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Molecules of Interest

Combinatorial biochemistry in plants: the case of *O*-methyltransferases

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Abstract

Combinatorial chemistry is common place today in chemical synthesis. Virtually thousands of derivatives of a molecule can be achieved by automated systems. The use of biological systems to exploit combinatorial chemistry (combinatorial biochemistry) now has multiple examples in the polyketide field. The modular functional domain structure of polyketide synthases have been recombined through genetic engineering into unnatural constellations in heterologous hosts in order to produce polyketide structures not yet discovered in nature. We present herein an example for a potential type of combinatorial biochemistry in alkaloidal systems using various combinations of *Thalictrum tuberosum* (meadow rue) *O*-methyltransferase subunits that result in heterodimeric enzymes with substrate specificities that differ from those of the homodimeric native enzymes. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *O*-Methyltransferases; Alkaloid biosynthesis; Phenylpropanoid biosynthesis; Combinatorial biochemistry

1. Introduction

S-Adenosyl-L-methionine-dependent methyltransferases are involved in the *O*-methylation of many plant natural products. For several alkaloids of the isoquinoline type, the complete biosynthetic pathway has been elucidated at the enzyme level, which provides us with insightful information into the substrate specificity of the various biosynthetic enzymes (reviewed in Kutchan, 1998). The biosynthesis of the central isoquinoline alkaloid intermediate (*S*)-reticuline requires the action of two *O*-methyltransferases, norcoclaurine 6-*O*-methyltransferase and 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (Rüffer et al., 1983; Frenzel and Zenk, 1990; Sato et al., 1994; Hara et al., 1995). Partial purification and characterization of these enzymes indicated that they might have relatively strict substrate specificity. It was thought that only minor variations to the presumed in vivo substrate could be tolerated by the enzymes. This was found not only to be true for alkaloid biosynthetic pathways, but for other types of natural products as well.

As in alkaloid biosynthesis, *O*-methyltransferases are central also to flavonoid biosynthesis. The first methylation in the early stages of lignin biosynthesis is of caffeic acid to ferulic acid (reviewed in Dixon and Paiva, 1995). Ferulic acid is hydroxylated to 5-hydroxyferulic acid, which can then be further methylated to sinapic acid. The modification of more complex structures, such as isoflavone and isoliquiritigenin, are also catalyzed by *O*-methyltransferases (Maxwell et al., 1993; He et al., 1998). A common structural feature, a catechol moiety, is found between the substrate of early isoquinoline alkaloid biosynthesis, norcoclaurine, and the substrate of early phenylpropanoid biosynthesis, caffeic acid.

2. Homology of plant *O*-methyltransferases

In an effort to isolate cDNAs encoding methyltransferases of isoquinoline alkaloid biosynthesis from berberine-producing cell suspension cultures of the meadow rue *Thalictrum tuberosum*, a polymerase chain reaction (PCR) strategy was used based upon consensus sequences found in methyltransferase amino acid sequences present in the EMBL/GenBank databases (Frick and Kutchan, 1999). These experiments yielded four very similar (93.2–99.7% identity) cDNAs that showed

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71% similarity to other plant *O*-methyltransferases. A large number of the plant *O*-methyltransferase sequences present in the database has been annotated as caffeic acid *O*-methyltransferase of phenylpropanoid metabolism based upon sequence similarity. Despite this high similarity to caffeic acid *O*-methyltransferase, the four *T. tuberosum* methyltransferases were functionally expressed in insect cell culture and were tested for enzyme activity (Fig. 1). Two of these four nearly identical methyltransferases were able to methylate norcoclaurine to coclaurine, and two were unable to catalyze this transformation. This surprising result initiated a more thorough investigation of the substrate specificity of the recombinant enzymes.

3. Substrate specificity of plant *O*-methyltransferases

A series of 38 potential substrates were tested with the four recombinant methyltransferases of *T. tuberosum* (Frick and Kutchan, 1999). All four enzymes were demonstrated to be functional *O*-methyltransferases with overlapping, but not identical, substrate specificity. The *O*-methyltransferases were found to be surprisingly permissive with respect to the broad range of substrates that could be methylated. The structures tested varied from simple catechols, such as catechol and guajacol to complex tetrahydrobenzylisoquinoline alkaloids. The most unexpected result for us was that all four recombinant methyltransferases could *O*-methylate caffeic acid, while two of the four could methylate the alkaloidal biosynthetic intermediate norcoclaurine. The sequences of one of the norcoclaurine methylating enzymes and one of the caffeic acid methylating enzymes differed by only one amino acid. This was the first time that we have observed that an enzyme exhibits a functional overlap between alkaloid and phenylpropanoid biosynthesis. The K_m values for the three substrates catechol, caffeic acid and norcoclaurine were similar enough that it was not possible to conclude which substrate might have preferential in vivo significance.

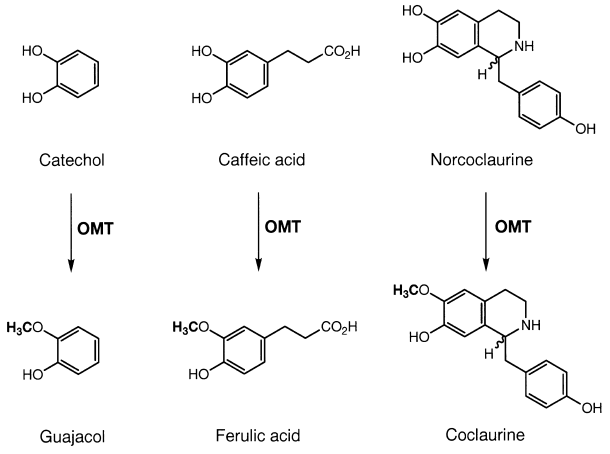


Fig. 1. Reactions catalyzed by heterologously expressed *O*-methyltransferases of *T. tuberosum*.

4. Recombination of *O*-methyltransferase subunits

During characterization, the *O*-methyltransferases were determined to be dimeric enzymes. The question was posed whether recombinant heterodimers would have a substrate specificity that was different from recombinant homodimers. In other words, would it be possible to create new enzyme activities by recombination of *O*-methyltransferase subunits?

To achieve some degree of heterologous expression as heterodimers, each cDNA was cloned into a baculovirus vector. All possible combinations of two were effected by co-infection of insect cell culture (Fig. 2). The 38 previously tested substrates were then tested again with the 10 potentially different recombinant *O*-methyltransferases. Several new substrates were methylated by heterodimers that were not methylated by homodimers. For example, protocatechuic acid was methylated by the heterodimer OMT 1.3, (*S*)-coclaurine by OMT 1.2, while (*S*)-norreticuline and (*R*,*S*)-6-*O*-methylnorlaudanosoline were methylated by the heterodimer OMT 2.3 (Fig. 3). The presence of new enzyme activities indicated that heterodimers had indeed to some degree formed in the insect cell culture heterologous expression system. Whether a mixture of heterodimers and homodimers are present in the native plant cannot be concluded at this point. But certainly in recombinant systems, it is possible to demonstrate a type of combinatorial biochemistry by shuffling *O*-methyltransferase monomers into novel combinations that result in methylation of additional substrates.

5. Variations in nature

A comparison of the substrate specificity of heterologously expressed norcoclaurine 6-*O*-methyltransferase from *T. tuberosum* (Frick and Kutchan, 1999), *Coptis japonica* (Morishige et al., 2000) and *Papaver somniferum* (A. Ounaroon and T.M. Kutchan, unpublished) suggests an evolution of *O*-methyltransferases. The most promiscuous enzymes are clearly those from *T. tuberosum*, which methylate at least 24 different substrates that vary from catechol to caffeic acid and phenethylisoquinoline alkaloids. The *C. japonica* enzyme has a much more restricted substrate range. Norcoclaurine is the substrate that is most efficiently methylated. A total of five alkaloids are methylated out of a group of 25 tested. The enzyme from *P. somniferum* demonstrates the least promiscuity of the three enzymes. Norcoclaurine serves as the best substrate and three other isoquinoline alkaloids

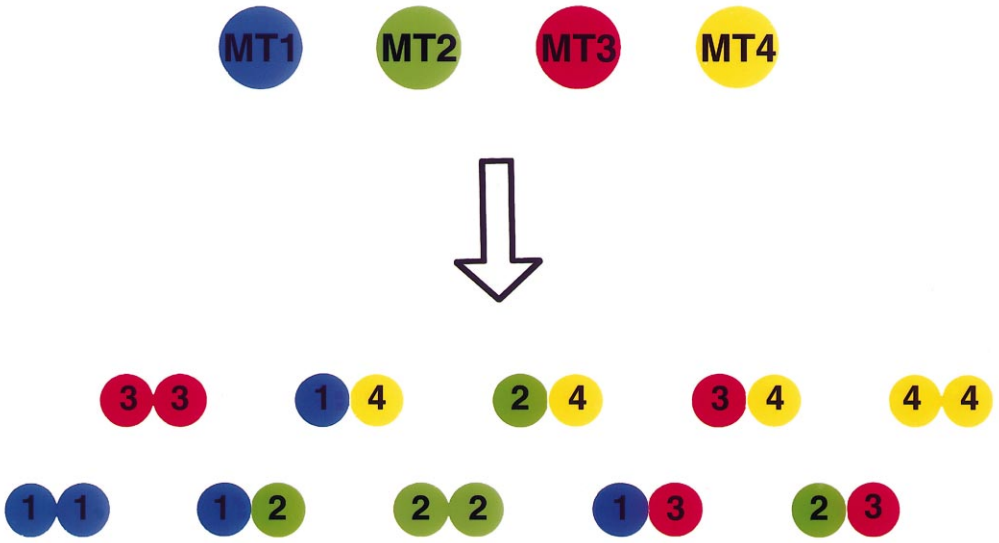


Fig. 2. Schematic indicating the homodimeric and heterodimeric *O*-methyltransferases than can be formed by co-infection of insect cells with recombinant baculovirus vectors containing the four *T. tuberosum O*-methyltransferase cDNAs.

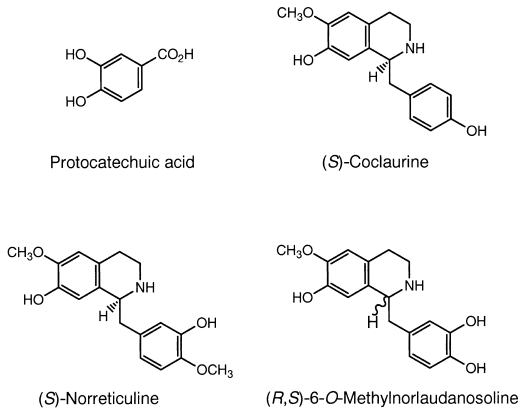


Fig. 3. Structures of four compounds that can be methylated by *T. tuberosum O*-methyltransferase heterodimers, but not by any of the four homodimers.

are methylated to a low extent. As for the *C. japonica* methyltransferase, the *P. somniferum* enzyme does not methylate caffeic acid.

Varying degrees of substrate specificity in *O*-methyltransferases has also been observed for enzymes of phenylpropanoid and flavonoid metabolism. For example, a methyltransferase from aspen and alfalfa can methylate both 5-hydroxyferulic acid and caffeic acid (Bugos et al., 1991; Gowri et al., 1991). The equivalent enzyme from loblolly pine methylates 5-hydroxyferulic acid and caffeic acid and their CoA esters (Li et al., 1997). Yet another from tobacco methylates caffeic acid and catechol (Pellegrini et al., 1993). There are also *O*-methyltransferases with broader substrate specificity, such as from the semi-aquatic freshwater weed *Chrysosplenium americanum* that methylates 5-hydroxyferulic acid, caffeic acid and the flavonoid aglycones luteolin and quercetin (Gauthier et al., 1998). Another from barley methylates flavonoid aglycones, but not caffeic acid (Christensen et al., 1998).

These enzymes with varying methylating capabilities show sequence similarities that might lead one to incorrectly classify them all as caffeic acid 3-*O*-methyltransferases. The sequence similarities of *O*-methyltransferases could relate only in the broadest sense to function, such as is the case with cytochromes P-450. It is clear that functional heterologous expression should be a prerequisite to the assignment of function. When the topic of homodimer versus heterodimer formation is considered, the picture can become even more complex.

Acknowledgements

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Review

The chemical diversity and distribution of glucosinolates and isothiocyanates among plants☆

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Abstract

Glucosinolates (β-thioglucoside-*N*-hydroxysulfates), the precursors of isothiocyanates, are present in sixteen families of dicotyledonous angiosperms including a large number of edible species. At least 120 different glucosinolates have been identified in these plants, although closely related taxonomic groups typically contain only a small number of such compounds. Glucosinolates and/or their breakdown products have long been known for their fungicidal, bacteriocidal, nematocidal and allelopathic properties and have recently attracted intense research interest because of their cancer chemoprotective attributes. Numerous reviews have addressed the occurrence of glucosinolates in vegetables, primarily the family Brassicaceae (syn. Cruciferae; including *Brassica* spp and *Raphanus* spp). The major focus of much previous research has been on the negative aspects of these compounds because of the prevalence of certain "antinutritional" or goitrogenic glucosinolates in the protein-rich defatted meal from widely grown oilseed crops and in some domesticated vegetable crops. There is, however, an opposite and positive side of this picture represented by the therapeutic and prophylactic properties of other "nutritional" or "functional" glucosinolates. This review addresses the complex array of these biologically active and chemically diverse compounds many of which have been identified during the past three decades in other families. In addition to the *Brassica* vegetables, these glucosinolates have been found in hundreds of species, many of which are edible or could provide substantial quantities of glucosinolates for isolation, for biological evaluation, and potential application as chemoprotective or other dietary or pharmacological agents. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Cancer; Chemoprotection; Edible plants; Functional food; Myrosinase; Crucifers

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☆ This paper is dedicated to Professor Anders Kjær, who, with his collaborators in Lyngby, Denmark, has contributed immeasurably to the scientific community's understanding of glucosinolates and to knowledge of their chemistry, biosynthesis, metabolism, and their relationship to the plants from which they were isolated; more glucosinolates have been isolated and characterized in Professor Kjær's laboratory than anywhere else in the world.

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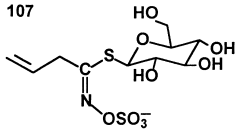
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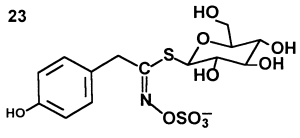
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1. Introduction

The first observations on the unique properties of glucosinolates and isothiocyanates or mustard oils, as they are commonly known, were recorded at the beginning of the 17th century as a result of efforts to understand the chemical origin of the sharp taste of mustard seeds. The discovery and early history of glucosinolates and the participation of the enzyme myrosinase (a β-thioglucosidase) in their conversion to isothiocyanates, are the subjects of an interesting and scholarly review by Challenger (1959). The glucosinolates known by the trivial names sinigrin (2-propenyl or allyl glucosinolate; **107**) and sinalbin (4-hydroxybenzyl glucosinolate; **23**) were isolated early in the 1830s from black (*Brassica nigra*) and white (*Sinapis alba*) mustard seeds, respectively.[2] (See Tables 1 and 2 for glucosinolate numbers used throughout this review.) The first general, although incorrect, structure for these compounds was proposed at the end of the nineteenth century by Gadamer (1897), who concluded that the side chain was linked to the nitrogen rather than the carbon atom of the "NCS" group. Despite certain difficulties the structure was generally assumed to be correct until 1956, when Ettlinger and Lundeen (1956a) pointed out the inadequacies of the Gadamer structure to explain certain properties of these compounds, proposed the now correct structure, and described the first chemical synthesis of a glucosinolate (Ettlinger and Lundeen, 1957). The remaining structural issue of the geometrical isomerism at the C=N bond was established to be *Z* (or anti-) by X-ray crystallographic analysis of sinigrin (see Fig. 1; Marsh and Waser, 1970).





Glucosinolates are β-thioglucoside *N*-hydroxysulfates [also known as (*Z*)-(or *cis*)-*N*-hydroximinosulfate esters or *S*-glucopyranosyl thiohydroximates], with a side chain (R) and a sulfur-linked β-D-glucopyranose moiety.

In the last 40 years, a succession of reviews have addressed the biology and chemistry of glucosinolates (e.g. Kjær, 1961, 1974; Ettlinger and Kjær, 1968; Kjær and Olesen Larsen, 1973, 1976; Underhill et al., 1973;

[2] Glucosinolates are arranged alphabetically by chemical name and assigned unique sequential identifying bold numbers in Table 1 which also provides the common (trivial) names. These numbers are used throughout this paper. Glucosinolates are listed alphabetically by common name in Table 2. Note that not all glucosinolates have been assigned common names. Chemical structures are shown in Fig. 1 in which the compounds are grouped by structural classes. Since the stereochemistry of sulfinyl groups and at carbon centers has not been established for many glucosinolates, the isomeric nature of the compounds is not specified. In Appendix A glucosinolates are referred to by bold number only. The chemical and common names provided by the original authors have not been altered to reflect current IUPAC systematic names, since this would make it too confusing for those interested in checking the original references.

Table 1
Chemical and common names of glucosinolates identified in higher plants. Numerical designations are used in the review. Class assignments refer to structure-based assignment of glucosinolates to chemical classes (see Fig. 1)

		Glucosinolate	
No.	Class	Chemical name	Common names
1	F	3-Methoxycarbonylpropyl	Glucoerypestrin
2	I	1-Acetyl-indol-3-ylmethyl	1-Acetyl-glucobrassicin
3	J	4-(4′-*O*-Acetyl-α-L-rhamnopyranosyloxy)benzyl	
4	J	2-(α-L-Arabinopyranosyloxy)-2-phenylethyl	
5	H	4-(Benzoyloxy)butyl	
6	H	2-(Benzoyloxy)ethyl	
7	H	2-Benzoyloxy-1-ethylethyl	Glucobenzsisaustricin
8	H	Benzoyloxymethyl	
9	H	2-Benzoyloxy-1-methylethyl	Glucobenzosisymbrin
10	H	3-(Benzoyloxy)propyl	Glucomalcomiin
11	G	Benzyl	Glucotropaeolin
12	D	3-Butenyl	Gluconapin
13	B	*n*-Butyl	
14	G	3,4-Dihydroxybenzyl	Glucomatronalin
15	G	3,4-Dimethoxybenzyl	
16	B	Ethyl	Glucolepidiin
17	E	1-Ethyl-2-hydroxyethyl	Glucosisaustricin
18	D	6-Heptenyl	
19	D	5-Hexenyl	
20	B	*n*-Hexyl	
21	G	2-Hydroxybenzyl	
22	G	3-Hydroxybenzyl	Glucolepigramin
23	G	4-Hydroxybenzyl	[Gluco]sinalbin
24a	D	2(*R*)-2-Hydroxy-3-butenyl	Progoitrin
24b	D	2(*S*)-2-Hydroxy-3-butenyl	Epiprogoitrin
25	E	3-Hydroxybutyl	
26	E	4-Hydroxybutyl	
27	E	2-Hydroxyethyl	
28	I	4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin
29	E	2-Hydroxy-2-methylbutyl	Glucocleomin
30	E	1-(Hydroxymethyl)propyl	
31	E	2-Hydroxy-2-methylpropyl	Glucoconringiin
32	A	3-Hydroxy-6-(methylsulfinyl)hexyl	
33	A	3-Hydroxy-5-(methylsulfinyl)pentyl	
34	A	3-Hydroxy-6-(methylsulfonyl)hexyl	
35	A	3-Hydroxy-5-(methylsulfonyl)pentyl	
36	A	3-Hydroxy-6-(methylthio)hexyl	
37	A	3-Hydroxy-5-(methylthio)pentyl	
38	D	2-Hydroxy-4-pentenyl	[Gluco]napoleiferin
39	E	2-Hydroxypentyl	
40	G	2(*R*)-Hydroxy-2-phenylethyl	Glucobarbarin
41	E	2-Hydroxypropyl	
42	E	3-Hydroxypropyl	
43	I	Indol-3-ylmethyl	Glucobrassicin
44	G	2-Methoxybenzyl	
45	G	3-Methoxybenzyl	Glucolimnanthin
46	G	4-Methoxybenzyl	Glucoaubrietin
47	I	1-Methoxyindol-3-ylmethyl	Neoglucobrassicin
48	I	4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin
49	G	2-(4-Methoxyphenyl)-2,2-dimethylethyl [or 2,2-dimethyl-2-(4-methoxyphenyl)ethyl]	
50	G	2-(4-Methoxyphenyl)-2-hydroxyethyl [or 2-hydroxy-2-(4-methoxyphenylethyl)]	
51	B	Methyl	Glucocapparin
52	D	3-Methyl-3-butenyl	
53	C	1-Methylbutyl	
54	C	2-Methylbutyl	
55	C	3-Methylbutyl	
56	C	1-Methylethyl	Glucoputranjivin, isopropyl
57	E	1-Methyl-2-hydroxyethyl	Glucosisymbrin
58	C	3-Methylpentyl	
59	C	4-Methylpentyl	

(*continued on next page*)

Table 1 (*continued*)

No.	Class	Glucosinolate: Chemical name	Common names
60	D	2-Methyl-2-propenyl	
61	C	1-Methylpropyl	Glucococchlearin, glucojiabutin, sec-Butyl, 2-Butyl
62	C	2-Methylpropyl	Isobutyl
63	A	4-Methylsulfinyl-3-butenyl	Glucoraphenin
64	A	4-(Methylsulfinyl)butyl	Glucoraphanin
65	A	10-(Methylsulfinyl)decyl	Glucocamelinin
66	A	7-(Methylsulfinyl)heptyl	Glucoibarin
67	A	6-(Methylsulfinyl)hexyl	Glucohesperin
68	A	9-(Methylsulfinyl)nonyl	Glucoarabin
69	A	8-(Methylsulfinyl)octyl	Glucohirsutin
70	A	7-Methylsulfinyl-3-oxoheptyl	
71	A	8-Methylsulfinyl-3-oxooctyl	
72	A	5-(Methylsulfinyl)pentyl	Glucoalyssin
73	A	3-(Methylsulfinyl)propyl	Glucoiberin
74	A	11-(Methylsulfinyl)undecyl	
75	A	4-Methylsulfonyl-3-butenyl	
76	A	4-(Methylsulfonyl)butyl	Glucoerysolin
77	A	10-(Methylsulfonyl)decyl	
78	A	6-(Methylsulfonyl)hexyl	
79	A	9-(Methylsulfonyl)nonyl	
80	A	8-(Methylsulfonyl)octyl	
81	A	5-(Methylsulfonyl)pentyl	
82	A	3-(Methylsulfonyl)propyl	Glucocheirolin
83	A	4-Methylthio-3-butenyl	Dehydroerucin
84	A	4-(Methylthio)butyl	Glucoerucin
85	A	10-(Methylthio)decyl	
86	A	2-(Methylthio)ethyl	Glucoviorylin
87	A	7-(Methylthio)heptyl	
88	A	6-(Methylthio)hexyl	Glucolesquerellin
89	A	9-(Methylthio)nonyl	
90	A	7-Methylthio-3-oxoheptyl	
91	A	6-Methylthio-3-oxohexyl	
92	A	8-(Methylthio)octyl	
93	A	8-Methylthio-3-oxooctyl	
94	A	5-(Methylthio)pentyl	Glucoberteroin
95	A	3-(Methylthio)propyl	Glucoiberverin
96	F	4-Oxoheptyl	Glucocapangulin; glucopangulin
97	F	5-Oxoheptyl	Gluconorcappasalin
98	F	5-Oxooctyl	Glucocappasalin
99	F	4-Oxopentyl or 3-(Methylcarbonyl)propyl	
100	D	1-Pentenyl	
101	D	4-Pentenyl	Glucobrassicanapin
102	B	*n*-Pentyl	
103	G	Phenyl	
104	G	4-Phenylbutyl	
105	G	2-Phenylethyl	Gluconasturtiin; phenethyl
106	G	3-Phenylpropyl	
107	D	2-Propenyl	Allyl, Sinigrin
108	B	*n*-Propyl	
109	J	2-(α-L-Rhamnopyranosyloxy)benzyl	
110	J	4-(α-L-Rhamnopyranosyloxy)benzyl	
111	J	6-Sinapoyl-β-D-1-thioglucoside of 4-methylsulfinylbut-3-enyl	
112	I	1-Sulfo-indol-3-ylmethyl	Glucobrassicin-1-sulfate
113	E	4,5,6,7-Tetrahydroxydecyl	
114	G	3,4,5-Trimethoxybenzyl	
115[a]		"*iso*"-Heptyl	
116[a]		"*iso*"-Hexyl	
117[b]	H	5-(Benzoyloxy)pentyl	
118[b]	H	6-(Benzoyloxy)hexyl	
119[c]		3-*O*-Apiosylglucomatronalin	
120[c]		3-*O*-Apiosylglucomatronalin 3,4-dimethoxybenzoyl ester	

[a] Structures unresolved; Grob and Matile (1980).
[b] Added in proof; Haughn et al. (1991); identified in *Arabidopsis* sp.
[c] Larsen et al. (1992); however, the identification of these compounds references only unpublished work.

Table 2
Alphabetical listing of common names of glucosinolates identified in higher plants[a]

Common name	Number	Chemical class	Common name	Number	Chemical class
1-Acetylglucobrassicin	**2**	I	Glucoiberverin	**95**	A
Dehydroerucin	**83**	A	Glucojiabutin	**61**	C
Epiprogoitrin	**24b**	D	Glucolepidiin	**16**	B
Glucoalyssin	**72**	A	Glucolepigramin	**21**	G
Glucoarabin	**68**	A	Glucolesquerellin	**88**	A
Glucoaubrietin	**46**	G	Glucolimnanthin	**45**	G
Glucobarbarin	**40**	G	Glucomalcomiin	**10**	H
Glucobenzosisymbrin	**9**	H	Glucomatronalin	**14**	G
Glucobenzsisaustricin	**7**	H	Gluconapin	**12**	D
Glucoberteroin	**94**	A	Gluconapoleiferin	**38**	D
Glucobrassicanapin	**101**	D	Gluconasturtiin	**105**	G
Glucobrassicin	**43**	I	Gluconorcappasalin	**97**	F
Glucobrassicin-1-sulfate	**112**	I	Glucoputranjivin	**56**	C
Glucocamelinin	**65**	A	Glucoraphanin	**64**	A
Glucocapangulin; glucopangulin	**96**	F	Glucoraphenin	**63**	A
Glucocapparin[b]	**51**	B	Glucosinalbin	**23**	G
Glucocappasalin	**98**	F	Glucosisaustricin	**17**	E
Glucocheirolin	**82**	A	Glucosisymbrin	**57**	E
Glucocleomin	**29**	E	Glucotropaeolin	**11**	G
Glucocochlearin	**61**	C	Glucoviorylin	**86**	A
Glucoconringiin	**31**	E	4-Hydroxyglucobrassicin	**28**	I
Glucoerucin	**84**	A	4-Methoxyglucobrassicin	**48**	I
Glucoerypestrin	**1**	F	Napoleiferin	**38**	D
Glucoerysolin	**76**	A	Neoglucobrassicin	**47**	I
Glucohesperin	**67**	A	Progoitrin	**24a**	D
Glucohirsutin	**69**	A	Sinalbin	**23**	G
Glucoibarin	**66**	A	Sinigrin	**107**	D
Glucoiberin	**73**	A			

[a] Note that not all isolated glucosinolates have been assigned common names.

[b] Also designated glucoapparin by Benn (1964b).

Underhill, 1980; Fenwick et al., 1983; Chew, 1988; Duncan and Milne, 1989; Brown and Morra, 1997; Halkier, 1999; Mithen et al., 2000), and their distribution among plants (Rodman, 1981). More narrowly focused reviews have examined the indole glucosinolates (McDanell et al., 1988), or specifically glucosinolates in the family Brassicaceae (Kjær, 1976). Similar coverage (i.e. of glucosinolates of crop plants, primarily the *Brassica* vegetables) has been provided by Stoewsand (1995) and Rosa et al. (1997). Many other even more narrowly focused reviews have concentrated on specific plant families or on specific aspects of glucosinolate biology and they are referenced herein, as appropriate.

The present review provides a comprehensive survey of the chemical structures of all known glucosinolates and the plant families from which they have been isolated. It provides a single source of their chemical structure, their trivial names, and groups these compounds into families according to their structural similarities. We discuss, mostly by reference, the state of scientific understanding of the synthesis, biosynthesis and ecological importance of glucosinolates and their conversion to isothiocyanates and other products by myrosinase. To our knowledge, there has been no recent effort to provide a comprehensive compilation and cataloging of isolated glucosinolates, their structures, systematic and trivial (common) names, and their distribution among plant species. Although we have attempted to do so herein, undoubtedly there are omissions. Since many of these compounds were identified before modern spectroscopic techniques were available, some of the structural assignments of glucosinolates to plant taxa may require revision.

2. Glucosinolate distribution among plants

There is now a voluminous literature on the glucosinolates of the plant family, Brassicaceae, which alone contains more than 350 genera and 3000 species. Of the many hundreds of cruciferous species investigated, all are able to synthesize glucosinolates (Kjær, 1976). Among the Brassicaceae, the genus *Brassica* contains a large number of the commonly consumed species. *Brassica* sp. glucosinolates have been the subject of scholarly reviews by Kjær (1974, 1976), Fenwick et al. (1983), Chew (1988), McDanell et al. (1988), Duncan and Milne (1989), Stoewsand (1995) and most recently by Rosa et al. (1997). Glucosinolates are by no means confined to

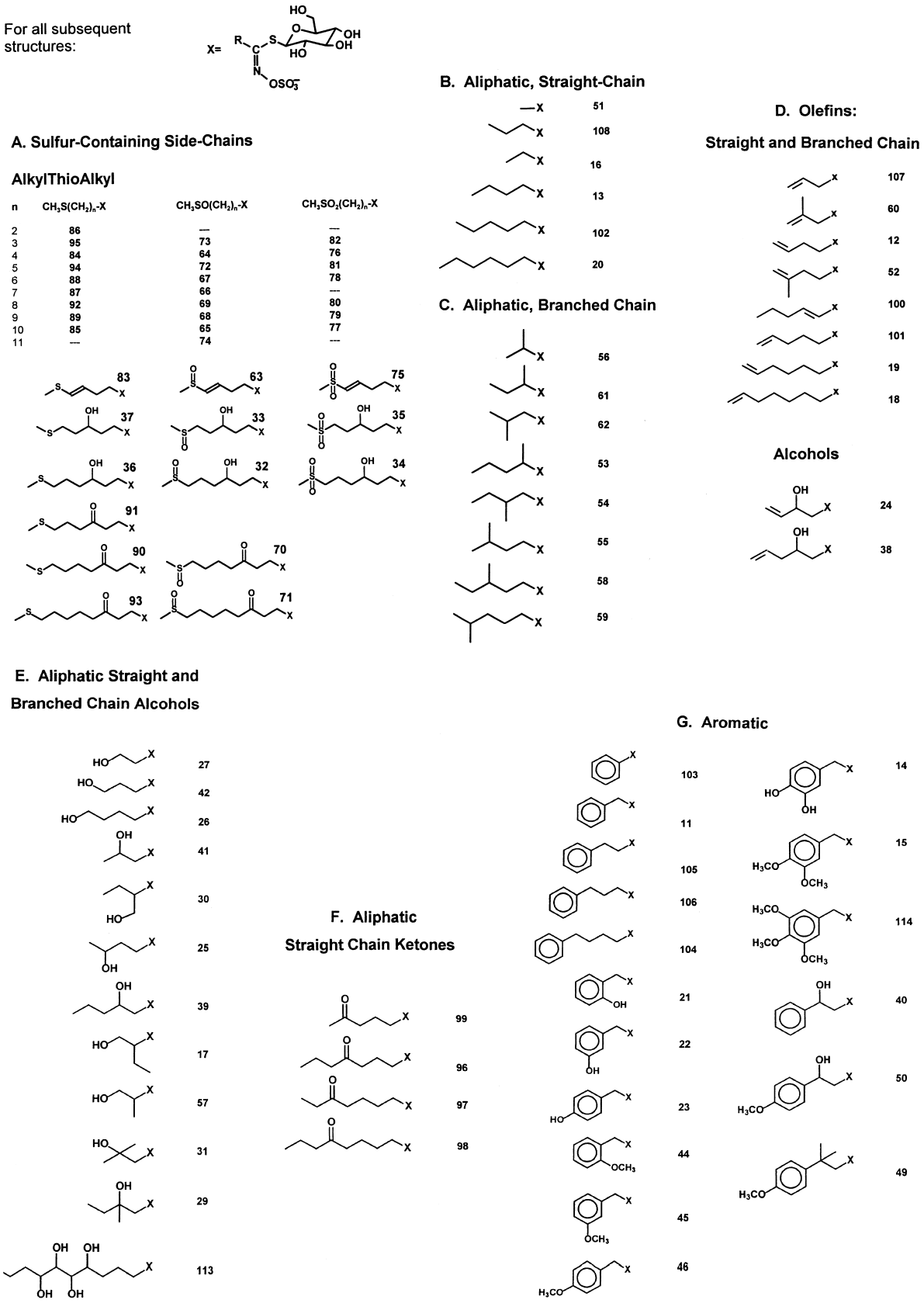


Fig. 1. Classification of glucosinolates according to chemical structures. [a] Elliott and Stowe (1970) have isolated the sulfate derivative of glucobrassicin (**112**) from *Isatis tinctoria* and Schraudolf and Bauerle (1986) have isolated the acetyl derivative (**2**) from *Tovaria pendula*, but structural assignment should be regarded as tentative (McDanell et al., 1988) (*continued on next page*).

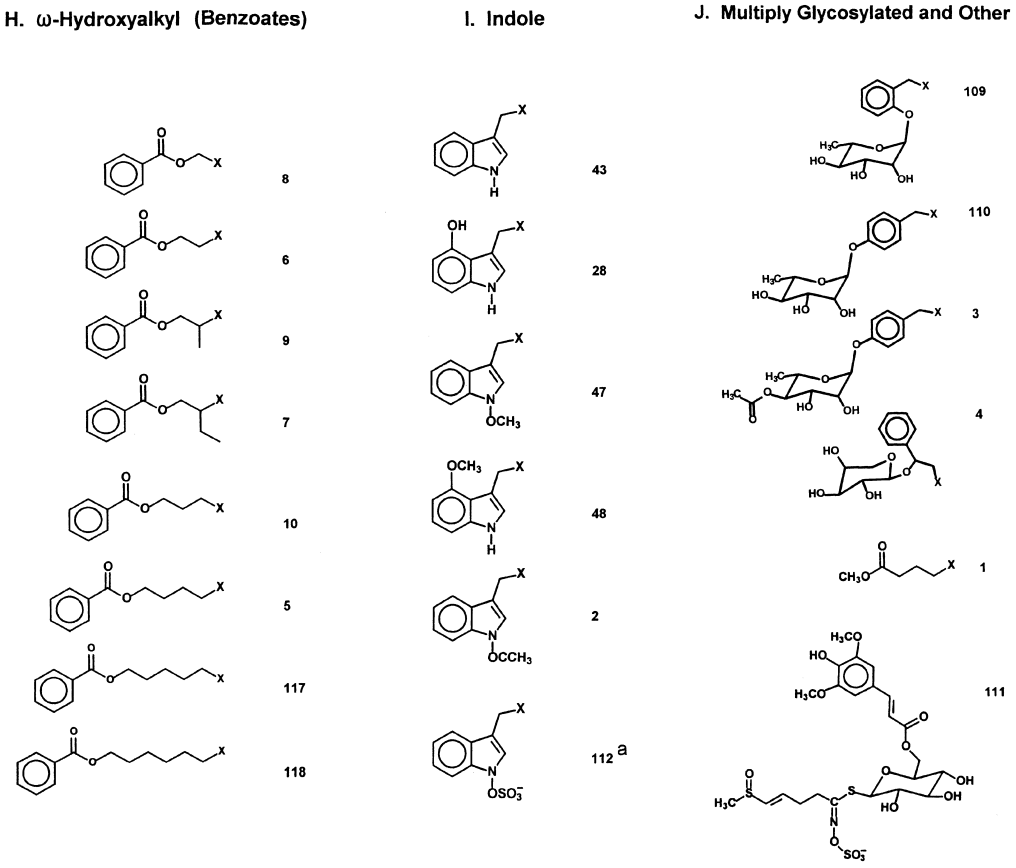


Fig. 1 (*continued*).

crucifers; at least 500 species of non-cruciferous dicotyledonous angiosperms have been reported to contain one or more of the over 120 known glucosinolates (Table 1). Most glucosinolate-containing genera are clustered within the Brassicaceae, Capparaceae and Caricaceae; of the sixteen families listed in Table 3, these include the largest number of glucosinolate-containing species (Rodman, 1981). Indeed, the capacity to biosynthesize glucosinolates has been used as a taxonomic marker to support evolution-based classification schemes (Rodman, 1981, 1991a,b; Mithen et al., 1987a; Rodman et al., 1993). Methyl glucosinolate, for example, is not found in the Brassicaceae, but is a distinctive component of the closely related Capparaceae. Glucosinolates with glycosylated R-groups appear limited to the Resedaceae and Moringaceae. Although the idea that there are taxon-specific glucosinolates is intriguing, this view is not highly developed and it is beyond the scope of this review. Rodman (1981) maintained that by the early 1980s, the taxonomic distribution of glucosinolates was fairly well known and that surprising discoveries of additional glucosinolate-containing plants would be likely to come from less-well-studied tropical plant families. For example the Euphorbiaceae, a vast family found primarily in the warmer parts of the world, was then and still is the only glucosinolate-containing family in which all genera except one (*Drypetes*; syn. *Putranjiva*) explicitly lack the capacity to produce glucosinolates and myrosinase. However, these compounds have only been searched for in a very small number of the estimated 5000 or more species within this family.

3. Glucosinolate chemistry

3.1. Types of glucosinolates

We have grouped glucosinolates into a number of chemical classes on the basis of structural similarities. The most extensively studied glucosinolates are the aliphatic, ω-methylthioalkyl, aromatic and heterocyclic (e.g. indole) glucosinolates, typified by those found in the *Brassica* vegetables (e.g. compounds **12, 23, 24, 38, 43, 47, 56, 61, 63, 64, 72, 73, 84, 94, 95, 100, 101, 105, 107** in Table 1 and Fig. 1). Glucosinolate side chains, however, are characterized by a wide variety of chemical structures (Fig. 1). The most numerous glucosinolates are those containing either straight or branched carbon chains. Many of these compounds also contain double bonds (olefins), hydroxyl or carbonyl groups, or sulfur

Table 3
Sixteen families of glucosinolate-containing angiosperms[a]

Family	Chemical class	Glucosinolates
Bataceae	I	**28, 43**
Brassicaceae	A–J	**1, 5–20, 22–26, 28–48, 50, 51, 53–69, 72–84, 86–89, 91–95, 99–107, 111, 112, 114**
Bretschneideraceae	E, G	**14, 31**
Capparaceae	A, B, C, D, E, F, G, I	**12, 13, 23, 24, 28, 29, 43, 47, 48, 51, 52, 54, 56, 73, 96–98, 107, 108**
Caricaceae	G	**11**
Euphorbiaceae	C, E	**26, 29, 56**
Gyrostemonaceae	C	**61, 62**
Limnanthaceae	E, G	**31, 45**
Moringaceae	C, E, G, J	**3, 11, 23, 31, 56, 61, 62, 110**
Pentadiplandraceae	G	**11, 49**
Phytolaccaceae	C, E, G	**11, 22, 23, 29, 61**
Pittosporaceae	G	**23**
Resedaceae	E, G, I, J	**4, 11, 21–23, 31, 40, 43, 47, 105, 109**
Salvadoraceae	C, G	**11, 23, 56**
Tovariaceae	C	**2, 11, 43, 47, 56**
Tropaeolaceae	B, C, E, G	**11, 16, 23, 31, 46, 56, 61, 62**

[a] Isolation and identification probably reflects a bias, based upon the quantity and ease of isolation of these compounds and the availability of plant sources. Very likely, there are many more undiscovered glucosinolates.

linkages in various oxidation states. The largest single group (one-third of all glucosinolates) contain a sulfur atom in various states of oxidation (e.g. methylthioalkyl-, methylsulfinylalkyl-, or methylsulfonylalkyl). Another small group of benzyl glucosinolates have an additional sugar moiety, rhamnose or arabinose, in glycosidic linkage to the aromatic ring. The presence of these sugars is of unknown significance, although it is intriguing that they are present in two families of plants (the Moringaceae and Resedaceae) containing certain genera that are widely exploited for their pharmacological properties. There has been an unconfirmed report that the 5-carbon sugar, apiose, may be linked to the hydroxybenzyl glucosinolate side chain in *Hesperis matronalis*, a member of the Brassicaceae family (Larsen et al., 1992; Halkier, 1999). Additionally, a number of sinapoyl and cinnamoyl salts and esters of some of the common glucosinolates are substituted on the thioglucoside moiety (Linscheid et al., 1980; Sakushima et al., 1995). Bjerg and Sørensen (1987) claim that cinnamoyl derivatives of glucosinolates predominate in some plants and plant parts, and they present hypothetical structures of *p*-coumaroyl, caffeoyl, feruloyl, sinapoyl and isoferuloyl glucosinolates with these phenylpropenyl moieties esterified to the S-β-glucose at positions C-2′ and C-6′. These compounds are not discussed further in this review. With few exceptions, the configuration at chiral centers of a number of the compounds represented in Fig. 1 is not fully characterized.

3.2. Isolation and crystallization

Early isolations of glucosinolates used paper and thin-layer chromatography almost exclusively (Greer, 1962; Bjorkman and Janson, 1972). High voltage electrophoresis combined with paper chromatography has been used, but with low yield and considerable complications (Elliott and Stowe, 1970; Wetter and Dyke, 1973; Olsen and Sørensen, 1980a). This early work is reviewed by Olsen and Sørensen (1981). Isolations of indole and aryl glucosinolates have been reported in which acidic alumina was used as the initial step, followed by either ion exchange chromatography on DEAE–Sephadex A-25 (Hanley et al., 1983) or Sephadex G10 size exclusion chromatography (Hanley et al., 1984). Excellent results in isolating preparative quantities of diverse glucosinolates from crude extracts, by reversed phase (C-18) solid phase extraction or flash-chromatographic reversed phase techniques are described in detail by Bjerg and Sørensen (1987) and Peterka and Fenwick (1988). A disadvantage of most of this work, however, was that the products were rarely of established purity and crystallized. Thies (1988) described a method by which gram quantities of glucosinolates can purportedly be obtained rapidly. Although this paper provides explicit and easy to understand protocols for two glucosinolates (sinigrin and glucotropaeolin), crystallizing other glucosinolates still remains problematic. Very few additional glucosinolates have therefore been crystallized (Thies, 1988). Early attempts to isolate naturally occurring glucosinolates included the crystallization of sodium salts of 2-hydroxy-2-methylpropyl glucosinolate and of the tetraacetate and pentaacetate (derivatives of the thioglucoside moiety) forms of this compound (Kjær et al., 1956; Schultz and Wagner, 1956). Kjær listed nine glucosinolates that had been crystallized as either potassium, sodium or rubidium salts, and another seven that had

been characterized as crystalline acetates by the year 1959 (Kjær, 1961).

3.3. Chemical synthesis

Although methods for the synthesis of a number of glucosinolates (e.g. benzyl-, methyl-, phenethyl-, 2-propenyl-, 3-butenyl-), were reported over four decades ago (Ettlinger and Lundeen, 1957; Benn, 1963, 1964a–c; Benn and Ettlinger, 1965; Benn and Yelland, 1967; Kjær and Jensen, 1968; Matsuo, 1968), these compounds were not routinely synthesized, nor is their synthesis straightforward. Synthetic routes to naturally occurring indole glucosinolates have recently been developed by Rollin and colleagues (Viaud and Rollin, 1990; Viaud et al., 1992; Chevolleau et al., 1993; Gardrat et al., 1993). Gram-scale synthetic protocols were developed for phenethyl glucosinolate in 1980 (Gil and MacLeod, 1980e), a milligram-scale synthesis for ethyl glucosinolate (Keller et al., 1984), the gram-scale synthesis of sinigrin (Abramski and Chmielewski, 1996), and the first synthesis [milligrams] of ω-methylthioalkyl glucosinolates, 2-methylthioethyl-, 3-methylthiopropyl- and 4-methylthiobutyl-glucosinolate (Mavratzotis et al., 1996) have been reported. Synthesis of a group of α-glucosinolates (e.g. anomers of the naturally occurring phenyl-, benzyl-, 2-phenethyl- and indol-3-ylmethyl-glucosinolates, as well as (*E*)-styryl glucosinolate which has not been found to occur naturally) was reported by Blanc-Muesser et al. (1990). Other analogues (e.g. deoxyglucotropaeolins and 2-fluoro-2-deoxyglucotropaeolins) were synthesized to establish the importance of the $-$OH group at carbon-2 for glucosinolate–myrosinase binding but also to study the molecular mechanism of this reaction (Cottaz et al., 1996, 1997). Further, Lazar and Rollin (1994) developed a glucosinolate analog in which the anionic OSO_3^- was replaced by an OPO_3^{2-} moiety, in order to observe the changes in myrosinase hydrolysis kinetics after alteration of a site deemed critical in the glucosinolate recognition process. Most recently, Aucagne et al. (1999) have reported the synthesis of "C-glucotropaeolin," benzyl glucosinolate in which the thioglucoside sulfur atom is replaced by a carbon. Rollin and colleagues, leaders in modern day glucosinolate synthetic chemistry (Blanc-Muesser et al., 1990; Joseph and Rollin, 1993a,b; Lazar and Rollin, 1994; Cottaz et al., 1996, 1997; Aucagne et al., 1999), have reported a new approach to the synthesis of glucosinolate precursors that may broaden the range of synthetically accessible compounds (Cassel et al., 1998). Glucoraphanin, the member of this family of compounds that is to us most interesting from a nutritional standpoint, has not yet been synthesized, but controlled oxidation of glucoerucin to yield glucoraphanin and sulforaphane has been recently reported by Iori et al. (1999), and a series of synthetic analogs of sulforaphane were developed by Posner et al. (1994).

3.4. Biosynthesis

Most of our knowledge of the biosynthesis of glucosinolates is based on elegant studies by Underhill and colleagues at the Prairie Regional Laboratories in Saskatoon, Canada (see Underhill et al., 1973 for details), and more recent genetic studies by Mithen and collaborators at the Institute of Food Research, Norwich, UK (Dawson et al., 1993; Magrath and Mithen, 1993; Magrath et al., 1993, 1994; Parkin et al., 1994; Mithen et al., 1995b; Toroser et al., 1995; Giamoustaris and Mithen, 1996). Taken together, this body of work has provided strong evidence that elongation of amino acid side chains (e.g. α-amino acid homologues, derived from common amino acids by acetate addition to the α-keto acid and decarboxylation), occurs before *S*-glycosylation, whereas side chain modification (e.g. desaturation, hydroxylation) probably occurs after addition of the glycone moiety.

The initial step in the biosynthesis of the glucosinolates proceeds, as in that of cyanogenic glucosides, by *N*-hydroxylation of a precursor amino acid, followed by decarboxylation to form an aldoxime. In brief, the widely accepted model for glucosinolate biosynthesis involves three major steps: (a) side chain elongation; (b) glucone biosynthesis; and (c) side chain modification. Early evidence for the chain elongation of aliphatic glucosinolates came from in-vivo radiolabelling studies almost 40 years ago. Administration of ^{14}C-labelled amino acids and ^{14}C-acetate to horseradish, nasturtium and watercress resulted in the isolation of ^{14}C-labelled glucosinolates (Underhill et al., 1962; Chisholm and Wetter, 1964). Subsequent studies with *Arabidopsis thaliana* and *Brassica napus* have examined genetic variants in side chain length. This has led to the mapping of a number of "*Gsl-elong*" loci, allelic variation at which has been proposed to determine the length of the glucosinolate side chain, and to the isolation of a yeast artificial chromosome clone that hybridizes to RFLP markers near the *Gsl-elong* gene in *Arabidopsis* (Magrath et al., 1994; Mithen and Campos, 1996).

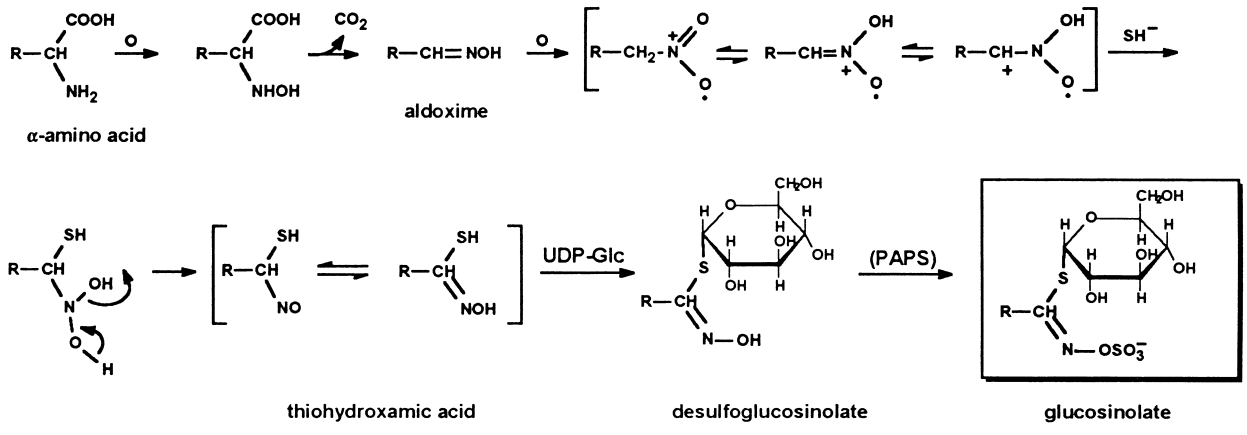
Glycone biosynthesis is initiated by the conversion of protein amino acids (e.g. alanine, methionine, valine, leucine or isoleucine for the aliphatic glucosinolates; phenylalanine or tyrosine for the aromatic glucosinolates and tryptophan for the indole glucosinolates) or chain elongated amino acids (e.g. many of the precursors of aliphatic glucosinolates such as homomethionine, dihomo-methionine, trihomo-methionine) to aldoximes (reviewed by Kjær and Olesen Larsen, 1973, 1976; Halkier and Du, 1997; Halkier, 1999; Mithen et al., 2000). Conclusive evidence has only recently been developed that cytochromes P450 catalyze the conversion of amino acids to aldoximes, a process long known also to be required by cyanogenic glycoside-producing plants (Hull et al., 2000; Wittstock and Halkier, 2000).

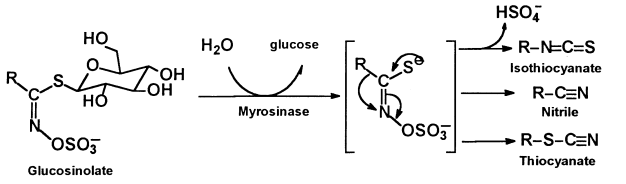
Biosynthetic steps after aldoxime formation are believed to involve conversion to a thiohydroximic acid, introduction of the thioglucoside sulfur from cysteine, *S*-glycosyl transfer from UDP-glucose, and sulfation by the universal high energy sulfate donor, 3′-phosphoadenosine-5′-phosphosulfate [PAPS] (Underhill, 1980; Haughn et al., 1991; Reed et al., 1993; Bennett et al., 1993, 1995; GrootWassink et al., 1994; Halkier and Du, 1997; Du and Halkier, 1998; Witczak, 1999; Halkier, 1999; Mithen et al., 2000). Neither biochemical evidence for the proposed intermediates between aldoxime formation and thiohydroximic acid nor purification and characterization of many of the enzymes in these steps has been attained. *S*-Glycosylation of thiohydroximic acids is catalyzed by a soluble UDPG:thiohydroximate glucosyltransferase and results in a desulfoglucosinolate. This enzyme has been purified from *Brassica napus, B. juncea, B. oleracea* and *Arabidopsis thaliana* (Jain et al., 1990b; Reed et al, 1993; GrootWassink et al., 1994; Guo and Poulton, 1994). Both the *B. oleracea* and the *B. napus* enzyme had high substrate-specificity for thiohydroximates, but had very low specificity for side chain structure. The final step in glycone formation is the sulfation of desulfoglucosinolates. This occurs via a soluble 3′-phosphoadenosine 5′-phosphosulfate (PAPS):desulfoglucosinolate sulfotransferase. This too has been purified and characterized but it is extremely unstable and it has a highly variable and selective substrate specificity (Glendening and Poulton, 1988; Jain et al., 1990a).

The precise mechanism of side chain modification of glucosinolates has been the source of much speculation and little experimental work. Initial oxidation of the side chain sulfur of methionine and its chain elongated homologues is expected to give rise to the large family of methylsulfinyl- and methylsulfonyl-glucosinolates. The Mithen group has proposed models for side chain modification of aliphatic and alkylthioalkyl glucosinolates based upon allelic variation at three loci: Gsl-oxid, Gsl-alk and Gsl-oh, resulting in oxidation (of the methylthio group), desaturation (of alkyl to alkenyl side chains) and hydroxylation (of alkenyl groups) respectively (Parkin et al., 1994; Mithen et al., 1995b; Giamoustaris and Mithen, 1996). Presumably, similar hydroxylations, desaturations and oxidations occur to the branched chain, aromatic and indole glucosinolates since they present a similar range of structural diversity to the aliphatic glucosinolates. The exception to this is the fact that at least ten of the aromatic and indole glucosinolates are singly or multiply methoxylated. It has been suggested that benzoyloxyalkyl glucosinolates arise from the conjugation of a hydroxylalkyl glucosinolate with benzoic acid (Mithen et al., 2000).

4. Hydrolysis of glucosinolates by plant and microbial myrosinases

Glucosinolates are very stable water-soluble precursors of isothiocyanates, and are typically present in fresh plants at much higher levels than their cognate isothiocyanates. Under carefully controlled conditions designed to extract glucosinolates and isothiocyanates completely, while preventing myrosinase activity, some fresh plants have been shown to contain almost exclusively glucosinolates (Fahey et al., 1997). The relatively nonreactive glucosinolates are converted to isothiocyanates upon wounding of the plant, mastication of fresh plants (i.e. vegetables) or by tissue damage caused by bruising or freeze–thawing during cultivation, harvest, shipping or handling (Bones and Rossiter, 1996; Rosa et al., 1997). This tissue damage releases myrosinase (EC 3.2.3.1), a glycoprotein that coexists with, but is physically segregated from its glucosinolate substrates. Myrosinase has long been thought to be localized in specialized 'myrosin' cells (Bones and Iversen, 1985; Bones et al., 1991; Drozdowska et al., 1992). An abundance of histological and immunochemical evidence (Luthy and Matile, 1984; Lenman et al., 1990; Bones, 1990; Bones et al., 1991; Höglund et al.,





1991; Bones and Rossiter, 1996) suggests that this enzyme is normally sequestered within aqueous vacuoles, although it is present in dormant mature seeds, so that unresolved questions remain about its localization in those organs.

