β-xylose, or α-rhamnose) or an *S*-alcohol with *S*-sugar (α-glucose, α-xylose, or β-rhamnose) induces larger glycosylation shift (Δδ).[21] While *SR* combination results in a smaller Δδ. The C-26 rhamnosidic bond of osladin is a glycoside of hemiacetalic alcohol. Thus it is interesting to investigate the

glycosylation shift for the case of hemiacetal, namely 1,1'-disaccharide. Thus we have prepared a variety of (*R,R*)-1,1'-disaccharides such as β-glc-β-glc, β-xyl-β-xyl, α-rhα-α-rha, β-glc-β-xyl, β-glc-α-rha and β-xyl-α-rha as well as (*S,S*)-1,1'-disaccharides such as α-glc-α-glc, α-xyl-α-xyl, α-glc-α-xyl, α-glc-β-rha, and α-xyl-β-rha by using AgOTf promoted glycosylation of glycosyl chloride with 1-hydroxy sugar. The Δδ values for anomeric carbons (δ value of disaccharide - δ value of original hemiacetal) of all of these disaccharides were less than 3 ppm. While *S-R* combination such as α-glc-β-glc, α-xyl-β-xyl, β-rhα-α-rha, α-glc-β-xyl, α-glc-α-rha, β-rhα-β-glc, α-xyl-α-rha, and β-rhα-β-xyl gave Δδ value larger than 5 ppm. Thus the glycosylation shift is possible to extend for the glycoside of hemiacetal.[30]

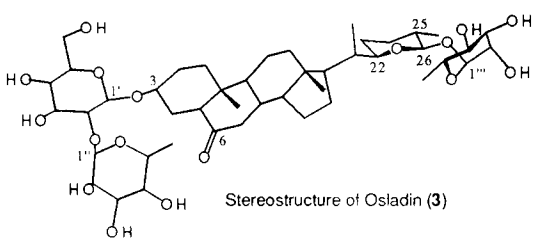
The chemical shift at the anomeric carbon of α-rhamnose itself is δ 95.5. Thus in **2** the glycosylation shift at C-1''' is 1.8 ppm indicating a glycoside originated from *RR* or *SS* combination. Since α-rhamnose itself is *R*, the stereochemistry at C-26 of the aglycone **2** is *R*; C-26 of glycoside **2** itself is *S* due to change of the priority orders. On the other hand, the glycosylation shift at C-1''' of the sweet osladin (**3**) is 6.3 ppm. The value suggests *R* configuration at C-26 of glycoside **3**.

Furthermore to reveal the real structure of osladin (**3**), we have prepared monoclinic single crystals by repeated recrystallizations from a mixture of acetone and water. According to the ORTEP drawing as well as the stereostructure, the sweet osladin should be represented by structure **3**. Thus, the C-22*S*, 25*R*, 26*S* stereochemistry assigned by Havel and Cerny[16] needs to be revised to C-22*R*, 25*S*, 26*R*, respectively. In addition, it is important to note that **3** is intensively sweet while **2** is totally free from any taste even though the configurational change is minor.

In the original paper on the structure determination of osladin, Jizba, Dolejs, Herout, and Sorm, did not mention the intensity of the sweetness.[3] Although osladin has been claimed to be 3000 times sweeter than sucrose by Farnsworth,[17] reexamination of sweetness by Ajinomoto Co. suggests it is only 500 times sweeter.[6b]



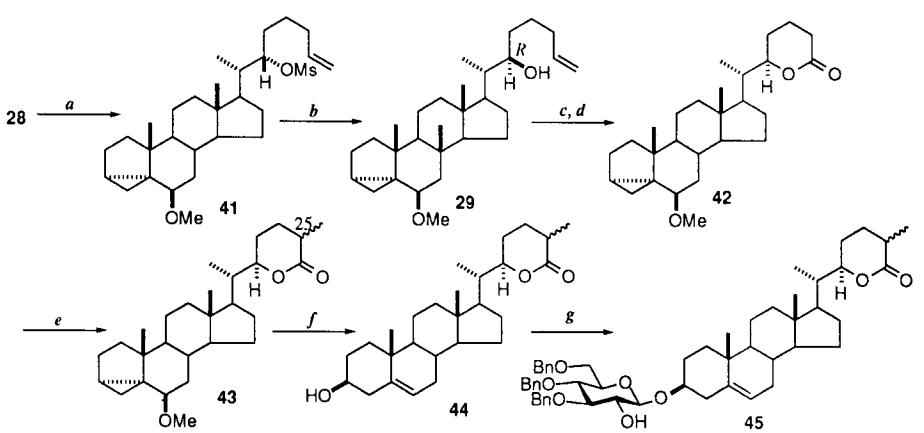
ORTEP Drawing of Osladin (**3**)

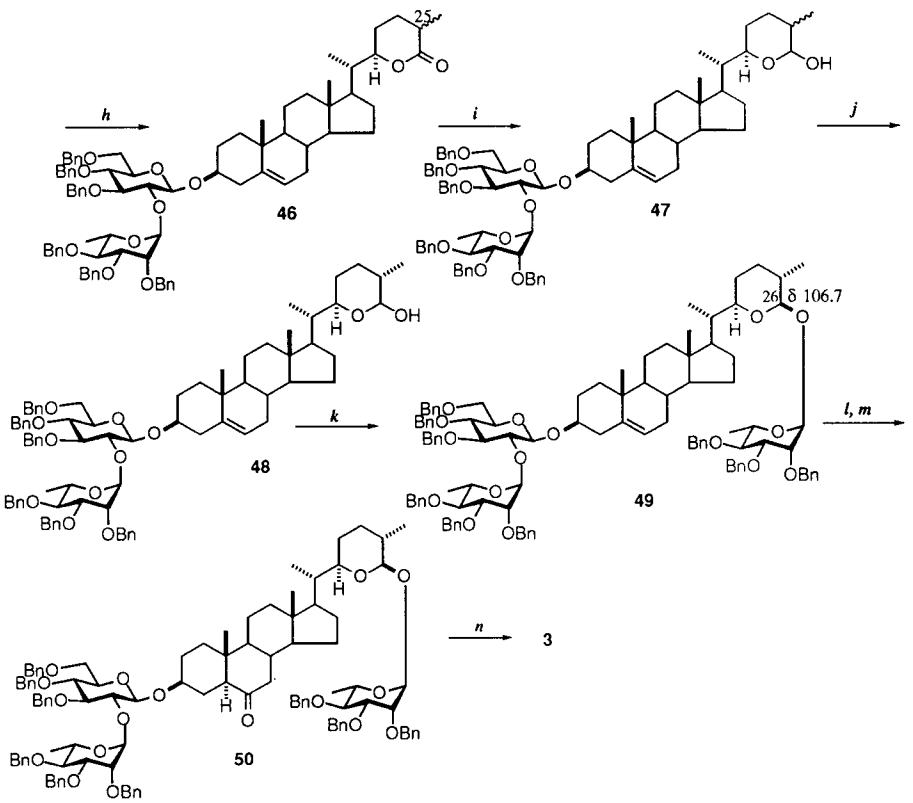


Stereostructure of Osladin (**3**)

5. Synthesis of The Real Osladin

To complete the synthesis of sweet osladin (**3**), we needed to modify the synthesis of **2**. As already discussed, Grignard reaction of the aldehyde **27** generates the C-22 *S* product **28** predominantly. Since the C-22 stereochemistry of real osladin (**3**) is *R*, we needed to invert the stereochemistry at C-22 of **28**. Although a variety of Mitsunobu reaction conditions failed to give any inversion product, Corey's S_N2 reaction of mesylate **41** with KO_2 achieved clean inversion at C-22 to give C-22 *R* carbinol **29** in 88% yield.[23] The alcohol **29** was transformed into lactone **42** by ozonolysis and subsequent PDC oxidation. Methylation at the α-position of the lactone afforded monomethylated product **43** as a 1:1 mixture of stereoisomers. Since it is possible to isomerize the configuration of the C-25 methyl group at a later stage, this mixture was employed for the solvolysis and glycosylation reactions. Triflic acid catalyzed solvolysis of **43** afforded homoallylic alcohol **44**. Condensation of **44** and glucosyl chloride **34** catalyzed by triflic acid in the presence of TMU took place in a β-selective manner to give 2' hydroxyl group discriminated glucoside **45** in 57% yield. By means of an α-selective thermal rhamnosylation reaction, the L-rhamnosyl residue was coupled to **45** to give disaccharide **46** in 81% yield.[6a] The hemiacetals **47** obtained by DIBALH reduction of **46** were treated with base to give the more stable equatorial methyl product **48**. Glycosylation of the hemiacetal hydroxyl group at C-26 was achieved by using rhamnosyl chloride **36**, AgOTf, and TMU to yield trisaccharide **49** stereoselectively in 61% yield. The characteristic low field shift (δ 106.7) of the C-26 anomeric carbon was observed in the ^{13}C NMR of **49**.[22] The trisaccharide **49** was subjected to hydroboration, and subsequent PDC oxidation to give ketone **50**. The benzyl groups of **50** were cleaved by $Pd(OH)_2$ catalyzed hydrogenolysis to give osladin **3**. This product was very sweet and showed indistinguishable spectral properties with those of natural osladin. Thus we have shown that the structure **3** represents the real sweet principle of *P. vulgare*.[6]





a: MsCl, Piridine *b*: KO_2, 18-Crown-6, DMSO-DME (1:1) *c*: O_3, Sudan III, EtOH-H_2O, then Me_2S *d*: PDC, CH_2Cl_2 *e*: LDA, HMPA, THF then MeI *f*: TfOH (0.005 equiv), dioxane-H_2O (9:1) *g*: **34**, TfOH, TMU, CH_2Cl_2 *h*: **36**, TMU, neat, 80 °C, 56 h *i*: DIBALH, ether *j*: MeONa, MeOH *k*: **36**, AgOTf, TMU, CH_2Cl_2 *l*: BH_3•THF then H_2O_2, NaOH *m*: PDC, CH_2Cl_2 *n*: H_2, $Pd(OH)_2$, MeOH-EtOAc-H_2O

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CONVERSION OF (-)-ISOPIPERITENONE INTO 7-HYDROXY-ISOPIPERITENONE 7-*O*-β-D-GLUCOPYRANOSIDE BY SUSPENSION CELL CULTURE OF *MENTHA PIPERITA*

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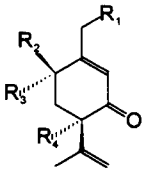
Key Word index -- *Mentha piperita*; Labiatae; peppermint; cell suspension culture; isopiperitenone; 7-*O*-β-D-glucopyranoside; absolute stereochemistry

Abstract -- (-)-Isopiperitenone (**1**) is a normal biosynthetic intermediate in menthol biosynthesis of *M .piperita*. It was hoped that feeding of this intermediate could enhance biosynthesis of menthol in the suspension cell culture of the plant. When this compound was fed to the suspension culture, it was converted to menthol as expected albeit in a lower extent. However, its major metabolites were unexpected oxygenated products, (-)-7-hydroxy-isopiperitenone (**2**), (-)-(4*R*,6*R*)- and (+)-(4*R*,6*S*)-6-hydroxyisopiperitenone (**3** and **4**), (+)-(4*S*)-4-hydroxyisopiperitenone (**5**), and (-)-(4*R*,8*R*)- and (-)-(4*R*,8*S*)-8,9-epoxyisopiperitenone (**6** and **7**), to be excreted into the culture medium. Among these, (-)-7-hydroxyisopiperitenone was further converted into a polar compound, which was accumulated in the cells. The polar compound was purified and identified as (4*R*)-7-hydroxyisopiperitenone 7-*O*-β-D-glucopyranoside (**8**). (+)-Isopiperitenone (**9**) was also biotransformed into (+)-7-hydroxy-isopiperitenone (**10**) and then eventually to the corresponding glucoside (**11**). About 50% of **1** was converted into the **8** in four days and remained constant thereafter. The glycoside of **9** was, however, continued to be accumulated until 10th day after feeding. Previously reported conversion of menthol into menthyl glucoside by *M. piperita* cell culture and the present results suggest strong glucosyl transferase activity in the cells, which could be utilized for production of glucoside through biotransformation technique.

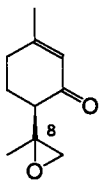
Introduction

Several studies have been done to produce essential oils by plant cell cultures [1,2]. In the case of peppermint, *Mentha piperita* L. (Lamiaceae), there have been attempts to produce major components of peppermint oils through biotransformations of biosynthetic intermediates [3-5]. However, feeding of (-)-isopiperitenone (**1**), a intermediate at branching point leading either to menthol or piperitone, to the cell culture of *M. piperita* results in 7-hydroxylation [6,7].

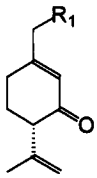
In the leaves of the peppermint, menthol and neomenthol are known to undergo acetylation and glucosylation, respectively, for transport to and degradation in root [8]. Glucoside formation of the exogenous terpenoids, including menthol and neomenthol as well as the aromatic alcohols, has also been known in the cell suspension culture of the peppermint [9]. The formation of (-)-(4*R*)-7-hydroxyisopiperitenone from (-)-(4*R*)-isopiperitenone thus strongly suggests further metabolism into its glucoside. This paper now describes the isolation and characterization of the other minor oxygenated products and glucoside produced by the suspension cells of the peppermint.



1, R_1=R_2=R_3=R_4=H ; **2**, R_1=OH, R_2=R_3=R_4=H
3, R_1=R_3=R_4=H, R_2=OH ; **4**, R_1=R_2=R_4=H, R_3=OH
5, R_1=R_2=R_3=H, R_4=OH ;
8, R_1=7-O-β-glucopyranosyl,
R_2=R_3=R_4=H



6, 8*R*
7, 8*S*



9, R_1= OH
10, R_1=
7-O-β-glucopyranosy

Results and Discussions

EIMS of **3** showed $[M]^+$ at *m/z* 166 consistent with the molecular formula $C_{10}H_{14}O_2$. IR and NMR data considered with MS data suggested 6-hydroxyisopiperitenone structure. The stereochemistry of C-6 in **3** would have been easily determined if H-5 siganal were resolved into multiplets. Nevertheless, the stereochemistry at C-6 was determined to be *R* on the basis of a ^1H NMR coupling pattern analysis at H-6 as follows. The H-5ax signal had three large coupling constants, one (J = 12.4 Hz) due to geminal coupling, the other (J = 13.6 Hz) coupling with H-4. And the last (J = 10.2 Hz) must be then due to coupling with H-6. Assuming axial orientation of H-4 due to preference of propenyl side chain toward equatorial position, we concluded that H-6 was in axial orientation. Therefore, the absolute configuration at C-6 was *R*.

Spectral data of **4** were very close to **3**, and indicated it was a stereoisomer of **5**. The stereochemistry at C-6 was determined indirectly as in the case of the compound **3**. Assuming axial orientation of H-4, J = 9.1 Hz could be assigned to axial-axial coupling between H-4 and H-5ax. Starting from this, J = 4.2 Hz then must be due to axial-equatorial coupling H-5ax and H-6. Therefore, the configuration of the hydroxyl group at C-6 was concluded to be *S*.

EIMS of **5** showed a molecular ion at *m/z* 166 corresponding to the molecular formula, $C_{10}H_{14}O_2$. NMR and MS data suggested the structure of 4-hydroxyisopiperitenone. The stereochemistry of C-4 in **5** was deduced to be *S* by comparison with optical rotation values of 4-hydroxymenthones. Compound **5** was reduced to a mixture of 4-hydroxymenthones by palladium on activated carbon and H_2. The mixture, with specific optical rotation value of +104°, contained (1*R*)- and (1*S*)-4-hydroxymenthone with unknown configuration at C-4. Optical rotation sign of (4*S*)-4-hydroxymenthones is known to be positive and that of (4*R*)-4-hydroxymenthones negative regardless of configuration at C-1 [10]. Therefore, the reduced mixture must be composed of (1*R*,4*S*)- and (1*S*,4*S*)-4-hydroxymenthone, finally confirming the configuration of compound **5** at C-4 as *S*.

EIMS of **6** and **7** showed a molecular ion at *m/z* 166 corresponding to the molecular formula $C_{10}H_{14}O_2$. These compounds were thus also oxygenated derivatives of **1**. The IR and NMR spectra indicated the presence of an oxirane ring introduced between C8 and C-9 in compound **6** and **7**. Thus the structure of **6** and **7** were determined as 8,9-epoxyisopiperitenones.

The stereochemistry of **7** and **8** is identical at C-4 but opposite at C-8. The stereochemistry of **7** and **8** was determined by comparison of optical rotation values and NMR data between the isolated products and synthetic sample of known confguration. (1*R*,2*R*,4*S*)-1,2-Dibromlimonene was synthesized by monobromination of (-)-limonene. The

dibromolimonene was epoxidized with *m*CPBA to yield a mixture of two epoxides. Separation of the diastereomeric pair was achieved by column chromatography over silica gel. One of the dibromoepoxides was debrominated with zinc powder to give a 8,9-epoxy-*p*-menth-1-ene. Its physico-chemical data were identical to those of (4*R*,8*S*)-8,9-epoxy-p-menth-1-ene as appeared in the reference [11], except for the sign of optical rotation. From these results, configuration of the compound was clearly identified as (4*S*,8*R*). This compound was oxidized with CrO3 and *t*BuOOH to yield (4*R*,8*R*)-epoxyisopiperitenone whose physico-chemical properties were identical to those of the biotransformed compound **6**. Therefore, the absolute configuration of compound **6** was finally determined to be (4*R*,8*R*).

The stereochemistry of **7** was determined in the same manner as **6**. (4*S*,8*S*)-8,9-Epoxy-*p*-menth-1-ene was obtained as above. This compound was then oxidized to yield (4*R*,8*S*)-epoxyisopiperitenone, whose physico-chemical properties were identical to those of compound **7**, a product of biotransformation. The absolute configuration of compound **6** was now finally determined to be (4*R*,8*R*).