Myrosinase has recently been cloned and sequenced (Xue et al., 1992; Henrissat et al., 1995; Rask et al., 2000) and its X-ray structure has been mapped (Burmeister et al., 1997, 2000). It is a β-thioglucosidase with an amino acid sequence that has strong similarities to that of the glycosylhydrolase family [EC 3.2.1–3.2.3]. The enzyme thus makes contact with the substrate and appears to require an hydroxyl group at C-2 on the glucose moiety for glucosinolate binding. There is a single nucleophilic glutamate (Glu-426), which is required for catalytic activity, in contrast to two glutamate moieties at the active sites of the related family 1 *O*-glycosidases (Cottaz et al., 1996). After hydrolytic cleavage of the β-glucosyl moiety, the sulfate moiety is released nonenzymatically to form the thiohydroxamate-*O*-sulfonate from both aliphatic and aromatic glucosinolates. This unstable intermediate then rearranges to form isothiocyanates, or other breakdown products (e.g. thiocyanates, nitriles, epithionitriles, oxazolidine-2-thiones) in a manner that depends upon the glucosinolate substrate as well as the reaction conditions (e.g. pH, or the presence of Fe^{2+} or epithiospecifier protein). The extensive and confusing literature on myrosinase products will not be discussed except for those formed from the indole and goitrogenic glucosinolates (see Sections 5.1 and 5.2).

The kinetics of the myrosinase reaction differs widely from species to species, and multiple forms of the enzyme can exist even within the same plant (James and Rossiter, 1991). There are fungal (Reese et al., 1958; Ohtsuru and Hata, 1972) and bacterial myrosinases (e.g. *Enterobacter cloacae*; Tani et al., 1974), in addition to the plant enzymes. Myrosinases are also present in many bacteria commonly associated with human and animal gut microflora (Campbell et al., 1987; Diedrich and Kujawa, 1987; Nugon-Baudon et al., 1988, 1990; Rabot et al., 1993) and in the crucivorous aphids *Brevicoryne brassicae* and *Lipaphis erisimi* (MacGibbon and Beuzenberg, 1978). An early report of the occurrence of myrosinase-like activity in mammalian tissues (Goodman et al., 1959) probably reflects the activities of the intestinal microflora, a hypothesis that is strongly supported by recent evidence from a number of laboratories (Oginsky et al., 1962; Rabot et al., 1993; Shapiro et al., 1998; Getahun and Chung, 1999). Myrosinases are activated to various degrees by ascorbic acid, and in some instances the enzyme is almost inactive in its absence (Shikita et al., 1999). Activation is not dependent on the redox reactivity of ascorbate, however, and it has been suggested that ascorbate provides a nucleophilic catalytic group (Ettlinger et al., 1961; Burmeister et al., 2000). The activation of ascorbate is "uncompetitive," i.e. ascorbate raises both V_{max} and K_m for the glucosinolate substrates (Shikita et al., 1999). Myrosinase has been purified and characterized from several sources, including white mustard (*Sinapis alba*; Björkman and Janson, 1972; Palmieri et al., 1986), cress (*Lepidium sativum*; Durham and Poulton, 1989), yellow mustard (*Brassica juncea*; Ohtsuru and Hata, 1972), rapeseed (*Brassica napus*; Lönnerdal and Janson, 1973), and wasabi (*Wasabia japonica*; Ohtsuru and Kawatani, 1979). The enzyme has also been reported to occur in *Raphanus sativus* (daikon) vegetative tissue (Iversen and Baggerud, 1980; El-Sayed et al., 1995) and sprouts (Shikita et al., 1999). Large variations in myrosinase specific activity have been reported in various cruciferous plant sources; Wilkerson and colleagues examined twelve cruciferous vegetables [including red, white, Chinese, and Savoy cabbage, Brussels sprouts, cauliflower, calabrese broccoli, radish, swede, turnip and watercress] and found that the specific activity of partially purified myrosinase ranged from 0.3 μmol/min/mg protein (watercress) to 10.5 μmol/min/mg protein (radish) (Wilkinson et al., 1984). Myrosinase, purified to homogeneity from daikon sprouts, has a specific activity of 280 μmol/min/mg protein with sinigrin as a substrate (Shikita et al., 1999).

To our knowledge only Björkman and Lönnerdal (1973) have evaluated the differential activity of purified myrosinase on a range of different glucosinolate substrates. This work only examined the differential hydrolysis of six glucosinolates, and must be repeated using modern analytical techniques on a wider range of glucosinolates. Evidence strongly suggests, however, that upon ingestion by humans, β-thioglucosidase activity of gut microflora is largely responsible for converting ingested glucosinolates to their cognate isothiocyanates (Shapiro et al., 1998; Getahun and Chung, 1999). Similar observations have also been made in numerous animal studies. For example, presumptive myrosinase activity was demonstrated in chickens (Campbell et al., 1987), rats (Diedrich and Kujawa, 1987; Nugon-Baudon et al., 1990), and in gnotobiotic animals harboring either mixed populations or single bacterial strains of human fecal origin (Nugon-Baudon et al., 1990; Rabot et al., 1993). Almost all of the mammalian chemoprotective activity from crucifers (discussed later in this review) is due to these isothiocyanates. Their formation from glucosinolates by myrosinase is presumably required to generate this biological activity.

Considerable effort has gone into the study of the catabolism of glucosinolates by microbes (Brabban and Edwards, 1994).

5. Glucosinolate content of plants

Glucosinolate content in plants is about 1% of dry weight in some tissues of the *Brassica* vegetables (Rosa et al., 1997), although the content is highly variable (Kushad et al., 1999; Farnham et al., 2000), and can approach 10% in the seeds of some plants, where glucosinolates may represent one-half of the sulfur content of the seeds (Josefsson, 1970). Most species contain a limited number of glucosinolates (generally less than one dozen) although as many as 23 different glucosinolates have been identified in *Arabidopsis thaliana* (Hogge et al., 1988; Haughn et al., 1991). Distribution of the glucosinolates that have been examined varies among plant organs, with both quantitative and qualitative differences between roots, leaves, stems and seeds. For example, seeds or young sprouts of broccoli (*Brassica oleracea* var. *italica*) can contain 70–100 μmol total glucosinolates per g fresh wt, with $<1\%$ contributed by indole glucosinolates and the balance consisting almost entirely of the aliphatic glucosinolates, glucoraphanin, glucoerucin and glucoiberin (Fahey et al., 1997). Late vegetative to reproductive stage plants of the same cultivar typically may contain only about 1–4 μmol of total glucosinolates per g fresh wt, with aliphatic and indole glucosinolates present at roughly equivalent levels (Fahey et al., 1997; Fahey and Stephenson, 1999). A small number of glucosinolates constitute about 0.05–0.1% of the fresh weight of broccoli or about 50–100 mg of glucosinolates per 100 g portion (Kushad et al., 1999; Farnham et al., 2000). Plant age is therefore a major determinant of the qualitative and quantitative glucosinolate composition of plants. Environmental factors such as soil fertility (Booth and Walker, 1992; Fahey and Stephenson, 1999), pathogen challenge (Butcher et al., 1974), wounding (Bodnaryk, 1992) or plant growth regulators (Bodnaryk, 1994; Bodnaryk and Yoshihara, 1995) also have significant effects on levels of specific glucosinolates in the growing plants and may affect distribution among plant organs.

5.1. Special properties of indole glucosinolates

The isothiocyanates formed from indole glucosinolates are unstable, and decompose spontaneously to indole-3-carbinol, indole-acetonitrile, thiocyanate ions and 3,3′-diindolylmethane. Indole-3-carbinol may then spontaneously condense under the acid conditions of the stomach to form compounds that closely resemble 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) in structure, toxicity and carcinogenicity (Bjeldanes et al., 1991). Despite this toxicity, indole glucosinolate metabolites, in particular indole-3-carbinol, have been investigated for their potential as cancer chemoprotective agents (e.g. Bradlow et al., 1991; Coll et al., 1997). For more detailed treatment of the potentially carcinogenic and anticarcinogenic dual nature of these compounds, see Kim et al. (1997) and reviews by Broadbent and Broadbent (1998a,b), Fenwick et al. (1983), McDanell et al. (1988), Rosa et al. (1997) and Stoewsand (1995).

5.2. Goitrogenic and antinutritional effects of glucosinolates

Hydrolysis of β-hydroxyalkenyl glucosinolates (e.g. progoitrin and epi-progoitrin), gives rise to β-hydroxyalkenyl isothiocyanates. These compounds cyclize to oxazolidine-2-thiones which may have goitrogenic effects in mammals — first observed in rabbits and designated "cabbage" goiter by Webster and Chesney (1930). The "antinutritional" nature of the β-hydroxyalkenyl glucosinolates is discussed in Bille et al. (1983), Vermorel et al. (1988), Mawson et al. (1993a,b, 1994a,b, 1995a,b) and Yong-Gang et al. (1993) as well as in the more general reviews of Fenwick et al. (1983), Rosa et al. (1997) and Griffiths et al. (1998). Efforts to avoid the goitrogenicity of rapeseed (*Brassica napus*), one of the most important oilseed crops in the world, led to the highly successful development of the oilseed crop "Canola." Canola (a contraction of "Canadian" and "oil") was developed in the 1970s by a plant breeding program designed to develop cultivars of oilseed rape with low levels of glucosinolates and erucic acid. The designation "Canola" is not specific, nor does it even refer to a single variety. Two types of Canola are now grown: a short season, yellow seeded, Polish (*Brassica rapa*) type and a longer season, black seeded (*Brassica napus*) variety. Canola seed contains about 40% oil and by regulation this oil must contain $<2\%$ erucic acid. The seed meal, which is fed to animals after oil is expressed, must have <30 μmol of glucosinolates per gram of meal. The value of the Canadian Canola crop now exceeds that of their wheat crop (over 3 million tons produced on about 3 million hectares in Saskatchewan alone, according to the Saskatchewan Canola Development Commission).

5.3. Naturally occurring isothiocyanates outside the plant kingdom

Although isothiocyanates have been reported outside the plant kingdom (e.g. long chain α,ω-bisisothiocyanates from marine sponges; Karuso and Scheuer, 1987), it has been assumed that the route of synthesis in this case is not via glucosinolates (Hagadone et al., 1984). Isothiocyanates from fungi have long been recognized

for their antibiotic properties (e.g. paulomycin) but their biosynthesis in fungi appears not to occur through a glucosinolate intermediate (Wiley et al., 1986).

6. Biotic interactions of glucosinolates and isothiocyanates

The antibacterial activities of glucosinolates/isothiocyanates (Kjær and Conti, 1954; Procházka and Komersová, 1959; Virtanen, 1962; Wagner et al., 1965; Dornberger et al., 1975; Johns et al., 1982; Uda et al, 1993; Brabban and Edwards, 1995; Delaquis and Mazza, 1995; Hashem and Saleh, 1999; Lin et al., 2000) and their antifungal activity (Drobinca et al., 1967; Kojima and Ogawa, 1971; Mari et al, 1993; Delaquis and Mazza, 1995; Mayton et al., 1996; Manici et al., 1997; Hashem and Saleh, 1999) have been recognized for many decades. The activity of isothiocyanates such as sulforaphane against numerous human pathogens (e.g. *Escherichia coli, Salmonella typhimurium, Candida* sp.) could even contribute to the medicinal properties ascribed to cruciferous vegetables, such as cabbage and mustard, which have been used as wound poultices and antitumor agents for centuries (Hartwell, 1982). Activity against a range of soil-borne fungal and bacterial plant pathogens is profound, and has been extensively characterized (see reviews by Brown and Morra, 1997; Rosa and Rodrigues, 1999).

Antagonistic interactions are not confined to microbes, since nematocidal activity of glucosinolates has been demonstrated (Lazzeri et al., 1993; Mayton et al., 1996) as has activity as a feeding deterrent to caddisflies, snails and amphipods (Newman et al., 1992). One of the more complex interactions of glucosinolates/isothiocyanates is their activity as allelochemicals, compounds that affect successive plant communities and/or those growing simultaneously, in close proximity (e.g. Brown and Morra, 1995; Charron and Sams, 1999; Smith, 2000). This area has long been the subject of scientific investigation, and has recently been extensively reviewed by Brown and Morra (1997). In addition, glucosinolates are widely recognized as defensive compounds against generalist herbivores and are likely to be involved in host plant recognition by specialist predators, thus acting both as an insecticide and as an insect feeding attractant (e.g. Rodman and Chew, 1980; Louda and Rodman, 1983; Mithen et al., 1986, 1987b; Hammond and Lewis, 1987; Louda et al., 1987; Tsao et al., 1996; Rask et al., 2000). For example, glucosinolates provide important feeding cues to insects including *Pieris* sp. caterpillars and other specialist feeders (e.g. *Plutella* sp., seed weevils, flea beetles), which are differentially stimulated to feed by various glucosinolates (Renwick et al., 1992). Specific tarsal contact chemoreceptors on larvae respond differentially to the glucosinolates rather than the cognate aglycone (e.g. the isothiocyanate) and ovipositing females utilize glucosinolate clues in selecting suitable plants (Rodman and Chew, 1980). Larsen et al. (1992) report that certain glucosinolates found within the host plant (*Hesperis matronalis*) of a monophagous weevil (*Ceutorhynchus inaffectatus*) were powerful feeding stimulants. MacGibbon and Beuzenberg (1978) have demonstrated intense zones of myrosinase activity within the gut of the aphid *Brevicoryne brassicae* that is probably due to bacterial activity. Activity of protective enzymes such as glutathione transferase was induced in *Spodoptera frugiperda* (fall armyworm) and *Trichoplusia ni* (cabbage looper) by feeding on allyl and benzyl isothiocyanates (Chew, 1988). Mithen et al. (1995a) provide insight into the effects of genetically mediated changes in plant chemistry (e.g. glucosinolate content) on plant–herbivore interactions. A synthesis of the extensive literature on insect feeding interactions with glucosinolate-containing plants can be found in Chew (1988), Rask et al. (2000), and in the review by Brown and Morra (1997).

7. Analytical methods

Since the work of Ettlinger and Lundeen (1956a,b, 1957), much effort has been devoted to developing methods for the efficient isolation and identification of glucosinolates (Betz and Fox, 1994). Most early identifications relied on paper or thin-layer chromatography of the glucosinolates or of their presumptive hydrolysis products (e.g. an investigation of the glucosinolates from the seeds of 151 different crucifers by Danielak and Borkowski, 1969). Numerous techniques were utilized for the quantification of ''total'' glucosinolates. For example, McGregor (1980) examined the environmental and within- and between-laboratory variation of six different analytical methods for glucosinolate determination (e.g. steam distillation and titration of volatile isothiocyanates; UV spectroscopy of oxazolidinethiones; gas chromatography of volatile isothiocyanates; gas chromatography/UV spectroscopy; UV spectroscopy of thiourea derivatives of isothiocyanates; and gas chromatography of trimethylsilyl derivatives of glucosinolates). Glucosinolate separations were then performed by gas liquid chromatography of trimethylsilylated derivatives of glucosinolates from which the sulfate group had been removed (Underhill and Kirkland, 1972a) and this technique was subsequently coupled with mass spectrometry (Christensen et al., 1982). Enzymatic removal of the sulfate prior to derivatization led to multiple products (Heaney and Fenwick, 1987; Theis, 1988). This method was used as recently as 10 years ago by Daxenbichler et al. (1991), who undertook an extensive survey of the glucosinolate composition of seeds from about 300 wild plant species

using gas chromatographic detection of desulfoglucosinolate hydrolysis products.

In 1984, G.R. Fenwick and colleagues (Norwich, UK) developed the reversed-phase HPLC method for quantitative analysis of desulfoglucosinolates which is most widely used today (Spinks et al., 1984). This method utilizes an on-column enzymatic desulfation treatment of plant extracts followed by HPLC detection of the resultant desulfoglucosinolates. Adaptation of the sulfohydrolase desulfation method as an HPLC method, although the most widely used method for glucosinolate separation, is still subject to difficulties in interpretation because of the effects of pH, time and enzyme activity on the desulfation products (Minchinton et al., 1982; Spinks et al., 1984; Sang and Truscott, 1984). Typically, this method uses response factors determined with purified desulfosinigrin and uses desulfobenzyl glucosinolate as an internal standard. Correspondence of glucosinolate retention times, and comparison to standardized rapeseed extracts are typically used to validate chromatographic profiles. Unfortunately, the biological activity of these molecules is compromised by the removal of the sulfate. After desulfation, they can no longer serve as substrates for myrosinase and thus their cognate isothiocyanates are not available for bioassay or for direct measurement by cyclocondensation — key tools in the study of the pharmacokinetics, pharmacodynamics and bioactivity of these compounds.

To our knowledge, many plant glucosinolates have not been rigorously identified by modern analytical and spectroscopic methods such as HPLC, NMR, mass spectroscopy or supercritical fluid chromatography with light scattering detection (Fenwick et al., 1980; Eagles et al., 1981; Fenwick et al., 1982; Bjerg and Sørensen, 1987; Bradfield and Bjeldanes, 1987; Lafosse et al., 1990; Prestera et al., 1996). There was, and still is, an extreme paucity of high purity chromatographic standard glucosinolates available to researchers. Only the generosity of a handful of leaders in this field has permitted investigators who do not isolate and purify their own standards to perform meaningful research on these compounds.

7.1. Characterizing and quantifying glucosinolates (HPLC/MS)

We have recently improved upon the methods developed by Helboe and others (Helboe et al., 1980; Betz and Fox, 1994) for the separation and identification of individual glucosinolates in plant extracts (Prestera et al., 1996). These improvements exploit: (1) paired ion chromatography of alkylammonium salts (e.g. tetraoctyl- or tetradecylammonium bromide) used in conjunction with myrosinase hydrolysis and isothiocyanate assay by cyclocondensation with vicinal dithiols (Zhang et al., 1992, 1996; see below); (2) a novel method for replacement of the counter ion by NH_4^+ which is critical for bioassay and mass spectroscopy; (3) improvements in mass spectroscopic analysis by combined fast atom bombardment and chemical ionization techniques; and (4) high resolution nuclear magnetic resonance (NMR) spectroscopy, which provides final confirmation of identity (Prestera et al., 1996). This combination of steps provides a powerful method for rapidly characterizing and quantifying glucosinolates.

7.2. Identifying and quantifying isothiocyanates

Separation and identification of isothiocyanates from plant extracts is typically accomplished by HPLC (Zhang et al., 1992; Kore et al., 1993; Bertelli et al., 1998). We have also developed a sensitive assay for quantification of total isothiocyanates in plant extracts that exploits the ability of isothiocyanates to react with 1,2-benzenedithiol to form a cyclic thiocarbonyl reaction product, 1,3-benzodithiole-2-thione, with a very high extinction coefficient in the near ultraviolet range (Zhang et al., 1992, 1996). We can now measure as few as 10 pmol of isothiocyanates in complex biological fluids such as plant extracts by modification of this technique for HPLC (Zhang et al., 1996). There is excellent correlation between total glucosinolate titer as determined by measuring isothiocyanates produced by the action of exogenous purified myrosinase on extracted glucosinolates, and the levels of these glucosinolates measured directly by the paired ion chromatography techniques referred to above (Fahey and Stephenson, 1999). This method can therefore be used to quantify either total or individual glucosinolates or isothiocyanates from plant extracts, from separate HPLC peaks or from clinical samples such as urine or blood (Zhang et al., 1996; Fahey et al., 1997; Bertelli et al., 1998; Shapiro et al., 1998; Zhang and Talalay, 1998; Getahun and Chung, 1999).

8. Glucosinolates/isothiocyanates and cancer chemoprotection

Over the past 20 years, compelling evidence has been obtained linking increased consumption of fruits and vegetables, especially cruciferous vegetables, to reduced incidence of many types of cancer (Steinmetz and Potter, 1991, 1996; Block et al., 1992; Doll, 1992; Verhoeven et al., 1996; Michaud et al., 1999; Talalay, 1999). Ingestion of about two servings per day of cruciferous vegetables may result in as much as a 50% reduction in the relative risk for cancer at certain sites (Graham et al., 1978, and as calculated from the data of Kune et al., 1987 and Kohlmeier and Su, 1997). At least some of the cancer chemoprotective activity of these vegetables is widely believed to be due to their content of minor

dietary components such as glucosinolates. Certain glucosinolates (e.g. benzyl-, *p*-hydroxybenzyl- and 2-hydroxybut-3-enyl glucosinolates) have themselves been reported to induce mammalian Phase 2 enzymes of detoxication (Wattenberg et al., 1986, Tawfiq et al., 1995; Fahey et al., 1997). The enzyme myrosinase — activated in damaged plant tissue and also present in the microflora of the human digestive tract — converts these glucosinolates to a number of compounds including thiocyanates, nitriles and isothiocyanates. Most attention has been focused on the cancer-preventive potential of these metabolites, primarily as inducers of Phase 2 enzymes but with potential antiproliferative, apoptosis-promoting, redox regulatory and Phase 1 enzyme inhibiting roles as well (Zhang and Talalay, 1994, 1998; Barcelo et al., 1996; Nastruzzi et al., 1996; International Life Sciences Institute, 1999; Gamet-Payrastre et al., 2000; Nakamura et al., 2000). A few examples of these cancer-preventive studies follow: (1) Sulforaphane has been shown to elevate levels of mammalian Phase 2 enzymes by ARE (Antioxidant Response Element)-mediated transcriptional activation (Zhang et al., 1992, 1994; Prestera et al., 1993; Talalay et al., 1995; Talalay and Zhang, 1996; Fahey et al., 1997). Sulforaphane reduced the incidence, delayed the appearance of, and reduced the size of tumors in a rat mammary tumor model (Zhang et al., 1994; Fahey et al., 1997), serves as an indirect antioxidant (Fahey and Talalay, 1999), exerts selective cytostatic and cytotoxic effects on human colon cancer cells in vitro (Gamet-Payrastre et al., 1998), inhibits cytochrome P450 (Mahéo et al., 1997; Morel et al., 1997), in particular CYP2E1 (Barcelo et al., 1996) and induces cell cycle arrest and apoptosis in human colon cancer cells in vitro (Gamet-Payrastre et al., 2000). (2) Phenethyl isothiocyanate has been shown to inhibit induction of lung and esophageal cancer in both rat and mouse tumor models (Morse et al., 1993; Stoner and Morse, 1996, 1997; Hecht, 1996; Stoner et al., 1999). These effects correlated well with a reduction in carcinogen–DNA adduct formation and strongly suggested inhibition of cytochromes P450 as a mechanism of action. An analogous effect on NNK metabolism was observed in smokers who consumed watercress (a source of phenethyl glucosinolate) (Hecht, 1999), as well as a significant increase in the glucuronidation of nicotine metabolites, thus suggesting induction of the Phase 2 detoxication enzyme UDP-glucuronosyltransferase activity in humans by phenethyl isothiocyanate (Hecht et al., 1999). Adesida et al. (1996) demonstrated a pronounced antiproliferative effect of phenethyl isothiocyanate metabolites on human leukemia cells in vitro. (3) Crambene (cyanohydroxybutane), glucoiberin and indole-3-carbinol have been shown to elevate quinone reductase and glutathione transferase (Phase 2 detoxication enzymes) and CYP1A (a Phase 1 enzyme) and, in some cases, to do so synergistically; crambene has been identified as the most potent inducer in this system (Staack et al., 1998; Wallig et al., 1998). (4) The metabolism of isothiocyanates in human volunteers has been examined after ingestion of a plant source of sulforaphane (Shapiro et al., 1998), and phenethyl isothiocyanate (Getahun and Chung, 1999), and both studies strongly suggested a role for microflora in the digestive tract in the hydrolysis of glucosinolates to isothiocyanates. Seow et al. (1998) and Fowke et al. (2001a,b) have utilized the cyclocondensation assay developed by Zhang et al. (1992, 1996) in order to follow the metabolism of dietary isothiocyanates and to demonstrate the correlation of urinary isothiocyanate levels with reported cruciferous vegetable intake obtained from food frequency questionnaires, in free living populations in Singapore and the US, respectively.

9. Concluding remarks

The genus *Brassica*, represents only 1 of over 350 genera in the Brassicaceae family which, in turn, is only 1 of 16 families of glucosinolate-containing higher plants (Table 3). Many glucosinolate-containing genera contain plants that have been used for food or medicinal purposes by various cultures for many centuries (e.g. capers, *Capparis spinosa*; wasabi, *Wasabia japonica*; Arugula, *Eruca sativa*; Radish, *Raphanus sativus*) and are currently being investigated for their fungicidal, bacteriocidal, nematocidal and allelopathic properties (e.g. Chew, 1988; Lazzeri et al., 1993; Palada, 1996; Charron and Sams, 1999). The glucosinolates in species such as *Crambe abyssinica* and *Brassica napus* that can be grown as field and row crops are being investigated as sources of starting material for the production of high value fine chemicals (Daubos et al., 1998) as well as for their use as feedstocks and sources of chemoprotective compounds (Barrett et al., 1998). Nutritive value of defatted seed cake from lesser grown glucosinolate-containing plants such as the tropical annual *Cleome viscosa* (already eaten as a leafy green and a condiment) have been explored (Rukmini and Deosthale, 1979). Other tropical and subtropical species have such a compelling ethnopharmacology and such manifold food and medicinal uses that a more rigorous investigation of the properties of their glucosinolates seems promising.

It may thus be of more than academic interest to re-examine some of the "non *Brassica*" glucosinolate-containing plants for their potential pharmacological value — in particular for cancer chemoprotection. Therefore in Appendix A we list the glucosinolate-containing angiosperms. An unconfirmed report of the occurrence of glucosinolates in the common mushroom

(*Agaricus bisporus*; MacLeod and Panchasara, 1983) is omitted. Likewise omitted are reports claiming to have identified glucosinolates in plantain (*Plantago major*; Cole, 1976) and cocoa (*Theobroma cacao*; Gill et al., 1984), because these conclusions were strongly questioned in subsequent more detailed work (Larsen et al., 1983; Bjerg et al., 1987). The glucosinolates of the vegetable *Brassica* species are not listed by species and variety; rather, the reader is referred to the excellent reviews by Fenwick and colleagues (1983) and by Rosa and colleagues (1997) for a detailed treatment of the glucosinolates of *Brassica* vegetables.

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Appendix on next page

Appendix A

Distribution of glucosinolates among plant species[a,b]

FAMILY		Aliphatic					Aromatic				
Genus→species subspecies/variety	Sulfur-containing A	Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
BATACEAE											Rodman, 1991a
Batis maritima									**43, 28**[c]		Schraudolf et al., 1971
BRASSICACEAE											
Aethionema											
armenum	**73, 82**										Daxenbichler et al., 1991
Alliaria											
petiolata				**107**			**11**				Cole, 1976, Daxenbichler et al., 1991
Alyssoides											
utriculata	**64, 84**			**12**			**23**				Daxenbichler et al., 1991
Alyssum											
alyssoides	**67, 72, 88, 94**			**12, 24, 101**							Daxenbichler et al., 1991
argenteum	**72, 84, 94**										Danielak and Borkowski, 1969, Daxenbichler et al., 1991
bertolonii ssp. *scutarinum*	**72, 94**										Daxenbichler et al., 1991
chondrogynum	**84, 94**										Hasapis et al., 1981
constellatum	**73**										Daxenbichler et al., 1991
dasycarpum				**24**			**23**				Daxenbichler et al., 1991
desertorum	**67, 88**						**23**				Daxenbichler et al., 1991
granatense	**67, 72, 88, 94**			**24**							Daxenbichler et al., 1991
minus	**72, 94**										Daxenbichler et al., 1991
minutum	**67, 72, 88, 94**			**24**							Daxenbichler et al., 1991
murale	**67, 72**										Daxenbichler et al., 1991
peltarioides	**73, 95**										Daxenbichler et al., 1991
perenne			**56**				**12**				Cole, 1976
saxatile	**72, 94, 95**		**56**	**12, 101**			**12**				Cole, 1976, Danielak and Borkowski, 1969
sibiricum	**73, 95**										Daxenbichler et al., 1991
tortuosum	**72, 84, 94**						**105**				Daxenbichler et al., 1991
troodi	**87, 88, 94**										Hasapis et al., 1981
Anastatica											
hierochuntica	**73**										Daxenbichler et al., 1991

(*continued on next page*)

Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Arabidopsis											
thaliana	**64, 66, 67, 69, 72, 73, 84, 87, 88, 92, 94, 95**		**56**	**12, 107**	**26, 42**		**12, 105**	**5, 6, 10**	**43, 47, 48**		Cole, 1976, Daxenbichler et al., 1991, Haughn et al., 1991, Hogge et al., 1988, Ludwig-Müller et al., 1999
wallichii	**66, 69, 87, 92**			**12, 107**							Daxenbichler et al., 1991
Arabis											
alpina L.	**65, 68, 89, 95**										Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991
var. *grandiflora*	**65, 68**										Daxenbichler et al., 1991
amplexicaulis	**68, 69, 85, 89, 92**						**23, 50**				Daxenbichler et al., 1991
caucasica	**95**										Cole, 1976
drummondii	**67, 72, 78, 88**		**56, 61, 62**		**57**		**11**				Rodman and Chew, 1980
glabra	**66, 67, 87, 88**						**23**				Daxenbichler et al., 1991
hirsuta[f]	**69, 71, 87, 92, 93**		**56**				**50**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Kjær and Schuster, 1972a
ssp. *hirsuta*	**69, 89, 92**						**23, 50**				Daxenbichler et al., 1991
holboellii	**67, 92**		**56, 61**								Daxenbichler et al., 1991
kennedyae	**87, 88, 95**			**107**							Hasapis et al., 1981
laevigata	**69, 82, 87, 92**		**56**	**107**							Daxenbichler et al., 1991
nipponica	**69, 92**						**23, 50**				Daxenbichler et al., 1991
petiolaris				**107**							Daxenbichler et al., 1991
purpurea	**87, 89**										Hasapis et al., 1981
sparsiflora	**66, 67, 87, 88, 94**		**56, 61**				**105**				Daxenbichler et al., 1991
stelleri var. *japonica*	**69, 92, 95**						**23, 50**				Daxenbichler et al., 1991
turrita	**68, 69, 77, 79, 80, 92, 95**										Cole, 1976, Daxenbichler et al., 1991
Armoracia											
lapathifolia Gilib.[g]	**37, 73, 82, 84, 86, 88, 94, 95**	**13, 16, 51, 102**	**54, 55, 56, 61, 62**	**12, 19, 38, 101, 107**	**25, 39, 41**		**11, 44, 103, 104, 105, 106**		**43**		Ettlinger and Kjær, 1968, Fenwick et al., 1983, Grob and Matile, 1980

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Aubrietia											
deltoidea	**95**		**61**		**57**		**46**	**9**			Ahmed et al., 1972b, Cole, 1976, Danielak and Borkowski, 1969, Kjær et al., 1956, Kjær et al., 1971
Aurinia											
saxatilis	**73, 94**			**12, 101**							Daxenbichler et al., 1991
ssp. *orientalis*	**72, 84, 94**		**61**	**12, 101**							Daxenbichler et al., 1991
Barbarea											
intermedia	**95**		**56**	**107**			**105**				Cole, 1976
orthoceras							**40, 105**				Daxenbichler et al., 1991
praecox									**47**		Danielak and Borkowski, 1969
stricta			**56**	**107**			**105**				Cole, 1976
verna							**23, 105**				Daxenbichler et al., 1991
vulgaris	**95**						**40, 105**		**43, 47**		Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Huang et al., 1994
Berteroa											
incana	**94**			**101**							Cole, 1976
Biscutella											
auriculata	**68, 69**										Daxenbichler et al., 1991
didyma	**64, 95**						**11, 105**				Lockwood and Belkhiri, 1991
laevigata	**68, 69**						**23, 105**				Cole, 1976, Daxenbichler et al., 1991
Boreava											
aptera				**12**			**23**				Daxenbichler et al., 1991
orientalis				**12, 24**			**23**				Daxenbichler et al., 1991
Bornmuellera											
dieckii	**67, 72, 94**						**23**				Daxenbichler et al., 1991
Brassica sp. (predominant GS's)	**63, 64, 72, 73, 84, 94, 95**		**56, 61**	**12, 24a, 38, 100, 101, 107**			**23, 105**		**43, 47**		
Bunias											
orientalis	**63, 64**		**56**				**23**				Daxenbichler et al., 1991
Cakile											
arabica			**56, 61**	**107**							Rodman, 1981
artica	**84, 95**			**12, 107**			**105**				Rodman, 1981
constricta	**84, 94, 95**		**61**	**12, 107**							Rodman, 1981

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
edentula ssp. *eden* v. *eden,*											
Northern	**95**		**56, 61**	**12, 107**			**11**				Rodman, 1981
ssp. *eden* v. *eden, Southern*	**73, 95**		**56, 61**	**12, 107**			**11**				Rodman, 1981
ssp. *eden* v. *lacustris*	**95**		**56, 61**	**12, 107**			**11**				Rodman, 1981
ssp. *harperi*	**64, 84, 94, 95**		**61**	**12, 101, 107**			**105**				Rodman, 1981
geniculata	**84, 95**		**61**	**12, 107**							Rodman, 1981
lanceolata ssp. *alacranensis*	**84**			**12**							Rodman, 1981
ssp. *fusiformis*	**84, 94, 95**		**61**	**12, 101, 107**							Rodman, 1981
ssp. *lanceolata*	**84, 94, 95**		**61**	**12, 101, 107**			**105**				Rodman, 1981
ssp. *pseudoconstricta*	**84, 94, 95**		**61**	**12, 101, 107**							Rodman, 1981
maritima	**84, 95**		**56, 61**	**12, 101, 107**			**23**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991
ssp. *baltica*	**95**		**56, 61**	**12, 107**							Rodman, 1981
ssp. *euxina*	**95**			**107**							Rodman, 1981
ssp. *maritima* (W.Med.)	**64, 73, 81, 84, 88, 94, 95**		**56, 61**	**12, 107**							Rodman, 1981
ssp. *maritima* (W.Eur.)	**95**		**56, 61**	**107**							Rodman, 1981
Calepina											
irregularis	**73, 82**										Daxenbichler et al., 1991
Camelina											
microcarpa	**65, 74**										Daxenbichler et al., 1991
rumelica	**65, 68, 74**										Daxenbichler et al., 1991
sativa	**65, 68, 84**										Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Fenwick et al., 1983
Capsella											
bursa-pastoris	**65, 68, 84, 95**			**24, 101, 107**			**23**				Daxenbichler et al., 1991
cordifolia			**56, 61, 62**		**30, 31, 57**		**11, 105**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Lockwood and Belkhiri, 1991
Cardamine											
armara			**54**								Danielak and Borowski, 1969
flexuosa			**56, 61**	**12**			**11, 105**				Daxenbichler et al., 1991
hirsuta			**61**	**12, 101**			**11, 23, 105**				Cole, 1976, Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
impatiens				**12, 101**			**11**				Cole, 1976, Daxenbichler et al., 1991
var. *pectinata*				**12, 101**							Daxenbichler et al., 1991
pratensis			**54, 61**								Cole, 1976, Danielak and Borkowski, 1969
Cardaria											
chalapensis	**64, 67, 72, 84, 88, 94**						**11, 23, 105**				Daxenbichler et al., 1991
draba	**64, 76, 84**	**13**	**61**	**12, 101, 107**			**23**				Cole, 1976, Daxenbichler et al., 1991, Hasapis et al., 1981, Lockwood and Belkhiri, 1991
ssp. *chalapensis*	**64, 67, 76, 88**						**23**				Daxenbichler et al., 1991
Carrichtera											
annua	**72, 84, 94**			**12, 101**							Daxenbichler et al., 1991
Caulanthus											
lasiophyllus	**72, 94**		**56**								Daxenbichler et al., 1991
Cheiranthus											
cheiri	**82, 84**										Cole, 1976, Daxenbichler et al., 1991, Kjær, 1959
kewensis	**73, 95**										Chisholm, 1972
Chorispora											
purpurascens				**12, 24**			**23**				Daxenbichler et al., 1991
tenella	**64, 73, 84, 95**			**107**							Daxenbichler et al., 1991, Rodman and Chew, 1980
Christolea											
crassifolia	**67, 88**		**56, 61**								Daxenbichler et al., 1991
Cochlearia											
anglica			**54, 56, 61**		**31**						Cole, 1976, Kjær et al., 1956
danica			**54, 56, 61**		**31**						Cole, 1976, Kjær et al., 1956
officinalis			**54, 56**		**31**						Ahmed et al., 1972b, Brown and Stuart, 1968, Danielak and Borkowski, 1969, Kjær et al., 1956
Coincya											
longirostra				**12, 24a, 24b, 107**			**23**				Cole, 1976, Vioque et al., 1994

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
monensis ssp. *cheiranthos*											Vioque et al., 1994
var. *granatensis*				**12, 24a, 24b**			**23**				Vioque et al., 1994
var. *johnstonii*				**24a, 24b**			**23**				Vioque et al., 1994
var. *recurvata*				**12, 24a, 24b**			**23**				Vioque et al., 1994
var. *setigera*				**24a, 24b**							Vioque et al., 1994
ssp. *nevadensis*				**12, 24a, 24b**			**23**				Vioque et al., 1994
ssp. *orophila*				**12, 24a, 24b**			**23**				Vioque et al., 1994
ssp. *puberla*				**12, 24a, 24b**			**23**				Vioque et al., 1994
rupestris ssp. *leptocarpa*				**12, 24a, 24b**			**23**				Vioque et al., 1994
ssp. *rupestris*				**12, 24a, 24b**			**23**				Vioque et al., 1994
transtagana				**24a, 24b**			**23**				Vioque et al., 1994
Conringia											
orientalis	**95**		**62**	**12, 24**	**31**				**28, 43, 47**		Ahmed et al., 1972b, Boufford et al., 1989, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Gmelin and Virtanen, 1959b, Lockwood and Belkhiri, 1991, Underhill and Kirkland, 1972b
planisiliqua				**12, 107**			**23**				Daxenbichler et al., 1991
Coronopus											
didymus							**11**				Daxenbichler et al., 1991
squamatus							**23, 114**				Daxenbichler et al., 1991
Crambe											
abyssinica				**24b**			**23**				Daubos et al., 1998, Daxenbichler et al., 1965, Daxenbichler et al., 1991
cordifolia				**24**							Daxenbichler et al., 1991
filiformis				**12, 24**			**23**				Daxenbichler et al., 1991
juncea				**12, 24**			**23**				Daxenbichler et al., 1991
koktebelica				**12, 24**			**23**				Daxenbichler et al., 1991
maritima				**12, 24b, 107**			**23**				Danielak et al., 1969, Daxenbichler et al., 1991, Quinsac et al., 1994
orientalis				**12, 24**			**23**				Daxenbichler et al., 1991
tataria				**24**			**23**				Daxenbichler et al., 1991
Dentaria											
laciniata					**29, 31, 57**		**23**				Daxenbichler et al., 1991
Descurainia											
appendiculata				**12, 107**							Daxenbichler et al., 1991
pinnata				**12, 107**							Daxenbichler et al., 1991
ssp. *ochroleuca*				**12, 24, 101, 107**							Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
richardsonii	**76, 94**		**56, 61, 62**	**12, 101, 107**			**11**				Rodman and Chew, 1980
sophia			**61**	**12, 107**			**23**				Cole, 1976, Daxenbichler et al., 1991
Dimorphocarpa											
wislizenii	**67, 72, 87, 88, 94**										Daxenbichler et al., 1991
Diplotaxis											
catholica				**12**			**23**				Daxenbichler et al., 1991
crassifolia							**23**				Daxenbichler et al., 1991
erucoides	**84, 95**			**12, 107**			**11, 23, 105**		**28, 43, 47, 48**		Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Hasapis et al., 1981, Lockwood and Belkhiri, 1991
griffithii	**64, 84**						**23**				Daxenbichler et al., 1991
muralis				**107**							Danielak and Borkowski, 1969
siifolia				**12, 101**			**23**				Daxenbichler et al., 1991
tenuifolia	**64, 84**										Cole, 1976, Daxenbichler et al., 1991
viminea	**84**		**56, 61**	**107**			**23**				Cole, 1976, Daxenbichler et al., 1991
virgata			**56, 61**	**12, 24**			**11, 105**				Daxenbichler et al., 1991, Lockwood and Belkhiri, 1991
Dithyrea											
californica	**67, 72, 87, 88, 94**			**107**							Daxenbichler et al., 1991
Draba											
aizoides	**84, 95**										Cole, 1976
nemorosa var. *hebecarpa*	**68**		**61**	**12, 107**							Daxenbichler et al., 1991
spectabilis	**69**		**56, 61**								Rodman and Chew, 1980
Enarthrocarpus											
strangulatus				**24**			**23**				Daxenbichler et al., 1991
Erophila											
verna			**56, 61**				**11, 23**				Daxenbichler et al., 1991
Eruca											
longirostris	**64, 84**			**12**							Daxenbichler et al., 1991
sativa	**84, 95**		**61**								Ahmed et al., 1972b, Cole, 1976, Danielak and Borkowski, 1969, Fenwick et al., 1983
vesicaria	**64, 84**										Daxenbichler et al., 1991
ssp. *sativa*	**64, 84**										Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Erucaria											
hispanica				**24**			**23**				Daxenbichler et al., 1991
Erucastrum											
gallicum				**12, 107**							Danielak and Borkowski, 1969
laevigatum				**12, 107**			**23, 105**				Daxenbichler et al., 1991
nasturtiifolium	**63**						**23**				Daxenbichler et al., 1991
strigosum	**73**						**23**				Daxenbichler et al., 1991
Erysimum						**99**					Chisholm, 1973
allionii	**64, 76, 82, 84**										Daxenbichler et al., 1991
asperum	**33, 35, 37, 67, 72, 73, 76, 82, 88, 91, 94**		**61**	**107**			**23**	**8**			Daxenbichler et al., 1991, Rodmann and Chew, 1980
aureum	**95**		**61**								Cole, 1976
capitatum	**73, 82**			**107**							Daxenbichler et al., 1991
cheranthoides	**84, 95**										Cole, 1976
cuspidatum	**73, 82**										Daxenbichler et al., 1991
diffusum	**73, 82**										Daxenbichler et al., 1991
hieracifolium	**33, 35, 37, 72, 73, 82, 84, 94, 95**			**107**	**42**						Cole, 1976, Daxenbichler et al., 1980, Daxenbichler et al., 1991, Kjær and Schuster, 1970
linifolium	**73, 82**										Daxenbichler et al., 1991
ochroleucum	**82**					**1**					Danielak and Borkowski, 1969
odoratum	**73**			**107**		**1**[h]					Blua et al., 1988, Chisholm, 1973, Daxenbichler et al., 1991, Kjær and Gmelin, 1957
perofskianum	**64, 76, 82, 84, 95**										Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991
repandum	**73, 84, 95**										Cole, 1976, Daxenbichler, 1991
rhaeticum	**32, 34, 36, 37, 67, 72, 78, 81, 88, 94**										Kjær and Schuster, 1973
rupestre	**82**					**1**					Chisholm, 1973, Danielak and Borkowski, 1969, Kjær and Gmelin, 1957
sisymbrioides	**73, 82**						**23**				Daxenbichler et al., 1991
sylvestre	**73, 95**										Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Euzomodendron											
bourgaeanum	**64, 95**										Daxenbichler et al., 1991
Farsetia											
aegyptia				**107**							Gil and MacLeod, 1980b
clypeata	**84**										Gil and MacLeod, 1980b
hamiltonii	**64, 72, 73, 84, 94, 95**		**53, 61**	**12, 107**			**11, 105**				Daxenbichler et al., 1991
jacquemontii	**64, 84**		**56**	**12**			**23**				Daxenbichler et al., 1991
ramosissima	**84**			**12**							Gil and MacLeod, 1980b
Fibigia											
macrocarpa	**64**			**12, 24**							Daxenbichler et al., 1991
Goldbachia											
laevigata	**82**						**23**				Daxenbichler et al., 1991
Heliophila											
amplexicaulis	**79, 80**						**23**				Daxenbichler et al., 1991
longifolia							**15, 23**				Daxenbichler et al., 1991
Hesperis											
matronalis	**67, 72, 84, 94, 95**		**61**	**12, 24**			**14, 23, 105**			**119, 120**	Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Larsen et al., 1992
pendula	**67**						**23**				Daxenbichler et al., 1991
Hirschfeldia											
incana	**95**			**12, 24, 101, 107**			**23, 105**				Cole, 1976, Daxenbichler, 1991, Lockwood and Belkhiri, 1991
Iberis											
amara	**73, 84, 95**			**12**							Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Kjær, 1959
crenata	**73, 82**			**12**							Daxenbichler et al., 1991
linifolia	**73, 82**										Daxenbichler et al., 1991
sempervirens	**84, 95**										Danielak and Borkowski, 1969
simplex	**73, 82**										Daxenbichler et al., 1991
umbellata	**73, 95**			**107**							Daxenbichler et al., 1991
Isatis											
aleppica				**12**							Cole, 1976
cappadocica ssp. *steveniana*				**12, 24**			**23**				Daxenbichler et al., 1991
costata				**12, 24**			**23**				Daxenbichler et al., 1991
djurdjura	**64**			**12, 24**					**28, 43, 47, 48**		Lockwood and Belkhiri, 1991

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Appendix A. (*continued*)

FAMILY	Sulfur-containing	Aliphatic: Straight-	Aliphatic: Branched-	Aliphatic: Olefins	Aliphatic: Alcohols	Aliphatic: Ketones	Aromatic: Aryl	Aromatic: Benzoate	Aromatic: Indole	Multiply glycosylated	Reference
Genus→species subspecies/variety	A	B	C	D	E	F	G	H	I	J	
glauca				**12**			**23**				Daxenbichler et al., 1991
iberica				**12**			**23**				Daxenbichler et al., 1991
tinctoria	**64, 95**			**12, 24**			**23**		**28, 43, 47, 48, 112**		Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Elliott and Stowe, 1971, Lockwood and Belkhiri, 1991
Leavenworthia											
alabamica					**57**		**23, 105**				Daxenbichler et al., 1991
torulosa				**101**			**105**				Daxenbichler et al., 1991
Lepidium											
apetalum							**11**				Daxenbichler et al., 1991
austrinum	**64**	**16**					**11, 23, 45**				Daxenbichler et al., 1991
bonariense							**46**				Kjær and Wagnières, 1971, Kjær et al., 1971
campestre							**23**				Danielak and Borkowski, 1969
densiflorum							**11, 23**				Daxenbichler et al., 1991
draba	**82**										Danielak and Borkowski, 1969
graminifolium	**95**						**11, 15, 22, 23, 44, 114**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Olsen and Sørensen, 1980a
heterophyllum	**95**										Cole, 1976
hyssopifolium							**11, 114**				Kjær and Wagnières, 1971, Kjær et al., 1971
iberis	**84**										Daxenbichler et al., 1991
lasiocarpum							**11, 23**				Daxenbichler et al., 1991
latifolium	**95**										Cole, 1976
montanum v. *angustifolium*			**56, 61**				**11, 23, 45**				Daxenbichler et al., 1991
perfoliatum	**64, 84, 94, 95**						**11, 45**				Daxenbichler et al., 1991
pinnatifidum							**11**				Daxenbichler et al., 1991
ruderale							**11**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY	Sulfur-containing	Aliphatic: Straight-	Aliphatic: Branched-	Aliphatic: Olefins	Aliphatic: Alcohols	Aliphatic: Ketones	Aromatic: Aryl	Aromatic: Benzoate	Aromatic: Indole	Multiply glycosylated	Reference
Genus→species subspecies/variety	A	B	C	D	E	F	G	H	I	J	
sativum	**69**			**24, 107**			**11, 23, 105**		**43, 47**		Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Gil and MacLeod, 1980a, Gil and MacLeod, 1980c, Gil and MacLeod, 1980d, Gmelin and Virtanen, 1959a, Schraudolf, 1965
sordidum							**114**				Kjær and Wagnières, 1971
subulatum				**12, 101**			**11, 23, 45**				Daxenbichler et al., 1991
thurberi							**11, 45**				Daxenbichler et al., 1991
vesicarium							**11, 15, 22, 45**				Daxenbichler et al., 1991
virginicum							**11, 23**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991
v. *medium*							**11, 23**				Daxenbichler et al., 1991
Lesquerella											
angustifolia	**64, 84**										Daxenbichler et al., 1991
argyraea ssp. *argyraea*	**73**						**23**				Daxenbichler et al., 1991
auriculata	**67, 88**										Daxenbichler et al., 1991
densipila	**67, 88**										Daxenbichler et al., 1991
douglasii	**82**										Daxenbichler et al., 1991
engelmannii	**64, 76, 84**										Daxenbichler et al., 1991
fendleri	**73**										Daxenbichler et al., 1991
globosa	**73, 84, 95**										Daxenbichler et al., 1991
gordonii	**73**										Daxenbichler et al., 1991
gracilis ssp. *gracilis*	**73, 95**						**23**				Daxenbichler et al., 1991
lasiocarpa ssp. *lasiocarpa*	**67, 88**										Daxenbichler et al., 1991
lescurii	**67, 88**										Daxenbichler et al., 1991
lindheimeri	**73, 95**										Daxenbichler et al., 1991
ludoviciana	**64, 72, 73**		**56**	**107**							Daxenbichler et al., 1991
lyrata	**67, 88**										Daxenbichler et al., 1991
mirandiana	**72**										Daxenbichler et al., 1991
ovalifolia ssp. *ovalifolia*	**67**						**23**				Daxenbichler et al., 1991
perforata	**67, 88**										Daxenbichler et al., 1991
pinetorum	**73, 88**										Daxenbichler et al., 1991
stonensis	**67, 88**										Daxenbichler et al., 1991
tenella	**73**										Daxenbichler et al., 1991
Lobularia											
maritima	**67, 88**			**12, 101, 107**			**11, 105**				Cole, 1976, Daxenbichler et al., 1991, Hasapis et al., 1981

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Lunaria											
annua	**67, 94**		**54, 56, 61**								Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Kjær, 1959
rediviva	**64, 66, 67, 69, 72, 84, 94, 95**		**56, 61**	**100, 107**							Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991
Malcolmia											
africana	**64, 67, 72, 73, 82**			**100**			**23**				Daxenbichler et al., 1991
cabulica	**82**										Daxenbichler et al., 1991
littorea	**82, 95**						**23**				Daxenbichler et al., 1991
maritima	**82**				**42**			**10**			Danielak and Borkowski, 1969, Daxenbichler et al., 1980, Daxenbichler et al., 1991
Matthiola											
annua	**63**										Gmelin and Kjær, 1970a
bicornis	**63, 67, 72, 82, 83, 84**										Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Kjær, 1959
fruticulosa	**63, 83, 84**		**56**				**105**				Daxenbichler et al., 1991, Gmelin and Kjær, 1970a
incana	**63, 95**						**105**				Cole, 1976, Gmelin and Kjær, 1970a
parviflora	**63, 83**						**23, 105**				Daxenbichler et al., 1991
sinuata	**63, 83**						**15**				Cole, 1976, Daxenbichler et al., 1991
Moricandia											
arvensis	**95**			**12, 24, 107**			**11, 105**	**6**	**28, 43, 47, 48**		Daxenbichler et al., 1991, Lockwood and Belkhiri, 1991
baetica				**24**			**23**				Daxenbichler et al., 1991
foetida				**24**							Daxenbichler et al., 1991
moricandioides				**24**							Daxenbichler et al., 1991
Nasturtiopsis											
arabica					**29, 31**						Daxenbichler et al., 1991
Nasturtium											
officinale	**66, 69, 87, 89, 92**						**11, 23, 105**				Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Lockwood and Belkhiri, 1991
Nerisyrenia											
camporum	**66, 67, 72, 87, 88, 94**										Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Neslia											
paniculata	**65, 68, 73, 74, 82**										Daxenbichler et al., 1991, Kjær and Schuster, 1972b
Notoceras											
bicorne	**67, 88, 94**			**12, 24**							Daxenbichler et al., 1991
Peltaria											
alliaceae				**12, 24, 60, 107**							Daxenbichler et al., 1991
angustifolia				**12**			**23**				Daxenbichler et al., 1991
Physaria											
floribunda	**64, 67, 72**		**56**								Daxenbichler et al., 1991
Raphanus											
raphanistrum	**83, 95**			**107**							Cole, 1976
sativus	**63, 64, 75, 83, 95**	**20, 102**	**56, 58, 59**	**19, 107**			**23, 105**			**111**	Ahmed et al., 1972b, Cole, 1976, Daxenbichler et al., 1991, Fenwick et al., 1983, Kjær et al., 1978
var. *caudatus*	**63, 64, 83**		**56**	**107**			**23**				Daxenbichler et al., 1991
Rapistrum											
rugosum	**73, 82, 83, 95**			**12**			**23, 105**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Lockwood and Belkhiri, 1991
ssp. *orientale*	**82**			**12**			**23**				Daxenbichler et al., 1991
Reboudia											
pinnata				**12**			**11**				Daxenbichler et al., 1991
Rhynchosinapis											
hispida				**12, 24**			**23**				Daxenbichler et al., 1991
longirostra				**24**			**23**				Daxenbichler et al., 1991
monensis							**11**				Cole, 1976
Rorippa											
dubia	**68, 69**						**23**				Daxenbichler et al., 1991
globosa	**66, 68, 69**						**23**				Daxenbichler et al., 1991
hilariana	**69**			**12, 107**			**23**				Daxenbichler et al., 1991
indica	**69, 92**						**105**				Daxenbichler et al., 1991
islandica	**66, 69**			**12**			**23**				Daxenbichler et al., 1991
nasturtium-aquat.							**105**				Cole, 1976
sylvestris	**69, 92**										Daxenbichler et al., 1991
Rytidocarpus											
moricandioides				**24**			**23**				Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	 Branched- C	 Olefins D	 Alcohols E	 Ketones F	Aromatic Aryl G	 Benzoate H	 Indole I	Multiply glycosylated J	Reference
Savignya											
parvifloria							**23**				Daxenbichler et al., 1991
Schimpera											
arabica				**12**			**23**				Daxenbichler et al., 1991
Schouwia											
purpurea				**12, 24, 101, 107**					**28**		Ghaout et al., 1991
Selenia											
aurea							**11, 40, 105**				Daxenbichler et al., 1991
grandis				**24**			**23, 105**				Daxenbichler et al., 1991
Sibara											
virginica	**66, 69, 87, 92**						**23, 40, 105**				Daxenbichler et al., 1991, Gmelin et al., 1970
Sinapis											
alba	**84, 95**		**61**	**12, 24, 101**			**11, 23, 105**		**43, 47**		Ahmed et al., 1972b, Cole, 1976, Danielak and Borkowski, 1969, Fenwick et al., 1983, Lockwood and Belkhiri, 1991, Olsen and Sørensen, 1980a, Schraudolf, 1965
arvensis	**73, 79, 80, 82, 95**			**12, 24, 107**			**23, 105**				Cole, 1976, Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Hasapis et al., 1981, Lockwood and Belkhiri, 1991
Sisymbrella											
aspera	**66, 69**			**12**			**23**				Daxenbichler et al., 1991
Sisymbrium											
alliaria				**107**							Danielak and Borkowski, 1969
altissimum	**95**		**56, 61**	**12, 24, 38**	**57**		**23, 105**				Cole, 1976, Daxenbichler et al., 1991
austriacum	**95**			**38**	**57**			**7, 9**			Ahmed et al., 1972b, Cole, 1976, Danielak and Borkowski, 1969, Kjær and Christensen, 1962
ssp. *contortum*					**57**			**9**			Daxenbichler et al., 1991
confertum					**17, 57**		**23**				Daxenbichler et al., 1991
crassifolium					**31, 57**		**23**				Daxenbichler et al., 1991
erysimoides			**56, 61**								Daxenbichler et al., 1991
gariepinum				**24**			**23**				Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
irio			**56, 61**	**24, 38**							Cole, 1976, Daxenbichler et al., 1991
loesilii				**38, 107**	**17, 57**						Cole, 1976, Daxenbichler et al., 1991
officinale			**56, 61**	**38, 107**			**23**				Cole, 1976, Daxenbichler et al., 1991
orientale	**95**			**12, 24, 38**			**11, 23, 105**				Cole, 1976, Daxenbichler et al., 1991, Hasapis et al., 1981
polyceratium					**17, 31, 57**						Daxenbichler et al., 1991
sophia				**107**							Danielak and Borkowski, 1969
strictissimum	**95**		**56, 61**	**38**							Cole, 1976
Stanleya											
pinnata				**12, 24**							Daxenbichler et al., 1991
Sterigmostemum											
incanum	**73, 95**										Daxenbichler et al., 1991
Streptanthella											
longirostris	**64**			**12**			**23**				Daxenbichler et al., 1991
Streptanthus											
arizonicus				**12**							Daxenbichler et al., 1991
Synthlipsis											
greggii	**94**										Daxenbichler et al., 1991
Tchihatchewia											
isatidea	**69**						**23**				Daxenbichler et al., 1991
Teesdalia											
nudicaulis	**73**				**29, 42**		**105**				Cole, 1976, Daxenbichler et al., 1991
Thelypodium											
ambiguum			**61**	**107**							Daxenbichler et al., 1991
brachycarpum	**72**		**61**	**12, 107**			**11**				Al-Shehbaz, 1973
crispum	**64, 72, 76, 84, 94**		**61**	**12, 101, 107**			**11**				Al-Shehbaz, 1973
eucosmum	**84**		**56, 61**	**12, 101, 107**			**11**				Al-Shehbaz, 1973
flexuosum				**24**							Al-Shehbaz, 1973
howellii ssp. *spectabilis*	**95**		**56**	**12, 101**			**11**				Al-Shehbaz, 1973
integrifolium ssp. *affine*				**24**							Al-Shehbaz, 1973
ssp. *complanatum*				**24**							Al-Shehbaz, 1973
ssp. *gracilipes*				**24**							Al-Shehbaz, 1973
ssp. *integrifolium*				**24**							Al-Shehbaz, 1973
ssp. *longicarpum*				**24**							Al-Shehbaz, 1973
laciniatum	**72, 84, 94**		**56, 61, 62**	**12, 101, 107**			**11, 105**				Al-Shehbaz, 1973
laxiflorum	**84**		**56, 61**	**12, 101, 107**			**11, 105**				Al-Shehbaz, 1973

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
milleflorum			**56, 61, 62**				**11, 105**				Al-Shehbaz, 1973
paniculatum			**56, 61**	**12, 101, 107**							Al-Shehbaz, 1973
repandum	**72**			**24**							Al-Shehbaz, 1973
rollinsii			**61**	**12, 107**			**11**				Al-Shehbaz, 1973
sagittatum											
ssp. *ovalifolium*			**56, 61**		**30, 57**		**11, 105**				Al-Shehbaz, 1973
ssp. *sagittatum*			**56, 61**		**30, 57**						Al-Shehbaz, 1973
stenopetalum			**56, 61**								Al-Shehbaz, 1973
texanum				**12**							Daxenbichler et al., 1991
wrightii	**72**			**12, 24**			**11**				Al-Shehbaz, 1973
Thlaspi											
alpestre					**31, 42**		**23**				Daxenbichler et al., 1991
arvense	**73**			**107**			**11**				Danielak and Borkowski, 1969, Daxenbichler et al., 1991, Rodmann and Chew, 1980
avalanum					**31**		**23**				Daxenbichler et al., 1991
perfoliatum							**23**				Daxenbichler et al., 1991
Thysanocarpus											
montanum			**56, 61**								Rodman and Chew, 1980
radians	**73**						**23, 105**				Daxenbichler et al., 1991
Turritis											
glabra	**95**			**107**							Cole, 1976
Wasabi											
japonica	**67**	**51**	**56, 58, 61**	**12, 18, 19, 101, 107**			**105**				Fenwick et al., 1983, Fuke et al., 1997
Zilla											
spinosa				**24**			**23**				Daxenbichler et al., 1991
BRETSCHNEIDERACEAE											Rodman, 1991a
Bretschneidera											
sinensis					**31**		**14**				Boufford et al., 1989
CAPPARACEAE											Rodman, 1991a
Boscia											
fischeri		**51**									Kjær and Thomsen, 1963b
senegalensis		**51**									Seck et al., 1993
Capparis											
angulata						**96**					Kjær and Thomsen, 1963b
baducca		**51**									Gremlin and Kjær, 1970d
cartilagenea		**108**[d]	**54, 56**								Fenwick et al., 1983, Kjær and Thomsen, 1963b

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
ferruginea						**97**					Brown and Stuart, 1968
flexuosa		**13, 51**	**56**	**12, 24**	**25, 26**	**97**	**11**				Ahmed et al., 1972b, Brown and Stuart, 1968, Gaind et al., 1975, Gmelin and Kjær, 1970d, Kjær and Schuster, 1971
galeata			**54, 56**								Kjær and Thomsen, 1963b
grandis					**113**						Gaind et al., 1975
hastata		**51**									Gmelin and Kjær, 1970d
inermis		**13, 51**		**12, 24**	**25**						Kjær and Thomsen, 1963b
linearis				**52**							Brown and Stuart, 1968, Gaind et al., 1975, Kjær and Wagnières, 1965
masaikai					**27**						Hu et al., 1989
Mitchellii			**54, 56**								Kjær and Thomsen, 1963b
nobilis		**51**									Kjær and Thomsen, 1963b
odoratissima		**51**									Gremlin and Kjær, 1970d
ovalifolia		**51**									Kjær and Thomsen, 1963b
ovata											
var. *deserti*									**47**		Ahmed et al., 1972a
var. *palaestina*	**73**	**51**		**107**	**29**	**97**			**43**		Ahmed et al., 1972a, Ahmed et al., 1972b
quiniflora		**51**									Kjær and Thomsen, 1963b
rupestris		**51**									Kjær and Thomsen, 1963b
salicifolia						**97, 98**					Brown and Stuart, 1968, Kjær and Thomsen, 1962b, Kjær and Thomsen, 1963a, Kjær and Thomsen, 1963b
spinosa	**73**	**51**		**107**	**29**	**97**	**23**		**28, 43, 47, 48**		Daxenbichler et al., 1991, Kjær and Thomsen, 1962a, Kjær and Thomsen, 1963b, Shraudolf, 1989
var. *aegyptia*	**73**	**51**		**107**	**29**	**97**					Ahmed et al., 1972a, Ahmed et al., 1972b
var. *deserti*	**73**	**51**		**107**	**27**	**96**			**47**		Ahmed et al., 1972a, Ahmed et al., 1972b
Tuereckheimii		**51**									Kjær and Thomsen, 1963b
Tweediana		**51**									Kjær and Thomsen, 1963b

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
Cleome											
anomala					**29**		**23**		**43, 47**		Daxenbichler et al., 1991, Schraudolf, 1965
arabica		**51**			**29**		**23**				Daxenbichler et al., 1991, Kjær and Thomsen, 1963b
arborea		**51**			**29**				**43, 47**		Ahmed et al., 1972b, Kjær and Thomsen, 1963b, Schraudolf, 1965
diandra					**17, 29, 31**						Daxenbichler et al., 1991
gigantea		**51**			**29**						Kjær and Thomsen, 1963b
graveolens		**51**			**29**				**43, 47**		Ahmed et al., 1972b, Kjær and Thomsen, 1963b, Schraudolf, 1965
gynandra[e]											Daxenbichler et al., 1991
integrifolia		**51**			**29**						Kjær and Thomsen, 1963b
isomeris							**23**				Daxenbichler et al., 1991
machycarpa		**51**			**29**						Kjær and Thomsen, 1963b
monophylla		**51**							**43, 47**		Ahmed et al., 1972b, Kjær and Thomsen, 1963b, Schraudolf, 1965
ornithopodioides		**51**			**29**						Kjær and Thomsen, 1963b
papillosa							**23**				Daxenbichler et al., 1991
pilosa		**51**			**29**						Daxenbichler et al., 1991
pungens		**51**									Kjær and Thomsen, 1963b
rosea		**51**									Kjær and Thomsen, 1963b
serrulata		**51**			**29**						Daxenbichler et al., 1991
sonorae							**23**				Daxenbichler et al., 1991
speciosissima		**51**			**29**						Kjær and Thomsen, 1963b
spinosa		**51**			**29**						Kjær and Thomsen, 1962a, Kjær and Thomsen, 1963b
tenuis					**29**		**23**				Daxenbichler et al., 1991
trachycarpa[e]											Daxenbichler et al., 1991
trachysperma		**51**			**29**				**43, 47**		Ahmed et al., 1972b, Kjær and Thomsen, 1963b, Schraudolf, 1965
viscosa		**51**			**29**		**23**				Hasapis et al., 1981, Kjær and Thomsen, 1963b, Rukmini and Deosthale, 1979
Courbonia											
virgata							**23**				Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
Crataeva											
roxburghii		**51**									Kjær and Thomsen, 1963b
tapia		**51**			**29**		**23**				Daxenbichler et al., 1991, Kjær and Thomsen, 1963b
Dhofaria											
macleishii		**51**									Lüning et al., 1992
Dipterygium											
glaucum		**51**									Lüning et al., 1992
Gynandropsis											
gynandra		**51**							**43, 47**		Ahmed et al., 1972b, Hasapis et al., 1981, Kjær and Thomsen, 1963b, Schraudolf, 1965
pentaphylla		**51**							**43, 47**		Kjær and Thomsen, 1963b, Schraudolf, 1965
speciosa		**51**									Kjær and Thomsen, 1963b
Isomeris											
arborea		**51**									Blua and Hanscom, 1986, Blua et al., 1988
Maerua											
aethiopica		**51**									Kjær and Thomsen, 1963b
hoehnelii		**51**			**29**						Kjær and Thomsen, 1963b
ovalifolia		**51**			**29**		**23**				Daxenbichler et al., 1991
pubescens[e]											Kjær and Thomsen, 1963b
Polanisia											
dodecandra					**29**		**23**				Daxenbichler et al., 1991
ssp. *tracysperma*					**29**						Daxenbichler et al., 1991
viscosa					**29**		**23**				Daxenbichler et al., 1991
Puccionia											
macradenia		**51**									Lüning et al., 1992
Ritchiea											
albersii		**51**									Kjær and Thomsen, 1963b
Thylachium											
africanum		**51**									Kjær and Thomsen, 1963b
thomasii		**51**									Kjær and Thomsen, 1963b
Wislizenia											
refracta							**23**				Daxenbichler et al., 1991

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
CARICACEAE											Rodman, 1991a
Jarilla											
chocola							**11**				Daxenbichler et al., 1991, Gmelin and Kjær, 1970b
Carica											
cauliflora							**11**				Gmelin and Kjær, 1970b
chilensis							**11**				Gmelin and Kjær, 1970b
papaya							**11**				Blua et al., 1988, Chan et al., 1978, Flath and Forrey, 1977, Gmelin and Kjær, 1970c, MacLeod and Pieris, 1983, Tang, 1973, Tang, 1974
pennata							**11**				Gmelin and Kjær, 1970b
quercifolia							**11**				Gmelin and Kjær, 1970b
Cycliomorpha											
solmsii							**11**				Tang and Hamilton, 1976
EUPHORBIACEAE											
Drypetes (syn. *Putranjiva*)											
roxburghii			**56**		**29**						Ahmed et al., 1972b, Benn and Meakin, 1965, Kjær and Thomsen, 1963b
gossweileri					**26**						Ettlinger and Kjær, 1968
GYROSTEMONACEAE											Rodman, 1991a
Codonocarpus											
continifolius			**61**								Kjær and Malver, 1979
Tersonia											
brevipes			**61, 62**								Kjær and Malver, 1979
LIMNANTHACEAE											Rodman, 1991a
Limnanthes alba var. *alba*					**31**						Boufford et al., 1989, Daxenbichler and Van Etten, 1974
douglasii							**45**				Ettlinger and Lundeen, 1956b
MORINGACEAE											Rodman, 1991a
Moringa oleifera (syn. *aptera, pterygosperma*)			**56, 61, 62**		**31**		**11, 23**			**3, 110**	Daxenbichler et al., 1991, Kjær et al., 1979, Sørensen, 1970
peregrina			**61, 62**				**11**			**3, 110**	Kjær et al., 1979

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Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
PENTADIPLANDRACEAE											Rodman, 1991a
Pentadiplandra											
brazzeana							**11, 49**				El Migirab et al., 1977, Fenwick et al., 1983
PHYTOLACCACEAE											
Codonocarpus											
cotinifolia			**61**		**29**		**23**				Daxenbichler et al., 1991
Phytolacca											
americana							**23**				Daxenbichler et al., 1991
dioica							**11, 22, 23**				Daxenbichler et al., 1991
PITTOSPORACEAE											Rodman, 1991a
Bursaria spinosa var. *incana*							**23**				Daxenbichler et al., 1991
RESEDACEAE											Rodman, 1991a
Caylusea											
abyssinica							**23, 40**				Daxenbichler et al., 1991
Reseda											
alba					**31**		**23, 105**		**43**		Boufford et al., 1989, Daxenbichler et al., 1991, Olsen and Sørensen, 1979, Schraudolf, 1965, Underhill and Kirkland, 1972b
complicata									**43, 47**		Schraudolf, 1965
crystallina									**43**		Schraudolf, 1965
lutea							**11, 23**		**43**		Cole, 1976, Daxenbichler et al., 1991, Schraudolf, 1965
luteola							**23, 40, 105**		**43**		Björkqvist and Hase, 1988, Cole, 1976, Daxenbichler et al., 1991, Ettlinger and Kjær, 1968, Kirkland et al., 1971, Kjær and Gmelin, 1958
media							**11, 22, 105**		**43**		Olsen and Sørensen, 1980b
odorata							**21**		**43**	**109**	Ahmed et al., 1972b, Olsen and Sørensen, 1979
phyteuma							**23**		**43**		Daxenbichler et al., 1991, Schraudolf, 1965
stricta							**23, 105**				Daxenbichler et al., 1991
Sesamoides canescens var. *canescens*							**40, 105**			**4**	Olsen et al., 1981
pygmaea							**105**				Olsen et al., 1981

(*continued on next page*)

Appendix A. (*continued*)

FAMILY Genus→species subspecies/variety	Sulfur-containing A	Aliphatic Straight- B	Branched- C	Olefins D	Alcohols E	Ketones F	Aromatic Aryl G	Benzoate H	Indole I	Multiply glycosylated J	Reference
SALVADORACEAE											Rodman, 1991a
Salvadora											
persica							**11, 23**				Daxenbichler et al., 1991
pendula			**56**								Ahmed et al., 1972b
TOVARIACEAE											Rodman, 1991a
Tovaria											
pendula			**56**				**11**		**2, 43, 47**		Chistensen et al., 1982, Schraudolf, 1965, Schraudolf and Bauerle, 1986
TROPAEOLACEAE											Rodman, 1991a
Tropaeolum											
boliviense			**56, 61**				**11**				Kjær et al., 1978
cochabambae			**56, 61, 62**				**11, 46**				Kjær et al., 1978
hjertingii							**11**				Kjær et al., 1978
longiflorum			**56, 61**				**11**				Kjær et al., 1978
majus							**11, 23, 46**				Daxenbichler et al., 1991, Kjær et al., 1978
minus							**11**				Kjær et al., 1978
peregrinum		**16**	**56, 61, 62**		**31**		**11, 23, 46**				Daxenbichler et al., 1991, Ettlinger and Kjær, 1968, Kjær et al., 1978
seemannii			**56, 61**				**11**				Kjær et al., 1978
tuberosum			**56, 61**				**11**				Kjær et al., 1978

[a] Descriptions of many of the compounds specifically referenced herein may also be found in Fenwick et al. (1983), Ettlinger and Kjær (1968) and Kjær and Olesen Larsen (1973, 1976). The taxonomic designations provided by the original authors have not been altered to reflect changes in the taxonomic literature that have occurred since initial discovery, nor have they been adjusted to concur with the International Code of Botanical Nomenclature (1993), since this would make it too confusing for those interested in checking the original references.

[b] Danielak and Borkowski (1969) provide numeous tentative identifications, not all of which are included in this summary. They report finding glucosinolates in a total of 151 cruciferous species, but provide only R_f values (thin-layer chromatography) for many of these.

[c] Schraudolf et al. (1971) describe an hydroxyindole of ambiguous identity.

[d] The designation of *n*-propyl glucosinolate [**108**] by Fenwick et al. (1983) appears to be incorrect since the original reference (Kjær and Thomsen, 1963b) indicates a structure consistent with the name isopropyl glucosinolate [**56**].

[e] Total glucosinolates reported, but no specific compounds identified.

[f] "MeSOcOx" and "MeSOOcOx" reported, but structure not clarified.

[g] "iso-Heptyl" [**115**] and "iso-hexyl" [**116**] reported, but structure not clarified (see Table 1).

[h] 4-Acetylbutyl glucosinolate (glucoerypestrin or 3-methoxycarbonylpropyl glucosinolate, [**1**]) reported in *E. odoratum* or *E. rupestre* — confusion exists about identity of source (see Chisholm, 1973).

[i] The level of analytical resolution provided in most of the studies cited herein does not permit discrimination between progoitrin (**24a**) and epiprogoitrin (**24b**). Clear exceptions are: (a) the well documented predominance of **24b** in *Crambe abyssinica* and *C. maratima*; (b) the well documented presence of **24a** in many *Brassica* sp., especially rapeseed (*B. campestris* and *B. napus*); (c) a report of the presence of both **24a** and **24b** in numerous *Coincya* species (Vioque et al., 1994). With these three exceptions, we have therefore not distinguished between enantiomorphs and use the designation "**24**".

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Purification and characterization of a lectin from the mushroom *Mycoleptodonoides aitchisonii*

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Abstract

A lectin was isolated from the mushroom *Mycoleptodonoides aitchisonii* by means of affinity chromatography on bovine submaxillary mucin (BSM)-Toyopearl and gel filtration on Superose 12 HR10/30 using a FPLC system. This lectin is composed of four identical 16 kDa subunits and the molecular mass of the intact lectin was estimated to be 64 kDa by gel filtration. In a hemagglutination inhibition assay, it exhibited strong sugar-binding specificity towards asialo-BSM among the mono- or oligo-saccharides and glycoproteins tested. The binding specificity of the lectin was also examined by surface plasmon resonance analysis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Mycoleptodonoides aitchisonii*; Climacodontaceae; Mushrooms; Fruiting body; Lectin; Surface plasmon resonance

1. Introduction

Mushrooms can be defined as macrofungi with distinctive fruiting bodies which are either epigenous or hypogenous and are sufficiently conspicuous in size to the naked eye to be hand-picked (Chang and Miles, 1992; Wang et al., 1998). Many lectins have been isolated from mushrooms (Kawagishi, 1995; Wang et al., 1998) and some macrofungi produce them not only at their fruiting body-stages but also at the mycelium-stages (Kino et al., 1989; Tanaka et al., 1989; Guillot et al., 1991; Kawagishi et al., 1997). However, the roles of lectins in fungi are still unknown. In the course of our continuing screening for mushroom lectins with novel carbohydrate specificity, we found strong lectin activity in the extract of the edible mushroom *Mycoleptodonoides aitchisonii*. This paper reports the isolation and characterization of this lectin from this source.

2. Results and discussion

The isolation procedure employed is summarized in Table 1. Thus, initially a saline extract of the fruiting bodies of *M. aitchisonii* was adjusted to 80% saturation with ammonium sulfate. Since the resulting extract exhibited no remarkable binding specificity to any monosaccharides but showed specificity against asialo-BSM and BSM in an hemagglutination inhibition assay, BSM-Toyopearl was considered as an affinity support. Accordingly, after centrifugation, the precipitate obtained by ammonium sulfate precipitation was applied to a BSM-Toyopearl column. All the lectin activity was adsorbed onto the column but could be eluted with 1 M NaCl. The lectin-containing fraction was then further purified by FPLC using Superose 12, to give the purified *M. aitchisonii* lectin (MAL).

MAL displayed a single band on SDS-PAGE regardless of the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol (Fig. 1). Gel filtration of the lectin on Superose 12 gave a symmetrical single peak and the molecular weight of the intact protein was estimated to be 64,000 by gel filtration (Fig. 2). These results suggested the lectin to be composed of four identical subunits without any S–S linkages.

Amino acid analysis using acid hydrolysis revealed a high content of acidic amino acids and Gly, a low

Abbreviations: MAL, *Mycoleptodonoides aitchisonii* lectin; PBS, 10 mM phosphate-buffered saline (pH 7.4); HBS, 10 mM HEPES buffered saline (pH 7.4); BSM, bovine submaxillary mucin; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ABEE, *p*-aminobenzoic ethyl ether; TFA, trifluoroacetic acid; NP, *p*-nitrophenyl; All sugars are of D-configuration unless otherwise stated.

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content of Met and Phe, and no detectable Tyr (Table 2). Although as much as 3 nmol of the protein was applied to a protein sequencer, the N-terminal amino acid could not be determined, suggesting that its N-terminus was blocked. Isoelectric focusing gave a few bands in the pH zone 4.3–4.5 (Fig. 3) and the carbohydrate content of the protein was circa 4.9%. These results showed that the lectin was an acidic glycoprotein. The components of the sugar in the protein were determined as GlcNAc, Gal, Man, and Xyl (1.56, 4.89, 3.68, 1.00 in molar ratio)

Table 1
Purification of MAL (from 6 kg of the fruiting bodies)

Step	Total protein (mg)	Total agglutination activity (titer)[a]	Specific agglutination activity (titer/mg)	Recovery of activity (%)
1. 80% Ammonium sulfate precipitation	328.0	83,968	256	100
2. Eluate from BSM-Toyopearl	37.0	37,888	1024	45.1
3. Eluate from Superose 12	29.8	30,515	1024	36.3

[a] Measured with type A neuraminidase-treated blood cell.

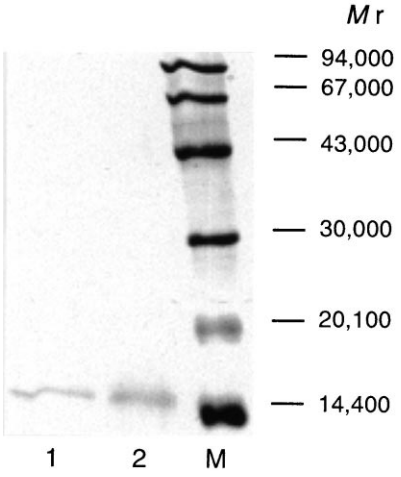


Fig. 1. SDS-PAGE of MAL. Lane 1 MAL (reduced with 2-mercaptoethanol); lane 2, MAL (non-reduced); lane M, marker.

Table 2
Amino acid composition of MAL (see Section 3)

Amino acid	Mol%	Amino acid	Mol%
Asx	12.1	Ile	3.4
Thr	5.9	Leu	4.4
Ser	7.3	Tyr	0.0
Glx	10.2	Phe	1.8
Gly	17.7	Lys	5.9
Ala	7.5	His	2.7
Val	6.2	Arg	3.5
Cys	3.8	Pro	7.3
Met	0.3	Trp	n.d.[a]

[a] Not determined.

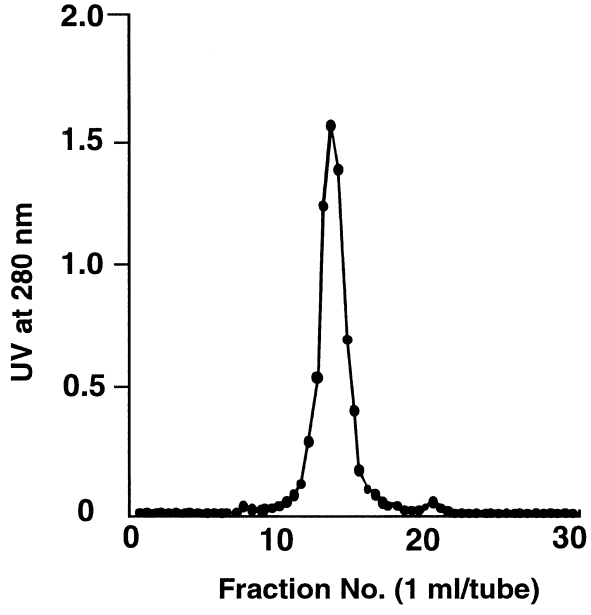


Fig. 2. Gel filtration of MAL on a Superose 12 HR10/30 column.

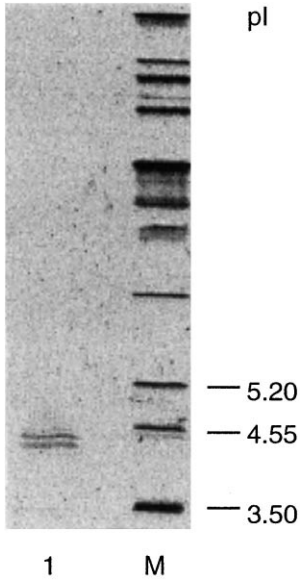


Fig. 3. Isoelectric focusing of MAL; Lane 1, MAL, lane M, marker.

by the analysis of their ABEE-derivatives. As far as we are aware, among mushroom lectins, there are only 2 lectins whose sugar compositions have been reported (Sueyoshi et al., 1985; Zhuang et al., 1996); ABA-I to IV from *Agaricus bisporus* had Fuc, Man, Gal and GlcNAc and APL from *Amanita pantherina* contained Glc, Man, Xyl and GlcNAc. Thus, the type of sugar chain observed for MAL has not yet been known as a sugar chain of mushroom lectins.

The lectin activity was stable between pH 4.0 and 9.0, and below 45°C, but was rapidly deactivated over 50°C (not shown). EDTA treatment, or addition of metal cations such as $CaCl_2$, $MgCl_2$ and $MnCl_2$, showed no effect on lectin activity, suggesting that MAL activity was not dependent on metal cations or that the lectin was already bound very tightly to metal cations.

While MAL did not agglutinate any type of human erythrocytes, it was bound tightly to sialidase-treated erythrocytes regardless of blood type; the titer of the lectin (10 μg/ml) was 256 for all types of the enzyme-treated erythrocytes.

The sugar-binding specificity of MAL was assayed by the hemagglutination inhibition method (Table 3), but it did not bind to any of the mono- or oligo-saccharides tested. Among the glycoproteins examined, asialo-BSM was the strongest inhibitor. BSM also inhibited hemagglutination by the lectin. Asialofetuin was bound to the lectin at a much higher concentration than asialo-BSM and BSM. Transferrin, α_1-acid glycoprotein, and fetuin did not inhibit the hemagglutination by MAL. In addition, asialo-BSM was treated with trypsin, pronase, glycopeptidase A, and hydrazine. The resulting reaction mixtures showed a much weaker inhibitory activity toward MAL-mediated hemagglutination at the same concentrations than intact asialo-BSM (data not shown). From this result, we could not therefore elucidate the detailed carbohydrate structure recognized by MAL. However both this and the hemagglutination assay indicated that MAL mainly recognized asialo-mucin type sugar chains, and that more than one sugar chain (close to each other in the glycoprotein) were necessary for the binding of MAL to the glycoprotein. This speculation was confirmed by surface plasmon resonance analysis using BIAcore 2000. Four glycoproteins were immobilized on the sensor chip CM-5 by amine coupling. The sensorgrams and the kinetic data of the binding are summarized in Fig. 4 and Table 3, respectively. The binding of the lectin to all the glycoproteins fitted best a heterogeneous ligand model among the various models displayed in the evaluation software. Therefore two kinetic parameters were given for each glycoproteins. The association rates (Kon) of the binding between four immobilized glycoproteins and the lectin were similar to each other. However, asialo-BSM and BSM showed slower dissociation rates (Koff) than asialo-fetuin and fetuin. The results of degradation of asialo-BSM, the hemagglutination inhibition assay and the BIAcore analysis may thus be explained in terms of either multivalent binding of MAL to glycoproteins, or an allosteric effect of the lctin; indeed, MAL caused a conformational change after binding of one sugar binding site to the lectin to the first sugar chain of the glycoprotein, as well as being bound to the second different sugar chain. Accordingly, the binding was considered to be through a heterogeneous ligand. Interestingly, the degraded asialo-BSM did not bind to the lectin strongly.

Table 3
Comparison of kinetic parameters obtained from the interactions of immobilized glycoproteins with MAL by using BIAcore with the result of inhibition of MAL-mediated hemagglutination by the glycoproteins

Ligands (inhibitors)[a]	Kon (M^{-1} S^{-1})	Koff (S^{-1})	Rmax (RU)[b]	KD (M)	Hemagglutination inhibition activity (μg/ml)[c]
Asialo-BSM	1.74×10^3	6.33×10^{-4}	7.77×10	3.63×10^{-7}	0.015
	1.71×10^2	3.00×10^{-5}	2.42×10^2	1.76×10^{-7}	
BSM	5.09×10^5	2.32×10^{-4}	1.93×10	4.57×10^{-10}	0.49
	2.43×10^3	2.57×10^{-3}	3.33×10	1.06×10^{-6}	
Asialo-fetuin	5.68×10^3	4.58×10^{-2}	1.02×10^2	8.06×10^{-6}	62.5
	4.54×10^3	2.93×10^{-3}	9.92×10	6.45×10^{-7}	
Fetuin	6.77×10^3	3.44×10^{-3}	3.14×10	5.08×10^{-7}	n.i.[d]
	1.61×10^3	2.10×10^{-3}	4.43×10^2	1.31×10^{-6}	

[a] Glucosec, galactose, mannose, fucose, L-fucose, xylose, methyl α-glucoside, methyl β-glucoside, *N*-acetylglucosamine, *N*-acetylgalactosamine, methyl α-mannoside, methyl β-mannoside, L-arabinose, rhamnose, mannitol, *N*-acetylneuramic acid, lactose, *N*-acetyllactosamine, lactitol, lactulose, riburose, maltose, and sorbitol did not inhibit at all at concentrations up to 400 mM. Hyaluronic acid, α_1-acid glycoprotein,transferrin, fetuin, Galβ (1→3) GlcNAc, Galβ(1→3)GalNAc, Galβ (1→4) GlcNAc, GlcNAcβ (1→3) Gal-βpNP, GlcNAcβ (1→6) Gal-βpNP, Fucα (1→3) GlcNAc, Galβ (1→3) Galβ (1→4) GalNAcl-βpNP and Galβ (1→4)Galβ (1→4) GalNAcl-βpNP did not inhibit at all at concentrations up to 1 mg/ml.

[b] Maxumum binding capacity of MAL to the surface.

[c] Minimum inhibitor concentration required for inhibition of 4 hemagglutination doses of the lectin.

[d] n.i.; not inhibited at all at concentrations up to 1.0 mg/ml.

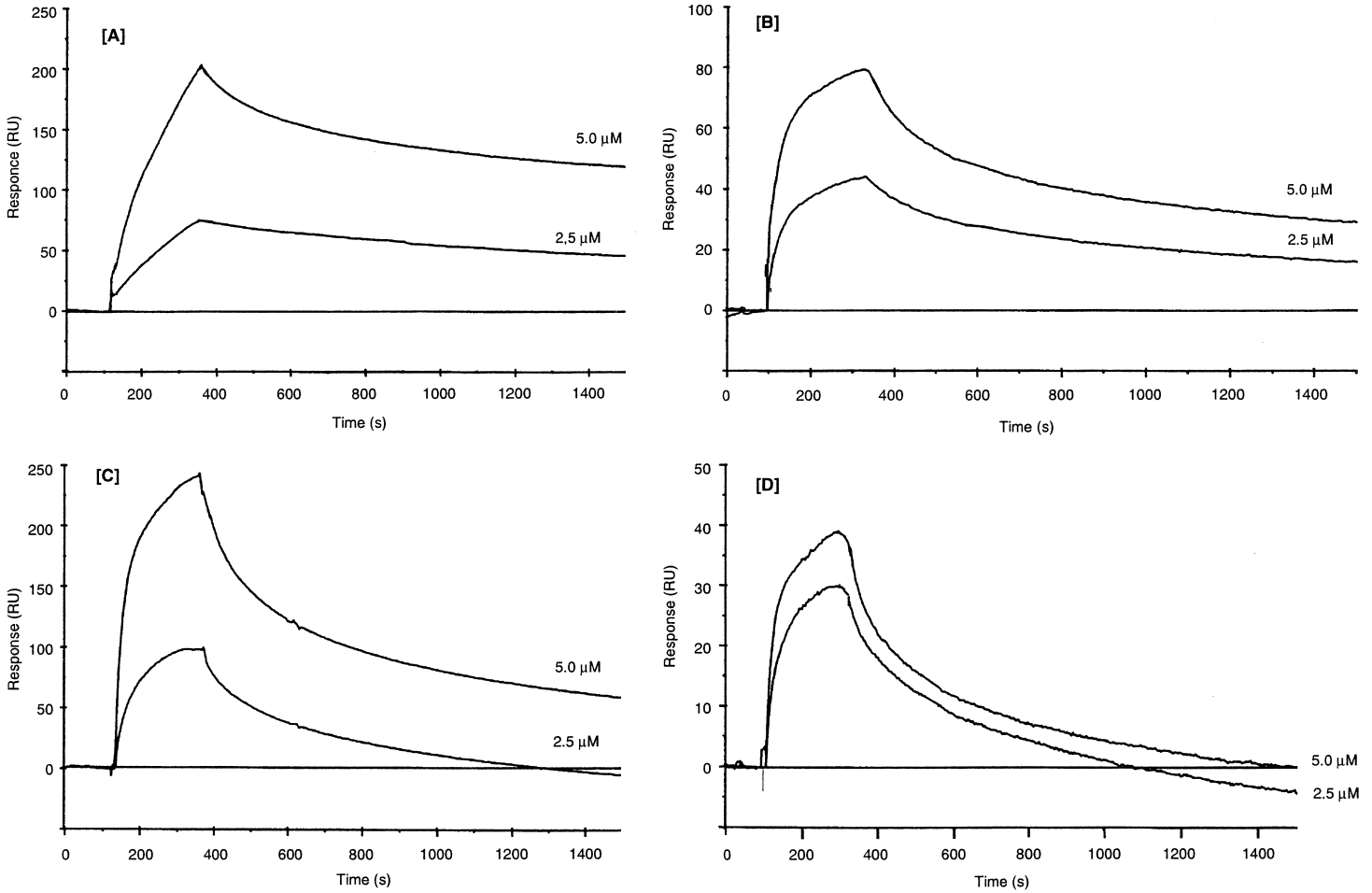


Fig. 4. Sensorgrams showing the interaction between MAL and immobilized glycoproteins. [A] asialo-BSM, [B] BSM, [C] asialo-fetuin, [D] fetuin.

To our knowledge, this is the first lectin isolated from a family Climacodontaceae.

3. Experimental

3.1. Materials

M. aitchisonii fruiting bodies were collected at Narusawa village, Yamanashi Prefecture, Japan, frozen upon collection and stored at −30°C. The fungus was identified by one of the authors (H.K.). Amino-Toyopearl 650M was purchased from Tosoh (Japan). FPLC system and Superose 12 HR10/30 column were products of Pharmacia (Sweden). BIAcore 2000 was a product of Biacore AB (Sweden). Galβ1→3GlcNAc, Galβ1→4GlcNAc, Galβ1→6GlcNAc, Galβ1→3GalNAc, Galβ1→4GalNAc, Galβ1→6GalNAc, and Fucβ1→3GlcNAc were synthesized by enzymatic methods (Hedbys, et al., 1989; Sakai et al., 1992; Usui et al., 1993). All other sugars for the hemagglutinating inhibition tests were products of Nacalai Tesque (Japan), Wako Pure Chemical (Japan) or Sigma (USA). All glycoproteins used were purchased from Sigma (USA). ABEE reagent and the Honenpak C_{18} reveresed-phase HPCC column were obtained from Honen Corporation (Japan).

3.2. Preparation of the affinity adsorbent

BSM was conjugated to Amino-Toyopearl 650M by following the instructions of manufacturer.

3.3. Isolation of MAL

All procedures were carried out at 4°C except for defrosting of the fruiting bodies. After defrosting, fruiting bodies of *M. aitchisonii* were homogenized with saline (1.0 l per 100 g fruiting bodies) in a blender and extracted with stirring overnight. The resulting suspension was filtered through the filtrate was centrifuged (10,000 × *g*) to remove insoluble residues. Solid ammonium sulfate was next added to the supernatant to 80% saturation. The resulting precipitate was collected by centrifugation (10,000 × *g*, 20 min), resuspended in distilled water, dialyzed against distilled water and lyophilized. The lyophilized extract was redissolved in 10 mM PBS (pH 7.4) and applied to a BSM-Toyopearl column equilibrated in the same buffer. After all unbound substances were removed by washing the column with buffer, the bound fraction was then desorbed by elution with 1 M NaCl. The eluates were concentrated by ultrafiltration, dialyzed against PBS, and further purified by gel filtration on Superose 12 HR10/30 using a FPLC system in PBS. The hemagglutinating fraction was dialyzed against distilled water and lyophilized, to give the purified (MAL) lectin.

3.4. Hemagglutination test

A 10% suspension of erythrocytes in PBS (10 ml) was treated with sialidase (10 ml, 1 U/ml) for 1 h at 37°C, then washed three times with PBS and suspended at a concentration of 3% in PBS. Agglutination of the erythrocytes by the lectin and inhibition of the agglutination by sugars and glycoproteins were carried out using microtiter U-plates. For the hemagglutination assay, erythrocyte-suspensions were added to lectin solutions of various concentrations in the wells, with reaction mixtures incubated for 1 h at room temperature. The titer was defined as the reciprocal of the end-point dilution causing hemagglutination. For the hemagglutination inhibition assay, lectin solutions of titer 4 were added to each sugar solutions of various concentrations, and the reaction mixtures were incubated for 1 h at room temperature. To the mixtures, erythrocyte-suspensions were added and further incubated for 1 h at room temperature. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of titer 4 of the lectin.

3.5. SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (Laemmli, 1970). Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°C. Gels were stained with Coomassie Brilliant Blue. The molecular weight standards (Pharmacia, Sweden) used were phosphorylase b (Mr 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and a-lactalbumin (14,400).

3.6. Gel filtration for molecular weight estimations

Gel filtration for measuring the molecular weights of native lectin was carried out on a Superose 12 HR10/30 column with a FPLC system. The molecular weight standards (Pharmacia, Sweden) used were ferritin (440,000), catalase (232,000), bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700).

3.7. Sugar analysis

Sugar contents were measured by the phenol-sulfuric acid method with reference to glucose.

Sugar compositions were determined as follows; purified lectin (200 μmg) was dissolved in 20 ml distilled water in a test tube to which 4 M TFA (20 μl) was added. The test tube was incubated at 100°C in a hot block bath. After 4 h, the tube was cooled to room temperature and the acid was removed by using a centrifugal concentrator at 35°C. The dried sample was

derivatized with ABEE in the presence of borane–pyridine complex at 80°C. After 1 h, the reaction mixture was cooled to room temperature. The distilled water (0.2 ml) and an equal volume of chloroform were added to the reaction mixture. After vigorous vortexing, it was centrifuged (6000 × *g*, 1 min). The upper aqueous layer was analyzed by reversed–phase HPLC under the condition as follows; column, Honenpak C_{18} (75 mm × 4.6 mm I.D.); solvent, A 0.02% TFA/CH3CN(90/10), B 0.02% TFA/CH_3CN (50:50); program, 0–45 min (B conc. 0%), 45–55 min (B conc. 100%), 55–70 min (B conc. 0%); flow rate, 1 ml/min; temp., 45°C; detection, absorbance at 305 nm. The monosaccharide and amino monosaccharide standards used were *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, glucose, mannose, xylose, and fucose.

3.8. Amino acid analysis

The lectin was hydrolyzed with 6 M HCl at 110°C for 24 h in a sealed evacuated tube and analyzed on a Hitachi L-8500A amino acid analyzer. The cysteine and methionine contents were determined by oxidation of the lectin with performic acid followed by hydrolysis under the same condition to that of the intact protein and analysis on the analyzer.

3.9. N-terminal amino acid analysis

Attempts to obtain the N-terminal amino acid of the protein used a PPSQ-10 Protein Peptide Sequencer (Shimadzu, Japan).

3.10. Thermostability

Samples in PBS were heated for 30 min at the temperatures indicated, cooled on ice, and titrated.

3.11. pH stability

The pH dependence of the lectin was measured by incubating the samples in the following buffers for 24 h at room temperature, 0.02 M sodium acetate buffer (pH 3.5–5.5), 0.02 M sodium phosphate buffer (pH 6.0–7.5), 0.02 M Tris–HCl Buffer (pH 8.0–9.0), and 0.02 M glycine–NaOH buffer (9.5–11.0).

3.12. Effect of metal cations on lectin activity

To examine metal cation requirements of the hemagglutination by the lectin, the sample (100 mg/ml) was incubated in 2 mM EDTA for 1 h at room temperature and titrated.

3.13. Surface plasmon resonance analysis

Real time detection of MAL binding to glycoproteins was recorded by using a BIAcore 2000. Glycoproteins were immobilized covalently via their primary amines to a carboxyl group within a dextran layer on the sensor chip CM-5 according to the manufacture's specifications and our previous reported method (Zeng et al. 1998). MAL at various concentrations in HBS were injected over immobilized glycoproteins at 20 μl/min. After injection of the protein, HBS was introduced onto the sensor surface to initiate dissociation. The data were analyzed by BIAevaluation 3.0 software (Biacore AB, Sweden).

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Tropane alkaloid production by shoot culture of *Duboisia myoporoides* R. Br.

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Abstract

This work demonstrates the presence of hyoscyamine and scopolamine at different stages of shoot regeneration from non-organogenic and organogenic calli. The 11-week-old non-organogenic calli contained 0.41±0.03 and 0.23±0.02 μg g^{-1} dry wt hyoscyamine and scopolamine respectively. However, no root meristem was found in the calli. The alkaloids were absent in 2-week-old organogenic calli. The shoot-buds induced on the non-organogenic and organogenic calli did not contain these alkaloids. Hyoscyamine and scopolamine contents of the 6-week-old non-rooted shoots regenerated from non-organogenic calli were 7.8±0.1 and 6.5±0.4 μg g^{-1} dry wt respectively and those in the 9-week-old non-rooted shoot regenerated from organogenic calli were 38.5±0.4 and 3.6±0.1 μg g^{-1} dry wt respectively. Hyoscyamine and scopolamine contents of the 4-week-old roots regenerated from non-organogenic and organogenic calli were higher than those in the non-rooted shoots. Since the presence of hyoscyamine and scopolamine in the non-rooted shoot depends on the stage of differentiation, manipulation of culture environment may improve hyoscyamine and scopolamine contents of the non-rooted shoots. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Duboisia myoporoides*; Solanaceae; Shoot culture; Tropane alkaloid; Hyoscyamine; Scopolamine

1. Introduction

The plant species *Duboisia myoporoides* R. Br. of the Solanaceae family is indigenous to Australia and has long been a major commercial source of the tropane alkaloids hyoscyamine and scopolamine (Griffin, 1985). Tropane alkaloids are extensively used in medicines for their mydriatic, analgesic and antispasmodic actions in humans (Hashimoto and Yamada, 1992). However, the commercial cultivation of this plant is limited due to its restricted distribution in tropical areas (Griffin, 1985).

In the complete plant of some tropane alkaloid producing plant species, alkaloids are biosynthesized in the root cells and translocated to the aerial parts for storage (Waller and Nowacki, 1978). Kitamura et al. (1985) reported that the leaves of the *D. myoporoides* seedlings always contained the main alkaloids throughout the development whereas the leaves of the plants regenerated from callus contained no or few alkaloids at the early stages of development. After development, the leaves of the regenerated plant contained the main alkaloids.

Tropane alkaloid production is possible in the untransformed and genetically transformed cultured roots of different tropane alkaloid-producing species such as *Duboisia myoporoides, Hyoscyamus muticus, Datura stramonium, Atropa belladonna* (Endo and Yamada, 1985; Flores and Filner, 1985; Deno et al., 1987; Sharp and Doran, 1990; Maldonado-Mendoza et al., 1993; Yukimune et al., 1994; Mahagamasekera and Doran, 1998). Tropane alkaloids are detected in the cultured shoots of various *Duboisia* species but only after root formation (Yamada and Endo, 1984; Lin and Griffin, 1992a). However, untransformed and genetically transformed shoots of different *Duboisia* species have the ability to transform hyoscyamine to scopolamine without root formation (Hashimoto and Yamada, 1989; Kitamura et al., 1997; Mahagamasekera and Doran, 1998). The reason for the absence of tropane alkaloids in the cultured non-rooted *D. myoporoides* shoots is not clear.

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Depending on the cytokinin/auxin combinations used at the callus induction stage, two different types of calli, non-organogenic and organogenic, can be induced on different explants of *D. myoporoides*. Kitamura et al. (1980) reported that non-organogenic calli did not regenerate any shoot-bud or root when incubated in the induction medium. Shoot-buds were induced when the calli were transferred to a medium supplemented with cytokinin/auxin combinations different to that used at the calli induction stage. Kukreja et al. (1986) reported that depending on the cytokinin/auxin combinations used at the calli induction stage both shoot-bud and root producing organogenic calli can be induced. The authors reported regeneration of shoots from organogenic calli. They used the same cytokinin and auxin combination for calli induction and at different stages of shoot regeneration. These studies did not test for the alkaloid biosynthetic abilities of the non-rooted shoots.

Our work was aimed at investigating the production of tropane alkaloids at different stages of *D. myoporoides* shoot regeneration from non-organogenic and organogenic calli without any root initiation. The presence of root meristem in the non-organogenic and organogenic calli was also studied histochemically. Hyoscyamine and scopolamine contents of the non-rooted shoots were compared to those of the cultured roots without any shoot, regenerated from non-organogenic and organogenic calli. Tropane alkaloid contents of the cultured separated organs were compared to those from the mature plant.

2. Results and discussion

Non-organogenic calli were induced within 7–10 days. The mass of the calli increased but no shoot-buds or roots were induced during 12 weeks of incubation (Fig. 1). Shoot regeneration from these calli needed different cytokinin/auxin combinations at different stages. These results are consistent with those of Lin and Griffin (1992b) who also reported shoot regeneration of a *Duboisia* hybrid using the same cytokinin/auxin combinations. Kitamura et al. (1980) however, reported shoot regeneration of *Duboisia myoporoides* from non-organogenic callus. They used kinetin as a cytokinin in combination with IAA or NAA as auxins for callus induction. Our results indicate that *Duboisia myoporoides* shoots can be regenerated from non-organogenic calli induced with cytokinin and auxins other than kinetin + IAA or NAA.

Organogenic calli were slow growing and induced after 4 weeks. Within 2 weeks after calli induction, extensive shoot-bud induction was evident on the calli (Fig. 3). It was difficult to separate the shoot-buds induced on the calli. Some of the shoot-buds were incubated without separating from the calli. At the end of the incubation period (18 weeks) the differentiated shoots were attached to the calli on which they were induced. With a longer incubation period (> 15 weeks) the shoot-bud regenerating capacity of the calli decreased but never disappeared entirely. *Duboisia myoporoides* shoot regeneration from organogenic calli induced with BA 10 μM + IBA 0.1 μM has not been reported before. However, Badaoui et al. (1996) reported *Solanum paludosum* shoot regeneration from organogenic calli induced with the same cytokinin and auxin combination.

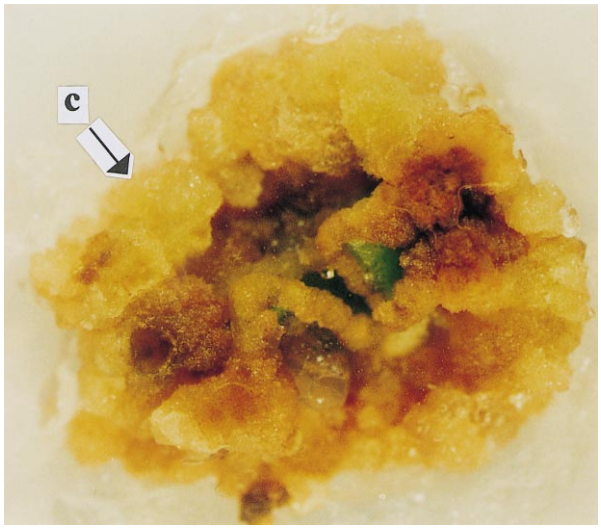


Fig. 1. Non-organogenic callus induced with BA 10 μM + NAA 1 μM. *Abbreviation: c*, non-organogenic callus.

Organogenic calli which produced only roots were induced after 6–7 weeks. The calli were compact and slow growing. After 6 weeks of incubation (starting after calli induction), small roots were appeared on the calli. The roots had many root hairs and did not grow well during the incubation period (Fig. 5). Root induction on the organogenic calli is consistent with the results of Kukreja et al. (1986) who reported *D. myoporoides* root induction with various cytokinin and auxin combinations. Our results showed that roots can be cultured in the induction medium of the calli. However, an investigation using various BA + IBA concentrations is necessary to overcome the problem of the slow growth of the roots.

Histochemical study showed the presence of only meristemoid regions in the 11-week-old non-organogenic calli (Fig. 2). Thorpe (1980) reported the presence of meristemoid regions in the calli tissues of various plants. Our results showed that after 11 weeks incubation, although shoot-buds and roots were not induced on the non-organogenic calli, the presence of meristemoid regions indicated active cell division in the calli. It appears that the cytokinin and auxin combinations used at the induction stage were not suitable for shoot-bud or root induction.

An organized cell arrangement was observed in the 2-week-old organogenic calli which produced shoot-buds and those which produced roots indicating the initial stage of shoot-bud (shoot-bud meristem) (Fig. 4) and root (root meristem) (Fig. 6) formation respectively in the calli. Since the organogenic calli were induced in the basal medium supplemented with BA + IBA (concentrations were varied), it appears that either cytokinin BA or BA + IBA combination has some effect on organogenic calli induction. Patel and Thorpe (1984) found that cell division mediated by BA leads to shoot formation in radiata pine.

Histochemical study of the non-organogenic and organogenic calli showed that cell organization for shoot-bud and root formation starts at the calli induction stage. It appears that by varying cytokinin/auxin combinations at the calli induction stage, shoot-bud or root induction can be enhanced. Kukreja et al. (1986) reported shoot-bud induction directly on *D. myoporoides* leaf explants without any callus induction.

Hyoscyamine and scopolamine were detected in the 11-week-old non-organogenic calli (Table 1). Sipply and Friedrich (1975) detected atropine and scopolamine in the 15-week-old leaf callus of *D. myoporoides*. Yamada and Endo (1984) found hyoscyamine and scopolamine in the 12-week-old leaf-derived non-organogenic callus of *D. leichhardtii*. These alkaloids were no longer detected in the 16-week-old calli. Since we did not analyze calli after 11 weeks it is not clear whether tropane alkaloid biosynthetic pathway expressed in the non-organogenic calli continued for a certain period of time. The alkaloids were absent in the 2-week-old green calli produced from the 11-week-old non-organogenic calli (Table 1). As green calli was the initial stage of shoot-

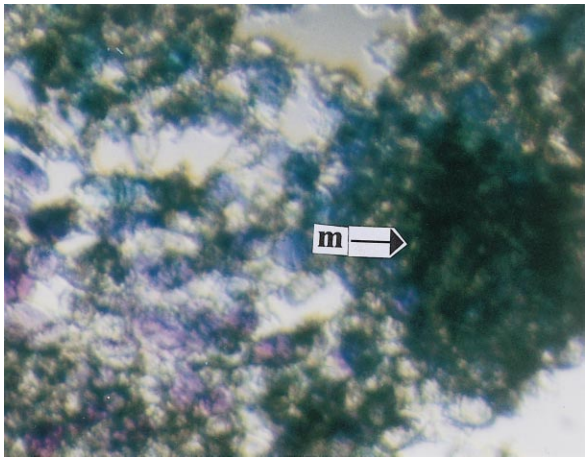


Fig. 2. Meristemoid region in 11-week-old non-organogenic callus (×100). Section stained with toluidine blue O. *Abbreviation: m*, meristemoid region.

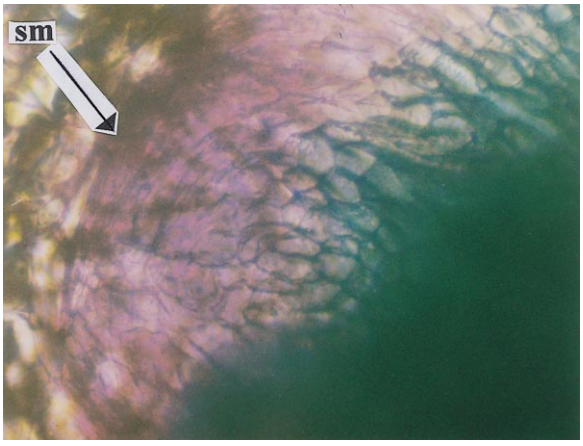


Fig. 4. Organized cell arrangement (shoot-bud meristem) from 2-week-old organogenic callus which produced only shoot-buds (×400). Section stained with toluidine blue O. *Abbreviation:* sm, shoot–bud meristem.

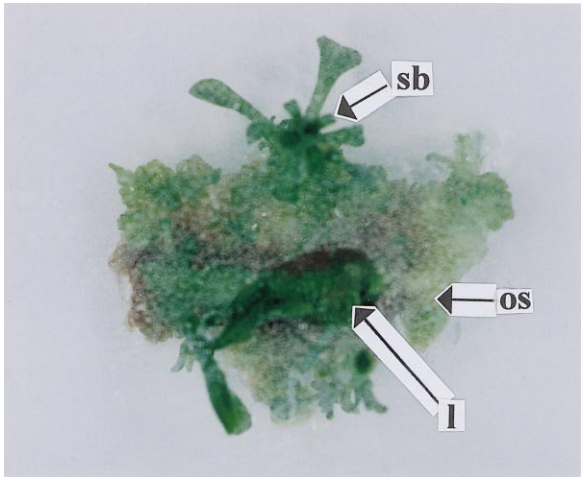


Fig. 3. Only shoot-buds on the organogenic callus induced with BA 10 μM + IBA 0.1 μM. *Abbreviations:* l, leaf explant; sb, shoot–bud; os, organogenic callus which produced only shoot–buds.