After the administration of **1** to the peppermint cell suspension cultures, the content of 7-hydroxyisopiperitenone (**2**) in the medium was determined. The content reached its maxima on the 3rd day after the administration. The enantiomer of **1**, (+)-(4*S*)-Isopiperitenone (**9**), was also fed to the culture. The isolated major product was identified as (+)-(4*S*)-7-hydroxyisopiperitenone (**10**).

The content of 7-hydroxyisopiperitenone reached its maximum on the 3rd day after the administration and decreased thereafter. This suggested further metabolism of the 7-hydroxy compound. Since the glycosylation of the exogenous alcohols administered to the plant cell cultures is known, the polar fraction of the cells and the incubation broth were analyzed to demonstrate the formation of the glycoside. The culture broth did not contain any new metabolites form **1** and **2**. However, the initial MeOH extracts of the cells incubated with the isopiperitenone were shown to contain a new polar compound which had not been found in the control experiment. The polar compound was vigorously purified from the crude MeOH extracts through the adsorption to XAD-2 resin, silica gel CC and finally preparative HPLC. The resulting compounds **8** was unequivocally identified as the novel β-glucoside of **2** as follows.

Its IR, NMR and MS spectra indicated that the isolated compounds was glycoside. Anomeric proton signal was observed at 4,32 (*d*, *J*=7.7), the coupling constant being typical in β-glucosides. The ^{13}C NMR spectra also showed the signals due to the β-glucoside moiety, typical anomeric carbon signal being detected near δ 104.

Feeding of (+)-isopiperitenone (**9**) also was found to produce (+)-7-hydroxyisopiperitenone which was further metabolized to the corresponding glucoside (**11**). The identity of the sugar of the glycosides and the configuration at the anomeric position of the sugar moiety were further confirmed by the enzymatic hydrolysis using β-glucosidase. The released sugar was analyzed with HPLC. The retention time of the sugar from the glycosides was 5.74 min under the given conditions, matching exactly to that of the glucose. The analysis of the organic layer by GC also confirmed the presence of the terpene alcohol in **8** and **11**.

The metabolism of the terpene alcohols into β-D-glucoside has been well documented. The peppermint cells, especially, are known to possess a high glucosyl transferase activity [9]. This work clearly demonstrates the accumulation of the glucosides in the cell rejecting the implied extracellular accumulation [12].

Fate of the isopiperitenones after feeding to the culture was followed in the medium and the cells, and representative results showing the general trend is shown in Figure 1. The content of the fed substrates decreased rapidly for the first 3 days with a concomitant increase in the alcohol and the β-D-glucoside content. More than 95% of the isopiperitenones were consumed within one week. However, the kinetics of the glucoside formation from **9** was somewhat

different from **1**. For **1**, the formation of the glucoside **8** leveled off after the 7th day of incubation. The maximum yield of the glucoside **8** was about 35 %. In the case of the (4*S*)-glucoside, a continuous accumulation until the 10th day after the incubation to reach at maximal 60 % conversion rate was observed. This difference in the production patterns between the glucosides **8** and **11** could be explained by the further metabolism of the (4*R*)-glucoside, as opposed to the (4*S*)-glucoside which kept accumulating until 10 days after the feeding. Glucosylation of the menthol in the leaves of the peppermint was indicated as a transport form to be degraded in the rhizome. It is known that the glucosyl transferase from the peppermint leaves does not discriminate the (-)-menthol over the (+)-neomenthol. The discrimination of the hydroxylation enzyme between the substrates **1** and **9**, not of the glycosylation enzyme, could explain the differences in the maximal amount of the glycosides. Further study on the differences in the metabolic pattern should be pursued to clarify the above observation.

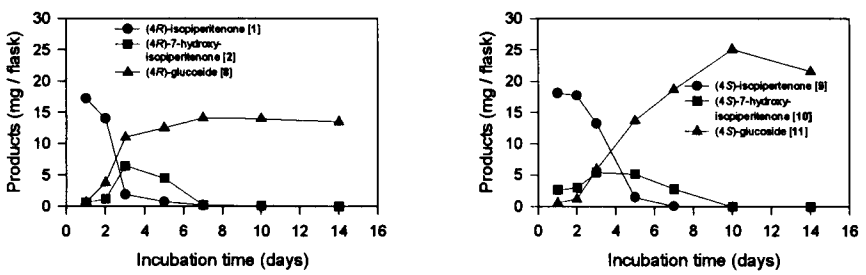


Fig. 1. Changes in the contents of the isopiperitenones, the 7-hydroxyisopiperitenones and their glucosides in the peppermint cell suspension culture during the incubation of (left) (-)-(4*R*)-isopiperitenone and (right) (+)-(4*S*)-isopiperitenone.

Experimental

Plant cell culture and biotransformation procedure. Suspension cultures of *M. piperita*, established in Lin and Staba medium with 2,4-D (2 mg/liter), were gift from professor H. J. Lee, Department of Food Science and Technology, Seoul National University. The culture was incubated in a 250 ml Erlenmeyer flask containing 100 ml of the medium on a rotary shaker (120 rpm) at 27°C with 16-8 hrs light-dark cycle. The culture was subcultured monthly with the initial inoculum of 1g fresh cell per flask. Twenty milligrams of (-)-isopiperitenone (**1**) was administered per flask containing 3-week-old suspension cells and subsequently incubated for 48 hrs to isolate monoterpenes.

Extraction and isolation. The cells were filtered through filter paper and carefully washed with distilled water. The filtrate was extracted with CH_2Cl_2 (500 ml x3). The CH_2Cl_2 layer was dried with anhydrous $MgSO_4$ and concentrated *in vacuo*. The oily residue was chromatographed on silica gel column (2 x 50 cm, hexane-diethyl ether, 4:1) and further purified by HPLC. The HPLC conditions for isolation of monoterpenes were as follows: column, LiChrosorb Si60 (Merck); 10 mm x 25 cm, solvent; hexane-diethyl ether 1:1, flow rate; 3.0 ml/min, UV detector; at 240 nm. The harvested cells (113 g fr. wt from 10 flasks) were soaked in MeOH (500 ml) and ground. The cells were separated and extracted a second time with MeOH (500ml). The MeOH extracts were evaporated to dryness, and the residue was dissolved in 50 ml of H_2O. The residue was chromatographed on XAD-2 column (2.5 x 30 cm)

with a successive elution of 200ml of H2O, 200 ml of 50% aqueous MeOH and finally 200ml of MeOH. The fraction eluted by 50% MeOH was concentrated and subjected to silica gel CC (100 g, Silica gel 60) eluted successively with $CHCl_3$, $CHCl_3$-MeOH (4:1), $CHCl_3$-MeOH (2:1) and $CHCl_3$-MeOH (1:1). A fraction eluted with $CHCl_3$-MeOH (2:1) was finally purified with HPLC. The HPLC conditions were as follows: Column, Bondapack C18, 19 x 300 mm; solvent, H_2O-MeOH (65 : 35); detection, UV 234 nm; flow rate, 10.0 ml/min. The fraction corresponding to the peak at 15.7 min was concentrated to give a colorless solid,

(-)-(4R,6R)-6-Hydroxyisopiperitenone **(3).** colorless oil (8 mg); $[\alpha]^{20}_D$ = -7.2 ° (c 0.08, CH_2Cl_2); UV λ^{MeOH}_{max} nm 231 (3.9) nm; IR (neat) ν^{KBr}_{max} cm^{-1} 3310, 2940, 1640; ^1H NMR ($CDCl_3$, 400 MHz) 1.74 (3H, s, H-10), 2.06 (3H, s, H-7), 2.14 (1H, ddd, *J* = 12.4, 13.6, 10.2 Hz, H-5ax), 2.31 (1H, ddd, *J* = 12.4, 4.5, 4.7 Hz, H-5eq), 2.46 (1H, br. s, -OH), 3.05 (1H, dd, *J* = 13.6, 4.5 Hz, H-4), 4.51 (1H, br. s, H-6), 4.81 (1H, m, H-9a), 4.98 (1H, m, H-9b), 5.88 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) 19.67 (C-7), 19.94 (C-10), 37.94 (C-5), 53.88 (C-4), 69.74 (C-6), 114.49 (C-9), 126.82 (C-2), 143.00 (C-8), 163.82 (C-1), 198.21 (C-3); EIMS *m/z* $[M]^+$ 166 (8), $[M\text{-}H2O]^+$ 148 (27), 133 (16), 105 (22), 98 (100), 69(46).

(+)-(4R,6S)-6-Hydroxyisopiperitenone **(4).** white powder (8 mg); $[\alpha]^{20}_D$ +17.4 ° (c 0.08, CH_2Cl_2); UV λ^{MeOH}_{max} nm 231 (3.9); IR (KBr) ν^{KBr}_{max} cm^{-1} 3410, 2920, 1660 ; ^1H NMR ($CDCl_3$, 400 MHz) 1.77 (3H, s, H-10), 2.05 (3H, s, H-7) 2.39 (1H, ddd, *J* = 13.6, 9.1, 4.2 Hz, H-5ax), 2.10 (1H, ddd, *J* = 13.6, 4.8, 5.6 Hz, H-5eq), 3.30 (1H, dd, *J* = 9.1, 4.8 Hz, H-4), 4.35 (1H, br. s, H-6), 4.76 (1H, m, H-9a), 4.96 (1H, m, H-9b), 5.88 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) 20.96 (C-7), 21.08 (C-10), 36.11 (C-5), 50.01 (C-4), 67.28 (C-6), 113.73 (C-9), 127.29 (C-2), 142.42 (C-8), 159.82 (C-1), 198.81 (C-3); EIMS *m/z* $[M]^+$ 166 (10), $[M\text{-}H_2O]^+$ 148 (58), 133 (35), 105 (47), 98 (100), 69 (46).

(+)-(4S)-4-Hydroxyisopiperitenone **(5).** colorless oil (7 mg); $[\alpha]^{20}_D$ +136° (c 0.05, CH_2Cl_2); UV λ^{MeOH}_{max} nm 236 (4.3) nm; IR (neat) ν^{KBr}_{max} cm^{-1} 3475, 2930, 1670 ; ^1H NMR (CDCl3, 400 MHz) 1.84 (3H, s, H-10), 1.95 (1H, m, H-5a), 1.96 (3H, s, H-7), 2.30 (2H, m, H-6), 2.33 (1H, m, H-5b), 3.78 (1H, s, OH-4), 4.69 (1H, m, H-9a), 4.94 (1H, m, H-9b), 5.98 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) 18.09 (C-10), 24.22 (C-7), 29.97 (C-6), 32.62 (C-5), 77.14 (C-4), 113.49 (C-9), 124.42 (C-2), 144.36 (C-8), 164.22 (C-1), 201.16 (C-3); EIMS *m/z* $[M]^+$ 166 (5), $[M\text{-}H_2O]^+$ 148 (59), 133 (32), 105 (46), 97 (22), 82 (100), 69 (16).

(-)-(4R,8R)-8,9-Epoxyisopiperitenone **(6).** colorless oil (10 mg); $[\alpha]^{20}_D$ -110.0° (c 0.70, CH_2Cl_2) ; UV λ^{MeOH}_{max} nm 235 (4.1) nm; IR (neat) ν^{KBr}_{max} cm^{-1} 2930, 1670, 1200; ^1H NMR (CDCl3, 400 MHz) 1.26 (3H, s, H-10), 1.97 (3H, s, H-7), 2.01 (1H, m, H-4), 2.04 (1H, m, H-5a), 2.24 (1H, m, H-5b), 2.37 (2H, m, H-6), 2.73 (1H, d, *J* = 4.5 Hz, H-9a), 2.82 (1H, d, *J* = 4.5 Hz, H-9b), 5.87 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) 17.15 (C-10), 24.08 (C-7), 24.78 (C-5), 30.81 (C-6), 53.44 (C-4), 55.77 (C-9), 56.57 (C-8), 126.45 (C-2), 162.40 (C-1), 197.82 (C-3); EIMS *m/z* $[M]^+$ 166 (19), $[M\text{-}H2O]^+$ 148 (19), 136 (24), 121 (30), 108 (28), 93 (30), 82 (100), 67 (13).

(-)-(4R,8S)-8,9-Epoxyisopiperitenone **(7).** colorless oil (4 mg); $[\alpha]^{20}_D$ -41.2 ° (c 0.20, CH_2Cl_2); UV λ^{MeOH}_{max} nm (log ε) 234 (4.1) nm; IR (neat) ν^{KBr}_{max} cm^{-1} 2930, 1670, 1200; ^1H NMR (CDCl3, 400 MHz) 1.44 (3H, s, H-10), 1.74 (1H, m, H-5a), 1.95 (3H, s, H-7), 2.08 (1H, m, H-5b), 2.35 (2H, m, H-6), 2.37 (1H, m, H-4), 2.53 (1H, d, *J* = 4.6 Hz, H-9a), 2.59 (1H, d, *J* = 4.6 Hz, H-9b), 5.87 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) 21.31 (C-10), 24.12 (C-7), 24.75 (C-5), 30.67 (C-6), 50.89 (C-4), 50.92 (C-9), 56.40 (C-8), 126.84 (C-2), 162.00 (C-1), 198.40 (C-3); EIMS *m/z* $[M]^+$ 166 (19), $[M\text{-}H_2O]^+$ 148 (19), 136 (24), 121 (30), 108 (27), 93 (28), 82 (100), 67 (12).

(4R)-7-Hydroxyisopiperitenone 7-O-β-D-glucopyranoside **(8).** $[\alpha]^{22}_D$ = -53.9 °(c 0.857, MeOH). UV λ^{MeOH}_{max} nm (log): 234 (4.10); IR ν^{KBr}_{max} cm^{-1} : 3390 (-OH), 2920 (C-H), 1660

(C=O), 1040 (C-O), 890 (C=C); ^{1}H NMR (400 MHz, CD_3OD) 1.73 (3H, *s*, H-10), 2.04 (1H, *dddd*, *J*=4.8, 4.9, 4.9, 13.3, H-5a), 2.15 (1H, *dddd*, *J*=4.9, 9.0, 10.8, 13.3, H-5b), 2.41 (2H, *m*, H-6), 3.07 (1H, *dd*, *J*=4.8, 10.8, H-4), 3.25 (1H, *m*, H-2'), 3.28 (1H, *m*, H-5'), 3.30 (1H, *m*, H-4'), 3.36 (1H, *m*, H-3'), 3.66 (1H, *dd*, *J*=5.5, 11.0, H-6'a), 3.87 (1H, *d*, *J*=11.0, H-6'b), 4.29 (1H, *d*, *J*=16.0, H-7a), 4.32 (1H, *d*, *J*=7.7, H-1'), 4.51 (1H, *d*, *J*=16.0, H-7b), 4.75 (1H, *br. s*, H-9a), 4.91 (1H, *t*, *J*=1.6, H-9b), 6.19 (1H, *t*, *J*=1.5, H-2); ^{13}C NMR (100MHz, CD_3OD) , 20.9 (C-10), 26.6 (C-6), 28.8 (C-5), 55.8 (C-4), 62.8 (C-6'), 71.4 (C-7), 71.6 (C-4'), 75.0 (C-2'), 78.0 (C-3'), 78.1 (C-5'), 104.0 (C-1'), 114.1 (C-9), 124.9 (C-2), 144.8 (C-8), 164.2 (C-1), 202.2 (C-3); EIMS 70 eV, m/z (rel. int), 328[M]$^+$ (11), 195 (35), 166 (76), 148[M-Glucose]$^+$ (74), 98 (100)

(4S)-7-Hydroxyisopiperitenone 7-O-β-D-glucopyranoside (**11**). $[\alpha]^{22}_D$ = -14.4° (c 0.457, MeOH). UV λ^{MeOH}_{max} nm (log): 234 (4.10); IR ν^{KBr}_{max} cm^{-1}: 3390 (-OH), 2920 (C-H), 1660 (C=O), 1040 (C-O), 890 (C=C); ^{1}H NMR (400MHz, CD_3OD) , 1.73 (3H, *s*, H-10), 2.04 (1H, *dddd*, *J*=4.8, 4.9, 4.9, 13.4, H-5a), 2.13 (1H, *dddd*, J=4.9, 9.0, 10.8, 13.4, H-5b), 2.41 (2H, *m*, H-6), 3.08 (1H, *dd*, *J*=4.8, 10.8, H-4), 3.24 (1H, *m*, H-2'), 3.27(1H, *m*, H-5'), 3.28 (1H, *m*, H-4'), 3.34 (1H, *m*, H-3'), 3.66 (1H, *dd*, *J*=5.5, 11.9, H-6'a), 3.87 (1H, *d*, *J*=11.9, H-6'b), 4.28 (1H, *d*, *J*=16.0, H-7a), 4.31 (1H, *d*, *J*=7.7, H-1'), 4.51 (1H, *d*, *J*=16.0, H-7b), 4.74 (1H, *br. s*, H-9a), 4.91 (1H, *t*, *J*=1.5, H-9b), 6.19 (1H, *t*, *J*=1.5, H-2), ^{13}C-NMR (100MHz, CD_3OD) , 20.9 (C-10); 26.7 (C-6), 28.8 (C-5), 55.8 (C-4), 62.8 (C-6'), 71.2 (C-7), 71.6 (C-4'), 75.0 (C-2'), 78.1 (C-3'), 78.1 (C-5'), 103.8 (C-1'), 114.1 (C-9), 125.0 (C-2), 144.8 (C-8), 164.2 (C-1), 202.2 (C-3); EI-MS 70 eV, m/z (rel. int), 328 [M]$^+$ (14), 195 (31), 166 (77), 148[M-Glucose]$^+$ (79), 98 (100)

Enzymatic hydrolysis of the glucosides. A reaction mixture containing the isolated glucoside (0.5 mg), 1 unit of β-glucosidase from the almond, and 100 mM NaOAc buffer (pH 5.0) in a total volume of 0.3 ml was incubated for 20 h at 35°. After the incubation, the reaction mixture was extracted with Et_2O for the analysis of the monoterpene alcohols. The ether layer was subjected to GC, the GC conditions being as described above. Released sugar from the enzymatic hydrolysis of the glucoside was analyzed by HPLC. The HPLC conditions were as follows: Column, Amide-80, 4.6 x 250 mm; solvent, H_2O-MeCN (30:70); flow rate, 1.0 ml/min; oven temperature, 70°; detection, refractive index.