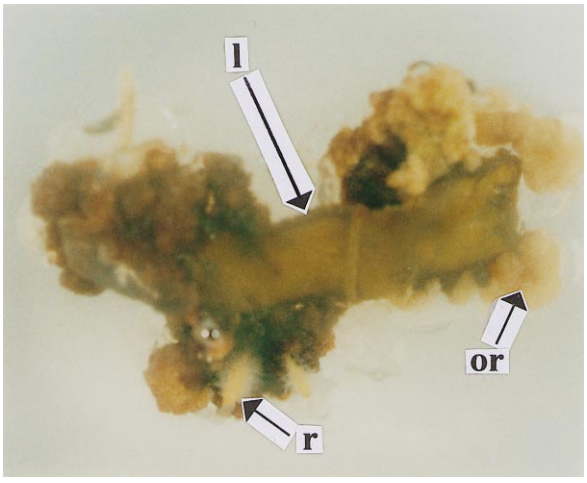


Fig. 5. Only roots on the organogenic callus induced with BA 1 μM + IBA 10 μM. *Abbreviations:* l, leaf explant; r, root; or, organogenic callus which produced only roots.

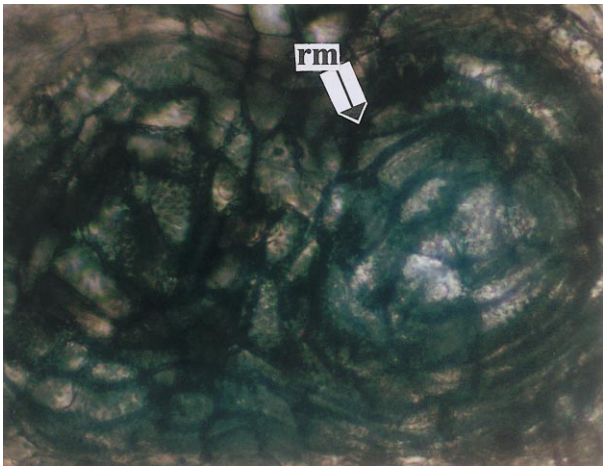


Fig. 6. Organized cell arrangement (root meristem) in the 2-week-old organogenic callus which produced only roots (×400). Section stained with toluidine blue O. *Abbreviation*: rm, root meristem.

bud induction, it appears that tropane alkaloid biosynthesis was repressed by shoot-bud organogenesis.

Hyoscyamine and scopolamine were absent in the 2-week-old shoot-buds induced on the green calli (Table 1). Similar results were found in other *Duboisia* species (Lin and Griffin,1992a). Tropane alkaloids were detected in the 6-week-old non-rooted shoot regenerated from non-organogenic calli (Table 1). Missaleva et al. (1993) also reported tropane alkaloids in the non-rooted *Datura innoxia* shoot. Absence of the alkaloids in the 2-week-old shoot-buds and their presence in the 6-week-old non-rooted shoots found in our study indicates that, hyoscyamine and scopolamine formation in the non-rooted shoot is related to differentiation.

Hyoscyamine and scopolamine were absent in the 2-week-old organogenic calli and 3-week-old shoot-bud (Table 1). It appears that formation of hyoscyamine and scopolamine may be repressed by shoot-bud organogenesis. Tropane alkaloids were also absent in the 4-week-old shoots (Table 1). The alkaloids were detected in the 9-week-old shoot regenerated from the organogenic calli (Table 1). These results indicate that tropane alkaloid production in the non-rooted shoot was related to the stage of differentiation. However, the stage of differentiation necessary for tropane alkaloid biosynthesis in the regenerated non-rooted shoots may depend on the incubation period or on the other factors related to differentiation such as the cytokinin and auxin combination, type of explant and the plant species. Our results also indicated that tropane alkaloids may be produced in the non-rooted shoots regenerated from both non-organogenic and organogenic calli.

Fourteen-week-old organogenic calli collected from the base of the differentiated shoot contained only hyoscyamine (Table 1). However, the 9-week-old differentiated shoot attached to the same calli contained both hyoscyamine and scopolamine. Hashimoto et al. (1991) detected hyoscyamine 6β-hydroxylase (H6H) the enzyme responsible for the conversion of hyoscyamine to scopolamine in the aerial parts of some tropane alkaloid-producing plant species. The authors reported that in the presence of root, conversion of hyoscyamine to scopolamine takes place in the aerial parts. As hyoscyamine and scopolamine were detected in the non-rooted shoot of our study, it appears that the complete biosynthetic pathway was expressed in the non-rooted shoot but not in the calli. However, larger amounts of hyoscyamine biosynthesized in the non-rooted shoot may be translocated to the calli for storage. Kitamura

Table 1
Tropane alkaloid contents in cultured and mature plant materials

Origin of plant material	Tissue analysed[a]	Tropane alkaloid contents (μg g^{-1} dry weight±standard deviation)	
		Hyoscyamine	Scopolamine
Shoot regenerated from non-organogenic calli	11-week-old calli	0.41±0.03	0.23±0.02
	2-week-old green calli	ND[b]	ND
	2-week-old shoot-bud	ND	ND
	6-week-old shoot	7.8±0.1	6.5±0.4
Shoot regenerated from organogenic calli	2-week-old calli	ND	ND
	3-week-old shoot-bud	ND	ND
	4-week-old shoot	ND	ND
	9-week-old shoot	38.5±0.4	3.6±0.1
	14-week-old calli	6.9±0.2	ND
Root regenerated from non-organogenic calli	4-week-old root	69±1	37.3±0.3
Root regenerated from organogenic calli	4-week-old root	82±3	48.2±0.6
Mature plant	Leaves	3050±40	2250±60
	Roots	920±20	190±10

[a] Details of how the 'tissues analysed' are obtained described in Section 3.
[b] ND: Not detected.

et al. (1996) reported cycling of tropane alkaloids in the cultured rooted shoot of *D. myoporoides*. Our results indicate that cycling of tropane alkaloids is possible in the cultured non-rooted shoots attached to the calli. Scopolamine may also have been translocated to the callus but the amount may be below the detection limit of the analytical procedure used.

The 4-week-old roots regenerated from non-organogenic and organogenic calli contained significantly higher amounts of hyoscyamine and scopolamine than those produced in the non-rooted shoots regenerated from the non-organogenic and organogenic calli ($P < 0.001$) (Table 1). Missaleva et al. (1993) also reported higher amounts of tropane alkaloids in the cultured root than those produced in the non-rooted shoots of *Datura innoxia*. Comparison of the incubation period for tropane alkaloid production in the regenerated non-rooted shoots with that for the regenerated roots of our study indicates that the differentiation necessary for tropane alkaloid production takes place earlier in the cultured roots than in the cultured non-rooted shoots. These results are in contrast to previous reports that tropane alkaloid formation in the cultured shoots of *Duboisia* species takes place only after root formation (Lin and Griffin, 1992a). Our findings show that although the root is the main site for tropane alkaloid biosynthesis, under certain conditions tropane alkaloids are also biosynthesized in the regenerated shoots of *D. myoporoides* without root formation.

Hyoscyamine and scopolamine contents of the cultured shoots and roots regenerated from non-organogenic and organogenic calli were compared to those of the leaves and roots of the mature plant (Table 1). The amount of hyoscyamine was higher than the amount of scopolamine in the non-rooted shoots and roots without aerial organ. A similar alkaloid observation was also found in the leaves and roots of the mature plant. The amounts of hyoscyamine and scopolamine in the non-rooted shoots were lower than those in the roots without aerial organ. As root to shoot or shoot to root translocation of alkaloids was not possible in the separated cultured organs, the amounts of alkaloids detected must be biosynthesized in the particular organ. The higher amounts of hyoscyamine and scopolamine detected in the leaves compared to those in the roots of the mature plant indicates that the alkaloids biosynthesized in the roots were translocated to the aerial parts as well as showing that alkaloid biosynthesis takes place in the aerial parts independently.

In conclusion, this paper reports the first production of tropane alkaloids from non-rooted shoots of *D. myoporoides*, whereas all previous reports of tropane alkaloids are from root culture. Although the amount of tropane alkaloid production from the non-rooted shoots is lower than from root culture, further studies on manipulation of the culture environment may improve shoot culture production.

3. Experimental

All tissue culture experiments were carried out duplicate with 20 replicates per experiment. All cultures were transferred to fresh media at 4–5 week intervals and incubated at 25±2°C. For light incubation, a 14 h photo-period from a fluorescent tube with a light intensity 15.2 μmol $s^{-1}m^{-2}$ at a distance 40 cm from the light source was used. At the calli induction stage plastic Petri dishes (90×14 mm) and for all other stages glass and plastic tissue culture vials (5.5×5.0 cm; 7.0×6.5 cm) were used.

3.1. Plant materials and callus induction

Duboisia myoporoides R. Br. plant materials were collected from Mount Annan Botanic Gardens, Campbelltown NSW, Australia. Fresh, healthy and mature leaves collected from the tree were surface sterilized by placing the leaves in 70% v/v ethanol for 30 s followed by two treatments of 10 min in 1% v/v hypochlorite solution and three washes in sterile water. Segments of 1 cm surface sterilized leaf pieces were placed on the MS (Murashige and Skoog, 1962) basal medium adjusted to pH 5.7 supplemented with 3% w/v sucrose and 0.9% w/v agar with BA 10 μM + NAA 1μM and with BA 10 μM + IBA 0.1 μM independently. The cultures were incubated in the dark. The leaves and roots from mature plant were harvested (old winter) for an alkaloid assay.

The non-organogenic calli induced with BA 10 μM + NAA 1 μM were incubated in the same medium for 12 weeks (from the date of calli induction). Some of the 11-week-old calli (calli induction started after 1 week) were analyzed for the alkaloids and the rest were used for shoot culture.

3.2. Shoot culture from non-organogenic calli

The following steps (a, b, c) were carried out for shoot regeneration from non-organogenic calli by using the procedure of Lin and Griffin (1992b).

3.2.1. (a) Production of green calli

Eleven-week-old non-organogenic calli were transferred to the basal medium supplemented with BA 0.1 μM + NAA 27 μM and incubated in the light for 2 weeks. Some of the green calli were harvested for chemical analysis and the rest were used for shoot-bud induction.

3.2.2. (b) Shoot-bud induction

The green calli developed from 11-week-old calli were transferred to the basal medium supplemented with BA 22 μM and incubated in the light for 8 weeks. Induced shoot-buds were incubated for one more week. Some of

the 1–2-week-old shoot-buds were harvested for chemical analysis and the rest were used for shoot elongation.

3.2.3. (c) Shoot elongation

The shoot-buds were separated from calli and transferred independently to basal medium supplemented with BA 5 μM + NAA 0.5 μM and incubated in the dark for 2 weeks. The elongated shoots were incubated in a medium supplemented with the same cytokinin and auxin combination in the light for 4 weeks. The 6-week-old non-rooted shoots were analyzed for hyoscyamine and scopolamine.

3.3. Shoot culture from organogenic calli

The organogenic calli induced with BA 10 μM + IBA 0.1 μM were incubated in the same medium for shoot-bud induction and shoot elongation. Incubation was carried out for 18 weeks from the date of calli induction. The 2-week-old organogenic calli, 3-week-old shoot-buds, 4 and the 9-week-old non-rooted shoots were analyzed for hyoscyamine and scopolamine. Some of the 14-week-old callus carrying differentiated shoots were separated and analyzed independently for the tropane alkaloids.

3.4. Root culture from non-organogenic calli

Eleven-week-old leaf-derived non-organogenic calli induced with BA 10 μM + NAA 1 μM were transferred to new medium supplemented with full MS salt + full MS vitamin + IBA 25 μM and incubated in the dark for 6 weeks. After formation of roots the cultures were incubated for another 4 weeks and the 4-week-old roots were analyzed for tropane alkaloids.

3.5. Root culture from organogenic calli

Surface sterilized 1 cm segment of leaf pieces were placed on the semi-solid MS basal medium supplemented with 3% w/v sucrose, 0.9% w/v agar and BA 1 μM + IBA 10 μM and incubated in the dark for 6 weeks. After formation of roots, the cultures were further incubated for 4 weeks. The 4-week-old roots were analyzed for tropane alkaloids.

3.6. Statistical analysis

Hyoscyamine and scopoalmine contents of the non-rooted shoots and roots regenerated from non-organogenic and organogenic calli were compared by using one-tailed two sample *t*-test.

3.7. Histochemical analysis

Cultured tissues were collected from five replicate culture vials and 50–60 sections were prepared. The results are presented as the average response of 30 randomly selected sections.

Free-hand fresh sections of the 11-week-old non-organogenic and 2-week-old organogenic calli were stained with toluidine blue O for 1–2 min and then destained with water (O'Brien and McCully, 1981). The stained sections were examined microscopically for the presence of shoot or root meristem.

3.8. Extraction and analysis of alkaloids

For each of the 20 replicate vials per experiment, the harvested samples were combined, air-dried and divided into four portions for individual extraction and analysis.

Following analytical method was validated by testing for precision, accuracy, recovery, linearity, limits of detection, limit of quantification and sample and standard stability (Nou, 1997).

About 0.1–0.5 g of the air-dried sample was sand ground and 1 ml of 10 ppm homatropine solution (internal standard) and 10 ml of 0.5 M HCl added. After 15 min the solution was filtered and the filtrate was made alkaline with ammonia solution and passed through an "Extrelut 20" (Merck, Germany) column and the alkaloids eluted with 40 ml dichloromethane:isopropanol (4:1 v/v). It was necessary to repeat the whole procedure 2–4 times to obtain a combined extract which was evaporated to dryness. The residue was dissolved in 1 ml methanol for GC–MS analysis using a SGE BPX5 (30 m×22 mm, 0.25 μm film thickness) column set at 85°C for 3 min, rising to 280°C at 12°C min^{-1}. The injector and detector temperatures were 220 and 280°C respectively and helium was used as the carrier gas. The mass spectrometer was run in the electron impact mode.

Quantification was achieved by comparing the total ion chromatograms of the standard and sample. The following typical mass spectra were obtained for the standard hyoscyamine: 289 [M^+] (7), 124 (100), 140 (8), 82 (25), 67 (11) and scopolamine: 303 [M^+] (21), 94 (100), 154 (30), 138 (74), 108 (47) and those of the sample hyoscyamine: 289 [M^+] (7), 124 (100), 140 (13), 82 (25), 67 (12) and scopolamine: 303 [M^+] (21), 94 (100), 154 (32), 138 (67), 108 (51).

Acknowledgements

We thank the staff at the Mount Annan Botanic Gardens for supplying *Duboisia myoporoides* R. Br. plant materials, Dr. Philip Tong of AGAL for carrying out the GC–MS analysis and Darlene Williams for her generous assistance. The senior author acknowledges the financial support from her husband and UWS Macarthur during PhD candidature. This report is based on the senior author's PhD dissertation "Relationship

between organogenesis, differentiation and histolocalization of selected alkaloids in *Duboisia myoporoides* R. Br.'' presented to the University of Western Sydney, Macarthur, Australia, June, 1999.

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Metabolism of gibberellins A_1 and A_3 in fruits and shoots of *Prunus avium*

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Abstract

Isotope-labelled GA metabolites were identified by GC–MS, following HPLC fractionation of extracts derived from fruits or shoots, that had been incubated with [^{2}H]- and [^{3}H]- GA_1 or [^{2}H]- and [^{3}H]- GA_3. GA_1 (**1**) was converted into GA_8 (**10**) by developing fruits and vegetative shoots of sweet cherry (*Prunus avium* cv. 'Stella'), while GA_3 (**4**) was converted into GA_3-isolactone (**17**). Other metabolites of each GA were detected but were not identified unequivocally. These included a metabolite of GA_1 (**1**) in fruitlets that was more polar (by reverse phase HPLC) than GA_8 (**10**) and a metabolite of similar polarity to GA_{87} (**6**), was obtained after incubating fruitlets with GA_3 (**4**). However, no evidence was obtained to suggest that GA_{87} (**6**) was a metabolite of GA_3 (**4**) or that GA_{85} (**2**) was a metabolite of GA_1 (**1**) in these tissues, under the conditions used. The pattern of metabolites obtained from vegetative tissues was similar to that from fruitlets. However, the results suggested that GA_1 (**1**) and GA_3 (**4**) were metabolised at a greater rate in shoots from mature trees than in shoots from seedlings, and that GA_1 (**1**) was metabolised more rapidly than GA_3 (**4**) in juvenile and mature shoots. We conclude from these observations that GA_3 (**4**) is not a precursor of GA_{87} (**6**) and GA_{32} (**5**), also, that GA_1 (**1**) is not a precursor of GA_{85} (**2**) and GA_{86} (**3**) in developing fruits or in vegetative shoots of sweet cherry. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Prunus avium*; *Rosaceae*; Sweet cherry; Metabolism; Identification; Gibberellins; Developing fruits; Growing shoots; GC–MS

1. Introduction

Previous work in this laboratory identified the trihydroxy **C**-19 gibberellin, GA_{87}, (**6**, see Fig. 4) (Blake et al., 1993). Also, 11 other GAs, including GA_1 (**1**), GA_3 (**4**), GA_{32} (**5**), GA_{85} (**2**) and GA_{86} (**3**) were characterised in developing fruitlets of sweet cherry (*Prunus avium* L.) (Blake and Browning, 1994). While, GA_1 (**1**), GA_3 (**4**), GA_5 (**7**), GA_8 (**10**), GA_{19} (**13**), GA_{20} (**11**), GA_{29} (**12**), GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**) were identified in young seedlings of *P. avium*, only GA_1 (**1**), GA_3 (**4**), GA_5 (**7**), GA_8 (**10**), GA_{19} (**13**), GA_{20} (**11**), and GA_{29} (**12**) were detected in shoot tips of mature, florally-competent tissues (Blake et al., 2000a).

Structure–activity studies showed that GAs, unsaturated in the **C**-1,2 or **C**-2,3 positions, inhibited flowering and stimulated shoot elongation of mature *P. avium* trees and increased growth of *P. avium* seedlings (Olivera and Browning, 1993). These effects were enhanced by an increase in the degree of hydroxylation of the GA and thus GA_3 (**4**) was more active than GA_5 (**7**) or GA_7 (**16**). Olivera and Browning (1993) noted that exogenous GA_3 (**4**) stimulated 44% of floral spurs to produce vegetative shoots as well as inhibiting flowering. While applications of GA_{87} (**6**) to fruiting spurs of *P. avium* were as active as GA_3 (**4**) in inhibiting flowering, up to 80% of floral spurs treated with GA_{87} produced vegetative shoots (P.S. Blake, A.W.L. Chu and L.N. Mander, unpublished results).

These observations suggest that the polyhydroxy GAs (e.g. GA_{32}, GA_{87}, etc.) detected in tissues of sweet cherry may be important in the early enforcement of the juvenile, non-flowering state in *P. avium* seedlings. However, the biosynthetic route to these tri- and tetrahydroxylated GAs has not been elucidated in *P. avium* or any other *Prunus* species in which they have been identified. These include apricot, (*P. armeniaca* L.) (Bottini et al., 1985), sour cherry (*P. cerasus* L.) (Nakayama et al., 1996) and peach (*P. persica* L.) (Yamaguchi et al., 1975).

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Metabolism studies with *Prunus* species have demonstrated that, following incubation of cell suspension cultures of peach leaves with [^{2}H]- and [^{3}H]-GA_5, the major isotope-labelled metabolites were identified as GA_3 (**4**) and GA_6 (**15**). Smaller amounts of GA_1 (**1**), GA_8 (**10**) and GA_{22} (**8**) were detected also (Koshioka et al., 1988). Immature fruits of apricot metabolised [^{2}H]- and [^{3}H]-GA_5 to labelled GA_1 (**1**), GA_3 (**4**) and GA_6 (**15**) (De Bottini et al., 1987). Blake et al. (1993) suggested that since GA_{87} (**6**) is intermediate in structure between GA_3 (**4**) and GA_{32} (**5**), it may also be an intermediate in the biosynthesis of GA_{32} (**5**) from GA_3 (**4**). In this hypothetical pathway, GA_3 (**4**) would be C-12 hydroxylated to GA_{87} (**6**) and this in turn would be C-15 hydroxylated to GA_{32} (**5**). Similarly, the biosynthesis of GA_{86} (**3**) would proceed from GA_1 (**1**), with GA_{85} (**2**) as the **C**-12 hydroxylated intermediate.

GA_1 (**1**) and GA_3 (**4**) are well established products of the early 13-hydroxylation pathway in higher plants (for review, see MacMillan, 1997). Although, an alternative pathway to GA_3 (**4**), via GA_{95} (**14**) has been proposed in *P. cerasus* (Nakayama et al., 1996), but not investigated.

The objectives of this work were two-fold, firstly to determine if GA_{85} (**2**) and GA_{86} (**3**) are biosynthesised from GA_1 (**1**) and whether GA_{87} (**6**) and GA_{32} (**5**) are biosynthesised from GA_3 (**4**) by developing fruitlets and shoots of sweet cherry. Secondly, to compare the metabolism of GA_1 (**1**) and GA_3 (**4**) by actively growing shoots of juvenile and mature *P. avium* trees.

2. Results

For metabolism studies we used a mixture of [^{2}H]- and [^{3}H]- GAs as a 'dual-label' and we assumed that the metabolic pathways for the [^{2}H]- and [^{3}H]-labelled substrates were identical to each other and to those of the endogenous GAs. Samples were extracted and purified using procedures known to minimise losses of polar GAs (Blake et al., 1993). Extracts were fractionated by reverse phase HPLC and an aliquot of each fraction was taken for liquid scintillation counting to identify the fractions that contained metabolites. The remainders of these fractions were then derivatised and subjected to full-scan gas chromatography–mass spectrometry (GC–MS) or GC with selected ion monitoring (SIM) to characterise the [$^2H_2$]-metabolites present. Ion cluster analysis (Gaskin and Macmillan, 1991) of control samples, showed that the amounts of exogenous GAs used were ca. 20 times and 8 times the endogenous concentrations of GA_1 (**1**) and GA_3 (**4**), respectively, in fruitlets, at the time treatments were applied. Concentrations of endogenous GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**) were not measured (stable-isotope-labelled standards were not available). However, bioassays demonstrated that greater GA-like activity was associated with HPLC fractions containing GA_{32} (**5**) and GA_{87} (**6**) than fractions containing GA_1 (**1**) and GA_3 (**4**) in extracts of developing fruitlets (Blake et al., 1993) as well as in extracts of mature fruits (Blake et al., 2000a).

In control experiments, tissue samples were plunged into liquid N_2 immediately following injection of the isotope-labelled substrates and minimal metabolism or conversion of [^{3}H]-GA_1 or [^{3}H]-GA_3 was observed during the purification procedures (Figs. 1–3). Circa 95% of the radioactivity injected into fruits or shoots was recovered by the extraction procedure at time 0 and over 70% was recovered as a single 'peak' after HPLC. The recovery of [^{3}H] by the solvent extraction was reduced as the incubation time increased and the total amount of radioactivity recovered in fractions 10–30, (those known to contain the di-, tri- and tetrahydroxy GAs of interest), from HPLC decreased as the incubation time increased (data not shown). These losses during sample purification were assumed to be as a result of the formation of polar conjugates of the GAs, but this was not investigated.

2.1. Metabolism of GA_1 in fruitlets

Following HPLC fractionation and scintillation counting of extracts, derived from fruitlets that were injected with [^{3}H]- and [^{2}H]-GA_1 and then incubated for 48 or 96 h, two main peaks of radioactivity were identified (Fig. 1). The first peak eluted in fraction 16, the retention time expected for GA_{85} (**2**) and GA_{87} (**6**) and the second eluted in fractions 18 and 19, the retention time expected for GA_8 (**10**) and GA_{29} (**12**) using these HPLC conditions (Blake et al., 2000). After methylation of these fractions and further purification by TLC, scintillation counting revealed that most of the applied radioactivity was centred at the same R_f as the methyl esters of GA_8 (**10**), GA_{85} (**2**) and GA_{87} (**6**). [$^2H_2$]-GA_8 was identified after trimethylsilylation and GC–MS of the purified fractions and data derived from full-scan GC–MS are presented (Table 1). In fraction 16, endogenous GA_{85} (**2**) and GA_{87} (**6**) were detected by the intensity of characteristic ions maximising at the correct KRIs and incorporation of [$^2H_2$] was measured by comparing the ratio of the $(M)^+$ and $(M+2)^+$ of the GAs by SIM. However, no enrichment of GA_{85} (**2**) or GA_{87} (**6**) with [$^2H_2$] was detected (Table 2). Similarly, GC–SIM showed that endogenous GA_{32} (**5**) and GA_{86} (**3**) were present in fractions 13 and 14 but without [$^2H_2$] enrichment. After derivatisation and GC–MS, the small amount of radioactivity in fraction 24 was confirmed as unmetabolised GA_1 (**1**) (Table 1).

2.2. Metabolism of GA_3 in fruitlets

HPLC of extracts of fruitlets fed with [^{3}H]- and [^{2}H]-GA_3 then incubated for 48 or 96 h, yielded one clear

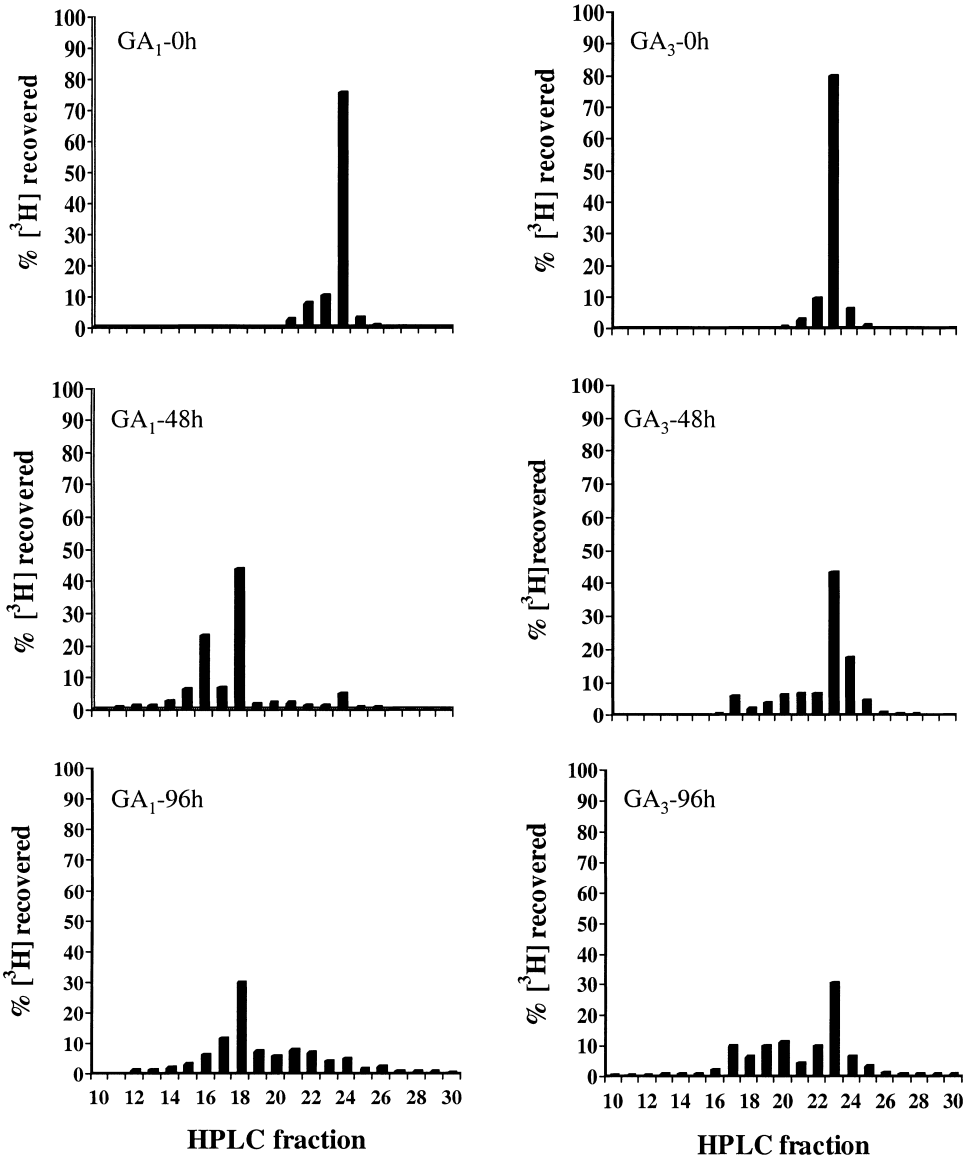


Fig. 1. HPLC separation of [^{3}H]-labelled metabolites of GA_1 (left) and GA_3 in cherry fruitlets after 0, 48 and 96 h incubation, respectively. The data are expressed as percent of total [^{3}H]- recovered from HPLC fractions 10–30.

peak and two poorly resolved peaks of radioactivity (Fig. 1). The largest amount of radioactivity recovered (47% after 48 h; 32% after 96 h) was in fraction 23, equivalent to the retention time of GA_3 (**4**) and GC–MS of the derivatised fraction confirmed the presence of unmetabolised GA_3 (**4**) (Table 1). The peak in fractions 19 and 20 was identified after derivatisation and GC–MS as [$^2H_2$] derivative of GA_3-isolactone (**17**) (Table 1). The metabolite recovered in fractions 16 and 17 (Fig. 1) was identified tentatively as the [$^2H_2$]-derivative of 16,17-dihydro,17-hydroxygibberellinA_3 (**18**) by comparison of full-scan GC–MS spectral data for the MeTMSi derivative with literature values (Gaskin and MacMillan, 1991); data derived from the full-scan GC–MS data are presented (Table 1). GC–SIM demonstrated the presence of GA_{32} (**5**) and GA_{86} (**3**) in fractions 13 and 14, and GA_{87} (**6**) and GA_{85} (**2**) in fractions 16 and 17, but no incorporation of [$^2H_2$]- into these GAs was detectable (Table 2).

2.3. Metabolism of GA_1 in shoots

The patterns of metabolites obtained by HPLC and scintillation counting, after incubation of [^{3}H]- and [$^2H_2$]-GA_1 with actively growing shoots from flowering (mature) and from non-flowering (juvenile) trees were similar (Fig. 2). The major metabolite of [$^2H_2$]-GA_1 was identified by GC–MS of the MeTMSi derivative as [^{2}H]-GA_8 (Table 1). The amount of [^{2}H]-GA_8 detected, by comparison of the areas of peaks in the SIM chromatograms, was greater in extracts of mature shoots than juvenile shoots (Table 3). Furthermore, a smaller

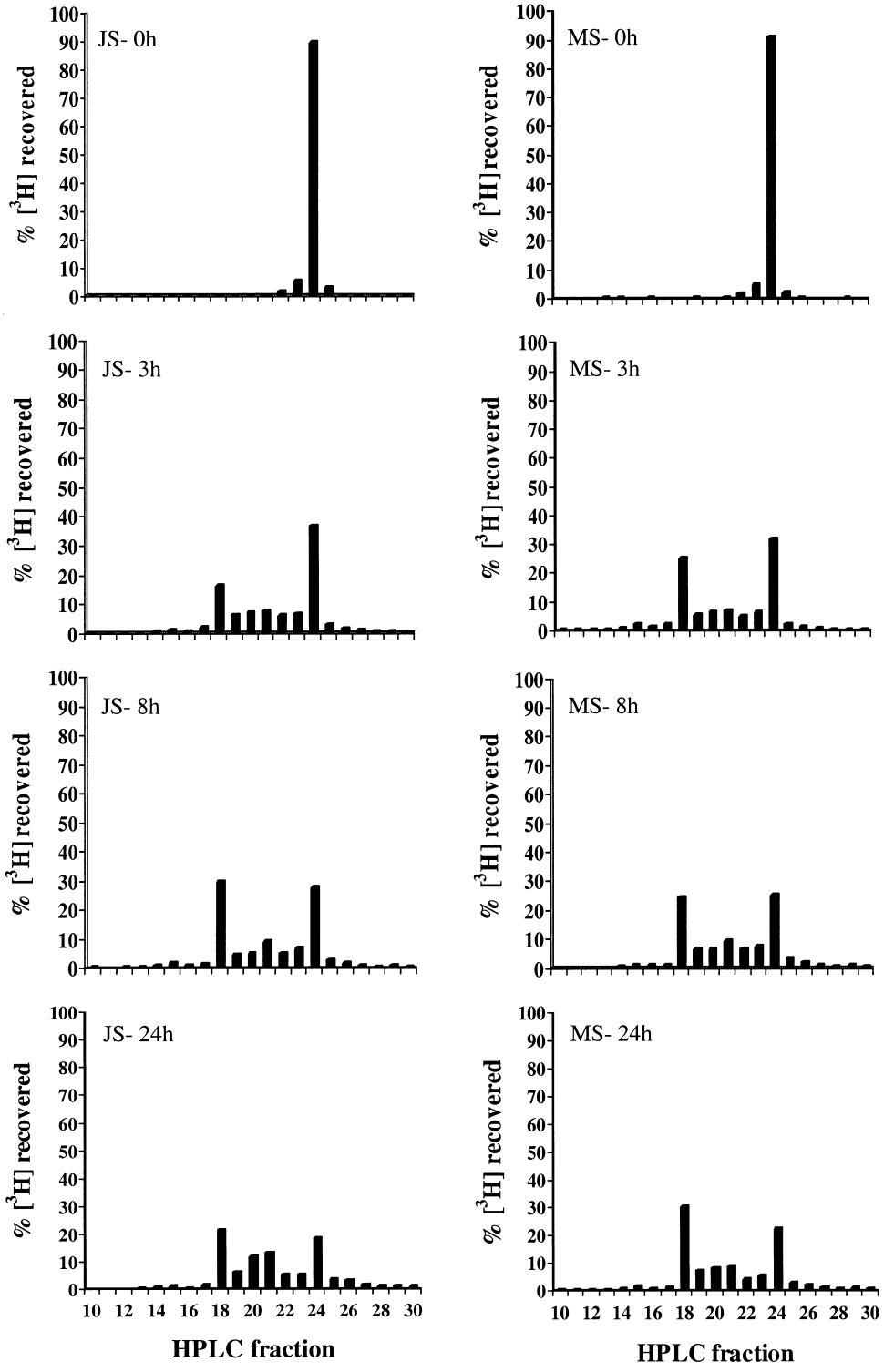


Fig. 2. HPLC separation of [^{3}H]-labelled metabolites of GA_1 in juvenile (JS) and mature (MS) shoots of cherry after different incubation periods: 0, 3, 8 and 24 h. The data are expressed as percent of total [^{3}H]- recovered from HPLC fractions 10–30.

amount of unmetabolised GA_1 (**1**) was detected in mature shoots than in juvenile shoots (Table 3). Taken together, these data suggest that the rate of metabolism of GA_1 (**1**) to GA_8 (**10**) may be greater in mature shoots than in juvenile shoots.

2.4. Metabolism of GA_3 in shoots

The rate of metabolism of GA_3 (**4**) appeared to be lower than the rate of metabolism of GA_1 (**1**) by juvenile and mature shoots. Greater amounts of unmetabolised

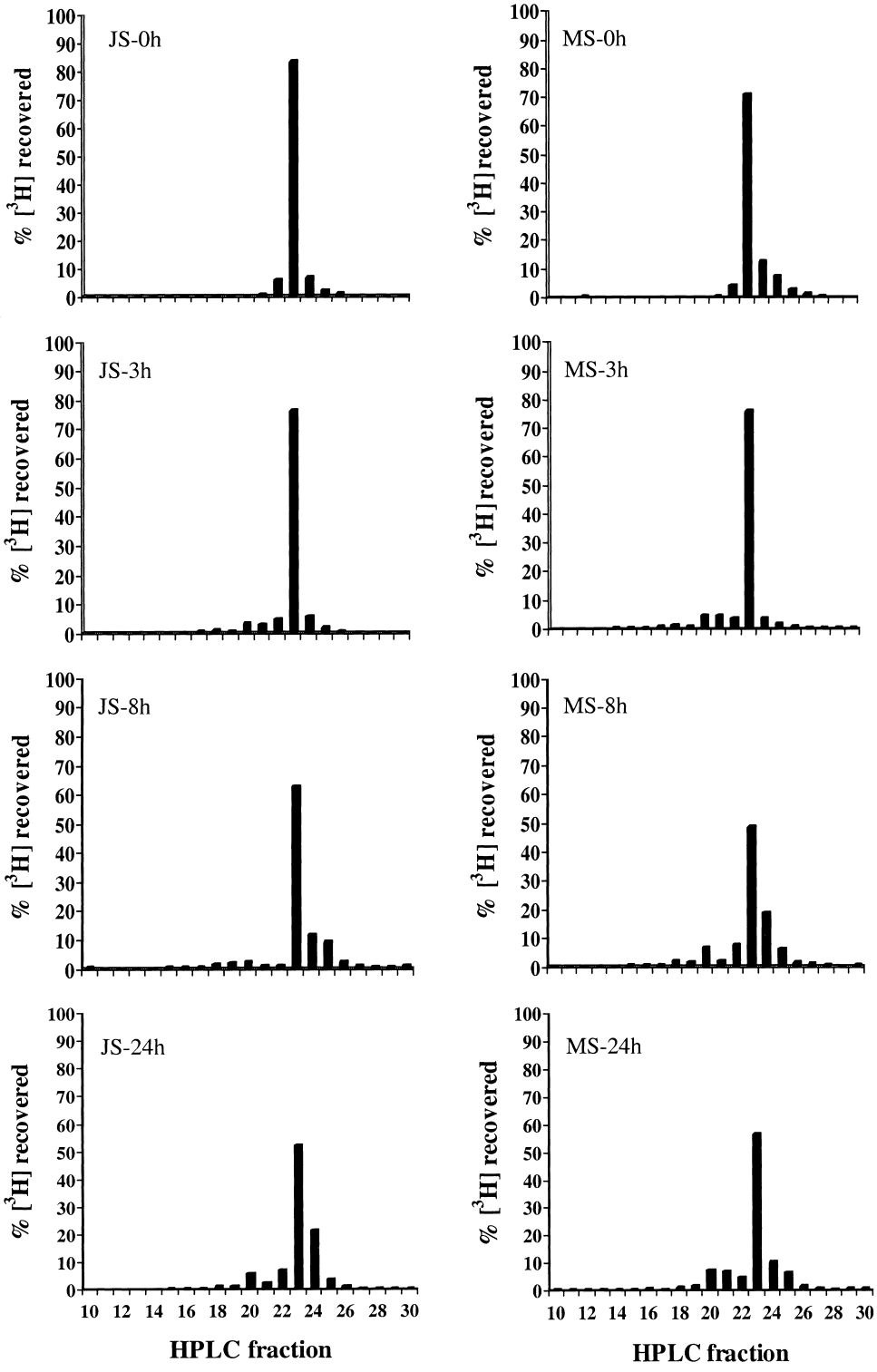


Fig. 3. HPLC separation of [^{3}H]-labelled metabolites of GA_3 in juvenile (JS) and mature (MS) shoots of cherry after different incubation periods: 0, 3, 8 and 24 h. The data are expressed as percent of total [^{3}H]- recovered from HPLC fractions 10–30.

GA_3 (**4**) than unmetabolised GA_1 (**1**) were detected after each time of incubation (Table 3). The only product identified by GC–MS was GA_3-isolactone and the amount of GA_3-isolactone (**17**), measured by comparison of areas of peaks in the SIM chromatograms, was slightly greater in mature shoots than in juvenile of unmetabolised GA_3 (**4**) shoots (Table 3). Also, greater amounts of unmetabolised GA_3 (**4**) were recovered from juvenile shoots than from mature shoots (Table 3). Taken together these results suggest that mature shoots

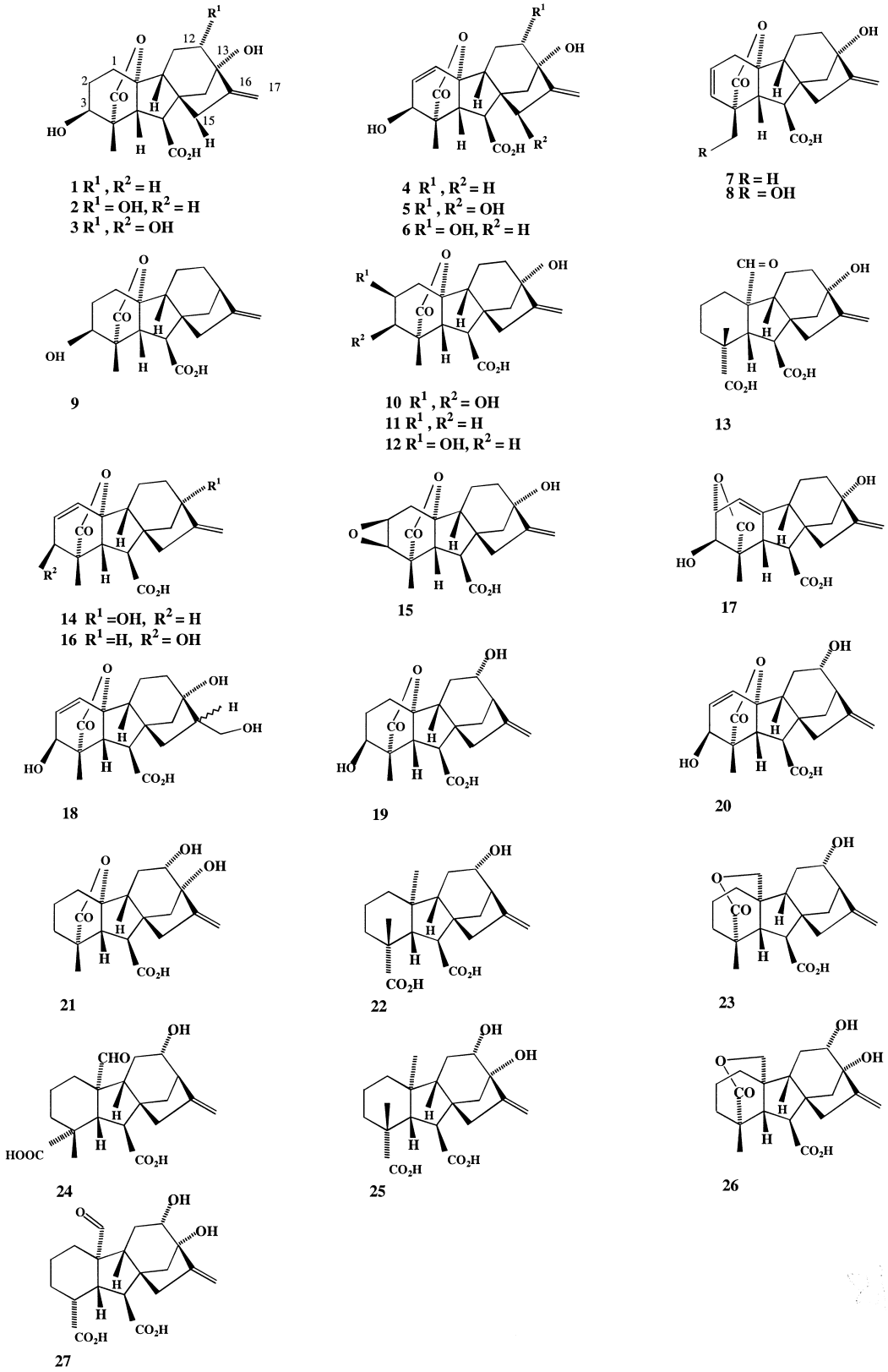


Fig. 4. Structures of gibberellins.

Table 1
Intensities of characteristic ions and KRI for MeTMSi derivatives of standard compounds and isotopically-enriched GAs isolated following feeds of $[^2H_2]$-GA_1 and $[^2H_2]$-GA_3 to fruits and shoots of sweet cherry

Compound identified	KRI	Diagnostic ions (m/z) with % abundance in standard and sample					HPLC Fraction
$[^2H_2]$-GA_1		Ion	508 (M^+)	493	450	378	209	
	2678	Standard	100	15	27	26	73	
	2678	Sample	100	18	29	28	75	24
$[^2H_2]$-GA_8		Ion	596 (M^+)	581	537	450	209	
	2819	Standard	100	11	14	23	71	
	2819	Sample	100	10	14	26	69	18
$[^2H_2]$-GA_3		Ion	506 (M^+)	491	477	240	210	
	2701	Sample	100	12	19	38	46	
	2701	Standard	100	11	18	36	44	22–23
$[^2H_2]$-GA_3-isolactone		Ion	506 (M^+)	491	477	240	210	
	2644	Sample	100	14	18	53	21	
	2644	Standard	100	12	17	49	20	19–20
17-$[^2H_2]$-16,17H_2,17 (OH)-GA_3 [a]		Ion	596 (M^+)	581	553	506	465	
	2963	Sample	36	8	11	59	100	
								16–17

[a] Tentative identification based on comparison with spectral and KRI data for 16,17dihydro,17hydroxy-GA_3 in Gaskin and MacMillan (1991).

Table 2
Incorporation of deuterium into GA_{85}, GA_{86}, GA_{87} and GA_{32}, 0, 48 and 96 h after feeds of 17,17$[^2H_2]$-GA_1 and 17,17$[^2H_2]$-GA_3 to *P. avium* fruitlets, expressed as (M + 2)/(M)

GA	17,17$[^2H_2]$-GA_1			17,17$[^2H_2]$-GA_3		
	0 h	48 h	96 h	0 h	48 h	96 h
GA_{85}	0.22	0.21	0.22	0.22	0.21	0.21
GA_{86}	0.40	0.40	0.39	0.40	0.39	0.39
GA_{87}	0.21	0.21	0.21	0.22	0.23	0.21
GA_{32}	0.38	0.38	0.38	0.39	0.38	0.38

may metabolise GA_3 (**4**) at a greater rate than juvenile shoots.

3. Discussion

These experiments did not support our earlier hypothesis (Blake et al., 1993) that GA_3 (**4**) is metabolised to GA_{87} (**6**) and then to GA_{32} (**5**). However, it is possible that GA_{87} (**6**) and GA_{32} (**5**) are only biosynthesised in the early stages of fruit and/or seed development. The fruitlets used in these experiments were collected 35–40 days after anthesis and they may have lost the ability to synthesise GA_{32} (**5**) and GA_{87} (**6**). Alternatively, the rate of metabolism of GA_3 (**4**) to GA_{87} (**6**) may have been very slow in sweet cherry fruitlets and the four-day period during which these experiments were performed was insufficient for incorporation of $[^2H_2]$ into endogenous GA_{87} (**6**) to be apparent. However, since other labelled products were detected, it is more likely that GA_3 (**4**) is not converted to GA_{87} (**6**) as an intermediate in the biosynthesis of GA_{32} (**5**) by immature fruits or vegetative tissues of sweet cherry. Similarly, evidence was obtained that GA_1 (**1**) is converted into GA_8 (**10**) and it is not an intermediate in the biosynthesis of GA_{86} (**3**). To our knowledge this is the first report in which GA_8 (**10**) has been identified as a metabolite of GA_1 (**1**) in cherry fruitlets (and shoots), although it has been demonstrated previously in other species (for review, see MacMillan, 1997).

Our findings suggest that other GA-biosynthetic pathways, that yield GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**) as products, are operating in sweet cherry fruitlets. For instance, biosynthesis may be via 12α-hydroxylated intermediates, such as GA_{58} (**19**) and GA_{30} (**20**) these being 13-hydroxylated to GA_{85} (**2**) and GA_{87} (**6**), respectively. Early 12α-hydroxylation has been demonstrated in cell-free systems derived from *Cucurbita maxima* endosperm (Lange et al., 1993). While the 12α-hydroxy GAs, GA_{77} (**21**), GA_{111} (**22**), GA_{113} (**23**), and GA_{115} (**24**) were identified in *Raphanus sativus* (Nakayama et al., 1990; Nakayama et al., 1998). Also, GA_{30} (**20**) has been identified in immature seeds of *P. cerasus* (Nakayama et al., 1996), but was not detected in immature fruits of *P. avium* (Blake and Browning, 1994). Alternatively, GA_{111} (**22**) and the 12α,13 dihydroxyGAs; GA_{123} (**25**), GA_{124} (**26**), GA_{125} (**27**) and GA_{77} (**21**) were detected in immature strawberries and suggest that an early 12, 13 hydroxylation pathway may exist (Blake et al., 2000b). GA_{77} (**21**) would be **C**-3 hydroxylated to GA_{85} (**2**) and **C**-15 hydroxylated to GA_{86} (**3**) and it is possible that GA_{87} (**6**) is biosynthesised from GA_{77} (**21**) via 12α hydroxyGA_5 (**28**) by analogy with the biosynthesis of GA_3 (**4**) from GA_{20} (**11**) via GA_5 (**7**) (for review see MacMillan, 1997).

The occurrence of GA_3-isolactone (**17**) and 16,17-dihydro,17-hydroxygibberellinA_3 (**18**) following metabolism of GA_3 (**4**) by sweet cherry fruitlets is of interest. GA_3-isolactone (**17**) is usually considered to be an

Table 3
Comparison of normalised peak areas in SIM chromatograms for MeTMSi derivatives of GA_1, GA_3, GA_3-isolactone (GA_3-I) and GA_8 in extracts of juvenile and mature shoots after 0, 3, 8 and 24 h incubation[a]

Incubation time	Juvenile shoots				Mature shoots			
	GA_1[b]	GA_8[c]	GA_3[d]	GA_3-I[e]	GA_1[b]	GA_8[c]	GA_3[d]	GA_3-I[e]
0 h	100	0	100	0	97	0	109	0
3 h	73	43	95	13	59	61	89	52
8 h	45	117	87	43	33	142	70	85
24 h	26	100	74	100	9	126	51	189

[a] For GA_1, fractions 23, 24 and 25 were combined; for GA_3 fractions 22, 23 and 24 were combined; for GA_3-I fractions 19, 20 and 21 were combined; for GA_8 fractions 17, 18 and 19 were combined.
[b] Normalised to 0 h incubation by juvenile shoots.
[c] Normalised to 24 h incubation by juvenile shoots.
[d] Normalised to 0 h incubation by juvenile shoots.
[e] Normalised to 24 h incubation by juvenile shoots.

artefact, created either during the extraction procedure or during the GC run (Grünzweig et al., 1997). Our results suggest that in sweet cherry fruitlets these compounds result from metabolism of the exogenous GA_3 (**4**), as they were not detected in equivalent control samples by full-scan GC–MS or by SIM. However, isotope-dilution analysis indicated that protio-GA_3 (**4**) had not been metabolised to these products. Taken together this suggests that exogenous GA_3 (**4**) was metabolised to GA_3-isolactone (**17**) and 16,17-dihydro,17-hydroxygibberellinA_3 (**18**), while endogenous GA_3 (**4**) was not converted to these products. This may be accounted for in several ways; firstly, perhaps the amount of exogenous GA_3 (**4**) was too great and only exogenous GA_3 (**4**) was metabolised to reduce the 'pool' size to normal levels, quantitations indicated that the amount of exogenous GA_3 (**4**) was ca. 8 times the endogenous concentration. Secondly, the injection caused cell damage and GA_3-isolactone (**17**) and 16,17-dihydro,17-hydroxygibberellinA_3 (**18**) were formed as part of a wound-response reaction from the exogenous GA_3 (**4**), while control samples were frozen immediately after the injection preventing the accumulation of these metabolites. A third possibility is that the injection 'forced' exogenous GA into tissues from which endogenous GA is excluded and GA_3-isolactone (**17**) and 16,17-dihydro,17-hydroxygibberellinA_3 (**18**) were formed as part of a GA-deactivation process.

No qualitative differences were detected in the products formed by juvenile and mature shoots from [$^2H_2$]-GA_1 and [$^2H_2$]-GA_3, but there appeared to be small quantitative differences in the amounts of metabolite formed and the amount of substrate remaining. These findings suggest that there may be a greater rate metabolism of GA_1 (**1**) and GA_3 (**4**) in mature shoots of sweet cherry than in juvenile shoots. Evidence to support this view is available elsewhere. It is widely recognised that GA_1 (**1**) is an important factor in the regulation of shoot elongation (for review, see MacMillan, 1997) and rapid metabolism of GA_1 (**1**) has been correlated with the reduced shoot growth (Rood, 1986). While, Olivera and Browning (1993) noted reduced shoot growth of mature sweet cherry trees compared to juvenile trees and that exogenous GA_1 (**1**) did not promote spur shoot growth on mature cherry trees, while this GA promoted shoot growth of juvenile trees. These observations may be explained at least in part, by a greater rate of conversion of GA_1 (**1**) to GA_8 (**8**) in shoots of mature trees than in shoots from juvenile trees, but further investigation is required to confirm this hypothesis.

4. Experimental

4.1. Plant material

Rapidly expanding, immature fruitlets in which the embryo nucellus was invisible and the integument was fully expanded were collected 35–40 days after anthesis from trees of the self-fertile sweet cherry cv. 'Stella' grown on 'Colt' rootstocks in the orchard at East Malling. The pedicels of the fruitlets were cut under water and any fruitlets that floated were rejected. The cut surface of the pedicel was coated with anhydrous lanolin in the field, and the fruitlets placed into a coolbox lined with moist, absorbent paper for transfer to the laboratory.

Actively growing shoots with five fully expanded leaves were collected from 2-year-old seedlings raised from seeds collected from self-pollinated cv. 'Stella' fruits and 11-year-old mature trees (cv. 'Stella' on 'Colt' rootstocks), respectively. The shoots were cut under water and transported to the laboratory with their cut-ends in water.

4.2. Isotope-labelled GAs

17,17[$^2H_2$]-GA_1 and 17,17[$^2H_2$]-GA_3 were purchased from Professor L.N. Mander, Australian National University, Canberra, Australia. [1,2-^{3}H]-GA_1 (1406 GBq mmol^{-1}) was obtained from Du Pont de Nemours GmbH, NEN Division, Dreiech, Germany and [^{3}H]-GA_3 was a generous gift from Dr. Peter Hedden, IACR, Long Ashton Research Station. Both radioactive GAs were purified by reverse-phase HPLC using a Nucleosil 5 C18 column (250×8 mm) eluted at a flow rate of 2.0 ml min^{-1} with a linear gradient of 10% MeOH–80% MeOH, containing 50 μl l^{-1} glacial acetic acid, over 35 min. Fractions were collected every 30 s. and aliquots of these were monitored for radioactivity by liquid scintillation counting. Fractions were combined as necessary and lyophilised. Samples were redissolved in EtOH prior to use.

4.3. Application of treatments

For each of the GA treatments, 75 fruitlets collected from juvenile and mature plants. Fruitlets were injected with 2 μl of EtOH containing either 90 ng [$^2H_2$]-GA_1 and 1816 Bq [^{3}H]-GA_1 or 90 ng [$^2H_2$]-GA_3 and 1493 Bq [^{3}H]-GA_3. The injection was made into the suture one third of the way round the fruit from the pedicel. The needle at angle of 45° penetrated the endocarp and into the endosperm. The fruitlets were incubated in a humid atmosphere at 25°C, under a 16 h photoperiod. Subsamples of 25 fruitlets from each treatment were removed after 0, 48 or 96 h incubation. The fruitlets were rinsed in water, dried on absorbent paper and then frozen in liquid N_2 and stored at −20°C until analysis.

Shoots were treated by recutting the stem under water to just above the node of the third fully expanded internode. The freshly cut ends of the shoots were placed quickly into 0.5 cm^3 water in vials containing either 2150 Bq [^{3}H]-GA_1 and 2 ng [$^2H_2$]-GA_1 or 2967 Bq [^{3}H]-GA_3 and 2 ng [$^2H_2$]-GA_3. Two shoots were placed in each vial and ten vials were used for each treatment. Shoots were incubated in a growth room at 25°C under a 16 h photoperiod. Most of the water in the vials was absorbed by the shoots after 30 min and a further 0.5 cm^3 water was added. Further water was added to the vials in 2 cm^3 portions as necessary. After 3, 8 or 24 h incubation the shoots were removed from the vials, the cut ends rinsed in water and dried on absorbent paper, then weighed and frozen in liquid N_2 and stored at −20°C until extraction.

Fifty control shoots (time 0 h) for each treatment were prepared as above, but were then treated by injecting 2 μl of EtOH containing 2450 Bq [^{3}H]-GA_1 and 2 ng [$^2H_2$]-GA_1 or 2566 Bq [^{3}H]-GA_3 and 2 ng [$^2H_2$]-GA_3 near the base of each re-cut shoot. The shoots were plunged into liquid N_2 immediately after injection.

4.4. Extraction and purification

The frozen samples were homogenised with cold (4°C) 80% MeOH containing 20 mg l^{-1} BHT (1.0 g fw to 10.0 cm^3) and the extracts purified using methods described previously (Blake et al., 1993). After HPLC, 50 μl aliquots were taken for scintillation counting from each of the 50, 1.0 cm^3 fractions collected and the remainder evaporated to dryness on a centrifugal vacuum concentrator (CVC), redissolved in 100 μl MeOH and methylated with excess ethereal diazomethane.

The Me esters were taken to dryness under a stream of O_2–free N_2 and redissolved in 20 μl MeOH and 80 μl water added. Fractions containing radioactivity were further purified by HPLC using the same solvent gradient as was used for the free acids. An aliquot of 50 μl was taken from each fraction for scintillation counting and then the fractions containing radioactivity were derivatised. The TMSi ethers were prepared for GC–MS by redissolving in 25 μl BSA/pyridine (50:50 vol/vol), heating to 100°C for 30 min, evaporating to dryness and dissolving in 10 μl BSTFA. Some samples were further purified by normal phase TLC and were dissolved in 50 μl anhydrous CH_2Cl_2. For TLC, extracts were applied in a narrow band to 20×20 cm aluminium-backed, silica gel coated (0.2 mm; Merck 5553), pre-washed plates and Me-GA standards applied within a scored strip next to each vertical edge of the plate. The plates were developed using $CHCl_3$/MeOH (9:1 v/v) as the solvent, the scored strips cut from each side of the plate, which were sprayed with 10% H_2SO_4 in EtOH, heated at 110° for 10 min, and the position of GAs marked under UV light (375 nm). Silica gel was removed in broad bands from the remainder of the plates as appropriate, placed in plugged Pasteur pipettes and eluted with EtOH, which was then taken to dryness (CVC), and the extract derivatised for GC–MS as above.

4.5. Capillary column GC–MS

The MeTMSi derivatives were analysed using an HP 5890 GC coupled to a VG TRIO_1 MS using the conditions described previously (Blake et al., 1993). Samples (1.0 μl) were co-injected with 0.1 μl of a parafilm extract for calculation of KRIs (Gaskin and MacMillan, 1991). Samples for comparison of peak areas by SIM were combined as necessary and diluted with BSTFA to a final concentration equivalent to 2.0 g f.wt. μl^{-1}.

Acknowledgements

This paper is dedicated to the memory of Dr. Gordon Browning who sadly died during the course of this investigation. The authors are grateful to government of The People's Republic of China for financial support of Ma Huanpu and to the UK Ministry of Agriculture, Fisheries and Food for supporting P.S.B., G.B. and J.M.T. in this work.

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Profiling changes in metabolism of isoflavonoids and their conjugates in *Lupinus albus* treated with biotic elicitor

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Abstract

Liquid chromatography with ultraviolet and mass spectrometric detection was applied to monitor changes in profiles of isoflavonoid glycosides and free isoflavonoid aglycones in *Lupinus albus* L. Four isoflavonoid aglycones, fourteen isoflavonoid glycosides, four flavonol glycosides and flavone glycoside were identified in lupin tissue after LC/ESI/MS analyses. An elicitor preparation from purified yeast cell wall was used to inject the shoots of 3-week old seedlings or to infiltrate the cut lupin leaves. Qualitative and quantitative changes of isoflavonoids were measured at different time points after elicitation. In elicited lupin seedlings increased amounts of prenylated isoflavone aglycones were identified. The concentrations of glycosidic conjugates of isoflavones present in plant tissue were less affected. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Lupinus albus*; White lupin; *Leguminosae*; Flavone, flavonol and isoflavone glycosides; Isoflavone aglycones; Liquid chromatography–mass spectrometry; Desorption ionisation techniques; Electrospray; Elicitor

1. Introduction

Various classes of secondary metabolites play very important roles in the plant defence during infection (Hammerschmidt, 1999). In the plants belonging to family Leguminosae isoflavonoid derivatives possessing different structures are synthesised and accumulated as phytoalexins in response to infection with pathogenic microbes (Dakora and Phillips, 1996; Stafford, 1997). On the other hand, the different classes of phenylpropanoid compounds act as regulators of *nod* genes during interactions of legumes with nitrogen fixing, symbiotic bacteria collectively known as rhizobia (Lerouge, 1994; Spaink, 1995).

In many agronomically important species (soybean, pea, alfalfa, clover and bean) phytoalexins possessing a pterocarpan-type structure are synthesised de novo after pathogenic infection (Dixon, 1986; Dakora and Phillips, 1996). In lupin plants, prenylated derivatives of genistein and 2′-hydroxygenistein play a role if antimicrobial substances present constitutively in plant tissue (Ingham et al., 1983; Wojtaszek and Stobiecki, 1997); they can be considered as phytoanticipins (Van Etten et al., 1994). In addition many other minor isoflavonoids, with different patterns of prenylation on the isoflavone skeleton, or additional pyran or furan ring condensed to A-rings were identified in the white lupin (Tahara et al., 1984, 1989; Shibuya et al., 1991).

Graham and coworkers published a series of papers on application HPLC with UV detection for profiling of qualitative and quantitative changes of phenylpropanoid compounds in soybean cotyledons subjected to elicitation with cell wall glycans from *Phythophtora sojae*, a pathogen of soybean (Graham et al., 1990; Graham, 1991a,b, 1995; Graham and Graham 1991a,b, 1994). Distinct changes in concentration of glyceolin and the glycosylated form of its precursor — malonylated daidzin and daidzin itself were observed. The increase of genistein glycoside concentration was also registered (Graham and Graham, 1991a). Different physico–chemical methods (NMR, MS) were used for structural elucidation of flavonoid glycosides from *Arabidopsis thaliana* (Graham, 1998). Compounds identified were further used as standards for profiling flavonoid glycosides in putative phenylpropanoid mutants of

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Arabidopsis, according to the HPLC/UV protocol published by Graham (1991a,b).

LC with UV detection was used for studies of isoflavonoid profiles in roots of white and yellow lupin (*Lupinus albus, Lupinus luteus*) seedlings elicited with biotic or abiotic elicitors (Gagnon and Ibrahim, 1997; Kneer et al., 1999). In these studies the main interest was towards analyses of free aglycones present in the plant tissue.

In the leguminous plants isoflavonoid glycosides are partially present as acylated conjugates (Köster et al., 1983; Kessman and Bartz, 1986; Shibuya et. al., 1991; Graham, 1995; Sumner et al., 1996; Lin et al., 2000). Glycosides acylated with aliphatic acids are labile compounds and their deacylation occurs easily during isolation and purification.

Liquid chromatography coupled with a mass spectrometer equipped with different ionization techniques [thermospray (TSP), electrospray (ESI), continuous-flow liquid secondary mass spectrometry (CF-LSIMS)] was applied for analyses of secondary metabolites from biological material (Niessen, 1999; Stobiecki 2000). Utilization of LC/MS systems may overcome the problems related to the identification of acylated flavonoid glycosides. On the basis of m/z values of protonated molecules $[M+H]^+$ and fragment ions, it is possible to identify an acyl substituent linked to a sugar moiety in an isoflavonoid glycoside.

LC/CF-LSIMS was used for the identification of flavonoid glycosides and their acylated conjugates in extracts from alfalfa (*Medicago sativa*) cell cultures and cotyledons or hypocotyls of chickpea (*Cicer arietinum*) by Sumner et al. (1996). On the other hand LC/ESI/MS systems were also used for the detection of isoflavonoid glycosides and free aglycones in extracts from white lupin roots (Stobiecki et al., 1999) and in red clover (*Trifolium pratense*) Lin et al., 2000). Different malonylated or acetylated isoflavonoid glycosides were detected in the above mentioned sources.

During our research we demonstrated the applicability of LC/ESI/MS for direct identification of acylated and non-acylated isoflavonoid glycosides present among other compounds in white lupin. These conjugates play an important role as substrates for synthesis in plants of antimicrobial compounds in response to infection with pathogenic fungi or bacteria, so their direct identification and possibilities of profiling and quantification in plant material is of great importance.

2. Results and discussion

2.1. Structural studies of flavonoid glycosides

For structural elucidation of flavonoid glycosides isolated from roots and green parts of *Lupinus albus* seedlings mass spectrometric techniques were used; this methodological approach was described earlier by Frański et al., (1999a,b). Ten unmodified mono- and diglycosides were identified in the plant material (Table 1, footnote a). Molecular masses of the glycosides were established by liquid secondary ions (LSI) mass spectra. Also linked scans (B/E mode) were recorded for unmodified and permethylated compounds. On the basis of fragment ion masses created after cleavage of glycosidic bonds, sizes of sugars and aglycone moieties were estimated. For GC/MS analysis, flavonoid glycosides were methylated, methanolysed and again methylated or acetylated; aglycones and sugars were identified by mass spectra and retention times. Placement of sugar substitution on the aglycones was also established for all studied glycosides. In the case of kaempferol 3-*O*-galactoside (**14**) and isorhamnethin 3-*O*-glucoside (**15**), structures of these compounds were determined, when both compounds were present in the mixture. Differences in concentrations of the flavonoid glycosides in the sample allowed us to estimate the substitution of glucose and galactose to the appropriate aglycones on the basis of the respective peak areas of sugars and aglycones in the total ion current chromatogram obtained by GC/MS analysis of methanolysis products.

After LC/UV analyses of the root, stem or leaf extracts from seedlings of white lupin, purified by solid phase extraction (SPE) on double cartridges with cation exchangers and RP C-18 silica gels (Stobiecki et al., 1997), ten peaks of flavonoid glycosides, identified earlier in white lupin or other lupin species, were recognised (Shibuya et al., 1991; Frański et al. 1999a,b), (Table 1, footnote a). Two groups of phenolic compounds (flavonoid and isoflavonoid conjugates) were detected on the basis of differeces in UV absorption at $\lambda = 260$ and 350 nm (Fig. 1a). Individual compounds were recognised according to R_t values and UV spectra of standards. A few additional peaks with UV absorption characteristic of isoflavonoids were also registered. Compounds characterised as the isoflavonoid glycoside conjugates were identified by LC/ESI/MS, according to the masses of ions recorded in the mass spectra. All flavonoid conjugates detected by LC/ESI/MS analyses of root, leaf and stem extracts are presented in Table 1 (footnote b). Comparison of the LC/UV chromatogram with single ion chromatograms of chosen protonated molecules $[M+H]^+$ of compounds from an extract of lupin leaves is presented in Fig. 1.

On the basis of m/z values of protonated molecules $[M+H]^+$ and fragment ions $[A+H]^+$ created after a cleavage of the glycosidic bond between sugar and aglycone, the peaks unidentified earlier by LC/UV, after comparison with standards, were ascribed as 6″-*O*-malonylated derivatives of genistein and 2′-hydroxygenistein mono- and diglycosides (**4**, **5**, **10**, **17**, **18**), 3′-*O*-methylorobol 7-*O*-glucoside (**9**) and its 6″-mal-

onylated derivative (**19**) respectively. The presence of 3′-*O*-methylorobol 7-*O* glucoside (**9**) and its malonylated derivative (**19**) was also suggested from the pattern of UV light absorbance in the LC/UV chromatogram characteristic for isoflavonoids (see Fig. 1a peaks 10 and 19). This isoflavone was earlier identified in lupin leaves (Tahara et al., 1989).

Complementary information about structures of flavonoid glycosides in lupin tissue was obtained by GC/MS. Flavonoid glycosides (from roots and leaves) were derivatized (methylated, methanolysed and again methylated (with CH_3I or CD_3I) or acetylated). The GC/MS analysis of free aglycone derivatives in these samples revealed the presence of genistein, 2′-hydroxygenistein, kaempferol and isorhamnetin. An additional isoflavone peak was recognised in the total ion chromatogram of permethylated hydrolysis products. Its mass spectrum demonstrated the presence of a tetrahydroxy flavone or isoflavone, with the molecular ion of methylated derivative at m/z 342. The presence of a single methyl group in the B ring of this compound was shown by the m/z value of molecular ion M^+ and the fragmentation pathway was elaborated from the mass spectrum registered during GC/MS analysis of the methanolysed leaf extract, methylated with deuterated methyl iodide (CD_3I). On the basis of fragmentation pathways similar to genistein and 2′-hydroxygenistein (Stobiecki and Wojtaszek, 1990) and R_t values, different from those of kaempferol and luteolin, the presence of 3′-*O*-methylorobol 7-*O*-glucoside in green parts of lupin has been proposed. In total ion chromatograms of methanolysed root extract samples, a peak of isoflavone with four *O*-CD_3 groups and with the R_t value almost exactly the same as 3′-*O*-methylorobol methylated with CD_3I was recognised. As the fragmentation pattern was similar, we identified the isoflavone present in the white lupin root as orobol, but orobol 7-*O*-glucoside (**7**) was not detected by LC/ESI/MS, probably because of coelution with 2′-hydroxygenistein 7-*O*-glycoside (**6**). However, the presence of compound (**7**) was reported earlier in white lupin roots by Laman and Oleksyuk (1982).

Profiles of the flavonoid glycosides differed in various organs of lupin seedlings. The highest total amounts of flavonoid glycosides, expressed as genistein 7-*O*-glucoside equivalents, were found in roots and leaves, reaching 650 and 600 μg/g fresh weight, respectively, only in stems this level was lowered to 300 μg/g fresh weight. Although total amounts of flavonoid compounds differed between experiments, the metabolic profiles and their quantitative ratios were the same (data not shown). We speculate that this variability was due to differences in light intensities during growth and development of seedlings in the greenhouse during different

Table 1
Flavonoid glycosides detected in roots, shoots and leaves of *Lupinus albus* L. by LC/ESI/MS

No	Compound	M_r	Root	Stem	Leaves
1	2′-hydroxygenistein 7,4′-*O*-diglucoside[a,b]	610	±	±	±
2	genistein 7,4′-*O*-diglucoside[a,b]	594	+	+	+
3	isorhamnetin diglucoside[b]	640	–	–	+
4	genistein 7-*O*-glucoside-4′-*O*-(6″-*O*-malonyl)-glucoside[b] and/or	680	+	±	±
5	genistein 7-*O*-(6″-*O*-malonyl)-glucoside)-4′-*O*-glucoside[b]	680	+	±	±
6	2′-hydroxygenistein 7-*O*-glucoside[a,b,c]	448	+	+	+
7	orobol 7-*O*-glucoside[b,c]	448	+	–	–
8	genistein 7-*O*-glucoside[a,b,c]	432	+	+	+
9	3′-*O*-methylorobol 7-*O*-glucoside[b,c]	462	–	+	±
10	2′-hydroxygenistein 7-*O*-(6″-*O*-malonyl)-glucoside[b,c]	534	+	+	+
11	kaempferol 3-*O*-glucoside[a,b]	448	–	±	+
12	apigenine 7-*O*-glucoside[a,b]	432	+	–	–
13	genistein 4′-*O*-glucoside[a,b,c]	432	+	–	±
14	isorhamnetin 3-*O*-glucoside[a,b]	448	–	±	+
15	kaempferol 3-*O*-galactoside[a,b]	478	–	±	+
16	isorhamnetin 3-*O*-galactoside[b]	478	–	±	+
17	genistein 4′-*O*-(6″-*O*-malonyl)-glucoside)[b,c]	518	+	+	+
18	genistein 7-*O*-(6″-*O*-malonyl)-glucoside[a,b,c]	518	+	–	+
19	3′-*O*-methylorobol 7-*O*-(6″-*O*-malonyl)-glucoside[b]	548	–	+	+
20	2′-hydroxygenistein[a,b,c]	286	+	+	+
21	genistein[a,b,c]	270	+	+	+
22	luteone[a,b,c]	354	+	±	±
23	wighteone[a,b,c]	338	+	±	±

[a] Compounds identified earlier in lupin (*Lupinus albus*) with mass spectrometric techniques in our laboratory.

[b] Presence in lupin organs detected on the basis of mass spectra and single ion currents monitoring of $[M+H]^+$ and $[A+H]^+$ during LC/ESI/MS analyses.

[c] Presence of the compound in lupin reported earlier in the literature.

experiments. Glycosides of kaempferol and isorhamnethin (**11**, **14–16**) were present only in the green parts of lupin seedlings; apigenin 7-*O*-glucoside (**12**) was detected only in the roots. Relative quantities of acylated isoflavonoid glucosides varied in different plant organs. The highest ratio of 6″-*O*-malonylated di- and monoglucosides of isoflavonoids to non acylated compounds was found in roots and stems; only in leaves

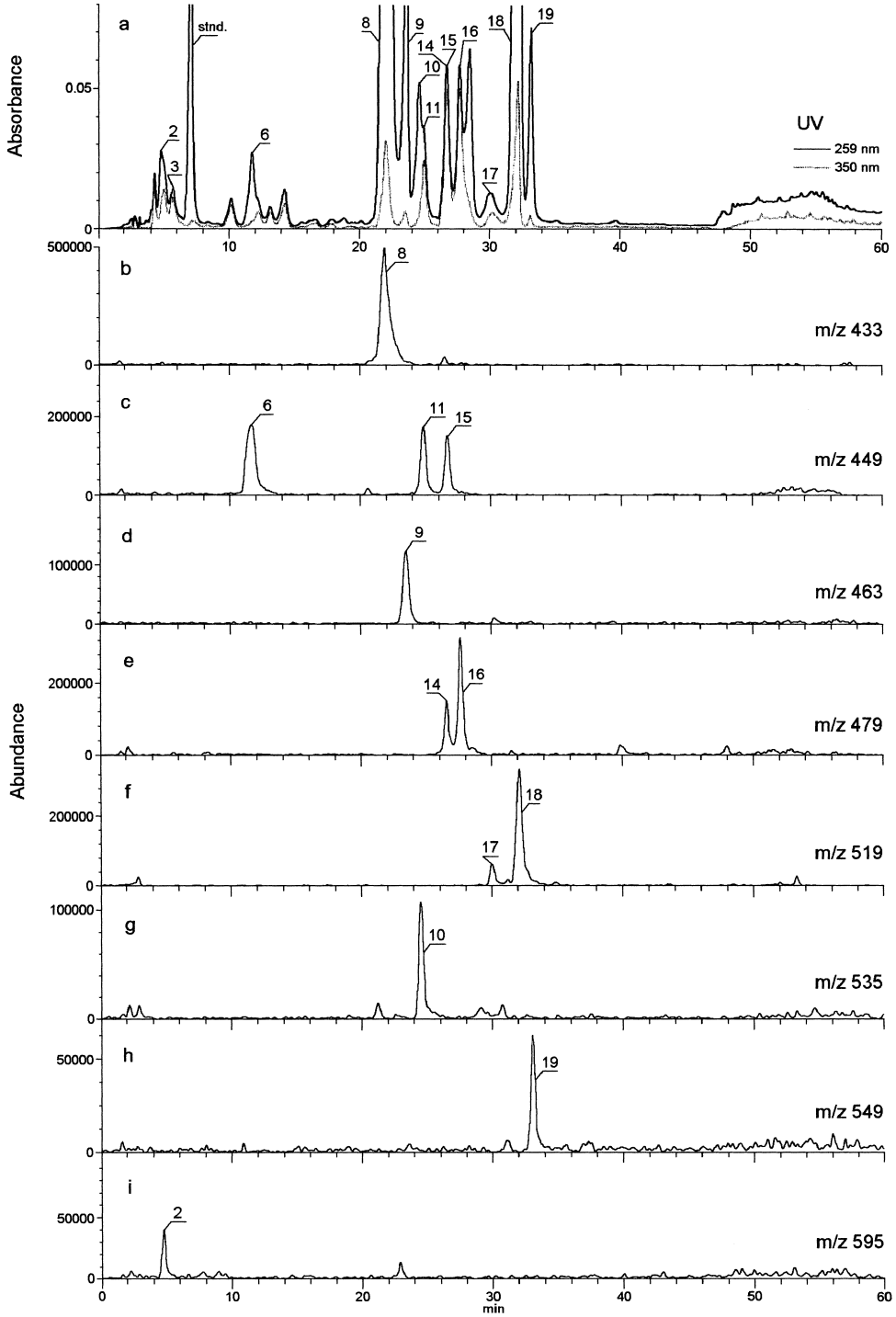


Fig. 1. Ultraviolet chromatogram of flavonoids identified in lupin leaves (a); single ion chromatograms of protonated molecules $[M+H]^+$ of chosen flavonoid and isoflavonoid glycosides: genistein 7-*O*-glucoside (b); 2′-hydroxygenistein 7-*O*-glucoside, kaempferol 3-*O*-glucoside and kaempferol 3-*O*-galactoside (c); 3′-*O*-methylorobol 7-*O*-glucoside (d); isorhamnetin 3-*O*-glucoside and isorhamnetin 3-*O*-galactoside (e); genistein 4′-*O*-(6″-*O*-malonylglucoside) and genistein 7-*O*-(6″-*O*-malonylglucoside) (f); 2′-hydroxygenistein 7-*O*-(6″-*O*-malonylglucoside) (g); 3′-*O*-methylorobol 7-*O*-(6″-*O*-malonylglucoside) (h); genistein 7,4′-*O*-diglucoside (i).

amounts of non-acylated glycosides exceed those of malonylated compounds. Free aglycones constituted at maximum a few percent of the total amount of isoflavonoids; the highest ratio of free aglycones to glycosides was found in the lupin roots (Wojtaszek and Stobiecki, 1997).

2.2. Profiling of flavonoids and their conjugates in healthy and elicitor treated white lupin

Two different experiments on elicited lupin plants were conducted. In the first one cut leaves were infiltrated with the elicitor preparation from yeast cell wall (YE) used at a concentration of 100 μg of glucose equivalents/ml. In the second experiment the elicitor solution was injected into the shoot of lupin seedlings, 10 μl (500 μg/ml) into each seedling. Quantitative changes of isoflavonoid glycosides and free isoflavonoid aglycones in elicited and control plant material were analysed with high performance liquid chromatography equipped with a diode array detector (DAD). Samples of phenolic compounds present in the extracts from lupin stems and infiltrated leaves were concentrated and purified by solid phase extraction (SPE).

In YE-infiltrated leaves a substantial two–four-fold increase in the concentration of free aglycones: genistein (**21**), 2′-hydroxygenistein (**22**) and their prenylated derivatives (luteone and wighteone) (**22**, **23**) was observed (Fig. 2). Concentrations of glycosides were on comparable levels in elicited and control leaves. However, slightly increased amounts of 2′-hydroxygenistein conjugates (**6**, **10**) were noted 24 and 48 h after infiltration. This was not the case for genistein glucosides (**8**, **18**). Although an effect of wounding was observed on the concentration of free aglycones, the elicitation appeared to be a stronger stimuli and affected the concentrations of aglycones to a higher degree. The increase observed in free aglycone concentration was higher between 24 and 48 h after the elicitation, than during first 24 h of the experiment (Fig. 2).

When lupin stems were elicited by the injection of YE, in the tissue proximal to the injection point, an increase of prenylated aglycones concentrations (luteone and wighteone (**22**, **23**)), was observed. It started from the 4th h of the experiment and further steady increase of aglycones quantities was noted up to 48 h. These data are consistent with the information of Graham and coworkers about changes of soybean phytoalexin–glyceolin concentration in tissue proximal to the point of elicitation with a *Phythophtora sojae* elicitor (1991a,b, 1994). Genistein and 2′-hydroxygenistein concentrations in control and elicited stem samples were on a very low level, slightly above the detection limit, and their quantification was not reliable (see Fig. 3). Amounts of 2′-hydroxygenistein glucoside (**6**) and its malonylated derivative (**10**) were almost two times higher 48 h after elicitation; however concentrations of genistein conjugates were more or less on the same level throughout the experiment (Fig. 3). In the other plant organs (roots, leaves or hypocotyls), analysed after injection of elicitor to the stem, significant differences in the concentration of isoflavonoid aglycones and their conjugates were not observed between control and elicited plants (data not presented).

3. Conclusion

Hyphenation of mass spectrometry with the electrospray ionisation technique to the detection system of liquid chromatography improves the possibilities of the detection of phenolic compound conjugates in the samples obtained from plant material. After elicitation of lupin plants with biotic factor (YE), substantial changes in the concentrations of modified isoflavonoids, mainly prenylated derivatives (wighteone and luteone (**22**, **23**) were detected; they take the role of phytoalexins in lupin because of their high antimicrobial activity (Ingham et al., 1983). Concentrations of glycosidic conjugates of isoflavonoids were less affected by elicitation with YE.

4. Experimental

4.1. Biological material

Seeds of white lupin (*Lupinus albus* L. cv. Bac) were sterilised with 1.5% KOCl for 20 min, washed several times with sterile deionised water and grown for 3 weeks under greenhouse conditions.

The yeast elicitor (YE) was prepared as described previously (Wojtaszek and Stobiecki, 1997). The concentration of the YE preparation was determined as glucose equivalents by the phenol–sulphuric acid method (Fry, 1994).

Two different kinds of tests with YE were carried out. In the first type of experiments YE solution (500 μg/ml) was injected (10 μl) into the plant stem. The same volume of water was injected into control plants. Fragments of stem 20 mm long (10 mm each side from the point of injection) were cut off at the defined time points between 0 and 48 h. The second test was performed on infiltrated white lupin leaves. Cut leaves were submerged into the YE preparation (100 μg/ml) and infiltration was performed in this solution during five cycles of reduced pressure, for 40 s each; control leaves were infiltrated in the same way with water. Infiltrated leaves were transferred to 10-cm diameter sterile Petri dishes leaves (four leaves per plate), covered with wet filter paper. The dishes were incubated in a chamber with a 16/8 h photoperiod. Leaves were collected after 24 and 48 h and profiles of phenolic compounds were analysed.

4.2. Isolation of phenolic compounds

The plant material was homogenised in 80% MeOH (20 ml per 1 g fresh weight) and placed in a ultrasonic bath for 30 min. These suspensions were filtered on a Büchner funnel and concentrated under vacuum (40°C). The samples of phenolic compounds for LC and GC analyses were purified and concentrated with solid

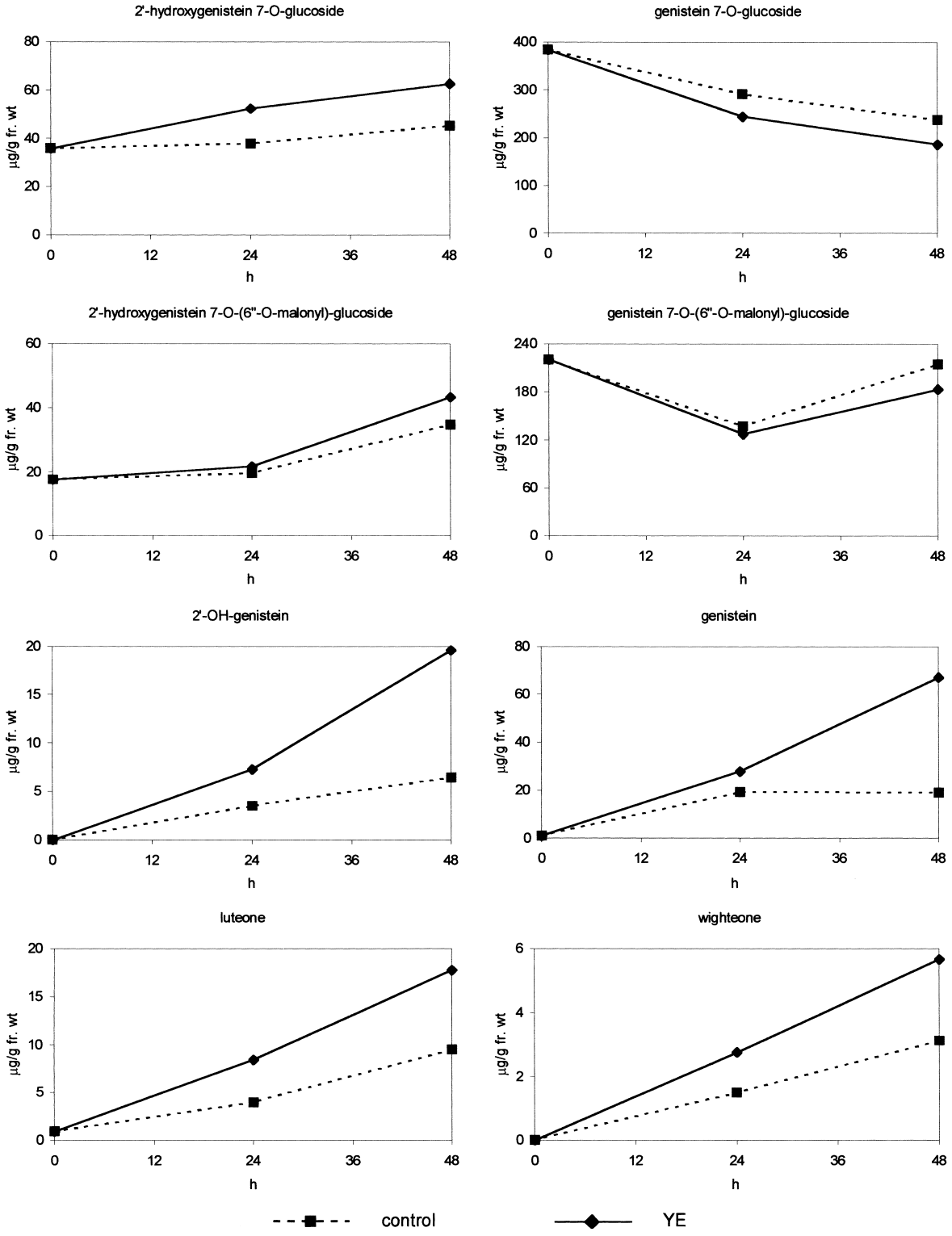


Fig. 2. Time related changes of concentrations of isoflavonoid glycosides and free isoflavonoid aglycones in lupin leaves infiltrated with YE. Determinations were repeated twice each for eight leaves. Similar results were obtained in both experiments.

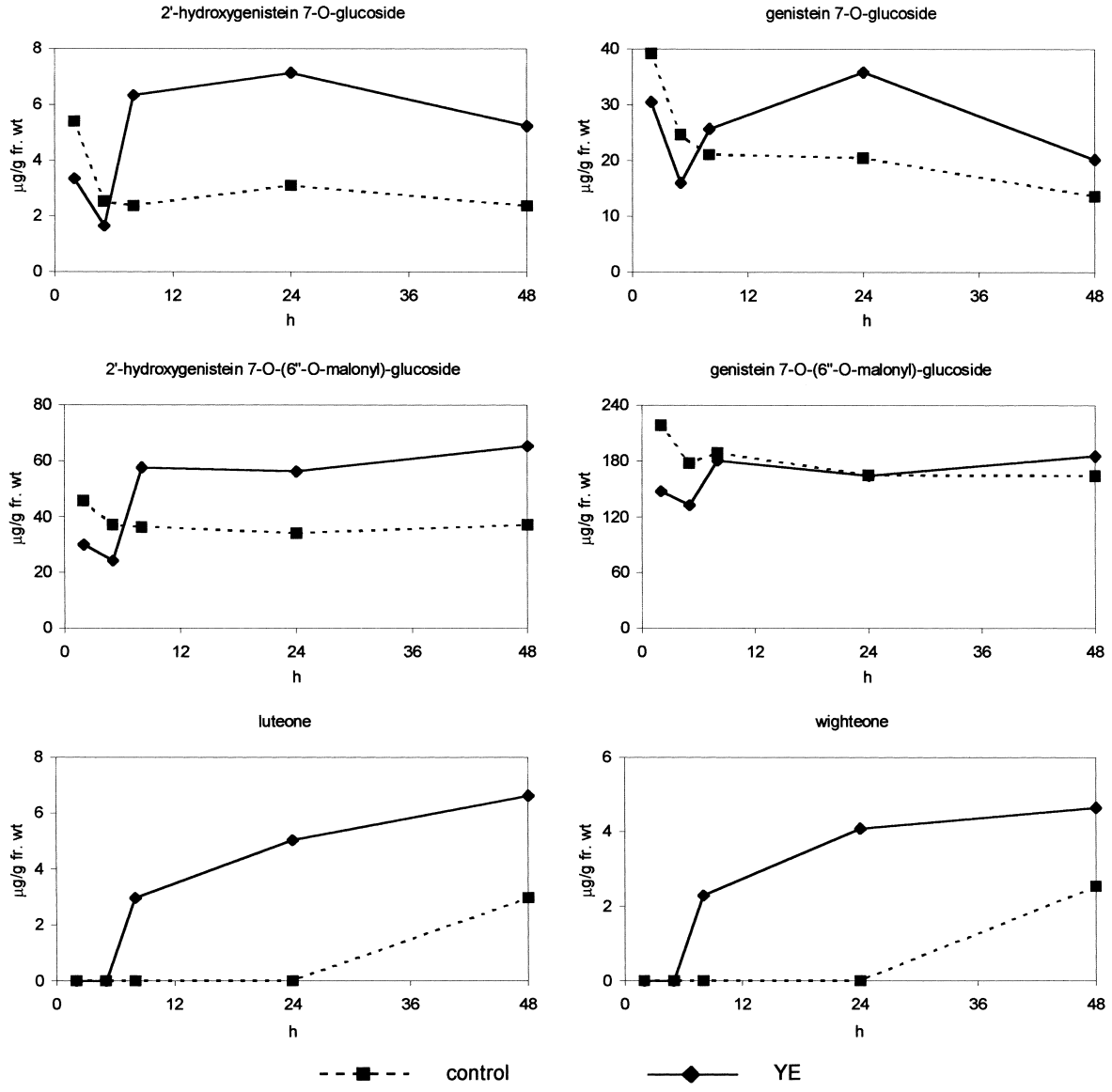


Fig. 3. Time related changes of concentrations of isoflavonoid glycosides and free isoflavonoid aglycones in lupin stem after injection of YE. Determinations were repeated twice each for eight leaves. Similar results were obtained in both experiments.

phase extraction (SPE) method on cartridges with cation exchanger and RP C-18 silica gel combined in tandem (Alltech, Carnforth, England) according to Stobiecki et al. (1997). Samples for GC analysis were methylated, methanolysed and again methylated or acetylated, as described previously (Frański et al., 1999a,b). Flavonoid glycosides standards prepared for LC analyses were isolated from healthy lupin plants (roots and green parts) (Frański et al., 1999a,b). Isolated and identified glycosides were used further as standards for LC analyses.

4.3. Liquid secondary ion mass spectrometry

Mass spectral analyses were performed on an AMD Intectra (Germany) double-focusing, reversed-geometry *B–E* mass spectrometer, which was fitted with a cesium ion gun operating at an ion energy of 12 keV and an accelerating voltage of 8 kV. Samples were dissolved in different matrices (glycerol, thioglycerol, nitrobenzyl alcohol, triethanolamine) and analysed in positive and negative ion modes. Collision-activated dissociation (CAD) in the linked-scan *B*/*E* mode was performed with helium as the collision gas in the cell in the first field-free region such that the intensity of the ion beam was reduced by 50% of its value. CAD mass spectra were recorded at a scan rate of 20 s per decade.

4.4. LC/UV and LC/ESI/MS analyses

LC/ESI/MS analyses were performed on a Nermag model R 10-10 (Quad Service, Argenteuil, France)

quadrupole mass spectrometer equipped with an electrospray ion source (Analytica, Brandford, CT, USA). The instrument was coupled with a Varian model 9012 HPLC pump (Harbour City, CA, USA). The samples were injected through a 2 μl injection loop onto a MB2-SP-C18 RP column (200×2 mm id.; Varian) using a 0.2 ml/min flow rate. At the outlet the effluent was split 3:1 so that 50 μl were delivered to the electrospray ion source and 150 μl passed through a variable-wavelength UV detector (model 481; Waters, Bedford, MA, USA) operating at a wavelength of 259 nm. MS were recorded in the positive ion mode. Nitrogen was used both as drying and auxiliary gas. Acquisition of the spectra was performed at 1.3 scan/s using an HP Chemstation data system. The elution protocol was carried out using two solvent mixtures: A (95% MeCN, 4.5% H_2O, 0.5% HOAc; v/v/v) and B (95% H_2O, 4.5% MeCN, 0.5% HOAc v/v/v). Elution steps were as follows: 0–5 min isocratic at 10% A, 5–40 min linear gradient from 10 to 30% of A, 40–48 min linear gradient up to 100% of A, 48–60 min isocratic at 100% of A. Flavonoids were identified by their MS spectra and/or by their retention times compared with data obtained for standards.

Quantitative analyses were performed on a Waters HPLC pump Model Alliance, equipped with a Waters diode array detector Model M996 (Bedford, MA, USA). To each analysed sample 125 μM *p*-hydroxybenzoic acid was added as internal standard. Quantification of total isoflavones was achieved by integration of UV chromatograms and comparison with the respective calibration curve for genistein 7-*O*–βD-glucoside prepared in the range 10–500 μM.

4.5. GC/MS analysis

Samples were subjected to GC/MS analyses on a Hewlett–Packard (Palo Alto, CA, USA) gas chromatograph Model 5890/II with a mass-selective detector Model 5971A. The instrument was equipped with an XTI-5 fused-silica capillary column (30 m×0.25 mm i.d.) (Restek, Bellefonte, PA, USA). The carrier gas was helium at a flow-rate 1 ml/min. The column temperature was programmed from 140°C (held for 2 min) at $5°C \times min^{-1}$ to 300°C, which was maintained for 5 min. The injector temperature was 250°C. Mass spectra were recorded in the range 50–650 amu.

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A distinctive flavonoid chemistry for the anomalous genus *Biebersteinia*

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Abstract

Leaf surface extracts of *Biebersteinia orphanidis* have yielded a complex mixture of five flavones with the unusual 5,7-dihydroxy-6,8-dimethoxy A ring substitution pattern. They are acerosin, hymenoxin, nevadensin, sudachitin and 5,7,4′-trihydroxy-6,8-dimethoxyflavone. Also present at the leaf surface are gardenin B, luteolin, apigenin, acacetin and the coumarin umbelliferone. The internal leaf flavonoids include the 7-glucosides of apigenin, luteolin and tricetin, together with the 7-rutinosides of apigenin and luteolin. This profile differs from those of *B. heterostemon* and *B. odora*. It appears that *B. orphanidis* is as highly distinctive in its flavonoid pattern as it is phytogeographically. The data also confirm the conclusion of other studies, including *rbc*L and *atp*B gene sequence analysis, that *Biebersteinia* is completely unrelated to the Geraniaceae, where it was once placed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Flavonoids; *Biebersteinia*; Chemotaxonomy

1. Introduction

Biebersteinia Stephan is a genus of five rare, herbaceous species distributed in the mountainous semi-arid areas of central and western Asia and the eastern Mediterranean as far as Greece. Its taxonomic position has been in dispute since its discovery in 1806. In 1841, Endlicher gave it familial status, while Boissier included it first in the Zygophyllaceae and then later (1867) in the Geraniaceae. Subsequent authors, notably Kunth (1931), have followed the latter classification. However, a palynological study by Bortenschläger (1967) suggested it was wrongly placed in the Geraniaceae and this was supported by later anatomical (Tutel, 1982), embryological (Kamelina and Komarova, 1990) and chemotaxonomic (Bate-Smith, 1973) studies. A recent phylogenetic analysis of *rbc*L and *atp*B gene sequences of *B. orphanidis* Boiss. (Bakker et al., 1998) indicated that the genus was nested in the Sapindales but with no link to any other clade within this order or within the Geraniales.

Phytochemically, the genus has not been analysed in detail. Bate-Smith (1973) in a phenolic survey of *Geranium* and related genera examined *Bierbersteinia multifida* DC. and *B. odora* Stephan and found that they had completely different flavonoid patterns from those known for any other genus of the Geraniaceae. He reported myricetin, prodelphinidin and procyanidin from *B. odora* and a number of unusual unidentified flavonoids from both taxa.

A later phytochemical paper included a study of the flavones (luteolin 7-glucoside and 7-rutinoside) (Omurkamzinova et al., 1991) of *B. multifida*. In the Tibetan species, *B. heterostemon* Maxim. (Zhang et al., 1995) a rare flavone, 8-hydroxytricetin 8,4′,5′-trimethyl ether was reported together with a luteolin *C*-glucoside, quercetin 7-glucoside and umbelliferone.

Biebersteinia orphanidis, which has not been investigated previously for its phenolic constituents, occupies a different phytogeographical position from the other four species, i.e. in Anatolian Turkey and Greece.

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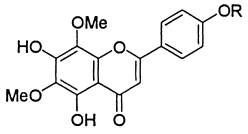
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Therefore, the present flavonoid analysis of *B. orphanidis* was undertaken in the hope it might reveal useful geographic differences and hence some insight into the evolution within the genus and the taxonomic position of *Biebersteinia* in the dicotyledons.

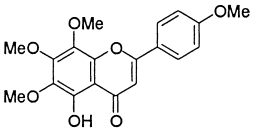
2. Results

2.1. Surface flavonoids

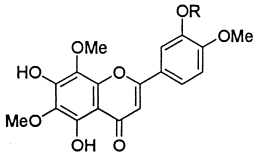
The acetone washings of uncut leaf tissue of *B. orphanidis* were concentrated, separated and purified by a combination of paper chromatography, TLC and HPLC to give nine flavones. Because of the rarity of the plant material and the complexity of the flavonoid mixture present, no compound was available in more than milligram amounts. Three were readily identified by direct comparison with authentic markers as luteolin, apigenin and acacetin. Ultraviolet, MS and NMR studies established that the remaining six flavones have a dimethoxy–dihydroxy substitution in the A-ring and are variously substituted in the B-ring with either 4′- or 3′,4′-hydroxy/methoxy substituents. One compound (**5**) has an additional methyl substitution in the A-ring (see below).



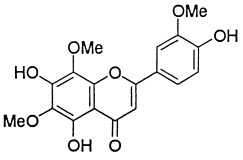
1 R = H
2 R = Me



3



4 R = H
6 R = Me



5

The only outstanding structural problem remaining was the location of the methoxyl substituents in the A-ring. Spectral shifts with aluminium chloride in the UV region showed that the 5-hydroxyl was free in all six compounds. Placement of the other free hydroxyl group at position 7 on spectral evidence alone was not possible, because 7-hydroxyflavones which are methoxylated at adjacent positions (6-, 8- or 6,8-) fail to undergo the normal bathochromic sodium acetate shift that would be expected. Proof that these six flavones have the 5,7-dihydroxy-6,8-dimethoxy A-ring substitution came from a detailed consideration of the neutral UV spectra (Table 1), when compared with model compounds.

The data in Table 1 show that in the apigenin series of flavones (compounds **1**–**3**) there are two signals in the short UV, a peak at 277–280 nm and a second peak or shoulder at 294–296 nm. These are exactly matched in the UV spectrum of nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone, **2**) but differ in the alternative structures (5,6-dihydroxy- and 5,8-dihydroxy). Similarly in the luteolin series, flavones **4**–**6**, there are two short wavelength UV bands at 255 and 278–280 nm. This is almost precisely matched by hymenoxin (5,7-dihydroxy-6,8,3′,4′-tetramethoxyflavone, **6**) but differs in the flavones with alternative structures (Table 1).

From these results, therefore, it is possible to conclude that **1** is 5,7,4′-trihydroxy-6,8-dimethoxyflavone, **2** is nevadensin, **3** is gardenin B, **4** is acerosin, **5** is sudachitin and **6** is hymenoxin. Authentic markers of nevadensin and gardenin B were available for comparison and co-TLC and co-HPLC confirmed the identities of **2** and **3**. Other relevant data for **1**–**6** are collected in Table 2. Additional evidence supporting methoxylation at positions 6- and 8- is present in the mass spectra of **1**–**6**. All the compounds measured have an M-15 fragment ion which is more intense than the molecular ion. This is a characteristic feature of 6- and 8-methoxylated flavones (Harborne et al., 1978).

Identification of **2** as nevadensin was also confirmed using 1D ^{1}H, 1D ^{1}H–^{1}H XSROESY and 2D ^{1}H–^{13}C HSQC NMR experiments acquired at 500 MHz. In particular, the location of the three methoxyl substituents was established using the 1D ^{1}H–^{1}H XSROESY experiment, which allows detection of 'through-space' ^{1}H–^{1}H ROE connectivities by site-selective excitation of individual resonances in the 1D ^{1}H NMR spectrum (Gradwell et al., 1997). Application of this procedure to nevadensin has been described

Table 1
UV spectral maxima of highly substituted flavone methyl ethers

Compound	UV spectral maxima (nm) in MeOH[b]
Apigenin-based flavones	
1	278, 296sh, 335
2 nevadensin	280, 294sh, 333
3 gardenin B	277, 296, 333
Model flavones[a]	
5,7-dihydroxy-6,8-dimethoxy	280, 294sh, 333
5,6-dihydroxy-7,8-dimethoxy	–, 297, 331
5,8-dihydroxy-6,7-dimethoxy	282, –, 332
Luteolin-based flavones	
4 acerosin	255, 279, 346
5 sudachitin	255, 278, 348
6 hymenoxin	254, 280, 344
5,7-dihydroxy-6,8-dimethoxy	250sh, 279, 336
5,6-dihydroxy-7,8-dimethoxy	252, 288, 344
5,8-dihydroxy-6,7-dimethoxy	254, 284, 298sh, 341

[a] Apigenin series: nevadensin, pilosin, pedunculin; luteolin series: hymenoxin, thymonin, leucanthoflavone. These spectral data are based on Grayer et al. (2000), Harborne and Baxter (1999) and references therein, and La Duke (1982).

[b] sh = shoulder.

recently, and allows the specific assignment of the methoxyl ^{1}H NMR resonances of the methoxyl substituents of **2** at δ 3.78, 3.87 and 3.88 (in DMSO-d_6) to 6-OCH_3, 4′-OCH_3 and 8-OCH_3, respectively (Grayer et al., 2000). The corresponding ^{13}C NMR resonances were obtained by inverse-detection using the HSQC experiment, as δ 59.7, 55.2 and 60.8, respectively. The amount of **1** available for NMR analysis was less than 0.1 mg, which did not permit use of the 1D XSROESY experiment over a reasonable timescale. However, analysis of the 1D ^{1}H NMR spectrum indicated that the compound contained only two methoxyl substituents (δ 3.78 and 3.86). These could be placed in the A-ring, as the chemical shift values of the H-2′,6′ (δ 7.92, 2H, *d*, $J=8.9$ Hz) and H-3′,5′ (δ 6.96, 2H, *d*, $J=8.9$ Hz) protons in the B-ring indicated that the 4′-OH group was free (Markham and Geiger, 1994). The chemical shift values of the ^{13}C resonances of the methoxyl groups at δ 3.78 and 3.86 were obtained as δ 60.1 and 61.1, respectively, by HSQC. The combined ^{1}H and ^{13}C NMR data indicated assignment of these resonances to 6-OCH_3 and 8-OCH_3, respectively, by comparison with data for the same substituents in **2**. Thus the NMR data for **1** supported its identification as 5,7,4′-trihydroxy-6,8-dimethoxyflavone. In the case of **5**, three methoxyl resonances at δ 3.78, 3.88 and 3.89 were detected in the ^{1}H NMR spectrum, the corresponding ^{13}C resonances of which were δ 59.9, 61.0 and 55.6, respectively, by HSQC. The B-ring was found to be substituted at both C3′ and C4′, according to the ^{1}H NMR spectrum, with characteristic resonances at δ 6.97 (*d*, $J=8.8$ Hz, H-5′), 7.55 (*dd*, $J=8.8$, 2.1 Hz, H-6′) and 7.56 (*d*, $J=2.1$ Hz, H-2′). Site-selective excitation of the methoxyl resonance at δ 3.89 using 1D XSROESY gave a ROE connectivity to H-2′ (δ 7.56), indicating that the corresponding methoxyl group must be located at C3′, rather than C4′. The remaining methoxyl groups were assigned to C6 and C8 on the basis of their ^{1}H and ^{13}C chemical shift values, again by analogy with corresponding data for **1** and **2**. The identity of **5** was thus confirmed to be 5,7,4′-trihydroxy-6,8,3′-trimethoxyflavone.

All these flavones, apart from **1**, have been reported before in nature (Harborne and Baxter, 1999) and have largely been found in plants of the Compositae. In general, methylated flavones with 5,6,7,8,4′-pentahydroxy or 5,6,7,8,3′,4′-hexahydroxy substitution are mainly confined to plants of the Rutaceae, Labiatae and Compositae. In *Biebersteinia*, there are two biogenetically related series present. In the apigenin series, there are the three compounds increasing in *O*-methylation from **1** to **2** to **3**. In the luteolin series, there is the so far undetected 5,7,3′,4′-tetrahydroxy-6,8-dimethoxyflavone, a putative precursor which on increasing *O*-methylation has yielded **4** and **5** and eventually **6**.

Table 2
Spectral and chromatographic data for flavone methyl ethers **1–6**

Compound	Structure	Mass spectra[a]		HPLC R_t (min)	TLC[b] $R_f\times100$	
		M	B-ring		1	2
1	5,7,4′-triOH,6,8-diOMe	330	121	7.04	16	66
2	5,7-diOH,6,8,4′-triOMe (nevadensin)	344	132	11.94	47	83
3	5-OH,6,7,8,4′-tetraOMe (gardenin B)	–	–	16.38	47	–
4	5,7,3′-triOH,6,8,4′-triOMe (acerosin)	360	–	8.11	31	61
5	5,7,4′-triOH,6,8,3′-triOMe (sudachitin)	360	149	7.37	31	61
6	5,7-diOH,6,8,3′,4′-tetraOMe (hymenoxin)	374	162	10.77	39	72

[a] Five of the six compounds show a fragment ion at 197 a.m.u., corresponding to a dihydroxydimethoxy A-ring. All five showed an M-15 ion uniformly more intense (ca. 117%) than the molecular ion.
[b] TLC system 1, toluene-HOAc (4:1) on silica gel plates. TLC system 2, 50% HOAc on cellulose.

Table 3
Internal flavonoids of *B. orphanidis* leaf

Compound	Identity	Neutral max[a]	HPLC R_t	R_f (×100)	
				BAW	15% HOAc
7	Luteolin 7-glucoside	255, 267, 350	17.87	44	07
8	Luteolin 7-rutinoside	255, 267, 350	17.70	38	24
9	Apigenin 7-glucoside	267, 336	21.02	56	17
10	Apigenin 7-rutinoside	267, 336	20.50	57	29
11	Tricetin 7-glucoside	249, 260, 268, 352	14.26	21	09

[a] Positive shifts with NaOH, NaOAc, $AlCl_3$, $AlCl_3$–HCl with all compounds and with H_3BO_3 in the case of **7**, **8** and **10**.

2.2. Vacuolar flavonoids

Leaf material of *B. orphanidis*, after acetone washing to remove surface flavonoids, was extracted into 80% methanol. The water-soluble flavonoids present were then separated and purified by standard procedures. Identifications were based on UV spectral analyses, hydrolyses to aglycone and sugar and, wherever possible, direct comparison by co-PC and co-HPLC with authentic markers. The results for the five major flavone glycosides are shown in Table 3. The compounds present are the 7-glucosides of apigenin, luteolin and tricetin (**9**, **7** and **11**) and the 7-rutinoside of apigenin and luteolin (**10** and **8**). The 7-rutinoside linkage and site of attachment in **8** was confirmed by ^{1}H NMR measurements and co-chromatography with an authentic sample in our collection.

Some free tricetin and free luteolin were present in the internal flavonoid extract. Three further flavone glycosides were detected, but none was present in sufficient quantity for complete characterisation. Two of the three compounds were provisionally recorded as luteolin and tricetin 7-diglucosides, on the basis of acid hydrolysis and chromatographic mobilities. The third compound appeared to be an apigenin 7-glucoside with aliphatic acylation.

3. Discussion

The present studies show that *B. orphanidis* has a highly distinctive flavonoid profile and uniquely contains a set of six closely related flavone methyl ethers, with dihydroxy, dimethoxy A-ring substitution. Related work on *B. heterostemon* (Zhang et al., 1995) and our own preliminary investigation on the flavonoids of *B. odora* suggest that this pattern in flavonoid production is common throughout this small genus. These plants are quite different from species in the Geraniaceae, which generally have flavonols, condensed and hydrolysable tannins (Bate-Smith, 1973).

The chemical data indicate that the earlier positioning of *Biebersteinia* in the Geraniaceae or Geraniales is misplaced and agree with the conclusions of an *rbc*L and *atp*B sequence comparison that *Biebersteinia* deserves to be placed in its own family. Furthermore, the DNA analyses provide support for a position for *Biebersteinia* in the Sapindales. What is particularly interesting is that it is placed near to Rutaceae species (Bakker et al., 1998). Such a link was proposed originally by De Candolle (1824). It is now apparent in the flavonoid data, since methylated flavones with 5,7-dihydroxy-6,8-dimethoxy A-ring substitution are quite uncommon in nature but do appear in a number of Rutaceae spp. In fact, sudachitin, here reported for the first time in *Biebersteinia*, was earlier isolated from unripe fruit of *Citrus sudachii* and fruit peel of *C. reticulata* (Horie et al., 1961). Thus, these chemical results are completely in harmony with the macromolecular phylogeny which places *Biebersteinia* fairly close to *Ruta* and *Xanthoxylum* of the Rutaceae, and within the same order Sapindales.

4. Experimental

4.1. Plant materials

Aerial parts of *B. orphanidis* were collected from the wild in Greece in May 1997 most of which were air-dried. It was verified by D. Vassiliades and a herbarium specimen has been deposited in the Herbarium of the University of Athens (ATHU).

4.2. Flavonoid analysis

4.2.1. Lipophilic flavonoids

Lipophilic flavonoids were rinsed from uncut leaf tissue by briefly dipping ×2 in Me_2CO at room temperature. The concentrated extracts were run on silica gel TLC in toluene:HOAc (4:1) and the eluted bands rerun on Whatman 3MM paper in 30% HOAc. Final purification was either on paper in H_2O or by CC on Sephadex LH-20. Compounds **1**, **2** and **5** were further purified by HPLC before ^{1}H and ^{13}C NMR analysis using a reverse phase Waters Bondapak Phenyl analytical column at 25°C and UV detection at 350 nm on a diode array detector. The solvents used were (A) 2% HOAc and (B) MeOH–HOAc–H_2O (18:1:1) using a flow rate of 1 ml/min and a gradient elution programme changing from 40% A/60%B to 100%B over 20 min in a linear mode. Molecular weights were determined by EIMS and LCMS. Known compounds were identified by comparison of UV spectral data and R_f values in six solvents with standard markers and/or literature data.