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CHEMOTAXONOMY OF THE GENUS NUXIA (BUDDLEJACEAE)

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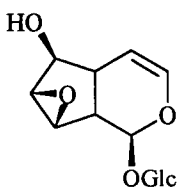
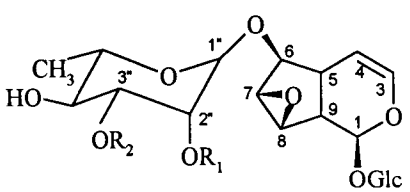
Abstract -- An investigation of two species of Nuxia (Buddlejaceae) showed that this genus is characterised by the presence of the eight-carbon iridoid glucoside unedoside and/or its derivatives. From N. floribunda was isolated unedoside, nuxioside (6-O-a-L-rhamnopyranosyl-unedoside) and 2"-acetyl-3"-cinnamoyl-nuxioside, while from N. oppositifolia was obtained 2"-acetyl-3"-benzoyl-nuxioside. Verbascoside was isolated from both plants. The biosynthesis of unedoside in N. floribunda was investigated and deoxyloganic acid was shown to be a precursor. The taxonomic position of Nuxia and Buddlejaceae is discussed.

Introduction

The genus Nuxia comprises fifteen species of shrubs and trees ranging from Southern Arabia, tropical Africa (including Madagascar, Comoro and the Mascarene Islands) to South Africa [1]. Nuxia is a member of the Buddlejaceae, traditionally included in Loganiaceae [1], but most contemporary taxonomists separate these taxa and have raised Buddlejaceae to family rank [2-5]. Continuing our work with Loganiaceae and related taxa [6-8], we have now investigated two species of Nuxia, namely N. oppositifolia Benth. and N. floribunda Benth.



1 Unedoside



2 R_1= R_2= H; Nuxioside
3 R_1=Ac; R_2= Cinnamoyl
4 R_1=Ac; R_2= Benzoyl

Results

Nuxia floribunda

Fractionation of the water-soluble part of an ethanol extract of N. floribunda by reverse phase chromatography gave, together with verbascoside, the iridoid glucoside unedoside (**1**), as well as two unknown iridoid diglycosides **2** and **3**.

The NMR spectra of **2** were similar to those of **1** but had additional features. Thus, the ^{13}C NMR spectrum had 20 signals of which 14 could be assigned to a unedoside moiety and 6 to an a-rhamnopyranosyl moiety similar to 6-O-a-L-rhamnopyranosylcatalpol. When comparing the ^{13}C NMR spectra of **1** and **2** it was obvious that the site of attachment for the

rhamno-pyranosyl auxiliary in **2** was at the C-6 oxygen atom of the unedoside moiety, since this carbon atom showed a downfield shift (4.9 ppm) while C-5 and C-7 had upfield shifts (ca. 2 ppm) relative to those seen for **1**. We have named the compound nuxioside.

Compound **3** was amouphous. The ^{13}C NMR spectrum contained 31 signals which could be assigned to (i) a unedoside moiety (14 peaks), (ii) a substituted a-rhamnopyranosyl moiety (6 peaks), (iii) an E-cinnamoyl group (9 peaks) and (iv) an acetyl group (2 peaks). Comparison with the spectrum of **2** showed that **3** was an acylated nuxioside derivative. The positions of the two acyl groups were determined from the ^1H NMR spectrum. In this, H-2" and H-3" of the rhamnosyl group were seen at d 5.32 and d 5.23, respectively, both approximately 1.5 ppm downfield when compared to the spectrum of **2**. The remaining signals were very similar in the two spectra and, therefore, the oxygen atoms of C-2" and C-3" had to be the sites of acylation. The positions of the individual acyl groups were determined by long-range selective proton decoupling (LSPD). Thus, selective irradiation at the acetyl methyl singlet at d 2.11 reduced the carbonyl signal at d 171.7 to a doublet (4 Hz) while decoupling at d 5.32 (H-2") similarly collapsed it to a quartet (7 Hz). This proved the acetyl group to be sited at the C-2"-oxygen atom. In the same way, the cinnamoyl group was shown to be in the 3"-position. Consequently, compound **3** is 2"-acetyl-3"-cinnamoyl-nuxioside.

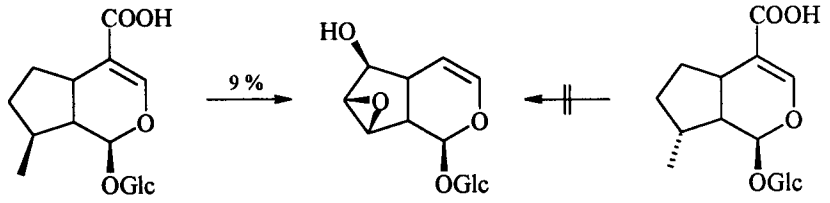
Nuxia oppositifolia

From the polar part of the ethanol extract of N. oppositifolia was obtained only two pure main components, namely verbascoside and the acylated diglycoside **4**. The ^1H NMR spectrum of **4** was very similar to that of **3**, except that the signals from the cinnamoyl group were replaced by those from a benzoyl group in the new compound. This was consistent with the ^{13}C NMR spectrum which showed 29 signals and was assigned by comparison with that of **3**. Also in this case the two acyl groups were positioned at the 2"- and 3"-oxygen atoms of the rhamnosyl auxiliary as shown by the low field positions of the corresponding proton signals. The position of each acyl group was also in this case settled by the LSPD-technique. Thus, the carbonyl signal at d 171.6 (acetyl-CO) showed couplings to the methyl singlet at d 2.10 (7 Hz) and to H-2" at d 5.32 (4 Hz). Therefore, the acetyl group of **4** was again attached at the 2"-position. Consequently, compound **4** is 2"-acetyl-3"-benzoyl-nuxioside.

Biosynthesis

Iridoid glucosides lacking both C-10 and C-11 are comparatively rare. Their biosynthesis has been investigated only in Thunbergia (Acanthaceae) [9], where it was found that deoxyloganic acid (**5**) was a precursor. This is surprising, since all other investigated iridoid glucosides lacking C-11 (including aucubin and catalpol) are derived from **6** [10,11]. Thus **6** was found to be a precursor of aucubin in Buddleja davidii [12] and B. albiflora [13] of the same family.

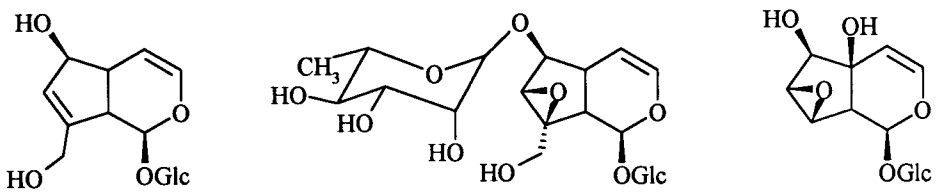
Deuterium-labelled precursors (**5** and **6**) were each fed to cuttings of N. floribunda during 3 days. Work-up gave unedoside (**1**) and the deuterium content was determined by ^2H NMR. Feeding with **5** gave a 9 % incorporation into **1**, while **6** was not significantly incorporated.



5 Deoxyloganic acid **1** Unedoside **6** epi-Deoxyloganic acid

Discussion

As stated in the introduction, Nuxia has earlier been included in Loganiaceae together with the other genera of Buddlejaceae. This was also the case for Retzia, a monotypic genus endemic to South Africa. Most contemporary botanists place Buddlejaceae and Retziaceae in the order Scrophulariales/Lamiales and even include Retziaceae in the small family Stilbaceae [14]. This fits very well with the chemical results since these taxa all produce decarboxylated iridoid glucosides (like **1-4** and **7-9**) and verbascoside or other caffeoyl phenylethanoid glycosides (CPG's). Conversely, compounds from these two groups have never been reported from Loganiaceae or even from the order Gentianales.



7 Aucubin **8** **9** Stilbericoside

The present results allow further chemotaxonomical deductions. Thus, in Buddlejaceae, the investigated genera Buddleja, Emorya and Gomphostigma, all contain the nine-carbon iridoids aucubin (**7**) and catalpol [6]. Some acylated rhamnosylcatalpol derivatives (esters of **8**) are also found in a few species of Buddleja. On the other hand, Nuxia have the eight-carbon iridoids (**1-4**). Therefore, the ability to produce acylated rhamnosyl iridoids may be seen as a chemical link between Nuxia and Buddleja. Finally, Retzia and Stilbaceae both contain the eight-carbon iridoids (**1** and **9**) and consequently, Nuxia could be seen as a link between Buddlejaceae and Retzia/Stilbaceae. Iridoids with a rhamnosyl group is otherwise restricted to a few sympetalous genera, namely Scrophularia and Verbascum in Scrophulariaceae and Premna in Verbenaceae [15, 16]. Recent work on chloroplast DNA sequences [17] furthermore demonstrates the close relationship between Buddlejaceae and Scrophulariaceae, and show that it is taxonomically distant to Loganiaceae, consistent with the chemotaxonomic information presented here.

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FIVE NOVEL SESQUITERPENE GLYCOSIDES FROM *DICTAMNUS DASYCARPUS*

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Key words--*Dictamnus dasycarpus*, Rutaceae, sesquiterpene glycosides, dictamnosides A-E.

Abstract----Five novel sesquiterpene glycosides named dictamnosides A-E (**1-5**) were isolated from the methanol extract of the root bark of *Dictamnus dasycarpus* (Rutaceae). Their structures were established on the basis of X-ray diffraction, spectroscopic and chemical methods.

Introduction

The root bark of *Dictamnus dasycarpus* Turcz. (Bai-Xian-Pi) (Rutaceae) is a traditional Chinese medicine used for treatment of jaundice, cough and rheumatism. It has also been widely used to treat some skin diseases [1]. A lot of phytochemical work has been done on the lipophilic components of the genus *Dictamnus*, and various compounds including furoquinoline alkaloids [2, 3], limonoids [4, 5] and a sesquiterpene [6] have been identified. In a previous paper, we have reported the isolation and identification of several antifungal components from the dichloromethane extract of the root bark of *D. dasycarpus* [7]. To our knowledge, the polar components of *Dictamnus* plants have not been well investigated. In order to understand more about the chemistry of the genus *Dictamnus*, we studied the methanol extract of the root bark of *D. dasycarpus*. Here, we report the isolation and structural determination of five novel sesquiterpene glycosides named dictamnosides A-E (**1-5**), respectively.

Results and Discussion

The root bark of *Dictamnus dasycarpus* (3 kg) was extracted with dichloromethane and methanol, successively. The methanol extract (85 g) was subjected to column chromatography on silica gel with a chloroform-methanol gradient (8:1→1:1). The fractions obtained from the chloroform-methanol (5:1→3:1) eluents were further filtered through Sephadex LH-20 columns with chloroform-methanol (1:1) and subsequently chromatographed on silica gel [chloroform-methanol-water (10:3:0.3)] and RP-18 Lobar [methanol-water gradient (1:4→3:7)] columns to give **1** (150 mg), **2** (50 mg), **3** (120 mg), **4** (30 mg) and **5** (15 mg).

Compound **1** was first purified as an amorphous powder. After crystallisation from a mixture of methanol and water (1:1), triclinic crystals were obtained. The positive ion mode D/CI mass spectrum of **1** exhibited a quasimolecular ion adduct at *m/z* 450 $[M+NH_4]^+$ and a fragment ion signal at *m/z* 288 $[M-162+NH_4]^+$. The signal at *m/z* 288 and the high polarity of **1** suggested it to be a glycoside. Acidic hydrolysis of **1** yielded glucose as its sugar component.

In the ^{13}C NMR spectrum of **1**, 21 carbon signals were observed as three methyls, six methylenes, nine methines and three quaternary carbon signals. Therefore, the aglycone of **1**

should contain fifteen carbons and was supposed to be a sesquiterpene. Combinational analysis of the ^{13}C NMR, DEPT and D/CI mass spectra led to the deduction of its molecular formula as $C_{21}H_{36}O_9$. Hence, the unsaturation degree of **1** is four. In the ^{13}C NMR spectrum of **1**, no signal due to a double bond was found. Thus, three ring systems exist in the aglycone of **1**.

According to the ^{13}C NMR data, five oxygen-connecting carbons were present in the aglycone of **1**. Acetylation of **1** gave compound **1a**. Analysis of the ^1H, ^{13}C NMR and mass spectra of **1a** revealed five acetyl groups in **1a**. In addition to the four easily acetylated hydroxyls in the glucose unit, only one hydroxyl in the aglycone of **1** was acetylated. Therefore, an ether bond should exist in the structure of **1**.

Single crystals of **1** were subjected to X-ray diffraction analysis. An ORTEP drawing of the structure of **1** is given in Figure 1. The crystal packing diagram is shown in Figure 2. Compound **1** is a new natural product named dictamnoside A.

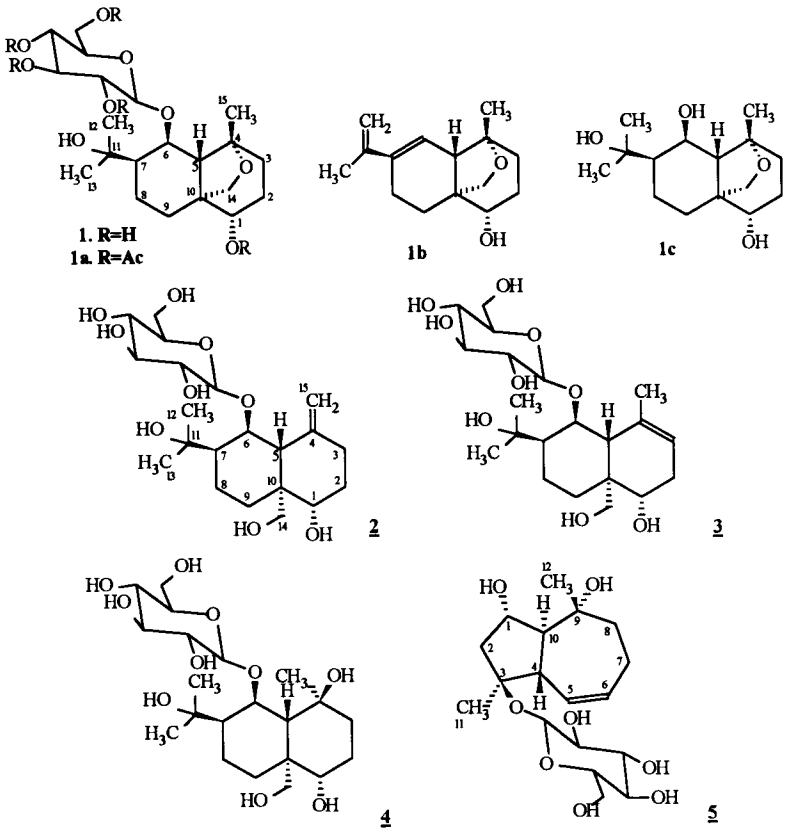
Acidic hydrolysis of **1** yielded **1b** as the major product. In the ^1H NMR spectrum of **1b**, only two methyl signals were observed, and one of them should be connected to an olefinic bond due to its chemical shift at δ 1.912 (3H, *br s*). Furthermore, in the ^{13}C NMR and DEPT spectra of **1b**, four carbon signals corresponding to two olefinic bonds were found at δ 143.14 (*s*), 137.03 (*s*), 121.28 (*d*) and 111.09 (*t*). According to the above evidences, **1b** was not the real aglycone of **1**, and the two double bonds were likely located at C-6/C-7 and at C-11/C-12 in the structure of **1b**. The above suggestion was further confirmed by ^1H-^1H COSY, HMQC and HMBC data of **1b**.

In order to obtain the aglycone, **1** was subjected to enzymatic hydrolysis with β-glucosidase. No obvious reaction happened after keeping the solution at 35 °C for three days. Further hydrolysis at room temperature for 30 days afforded **1c**. The structure of **1c** was established on the basis of the ^1H, ^{13}C NMR, ^1H-^1H COSY, HMQC and HMBC spectra, and was identified to be the real aglycone of **1**.

Compounds **2** and **3** were obtained as amorphous powders. The positive ion mode D/CI mass spectra of **2** and **3** gave the same quasimolecular ion adducts at *m/z* 450 $[M+NH_4]^+$ and the same fragment ion signals at *m/z* 288 $[M-162+NH_4]^+$, which also suggested them to be glycosides. Acidic hydrolysis of **2** and **3** yielded glucose as their sugar components.