4.2.2. NMR analyses

NMR spectra were recorded using a Varian 500 MHz instrument. All samples studied were dissolved in DMSO-d_6 and referenced to the residual solvent resonance at δ_H 2.50 ppm or δ_C 39.50 ppm as appropriate. All NMR experiments were carried out at 37°C.

*4.2.2.1. 5,7,4′-Trihydroxy-6,8-dimethoxyflavone (**1**).* ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.92 (2H, *d*, J = 2.1 Hz, H-2′), 6.96 (2H, *d*, J = 8.9 Hz, H-3′,5′), 6.78 (^{1}H, *s*, H-3), 3.86 (3H, *s*, 8-OCH_3), 3.78 (3H, *s*, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) (assignment of selected non-quaternary C atoms from HSQC): δ 61.1 (8-OCH_3), 60.1 (6-OCH_3).

*4.2.2.2. 5,7-Dihydroxy-6,8,4′-trimethoxyflavone (Nevadensin) (**2**).* ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.75 (^{1}H, *s*, 5-OH), 8.02 (2H, *d*, $J=8.8$ Hz, H-2′,6′), 7.15 (2H, *d*, $J=8.8$ Hz, H-3′,5′), 6.86 (^{1}H, *s*, H-3), 3.88 (3H, *s*, 8-OCH_3), 3.87 (3H, *s*, 4′-OCH_3), 3.78 (3H, *s*, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) (assignment of non-quaternary C atoms from HSQC): δ 127.9 (C-2′,6′), 114.5 (C-3′,5′), 102.9 (C-3), 60.8 (8-OCH_3), 59.7 (6-OCH_3), 55.2 (4′-OCH_3).

*4.2.2.3. 5,7,4′-Trihydroxy-6,8,3′-trimethoxyflavone (**5**)* ^{1}H NMR (500 MHz, DMSO-d_6): 7.56 (1H, *d*, $J=8.8$ Hz, H-2′), 7.55 (*dd*, $J=8.8$, 2.1 Hz, H-6′), 6.97 (*d*, $J=8.8$ Hz, H-5′), 6.86 (1H, *s*, H-3), 3.89 (3H, *s*, 3′-OCH_3), 3.88 (3H, *s*, 8-OCH_3), 3.78 (3H, *s*, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) (assignment of non-quaternary C atoms from HSQC): δ 120.1 (C-2′), 116.0 (C-5′), 110.0 (C-6′), 102.6 (C-3), 61.0 (8-OCH_3), 59.9 (6-OCH_3), 55.6 (3′-OCH_3).

4.3. Polar flavonoids

Leaf tissue of *B. orphanidis*, after removal of the surface flavonoids with acetone, was extracted with boiling 80% methanol. The concentrated extract was run on Whatman 3 MM paper in BAW (*n*-BuOH:HOAc:H_2O, 4:1:5, top layer) and the eluted bands separated in 15% HOAc. Further purification was carried out in BAW, CAW ($CHCl_3$:HOAc:H_2O, 10:10:1), 15% HOAc and H_2O. The known glycosides **7**–**11** were identified by standard procedures (TLC, HPLC, UV spectral data) including comparison with authentic markers. UV spectra, R_f and HPLC data are shown in Table 3. The HPLC programme for these flavone glycosides involved the use of 75% solvent A/25% solvent B to 35% A/65% B over 23 min in a linear gradient. Otherwise, HPLC details are similar to those described for the lipophilic flavonoids.

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Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*

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Abstract

The bisdesmoside oleanolic acid saponin, 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl-oleanolate, along with nine known compounds (two diterpenic acids, one chromene, three triterpenes, one steroidal glycoside, and two monodesmoside oleanolic acid saponins), were obtained from *Viguiera decurrens* roots. The chemical structure of the bisdesmoside oleanolic saponin was determined by chemical and NMR spectral evidence. A mixture of monodesmoside saponins displayed cytotoxic activity against P388 and COLON cell lines ($ED_{50} = 2.3$ and 3.6 μg/ml, respectively). Two of the known compounds showed insecticidal activity against the Mexican bean beetle larvae (*Epilachna varivestis*). © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Viguiera decurrens*; Asteraceae; Root; Oleanolic acid; Bisdesmoside; Saponin; Chikusetsusaponin methyl ester; Cytotoxic and insecticidal activity

1. Introduction

Chemical investigation of several species belonging to *Viguiera* (Asteraceae) have yielded diterpenes (Delgado et al., 1984a), sesquiterpene lactones (Alvarez et al., 1985) and flavonols (Delgado et al., 1984b) as the major constituents of their aerial parts. The following contribution is based upon our continuing study of the genus *Viguiera* and our search for bioactive natural products. *Viguiera decurrens* (Asteraceae) is a perennial herb endemic to the upper desert grasslands and lower oak-pine forests of the eastern Sierra Madre Occidental of western Chihuahua. The thick, elongated roots known to the Tarahumara Indians as "nakárori" ('naka' = ear, 'rori' = movement) are used medicinally and in fishing (Brambila, 1976). The distinctive decurrent leaves and winged stems (over one meter tall with yellow terminal flowers) appear as ears when the plant is blown by the wind. Upon placing the crushed roots (fresh or dried) in stream pools, the fish rise to the surface and are collected by the Indians as food. A poultice from the pungent root is also used to treat infections, wounds and boils, and an infusion of the root or leaves is drunk to alleviate gastric ulcers (Pennington, 1963; Bye, 1985).

This paper describes the bioactivity-isolation of oleanolic acid saponins from the roots of *V. decurrens*, the structure elucidation of the new saponin, and the cytotoxic and insecticidal evaluations of some of the isolated compounds.

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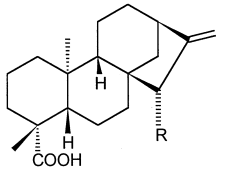
2. Results and discussion

Using a bioassay-directed fractionation of the roots of *V. decurrens*, three cytotoxic fractions and three non-cytotoxic fractions were obtained. Chromatographic purification of each cytotoxic fraction led to the isolation of two kaurenoic diterpenes, namely *ent*-kaur-16-en-19-oic acid (**1**) and 15α-angeloyloxy-*ent*-kaur-16-en-19-oic acid (**2**); three saponins: β-D-glucopyranosyl-oleanolate (**3**) (Yoshikawa et al., 1996a), β-sitosteryl-3-*O*-β-D-glucopyranoside, and oleanolic acid-3-*O*-methyl-β-D-glucuronopyranosiduronoate (**4**) (Sakai et al., 1994), and one chromene, 8-hydroxy-6-acetyl-2,2-dimethylchromene (**5**) (Bohlmann and Jakupovic, 1978). From the non-active fractions, the triterpenes friedelin, friedelan-3β-ol and oleanolic acid (**6**) as well as the oleanolic acid bisdesmoside saponin 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**) were also obtained.

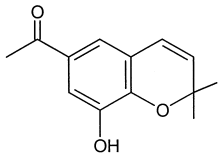
Compound **7** was obtained as a colorless powder (mp 216–218°C). In the positive mode FABMS, the quasi molecular ion-peak was observed at *m/z* 809 $M+H^+$, indicating a molecular formula of $C_{43}H_{68}O_{14}$. On acid hydrolysis compound **7** afforded oleanolic acid, glucose and 6-methylglucuronosiduronoate. The ^1H NMR ($CDCl_3$-DMSO-d_6) (see experimental) and ^{13}C NMR (see Table 1) spectra of (**7**) showed signals assignable to an oleanolic acid moiety [δ 5.26 (1H, *br t*, *J* = 3.5 Hz, H-12), 3.34 (1H, *m*, H-3), 2.83 (1H, *dd*, *J* = 13.5, 4.0 Hz, H-18], a β-D-methylglucuronopyranosiduronoate moiety [δ 4.35 (1H, *d*, *J* = 7.5 Hz, H-1′), 3.74 (3H, *s*, OCH_3], and a β-D-glucopyranosyl moiety [δ 5.43 (1H, *d*, *J* = 8.0 Hz, H-1″].

In order to clarify the type of cyclization of the sugar moieties, ^1H and ^{13}C NMR spectroscopic analysis was undertaken on the heptaacetyl derivative **8** by means of HMBC and HMQC experiments, and all the proton signals due to the sugar moieties were assigned. The profiles of these signals indicated that both methyl glucuronosiduronoate and glucose were β-pyranoses.

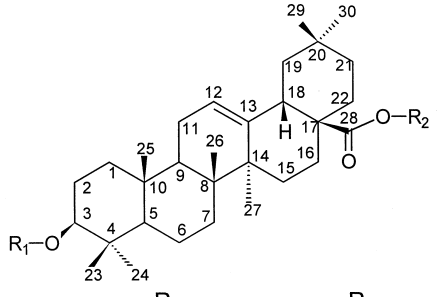
The bisdesmoside nature of 8 was demonstrated by the HMBC experiment which showed cross peaks between H-1′ of the methyl glucuronopyranosiduronoate moiety and C-3 of the oleanolic acid moiety, and between H-1″ of the β-D-glucopyranosyl moiety and C-28 of the genin. Similarly, the detection of all possible two- and three-bond correlations of the anomeric pro-



1 R = H
2 R = OAng



5



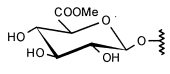
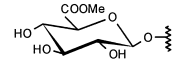
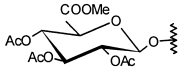
	R 1	R 2
3	H	β-D-Glcp
4		H
6	H	H
7		β-D-Glcp
8		β-D-Glcp

Table 1
^{13}C NMR spectral data for **7** ($CDCl_3$-DMSO-d_6) and **8** ($CDCl_3$) at 125 MHz, δ values

	7	8			7	8
	Sapogenol moiety				Sugar moieties	
1	38.6	38.9	6 Methyl-glucuronosiduronoate moiety	1′	104.8	102.9
2	27.1	27.7		2′	73.0	71.9
3	88.8	90.6		3′	71.8	72.9
4	39.6	39.4		4′	70.8	68.1
5	54.6	55.5		5′	76.3	72.5
6	17.3	18.1		6′	168.9	167.2
7	32.9	33.8		OCH_3	51.41	52.7
8	39.6	41.0				
9	46.7	47.6	Glcp moiety	1″	93.3	91.6
10	35.8	36.7		2″	74.4	71.6
11	24.9	23.4		3″	76.3	72.0
12	121.5	122.9		4″	69.4	69.6
13	142.7	142.9		5″	75.9	70.0
14	39.1	41.7		6″	62.9	61.6
15	29.8	29.7				
16	22.5	22.9				
17	45.7	45.8				
18	40.8	41.7				
19	45.7	46.8				
20	31.1	31.8				
21	32.9	33.8				
22	32.2	32.9				
23	27.1	27.7				
24	14.6	16.3				
25	15.8	15.3				
26	16.1	16.9				
27	26.6	25.6				
28	175.4	175.6				
29	32.2	32.9				
30	24.9	25.5				

tons confirmed the glycosidic linkages and the assignments for proton and carbon resonances.

Based on the above mentioned evidence and comparison of the ^{13}C NMR (Table 1) data for **7** and **8** with those for oleanolic acid 3, 28-*O*-bisdesmosides (Yoshikawa et al., 1995), the structure of this compound was confirmed as 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**).

The demethyl derivative of **7** has been reported as a natural product of *Aralia elata* (Lin et al., 1976), *Talinum triangulare* (Kohda et al., 1992), *Aralia elata* SEEM. (Yoshikawa et al., 1993), *Panax pseudoginseng* (Ida et al., 1994), and *Dolichos lablab* L. (Yoshikawa et al., 1998) and named chikusetsusaponin IVa; therefore, chikusetsusaponin IVa methyl ester (**7**) is a natural saponin from *V. decurrens*. The NMR spectral data of its heptaacetyl derivative (**8**) have not previously been recorded in the literature (see Table 1).

Although **7** could be considered an artifact due to the fact that MeOH was originally used for the isolation procedure, this possibility was ruled out from the following observations: (a) the corresponding acid was not isolated, and it is well known that the use of MeOH does not allow per se the formation of esters; (b) compound **7** was isolated from an EtOH extract of additional material of *Viguiera decurrens* roots (3.4 mg of compound) obtained from big root tissue (data not shown) and (c) the presence of methyl β-D-glucuronopyranosiduronoate triterpenoidal saponins as natural products, has been well documented recently in the literature (Sang et al., 1998; Zhang et al., 1999).

None of the six main fractions displayed antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Trycophyton mentagrophytes,* at concentrations up to 400 μg/ml.

The mixture of compounds **3**, **4** and β-sitosteryl-3-*O*-β-D-glucopyranoside obtained from fraction V (see Table 2) showed cytotoxic activity against the P388 cell line ($ED_{50} = 2.3$ μg/ml); however, the pure compounds were not toxic ($ED_{50} > 4$ μg/ml) (Geran et al., 1972).

Compounds **1**, **3**, **5**, **7** and friedelin were also evaluated for their insecticidal activity at different concentrations against *Epilachna varivestis* larvae, the Mexican bean beetle (Coleoptera: Coccinellidae), an important pest in Mexico (MacGregor and Gutiérrez, 1983). The results of these experiments indicated that friedelin and compounds **1** and **5** were not toxic ($LC_{50} > 5000$ μg/ml), while saponins **3** and **7** displayed toxicity ($LC_{50} = 1380$ and 80 μg/ml (calculated), respectively).

The presence of oleanolic acid mono- and bidesmoside saponins in the roots of *Viguiera decurrens* is noteworthy, and chemically validates the Tarahumara people's use of the roots to stupefy fish (Bye, 1985).

Recently, **4** has been reported to have in vivo inhibitory effects on ethanol absorption (Yoshikawa et al., 1996b), as well as hypoglycemic activity (Yoshikawa et al., 1996c), along with other oleanolic acid glycosides, isolated from *Aralia elata* and *Beta vulgaris*.

This is the first report of oleanolic acid saponins from the roots of a *Viguiera* species.

Table 2
Evaluation of cytotoxic activity

Fractions	ED_{50}[a] KB[b]	P388[c]	OVCAR[d]	COLON[e]	UISO[f]
I	>20	7.24	–	–	–
II	>20	3.3	–	–	–
III	>20	9.1	21.4	>20	22.4
IV	>20	12	25.1	>20	>20
V	>20	2.3	8.9	>20	3.02
VI	>20	5.7	47.9	>20	>20
Mixture of **3**, **4** and β-sitosteryl-3-*O*-β-D-Glcp	>4.0	2.3	–	3.6	–
3	>4.0	>4.0	–	–	–
4	>4.0	>4.0	–	–	–

[a] (ED_{50} μg/ml).
[b] KB, human epidermal carcinoma.
[c] P388, murine leukemia.
[d] OVCAR, ovarian cancer.
[e] COLON, colon carcinoma.
[f] UISO, uterine-cervix cancer.

3. Experimental

Mps are uncorr. NMR instrumentation: Varian VXR-200 and Varian Unity Plus-500. MS: JEOL JMS-AX 505 HA.

3.1. Plant material

The roots of *Viguiera decurrens* A. Gray were obtained in the eastern Sierra Tarahumara, Chihuahua, Mexico, on 12 October 1991 and identified by R. Bye. The herbarium voucher specimen (R. Bye 18338) is deposited in the National Herbarium of Mexico (MEXU).

3.2. Bioactivity-directed isolation of compounds

Dried and powdered roots (1.07 kg) of *V. decurrens* were extracted by percolation using a gradient of *n*-hexane–EtOAc–MeOH, yielding six fractions: Fr. I (100:0:0, 18.0 g); Fr. II (9:1:0, 1.6 g); Fr. III (7:3:0, 4 g); Fr. IV (1:1:0, 2.8 g), Fr. V (0:100:0, 12.0 g); Fr. VI (0:3:7, 30.6 g). These fractions were evaluated for their antimicrobial and cytotoxic activities (Table 2). Fractions I, II and V showed cytotoxic activity. Fr. I after filtration afforded 2.23 g of *ent*-kaur-16-en-19-oic acid (**1**, 0.1151%, mp 170–174°C). Fr. II was ground with *n*-hexane to yield 68 mg of 15α-angeloyloxy-*ent*-kaur-16-

en-19-oic acid (**2**, 0.104%). Fr. III was methylated with diazomethane and the residue was chromatographed on a silica gel column using mixtures of *n*-hexane–EtOAc; fractions 1–31 eluted with 95:5 (*n*-hexane–EtOAc) yielded 105 mg of friedelin (0.0098%, mp 242–245°C), from fractions 15–46 eluted with *n*-hexane–EtOAc 9:1 crystallized 50 mg of friedelan-3β-ol (0.0046%, mp 249–253°C), fractions 55–60 (8:2 *n*-hexane–EtOAc) were combined and the residue (700 mg) was subjected to silica gel column chromatography eluted with (CH_2Cl_2–MeOH 9:1) to yield 60 mg of oleanolic acid methyl ester (0.0056%, mp 198–200°C). Fr. IV was applied to a silica gel column using a gradient system of CH_2Cl_2–MeOH to yield 35.8 mg of oleanolic acid (**6**, 0.0033%, mp 196–198°C). Fr. V was subjected to chromatography with mixtures of CH_2Cl_2–MeOH, to obtain a cytotoxic fraction (see Table 2) which was composed of a mixture of saponins. The mixture was further separated by repeated column chromatography to yield 47 mg of β-D-glucopyranosyl olean-12-en-28-oate (**3**, 0.0043%, mp 235–237°C), 60 mg of β-sitosteryl-3-*O*-β-D-glucopyranoside (0.0056%, mp 267°C), 125 mg of oleanolic acid 3-*O*-methyl-β-D-glucuronopyranosiduronoate (**4**, 0.011%, amorphous powder), and 22 mg of 8-hydroxy-6-acetyl-2,2-dimethyl chromene (**5**, 0.002%, mp 135–137°C) (lit. mp 132°C, Bohlmann and Jakupovic, 1978). Fr. VI (30.6 g) was subjected to repeated CC on silica gel, eluting with EtOAc–MeOH (7:3), CH_2Cl_2–MeOH (95:5) and CH_2Cl_2–MeOH (85:15) respectively to afford 80 mg of 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**, 0.0074%).

Compounds **1**, **2**, **6**, β-sitosteryl-3-*O*-β-D-glucopyranoside, friedelin, friedelan-3-β-ol, were identified by direct comparison (IR, TLC) with authentic samples, while compounds **3**–**5** and **7** were characterized by means of physicochemical evidence.

3.2.1. 3-O-(Methyl-β-D-glucuronopyranosiduronoate)-28-O-β-D-glucopyranosyl oleanolate (7)

Colorless powder (MeOH, mp 216–218°C) (lit. mp 209–212°C, Sakai et al., 1994), $\alpha_D + 5.7$ ($CHCl_3$, *c* 0.28); IR ν_{max} cm^{-1}: 3420, 1720, 1700; positive FABMS *m*/*z*: 831 $[M+Na]^+$; high-resolution positive FABMS *m*/*z*: Calcd for $C_{43}H_{68}NaO_{14}$: 831.4506; found: 831.4478 $(M+Na)^+$, 809 $[M+H^+]$, 663 [M- Glc + Na^+], 475 [M-(Glu-Me)-Glc + Na]$^+$, 207 $[Glu]^+$, 179 $[Glc]^+$; ^{1}H NMR spectral data (500 MHz, $CDCl_3$-DMSO-d_6): δ 5.43 (1H, *d*, *J* = 8.0 Hz, H-1''), 5.26 (1H, *br t*, *J* = 3.5 Hz, H-12), 4.35 (1H, *d*, *J* = 7.5 Hz, H-1′), 3.78 (1H, *t*, *J* = 9.0 Hz, H-2″), 3.74 (3H, *s*, OCH_3), 3.72 (1H, *dd*, *J* = 11.0, 5.0 Hz, H-6a″), 3.63 (1H, *t*, *J* = 8.5 Hz, H-4′), 3.56 (1H, *dd*, *J* = 11.0, 5.5 Hz, H-6b″), 3.48 (1H, *t*, *J* = 8.5 Hz H-3″), 3.46 (1H, *d*, *J* = 9.0 Hz, H-5′), 3.44 (1H, *t*, *J* = 9.0 Hz, H-4″), 3.40 (1H, *t*, *J* = 8.5 Hz, H-3′), 3.36 (1H, *m*, H-5″), 3.34 (1H, *m*, H-2′), 2.83 (1H, *dd*, *J* = 13.5, 4.0 Hz, H-18), 1.11 (3H, *s*, H-30), 1.02 (3H, *s*, H-23), 0.92 (3H, *s*, H-29), 0.90 (3H, *s*, H-27), 0.89 (3H, *s*, H-24), 0.81 (3H, *s*, H-25), 0.71 (3H, *s*, H-26); ^{13}C NMR spectral data (125 MHz, $CDCl_3$-DMSO-d_6): see Table 1.

3.2.2. Compound (8)

Obtained by usual acetylation of **7**: powder, mp 99–101°C; positive FABMS *m*/*z*: 1125 $[M+Na]^+$. High-resolution positive FABMS *m*/*z*: calcd for $C_{57}H_{82}NaO_{21}$: 1125.5246; found: 1125.5318 $[M+Na]^+$, 769 [M- (Glu-Me)Ac_3 + H]$^+$, 741 [M- (Glu-Me)Ac_3 + K]$^+$, 331 [(Glc)Ac_4]$^+$; ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 5.58 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.31 (1H, *br t*, *J* = 3.5 Hz, H-12), 5.24 (1H, *t*, *J* = 9.5 Hz, H-3′), 5.22 (1H, *t*, *J* = 9.5 Hz, H-4′), 5.21 (1H, *t*, *J* = 9.5 Hz, H-3″), 5.19 (1H, *t*, *J* = 9.0 Hz, H-2″), 5.12 (1H, *t*, *J* = 8.5 Hz, H-4″), 5.04 (1H, *t*, *J* = 9.0 Hz, H-2′), 4.58 (1H, *d*, *J* = 8.0 Hz, H-1′), 4.27 (1H, *dd*, *J* = 12.5, 4.0, Hz, H-6a″), 4.04 (1H, *dd*, *J* = 12.5, 2.0 Hz, H-6b″), 3.98 (1H, *d*, *J* = 9.5 Hz, H-5′), 3.78 (1H, *ddd*, *J* = 10.5, 4.5, 2.5 Hz, H-5″), 3.77 (3H, *s*, OCH_3), 3.10 (1H, *dd*, *J* = 8.0, 4.5 Hz, H-3), 2.81 (1H, *dd*, *J* = 16.0, 4.5 Hz, H-18), 1.10 (3H, *s*, H-27), 0.90 (6H, *s*, H-23, H-30), 0.89 (6H, *s*, H-24, H-29), 0.73 (3H, *s*, H-25), 0.72 (3H, *s*, H-26); ^{13}C NMR spectral data (125 MHz, $CDCl_3$): see Table 1.

3.2.3. Acid hydrolysis of 7

Compound **7** (25 mg) was refluxed with 10% HCl-methanol (5 ml) for 2 h. The aglycone was extracted with EtOAc and identified by comparison with an authentic sample. The aqueous layer was adjusted to pH 6 with $NaHCO_3$. After evaporation to dryness, the sugars were extracted with pyridine and analyzed by TLC on silica gel with EtOAc–MeOH–H_2O–AcOH (11:2:2:2); detection was accomplished with *p*-anisidine phthalate.

3.3. Biological activity

3.3.1. Antimicrobial activity

Evaluations were performed with cultures of *Staphylococcus aureus* (ATCC 6538), *Salmonella typhimurium* (ATCC 06539), *Escherichia coli* (ATCC 8937), *Pseudomonas aeruginosa* (ATCC 9027); *Candida albicans* (ATCC 10231) and *Trycophyton mentagrophytes* (NRRL 1942), using the conventional disc assay procedure (Vanden Berghe and Vlietinck, 1991); nystatin (5–160 μg/ml) and gentamicin (2–128 μg/ml) were included as controls.

3.3.2. Cytotoxic activity

These studies were performed using the cell cultures listed in Table 2 according to Geran and Greenberg's screening protocols (Geran et al., 1972).

3.3.3. Insecticidal activity

This assay was carried out following the published protocol (Kubo, 1991), using first instar larvae of *Epilachna varivestis* (Coleoptera: Coccinellidae) which were

fed with different concentrations of compounds **1**, **3**, **5**, **7** and friedelin in contaminated leaf-discs. Leaves were obtained from greenhouse bean plants (criollo variety) free of pests and chemical insecticides. Concentrations ranged from 100 to 6000 μg/ml. The toxicity data were analyzed by the *Probit method* (Finney, 1962) and expressed as the LC_{50}.

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PHYTOCHEMISTRY

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Trianthenol: an antifungal tetraterpenoid from *Trianthema portulacastrum* (Aizoaceae)

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Abstract

An antifungal tetraterpenoid named trianthenol **1** has been isolated from the chloroform extract of *Trianthema portulacastrum*. Its structure was established as 15-hydroxymethyl-2,6,10,18,22,26,30-heptamethyl-14-methylene-17-hentriacontene on the basis of spectroscopic data including high resolution mass and two-dimensional NMR techniques. A benzaldehyde derivative **2,** a pentacyclic triterpenoid **3** and benzoic acid derivatives **4–5** are also reported for the first time from *Trianthema portulacastrum*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Trianthema portulacastrum*; Aizoaceae; Antifungal tetraterpenoid; Benzaldehyde derivative; Pentacyclic triterpenoid; Benzoic acid derivatives

1. Introduction

Trianthema portulacastrum Linn. belongs to the family Aizoaceae growing in South and tropical Africa, South America, W. Indies, Mediterranean and tropical Asia (Nasir and Ali, 1973). The plant has been used in the indigenous system of medicine for the obstruction of the liverasthma, amenorrhoea, dropsy, oedema, asceites and beri-beri. The extract of roots has cathartic and irritant properties and is used as an abortifacient. A decoction of the herb is used as a vermifuge and is useful in rheumatism. An ethanolic extract of the plant has shown some effect on blood-pressure of guinea-pigs and also on their ileum (Chadha, 1976). During the search for new bioactive compounds from medicinal plants, we found that chloroform extract of *T. portulacastrum* showed antifungal activity. This prompted us to investigate the activity-guided fractionation of the chloroform extract of this plant. Herein we report the isolation and structure elucidation of an antifungal tetraterpenoid **1** and four known compounds **2–5.**

2. Results and discussion

The chloroform extract after column chromatography over silica gel and repeated preparative TLC afforded antifungal tetraterpenoid **1** as an oil. Its IR spectrum showed characteristic peaks for hydroxyl group (3430) and isolated double bond (1640, 890 cm^{-1}). The HREIMS provided M^+ peak at m/z 574.6076 indicating the molecular formula $C_{40}H_{78}O$. The ^1H and ^{13}C NMR spectra were assigned on the basis of comparison with the related compound 13-hydroxymethyl-2,6,10,18,22,26,30-heptamethy-14-methylene-10-hentriacontene, (Mangoni et al., 1984) and ^{13}C-^1H COSY experiments. The ^1H NMR spectrum included signals for eight methyl doublets appearing as three groups in a 3:4:1 ratio at δ 0.86 ($J=6.5$), 0.84 ($J=6.6$), 8.2 ($J=6.1$) and one vinylic methyl singlet at δ 1.58. The allylic protons appeared as three systems in a 1:2:4 ratio at δ 2.24 (H-15), 2.12 (H_2-16) and 1.94 (H_2-13 and H_2-19), respectively. Further, two multiplets in a 2:4 ratio resonating at δ 1.51 and 1.28 were due to (H-2 and H-30) and (H-6, H-10, H-22, and H-26). The signal of two protons multiplet at δ 3.53 which shifted downfield to δ 4.03 in the corresponding monoacetate **1a** indicated the presence of hydroxymethylene. The downfield signals at δ 5.10, 4.91 and 4.82 were due to the vinylic protons of double

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bonds. The positions of functional groups were assigned on the basis of mass fragmentation pattern in the EI and HREIMS spectra (see Fig. 1). Their locations were confirmed by HMBC experiments, in which oxymethylene protons at δ 3.53 revealed ^2J correlations to C-15 (δ 48.78) and ^3J correlations to C-14 (δ 150.19) and C-16 (δ 29.06). The vinylic proton at δ 5.10 showed ^3J interactions to C-19 (δ 40.05) and C-15 (δ 48.78), while methylene protons at δ 4.91 and 4.82 showed ^3J interactions to C-13 (δ 35.07) and C-15 (δ 48.78). Inspite of the molecular formula, the ^{13}C NMR spectrum of **1** showed only 27 carbon signals. Quantitative measurements provided us the intensity values of these signals.

E-configuration was assigned to the trisubstituted double bond on the basis of shielding of the vinylic methyl group in the ^{1}H (δ 1.58) (Monaco et al., 1983) and ^{13}C NMR spectra (δ 16.08) (Duch and Grant, 1970). Therefore, the structure of **1** was elucidated as 15-hydroxymethyl-2,6,10,18,22,26,30-heptamethyl-14-methylene-17-hentriacontene.

Known compounds **2–5** were identified from their spectral data upon comparison with values reported in the literature as 5-hydroxy-2-methoxy benzaldehyde **2** (Yoshifumi et al., 1995), 3-acetyl aleuritolic acid **3** (McLean et al., 1987), *p*-methoxy benzoic acid **4** (Anon., 1994) and *p*-propoxy benzoic acid **5** (Ullah et al., 1998).

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded on Bruker AM-300 and AM-500 NMR spectrometers operating at 300 and 125.6 MHz, respectively. 2D NMR spectra were also recorded on same instruments using same solvent. Optical rotations were measured on Jasco DIP-30 polarimeter. IR spectrum was obtained on 460 Shimadzu spectrometer. EI and HREIMS were recorded on JMS HX-110 with a data system, and on JMS-DA 500 mass spectrometers. The chemical shifts are reported in ppm (δ) units and the coupling constants (J) are in Hz. Visualization of the TLC plates was achieved under UV at 254 and 266 nm by spraying with ceric sulfate reagent solution (with heating).

3.2. Plant material

Trianthema portulacastrum Linn. (Aizoaceae), whole plant was collected in June 1997, from Karachi (Pakistan) and identified by Miss Rubina Dawar, Department of Botany, University of Karachi, where a voucher specimen (no. 67339) has been deposited in the herbarium.

3.3. Extraction and isolation

The whole plant material (24 kg) of *T. portulacastrum* was shade-dried (5 kg), ground and extracted twice with chloroform at room temperature for a period of 2 weeks. The combined extracts were evaporated under reduced pressure to obtain a crude syrup. The total chloroform extract (151.13 g) was subjected to column chromatography over silica gel (70–230 mesh) successively eluting with *n*-hexane, *n*-hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH and finally MeOH in increasing order of polarity. The fractions (307–415) eluted with *n*-hexane–chloroform (9.3:0.7) were a mixture of two minor and one major components. The combined fractions were concentrated and the residue was subjected to column chromatography (flash silica 230–400 mesh) using *n*-hexane–chloroform (9.2:0.8) as eluent to afford **1** (60 mg). The fractions (950–1000) which were obtained through elution with *n*-hexane–chloroform (6.5:3.5) were combined and re-chromatographed over flash silica eluting with hexane–chloroform (3:2) in increasing order of polarity. The final purification of the resulting fractions by preparative TLC (F_{254}) using solvent system *n*-hexane–EtOAc (8.0:0.2) afforded **2** (17 mg). The fractions (1025–1050) which eluted with *n*-hexane–chloroform (4.0:6.0) comprised of one major component. It was re-chromatographed over flash silica eluting with *n*-hexane–chloroform (4.5:5.5) yielding compound **3** (22 mg). The fractions which eluted with *n*-hexane–chloroform (1.0:9.0) were mainly a mixture of two UV active compounds which could be resolved through preparative TLC using solvent system *n*-hexane–acetone (5.5:4.5) to obtain compounds **4** (23 mg) and **5** (25 mg).

3.4. Compound *1*

Compound **1** was obtained as an oil; $[\alpha]_D^{28}$: −5.2° (c = 0.38, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} : 3430, 1640, 1460, 1035, 890; EIMS m/z (% rel. int.): 574 M^+ (4.79), 544 (6.85), 543 (14.93), 349 (10.59), 322 (10.59), 321 (39.68), 304 (8.46), 303 (31.68), 57 (100); HREIMS m/z: 574.6076 (calcd for $C_{40}H_{78}O$, 574.6052); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.10 (1H, *t*, J = 6.2 Hz, H-17), 4.91 (1H, *s*, H_A-14′), 4.82 (1H, *s*, H_B-14′), 3.53 (2H, *m*, H-15′), 2.24 (1H, *m*, H-15), 2.12 (2H, *m*, H-16), 1.94 (4H, *m*, H-13, 19), 1.58 (3H, *s*, Me-18′), 1.51 (2H, *m*, H-2, 30), 1.28 (4H, *m*, H-6, 10, 22, 26), 0.86 (9H, *d*, J = 6.5 Hz, 3×Me), 0.84 (12H, *d*, J = 6.6 Hz, 4×Me) and 0.82 (3H, *d*, J = 6.1 Hz, Me); ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 150.19 (C-14), 136.70 (C-18), 122.12 (C-17), 110.50 (C-14′), 64.11 (C-15′), 48.78 (C-15), 40.05 (C-19) 39.40 (C-3, 29), 37.49 (C-5, 27), 37.37 (C-7, 25), 36.98 (C-9, 23), 36.73 (C-11, 21), 35.07 (C-13), 32.93 (C-26), 32.85 (C-6), 32.80 (C-22), 32.75 (C-10), 29.06 (C-16), 28.0 (C-2, 30), 25.44 (C-20), 25.38 (C-12), 24.83 (C-4,

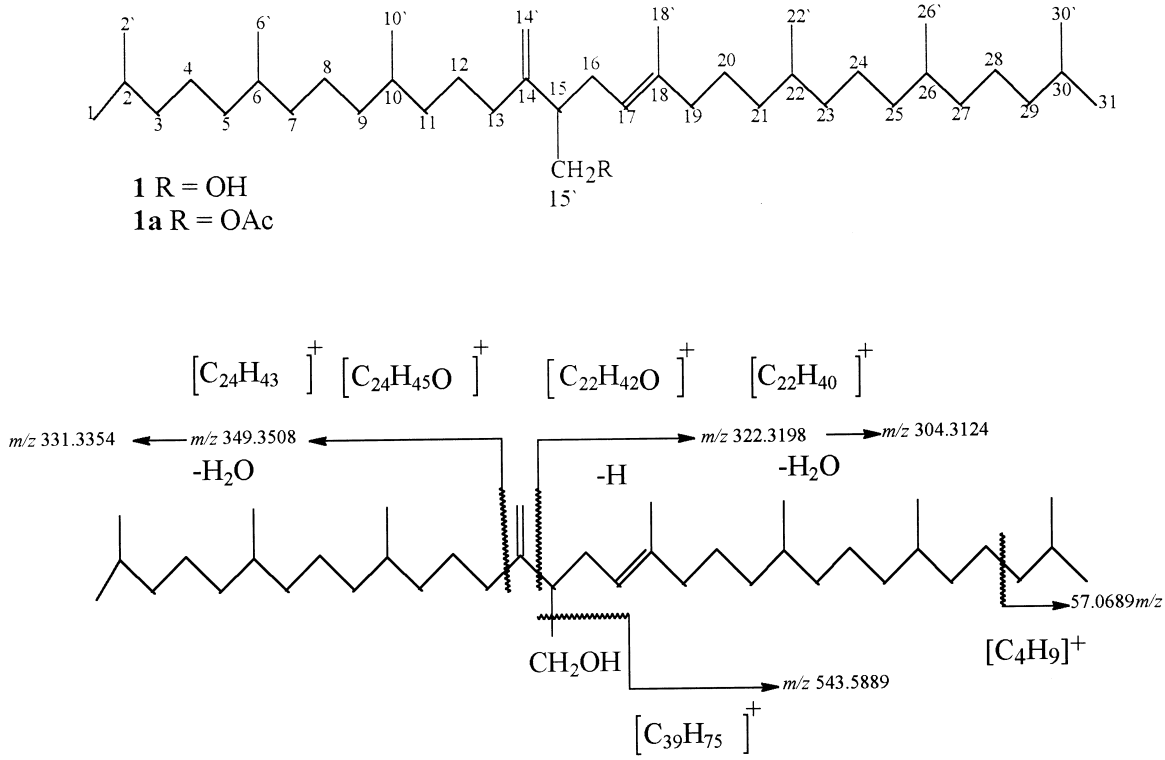


Fig. 1. Mass fragmentation (HREIMS).

Table 1
In vitro fungicidal bioassay of crude extract and trianthenol 1

Name of fungus	Inhibition (%) of crude extract	Inhibition (%) of trianthenol 1	Standard drugs	Inhibition % of Standard drugs
Human pathogens				
Trichophyton schoen leinii	71.4	50.7	Miconazole	90
			Ketoconazole	90
Pseudallescheria boydii	25.0	45.5	Miconazole	95
			Ketoconazole	
Animal pathogens				
Trichophyton simii	12.5	48.9	Miconazole	100
			Ketoconazole	
Plant pathogens				
Fusarium solani var. lycopersici (tomato)		58.3	Benlate	100
			Nabam	100
Macrophomina phaseolina		40.0	Benlate	100
			Nabam	
Rhizoctonia solani	75	69.5	Benlate	100

28), 24.73 (C-8, 24), 22.73 (C-2′,30′), 22.64 (C-1,31), 19.77 (C-6′, 10′, 22′, 26′) and 16.08 (C-18′).

3.5. Acetylation of *1*

A solution of **1** (14 mg) in pyridine (3 ml) and Ac_2O (3ml) was stirred for 12 h at room temperature. Usual workup provided the corresponding acetyl derivative **1a**. Compound **1a:** oil; IR ($CHCl_3$) ν_{max} cm^{-1}: 1724, 1640, 900; EIMS *m/z* (% rel. int.): 557(16), 556(36), 361(5), 360(18), 303(21), 57(100); ^1H NMR(400 MHz,$CDCl_3$): δ 5.06 (1H, *t*, $J=7.0$ Hz, H-17), 4.84 (1H, *s*, H_A-14′), 4.77 (1H, *s*, H_B-14′) 4.03 (2H, *m*, H-15′), 2.02 (3H, *s*, OAc), 1.53 (3H, *s*, Me-18) and 0.86–0.82 (24 H, 8×Me).

3.6. Bioassay evaluation

Crude extract, new tetraterpenoid trianthenol **1** and standard drugs (each 400 μg/ml of SDA) were subjected to antifungal activity against a number of pathogens, according to the established protocol (Shaukat et al., 1980). The % inhibition of the crude extract and trianthenol **1** was found to be moderate (Table **1**).

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PERGAMON

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PHYTOCHEMISTRY

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Cytotoxic labdane diterpenoids from *Croton oblongifolius*

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Abstract

Three labdane diterpenoids, 2-acetoxy-3-hydroxy-labda-8(17),12(*E*)-14-triene, 3-acetoxy-2-hydroxy-labda-8(17),12(*E*)-14-triene, and 2,3-dihydroxy-labda-8(17),12(*E*),14-triene were isolated from stem bark of *Croton oblongifolius*. Their structures were established by spectroscopic data, and each was also tested for cytotoxicity against various human tumor cell lines. The latter compound showed non-specific, moderate, cytotoxicities against all the cell lines; whereas the first two compounds were less active. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Croton oblongifolius*; Euphorbiaceae; Labdane; Diterpenoid; Cytotoxicity

1. Introduction

Croton oblongifolius Roxb. (Euphorbiaceae) has been used as a traditional medicine for many applications such as for dysmenorrhea, as a purgative, and to treat dyspepsia and dysenteria. Moreover, this plant has been used in conjunction with *C. sublyratus* to treat gastric ulcers and gastric cancers. Therefore, it is very interesting to identify the anti-tumor active principle(s) of this plant. Our continuing investigation on the chemical constituents of *C. oblongifolius* available in various parts of Thailand has yielded several new diterpenoids. For example, crotocembraneic acid, neocrotocembraneic acid, neocrotocembranal (Roengsumran et al., 1998, 1999a) were obtained from a *C. oblongifolius* specimen from Petchaboon Province in the central part of Thailand. Moreover, four labdane diterpenoids were isolated from another specimen from Prachuab Kirikhan Province in the southern part of Thailand (Roengsumran et al., 1999b). In this paper we describe the isolation and characterization as well as the cytotoxic activity against human tumor cell lines, of three new labdane diterpenoids **1**–**3** (Fig. 1) from a *C. oblongifolius* specimen from Loei Province in the northern part of Thailand.

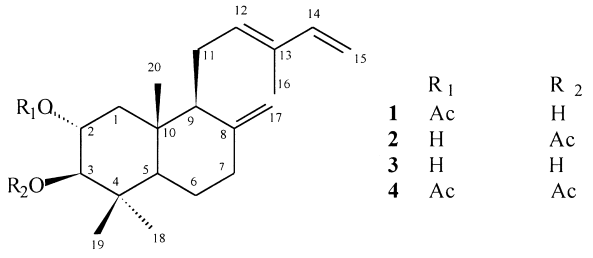


Fig. 1.

2. Results and discussion

The crude hexane extract of the stem bark of *C. oblongifolius* was separated by chromatography on a silica gel column using a hexane–ethyl acetate gradient system to obtain three labdane diterpenoids **1**–**3**. Treatment of **3** with acetic anhydride in pyridine gave **4**.

The molecular formula of compound **1** was assigned as $C_{22}H_{34}O_3$ based on elemental analysis, ^1H and ^{13}C NMR spectra (Table 1) and EIMS [M^+] (*m*/*z* 346). Its mass spectrum exhibited a peak at *m*/*z* 328 corresponding to [$M^+ - H_2O$]. The ion at *m*/*z* 286 was formed by

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Table 1
^{1}H and ^{13}C NMR spectral data (δ in ppm, J in Hz) for compounds **1**–**4** in $CDCl_3$

	Compound **1**		Compound **2**		Compound **3**		Compound **4**	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	42.3	1.25 *dd* (J = 11.7, 11.7) 2.12 *dd* (J = 4.3, 11.7)	46.3	1.29 *dd* (J = 12.1, 12.1) 2.21 *dd* (J = 4.3, 12.1)	45.0	1.18 *dd* (J = 11.7, 12.5) 2.10 *dd* (J = 4.6, 12.5)	42.4	1.27 *dd* (J = 11.9, 12.3) 2.08 *dd* (J = 4.6, 12.3)
2	73.2	4.96 *ddd* (J = 4.3, 10.0, 11.7)	67.8	3.81 *ddd* (J = 4.3, 10.1, 11.6)	69.1	3.69 *ddd* (J = 4.3, 9.6, 11.7)	70.0	5.04 *ddd* (J = 4.6, 10.5, 11.9)
3	80.5	3.23 *dd* (J = 5.2, 10.0)	84.5	4.55 *d* (J = 10.1)	83.2	3.02 *d* (J = 9.6)	80.2	4.71 *d* (J = 10.5)
4	39.9	–	39.4	–	39.3	–	39.3	
5	54.4	1.22 *dd* (J = 2.7, 12.6)	54.4	1.29 *dd* (J = 2.4, 12.8)	54.1	1.19 *dd* (J = 2.7, 12.5)	54.1	1.25 *dd* (J = 2.7, 12.8)
6	23.5	1.41 *dddd* (J = 4.3, 12.6, 12.6, 12.6) 1.74 *m*	23.5	1.41 *dddd* (J = 4.3, 12.8, 12.8, 12.8) 1.72 *dddd* (J = 2.6, 5.2, 5.2, 12.8)	23.4	1.40 *dddd* (J = 4.3, 12.5, 12.5, 12.5) 1.71 *m*	23.4	1.35 *dddd* (J = 4.3, 12.8, 12.8, 12.8) 1.65 *m*
7	37.6	2.00 *dd* (J = 5.2, 13.1) 2.42 *ddd* (J = 2.4, 4.3, 13.1)	37.6	2.02 *ddd* (J = 4.9, 13.1, 13.1) 2.41 *ddd* (J = 2.4, 4.3, 13.1)	37.4	1.99 *m* 2.39 *ddd* (J = 2.4, 4.0, 12.8)	37.4	1.96 *m* 2.34 *ddd* (J = 2.1, 4.0, 13.1)
8	146.3	–	146.9	–	146.6	–	146.6	
9	56.6	1.79 br.*d* (J = 11.6)	56.6	1.80 br.*d* (J = 10.4)	56.5	1.76 br.*d* (J = 10.7)	56.5	1.73 br.*d* (J = 9.8)
10	40.2	–	40.1	–	39.9	–	39.9	
11	23.4	2.19 *dd* (J = 6.4, 10.6) 2.29 br.*dd* (J = 4.3, 15.0)	23.3	2.19 *m* 2.36 *m*	23.3	2.17 *dd* (J = 6.7, 11.0) 2.34 br.*dd* (J = 5.5, 11.0)	23.3	2.12 *m* 2.20 *m*
12	133.1	5.35 *dd* (J = 6.4, 6.4)	133.0	5.40 *dd* (J = 6.4, 6.4)	132.8	5.38 *dd* (J = 6.1, 6.1)	132.8	5.28 *dd* (J = 6.4, 6.4)
13	133.7	–	133.8	–	133.8	–	133.8	
14	141.4	6.31 *dd* (J = 11.0, 17.4)	141.5	6.32 *dd* (J = 11.0, 17.4)	141.3	6.29 *dd* (J = 11.0, 17.4)	141.3	6.25 *dd* (J = 10.7, 17.4)
15	110.1	4.89 *d* (J = 11.0) 5.05 *d* (J = 17.4)	110.2	4.89 *d* (J = 11.0) 5.05 *d* (J = 17.4)	110.1	4.86 *d* (J = 11.0) 5.02 *d* (J = 17.4)	110.1	4.81 *d* (J = 10.7) 4.98 *d* (J = 17.4)
16	11.9	1.74 *d* (J = 1.0)	11.9	1.75 *d* (J = 0.9)	11.9	1.72 *d* (J = 0.9)	11.9	1.66 *d* (J = 0.9)
17	108.8	4.49 br.*d* (J = 1.5) 4.87 br.*d* (J = 1.5)	108.8	4.51 br.*d* (J = 1.5) 4.87 br.*d* (J = 1.5)	108.9	4.47 br.*d* (J = 1.2) 4.85 br.*d* (J = 1.2)	108.9	4.43 br.*d* (J = 1.2) 4.80 *m*
18	28.7	1.06 *s*	28.7	0.90 *s*	28.8	1.01 *s*	28.5	0.83 *s*
19	16.5	0.87 *s*	17.5	0.87 *s*	16.6	0.80 *s*	17.5	0.84 *s*
20	15.2	0.85 *s*	15.4	0.80 *s*	15.4	0.78 *s*	15.2	0.80 *s*
C=O	171.6	–	172.4	–	–	–	170.3	–
C=O	21.4	2.10 *s*	21.2	2.15 *s*	–	–	170.5	–
$\underline{CH_3}CO$	–	–	–	–	–	–	21.0	1.93 *s*
$\underline{CH_3}CO$	–	–	–	–	–	–	20.8	1.99 *s*

loss of acetic acid. A broad absorption band at 3463 cm^{-1} in the IR spectrum confirmed the presence of the hydroxy group. The IR stretching at 1722 cm^{-1} (C=O) and 1253 cm^{-1} (C−O) indicate the presence of an ester moiety in addition to the C=C stretching at 1639 cm^{-1}. The ^{13}C NMR spectrum and DEPT experiments revealed the presence of 22 non-equivalent carbons, of which 15 are sp^3 (five methyl, four methylene, four methine and two quaternary carbons) and six sp^2 (two methylene, two methine and two quaternary carbons) hybridized carbons, together with a carbonyl carbon of ester. The six vinylic (or sp^2) carbons were consistent with the presence of three double bonds in the molecule. The molecular formula, $C_{22}H_{34}O_3$, of compound **1** defined a degree of unsaturation of six; therefore, compound **1** must consist of two rings in addition to the three double bonds and one carbonyl group.

The ^1H and ^{13}C NMR spectra suggested that compound **1** possesses an acetate group (δ_H 2.10 and δ_C 21.4 for $\underline{C}H_3CO$), consistent with the EIMS data. The $-CH=C(CH_3)-CH=CH_2$ group gave a typical ^1H NMR spectrum for a labda-12(*E*),14-diene skeleton (δ 4.89, H-15, *d*, $J=11.0$; 5.05, H-15, *d*, $J=17.4$; 5.35, H-12, *dd*, $J=6.4$, 6.4; 6.31, H-14, *dd*, $J=11.0$ and 17.4; and 1.74, H3-16, *d*, $J=0.9$) (Bohlmann and Czerson, 1979; Noma et al., 1982; Roengsumran et al., 1998). The exomethylene group showed ^1H and ^{13}C NMR signals at δ_H 4.49 (H-17, br.*d*, $J=1.5$), 4.87 (H-17, br.*d*, $J=1.5$), and δ_C 108.8 (C-17, *t*). The ^1H NMR spectrum also showed three additional singlets due to three methyl groups at δ_H 0.85 (H_3-20), 0.87 (H_3-19) and 1.06 (H_3-18). Therefore, the OH and acetoxy moieties must be confined to the decalin ring system. A well-defined doublet of doublets at δ_H 3.23 (H-3, $J=5.2$, 10.0) was shown to be on C-3 carbon (δ_C 80.5) by HMQC experiment and was correlated with a quaternary carbon at δ_C 39.9 (C-4), two methyl carbons (δ_C 16.5, C-19 and 28.7, C-18) and an oxygen bearing methine carbon at δ_C 73.2 (C-2) as revealed by an HMBC experiment. Thus the two oxygen-bearing methine carbons must be adjacent to one another. The well-defined doublet of double doublets at δ_H 4.96 (H-2, $J=4.3$, 10.0 and 11.7) was shown to connect to δ_C 73.2 (C-2) by HMQC and correlated to δ_C 42.3 (C-1), 80.5 (C-3) and 171.6 (C=O) by the HMBC experiment. Therefore, the acetoxy group must be at C-2. A large coupling constant of 10.0 Hz between H-2 and H-3 indicated that both were in an axial orientation. Thus the OH and acetoxy moieties were in an equatorial orientation. The unequivocal assignment of compound **1** was established by analysis of data from HMQC, HMBC, COSY and NOESY experiments as shown in Table 1 and Fig. 2. The spectral data of the 2,3-dioxygenated decalin ring system with 8(17) exomethylene of compound **1** was also in agreement with those of methyl 2β, 3β-dihydroxy-labda-8(17),13*Z*-diene-15-oate isolated from *Nolana rostrata* (Garbarino et al., 1986). Thus compound **1** was 2-acetoxy-3-hydroxy-labda-8(17),12(*E*),14-triene.

Compound **2** was isolated as a white solid with a molecular formula $C_{22}H_{34}O_3$. The ^1H and ^{13}C NMR spectra (Table 1) of compound **2** were similar to those of compound **1**. The HMBC experiment revealed a correlation of the methine proton at δ_H 4.55 (H-3, *d*, $J=10.1$) to a quaternary carbon at δ_C 39.4 (C-4), an oxygen bearing methine carbon at δ_C 67.8 (C-2) and a C=O carbon at δ_C 172.4. Therefore, the acetoxy group was located on C-3. The two protons on C-2 and C-3 showed an axial relationship as suggested by the large coupling constant of 10.1 Hz as in compound **1**. Thus compound **2** was 3-acetoxy-2-hydroxy-labda-8(17),12(*E*),14-triene.

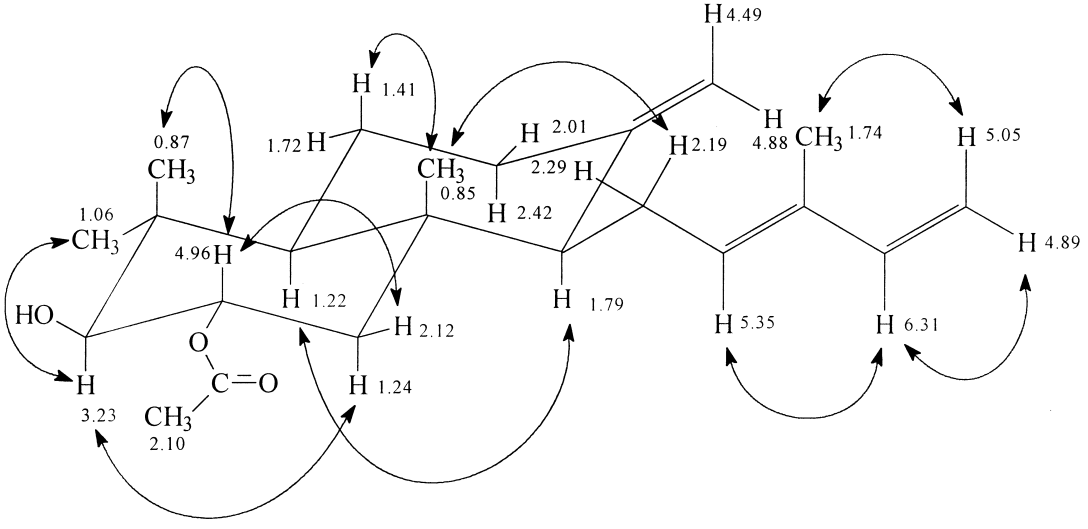


Fig. 2. Selected NOESY correlations for compound **1**.

Compound **3** has a formula of $C_{20}H_{32}O_2$, as established by elemental analysis. The ^1H and ^{13}C NMR spectral data for **3** (Table 1) were similar to those of compounds **1** and **2** except for the absence of an acetyl group. Therefore, compound **3** was 2,3-dihydroxy-labda-8(17),12(*E*),14-triene.

Compound **4** was obtained from peracetylation of compound **3**. It gave expected spectroscopic properties (IR, MS, ^1H and ^{13}C NMR).

Compounds (**1**–**4**) were tested for their cytotoxicity against human tumor cell lines (Table 2) and compound **3** showed non-specific moderate cytotoxicities against human gastric carcinoma (KATO-3, 2.2 μg/ml), colon adenocarcinoma (SW 620, 2.7 μg/ml), breast ductol carcinoma (BT474, 4.6 μg/ml), liver hepatoblastoma (HEP-G2, 3.7 μg/ml) and undifferentiated lung carcinoma (CHAGO, 3.3 μg/ml). Compound **4** was inactive against all cell lines (>10 μg/ml). Compound **1** showed weak activity against gastric (5.7 μg/ml) and colon adenocarcinoma (7.1 μg/ml) while compound **2** showed weak activity against gastric (3.3 μg/ml) and breast ductol carcinoma (5.9 μg/ml). It is very interesting to see that compounds **1** and **2** were more selective than compound **3**. Therefore, the presence of the acetyl group did not enhance cytotoxicity of compound **3**. Perhaps, monoacetylation (compounds **1** and **2**) and diacetylation (compound **4**) of compound **3** could render their ability to form hydrogen bond with certain receptor on tumor cells and made them more selective but less active.