The ^{13}C NMR spectra of both **2** and **3** exhibited 21 carbon signals with certain similarities to those of **1**. Therefore, compounds **2** and **3** were supposed to be analogues of **1**. On the basis of the ^{13}C NMR, DEPT and mass spectra, the molecular formulas of **2** and **3** were deduced to be $C_{21}H_{36}O_9$, the same as that of **1**. In the ^{13}C NMR spectrum of **2**, two olefinic carbon signals at δ 147.18 (*s*) and 106.59 (*t*) along with two methyl signals at δ 31.13 and 28.85 were observed, while in the ^{13}C NMR spectrum of **3**, signals of a trisubstituted double bond were found at δ 121.48 (*d*) and 134.86 (*s*) along with three methyl signals at δ 22.85, 30.12 and 30.63. The unsaturation degrees of **2** and **3** were both four according to their molecular formulas. Therefore, their aglycones should both consist of two ring systems, considering the existence of the olefinic bond and the glucose moiety in their structures. By comparison of the ^{13}C NMR data of **2** and **3** with those of **1**, and also from a biogenetic point of view, an exo-cyclic olefinic bond has to be located at C-4/C-15 in **2**, while a trisubstituted olefinic bond should be present at C-3/C-4 in **3**. No ether bond exist in the aglycones of **2** and **3.**

In order to confirm the above hypothesis, extensive 2D NMR measurements of **2** and **3** were performed. On the basis of the ^1H-^1H COSY, TOCSY and HMQC spectra, fragments containing coupled proton systems along with the one bond connections of proton and carbon signals were established (Table 1 and 2). In the HMBC spectra of **2** and **3**, a series of ^1H-^{13}C long range correlation signals were observed as shown in Figure 3. As a result, the linkages among all the fragments were established. Relative configurations of **2** and **3** were determined



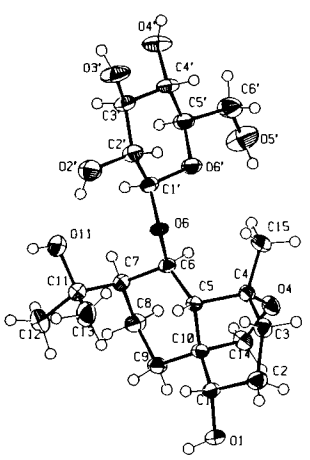


Fig. 1. Perspective view of the molecule of **1**; thermal ellipsoids at 50% probability level.

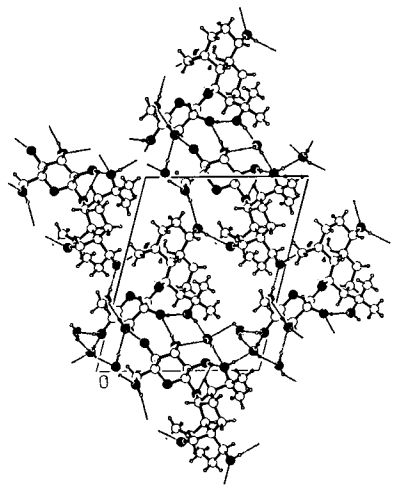


Fig. 2. Crystal packing diagram viewed down the A axis, H-bonds are represented by dashed lines.

according to the results of NOESY spectra as shown in Figure 4. Chemical shifts of all the α and β protons of each methylene were also assigned on the basis of nOe results (Table 1). Compounds **2** and **3** are new natural products, and named dictamnosides B and C, respectively.

Compound **4** was obtained as an amorphous powder. The positive ion mode D/CI mass spectrum of **4** exhibited a quasimolecular ion signal at *m/z* 468 $[M+NH_4]^+$. Due to its high polarity, **4** was also suggested to be a glycoside. Acidic hydrolysis of **4** yielded glucose as its sugar component.

The ^{13}C NMR spectrum of **4** exhibited 21 carbon signals. The fifteen carbon signals belonging to its aglycone were three methyls, five methylenes, four methines and three quaternary carbon signals, the same as those of **1**. On the basis of the ^{13}C NMR, DEPT and mass spectra, the molecular formula of **4** was deduced to be $C_{21}H_{38}O_{10}$, therefore, only three unsaturation degrees were present in the structure of **4**. The molecular weight of **4** was 18 dalton higher than that of **1**. Considering the similarities of the ^{13}C NMR data of **1** and **4**, and also from a biogenetic point of view, no ether bond between C-4 and C-14 should exist in **4**. On the basis of the ^1H-^1H COSY, TOCSY, HMQC and HMBC spectra of **4**, the above suggestion was confirmed (Table 2, Figure 3). However, the relative configuration of **4** was still unknown.

Many hydroxyl proton signals could be clearly observed at lowfield when the ^1H NMR spectrum of **4** was measured at -38°C. In order to use these hydroxyl proton signals in the determination of its relative configuration, a ^1H-^1H COSY spectrum of **4** was performed again at -38 °C, and the chemical shifts of all proton signals were assigned. The NOESY spectrum of **4** measured at the same temperature enabled then to establish the relative configuration (Table 1, Figure 4). Compound **4** is a new natural product named dictamnoside D.

Compounds **1-4** are glycosides with eudesmane-type sesquiterpene aglycones. Glycosides with the same type of aglycones were also found in another Chinese traditional medicine, *Atractylodes lancea* (Asteraceae) by a Japanese group [8].

Compound **5** was obtained as an amorphous powder. The positive ion mode D/CI mass spectrum of **5** gave a quasimolecular ion adduct at *m/z* 392 $[M+NH_4]^+$ and a fragment ion signal at *m/z* 230 $[M-162+NH_4]^+$, which also suggested it to be a glycoside. Acidic hydrolysis of **5** yielded glucose as its sugar component. In the ^1H NMR spectrum of **5**, one anomeric proton signal at δ 5.107 (*d*, 7.7) along with two methyls at δ 1.749 (*s*), 1.448 (*s*) and two olefinic proton signals at δ 6.085 (*dd*, 11.3, 1.9), 5.758 (*ddd*, 11.3, 5.5, 2.9) were observed. The ^{13}C NMR spectrum of **5** exhibited 18 carbon signals. Among the 12 carbon signals belonging to the aglycone were two methyls, three methylenes, five methines and two oxygen-connecting quaternary carbon signals. On the basis of the ^{13}C NMR, DEPT and mass spectra, the molecular formula of **5** was deduced to be $C_{18}H_{30}O_8$. Two ring systems should exist in the aglycone of **5** according to its unsaturation degree.

Combinational analysis of the ^1H-^1H COSY, TOCSY and HMQC spectra of **5** allowed the establishment of the following fragment: $-CH_2-CH(O)-CH-CH-CH=CH-CH_2-CH_2-$. The four remaining carbons of the aglycone were two methyls and two oxygen-connecting quaternary carbons. Since the two methyls appeared as singlets at δ 1.749 (3H, *s*) and 1.448 (3H, *s*) in the ^1H NMR spectrum, they should be linked to the two quaternary carbons, respectively. A HMBC spectrum allowed to establish the connection of the above fragments and the glucose moiety (Figure 3). The relative configuration of **5** was determined according to the results of the NOESY spectrum (Figure 4). Compound **5** is a new natural product and named dictamnoside E.

The aglycone of **5** possess a trinorguaiane type skeleton. A related compound, dictamnol, with the same trinorguaiane type skeleton was previously reported from *D. dasycarpus* [9].

Table 1. ^{1}H NMR (600 MHz, C_5D_5N) data of compounds **2-5**.

No.	**2**	**3**	**4***	**5**
1	3.698,*m*	3.968,*m*	3.795,*m*	4.827,*ddd*,8.0,6.8,4.8
2α	2.036,*m*	2.527,*m*	2.191,*m*	2.191,*dd*,13.6,4.8
2β	2.110,*m*	2.660,*br d*,16.1	2.015,*m*	2.910,*dd*,13.6,8.0
3α	2.377,*m*	5.404,*d*,1.8	2.319,*m*	
3β	2.377,*m*		2.087,*m*	
4				2.953,*ddd*, 12.1,2.5,2.2
5	2.840,*br d*, 6.6	3.405,*br s*	2.857,*m*	6.085,*dd*,11.3,1.9
6	4.946,*dd*,7.0,5.1	4.895,*dd*,4.4,4.1	5.140,*m*	5.758,*ddd*,11.3,5.5,2.9
7α	2.350,*m*	2.272,*m*	2.145,*m*	2.020,*m*
7β				2.430,*m*
8α	1.803,*m*	2.150,*m*	2.800,*m*	1.938,*m*
8β	2.110,*m*	2.489,*m*	2.492,*m*	1.938,*m*
9α	2.799,*ddd*,13.9,7.3,6.6	2.953,*br dd*,12.8,6.2	3.146,*m*	
9β	1.910,*ddd*,13.9,6.9,6.6	1.680,*ddd*,12.5,11.6,7.7	1.805,*m*	
10				2.461,*dd*,12.1,6.8
11				1.749,*s*
12	1.548,*s*	1.503,*s*	1.662,*s*	1.448,*s*
13	1.610,*s*	1.599,*s*	1.417,*s*	
14a	3.847,*d*,11.7	4.173,*dd*,11.0,5.9	4.672,*d*,9.9	
14b	4.332,*d*,11.3	4.406,*br d*,11.0	4.190,*m*	
15a	5.112,*br s*	2.127,*br s*	1.278,*s*	
15b	4.980 *br s*			
Glu-1	5.054,*d*,8.0	5.202,*d*,8.1	5.394,*m*	5.107,*d*,7.7
Glu-2	3.963,*dd*,8.8,8.1	4.036,*dd*,8.4,8.1	4.218,*m*	4.047,*dd*,8.5,8.0
Glu-3	4.178,*dd*,9.1,8.8	4.249,*m*	4.308,*dd*,12.5,8.5	4.298,*dd*,8.8,8.8
Glu-4	4.304,*dd*,9.6,9.1	4.218,*m*	4.006,*m*	4.254,*dd*,9.2,9.1
Glu-5	3.685,*ddd*,9.6,4.0,2.9	3.925,*ddd*,9.1,5.2,3.0	4.233,*m*	3.956,*ddd*,9.6,5.5,2.5
Glu-6a	4.365,*m*	4.441,*br d*,11.7	4.851,*m*	4.525,*dd*,11.7,2.5
Glu-6b	4.365,*m*	4.325,*m*	4.204,*m*	4.349,*dd*,11.8,5.5
1-OH		6.440,*d*,5.5	7.808,*br s*	
4-OH			5.011,*br s*	
11-OH	5.238,*s*		5.888,*s*	
14-OH		5.820,*br d*,4.8		
2'-OH			9.138,*br s*	
3'-OH			8.337,*br s*	
4'-OH			8.051,*br s*	
6'-OH			7.923,*br s*	

* ^{1}H NMR, ^{1}H-^{1}H COSY and NOESY spectra were measured at -38 °C.

Table 2. ^{13}C NMR (150 MHz, C_5D_5N) data of compounds **2-5**.

No.	**2**	**3**	**4**	**5**
1	81.43, *d*	78.68, *d*	80.39, *d*	70.47, *d*
2	33.72, *t*	35.19, *t*	30.40, *t*	48.24, *t*
3	36.18, *t*	121.48, *d*	42.31, *t*	84.88, *s*
4	147.18, *s*	134.86, *s*	72.01, *s*	49.75, *d*
5	51.26, *d*	51.44, *d*	59.90, *d*	131.55, *d*
6	77.37, *d*	79.07, *d*	80.47, *d*	130.79, *d*
7	46.12, *d*	44.88, *d*	46.08, *d*	24.03, *t*
8	20.10, *t*	18.16, *t*	18.12, *d*	43.52, *t*
9	28.35, *t*	26.52, *t*	28.53, *t*	74.11, *s*
10	42.90, *s*	42.16, *s*	44.07, *s*	60.10, *d*
11	72.65, *s*	72.36, *s*	71.42, *s*	23.63, *q*
12	31.13, *q*	30.12, *q*	30.35, *q*	22.98, *q*
13	28.85, *q*	30.63, *q*	30.12, *q*	
14	63.83, *t*	61.89, *t*	62.89, *t*	
15	106.59, *t*	22.85, *q*	24.12, *q*	
Glu-1	104.09, *d*	104.76, *d*	105.77, *d*	99.47, *d*
Glu-2	75.04, *d*	75.00, *d*	75.10, *d*	75.24, *d*
Glu-3	78.75, *d*	78.68, *d*	78.79, *d*	78.67, *d*
Glu-4	71.13, *d*	71.67, *d*	72.38, *d*	71.78, *d*
Glu-5	77.91, *d*	78.17, *d*	78.42, *d*	78.12, *d*
Glu-6	62.17, *t*	62.68, *t*	63.29, *t*	62.89, *t*

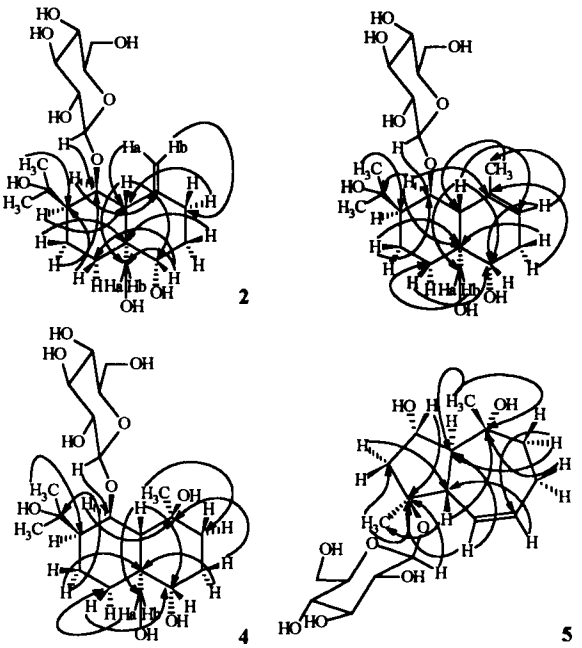


Fig. 3. Main ^{1}H-^{13}C long range correlation signals observed in HMBC spectra of **2-5**.

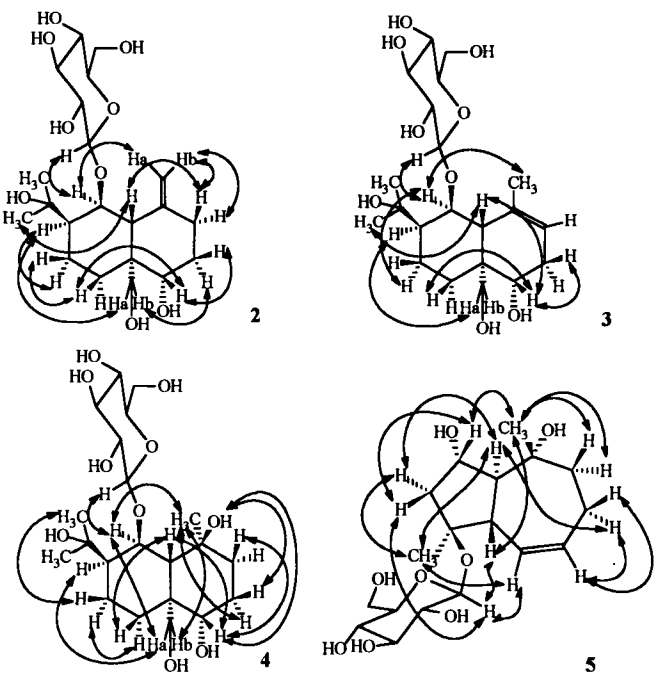


Fig. 4. Main nOe signals observed in NOESY spectra of **2-5**.

Dictamnol was also isolated during our investigation of the dichloromethane extract of the root bark of the plant [7].

Experimental

General: $[\alpha]_D$ were measured with a Perkin-Elmer 241 MC polarimeter. NMR spectra of **1** were obtained on Varian VXR 200 and Varian Unity 500 spectrometers. NMR spectra of **2-5** were obtained on a Jeol JNM-LA 600 spectrometer. Chemical shifts were reported in δ (ppm), with residual C_5D_5N signals (7.24/123.5) and $CDCl_3$ signals (7.25/77.0) as internal standards. A Nalorac inverse probe of 5 mm was used for all NMR experiments on **2-5** except for ^{13}C NMR measurements (Joel probe). D/CI mass spectra were recorded on a Finnigan MAT TSQ 700 instrument. β-Glucosidase from almonds (1000 IU/mg) was purchased from Fluka AG (No. 49290).

Plant material: The root bark of *Dictamnus dasycarpus* was purchased from Shanghai Medicine Materia Corporation, and identified by Prof. Jixian Guo of School of Pharmacy, Shanghai Medical University. A voucher specimen is deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Extraction and isolation: The root bark of *Dictamnus dasycarpus* (3 kg) was powdered and then percolated three times with dichloromethane and methanol successively at room temperature (3 x 10 l). The filtrate was evaporated to dryness to give dichloromethane (150 g) and methanol extracts (90 g), respectively.

The methanol extract (85 g) was subjected to column chromatography on silica gel with a chloroform and methanol gradient (8:1→1:1). The fraction eluted with chloroform-methanol (5:1) (6.5 g) was filtered through a Sephadex LH-20 column with chloroform-methanol (1:1), and then subjected to column chromatography on silica gel with chloroform-methanol-water (10:3:0.3) to give **2** (50 mg) as an amorphous powder. The fraction eluted with chloroform-methanol (4:1) (5.0 g) was first filtered through a Sephadex LH-20 column with chloroform-methanol (1:1). It was then subjected to chromatographies on silica gel columns with chloroform-methanol-water (10:3:0.3) and RP-18 Lobar columns with a methanol-water gradient (1:4→3:7) to give **1** (150 mg), **3** (120 mg) and **5** (15 mg) as amorphous powders. The fraction eluted with chloroform-methanol (3:1) (7.1 g) was filtered through a Sephadex LH-20 column with chloroform-methanol (1:1), and then subjected to column chromatography on silica gel with chloroform-methanol-water (10:3:0.3) to give **4** (30 mg) as an amorphous powder. Compound **1** was further crystallised from a mixture of methanol and water (1:1) to give triclinic crystals.

Acidic hydrolysis of 1-5. Compounds **1-5** (10 mg) were dissolved in 2N HCl (10 ml), respectively. After heating at 80°C in a water bath for 2 hrs, the reaction mixtures were extracted with ethyl acetate (3 x 10 ml). Then the aqueous phases were concentrated *in vacuo* and sugar components were identified by TLC chromatography on silica gel with ethyl acetate-methanol-water-acetic acid (65:15:15:20). Glucose was found as the only sugar component of **1-5**.