3. Experimental

Mps uncorr. were carried out using a Fisher Johns melting point apparatus, whereas optical rotation was measured on a Perkin–Elmer 341 polarimeter. UV spectra were recorded on a Hewlett Packard 8452A spectrophotometer, whereas IR spectra were obtained on a Nicolet Impact 410 Spectrophotometer. Low-resolution MS was measured at 70 eV employed a Fisons Instruments Trio 2000 mass spectrometer, and ^1H and ^{13}C NMR, HMQC and HMBC experiments were carried out on a JEOL JNM-A500 spectrometer. Microanalyses were performed at the Chulalongkorn Research Equipment Centre using a Perkin–Elmer PE 2400 Series II.

Table 2
Cytotoxicity data for compounds **1**–**4**[a]

Compound	Cell lines[b]				
	KATO-3	SW620	BT474	HEP-G2	CHAGO
1	5.7	7.1	>10	>10	>10
2	3.3	>10	5.9	>10	>10
3	2.2	2.7	4.6	3.7	3.3
4	>10	>10	>10	>10	>10
Doxorubicin hydrochloride	1.7	1.1	0.08	0.9	2.3

[a] Results are expressed as IC_{50} values (μg/ml).

[b] KATO-3, human gastric carcinoma ATCC No. HTB 103; SW620, human colon adenocarcinoma ATCC No. CCL 227; BT474, human breast ductol carcinoma ATCC No. HTB 20; HEP-G2, human liver hepatoblastoma ATCC No. HB 8065; CHAGO, human undifferentiated lung carcinoma.

3.1. Plant material

The stem bark of *C. oblongifolius* was collected from Ampur Wang Saphung, Loei Province, Thailand, in May 1998. Botanical identification was achieved through comparison with a voucher specimen No. BKF 084729 in the herbarium collection of the Royal Forest Department of Thailand.

3.2. Extraction and isolation

The powdered, sun-dried stem bark of *C. oblongifolius* (6.5 kg) was extracted with hexane. The hexane extract was filtered, and evaporated in vacuo to obtain a greenish yellow oil (300 g). The hexane extract (100 g) was fractionated by silica gel CC and eluted with hexane-EtOAc to obtain compounds **1** (120 mg, 0.005%), **2** (60 mg, 0.003%) and **3** (8.94 g, 0.41%) from 5%, 10% and 30% EtOAc in hexane, respectively.

3.3. Biological evaluation

Bioassay of cytotoxic activity against human tumor cell culture in vitro was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetic method (Carmichael et al., 1987; Twentyman and Luscombe, 1987). Doxorubicin hydrochloride was used as a positive control substance.

3.4. 2-Acetoxy-3-hydroxy-labda-8(17),12(E),14-triene (*1*)

White solid, m.p. 102–103°C (Found C, 76.24; H, 9.91 $C_{22}H_{34}O_3$ requires: C 76.26; H, 9.89%); $[\alpha]_D^{20}+50.17$ ($CHCl_3$; c 1.0); UV $\lambda^{CHCl_3}{}_{max}$ nm (log ε): 244sh (4.45); IR ν_{max}^{KBr} cm^{-1}: 3463 (OH), 2974, 2940, 2863, 1722 (C=O), 1639 (C=C), 1441, 1373, 1252 (C−O), 1030; ^1H and ^{13}C NMR: Table 1. EIMS *m/z* (rel. int.): 346 [M^+] (24), 331 [M^+-CH3] (6), 328 [M^+-H_2O] (4), 317(7), 304(8), 290(15), 286 [M^+-CH_3COOH] (75), 272(16), 271(72), 268(63), 255(25), 253(100), 243(49), 230(28), 229(43), 225(34), 213(33), 191(30), 187(46), 185(32), 173(37), 171(27), 147(31), 145(41), 133(32), 131(40), 119(40), 105(57), 91(70), 79(42), 77(66).

3.5. 3-Acetoxy-2-hydroxy-labda-8(17),12(E),14-triene (**2**)

White solid, m.p. 99–101°C (Found C, 76.25; H, 9.89 $C_{22}H_{34}O_3$ requires: C 76.26; H, 9.89%); $[\alpha]_D^{20}+9.46$ ($CHCl_3$; c 1.0); UV $\lambda^{CHCl_3}{}_{max}$ nm (log ε): 244sh (4.53); IR ν_{max}^{KBr} cm^{-1}: 3443 (OH), 2940, 2848, 1722 (C=O), 1644, 1605, 1460, 1373, 1248 (C−O), 1030; ^1H and ^{13}C NMR: Table 1. EIMS *m/z* (rel. int.): 346 [M^+] (9), 328 [M^+-H_2O] (4), 313(1), 286 [M^+-CH_3COOH] (19), 271(45), 268(32), 255(22), 253(78), 243(39), 229(44), 213(32), 203(27), 187(52), 173(67), 159(56), 147(68), 145(60), 135(73), 133(87), 121(80), 119(95), 107(82), 105 (100), 95(53), 93(68), 91(70), 81(67), 79(89).

3.6. 2,3-Dihydroxy-labda-8(17),12(E),14-triene (**3**)

White solid, m.p. 69–70°C (Found C, 78.87; H, 10.59 $C_{20}H_{32}O_2$ requires: C 78.90; H, 10.59%); $[\alpha]_D^{20}$-6.96 ($CHCl_3$; c 1.0); UV $\lambda^{CHCl_3}{}_{max}$ nm (log ε): 244.5sh (4.14); IR ν_{max}^{KBr} cm^{-1}: 3376 (OH), 2969, 2945, 2853, 1644, 1610, 1446, 1388, 1214(C−O), 1059; ^1H and ^{13}C NMR: Table 1. EIMS *m/z* (rel. int.): 304 [M^+] (68), 289 [M^+-CH3] (53), 286(22), 271(75), 253(33), 248(62), 233(45), 215(27), 201(26), 187(78), 173(42), 159(46), 147(61), 145(70), 133(76), 119(76), 105(94), 91(100), 79(84).

3.7. 2,3-Diacetoxy labda-8(17),12(E),14-triene (**4**)

Compound **4** was prepared by acetylation of **3** (100 mg, 0.33 mmol) with acetic anhydride (80 mg, 0.78 mmol) in pyridine. The reaction product was purified by silica gel CC eluting with 2% EtOAc in hexane to give **4** as viscous oil (85 mg, 66%); (Found C, 74.19; H, 9.34 $C_{24}H_{36}O_4$ requires: C 74.19; H, 9.34%); $[\alpha]_D^{20}+24.52°$ ($CHCl_3$; c 1.0); UV $\lambda^{CHCl_3}{}_{max}$ nm (log ε): 232sh (4.39); IR ν_{max}^{neat} cm^{-1}: 2979, 2955, 2850, 1732 (C=O), 1645, 1613, 1440, 1384, 1255 (C−O), 1040; ^1H and ^{13}C NMR: Table 1. EIMS *m/z* (rel. int.): 388 [M^+] (11), 373(1), 328 [M^+-CH_3COOH] (25), 313(13), 286(17), 268 [M^+-2CH_3COOH] (70), 253(100), 239(15), 225(33), 211(38), 197(38), 187(52), 173(33), 159(23), 145(39), 133(38), 119(51), 105(62), 91(60), 79(63).

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Phenylbutanoid dimers from the leaves of *Alpinia flabellata*

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Abstract

Three phenylbutanoid dimers, *cis*- and *trans*-1-(2,4,5-trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane and 1,2-bis(2,4,5-trimethoxy-*Z*-styryl)- cyclobutane, were isolated from the leaves of *Alpinia flabellata* Ridley, together with three known compounds (2,4,5-trimethoxybenzaldehyde, 2,4,5-trimethoxycinnamaldehyde and 3,5-dihydroxy-7,4′-dimethoxyflavone). The structures of these compounds were determined by spectroscopic analysis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Alpinia flabellata*; Zingiberaceae; Phenylbutanoid

1. Introduction

In our continuing investigation on the constituents of plants belonging to the family Zingiberaceae (Kikuzaki et al., 1991a,b, 1992; Nakatani and Kikuzaki, 1992; Kikuzaki and Nakatani, 1996), we have focused on the constituents of a plant of the genus *Alpinia*. *Alpinia* species are distributed in tropical and subtropical areas, and many are used to flavor foods and as ingredients in traditional medicine formulations (Sirat and Liamen, 1995). The seeds of some of these plants have been used as aromatic stomachic, carminative, astringent, tonic and sedative drugs (Itokawa et al., 1980, 1985, 1987; Hsu et al., 1994). Several biological and pharmacological activity studies on some of these plants have appeared in the literature. These showed that the leaves of some species act as plant growth inhibitors (Fujita et al., 1994) and have insecticidal and antifungal activities (Tawata et al., 1996). Their rhizomes also showed inhibitory activity against the actions of histamine and barium chloride on excised guinea pig ileum (Itokawa et al., 1981b; Hsu et al., 1994). Many species of *Alpinia* have also been phytochemically studied. Sesquiterpenes have been isolated from the rhizomes of *A. speciosa* (Morita et al., 1996), *A. japonica* (Morita et al., 1988) and *A. intermedia* (Itokawa et al., 1987), diterpenes have been isolated from the seeds of *A. speciosa* (Itokawa et al., 1980) and *A. japonica* (Sirat et al., 1994) and diarylheptanoids have been isolated from the aerial parts of *A. katsumadai* (Ngo and Brown, 1998) and *A. oxyphylla* (Itokawa et al., 1981a, 1982).

Alpinia flabellata Ridley is a rare species in Japan. The leaves of this plant have been used as a wrapping material for foods on the islands of Iriomote and Ishigaki, Okinawa, which is located at the southernmost tip of Japan. Alflabene (Mori et al., 1978), 2′,4′,5′-trimethoxyphenylbutadiene, and its dimers (Masuda et al., 1999) have been isolated from the rhizomes of *A. flabellata* Ridley. However, to our knowledge, there has been no previous investigation on the constituents of the leaves. In this paper, we describe the isolation and structural elucidation of three new dimeric phenylbutanoids.

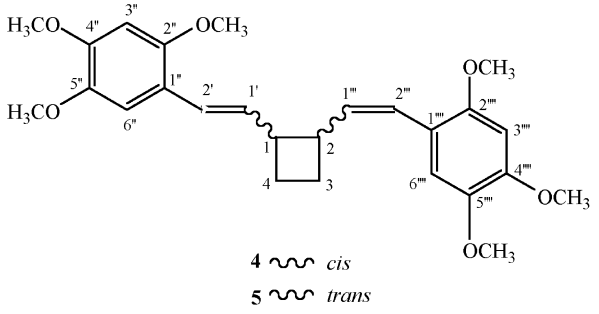
2. Results and discussion

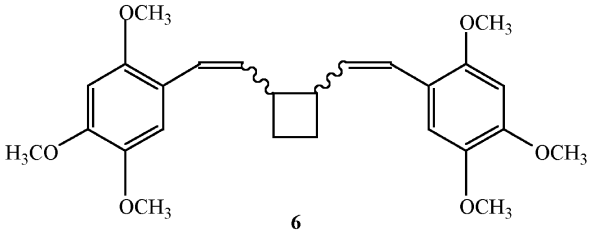
The dichloromethane extract of the leaves of *A. flabellata* was purified by repeated column chromatography (CC) using silica gel, Sephadex LH-20 and ODS gel and preparative HPLC to give six compounds, **1**–**6**.

Compounds **1–3** were determined to be 2,4,5-trimethoxybenzaldehyde, 2,4,5-trimethoxycinnamaldehyde and 3,5-dihydroxy-7,4′-dimethoxyflavone, respectively, based on their NMR spectral data and by comparison of these data with those reported in the literature

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(Kamaya and Ageta, 1990). This is the first isolation of these compounds from this plant.

Compound **4** showed a molecular ion peak at *m/z* 440 and a base peak at *m/z* 220, corresponding to half of the molecular ion in the EI-mass spectrum. The HR-mass spectrum gave an $[M]^+$ peak at *m/z* 440.2220, consistent with a molecular formula of $C_{26}H_{32}O_6$. The ^1H NMR spectrum of **4** showed signals due to two 1,2,4,5-tetra-substituted benzenes [δ 6.44 (1H, *s*), 6.46 (1H, *s*), 6.71 (1H, *s*) and 6.76 (1H, *s*)], six phenolic methoxyl groups [δ 3.68 (3H, *s*), 3.73 (3H, *s*), 3.791 (3H, *s*), 3.794 (3H, *s*), 3.85 (3H, *s*) and 3.86 (3H, *s*)] and four conjugated *Z*- and *E*-double bond protons [δ 5.70 (1H, *dddd, J* = 2.0, 2.0, 4.2, 10.0 Hz), 5.97 (1H, *br d, J* = 10.0 Hz), 5.78 (1H, *d, J* = 16.0 Hz) and 6.45 (1H, *dd, J* = 0.7, 16.0 Hz)]. As shown in Table 1, the ^{13}C NMR spectrum showed 26 carbon signals, including four olefinic, 12 aromatic and six phenolic methoxyl carbons. The HMQC spectrum of **4** indicated the presence of two methylene and two methine carbons. The mass spectrum was dominated by one major fragment corresponding to the 2,4,5-trimethoxyphenylbutanoid [*m/z* 220 $(C_{13}H_{16}O_3)^+$] moiety which suggested that **4** had a dimeric structure. The ^1H–^1H COSY spectrum showed that a methine proton [δ 4.11 (1H, *m*)] was coupled with methylene protons [δ 2.17 (1H, *m*) and 2.26 (1H, *m*)], and another methine proton [δ 2.80 (1H, *m*)] was also coupled with methylene protons [δ 1.75 (2H, *m*)]. Both methylene protons were also coupled with each other, suggesting a cyclobutane moiety. The cyclobutane structure was established by HMBC correlations between the methine proton at δ 2.80 (H-1) and C-2 and C-4, between the methine proton at δ 4.11 (H-2) and C-1, between the methylene protons at δ 2.17 (H-3) and δ 2.26 (H-3) and C-1 and C-4, and between the methylene protons at δ 1.75 (H-4) and C-1, C-2 and C-3. Furthermore, the signal of the

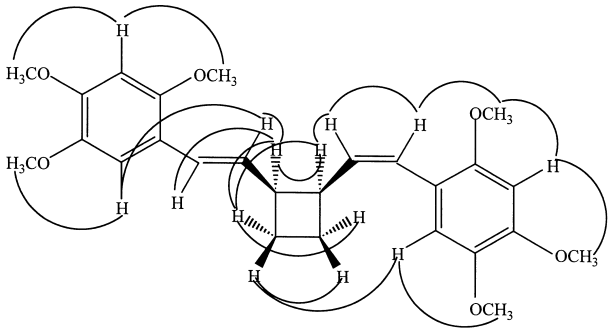
Table 1
^1H and ^{13}C NMR spectral data of compound **4** [δ (ppm) in $CDCl_3$ (500 MHz)][a]

Position	^1H	^1H–^1H COSY	^{13}C	HMBC
1	2.80 *m*	H-2, 4, 1′	41.5	H-2, 3, 4, 1′, 2′ 1‴
2	4.11 *m*	H-1, 3, 1‴, 2‴	37.2	H-1, 4, 1′, 1‴
3	2.17 *m*	H-2, 4	24.1	H-1, 4, 1‴
	2.26 *m*	H-2, 4		
4	1.75 (2H, *m*)	H-1, 3	25.4	H-1, 3, 1′
1′	5.78 *d* (16.0)	H-1, 2′	131.9	H-1′, 4, 2′
2′	6.45 *dd* (0.7 and 16.0)	H-1′, 6″	122.5	H-1, 1′, 6″
1″			119.5	H-1′, 2′, 3″, 6″
2″			150.7	H-3″, 6″
3″	6.44 *s*		98.1	
4″			148.6	H-3″, 6″
5″			143.2	H-3″, 6″
6″	6.71 *s*	H-2′	109.7	H-2′
1‴	5.70 *dddd* (2.0, 2.0, 4.2 and 10.0)	H-2, 2‴	129.6	H-1, 2, 3
2‴	5.97 *br d* (10.0)	H-2, 1‴	128.1	H-2
1⁗			122.4	H-3⁗, 6⁗
2⁗			151.7	H-3⁗, 6⁗
3⁗	6.46 *s*		97.0	
4⁗			147.8	H-3⁗, 6⁗
5⁗			142.3	H-3⁗, 6⁗
6⁗	6.76 *s*		114.9	
OMe	3.68 *s*		56.07	
	3.73 *s*		56.12	
	3.791 *s*		56.4	
	3.794 *s*		56.5	
	3.85 *s*		56.7	
	3.86 *s*		56.8	

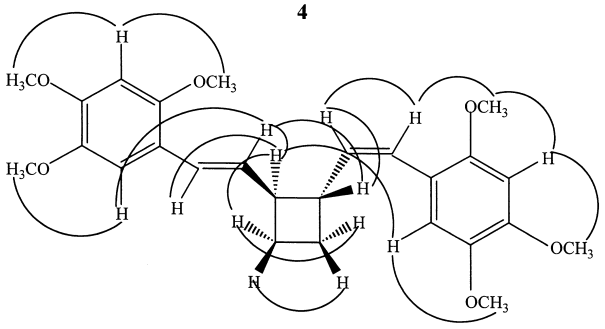
[a] Coupling constants (*J*) in Hz are given in parentheses.

aromatic carbon at δ 119.5 (C-1″) was coupled with the olefinic protons at δ 5.78 (H-1′) and δ 6.45 (H-2′), and the olefinic carbon at δ 122.5 (C-2′) was coupled with an aromatic proton at δ 6.71 (H-6″). These results suggested the presence of two trimethoxystyryl groups. In the HMBC spectrum, correlations were observed between the *Z*-olefinic proton [δ 5.70 (H-1‴)] and C-2 and C-3, between the *E*-olefinic proton [δ 5.78 (H-1′)] and C-1 and C-4, between a methine proton [δ 2.80 (H-1)] and C-1″, and between a methine proton [δ 4.11 (H-2)] and C-1‴. The ^{1}H-^{1}H COSY spectrum showed that the Z-olefinic proton at δ 5.70 (H-1‴) and the *E*-olefinic proton at δ 5.78 (H-1′) were coupled with a methine proton [δ 4.11 (H-2)] and another methine proton [δ 2.80 (H-1)], respectively. These results suggested that the *E*- and *Z*-styryl groups were attached to C-1 and C-2 of cyclobutane, respectively. The stereochemistries of the two trimethoxystyryl groups were assigned to be *cis*, based on the cross-peak observed between H-1 and H-2 in the NOESY spectrum of **4** (Fig. 1). Thus, compound **4** was determined to be *cis*-1-(2,4,5-trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane.

Compound **5** also showed a molecular ion peak at *m*/*z* 440 and a main fragment peak at *m*/*z* 220 in its EI-mass



4



5

Fig. 1. Observed NOE correlations in compounds **4** and **5**.

Table 2
^{1}H and ^{13}C NMR spectral data of compound **5** [δ (ppm) in $CDCl_3$ (500 MHz)][a]

Position	^{1}H	^{1}H-^{1}H COSY	^{13}C	HMBC
1	2.40 *ddd* (2.2, 8.3 and 18.5)	H-2, 4, 1′	45.3	H-2, 4, 1′, 2′ 1‴
2	3.75 *m*	H-1, 3, 1‴, 2‴	39.8	H-1, 4, 1′, 1‴, 2‴
3	2.21 (2H, *m*)	H-2, 4	24.5	H-1, 4
4	1.69 *m*	H-1, 3	28.2	H-1, 1′
	1.90 *m*	H-1, 3		
1′	6.03 *d* (16.1)	H-1, 2′	132.9	H-1, 4, 2′
2′	6.43 *dd* (0.7 and 16.1)	H-1′	122.4	H-1, 6″
1″			119.2	H-2′, 3″, 6″
2″			150.8	H-3″, 6″
3″	6.450 *s*		98.2	
4″			148.7	H-3″, 6″
5″			143.3	H-3″, 6″
6″	6.92 *s*		109.6	H-2′
1‴	5.60 *dddd* (2.2, 2.2, 4.5 and 10.0)	H-2, 2‴	130.5	H-1, 2
2‴	5.88 *br d* (10.0)	H-2, 1‴	127.5	H-2
1⁗			125.0	H-2, 3‴, 6‴
2⁗			151.4	H-3⁗, 6⁗
3⁗	6.454 *s*		97.6	
4⁗			143.0	H-3⁗, 6⁗
5⁗			147.6	H-3⁗, 6⁗
6⁗	6.74 *s*		112.8	
OMe	3.73 *s*		56.07	
	3.74 *s*		56.08	
	3.839 *s*		56.5	
	3.846 *s*		56.6	
	3.85 *s*		56.7	
	3.86 *s*		56.9	

[a] Coupling constants (*J*) in Hz are given in parentheses.

spectrum. The ^1H and ^{13}C NMR spectra of **5** were similar to those of **4** and suggested the presence of *E*- and *Z*-trimethoxystyryl groups and cyclobutane moiety (Table 2). The ^1H–^1H COSY spectrum showed that the *Z*-olefinic proton at δ 5.60 (1H, *dddd, J* = 2.2, 2.2, 4.5, 10.0 Hz, H-1‴) and the *E*-olefinic proton at δ 6.03 (1H, *d, J* = 16.1 Hz, H-1′) were coupled with the methine proton at δ 3.75 (H-2) and the methine proton at δ 2.40 (H-1), respectively. These results were also supported by HMBC correlations between the *Z*-olefinic proton [δ 6.43 (H-2′)] and C-6″, between an aromatic proton [δ 6.92 (H-6″)] and C-2′, and between a methine proton [δ 3.75 (H-2)] and C-1‴. Thus, the structure of **5** was proposed to be isomer of **4**. The relative stereochemistry at C-1 and C-2 was determined on the basis of NOESY experiments (Fig. 1). No NOE was observed between H-1 and H-2. Furthermore, an NOE was observed between the signal at δ 3.75 (H-2) and the *E*-olefinic proton [δ 6.03 (H-1′)]. Thus, compound **5** was determined to be *trans*-1-(2,4,5-trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane.

Several Diels–Alder dimeric phenylbutanoids and phenylcyclobutanes have been isolated from the rhizomes of *A. flabellata* and from another Zingiberaceae plant (Amatayakul et al., 1979; Fujita et al., 1994; Jitoe et al., 1993; Kuroyanagi et al., 1980; Masuda et al., 1999; Mori et al., 1978). In the analysis of these dimers by HPLC using ODS gel, the *trans* dimeric phenylbutanoids eluted faster than the *cis* isomers (Jitoe et al., 1993; Masuda et al., 1999). In the present study, when compounds **4** and **5** were analyzed by HPLC, they were clearly separated with retention times of 15.06 and 13.15 min, respectively.

Compound **6** also showed a molecular ion peak at *m/z* 440 and a main peak at *m/z* 220 in its EI-mass spectrum. The ^1H NMR spectrum showed only half of the signals corresponding to its molecular formula, indicating that **6** has a symmetrical structure. The ^1H NMR spectrum also suggested the presence of a trimethoxystyryl group. The two conjugated double bonds had a *Z*-orientation, which was deduced from the coupling constants of H-1′ and H-2′ [δ 5.56 (2H, *d, J* = 11.7 Hz) and 5.71(2H, *d, J* = 11.7 Hz)]. Since **6** was unstable in $CDCl_3$, we used C_6D_6 as a solvent in the ^{13}C NMR analysis. The ^{13}C NMR spectrum showed 13 carbon signals, including two *Z*-olefinic carbons, six aromatic, three phenolic methoxyl, one methine and one methylene carbons. Based on these data, compound **6** was determined to be 1,2-bis(2,4,5-trimethoxy-*Z*-styryl)cyclobutane.

3. Experimental

3.1. General

Mps: uncorr. NMR: ^1H at 500 MHz and ^{13}C at 125 MHz (Varian UNITY *plus* 500), TMS as an int. standard. MS: EI and HR MS 70 eV (Hitachi M-2000). CC, silica gel 60 (70–230 mesh, Merck), Sephadex LH-20 (Pharmacia) and ODS (100–200 mesh, Fuji Silysia).

3.2. Plant material

Leaves of *Alpinia flabellata* Ridley were collected in the forest on Iriomote island, Okinawa, Japan, and identified by one of the authors (S. Yonemori). A voucher specimen was deposited at the Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan.

3.3. Extraction and isolation

Dried leaves of *A. flabellata* (800 g) were chopped, pulverized and extracted 5 times with CH_2Cl_2 (1000 ml) at room temp. The combined CH_2Cl_2 soln was evaporated in vacuo to give 27.15 g of CH_2Cl_2 extract. The extract (25.78 g) was subjected to CC on silica gel using *n*-hexane–Me_2CO (3:1) as the eluting solvent to give 11 fractions (Frs 1-11). Fr 5 was evaporated (3.17 g) and subjected to silica gel CC with C_6H_6–MeOH (99:1) and CH_2Cl_2 to give a ppt (**1**, 11 mg) and 14 fractions (Frs A–N). Frs H–J were combined then subjected to repeated silica gel CC (C_6H_6–MeOH 98:2; C_6H_6–Me_2CO 90:5) to give 8 fractions (Frs H–J-1 to H–J-8). Fr H–J-5 was separated into an *iso*-PrOH soluble part and an *iso*-PrOH insoluble part. The insoluble part was recrystallized with MeOH to give **4** (67 mg). Gel filtration of the soluble part on Sephadex LH-20 (*iso*-PrOH), followed by repeated ODS gel CC (H_2O–MeOH 1:9, 1:4, 1:3) gave **2** (20.0 mg) and **3** (20 mg). Fr L was subjected to successive gel filtration on Sephadex LH-20 (*iso*-PrOH), silica gel CC (C_6H_6–Me_2CO 90:5) and ODS gel CC (H_2O-MeOH 1:3), and preparative HPLC using a reversed-phase column (ODS, H_2O–CH_3CN 3:7) to afford **6** (1 mg). Frs F–G were combined then purified by gel filtration on Sephadex LH-20 (*iso*-PrOH), silica gel CC (C_6H_6–Me_2CO 9:1; C_6H_6–EtOAc 90:5) to give **5** (57 mg).

3.4. *cis*-1-(2,4,5-Trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane (**4**)

White powder, mp 122–124°C, $[\alpha]_D^{21}$ −11.5° ($CHCl_3$; c 0.1). UV λ_{max}^{EtOH} nm (log ε): 205.8 (4.76), 229.0 (sh, 4.37), 261.0 (4.29), 298.2 (4.01), 313.4 (3.98). IR ν_{max} cm^{-1}: 1608, 1516, 1207, 1040, 873. EIMS 20 eV *m/z* (rel.int.%): 440 $[M]^+$ (37), 220 (100), 205 (19), 189 (93), 180 (16), 174 (13), 145 (14), 91 (8), 69 (8) and 45 (3). HR-EIMS 20 eV: found: *m/z* 440.2220 $[M]^+$, $C_{26}H_{32}O_6$ requires: 440.2197. ^{13}C NMR and ^1H NMR spectral data: Table 1.

Table 3
^1H and ^{13}C NMR spectral data of compound **6** [δ (ppm) in $CDCl_3$ and C_6D_6 (500 MHz)][a]

Position	^1H $CDCl_3$	^1H C_6D_6	^{13}C C_6D_6
1	4.93 *d* (4.6)	5.40 *m*	40.6
4	2.26 *m*	2.09 *m*	28.8
	2.92 *m*	2.93 *m*	
1′	5.56 *d* (11.7)	5.74 *d* (11.7)	124.3
2′	5.71 *d* (11.7)	5.89 *d* (11.7)	133.4
1″			125.1
2″			152.1
3″	6.36 *s*	6.27 *s*	98.6
4″			149.0
5″			143.8
6″	6.33 *s*	6.68 *s*	115.5
OMe	3.49 *s*	3.20 *s*	55.9
	3.63 *s*	3.45 *s*	56.3
	3.83 *s*	3.46 *s*	56.5

[a] Coupling constants (*J*) in Hz are given in parentheses.

3.5. *trans-1-(2,4,5-Trimethoxy-E-styryl)-2-(2,4,5-trimethoxy-Z-styryl)cyclobutane (**5**)*

Powder, mp 94–96°C, $[\alpha]_D^{21} + 0.2°$ ($CHCl_3$; c 0.1). UV λ_{max}^{EtOH} nm (log ε): 204.5 (4.64), 230.5 (sh, 4.21), 261.0 (4.14), 300.5 (3.87), 313.5 (3.84). IR ν_{max} cm^{-1}: 1613, 1515, 1205, 1125, 1038, 873. EIMS 20 eV *m/z* (rel. int.%): 440 $[M]^+$ (0.2), 220 (100) and 189 (26). HR-EIMS 20 eV: found: *m/z* 440.2240 $[M]^+$, $C_{26}H_{32}O_6$ requires: 440.2197. ^{13}C NMR and ^1H NMR spectral data: Table 2.

3.6. *1,2-bis(2,4,5-Trimethoxy-Z-styryl)cyclobutane (**6**)*

Oil, EIMS 20 eV *m/z* (rel.int.%): 440 $[M]^+$ (56), 220 (100), 205 (29), 189 (99), 181 (17), 174 (22), 145 (25), 115 (14), 91 (16), 69 (17) and 45 (8). HR-EIMS 20 eV: found: *m/z* 440.2163 $[M]^+$, $C_{26}H_{32}O_6$ requires: 440.2197. ^{13}C NMR and ^1H NMR spectral data: Table 3.

3.7. *HPLC analyses of **4** and **5***

The analytical conditions were as follows: analytical column: DEVELOSIL ODS-HG-5 (size: 4.6×250 mm); solvent: 70% CH_3CN in H_2O; flow rate: 1 ml/min; detection: 280 nm.

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Book review

Plant Genetic Engineering. Towards the Third Millennium. Proceedings of the International Symposium on Plant Genetic Engineering, 6–10 December 1999, Havana, Cuba

A.D. Arencibia, Elsevier, Amsterdam 2000, 272pp., ISBN: 0-444-50430-3, price $136.00

This is a publication relating to a conference held in Havana, Cuba in December 1999. The conference also honoured Professor Marc Van Montagu for his pioneering work in the genesis of plant transformation and his subsequent illustrious career in fostering plant biotechnology in a truly global way. There essentially is the essence of this book, for it is truly global with respect to various issues and besides the science therein, it gives an insight into the status of plant biotechnology outside the major industrial nations. This has already been described in a review of the meeting (Martin, C.R., 2000, Trends in Plant Science 5, 138–140). It is clear that many so-called developing countries have well established and affordable programmes in micropropagation technologies. With this sort of science base, the transfer of technology for genetic engineering and molecular breeding is a logical progression to aid socially-minded scientists deal with the many special needs of these regions. This is technology not only desired but also that can be put into practice with relatively limited resources as shown by the efforts of the host country.

However, the conference occurred right at the height of the onslaught by anti-GM movement in Europe and the beginning of the debate in the USA. Therefore it was probably refreshing for the scientists from industrialised countries to enjoy Caribbean hospitality and to witness scientists and senior international NGO representatives making the case of why the technology must proceed. The areas where it would be considered essential were in relation to global food security and in the development of renewable feed stocks for environmentally-friendly industrial processes. These needs are now in the forefront of the counter-arguments used by those making the case for GM.

The book falls into 39 thematic chapters. The first three chapters are a group giving the facts and figures for GM and other aspects of plant biotechnology globally. There are chapters here giving the views of ISAA (C. James), an economist specialising in global agriculture [J. van Wijk, see *Phytochemistry*, 2000, 55(2), 93–95] and the FAO (J. Izquierdo) and arguing the case for a beneficial transfer of technology to the countries of the south. The intervening chapters deal with the basic science and the technology development and describe many case studies that are well advanced. The final three chapters deal with safety and regulatory issues, which in one case shows that Monsanto did employ rigorous risk assessment in assessing their GM crops (W. Kaniewski et al.), even though they got the public relations drastically wrong.

The scientific chapters describe numerous case studies in engineering of tropical and subtropical crops for resistance to biotic and abiotic stresses. These are interspersed with familiar efforts by scientists from the industrialised nations to improve oil and fibre processing and quality, starch- and fructan- storage products and control of ripening. The interface between north and south was illustrated with a series of papers on the production of therapeutic products in plants, in which Cuba has an advanced programme.

The book was published early in 2000 which is commendable given the timing of the conference and ensures that this volume is timely and topical. It gives an accurate account of that seminal meeting since the reviewer had the privilege to be present and be educated on the broader issues outside the excellent science presented. These issues relating to the ethical, economic and risk to the environment of GM are now global. Yet, this was a meeting that was a passionate reaffirmation of the reasons why this technology was developed in the first instance, global food security being major among them. Some of the applications discussed have been already used in the debate against the detractors, including the now well publicised work of Luis Herrera-Estrella in engineering aluminium resistance into crops to allow growth on marginal lands in poor countries. If any phytochemists need reassurance on how to approach a debate with anti-GM lobbyists they can find much ammunition between these covers.

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PHYTOCHEMISTRY

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Editorial comment

Global phytochemistry: the ethnobotanist view☆

In the second of our Editorial Comments, we talk to Mark Plotkin who is both President of the Amazon Conservation Team and a Research Associate at the Smithsonian Institute, Museum of Natural History. Mark has worked for many years as an ethnobotanist with the indigenous people of the Amazon following study at Harvard, Yale and Tufts.

We also review his latest book "Medicine Quest" in this issue. Mark has written a number of non-specialist texts introducing natural product chemistry to a wider audience. In the light of public awareness of the commercialization of biotechnology, this interface is increasingly important for all of us. Insensitive exploitation of the global treasure chest of undeveloped phytomedicines without regard for social objectives may provoke reactions approaching a scale similar to that seen for GM. Mark has been in the forefront through his books and public lectures in raising the issues concerning interaction with the "phytochemists" amongst the indigenous peoples. We ask his views.

How do you see both your role, and that of others, in protecting the rights of indigenous people with the need for exploration for new phytomedicines?

It seems to me that there is a false dichotomy between ethnobotanical field research and Intellectual Property Rights (IPR) protection. Good research should entail protecting the rights of the people that have helped you — that is not rocket science, that is basic good manners! I believe that scientists working in the bush are uniquely positioned to be of assistance — whether it is direct involvement in terms of brokering deals (which is seldom the case) or, making sure the locals are not taken advantage of (in terms of helping them find and hire lawyers, ensuring that they are put in touch with non-governmental organizations that can advise them, etc.).

The fact is that Western medicine, no matter how successful it is, does not yet have all the answers (where are the cures for schizophrenia, deep depression, most cancers, AIDS, drug-resistant bacteria, etc.?) These new technologies make it easier to find, analyze, and manipulate molecules as never before which is why there is growing interest in natural products in many quarters to find treatments for these "incurable" ailments.. Ethnobotanists, anthropologists and others must be ever more diligent, even though the current interest is primarily focused on natural products that are not used by local peoples (e.g. ziconotide from cone snails, Synercid from soil fungi).

What does biopiracy mean to you and your colleagues?

To me, the term means testing and developing products for the market based on dishonest or illegal procedures — taking specimens out of a country without the proper permits or claiming that specimens are only destined for the herbarium or museum and then sending them to the laboratory. It is important to understand that some of what is considered "biopiracy" today may have been closer to standard operating procedure not so long ago. Only with the meetings in Rio de Janeiro in 1992 was there the beginning of a global effort to recognize the intellectual property rights of local peoples and develop an international framework to put this into practice.

Have you any personal experience of "biopiracy"?

The stories that keep coming up these days are of researchers who may have had the best of intentions but fell afoul of changing standards. Let me give you two recent stories I heard (perhaps apocryphal!) which outline potential pitfalls to be avoided.

1. A biologist has been studying the ecology of pond algae in Ecuador for 30 years. A colleague new to the department begins analyzing the most recent collection for anticancer compounds. Because the first researcher has never been interested in medicinal applications, his collecting permit has been

☆ The views expressed in Editorial Comment articles are the sole opinion of the authors, and publication shall not imply the concurrence of the Editors or Publisher.

approved every year. When the second researcher asks if these specimens were collected with a permit, she is told yes — but it wasn't the right permit for bio-prospecting!

2. A European botanist writes to a Brazilian colleague she met at a scientific congress, asking the Brazilian if he is interested in collaborating on a bio-prospecting project. The Brazilian gets written permission from the chief of a local Indian tribe, the Director of the local university, and the governor of the state in which all this occurs. Thinking all has been taken care of, the European travels to Brazil and begins collecting and ends up in a Brazilian jail. The accusation: she didn't have permission at the national level.

The point here is that one can run afoul of the law, even with the best of intentions (and I'm NOT assuming that everyone interested in medicinal compounds from nature automatically has the best of intentions!). What I'm saying is that the lack of standardization in legislation is a strong disincentive for the few companies willing to participate in an ethical manner.

How should conservationists, ethnobotanists and phytochemists be interacting in the future?

The complexity of interacting with — much less understanding an indigenous culture is extremely challenging to a researcher from the outside. I know that in my own work with the Tirio Indians in the northeast Amazon (which is a biocultural conservation effort, not bioprospecting) I have found it extremely fruitful to not only ask the help of anthropologists, but also physicians, cartographers, linguists, filmmakers and lawyers.

A personal example: a 1970′s publication based on work done with a different subtribe noted that the Indians used plant species x to treat "kananama" which is malaria. But a linguist later explained to me that "kananama" means "yellow" and a physician working in the village later told me that "kananama" is actually jaundice!

Do you think that there is still a high potential to detect new pharmaceutically interesting natural products in undeveloped phytomedicine and is there a potential to develop new drugs with unexpected pharmaceutical effects?

I believe the most promising frontiers for plant research entail testing fresh material (if that is what is used by local herbalists), rather than drying the material and sending it to a lab. We must play closer attention to the plant as used by local healers, particularly in terms of recipe and dosage. We should also work with the total recipe (including admixtures, where used), rather than searching for a single "magic bullet" compound.

Can indigenous people really be compensated for disclosure of their handed-down knowledge in the light of subsequent intellectual property?

I believe that this is possible, but the key is "culturalsensitivity. When we talk about "Indians" for example, that encompasses everything from tribes that have graduated lawyers and PhD biologists to groups in Amazonia that have little or no contact with the outside world. Compensation has to work in a way which clearly and materially benefits the culture — if it cannot be done in a particular case, the research should not go forward until proper arrangements can me made to ensure a fair and equitable return to the community for sharing their knowledge.

From your perspective, is the perceived lack of younger trained phytochemists/ethnobotanists, particularly in the west, real or not?

When I consulted with a small, start-up pharmaceutical company starting in the early 80's the CEO complained that they had to look for natural product chemists trained in India and Hong Kong because so many of the pharmacognosy programs in US universities had been closed down!

I've been criticized by colleagues who said that I shouldn't encourage students to go into ethnobotany or natural product chemistry because "there won't be any jobs" but I know unemployed lawyers and business people thrown out of work here in the US due to "corporate downsizing". And I know physicians who have quit practicing medicine because of their disgust with the health care system.

Given the rapidly changing marketplace, ALL students need to go to school with their eyes open to employment possibilities and with an eye towards getting a broad education which I believe will make them more employable than taking seven years of nothing but engineering courses.

How pessimistic are you about the future of the Amazonian rainforest and what needs to be done still?

Much will be lost, unfortunately, but (I believe) much will be saved. The battle to determine how much is still being waged!

What attracted you to this field, and when?

I had the great good luck to find a mentor when I was 19. I had dropped out of college and took a job at the Museum of Comparative Zoology at Harvard. At the suggestion of a friend, I enrolled in a night school class entitled "The Botany and Chemistry of Hallucinogenic Plants" taught by Dr. Richard Evans Schultes. One lecture and I was hooked on plants, Indians, and the Amazon...

And finally on a lighter note, a last question, what is your favorite phytochemical anecdote?

Two ethnobotanists had been investigating a West African shaman's potion, which appeared to be very effective in reducing blood sugar levels. They had carried out extensive work on the three plants in the potion in various combinations without being able to reproduce the effects. They were revisiting him after they failed to show any beneficial effects on diabetes of the plant mixture they had coerced from him and thus requested that they see how he made it up. He duly boiled the plants and right at the end added a crab, which elicited the response, "what is that?". The shaman pointed out that it was obviously a crab to which the scientist complained that he had not been told that part of the recipe. The shaman smiled and politely pointed out that the scientist had only asked him to give him all the plants in the recipe, which he had done! The supplemented potion was effective back in the lab.



Fig. 1. The subject of the interview, Mark J Plotkin is seen with a bushnegro guide in Suriname. (photo © Mark J. Plotkin, Amazon Conservation Team, 2334 Wilson Blvd, Arlington, VA 22201. USA: markplotkin@msn.com).

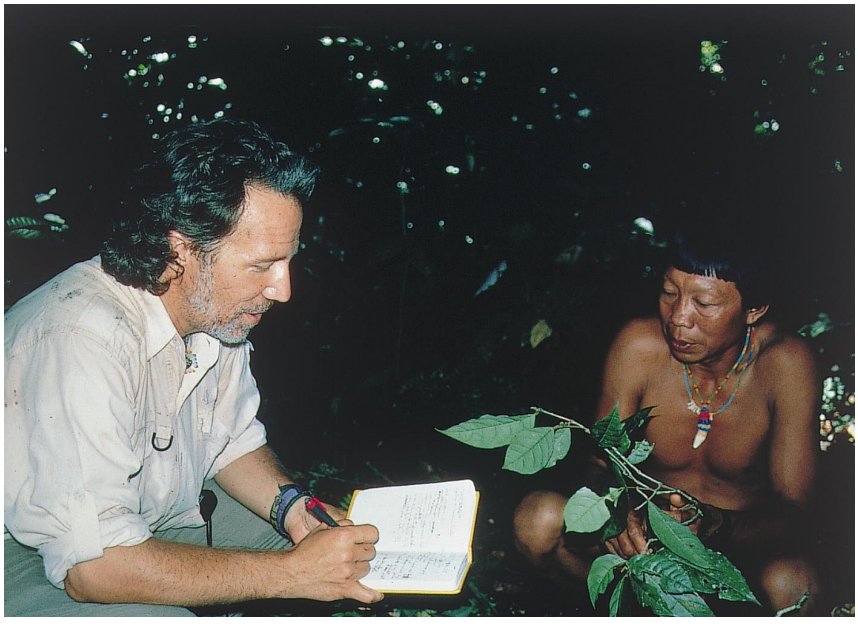


Fig. 2. Mark Plotkin with a Tirio guide in Suriname (© Mark J. Plotkin).

This anecdote is from Mark's book "Medicine Quest" which is reviewed on page 214. He has also written a children's book (The shaman's apprentice) which will hopefully inspire some of them to become the Phytochemistry faculty of the future.

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Investigation of avenacin-deficient mutants of *Avena strigosa*

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Abstract

The biosynthesis of cyclic triterpenoids in ten saponin-deficient (*sad*) mutant varieties of the diploid oat *Avena strigosa* is reported. Two mutants were found to be deficient in 2,3-oxidosqualene:β-amyrin cyclase (OSβAC) (EC 5.4.99) and thus unable to produce the β-amyrin necessary for the production of avenacins. The other mutants studied had post β-amyrin lesions. 2,3-Oxidosqualene:cycloartenol cyclase (OSCC) (EC 5.4.99.8) needed for sterol formation was present in all ten mutants. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Avena strigosa*; Poaceae; Biosynthesis; Mutants; Saponins; Avenacins; Phytosterols

1. Introduction

Saponins are common plant secondary metabolites. Many of these compounds have pronounced antifungal properties and it is possible that they act as preformed determinants of resistance to attack by fungi (Hostettmann et al., 1991). Four antifungal saponins, avenacins A-1, A-2, B-1 and B-2 (Fig. 1), have been isolated and characterised from oat roots by several groups of workers (Goodwin and Pollock, 1954; Burkhardt et al., 1964; Maizel et al., 1964; Tschesche et al., 1973; Crombie et al., 1984a,b; Begley et al., 1986; Crombie et al., 1986a,b; Crombie and Crombie, 1986). All four avenacins contain a pentacyclic triterpenoid skeleton which, in common with the phytosterol skeleton, is derived from mevalonic acid (MVA) by way of a series of reactions leading up to and including 2,3-oxidosqualene, which on cyclization gives rise to either β-amyrin, the precursor of the aglycone portion of the avenacins, or cycloartenol, the precursor of phytosterols and their derivatives (Scheme 1). Previous studies have confirmed that β-amyrin and the avenacins themselves are synthesised in the oat root tip (Trojanowska and Threlfall, 1998; Trojanowska et al., 2000).

Avenacins A-1 and B-1 each contain an *N*-methylanthranilic acid residue and are strongly fluorescent, while avenacins A-2 and B-2 each contain a benzoic acid residue and are weakly fluorescent. The trisaccharide component common to all four avenacins is formed from two glucose molecules and one arabinose molecule with the arabinose directly attached to the C-3 oxygen of the triterpenoid (Fig. 1).

"Take-all" is a disease of cereals caused by the fungus *Gaeumannomyces graminis* var. *tritici*. However, oats are resistant to this root-infecting fungus. Indirect evidence for a role for avenacins as a determinant for the resistance of oats to *G. graminis* has been provided by several groups of workers (Turner, 1940; Crombie and Crombie, 1986; Osbourn et al., 1994; Bowyer et al., 1995).

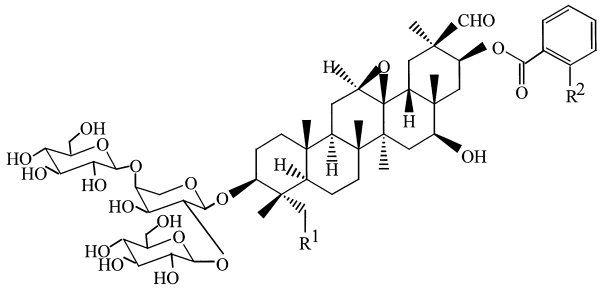
In order to evaluate further the contribution of avenacins to "take-all" resistance in oats, the diploid oat species *Avena strigosa* was treated with the chemical mutagen sodium azide, and a number of saponin-deficient (*sad*) mutants were isolated (Table 1) (Papadopoulou et al., 1999). Genetic analysis indicated that each of the mutant phenotypes was attributable to mutant alleles at single loci. In keeping with the postulated involvement of avenacins in disease resistance, these mutants all showed increased susceptibility to *G. graminis* and to a number of other root and stem-based diseases (Papadopoulou et al., 1999).

Here we report on the investigation of the biosynthesis of avenacins and phytosterols in the above mentioned

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Avenacin A-1 R^1 = OH, R^2 = NHMe
Avenacin A-2 R^1 = OH, R^2 = H
Avenacin B-1 R^1 = H, R^2 = NHMe
Avenacin B-2 R^1 = H, R^2 = H

Fig. 1. Structures of the four oat root avenacins (Crombie et al., 1984a).

sad mutants of *A. strigosa*. These investigations were of two types: (a) radiochemical feeding experiments employing *R*-[2-^{14}C]MVA and (b) radiochemical measurement of 2,3-oxidosqualene:cycloartenol cyclase (OSCC) (EC 5.4.99.8) and 2,3-oxidosqualene:β-amyrin cyclase (OSβAC) (EC 5.4.99) activities in crude cell-free preparations of oat roots.

A short report of some parts of this work has been published elsewhere (Trojanowska et al., 1998).

2. Results

2.1. Incubation of root tissue with R-[2-^{14}C]MVA

The patterns of incorporation of radioactivity from *R*-[2-^{14}C]MVA into triterpenoids by finely chopped roots of the ten *sad* mutants of *A. strigosa* listed in Table 1, *A. strigosa* (WT) and *A. sativa* are summarised in Fig. 2. Radiolabelled squalene, cycloartenol, 24-methylene cycloartanol, desmethylsterols and "steryl esters"[1] (were found to be present in all of the lipid extracts analysed (Fig. 2). ^{14}C-2,3-Oxidosqualene was detected in the lipid extracts of mutants 610, 791-3, 109-3 and 825-1. ^{14}C-β-Amyrin was present in the lipid extract of *A. strigosa* (WT), *A. sativa* and all of the mutants except 610 and 109-3.

^{14}C-Avenacins were detected by radio-TLC and radio-HPLC in *A. strigosa* (WT) and *A. sativa*, and appeared to be present in mutants 616-3, 1139-1 and 9 based on radio-TLC analysis (Fig. 2). However, on radio-HPLC of radio-TLC purified ^{14}C-avenacins from the three mutants, no radioactivity was found to be associated with the avenacin region of the radiochromatogram.

2.2. HPLC analysis of root extracts for the presence of avenacins

Aliquots of the root extracts were subjected to HPLC analysis (Table 2). In keeping with a previous study (Crombie and Crombie, 1986), the concentrations of avenacins in *A. strigosa* (WT) roots was found to be some nine times lower than that in *A. sativa* roots, and all four avenacins were present in both sets of roots. Furthermore, in agreement with the study of Papadopoulou et al. (1999) the roots of only two *sad* mutants (791-3 and 9) were found to contain avenacins.

Unidentified compounds with *N*-methylanthranilic acid residues were detected in *A. strigosa* (WT) and all of the mutants but not in *A. sativa* (Table 2). *A. strigosa* (WT) and all ten mutants contained fluorescent compounds with an R_t (min) value of 7.5, while only two mutants (1139-1 and 9) contained fluorescent compounds with R_t (min) values of 12 and 20. The peak at R_t 7.5 min does not correspond to the avenacin micelle peak (R_t (min) value of 10.0, in this system) observed in avenacin-containing extracts subjected to HPLC (Papadopoulou et al., 1999; Trojanowska, 1999). The peak ahead at R_t (min) 20.0 probably corresponds to monodeglucosylavenacin A-1 which is known to be produced by these two mutants (Papadopoulou et al., 1999).

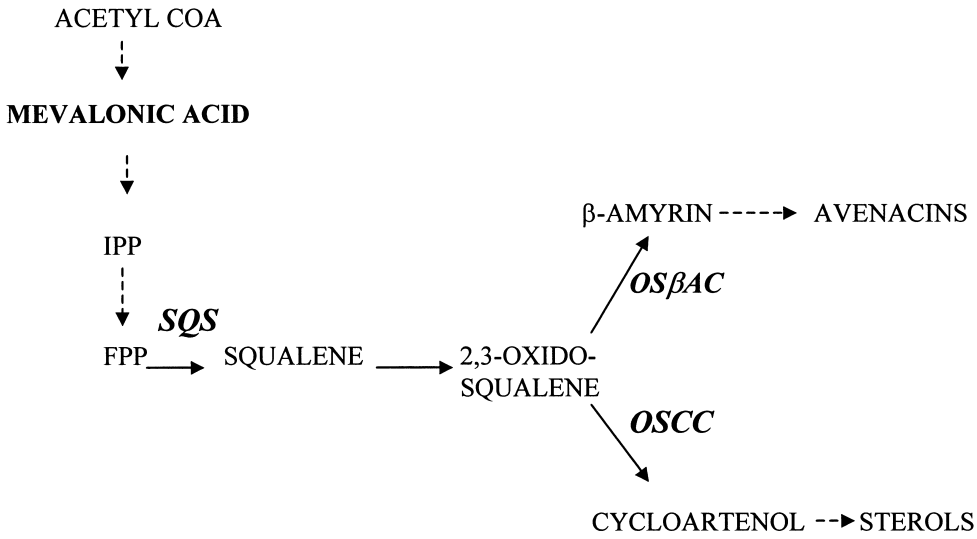
2.3. Assay of 2,3-oxidosqualene:cycloartenol cyclase (OSCC) and 2,3-oxidosqualene: β-amyrin cyclase (OSβAC) activities in cell-free preparations of mutants

OSβAC activity was not detected in mutants 610 and 109-3, but was present in the remaining mutants, in *A. strigosa* (WT) and in *A. sativa*. OSCC activity was detected in all ten mutants, in *A. strigosa* (WT) and in *A. sativa*, and was roughly similar to or higher than the OSβAC activity (Fig. 3).

3. Discussion

It must be stressed at the outset of the discussion that it is not valid to attach too much importance to differences in degrees of labelling or enzyme activity within or between the two oat species and the ten mutant lines of *A. strigosa*, unless the differences are very large. *A. strigosa* (WT) roots incorporated radioactivity from *R*-[2-^{14}C]MVA into the same range of terpenoids as *A. sativa* roots. However, they produced far less ^{14}C-"steryl esters" than *A. sativa* (Fig. 2). HPLC analysis showed that the total amount of avenacins (nmol g^{-1} fr.wt.) in A. strigosa (WT) roots was some nine times lower than that in *A. sativa* roots. In agreement with the

[1] Radioactive compounds which co-migrate with steryl esters on TLC but which on alkaline hydrolysis do not yield radioactive sterols (Trojanowska et al., 2000).



Scheme 1. Outline of the pathway for the biosynthesis of cyclic triterpenoids. **SQS**, squalene synthase; **OS**βAC, 2,3-oxidosqualene:β-amyrin cyclase; **OSCC**, 2,3-oxidosqualene:cycloartenol cyclase.

Table 1
Genetic classification and biochemical characteristics of the saponin-deficient (*sad*) mutants of *A. strigosa* (Papadopoulou et al., 1999)

Complementation group	Mutant number	Avenacins
sad 1	109-3	Absent
	610	Absent
sad 2	1027-5	Absent
	791-3	Reduced levels
sad 3	1139-1	Absent; mono-deglucosylavenacin A-1 present
sad 4	616-3	Absent
Unassigned	9	Reduced levels of avenacins; mono-deglucosylavenacin A-1 present
	376-1	Absent
	825-1	Absent
	1243	Absent

findings of Papadopoulou et al. (1999) none of the mutants contained HPLC-detectable amounts of avenacins, apart from 791-3 and 9, both of which contained reduced amounts of these compounds compared to *A. strigosa* (WT).

On the basis of the *R*-[2-^{14}C]MVA incorporation patterns, the mutants fell into three groups. In the first group, no ^{14}C-β-amyrin or ^{14}C-avenacins were produced but there was a substantial build up of ^{14}C-2,3-oxidosqualene in the two independently isolated mutants, 610 and 109-3, which fell into this group. This suggested that in these two *sad* 1 mutants the enzyme OSβAC was absent or impaired in some way. On performing an OSβAC assay on these two mutants, it was found that this enzyme activity was indeed absent as no ^{14}C-β-amyrin was detected whereas ^{14}C-cycloartenol was produced in both cases.

In the second group, radioactivity from *R*-[2-^{14}C]MVA was incorporated by the two *sad* 2 mutants (1027-5 and 791-3), one of which is only a partial mutation (791-3), and three of the unassigned *sad* mutants (1243, 825-1 and 376-1) into β-amyrin but not into avenacins. However, 825-1 and the *sad* 2 mutant (791-3) synthesised small amounts of ^{14}C-2,3-oxidosqualene. The enzyme assays showed that both ^{14}C-β-amyrin and ^{14}C-cycloartenol were produced by the three unassigned *sad* mutants which suggests that avenacin biosynthesis is either blocked, or in the case of the *sad* 2 mutant (791-3) producing reduced amounts of avenacins, is partially inhibited at some point on the pathway after OSβAC. This suggests that 791-3 has a partial mutation on the pathway.