Dictamnoside A (1). Colourless crystals. $[\alpha]_D^{25}$ -3.6° (MeOH, *c* 0.56). D/CI MS (Positive ion mode) *m/z*: 450 $[M+NH_4]^+$, 288 $[M\text{-glu}+NH_4]^+$. ^1H NMR (200 MHz, C_5D_5N): δ 1.41 (3H, *s*), 1.58 (3H, *s*), 1.65 (3H, *s*). ^{13}C NMR (50 MHz, C_5D_5N): δ 106.2 (*d*), 83.3 (*s*), 78.8 (*d*), 78.8 (*d*), 78.6 (*d*), 76.0 (*d*), 74.9 (*d*), 72.0 (*s*), 71.7 (*d*), 70.1 (*t*), 62.7 (*t*), 56.2 (*d*), 49.9 (*s*), 43.9 (*d*), 40.9 (*t*), 30.5 (*q*), 30.3 (*t*), 29.4 (*q*), 22.8 (*t*), 22.6 (*q*), 17.6 (*t*).

Compound 1a. **1** (20 mg) was dissolved in a mixture of acetic anhydride (3 ml) and anhydrous pyridine (2 ml). The solution was heated at 80 °C in a water bath for 2 hrs, and then evaporated to dryness *in vacuo*. The residue was purified on a silica gel column, with petrol-

acetone (1.5:1) as eluent to give **1a** (20 mg) as an amorphous powder. $[\alpha]_D^{25}$ -28.4° ($CHCl_3$, *c* 0.05). D/CI MS (Positive ion mode) *m/z*: 660 $[M+NH_4]^+$. ^1H NMR (200 MHz, C_5D_5N): δ 2.53 (1H, *br s*), 2.10 (6H, *s*), 2.02 (9H, *s*), 1.58 (3H, *s*), 1.65 (3H, *s*), 1.60 (3H, *s*). ^{13}C NMR (50 MHz, C_5D_5N): δ 170.4 (*s*), 170.3 (*s*), 170.3 (*s*), 169.8 (*s*), 169.5 (*s*), 100.0 (*d*), 83.4 (*s*), 78.6 (*d*), 76.3 (*d*), 73.5 (*d*), 72.46 (*d*), 72.3 (*d*), 72.0 (*s*), 70.5 (*t*), 69.2 (*d*), 62.5 (*t*), 55.5 (*d*), 48.9 (*s*), 44.0 (*d*), 40.6 (*t*), 30.7 (*q*), 28.8 (*q*), 26.5 (*t*), 22.5 (*q*), 22.1 (*t*), 20.9 (*q*), 20.7 (*q*), 20.6 (*q*), 20.4 (*q*), 20.4 (*q*), 19.1 (*t*).

Compound ***1b***. **1** (30 mg) was dissolved in 2N HCl (10 ml) and then 10 ml ethyl acetate were added. After heating at 80°C in a water bath for 2 hrs, the mixture was separated into ethyl acetate and water fractions by further extraction. The ethyl acetate fraction was subjected to column chromatography on silica gel with a chloroform-acetone gradient (5:1→3:1) to give **1b** (10 mg) as a colourless oil. $[\alpha]_D^{25}$ -7.0° ($CHCl_3$, *c* 0.72). D/CI MS (Negative ion mode) *m/z*: 233 $[M-H]^-$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.829 (1H, *s*), 5.007 (1H, *s*), 4.897 (1H, *s*), 3.835 (1H, *d*, 8.5), 3.649 (1H, *d*, 8.5), 1.912 (3H, *br s*), 1.278 (3H, *s*). ^{13}C NMR (125 MHz, $CDCl_3$): δ 143.14 (*s*), 137.03 (*s*), 121.28 (*d*), 111.09 (*t*), 81.69 (*s*), 75.39(*d*), 67.29 (*t*), 50.21 (*d*), 47.21 (*s*), 38.91 (*t*), 29.46 (*t*), 22.88 (*t*), 21.69 (*q*), 21.48 (*t*), 20.70 (*q*).

Compound ***1c***. **1** (40 mg) and β-glucosidase (60 mg) were dissolved in 5 ml distilled water. The solution was first kept at 35 °C for 3 days, and then at room temperature for 30 days until hydrolysis was complete as revealed by TLC analysis. The water solution was extracted with ethyl acetate (3 x 10 ml). The ethyl acetate extract was dried *in vacuo* and then chromatographed on a silica gel column with chloroform-acetone (2:1) to give **1c** (20 mg) as an amorphous powder. $[\alpha]_D^{25}$ +24.7° ($CHCl_3$, *c* 0.14). D/CI MS (Positive ion mode) *m/z*: 288 $[M+NH_4]^+$. ^1H NMR (500 MHz, C_5D_5N): δ 4.604 (1H, *dd*, 7.5, 5.5), 4.396 (1H, *d*, 8.5), 3.965 (1H, *dd*, 9.5, 7.0), 3.915 (1H, *d*, 8.5), 1.631 (3H, *s*), 1.573 (3H, *s*), 1.525 (3H, *s*).^{13}C NMR (50 MHz, C_5D_5N): δ 83.8 (*s*), 76.0 (*d*), 74.1 (*s*), 69.7 (*t*), 69.4 (*d*), 55.7 (*d*), 50.5 (*s*), 46.7 (*d*), 40.6 (*t*), 31.4 (*q*), 30.5 (*t*), 30.0 (*q*), 23.5 (*q*), 23.4 (*t*), 20.8 (*t*).

Crystallographic data for compound 1. $C_{21}H_{36}O_9 .2H_2O$, triclinic, space group P1 (No.2), a = 6.8317(7), b = 12.080(2), c = 14.9623(14) Å, a = 74.988(7), b = 81.253(8), g = 87.711(8) °, Z = 2, 5411 independent reflections, 4894 observed reflections [I > 2s(I)], final R1 0.041, Rw2 0.093 (observed data). Goodness of fit 1.118, residual density max/min: 0.223/-0.178 e $Å^{-3}$. Absorption coefficient m = 0.106 mm^{-1}.

Intensity data were collected at -50 °C on a Stoe AED2 4-circle diffractometre using MoKa graphite monochromated radiation with 2Q/w scans in the 2Q range 4-55 °. The structure was solved by direct methods using the program SHELXS-86 [10]. The refinement and all further calculations were carried out using SHELXL-93 [11]. The H-atoms of the water molecules were located from difference maps and refined isotropically. The hydroxyl H-atoms were included in idealised positions with X-O-H angles which maximize the electron density, a rotating group refinement was then applied. The remainder were included in idealised positions and treated as riding atoms. The non-H atoms were refined anisotropically using weighted full-matrix least-squares on F^2.

Bond lengths and angles are normal within experimental error. There are two independent molecules (1 and 2) in the unitcell together with four molecules of water of crystallisation. The molecular structure and crystallographic numbering scheme of Molecule 1 of **1** is illustrated in Figure 1. In the crystal, the two independent molecules and the water molecules are involved in an extensive hydrogen bonding network as shown in Figure 2.

Full tables of atomic parameters and bond lengths and angles may be obtained from the Cambridge Crystallographic Data Centre, UK., on quoting the full journal citation. Further details may be obtained from the author H. St-E.

acetone (1.5:1) as eluent to give **1a** (20 mg) as an amorphous powder. $[\alpha]_D^{25}$ -28.4° ($CHCl_3$, *c* 0.05). D/CI MS (Positive ion mode) *m/z*: 660 $[M+NH_4]^+$. ^1H NMR (200 MHz, C_5D_5N): δ 2.53 (1H, *br s*), 2.10 (6H, *s*), 2.02 (9H, *s*), 1.58 (3H, *s*), 1.65 (3H, *s*), 1.60 (3H, *s*). ^{13}C NMR (50 MHz, C_5D_5N): δ 170.4 (*s*), 170.3 (*s*), 170.3 (*s*), 169.8 (*s*), 169.5 (*s*), 100.0 (*d*), 83.4 (*s*), 78.6 (*d*), 76.3 (*d*), 73.5 (*d*), 72.46 (*d*), 72.3 (*d*), 72.0 (*s*), 70.5 (*t*), 69.2 (*d*), 62.5 (*t*), 55.5 (*d*), 48.9 (*s*), 44.0 (*d*), 40.6 (*t*), 30.7 (*q*), 28.8 (*q*), 26.5 (*t*), 22.5 (*q*), 22.1 (*t*), 20.9 (*q*), 20.7 (*q*), 20.6 (*q*), 20.4 (*q*), 20.4 (*q*), 19.1 (*t*).

Compound ***1b***. **1** (30 mg) was dissolved in 2N HCl (10 ml) and then 10 ml ethyl acetate were added. After heating at 80°C in a water bath for 2 hrs, the mixture was separated into ethyl acetate and water fractions by further extraction. The ethyl acetate fraction was subjected to column chromatography on silica gel with a chloroform-acetone gradient (5:1→3:1) to give **1b** (10 mg) as a colourless oil. $[\alpha]_D^{25}$ -7.0° ($CHCl_3$, *c* 0.72). D/CI MS (Negative ion mode) *m/z*: 233 $[M-H]^-$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.829 (1H, *s*), 5.007 (1H, *s*), 4.897 (1H, *s*), 3.835 (1H, *d*, 8.5), 3.649 (1H, *d*, 8.5), 1.912 (3H, *br s*), 1.278 (3H, *s*). ^{13}C NMR (125 MHz, $CDCl_3$): δ 143.14 (*s*), 137.03 (*s*), 121.28 (*d*), 111.09 (*t*), 81.69 (*s*), 75.39(*d*), 67.29 (*t*), 50.21 (*d*), 47.21 (*s*), 38.91 (*t*), 29.46 (*t*), 22.88 (*t*), 21.69 (*q*), 21.48 (*t*), 20.70 (*q*).

Compound ***1c***. **1** (40 mg) and β-glucosidase (60 mg) were dissolved in 5 ml distilled water. The solution was first kept at 35 °C for 3 days, and then at room temperature for 30 days until hydrolysis was complete as revealed by TLC analysis. The water solution was extracted with ethyl acetate (3 x 10 ml). The ethyl acetate extract was dried *in vacuo* and then chromatographed on a silica gel column with chloroform-acetone (2:1) to give **1c** (20 mg) as an amorphous powder. $[\alpha]_D^{25}$ +24.7° ($CHCl_3$, *c* 0.14). D/CI MS (Positive ion mode) *m/z*: 288 $[M+NH_4]^+$. ^1H NMR (500 MHz, C_5D_5N): δ 4.604 (1H, *dd*, 7.5, 5.5), 4.396 (1H, *d*, 8.5), 3.965 (1H, *dd*, 9.5, 7.0), 3.915 (1H, *d*, 8.5), 1.631 (3H, *s*), 1.573 (3H, *s*), 1.525 (3H, *s*).^{13}C NMR (50 MHz, C_5D_5N): δ 83.8 (*s*), 76.0 (*d*), 74.1 (*s*), 69.7 (*t*), 69.4 (*d*), 55.7 (*d*), 50.5 (*s*), 46.7 (*d*), 40.6 (*t*), 31.4 (*q*), 30.5 (*t*), 30.0 (*q*), 23.5 (*q*), 23.4 (*t*), 20.8 (*t*).

Crystallographic data for compound ***1***. $C_{21}H_{36}O_9 .2H_2O$, triclinic, space group P1 (No.2), a = 6.8317(7), b = 12.080(2), c = 14.9623(14) Å, a = 74.988(7), b = 81.253(8), g = 87.711(8)°, Z = 2, 5411 independent reflections, 4894 observed reflections [I > 2s(I)], final R1 0.041, Rw2 0.093 (observed data). Goodness of fit 1.118, residual density max/min: 0.223/-0.178 e $Å^{-3}$. Absorption coefficient m = 0.106 mm^{-1}.

Intensity data were collected at -50 °C on a Stoe AED2 4-circle diffractometre using MoKa graphite monochromated radiation with 2Q/w scans in the 2Q range 4-55 °. The structure was solved by direct methods using the program SHELXS-86 [10]. The refinement and all further calculations were carried out using SHELXL-93 [11]. The H-atoms of the water molecules were located from difference maps and refined isotropically. The hydroxyl H-atoms were included in idealised positions with X-O-H angles which maximize the electron density, a rotating group refinement was then applied. The remainder were included in idealised positions and treated as riding atoms. The non-H atoms were refined anisotropically using weighted full-matrix least-squares on F^2.

Bond lengths and angles are normal within experimental error. There are two independent molecules (1 and 2) in the unitcell together with four molecules of water of crystallisation. The molecular structure and crystallographic numbering scheme of Molecule 1 of **1** is illustrated in Figure 1. In the crystal, the two independent molecules and the water molecules are involved in an extensive hydrogen bonding network as shown in Figure 2.

Full tables of atomic parameters and bond lengths and angles may be obtained from the Cambridge Crystallographic Data Centre, UK., on quoting the full journal citation. Further details may be obtained from the author H. St-E.

Dictamnoside B (2). Amorphous powder. $[\alpha]_D^{25}$ -10.5° (MeOH, *c* 0.55). D/CI MS (Positive ion mode) *m/z*: 450 $[M+NH_4]^+$, 288 $[M\text{-glu}+NH_4]^+$. ^1H NMR (600 MHz, C_5D_5N) and ^{13}C NMR (150 MHz, C_5D_5N): Table 1 and 2.

Dictamnoside C (3). Amorphous powder. $[\alpha]_D^{25}$ -30.2° (MeOH, *c* 0.57). D/CI MS (Positive ion mode) *m/z*: 450 $[M+NH_4]^+$, 288 $[M\text{-glu}+NH_4]^+$. ^1H NMR (600 MHz, C_5D_5N) and ^{13}C NMR (150 MHz, C_5D_5N): Table 1 and 2.

Dictamnoside D (4). Amorphous powder. $[\alpha]_D^{25}$ -19.5° (MeOH, *c* 0.59). D/CI MS (Positive ion mode) *m/z*: 468 $[M+NH_4]^+$. ^1H NMR (600 MHz, C_5D_5N, room temperature): δ 3.828 (1H, *dd*, 9.1, 4.7, H-1), 2.040 (1H, *m*, H-2α), 2.180 (1H, *m*, H-2β), 2.153 (1H, *m*, H-3α), 2.060 (1H, *m*, H-3β), 2.816 (1H, *d*, 2.5, H-5), 5.132(1H, *dd*, 3.3, 2.5, H-6), 2.237 (1H, *m*, H-7), 2.590 (1H, *m*, H-8α), 2.336 (1H, *m*, H-8β), 1.704 (1H, *m*, H-9α), 3.055 (1H, *m*, H-9β), 1.652 (3H, *s*, H-12), 1.504 (3H, *s*, H-13), 4.604 (1H, *d*, 11.4, H-14a), 4.186 (1H, *m*, H-14b), 1.373 (3H, *s*, H-15), 5.308 (1H, *d*, 8.0, $H_{G\text{-}1}$), 4.040 (1H, *m*, $H_{G\text{-}2}$), 4.055 (1H, *m*, $H_{G\text{-}2}$), 3.940 (1H, *dd*, 9.5, 8.8, $H_{G\text{-}4}$), 4.160 (1H, *m*, $H_{G\text{-}5}$), 4.687 (1H, *dd*, 11.0, 1.9, $H_{G\text{-}6a}$), 4.178 (1H, *m*, $H_{G\text{-}6b}$). ^{13}C NMR (150 MHz, C_5D_5N): Table 2.

Dictamnoside E (5). Amorphous powder. $[\alpha]_D^{25}$ -6.2° (MeOH, *c* 0.29). D/CI MS (Positive ion mode) *m/z*: 392 $[M+NH_4]^+$, 230 $[M\text{-glu}+NH_4]^+$. ^1H NMR (600 MHz, C_5D_5N) and ^{13}C NMR (150 MHz, C_5D_5N): Table 1 and 2.

Acknowledgement--This work was supported by the Swiss National Science Foundation. The authors are grateful to Dr. A. Marston for his help during the investigation.

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TWO NEW SESQUITERPENE GLUCOSIDES FROM *IXSERIS* PLANTS

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Key Word Index--*Ixeris denticulata* f. *pinnatipartita* Kitag.; *I. sonchifolia* Hance; Compositae; sesquiterpene lactone glucoside; guaianolide; ixerin X; ixerin Z; cytotoxicity.

Abstract The whole plant of *Ixeris denticulata* f. *pinnatipartita* and *I. sonchifolia* respectively afforded a new guaianolide sesquiterpene lactone glucoside named ixerin X, 8(,15-dihydroxy-1(10),3,11(13)-guaiatriene-12,6-olide-15-O-β-D-glucopyranoside (**1**), ixerin Z, 1(10),3,11(13)-guaiatriene-12,6-olide-2-one-3-O- β -D-glucopyranoside (**2**), whose structure and stereo-chemistry were determined by spectroscopic methods. **1** inhibited the growth of human breast cancer MCF7 & MDA468 cell lines.

Introduction

The whole herb of *I. denticulata* f. *pinnatipartita* and *I. sonchifolia* were used as anti-inflammatory and haemostatic folk remedies in China. Chemical research of other species of the genus revealed the sesquiterpene lactones[1-7] as active principle which showed wide-spectral activities, such as cytotoxicity[8], anti-repellence & anti-feedance to some insects, etc. This paper intended to isolate any sesquiterpene lactones from *I. denticulata* f. *pinnatipartita* and *I. sonchifolia* from which sesquiterpene lactones had not been reported.

Results and Discussion

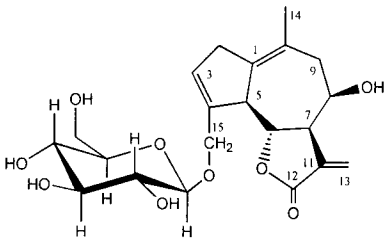
Two new sesquiterpene lactone glucosides ixerin X (**1**), Ixerin Z(**2**) were isolated from the whole herb of *I. denticulata* f. *pinnatipartita* and *I. sonchifolia*, respectively.

Ixerin X (**1**), has a molecular formula of $C_{21}H_{28}O_9$ as indicated by its high resolution EI-MS. The presence of an α-methylene-γ-lactone moiety was revealed by its IR absorption bands at 1750 and 1669cm^{-1}, and this was substantiated by a pair of characteristic low field ^{1}H NMR(CD_3OD, 250MHz) signals at δ6.13(1H, dd, J=3.3, 1.3Hz, H-13a), 6.23(1H,dd, J=3.3,1.3Hz, H-13b)(Table 1). An olefinic proton at δ6.05(1H,brs, H-3) and vinyl methyl at δ1.81(3H, brs, H-14) were also observed.

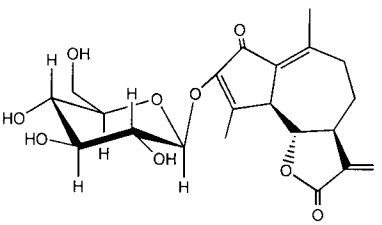
^{13}C NMR spectrum of **1** showed the presence of 21 carbons(Table 1), of which one was assigned to lactone carbonyl at δ 172.0, 6 were olefinic carbons indicating the existence of 3 double bonds, 9 oxygen bearing carbons including 6 from glucose moiety, others were 2 methylene , 2 methine and 1 methyl carbons at the upper field. In the ^{1}H NMR spectrum, a doublet at δ 3.75(1H,J=10.0Hz), was attributed to H-5 which coupled with H-6. The signal of

H-6 was at δ 3.71(1H,dd,J=10.0,12.5Hz). This indicated the trans-diaxial relationship between H5 and H6, H6 and H7. Since the H-7 in all other naturally occurring guaianolides has been assumed to be α -oriented, so, H-5 and H-6 would be α and β oriented in **1**, respectively. Signal at δ 3.03(1H,dt, J=3.3, 12.5Hz) was assigned to H-7, the large coupling constant between H-7 and H-8 (12.5Hz) indicative of an approximate dihedral angle of around 180°, allowed the assignment of H-8 as α configuration.

Based on the above evidence as well as by comparison with the data of previously reported crepidiaside E[9], **1** was finally determined to be 8β,15-dihydroxy-1(10),3,11(13)-guaiatriene-12,6-olide-15-β-D-glucopyranoside. The anomeric structure of **1** was thought to be β from its coupling constant (7.8Hz).



1



2

Ixerin Z (2), amorphous powder. FAB-MS and EI-MS indicated the formula of $C_{21}H_{26}O_9$. ^{1}H NMR (pyridine-d5, 400MHz) showed the characteristic signals at δ 6.19(1H, brs, H-13a), and δ 5.19(1H,brs, H-13b) which revealed the presence of α-methylene-γ-lactone moiety. Signals for 2 vinyl methyls at δ 2.31(3H, brs, H-15), δ 2.42(3H,brs, H-14), and for a sugar moiety were also observed. A double doublet at δ 3.17(1H, J=10.0, 12.2Hz) was assigned to H-6, which coupled with H-5 at δ 3.22(1H,d,J=10.0Hz) and H-7 at δ 2.74(1H, brt, J=12.2), this indicated the trans-diaxial relationship of respective vicinal 2 protons. The stereochemistry of these protons were considered to be H-5α, H-6β, since the H-7 in naturally occurring guaianolides have α-orientation. ^{13}C NMR data of **2** showed the presence of 21 carbons, of the 8 unsaturated carbons, 2 carbonyls were attributed to lactone carbonyl at δ 169.1 (C-12) and a α, β-unsaturated ketone carbonyl at δ 188.9(C-2), 6 were olefinic carbons; of 13 alkyl carbons, 7 were oxygen bearing carbons including 6 derived from glucose moiety, others were 2 methines, 2 methylenes and 2 methyls. The anomeric configuration of glucose moiety was determined to be β from $J_{H1'\text{-}H2'}$ value(7.0Hz). Based on above evidence, **2** was identified to be 1(10),3,11(13)-guaiatriene-12,6-olide-2-one-3-O-β-D-glucopyranoside as a previously not reported compound.

Ixerin X showed the inhibitory effect toward the growth of human breast cancer MCF7 and MDA468 cell lines, with the IC50 of 15 and 28μM, respectively (Fig. 1).

Table 1. ^{1}H NMR & ^{13}C NMRdata of compounds **1*** & **2****

(δ values from internal TMS, J in Hz)

	^{1}H NMR		^{13}C NMR	
	1	**2**	**1**	**2**
1			138.1 s	153.0 s
2	3.8(2H,m)		38.1 t	188.9 s
3	3.11(2H,brs)		131.1 d	153.7 s
4	6.05(1H,brs)		142.0 s	146.1 s
5	3.75(1H,d,J=10.0)	3.22(1H,d,J=10.0)	52.8 d	52.4 d
6	3.71(1H,dd,J=10.0,12.5)	3.17(1H,dd,J=10.0,12.2)	84.2 d	85.1 d
7	3.03(1H,dt, J=3.3, 12.5)	2.74(1H,brt, J=12.2)	59.2 d	47.9 d
8	3.76(1H,m, overlapped)	α: 2.29(1H,m, overlapped) β: 1.06(1H,dd,J=12.2)	69.7 d	24.1 t
9	α: 2.27(1H,dd, J=13.3, 2.5) β: 2.62(1H,dd, J=13.3, 11.5)	α: 2.08(1H,m) β: 1.89(1H,m)	46.6 t	36.9 t
10			127.5 s	124.6 s
11			140.2 s	139.5 s
12			172.0 s	169.1 s
13	a: 6.13(1H,dd, J=3.3, 1.3) b: 6.23(1H,dd, J=3.3, 1.3)	a: 6.19(1H, brs) b: 5.19(1H, brs)	122.0 t	117.9 t
14	1.83(3H, brs)	2.42(3H,s)	23.0 q	21.7 q
15	a: 4.52(1H,brd, J=13.0) b: 4.68(1H,brd, J=13.0)	2.31(3H,s)	68.8 d	14.9 q
1'	4.33(1H,d, J=7.8)	6.24(1H, d, J=7.0)	102.8 d	101.6 d
2'			75.1 d	75.3 d
3'			77.9 d	78.3 d
4'			71.7 d	71.1 d
5'			78.2 d	78.3 d
6'			62.8 t	62.3 t

* measured in CD_3OD at 250MHz for ^{1}H and 62.6MHz for ^{13}C

** measured in pyridine-d_5 at 400MHz for ^{1}H and 100MHz for ^{13}C

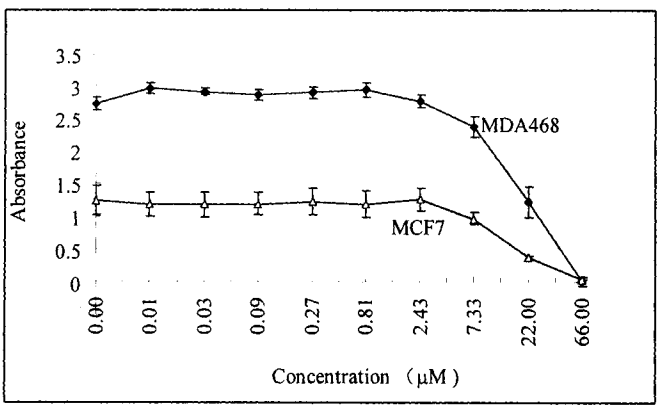


Fig. 1 Inhibitory Effect of Ixerin X on the Growth of Human Breast Cancer MCF7 and MDA468 Cell Lines

Experimental

General. Mps: uncorr.; ^{1}H NMR and ^{13}C-NMR: 250MHz and 62.5MHz, respectively for **1**, 400 MHz and 100MHz respectively, for **2**; 2D-NMR data (^{1}H-^{1}H COSY, HMQC, HMBC, NOESY): standard pulse sequences. CD_3OD and pyridine-d5 were used as solvent, respectively for **1** and **2**, with TMS as int. standard; EI-MS: 70ev, direct int.; FT-IR: KBr; CC: silica gel (coarse silica gel, 100~200 mesh), D-101 resin (16~50mesh); TLC: precoated silica gel plates (Merk, silica gel 60 F254).

Plant material. The plant of *I. denticulata* f. *pinnatipartita* Kitag.was collected in Dabie Mountains at Luotian county, Hubei province, China, in October 1992. *I. sonchifolia* Hance. was purchased from Nanjing Company of Traditional Chinese Medicine, in February 1991. Voucher specimen is deposited in the Herbarium of the Department of Pharmacognosy, China Pharmaceutical University.

Extraction & Isolation. The air-dried whole plant of *I. denticulata* f. *pinnatipartita* (4Kg) were extracted (x3) with hot (100°) water. After filtration, the solution was concentrated to 10 l , and precipitated by adding EtOH to a concentration of 60%. The supernatant was filtered after over night standing. The filtrate was applied to D-101resin column after the EtOH was removed off. The column was washed with water first, then with 20%, 40% and 85%EtOH successfully. The fraction from 40% EtOH was concentrated and was chromatographed on a silica gel column and eluted with chloroform-methanol (95:5~8:2). The eluates were monitored by TLC, and repeated chromatography afforded compound **1** (20mg).

The crude materials of *I. sonchifolia* (5Kg) were extracted with hot 95% EtOH. The extract was evaporated to dryness and extracted with petroleum ether, chloroform and methanol successively. The chloroform extract was applied to silica gel column and the column was eluted with n-hexane-chloroform-methanol(1:1:0~0:1:1). Repeated chromatography and purification gave compound 2 (25mg).

Ixerin X (**1**), fine needles(MeOH). Mp 180-183(dec.). UVλ^{MeCN}_{max}nm: 231. $[\alpha]^{28}_D$ -38.9° (MeOH, c0.11). IR ν^{KBr}_{max}cm-1: 3412(-OH), 2294, 2880, 1750(=C=O), 1669, 1448, 1384,1078, 1029. EI-MS m/z: 424[M]+ (3), 317(4), 294(4), 262(13), 244[M-$C_6H_{12}O_6$]+ (100), 227(43), 181(40), 119(56). ^{1}H NMR (CD_3OD, 250MHz) & ^{13}C NMR (CD_3OD, 62.5MHz): see Table 1.

Ixerin Z (**2**), amorphous powder. UV λ^{MeCN}_{max}nm: 267, 198. $[\alpha]^{28}_D$ +35.1° (MeOH, 0.11). FAB-MS m/z: 445[M+Na]+, 422[M]+. EI-MS m/z: 260 [M+1-$C_6H_{11}O_5$]+ (100), 245(13), 214(15), 189(53), 161(29), 151(28), 134(25), 107(22). ^{1}H NMR (pyridine-d5, 400MHz) & ^{13}C-NMR (pyridine-d5, 100MHz): see Table 1.

Bioassay. The regularly differentiated and cultured human breast cancer MCF7 and MDA468 cell lines (provided by Cancer Research Center, Nortingham University, U.K.) was incubated in a CO_2 filled incubator at 37 after the culture solution was removed and EDTA added. The suspended cells was diluted by medium (RPMI 1640 500ml, by adding 50ml of bovine serum) to be the concentration of 2.5(10^2/hole, transferred to a 96-holed culture plate (180μl/hole) and cultured for 4 hr. Ixerin X (1.4mg) was dissolved in 0.5ml of DMSO. The solution was 10 times diluted first, then 3 times diluted in turn with medium (RPMI 1640) to make 9 gradient concentration lines, and used as test solutions. 20μl of the test solution was added to above mentioned 96-holed culture plate which contains cultured cells, 8 holes was

used for one concentration and 20μl of the RPMT medium which was free of the sample (ixerin X) was used as control. The plate was cultured for a week. 50μl of MTT (2 mg/ml) i.e. 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide was added to each hole, and cultured for another 4hr. The purple sediment would be observed at the bottom of the hole, the culture solution that containing MTT was removed, and 125μl of DMSO-Glycine (100:25) buffer solution was added to each hole, the sediment was dissolved by oscillating the culture plate on a cradle. Subsequently, the absorbance of the solution was measured at 550nm, which was considered to correlate with the cell survival rate. The mean absorbance and the coefficient of variation for each concentration were calculated, and the relationship of absorbance versus concentrations was plotted, and IC_{50} calculated.

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TWO NEW DITERPENE DILACTONE GLYCOSIDES WITH A TRISACCHARIDE MOIETY FROM *PODOCARPUS NAGI*

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Key Word Index: *Podocarpus nagi;* Podocarpaceae; diterpene dilactone glycoside; nagilactosides F and G

Abstract - Another two diterpene dilactone glycosides, nagilactosides F and G were isolated from the polar fraction of *Podocarpus nagi*. Their structures were determined as 1-deoxy-nagilactone A-2α- O-β-D -glucopyranosyl - (1-3) -β-D-glucopyranosyl- (1-6) -β-D-glucopyranoside and 1-deoxy - nagilactone A-2α -O -β -D -glucopyranosyl - (1-6) - β - D -glucopyranosyl - (1-3)-β-D-glucopyranoside respectively by chemical and spectroscopic methods.

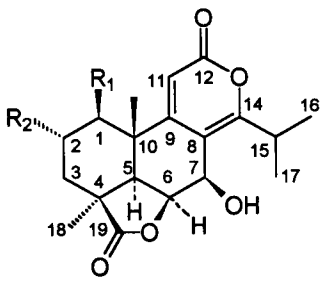
Introduction

Although the diterpene dilactones in Podocarpaceae were extensively studied because of their antitumor, plant inhibitory, insecticidal and other biological activities, their glycosides were reported very sparingly. We have found five diterpene dilactone glycosides, nagilactosides A-E(**1-5**)[1-3], with a monosugar or a disaccharide moiety from *Podocarpus nagi* (Podocarpaceae). Our further study led to the isolation of another two new glycosides with a trisaccharide moiety, nagilactosides F(**6**) and G(**7**), from the polar fraction of *P. nagi*.

Results and discussion

Nagilactosides F and G were all obtained as amorphous powders. Their UV and IR spectra showed the characteristic absorption of A-type diterpene dilactone[4] at 300 nm(α-pyranone), and 1750-1760 cm^{-1}(γ-lactone) and 1700-1715, 1620-1625, 1540 cm^{-1}(α-pyranone). Their positive FAB-mass spectra gived same molecular ion peaks at m/z 835$[M+H]^+$ and three fragment ion peaks at m/z 673[MH-162], 511[MH-2×162] and 349[MH-3×162], which indicated the successive loss of three sugar residues. In their ^1H NMR spectra (Table 1) the characteristic proton signals of A-type diterpene dilactone and the three anomeric proton of signals of sugars were assigned through the analysis of their ^1H-^1H COSY spectra.

From their ^{13}C NMR spectra the linkage positions and the configuration of the glycosidic bonds were determined. The diagnostic lowfield shift of two inner glucoses C-3 (δ 88.2 for **6** and δ 87.6 for **7**) and C-6 (δ 69.2 for **6** and δ 68.7 for **7**) indicated that the three sugars were linked through a 1→3 and 1→6 glycosidic bonds. The glycosidic configurations were assigned as β from the chemical shifts of the anomeric carbons (δ>100) and the coupling constants of the anomeric protons ($J \geq 7.5$ Hz). For finally determing the connection sequence of the sugars and the complete structure, partial hydrolysis with cellulase were carried out. **6** gived 1-deoxy-2α-hydroxy nagilactone A, nagilactoside A (**1**) and nagilactoside C (**3**). **7** afforded glucose, 1-deoxy-2α-hydroxy nagilactone A, nagilactoside A (**1**) and nagilactoside D (**4**). Accordingly, the structure of nagilactoside F (**6**) is 1-deoxy-nagilactone A - 2α - O - β - D - glucopyranosyl (1→6) - β - D-glucopyranosyl (1→3)-β-D-glucopyranoside and nagilactoside G (**7**) is 1 - deoxy - nagilactone A - 2α - O - β - D -glucopyranosyl (1→3)-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside.



	R_1	R_2
1	H	β-D-glc-
2	β-D-glc-	H
3	H	β-D-glc-(1→3)-β-D-glc-
4	H	β-D-glc-(1→6)-β-D-glc-
5	β-D-glc-(1→6)-β-D-glc-	H
6	H	β-D-glc-(1→6)-β-D-glc-(1→3)-β-D-glc-
7	H	β-D-glc-(1→3)-β-D-glc-(1→6)-β-D-glc-

Table 1. ^{1}H NMR Spectral Data of Compound 6 and 7 (400 MHz, DMSO-d_6) *

Proton	6	7
H-1α	1.77, dd (6.2, 13.7)	1.73, dd (5.9, 13.5)
H-1β	2.28, dd (9.7, 13.5)	2.28, dd (9.8, 13.1)
H-2β	4.08, m	4.10, m
H-3α	1.87, dd (5.2, 13.8)	1.87, dd (8.3, 13.5)
H-3β	1.99, t (13.8)	1.99, t (12.9)
H-5α	1.90, d (6.3)	1.93, d (6.4)
H-6α	5.00, m	5.02, m
H-7α	5.16, m	5.19, m
HO-7β	5.79, d (4.2)	5.78, d (4.1)
H-11	5.94, s	5.94, s
H-15	3.22, m	3.25, m
CH_3-16, 17	1.18, d (6.6)	1.19, d (6.8)
	1.15, d (6.6)	1.16, d (6.8)
CH_3-18, 20	1.35, s	1.37, s
	1.29, s	1.30, s
H-1',1'',1''' †	4.18, d (7.7)	4.22, d (7.7)
	4.35, d (7.8)	4.34, d (7.8)
	4.38, d (7.9)	4.38, d (7.7)

* Chemical shifts were reported in ppm, followed by signal multiplicity and coupling constants (Hz) in parentheses, the signals were assigned by ^{1}H-^{1}H COSY.

† Other sugar proton signals showed very complicated pattern and could not be distinguished.

Table 2. ^{13}C NMR Spectral Data of Compound 6 and 7 (25.05 MHz, DMSO-d_6) *

Carbon	6	7	Carbon	6	7
C-1	40.4	40.4	C-1'	101.6	101.1
C-2	72.1	71.2	C-2'	72.6	74.0
C-3	33.5	33.2	C-3'	88.2	76.8
C-4	42.4	42.4	C-4'	69.2	70.1
C-5	47.8	47.6	C-5'	75.5	75.4
C-6	74.0	73.6	C-6'	61.5	68.7
C-7	59.2	59.1	C-1"	103.2	103.1
C-8	110.7	111.2	C-2"	74.9	72.4
C-9	170.2	169.0	C-3"	76.9	87.6
C-10	36.9	36.8	C-4"	70.5	68.7
C-11	105.8	105.8	C-5"	76.4	76.8
C-12	162.9	161.5	C-6"	69.2	61.1
C-14	167.3	166.2	C-1"'	104.1	104.1
C-15	29.0	28.8	C-2"'	74.0	74.0
C-16, 17	20.7	20.3	C-3"'	76.9	76.8
	20.7	20.3	C-4"'	70.5	70.1
C-18, 20	27.0	26.5	C-5"'	76.4	76.3
	23.9	23.6	C-6"'	61.5	61.1
C-19	182.5	181.5			

* Chemical shifts were reported in ppm, and the signals were assigned by DEPT technique and comparison with known compounds.

Experimental

General. ^1H NMR spectral data were obtained on a Bruker AM-400 spectrometer and ^{13}C NMR on a Bruker AC-100 instrument. UV was recorded on a Shimadzu UV-300 instrument and IR were measured on a PE 599B spectrometer. Optical rotations were given with a Jasco DIP-181 polarimeter and CD spectra were measured with a Jasco-500C instrument. FAB MS data were recorded on a HP-5989 spectrometer. TLC were carried out on precoated Si gel 60 F_{254} plates (Merck, 0.2 mm thickness), while column chromatography were achieved on TSK gel Toyopearl HW-40F (30-60μ, Toso Co. Ltd.), MCI gel CHP 20P (25-150μ, Mitsubishi Chemical Industries Co. Ltd.) and Cosmosil 75 C_{18}-OPN (42-105μ, Nacalai Tesque Inc.) columns.The crude cellulase (12000γ/mg) was produced in our laboratory.

Plant material. The seed of *Podocarpus nagi* (Thunb.) Zoll. et Mor. ex Zoll. was collected in Renghua district, Guangdong, China. A voucher specimen is deposited at the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Extraction and isolation of the compounds. 15 Kg of defatted seed powder of *Podocarpus nagi* was extracted three times with ethanol at room temp.. After concentration, the extract (700 g) was subjected to a silica gel column with C_6H_6, CH_2Cl_2, Me_2CO and MeOH as eluent. MeOH fraction (150 g) was further applied to a MCI gel column (500 g), eluting with H_2O containing an increasing proportion of MeOH to afford two fractions. Fraction I (120 g), eluted with water, gave a large amount of sucrose, and fraction II (25 g), eluted with aqueous 40% MeOH, was further chromatographed again on a MCI gel column (300g) with H_2O-MeOH (0%-40,gradient) to afford fractions II-1 to II-5. Repeatedly chromatographed over Cosmosil 75 C_{18}-OPN, TSK gel Toyopearl HW-40F and MCI gel CHP 20P with 30%-40% MeOH, fraction II-2 and II-4 gave **6** (1.5g), **7** (0.6g).

Cellulase hydrolysis of **6** *and* **7**. A solution of **6** and **7** (2 mg) in 2 ml 0.1 M HOAc-NaOAc buffer (pH 4.5) was incubated at 40 °C with cellulase (1 mg) for 0.5 h. Partially hydrolysed products were identified by comparison with authentic samples on silica gel HPTLC with EtOH-EtOAc-H_2O (20:10:0.1) as developing solvent. 4 h later all glycosides were hydrolyzed and only glucose and aglycone were detected.

Compound **6**. Amorphous powder. Found: C, 49.09; H, 6.61. $C_{37}H_{54}O_{21}{\cdot}4H_2O$ requires: C, 49.00; H, 6.89. $[\alpha]_D^{26}$ +32.43 (H_2O; *c* 0.1). FAB-MS m/z: 835$[M+H]^+$, 673 $[M+H-(Glucose-H_2O)]^+$, 511$[M+H-2(Glucose-H_2O)]^+$, 349$[M+H-3(Glucose-H_2O)]^+$; UV $\lambda_{max}(H_2O)$ nm: 300; IR ν_{max}(KBr) cm^{-1}: 1750, 1700, 1615, 1540, 1080; CD: $\Delta\varepsilon_{208}$ +8.35°, $\Delta\varepsilon_{236}$ -1.10°, $\Delta\varepsilon_{293}$ +0.90°(H_2O; *c* 0.3); ^1H and ^{13}C NMR: Table 1 and 2.

Compound **7**. Amorphous powder. Found: C, 49.09; H, 6.61. $C_{37}H_{54}O_{21}{\cdot}4H_2O$ requires: C, 49.00; H, 6.89. $[\alpha]_D^{26}$ +25.07 (H_2O; *c* 0.1). FAB-MS m/z: 835$[M+H]^+$, 673 $[M+H-(Glucose-H_2O)]^+$, 511$[M+H-2(Glucose-H_2O)]^+$, 349$[M+H-3(Glucose-H_2O)]^+$; UV $\lambda_{max}(H_2O)$ nm: 300; IR ν_{max}(KBr) cm^{-1}: 1760, 1700, 1620, 1540, 1080; CD: $\Delta\varepsilon_{208}$ +8.50°, $\Delta\varepsilon_{236}$ -1.10°, $\Delta\varepsilon_{293}$ +0.93°(H_2O; *c* 0.3); ^1H and ^{13}C NMR: Table 1 and 2.

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CHEMICAL STUDIES OF ERYSIMUM CHEIRANTHOIDES L. II
Cardenolides from the Seeds of *Erysimum Cheiranthoides*

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Key Word Index -- *Erysimum cheiranthoides*; Cruciferae; cardenolide; cardenolide; cheiranthoside; strophanthidin

Abstract Two new cardiac glycosides were isolated along with one known aglycone of cardenolides from the seeds of *Erysimum cheiranthoides*. The new ones were characterized by spectral methods as strophanthidin, 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-3-O-acethyl-digitoxopyranoside, strophanthidin, 3-O-β-D-glucopyranosyl-(1→4)-α-L-rhanmopyranosyl (1→4)-β-D-3-O-acethyl-digitoxopyranoside, named cheiranthosides i and e, respectively.

Introduction

Erysimum cheiranthoides L. is a one-year or two-year herb belonging to *Erysimum* Genus, Cruciferae Family, distributed over the areas of China (except South China), the east of Mongolia, Korea, the center of Asia, Russia (the far east areas of Russia), some European countries and North America. As a folk drug in China, it is used mainly for diseases of cardiac failure, cardio palmus, edema, dyspepsia, etc. [1]. Our co-workers have reported that the EtOH ext. of E. cheiranthoides L. has the functions of strengthening the heart function and reducing the blood pressure [2]. Clinical observations indicate that the injection made from the herb without root has the functions of strengthening the heart function, slowing down the heart rate and reducing the blood pressure [3].

In the studies of its chemical components, Russian scientists Makarevich et al had isolated 9 cardenolides from the same herb growing in Russia [4]. Not long ago, we had isolated 5 cardenolides from the seeds of the plant, three of which were new compounds and were named Cheiranthoide I, II and III [5]. In this paper we are reporting another two new compounds isolated recently from the seeds of *E.cheiranthoides*, named cheiranthosides i and e , respectively.

Results and Discussion

The methanolic extract of the seeds of *E. cheiranthoides* was partitioned between hexane and water. Chromatographic analysis using a combination of MCI gel, silica gel and ODS provided two new glycosides, cheiranthosides i and e (1 and 2), along with one known aglycone.

Cheiranthoside i (1) showed a $[M-H]^-$ ion peak at m/z 721 and fragment peaks at m/z 679 $[M-H-CH_2CO]$ in the negative ion FAB-mass spectrum. By comparing the ^{13}C NMR spectrum of 1 with that of Cheiranthoside III(2), it was observed that the signals due to C'-2, C'-3, C'-4 and C'-5 of the sugar part were shifted by -2, +2, -2.5 and +2.3 ppm, respectively. The signals of carbonylic and methyliccarbons on acetyl group could be observed at 170.17 ppm and 20.9 ppm. The aglycone moiety signals of Cheiranthoside i(1) and Cheiranthoside III were identical. In the ^1H-NMR spectrum , proton signals of 3 methel groups at acetyl group of digitoxose C_3 due to 2.03 (3H, S) were observed.The proton signal due to 5.74 was a

proton connected with digitoxose C_3. The chemical shift of this proton,affected by acetyl group, moved, to the lower magnetic field,2-3ppm from the place of the proton without the affect of acetyl group. The signals of other protons of (1) were identical with those of CheiranthosideIII. Identified by TLC, compound (1) was inferred to be the acetylated digitoxose C_3 of Cheiranthoside III. The chemical structure of compound (1) was strophanthidin, 3-O- α -L-rhamnopyranosyl-(1 → 4)- β -D-3-O-acethyl-digitoxopyranoside, named Cheiranthoside i.

Table 1. ^{13} NMR Data for cheiranthosides i (1), e (2) and Strophanthidin (3) in C_5D_5N

	1	2	3
C-1	24.8	24.7	24.7
2	25.5	25.5	27.5
3	75.5	75.4	6.7
4	36.5	36.4	38.4
5	73.8	73.8	74.6
6	36.6	36.5	37.3
7	18.4	18.3	18.2
8	41.9	41.8	41.9
9	39.6	39.6	39.5
10	55.3	55.3	55.6
11	22.6	22.6	22.7
12	39.6	39.6	39.6
13	49.8	49.7	49.9
14	84.4	84.3	84.4
15	32.1	32.0	32.2
16	27.2	27.1	27.2
17	51.1	51.0	51.1
18	16.0	15.9	16.0
19	208.4	208.4	208.9
20	175.6	175.6	175.7
21	73.6	73.6	73.7
22	117.8	117.7	117.6
23	174.4	174.4	174.4
dig C-1	97.1	97.6	
2	37.0 [dig3-Ac	36.9	[dig3-Ac
3	70.8 20.9(Me)	70.2	20.9(Me)
4	78.8 170.17	70.4	170.1(C=O)]
5	69.6 (C=O)]	69.5	
6	18.5	18.3	
rha C-1	104.3	103.6	
2	72.3	72.3	
3	72.6	71.3	
4	73.6	84.8	
5	70.5	68.8	
6	18.5	18.3	
glc C-1		106.6	
2		76.1	
3		87.4	
4		71.7	
5		78.4	
6		62.4	

Cheiranthoside e (2) showed a $[M-H]^-$ ion peak at m/z 883 in the negative ion FAB - mass spectum. The ^{13}C NMR spectrum of(2) revealed that it had the same aglycone with that of (1) but the sugar moiety had one more glucose than that of (1), thus indicating that (2) was strophanthidin, 3 -O- β -D-glucopyranosyl - (1→4) - α -L-rhamnopyranosyl-(1→4)- β - D-3-O-acethyl-digitoxopyranoside. The spectrum data of (3) was the same as that of strophanthidin [4]. Therefore, (3) was identified strophanthidin.

Experiment

Optical rotations were taken with a JASCO DIP-360 digital polarimeter (l=0.5). ^1H(400 MHz) and ^{13}C(100 MHz) NMR: with TMS as an internal standard. FAB and EI-MS: JEOL JMS DX-303 HF mass spectrometer. TLC was performed on precoated silica gel 60 F_{254} (Merck) and detection was achieved by spraying10% H_2SO_4 following by heating. CC: silica gel (270-400 mesh, Merck), Chromatorex ODS (30-50 mesh, Fugi Silysia Chemical Ltd.) and MCI gel CHP-20P (Mitsubishi Chemical Ind.).

Extraction and separation. The seeds (2.5 Kg) of *E.cheiranthoides* were extracted with MeOH, and the extract (189 g) was partitioned between n-hexane and H_2O. The aq. layer (123 g) was subjected to CC over MCI gel CHP-20P, eluting sequentially with H_2O, 40%, 60%, 80% and 100% MeOH, to provide five frs (fr. 1-5). Fr 3 (60% MeOH eluate, 18g) was subjected to CC over silica gel ($CHCl_3$ -MeOH-H_2O, 8:2:0.2) and ODS (50% MeOH) to give cheiranthoside e (2,24mg). Fr. 4 (80%MeOH eluate,23g) was subjected to CC over silica gal ($CHCl_3$-MeOH-H_2O,8:2:0.1) and ODS (60 % MeOH) to give cheiranthoside I (1, 10.8mg). Fr.5 (100% MeOH eluate, 32g)was subjected to CC over silica gel ($CHCl_3$ -MeOH, 9.5:0.5-9:1) to give strophanthidin.

Cheiranthoside i (1) White amorphous powder, $[\alpha]^{24}_D$ + 0.00^0 (MeOH). Negative ion FAB-MS (m/z): 721 (M-H)$^-$, 679 (M-H-CH_2 CO)$^-$. ^{1}HNMR (pyridine d5): δ 1.01(3H, S, C_{18} -Me), 1.35 (3H, d, j=6.1 Hz, dig-6-Me),1.66 (3H, d, j=5.5 Hz, rha-6-Me), 2.03 (3H, S, dig-3-OAc-Me), 2.78 (1H, brd, j=8.0 Hz, C_{17}-H), 5.03, 5.29 (each 1H, d, j=17.7 Hz, C_{21} - H_2), 5.12 (1H, d, j=9.2 Hz, dig-1-H), 5.42 (1H, S, rha-1-H), 5.74 (1H, brd, dig-3-H), 6.13 (1H, S, C_{22} -H), 10.41 (1H, S, C_{19} -CHO).

Cheiranthoside e (2) White amorphous powder, $[\alpha]^{17}_D$ +10.24^0 (MeOH). Negative ion FAB-MS (m/z): 883 (M-H)$^-$,^{1} HNMR (pyridine d5): δ : 1.00 (3H, S, C_{18} -Me), 1.32 (3H, d, j=6.1 Hz, dig-6-Me), 1.71 (3H, d, j=6.10 Hz rha-6-Me), 2.00 (3H, S, dig-3-OAc-Me), 2.78 (1H, d, j=9.2 Hz, C_{17} -H), 5.11 (1H, dd, j=1.8,9.2 Hz,dig-1-H), 5.20 (1H, d, j=7.9Hz, glu-1-H), 5.37 (1H, S, rha-1-H), 5.67 (1H, d, j=3.1Hz dig-3-H), 6.13 (1H, S, C_{22} -H), 10.40 (1H, S, C_{19},CHO).

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DETERMINATION OF PAEONFLORIN IN QINGGAN INJECTION BY HPLC

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Key Word Index--HPLC, Paeoniflorin, Qinggan Injection, Determination

Abstract A high performance liquid chromatographic (HPLC) method for the analysis of paeoniflorin in Qinggan Injection was established. A Hypersil ODS (250 mm×4 mm ID) column was used as analytical column. The mobile phase was methanol-isopropanol-acetic acid-water (30: 2: 2: 66, v/v) and the flow rate was 0.8 ml. min^{-1}. The UV detection was performed at 233 nm. The column temperature was 30℃. Under this condition, the calibration curve was linear within the range from 0.2 to 1.6 ug· ml^{-1} with r = 0.9941. The recovery of paeoniflorin in Qinggan Injection was 98.5%. The relative standard deviations for within-day and between-day was 3.46% and 4.72%, respectively. This method is simple, rapid, sensitive, accurate and suitable for quantitative determination of single component in compounded preparations.

Qinggan Injection is a new Chinese medicine, which was made up with *Paeonia veitchii* Lynch, *Paeonia suffruticosa* Andr. and *Artemisia capillaris* Thumb. by Affiliated Third Hospital, Sun Yat-sen University of Medical Sciences. Paeoniflorin is the main component of *Paeonia veitchii*, and the pharmacological effect of paeoniflorin consists with the curative effect in the compounded preparation of *Paeonia veitchii*. Therefore, the accurate determination of paeoniflorin is an effective means for quality control in Qinggan Injection.

In this article, a high performance liquid chromatography (HPLC) method is described for the determination of paeoniflorin in Qinggan Injection.

Materials and Methods

1 Apparatus

The chromatographic system (Hewlett Packard 1100, USA) consisted of quaternary pumps with vacuum degasser (G1354A), a manual sample injector (G1328A), a variable wavelength detector (G1314A) and 2D chemistation bundle (G1317A).

2 Chromatographic conditions

A Hypersil ODS (250 mm×4 mm ID) column was used as analytical column. The mobile phase was methanol-isopropanol-acetic acid-water (30: 2: 2: 66, v/v) and the flow rate was 0.8 ml. min^{-1}. The UV detection was made at 233 nm. The column temperature was 30℃.

3 Materials

Standard paeonflorin was purchased from Natural Institute for the Control of Pharmaceutical and Biological Products. Qinggan Injection was made up by Affiliated Third Hospital, Sun Yat-sen University of Medical Sciences. All the solvents used were of analytical grade.

4 Sample preparation procedure

1 mg of paeonflorin was dissolved in 10 ml of 50%methanol; to 1 ml Qinggan Injection was dissolved in 9 ml of 50%methanol.

Results

1 Linearity

2, 4, 6, 8, 10, 12, 14, 16 ul of standard paeonflorin solution were directly injected into the HPLC system for analysis, respectively. The peak area of paeonflorin was calculated to make the calibration curve. A good linearity within the range from 0.2 to 1.6 ug. ml^{-1} with correlation coefficient of 0.9941 was obtained and the equation of the cure was Y = 51.3372+ 4662.7173X.

2 HPLC analysis

Standard paeonflorin, Qinggan Injection and sample without *Paeonia veitchi* were analyzed by HPLC. Their HPLC spectra were shown in Fig.

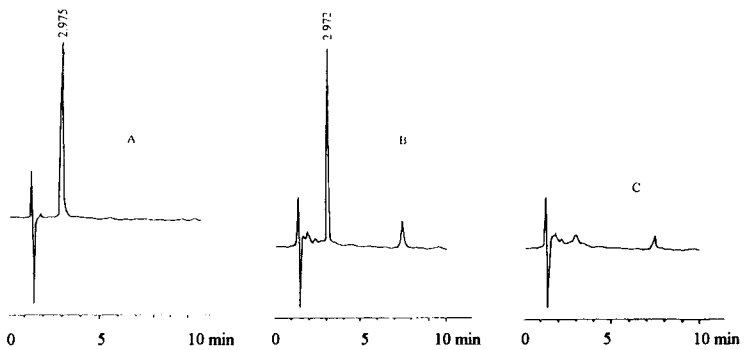

Fig. HPLC chromatograms of standard paeonflorin (A), Qinggan Injection (B) and sample without *Paeonia veitchi* (C).

Comparison of these t_R values indicated that t_R value(2.975 min) of standard paeonflorin was equal to t_R value (2.972 min) of Qinggan Injection. There was not peak of sample without *Paeonia veitchi* at 2.97 min. Therefore, paeoniflorin can be separated and determined satisfactorily without interference from impurities.

2.3 Precision

Precision was examined with 10 ul of paeonflorin which were directly injected into the HPLC system for 5 times. The within-day relative standard deviations was 3.46% (n=8). The between-day relative standard deviations was 4.72% over a period of 5 days (n=8).

2.4 Recovery

In order to check the recovery, the detected concentrations of paeoniflorin were compared with the practical value. The recovery rate of paeoniflorin from Qinggan Injection was 98.5% (RSD = 1.78%).

2.4 Sample check

The contents of paeoniflorin for Nine samples of Qinggan Injection were shown in table.

Table The results of paeoniflorin in Qinggan Injection

No.		Contents(mg/ml)	Average(mg/ml)
	1	0.0747	
970503	2	0.0732	0.0749
	3	0.0769	
	1	0.0784	
970505	2	0.0813	0.0801
	3	0.0805	
	1	0.0784	
970508	2	0.0756	0.0773
	3	0.0778	

Discussion

1 A thin layer chromatographic (TLC) scanner[3] or high performance liquid chromatography (HPLC)[1, 2] is often described for the determination of paeoniflorin in the crude drug. But the latter is more simple, more accurate and suitable for quantitative determination of single component in compounded preparations.

2 In this article, a HPLC method for the analysis of paeoniflorin in Qinggan Injection was established. The contents of paeoniflorin in tree lots of samples are steady. Therefore, It is an effective means for quality control in Qinggan Injection.

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THE STRUCTURE OF AN OLIGOSACCHARIDE AND ITS EFFECT ON CULTURED PLANT CELLS

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Key Word Index -- Oligosaccharide, Cultured plant cells, Effect

Abstract--The structure of an oligosaccharide and its effect on cultured plant cells are reported in this paper. The oligosacchride with different DP (degree of polymerization) were isolated from acid-hydrolyzed cell walls of cultured cells from *Panax ginseng*. The samples were passed through an active carbon column, a Dowex (H^+) column, and a Bio-Gel P-2 column and finally separated by HPLC. The oligosaccharide with different DP were obtained. The possible structure of an oligosaccharide DP-6 was characterized by TGC, GC-MS, FAB$^-$-MS and ^{13}C-NMR measurements.

Experiments showed that this oligosaccharide could increase the growth rate of T*axus yunnanensis* cultured cells by 36.7% and promote the taxol yielc by nearly 2 folds. Its effect was also found in other cultured plant cells such as panax notoginseng and *Carthamus tinctorius*.

Oligosaccharins are the oligosacchrides with regulatory activity derived from plant cell walls[1]. They have regulatory functions in growth, development, reproduction and defense against pathogenst[2], as a kind of elicitors, they also increase the rates of cell growth and the yields of secondary metabolites in cultured plant cells[3].

The structure of an oligosaccharide with DP-6 from the cell walls of cultured cells from *Panax ginseng* and its effect on cultured plant cells are reported in this paper. The possible structure of this oligosaccharide by characterized by GC, GC-MS, FAB$^-$-MS and ^{13}C-NMR measurements.

Materials and Methods

1. Cell culture of *Panax ginseng*

The callus of *P. ginseng* which had been subcultured in 50 ml flasks for 70 generations was incubated in MS medium supplemented with 2 mg/L 2,4-D and 0.1 mg/L KT and maintained at 26 ± 1 ^{0}C in darkness by subculturing at 25 days intervals. Suspension cultured cells were incubated on ratated shaker with 1/5 culture broth of the total capacity and rotative velocity of 120 rpm. The other cultured conditions were the same with the callus culture.

2.Extraction and isolation of oligosaccharedes

The broken cells from 4 repeating suspension culture were dissolved in 80% ethanol for 24 hours, and then their filtrates were disposed by Sevage method to remove the peptide portion. Oligosaccharides were obtained by partial acid-hydrolysis. Then the samples were neutralized with NaOH, filtered, treated, treated with active carbon and Dowex (H^+) to remove pigment and Na^+ concentrated, passed through a Bio-Gel P-2 column and three periods of oligosaccharides with different DP (degree of polymerization) were achieved. The second main peak fraction was concentrated and separated by Silica gel column. The 200ml eluate ($MeOH:H_2O=25:1$) was collected, concentrated. They were finally separated by HPLC

and three pure oligosaccharides with DP 6-8 which had the physiological effect [4] were obtained.

3.Identification of oligosaccharide DP-6

The structure of the oligosaccharide DP-6 was characterized by the methods of GC, GC-MS, FAB-MS and ^{13}C-NMR measurements described by the report.

4.Effect of oligosaccharide DP-6 on cultured plant cells

The cultured methods and conditions of cultured plant cells of T*axus yunnaensis, Panax notoginseng* and *Carthamus tinctorius*, and the experiment methods of oligosaccharide DP-6 on these cultured plant cells were reported previously [6-8].

Results and Discussion

1. Extraction and isolation of oligosaccharides

Using the way of 80% alcohol extraction and Sevage method could remove lipid-soluble and protein fraction in cells, to ensure isolated oligosacchides be the hydrolysate of semi-cellulose in primary cell walls of cultured cells from *Panax genseng*. During the course of acid hydrolysation, the factors influenced must be controlled strictly. The quantity of products of oligosaccharide could be affected on the condition of more high or too low of temperature.

Using active carbon column and Dowex (H^+) column could remove pigment, salt and saponin etc. This was good for later isolation of oligosaccharides. The oligosaccharide preparation then be further separated and purified by Bio-Gel P-2 column. The majority fraction of oligosaccharide with DP 6-8 was obtained by this isolation method (Fig.1, II peak).

Shown in studies on oligosaccharide isolation nowadays, all the last isolation and purification have gone through HPLC method. During the course of most underivative oligosacchride HPLC isolation, purified water was used as elution solvent. But every base line of oligosaccharide's peak could not be still separated completely. Especially, when the quantity of input smple was large. So the collection should be with great care. Fig. 2 showed that the II peak part isolated by Bio-Gel P-2 column processed in HPLC. Among them, the peak 7(oligosaccharide DP-6) indicated a one point on TLC (Rf=0.95) collected from the HPLC isolation.

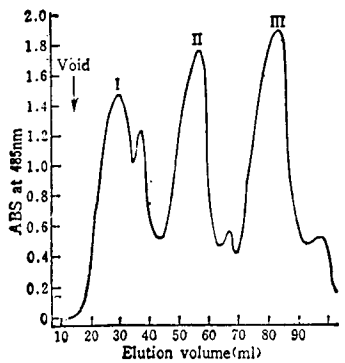

Fig. 1 Gel filtrated profileon Bio-Gel P-2 column Of the hydrolysats of oligosaccharides

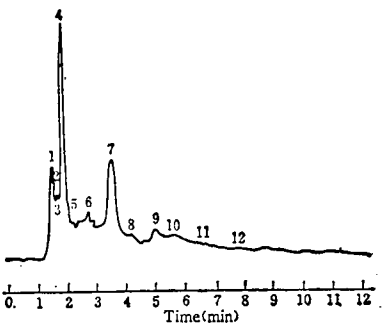

Fig. 2 HPLC of oligosaccharides

2. Identification of oligosaccharide DP-6

(1) FAB$^-$-MS

The FAB$^-$-MS spectra of oligosaccharide DP-6 (Fig. 3) appeared a fragment ion peak at *m/z* 989 (M-5 × H_2O-1)(losing 5 H_2O molecular), that showed it had not single saccharide which molecular weight was not *m/z* 180 (MW = 180), and did not appear a fragment ion peak decreasing by degree at *m/z* 162, that exhibited a branched chain in the molecular. The linked-scanning FAB$^-$-MS spectra of oligosaccharide DP-6 expressed branched chain at No. 4 single saccharide by signal at *m/z* 486(Fig.4).

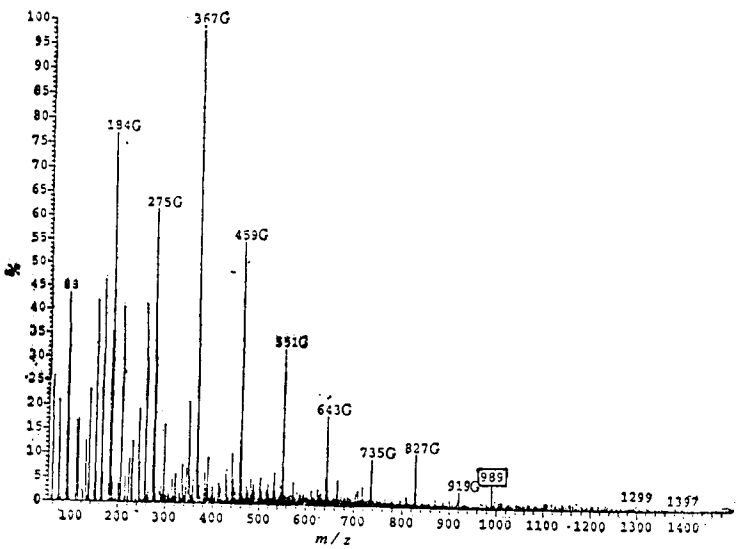

Fig. 3 FAB$^-$-MS spectrum of oligosccharide DP-6
G: mixed with the peaks of glycerin

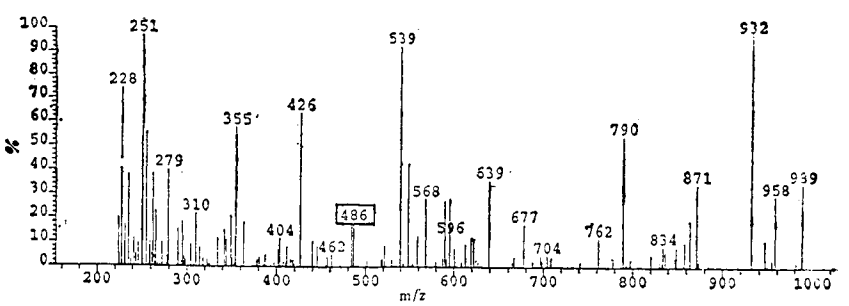

Fig. 4 Linked- scanning FAB$^-$-MS spectrum of oligosaccharide Dp-6

(2) Measurement of linked point among single saccharide of oligosaccharide DP-6

Acid-hydrolysis of hydrogenation of the whole methyl derivative oligosaccharide DP-6 yield acetyl derivatives, and tested by GC, GC-MS. The GC, GC-MS spectra of acetyl derivatives were determined by compared of standard spectra and index of computer. And the molar ratios of single saccharide were obtained by scanning integral of the peak area 9of chromatogram. The complete acid-hydrolyzed test showed that oligosaccharide DP-6 was consisted of Glc, Gal and Man (4:1:1)(Table 1).

Table 1 Methylation analysis of the linkage region of oligosaccharide DP-6

Methylation acetate derivative	Molar ratios
2,3,4,6-O-Me_4-Glc	0.9
2,3,6-O-Me_3-Glc	2.1
3,6-O-Me_2-Glc	1
2,3,4,6-O-Me_4-Gal	1.1
1,2,3,6-O-Me_4-Man	0.9

(3) ^{13}C-NMR

The ^{13}C-NMR spectra of oligosaccharide DP-6 (Fig. 5) appeared 6 beginning carbon signals at δ 95-107 ppm, the carbon signals at δ 107.41 ppm showed C_1 of α-D- galactocyl, at δ 103 ppm (4 × C) were C_1 carbon signals of β-glycosyl, at δ 98.87 ppm was C_1carbon signals of β-D- mannose by compared with the standard spectra of Glc, Gal, Man and their methyl derivatives. The other signals of oligosaccharide Dp-6 were showed in table 2.

By the analysis above, the structure of oligosaccharide DP-6 had been identified as in Fig.6

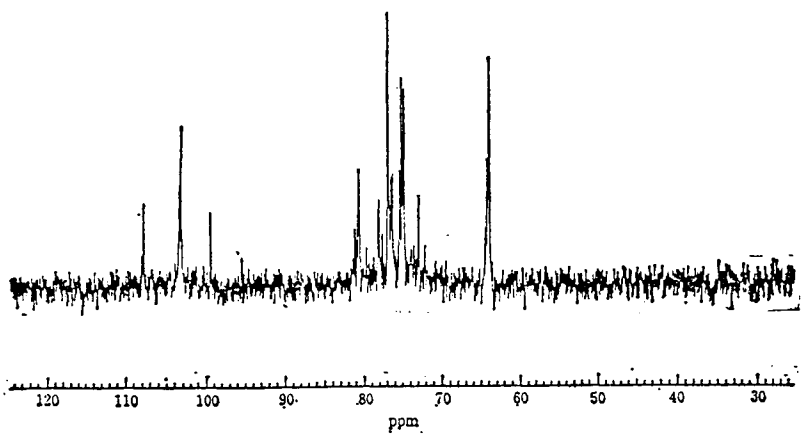

Fig. 5 ^{13}C-NMR data of oligosaccharide Dp-6

Table 2 ^{13}C-NMR data of oligosaccharide DP-6

δ (ppm)	107 (1 peak)	103 (4 peaks)	98 (1 peak)	80-79 (5 peaks)	77-75 (6 peaks)	74 (4 peaks)	73-71 (2 peaks)	64-63 (6 peaks)
Compd	Gal C_1	4×Glc C_1	Man C_1	3×Glc C_4 Man C_4 Glc C_2	Glc C_3, C_5 Man C_3, C_5 Gal C_3, C_5	3×Glc C_2 Gal C_4	Man C_2 Gal C_2	Glc C_6 Man C_6 Gal C_6

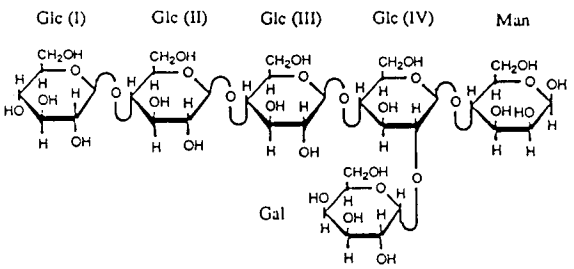

Fig. 6 Structure of oligosaccharide DP-6

3. Effect of oligosaccharide DP-6 on cultured plant cells

(1) Effect of oligosaccharide DP-6 on cultured cells of *Taxus yunnanesis*

The experiment result was showed in table 3. The 5.0mg/L of oligosaccharide DP-6 could increase the growth rate of *Taxus yunnanesis* suspension cells by 36.7% and promote the taxol yield by nearly 2 folds compared to the control.

Table 3 Effect of oligosaccharide DP-6 on cultured cells of *Taxus yunnanessis*

Oligosaccharide DP-6(mg/L)	Growth rate(g/L.d)	Taxol content(%)
0	0.26	0.026
1.0	0.32	0.047
5.0	0.35	0.079
10.0	0.27	0.025

(2) Effect of oligosaccharide DP-6 on cultured cells of *Carthamus tinctorius*

The previous study had showed that the 5.0 mg / L of oligosaccharide DP-6 could promote the α-tocopherol content by 1.1 folds and 20mg / L of oligosaccharide DP-6 increased the growth rate by 20.9% in the callus culture of *Carthamus tinctorius*. From a study of the effect of addition of oligosaccharide DP-6 on different days from the staring of cell growth, it was also seen that its effect on cell growth and α-tocopherol formation was evident the second day of its addition any time during the culture up to 20 days, but the total effect was the largest when it was added on the first day, because of the cumulative effect. When oligosaccharide DP-6 was added to a culture of suspended cells, the duration of exponential growth was shortened and the cells could be harvested earlier, because of the promotive effect on growth rate during exponential growth stage was increased by about 22% and the α-tocopherol content was promoted by about 1.6 folds by the addition of oligosaccharide DP-6

(3) Effect of oligosaccharide DP-6 on cultured cells of *Panax notoginseng*

In an appropriate concentration, the ologosaccharide DP-6 stimulated the saponin

formation and cell growth of *Panax notogensing* callus culture. The optimun concentration was about 15 mg/L, and promoted the growth rate and saponin yield by about 1 fold and 30% respectively.

In summary, the oligosaccharide DP-6 could increase the cell growth rate and the secondary metabolite yield in different cultured plant cells.

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