In the third group, radioactivity from *R*-[2-^{14}C]MVA was incorporated into β-amyrin and compounds with the radio-TLC properties of ^{14}C-avenacins by the *sad* 4 mutant (616-3), the *sad* 3 mutant (1139-1) and one of the unassigned *sad* mutants (9). However, on radio-HPLC no radioactivity was eluted with the avenacins present in the unassigned *sad* mutant (9) or with marker avenacins in the cases of the *sad* 3 mutant (1139-1) and the *sad* 4 mutant (616-3). In the enzyme assay experiments, both ^{14}C-β-amyrin and ^{14}C-cycloartenol were produced suggesting that some of the post β-amyrin enzymes of the pathway show reduced activity. As in the previous group, one of these mutants (9) accumulated reduced amounts of avenacins. In addition, the *sad* 3 mutant (1139-1) and the unassigned *sad* mutant (9) both contained mono-desglucoavenacin A-1 (Papadopoulou et al., 1999).

The *N*-methylanthranilic acid-containing compounds detected at R_t 7.5 min on HPLC of the extracts of *A.*

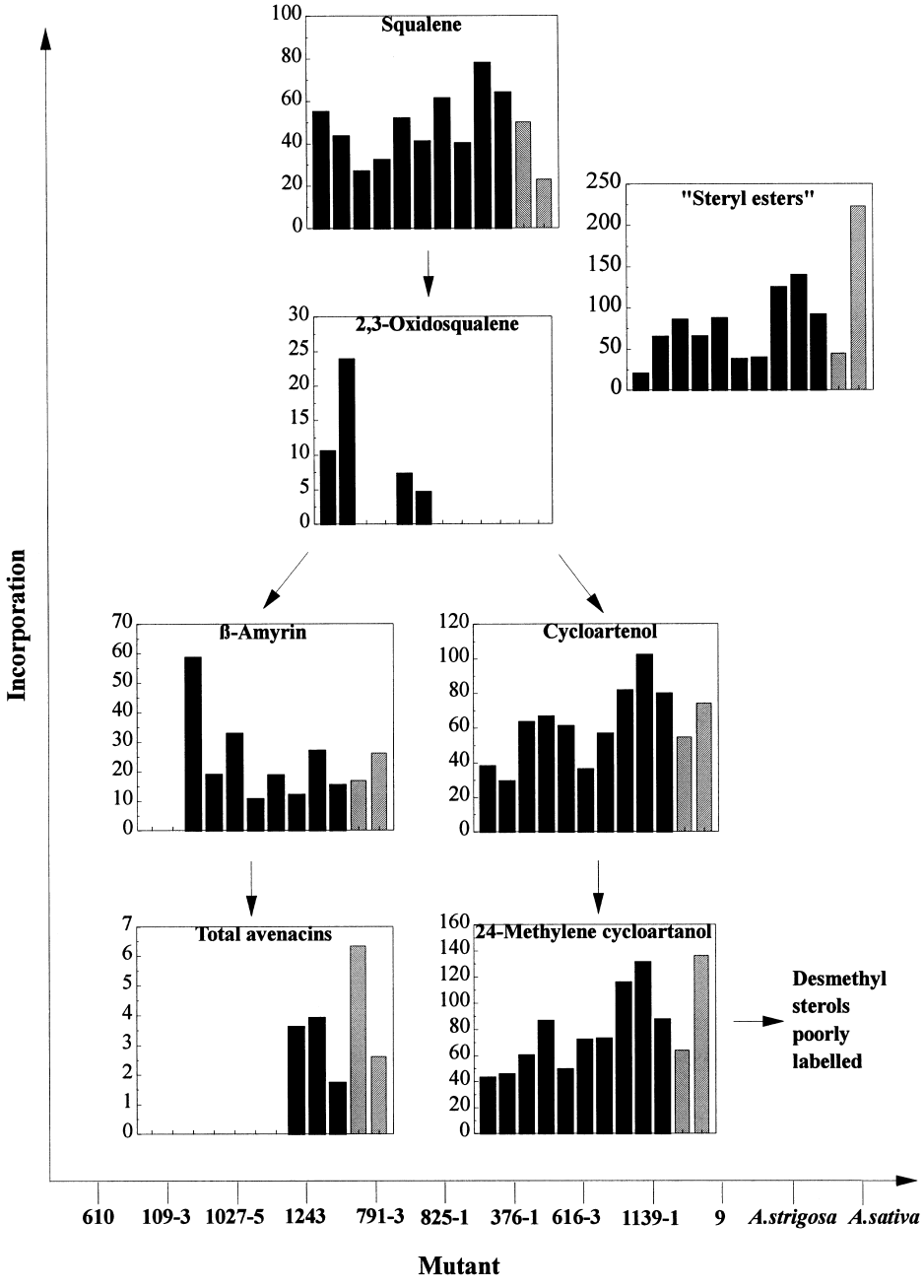


Fig. 2. Incorporation (nCi g^{-1} fr.wt) of *R*-[2-^{14}C]MVA into terpenoids of *A. strigosa* (WT) and its mutant lines. *A. sativa* was included for comparative purposes. N.B. The total incorporation into avenacins was quantified at the end of the TLC purification steps i.e. prior to radio-HPLC (see text).

strigosa (WT) and the ten mutant lines, are not micelles of avenacins which give rise to a peak with an R_t value of 10.0 min. These *N*-methylanthranilic acid compounds do not appear to be related to the avenacins apart from the fact that both sets of compounds contain a chromophore of *N*-methylanthranilic acid. It may be possible that as the avenacins are not being produced, free *N*-methylanthranilic acid or its CoA ester is available and is attached to atypical acceptors. Two of the mutants (9 and 1139-1) were distinguished by containing two *N*-methylanthranilic acid-containing compounds with R_t (min) values of 12 and 20, the latter of which is probably mono-deglucosylavenacin A-1.

The results show that there is an excellent measure of agreement between the genetic data, analytical data and biochemical data obtained by Papadopoulou et al. (1999). However, the nature of the defect(s) in most mutants remains a matter of conjecture.

In the cases of the two *sad* 1 mutants (109-3 and 610), the data are entirely consistent with the absence of OSβAC or production of an inactive form of OSβAC. Further experiments are needed to define whether the

Table 2
Summary of HPLC results

Mutant	Total avenacins (nmol g^{-1} fr.wt)	*N*-methylanthranilic acid-containing unknown compounds (nmole g^{-1} fr.wt)		
R_t (min)	12.1, 13.8, 16.0, 18.8	7.5	12	20
610	0	69.0	0	0
109-3	0	88.2	0	0
1027-5	0	161.1	0	0
1243	0	65.8	0	0
791-3	10.4[a]	163.4	0	0
825-1	0	146.5	0	0
376-1	0	113.4	0	0
616-3	0	97.2	0	0
1139-1	0	74.2	18.9	15.2
9	50.8[b]	36.8	12.1	6.7
A. strigosa (WT)	152.8[c]	79.0	0	0
A. sativa	1328.2[d]	0	0	0

[a] A-1, 10.4 nmol g^{-1} fr.wt.
[b] A-1, 42.4; B-1, 8.4 nmol g^{-1} fr.wt, respectively.
[c] A-1, 70.9; B-1, 9.2; A-2, 55.9; B-2 16.8 nmol g^{-1} fr.wt, respectively.
[d] A-1, 637.9; B-1, 22.4; A-2, 560.5; B-2 107.4 nmol g−1 fr.wt, respectively.

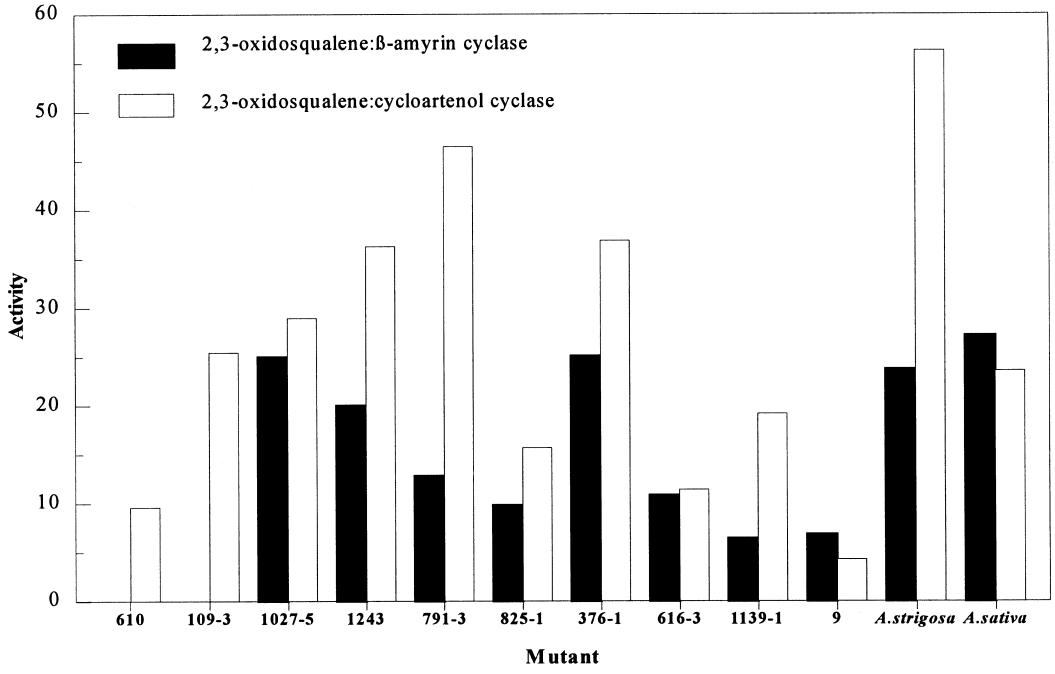


Fig. 3. Activity (pmol mg $protein^{-1}$ h^{-1}) of 2,3-oxidosqualene:β-amyrin cyclase and 2,3-oxidosqualene:cycloartenol cyclase in oat root preparations of *A. strigosa* (WT) and mutant lines. *A. sativa* was included for comparative purposes.

lack of OSβAC activity in these mutants is the result of a regulatory mutation. However, although the observed accumulation of ^{14}C-2,3-oxidosqualene in the *R*-[2-^{14}C]MVA feeding experiments provides evidence that the pathway up to but not including OSβAC is operating normally in these mutants, evidence now needs to be obtained that the post OSβAC enzymes are also operating normally. If this could be shown to be the case for one of the glucosyltransferases (one of the enzymes on the post OSβAC part of the pathway), then this would serve to confirm that the absence of OSβAC activity is the sole mutational event in *sad* 1 mutants (109-3 and 610). All of the other mutants were capable of the biosynthesis of β-amyrin.

The *sad* 2 mutant (1027-5), the *sad* 4 mutant (616-3), and three of the unassigned *sad* mutants (376-1, 825-1 and 1243) do not accumulate detectable amounts of avenacins. It seems entirely reasonable, therefore, that in each of these mutants, one of the post β-amyrin enzymes is either absent or inactive. In the case of the

sad 2 mutant 791-3, the presence of reduced avenacins could be taken to suggest that the enzyme which is inactive in the other *sad* 2 mutant (1027-5) is partially active in the *sad* 2 mutant 791-3.

The *sad* 3 mutant (1139-1) and the unassigned *sad* mutant (9) have in common the facts that: (i) they have stunted roots and, in the case of 1139-1, shoots as well; (ii) they accumulate amounts of mono-deglucosylavenacin A-1; and (iii) they incorporate radioactivity from *R*-[2-^{14}C]MVA into compounds with the radio-TLC properties of ^{14}C-avenacins. However, they differ in that 9 accumulates considerable amounts of avenacins as assessed by HPLC whereas 1139-1 contains no detectable amounts of avenacins. The presence of mono-deglucosylavenacin A-1 in these mutants possibly means that there is a mutation affecting the glucosyltransferase(s) needed to convert the mono-deglucosylavenacin form to avenacin A-1. It is noteworthy that two unique *N*-methylanthranilic acid-containing compounds were detected (by HPLC) in the above mutants in the course of the work carried out in Hull (Trojanowska, 1999). It may be that the one having an R_t greater than the R_t of the last avenacin to be eluted from the column (avenacin B-1) is in fact the mono-deglucosylavenacin A-1 detected by HPLC and SELDI-MS by Papadopoulou et al. (1999). Clearly, a great deal more work remains to be done on the biochemical changes which account for the absence or reduced levels of avenacins in these ten mutants of *A. strigosa*.

4. Experimental

4.1. Chemicals and general methods

All solvents were of AnalaR grade. Et_2O was dried over Na/Pb alloy and redistilled (bp 34°C) over reduced iron. Lanosterol, squalene, dihydrocholesterol, stigmasterol, β-sitosterol, benzoic acid methyl ester, NADPH and glucose-6-phosphate dehydrogenase, Type XII from *Torula* yeast, were purchased from Sigma Chemical Co., Poole, UK. Cycloartenol, α-amyrin and β-amyrin were purchased from Apin Chemicals Ltd, Abingdon, UK. N-methylanthranilic acid methyl ester was purchased from Lancaster Synthesis Ltd, Morecambe, UK. Mixtures of avenacins prepared using the method of Crombie et al. (1984a), were kindly donated by the Sainsbury Laboratory.

R-[2-^{14}C]MVA lactone (53 μCi μmol^{-1}) was obtained from Amersham International, Amersham, UK. *R*-[2-^{14}C]MVA lactone was converted to the sodium salt by removal of the toluene under a stream of nitrogen gas, adding 1 ml distilled H_2O and 10 mg sodium hydrogen carbonate. (*S*)-2,3-Oxido[3-$^3H_1$]squalene (30 μCi μmol^{-1}) in buffered aqueous solution was available from a previous study (Threlfall and Whitehead, 1988; Fulton, 1993). Protein estimation was performed using a modified method of Lowry (Peterson, 1977).

TLC was carried out on pre-coated silica gel G 20×20 cm aluminium backed plates (Merck) with a 2.5 cm concentrating zone at the base of each plate. For silver ion (Ag^+) chromatography, the plates were dipped in a solution of 10% (w/v) silver nitrate and then dried at 100°C for 1 h. Radioactivity on a developed plate was detected (prior to treating the plates with any reagent) by means of a radio-TLC scanner (Canberra Packard Bioscan Autochanger 4000, Bioscan System 200 Imaging Scanner). Compounds on a developed plate were located by examination of the plate under $UV_{254\ nm}$ light before and after spraying with 0.025% ANSA (Gitler, 1972) (non-destructive), by spraying with 1% anisaldehyde followed by heating (Waldi, 1965) (destructive), or by staining with iodine vapour. Compounds were recovered from the gel with Et_2O or MeOH. Radioactivity in solutions was measured by liquid scintillation spectrometry. Radioactive sterol and amyrin samples were analysed (after acetylation) by radio-Gas Chromatography (GC) (Brindle et al., 1988).

HPLC analysis of avenacins was performed on a Hewlett–Packard Series 1050 HPLC with a Hewlett–Packard 1040 M Series II HPLC Detection System. A 25 cm C18 reverse phase spherisorb ODS3 column was used with aq. 75% MeOH as the solvent system at a flow rate of 0.5 ml min^{-1}. The runs were monitored at six wavelengths, corresponding to the main avenacin UV absorption bands of 223, 225, 255, 274, 281 and 356 nm. For radio-HPLC analysis of avenacins, the same conditions were used as above with samples being collected every 15 s for liquid scintillation analysis. For the calculation of amounts of avenacins known amounts of benzoic acid methyl ester and *N*-methylanthranilic acid methyl ester dissolved in MeOH were injected onto the HPLC. The areas corresponding to these amounts were then used to calculate the molar amounts of avenacins in the samples. Correction for micelle formation was made by collecting the micelle peak and re-injecting the sample on the HPLC. The relative percentages of the areas for the four avenacins seen upon reinjection were added on to the previously resolved peaks.

4.2. Plant material, growth of oat seeds and preparation of root material for feeding and cell-free experiments

Seeds (M_2) of wild type (WT) *A. strigosa* (S75) and 10 mutant lines, 825-1, 1027-5, 109-3, 616-3, 791-3, 9, 1139-1, 1243, 610 and 376-1 generated by sodium azide mutagenesis (Papadopoulou et al., 1999), were bulked up as follows. The mutant seeds were placed on moist filter paper in Petri dishes at 4°C for 2 days and then left at room temperature for a further 2 days before transferring to a glasshouse (12–35°C). After 10–14 days the seedlings were separately placed into 1litre growing pots

along with John Innes Potting Compost Number 2 and grown in the glasshouse. During the winter months, supplementary lighting was used (Mercury Vapour Lamps MBF/V) to provide an 18 h photoperiod. After 12–16 weeks the plants, which are self-fertilising, had set seed and were ready for harvesting. The seeds were collected, dehulled, germinated and grown following the conditions described above.

A. sativa cv. *Image* was included in all of the experiments for comparative purposes.

Seeds of *A. sativa, A. strigosa* (WT) and *A. strigosa* mutants (200 of each) were dehulled, surface sterilised by soaking in 10% sodium hypochlorite for 10 min, then rinsed in distilled H_2O until free of chlorine. The seeds were then placed in Petri dishes containing two layers of dampened filter paper. The dishes were covered with aluminium foil and incubated at 22°C and 55% humidity for 3 days. At the end of this period the roots were on average 0.5–1.0 cm in length and the whole roots were excised.

4.3. Feeding experiments using R-[2-^{14}C]MVA

4.3.1. Incubation conditions

Roots (0.3 g) were excised, chopped finely and placed in 10 ml conical flasks containing 1 ml 0.1 M KH_2PO_4 buffer at pH 7.0 and 5μCi (95 nmol) *R*-[2-^{14}C]MVA. The flasks were incubated with gentle shaking in a water bath at 23°C for 9 h. The contents of each flask were then filtered through a sintered glass funnel and washed with 5 ml distilled H_2O. The retained tissue was homogenised (with an Ultra Turrax) with 2×5 ml hot MeOH and centrifuged at 4500 ***g*** for 10 min. The supernatants were pooled, taken to dryness in vacuo and the residue dissolved in MeOH (200 μl). Prior to analysis (see below) an aliquot (10 μl) was assayed for radioactivity.

4.3.2. Analysis of R-[2-^{14}C]MVA radiolabelled compounds

4.3.2.1. Initial separation by TLC. Prior to analysis by TLC, some of the samples had mass markers added to them as carriers to help prevent degradation and also to aid in visualising the bands on the TLC plates. Typically, 100 μg each of cycloartenol, β-amyrin, α-amyrin, stigmasterol, squalene, sitosterol and cholesteryl oleate were added. The extract (100 μl) was then separated by TLC with cyclohexane–EtOAc (1:1) as the developing solvent. The developed plate was scanned for radioactivity, sprayed with ANSA and examined under UV light. The bands containing squalene and steryl esters (R_f 0.69), dimethylsterols and β-amyrin (0.56), methylsterols (0.54) and desmethylsterols (0.52) were scraped off the plate individually, eluted with Et_2O and taken up in cyclohexane (200 μl). The compounds on the origin (avenacins, steryl glycosides, acylated steryl glycosides, *R*-[2-^{14}C]MVA lactone and *R*-[2-^{14}C]MVA) were eluted from the gel with MeOH and taken up in MeOH (200 μl). 1 μl of each of the samples was taken and counted.

4.3.2.2. Analysis of squalene and steryl esters (R_f 0.69). The sample containing squalene and steryl esters was rechromatographed (TLC) with petrol (40–60°C) as the developing solvent. The squalene (R_f 0.14) and steryl ester (0.00) bands were scraped off individually and eluted off the gel with Et_2O. The samples were dissolved in cyclohexane (200 μl). 1 μl of each was counted. The steryl ester sample was transferred to a screw topped glass tube, 5 ml 8% KOH in 80% ethanol added and the tube was placed in a heating block at 70°C for 2 h. The tube was then cooled and the free sterols extracted with 3×5 ml hexane. The hexane layers were pooled, dried, transferred to vials, acetylated and analysed following the procedure for free sterols iii).

4.3.2.3. Analysis of the dimethylsterol- and β-amyrin-containing fraction (R_f 0.56). After removal of the cyclohexane by evaporation, the sterols were acetylated (200 μl Ac_2O and 1 drop of pyridine) overnight at room temperature. Three ml ethanol were then added to destroy excess Ac_2O and the solvents removed under a stream of nitrogen. The acetylated samples were then dissolved in cyclohexane (200 μl) and separated by Ag^+-TLC with petrol–toluene (1:1) as the developing solvent. Acetylated markers were run at the same time. The developed plate was then scanned for radioactivity after which the acetylated markers were sprayed with anisaldehyde. Individual acetates were scraped off the plate, eluted with Et_2O and the samples taken up in cyclohexane (200 μl). Representative radioactive samples were further analysed by radio-GC. The R_fs and RR_ts (dihydrocholesteryl acetate) were as follows: cycloartenyl acetate, 0.31, 1.80; α-amyrin acetate, 0.44, 1.81; β-amyrin acetate, 0.44, 1.61; 24-methylene cycloartanyl acetate, 0.21, 1.96.

4.3.2.4. Analysis of avenacins, steryl glycosides and acylated steryl glycosides (R_f 0.00). 100 μl of the mixture of compounds recovered from the origin (of the preliminary TLC) was separated on TLC using $ChCl_3$-MeOH–H_2O (70:30:5.5) as the developing solvent. The area containing the avenacins (R_f 0.21–0.42), detected by blue fluorescence under UV light, was scraped off the gel, eluted with MeOH, dissolved in MeOH (200 μl) and 1 μl counted. The extracts were then analysed by HPLC and radio-HPLC. The R_t values (min) were as follows: avenacins, A-1, 16.1; A-2, 12.0; B-1, 18.8; B-2, 13.6.

4.4. OSCC and OSβA activities in cell-free preparations of mutants

Oat roots were ground in cold mortars for 30 s with 0.2 g acid-washed sand g^{-1}, 0.1 g insoluble PVP g^{-1} and

1 ml extraction buffer g^{-1} fr. wt of tissue. The extraction buffer comprised 0.1 M KH_2PO_4, 0.5 M sucrose, 10 mM Na_2EDTA, 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$ at pH 7.5. The resultant brei was squeezed through two layers of Miracloth into chilled Eppendorf tubes and centrifuged at 4500 ***g*** for 15 min. Cyclohexane (50 μl) containing 5 μl (2 nmol) (*S*)-2,3-oxido[3-$^3H_1$]squalene was transferred to the bottoms of silanized vials. The solvent was removed under a gentle stream of nitrogen gas and the (*S*)-2,3-oxido[3-$^3H_1$]squalene resuspended with gentle agitation in 2 μl of cellosolve (ethylene glycol monomethyl ether). The mixture was made up to 100 μl by the addition of 98 μl of cell-free extracts and incubated for 1 h at 30°C. The reactions were then quenched by the addition of 5 ml $CHCl_3$–MeOH (1:2) and the $CHCl_3$-soluble lipids extracted by the procedure reported by Threlfall and Whitehead (1988). The extracts were dissolved in acetone (100 μl) and 10 μl assayed for radioactivity. Aliquots (90 μl) of the extracts, along with standards of known concentration, were subjected to TLC with $CHCl_3$ as the developing solvent. The plate was then radioscanned, sprayed with ANSA and the bands visualised under UV light. Located compounds were recovered from the gel with Et_2O (unincorporated 2,3-oxidosqualene, R_f 0.27; cycloartenol and β-amyrin, 0.05). The cycloartenol and β-amyrin were dissolved in cyclohexane (100 μl), 10 μl counted and the remainder of the sample taken to dryness and acetylated overnight (for experimental details, see above). The acetylated sample was then subjected to Ag^+-TLC using petrol (40–60°C)-toluene (1:1) as the developing solvent, scanned for radioactivity and the plate either stained with I_2 vapour to locate bands of interest and allowed to fade, or the standards were sprayed with anisaldeyde while the rest of the plate was covered up. The areas of gel containing β-amyrin acetate (R_f 0.41) and cycloartenyl acetate (0.28) were then scraped off separately into scintillation vials and assayed for radioactivity.

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The study of a monocotyledon abscission zone using microscopic, chemical, enzymatic and solid state ^{13}C CP/MAS NMR analyses

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Abstract

We have investigated distinguishing features in cells of the abscission zone of a monocotyledon fruit, the oil palm *Elaeis guineensis*. The cell walls of the abscission zone and the subtending mesocarp and pedicel have been analysed by light and transmission electron microscopy, by chemical methods and by solid state ^{13}C CP/MAS NMR spectroscopy. Results show that these abscission zone cells have specific characteristics which include high levels of unmethylated pectin in the walls and an inducible (×35) polygalacturonase enzyme expression. Together these findings help to explain the localised precision of cell separation events. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Elaeis guineensis* Jacq. fruit; Oil palm; Abscission; Pectin; Uronic acids lignin; Polygalacturonase; Cellulase

1. Introduction

The shedding of leaves, flowers and fruits from the body of a plant is a precisely localised abscission event pre-determined by the positional differentiation of specific ethylene-responsive target cells formed days, months or even years before the act of shedding actually takes place (Osborne, 1989). What we do not know at present is whether the major inducible enzymes at these sites, which are linked to loss of cell to cell adhesion at shedding, reflect also any specificity in the walls or middle lamellae of the abscission cells.

Abscission cells are few in number and are generally differentiated as a plate, 2 or 3 cells in thickness only, across the junction of the organ in question and its point of attachment to the subtending part of the stem (Osborne, 1989). Until they start to separate from each other at shedding of the organ, these cells can be distinguished from their immediate neighbours on either side only as a rather uniform band of parenchyma cells, usually of smaller size. Once the organ immediately above them starts to undergo senescence and produce the relatively high levels of ethylene that are associated with yellowing of leaves, flower fading or fruit ripening, cells of the pre-determined site of shedding are signalled to a cascade of new gene expressions specific to those regions alone and absent from the cells of tissues distant to them. Perception of the ethylene signal by abscission cells causes them to enlarge, to produce abscission cell-specific isoforms of cell wall modifying enzymes and to undergo marked ultrastructural change. Nuclei enlarge as nuclear (transcriptional) activity rises, endoplasmic reticulum proliferates in relation to increased protein synthesis and the Golgi stacks dilate with high levels of vesicle production typifying secretion of newly synthesised components to the cell wall (Osborne, 1989).

These cellular events together with the ethylene induction of specific isoforms of polygalacturonases and cellulases and the activation of their respective genes have been widely studied in shedding leaves and fruits (Gonzalez-Carranza et al., 1998). Part of the understanding of abscission processes assumes that the composition and structure of the walls of the abscission target cells must possess a specificity not common to their neighbours on either side of the potentially separatable plate of cells. How else would only the abscission cells lose adhesion with each other

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under the influence of the newly secreted enzyme array induced by ethylene?

The major difficulty in studying the walls of abscission cells, either before or after their separation, has been their limitation in number and the difficulty of obtaining sufficient material for analysis that is free of contaminating wall products from tissue immediately above or below the target cell plate. Whereas the 2–3 rows of cells of the bean leaf abscission zone were ideal for the immunogold study that sought the distribution of a specific protein that is highly enriched in abscission zone cells (McManus and Osborne, 1990), it demonstrated only that the distribution of gold particles was particularly intense at the corners of the walls in the target cells but, in contrast, was thinly and more evenly distributed in the walls of cells immediately adjacent to them on either side (McManus and Osborne, 1989). Similarly, the use of in situ hybridization, which confirmed the expression of an inducible mRNA for the abscission-specific cellulase in cells that are part of the whole bean abscission region, could not explain why only the target cells of the zone would be caused to separate (Tucker et al., 1991).

We have now studied shedding of the fully ripe and ethylene-producing fruit of the oil palm, *Elaeis guineensis*, (Fig. 1A,B) a monocotyledon that possesses an unusually large and multi-layered plate of abscission cells between the fruit and the subtending pedicel tissue. Monocotyledon tissues generally (Jarvis et al., 1988), and those of oil palm specifically (Gallacher et al., 1994; Jarvis, 1994), are reported to have low levels of pectic polysaccharides in their cell walls. We have used the wide band of cells that comprises this fruit abscission zone to provide adequate wall material for analysis by chemical, cytochemical, enzymatic, light and electron microscopy and ^{13}C CP/MAS NMR spectroscopy. Distinctive features of these abscission cells compared with those of their immediate non-separating neighbour tissues have demonstrated the very high level of unmethylated pectins that walls of these target cells contain and the high level of inducible polygalacturonase activity that they can express. We suggest that, in abscission cells, the major polysaccharide degrading enzymes induced may be matched to specificities in their cell wall composition. This could help explain the positional preciseness of abscission cell separation.

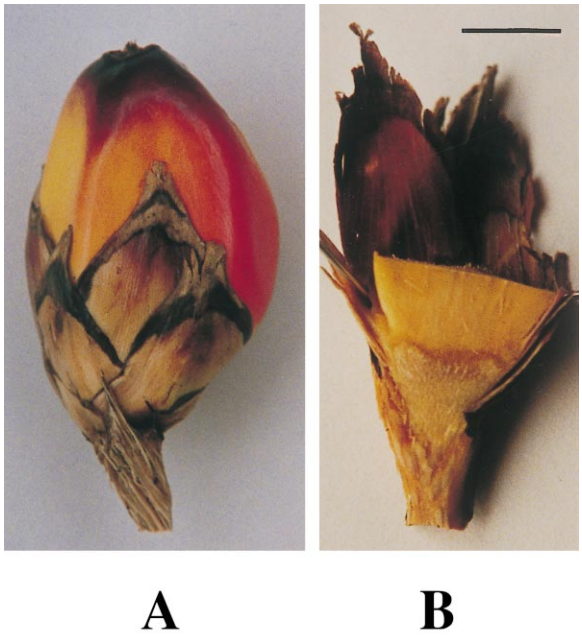


Fig. 1. Near ripe fruit of the oil palm (A) and longitudinal section to show the intact abscission zone (B). Bar = 1 cm.

2. Results

2.1. Microscopic analysis

Thin sections cut longitudinally across intact [Fig. 2(A)] and abscinded [Fig. 2(B)] abscission zones of the fruit show the small-celled and closely packed region of target tissue that is differentiated between the fruit mesocarp and the pedicel. The cells that separate one from another are turgid and intact [Fig. 2(B)] and can be readily collected for further analysis.

Transmission electron microscopy shows that the walls of the abscission cells separate at the middle lamellae but the primary walls remain intact with regions of plasmodesmatal connections being last to separate [Fig. 3(A)]. Like those of bean and other abscinding abscission cells, the cytoplasmic organelles are in a condition associated with high metabolic activity; enlarged mitochondria with multiple cristae, many polysomal clusters, expanded Golgi stacks with vesiculation, and evidence of the transfer of vesicle contents across the plasma membrane to the cell wall [Fig. 3(B)].

The mesocarp of oil palm fruit is highly fibrous (Henderson and Osborne, 1990). However, longitudinal sections through intact abscission zones of ripe fruits and stained for the presence of lignin (Fig. 4) show that, whilst the vascular connections between the fruit base and the pedicel are continuous across the whole abscission region, the vascular traces which actually pass through the abscission zone are extremely attenuated. Values for the proportion of lignified vascular fibre elements stained by phloroglucinol/HCl as a percentage of the non-stained parenchyma ground tissue are shown for the abscission zone and the mesocarp and pedicel tissue immediately above and below the abscission zone (Table 1). The low number of lignified fibres traversing the oil palm abscission zone (Fig. 4) must also contribute to the ready shedding of fruit once the parenchmya cells have separated one from another.

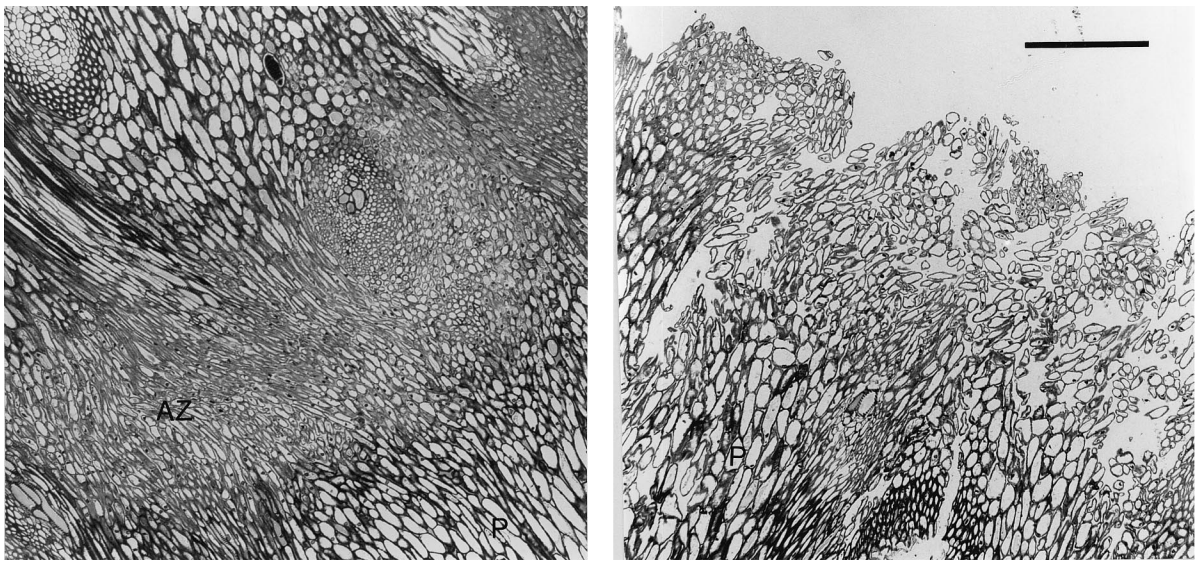


Fig. 2. Light microscopy of longitudinal sections through fruit abscission zones. Bar = 50 μm. (A) Intact abscission zone (AZ); (B) pedicel (P) side of abscission zone after fruit shedding showing the now independent cells.

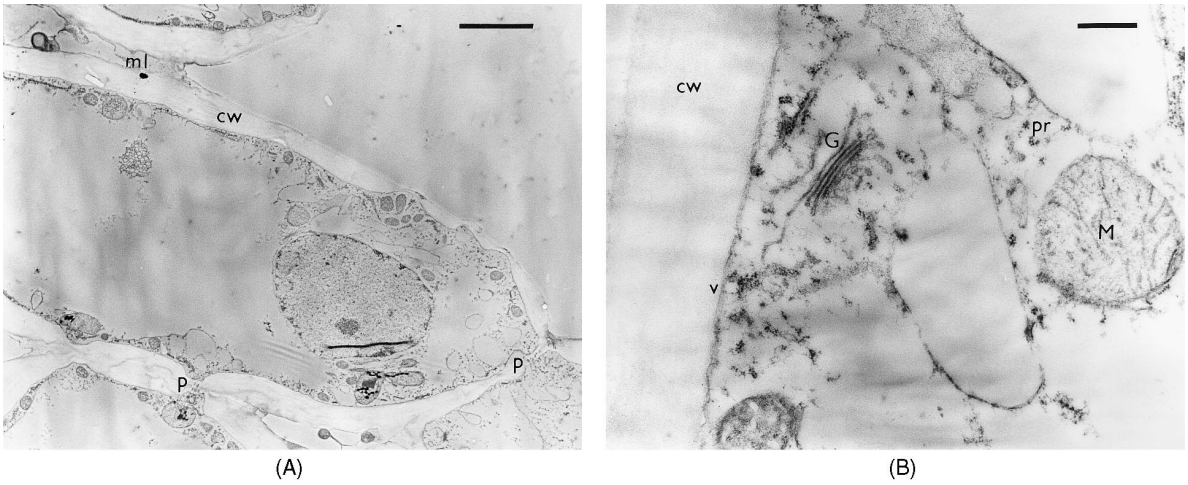


Fig. 3. Electron microscopy of abscission zone cells at the time of fruit shedding. (A) Abscission zone cells showing wall (cw) separation at the middle lamellae (ml) and continuity of plasmodesmatal connections (p). Bar = 4 μm; (B) higher magnification showing Golgi (G) with vesicles (v) at the plasma membrane, polyribosomal clusters (pr) and mitochondria (M) with cristae. Bar = 500 nm.

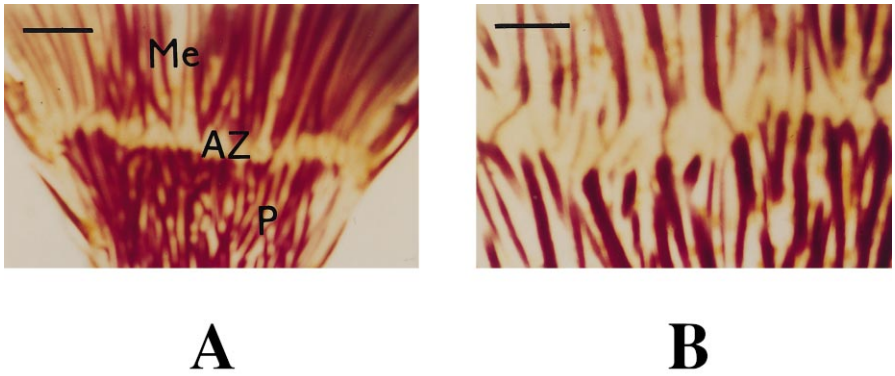


Fig. 4. Phloroglucinol-stained sections of longitudinal slices across the intact abscission zones of ripe fruit. A. Mesocarp (Me), abscission zone (AZ) and pedicel (P). Bar = 3 mm; B. higher magnification showing attenuated fibres through the abscission zone. Bar = 1 mm.

Table 1
The proportion of phloroglucinol-stained lignified vascular tissue (± S.D.) in longitudinal sections across intact (not abscinded) abscission zones of ripe fruit (Fig. 4)

Tissue[a]	% Phloroglucinol/ HCl-stained
Zone	7.1±2.5
Mesocarp	46.1±7.7
Pedicel	50.1±7.8

[a] Zone ($n=23$); mesocarp ($n=16$); pedicel ($n=16$).

Table 2
Analysis of total pectin content (galacturonic acid equivalents ± S.D.) in cells from abscinded abscission zones ($n=4$), mesocarp ($n=3$) and pedicel ($n=3$) tissue of ripe fruit

Tissue	Galacturonic acid equivalents mg g^{-1} dry weight
Zone	256.5±14.8
Mesocarp	173.8±31.8
Pedicel	135.7±33.9

2.2. Chemical analysis

That the abscission zone tissue is enriched with respect to the pectin content of the cell walls is seen from the chemical analyses of total pectin (as galacturonic acid equivalents) of wall material from the abscission zone and the adjacent mesocarp and pedicel (Table 2). The cytochemical location of high levels of polygalacturonate in abscission cells compared with mesocarp and pedicel was confirmed visually by the Ni^{2+} replacement of cations in the cell walls of longitudinal sections across the fruit (Fig. 5).

2.3. Polygalacturonase induction

Assays for polygalacturonase in extracts of freshly abscinded abscission zones of ripe fruit show increases of at least 35-fold compared with the value for intact abscission zones or for the mesocarp and pedicel tissues above or below the shedding position (Table 3).

2.4. NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectra of pectin standards from citrus illustrate the sharp carbonyl peak at 176-177 ppm for polygalacturonic acid and 171 ppm for methylesterified pectin (Fig. 6) and a decrease in the intensity of the resonance of methoxyl groups at 54 ppm with decreasing levels of pectin methylation from 70.8 to 31.8% to $<1.0\%$.

The spectrum for the loose abscission zone cells (uncontaminated by the mesocarp tissue above or pedicel below) from abscinded fruit (Fig. 7A) has a broad carbonyl peak at 170–180 ppm characteristic of pectin.

Table 3
Increase in polygalacturonase activity induced in abscission zone cells of abscinded ripe fruit compared with adjoining mesocarp and pedicel tissue ($n=3$, ±S.D.)

Tissue	Polygalacturonase activity nmol g Fw^{-1} min^{-1}
Intact abscission zone	19.2±7.7
Abscinded abscission zone	755.5±38.9
Mesocarp of abscinded fruit	18.1±1.7
Pedicel of abscinded fruit	21.7±8.7

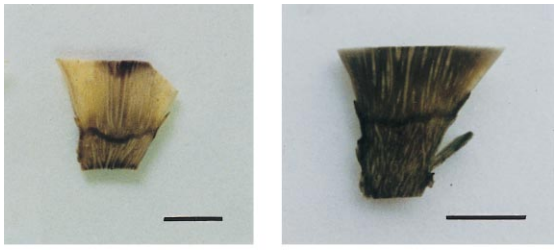


Fig. 5. Localisation of polygalacturonate by Ni^{2+}/Na_2S staining in longitudinal slices of the intact abscission zone of a ripe fruit. A. Direct staining. Bar = 1 cm. B. Staining after hydrolysis of methyl-esters (see methods). Bar = 1 cm.

The absence of aromatic resonances (115–165 ppm), as well as those resonances characteristic of protein, confirm that lignified vascular tissue in the abscission zone comprises $<10\%$ of the total sample as shown by the phloroglucinol staining (Fig. 4). However, the spectra of the mesocarp tissue above (Fig. 7B) and the pedicel tissue below (Fig. 7C) the abscission zone of ripe fruit do show resonances attributable to aromatic carbons. Although it is possible to excise tissue comprising mostly unseparated abscission zone cells from longitudinal sections of non-shed fruit, it inevitably contains a little tissue from above (mesocarp) and below (pedicel) the zone. The spectrum from non-abscinded, intact zone material (not shown) reflects the expected slightly higher signal for aromatic resonances from the small contribution of contaminating mesocarp and pedicel tissue.

3. Discussion

Both the ^{13}C CP/MAS NMR (Fig. 7) and the uronic acid analysis (Table 2) show that pectic polysaccharide is present at higher levels in the abscission zone of the oil palm fruit than in either the mesocarp and pedicel tissues above and below the zone. Although ^{13}C CP/MAS NMR spectra are not quantitative, the lower levels of methoxyl groups (54 ppm) in the abscinded

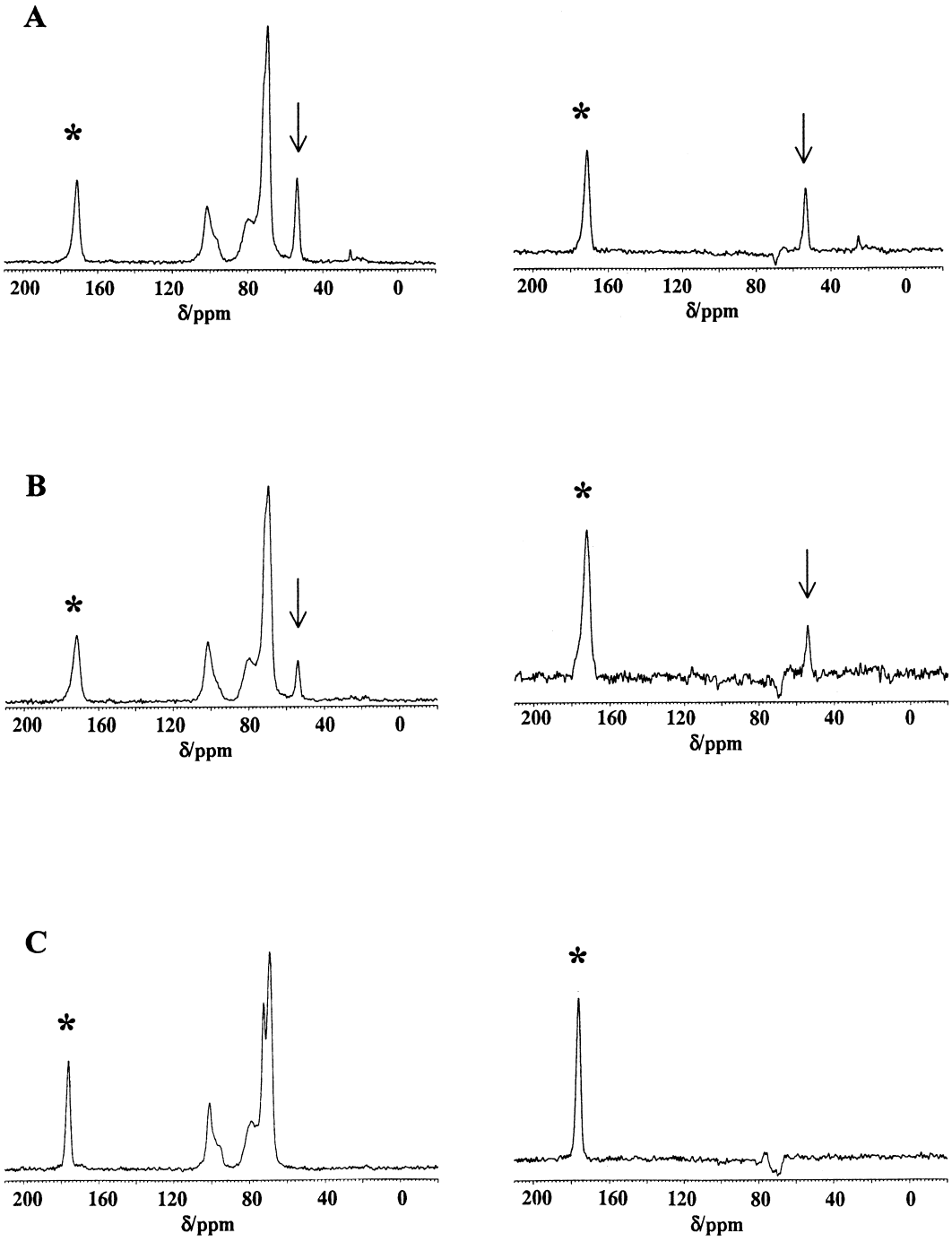


Fig. 6. ^{13}C CP/MAS NMR spectra of citrus pectin standards of different percentage methylation. Left, standard spectra; right, dipolar-dephased (50 μs) spectra. The resonance of the C-6 of methylesterified galacturonan is at ~171 ppm (asterisk in A and B) and at ~176–177 ppm in galacturonate potassium salt (asterisk in C). The intensity of the resonance for the methoxyl carbon at ~54 ppm (arrow in A and B) reflects the degree of methylation in the pectin (absent in polygalacturonic acid, C). A. Lime pectin, DM 70.8%. B. Lime pectin, DM 31.8%. C. Polygalacturonic acid (orange), DM < 1%.

zone tissue (Fig. 7A) compared with adjoining tissues from above or below the abscission zone (Fig. 7B and C) could partly reflect a lower level of pectin esterification and/or the numbers of methoxyl groups in the lignin present in each sample. In view of the fact that very little lignin is present in abscinded abscission zone samples (Table 1, Fig. 4), it is most likely that the signal at 54 ppm reflects a low level of methoxyl groups in the abscission zone cell wall material.

The intense nickel/sodium sulphide staining of the abscission zone also indicates that the walls of these cells are very pectin-rich (Fig. 5A). The fact that they

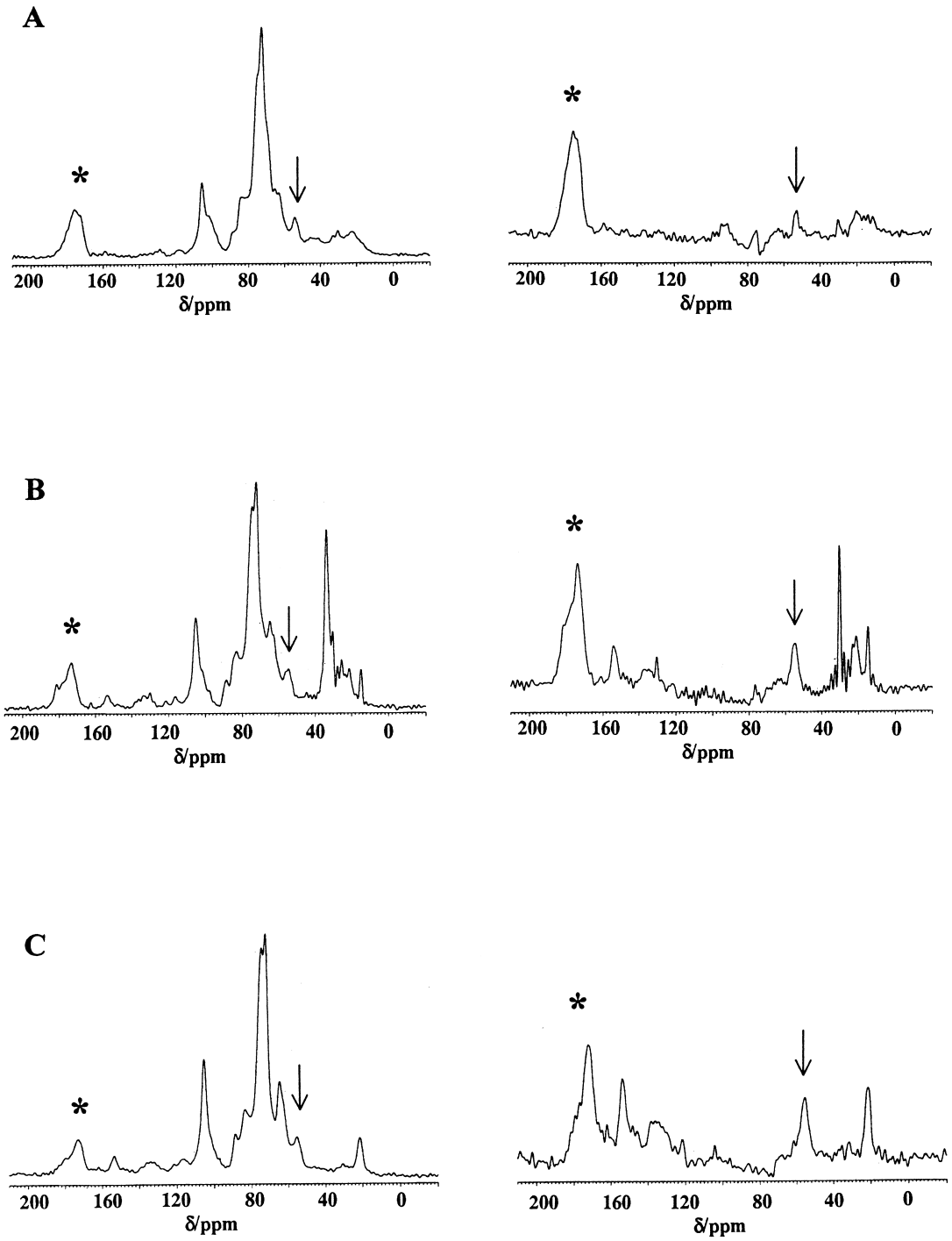


Fig. 7. ^{13}C CP/MAS NMR spectra of cell preparations from different parts of the fruit. Left, standard spectra; right dipolar-dephased (50 μs) spectra. A broad peak of carbonyl resonances lies between 170 and 180 ppm (asterisk) and methoxyl resonances lie between 50 and 60 ppm (arrow). A. Abscinded abscission zone cells. B. Mesocarp from directly above an intact abscission zone. C. Pedicel from directly below an intact abscission zone.

stain readily before bleach treatment indicates also a high level of unmethylated polygalacturonate. After bleach treatment, which hydrolyses the methylester groups in the pectin to form polygalacturonate, mesocarp, abscission zone and pedicel tissues all then stain with high intensity (Fig. 5B).

Monocotyledon leaf and stem cell walls (grasses, cereals, sedges, rushes and woodrushes) have all been reported to have low pectin contents with values of $\leqslant 50$ mg g^{-1} (Jarvis et al., 1988). However, ripe monocotyledon fruits (date and banana) show relatively high uronic acid contents of the pericarp (199 and 208 mg

g^{-1} respectively). These fruit values are close to those obtained by us for oil palm mesocarp and pedicel in the present study (174 and 136 mg g^{-1} respectively, Table 2) and also to those published for cell walls of ripe tomato, 185 mg g^{-1} (Koch and Nevins, 1989) and ripe pear, 137 mg g^{-1} (Ahmed and Labavitch, 1977). The uronic acid values we have shown for abscission zone cells of the oil palm fruit (257 mg g^{-1}) are, therefore, amongst the highest recorded for pectin levels in plants.

Cell from cell separation is achieved in abscission zone tissue by a cohort of wall hydrolases including inducible zone-specific isoenzymes of cellulases (β-1,4-glucanhydrolase) and polygalacturonases (Gonzalez-Carranza et al., 1998). In the pectin-rich abscission zone of the oil palm it is significant that the major inducible enzymes are polygalacturonases (Table 3). Inducible cellulase components in the fruit abscission zone of the oil palm and in the leaf-pulvinus abscission zone of the bean (*Phaseolus vulgaris)* have been assayed in parallel on a weight for weight basis. The value for oil palm is seen to be low in comparison (one third of the bean value, Table 4). In contrast, polygalacturonase activity in oil palm abscission zones rises over 35-fold but in bean there is no rise and the activity in both intact and abscinded abscission zones remains low (Table 4). This raises an interesting interpretation of certain tissue specific inductions that have been recorded for cellulase and polygalacturonase during the abscission of different organs of the same plant. In peach, for example, high levels of polygalacturonase and low levels of cellulase have been recorded for fruits and the reverse for the leaves (Bonghi et al., 1992). Three different abscission polygalacturonases that accumulate at different rates are expressed in tomato leaf and flower abscission but do not occur in the anthers in which the pollen cells separate; nor are significant levels of polygalacturonase mRNAs detectable in the anthers (Kalaitzis et al., 1997). In another relevant study with tomato, down-regulating the expression of fruit-specific polygalacturonase by anti-sensing the mRNA has led to a marked reduction in fruit polygalacturonase activity, whereas no effect was observed on this expression in leaf abscission zones which still abscinded normally (Taylor et al., 1990). In these and other examples where cellulase and/or polygalacturonase induction has been demonstrated a parallel assessment of polysaccharide components of the wall has not been available.

Table 4
Comparative polygalacturonase ($n=3$, ±S.D.) and cellulase ($n=2$, ±S.D.) activities in abscinded abscission zone tissue of the oil palm fruit and the bean leaf

Abscission zone tissue	Polygalacturonase activty nmol g Fw^{-1} min^{-1}	Cellulase activity % decrease viscosity in 30 min/50 mg Fwt
Oil palm fruit	709.5±29.5	27.6±0.5
Bean leaf	2.6±0.48	8.1±5.1

This study with the oil palm is the first example of a wide-ranging assessment of abscinding abscission zone cells compared with their non-separating immediate neighbours in an attempt to link the types of enzyme induction with certain properties of the target cell walls. Whereas in most studies to date the abscission zone tissue has been too limited to assess abscission cells independently of neighbour cells, this investigation has linked the localised high pectin levels found in the walls of abscission cells of the oil palm with the high level of inducible polygalacturonase expression that occurs when the ripe fruit is shed. We suggest that differences recorded for inducible enzyme expressions in abscission zones may reflect specific substrate-features of their wall composition and hence account for the precise positional localisation of cell from cell separation when an organ is shed.

4. Experimental

4.1. Plant material

Freshly harvested spikelets of clonal (271D) material were shipped air-freight from Malaysia in loose cotton bags enclosed in perforated polythene and boxed in perforated cardboard containers. At no time during transit did the temperature of the fruits fall below 12°C nor did it rise above that experienced by fruit in the field (maximum 40°C). Spikelets were received in Oxford within 24–30 h of excision from the parent palm and were used as the experimental material. Each spikelet was a known number of days from anthesis and consisted of 10–25 fruits. In near-ripe fruit the abscission zone is intact (Fig. 1B) with no sign of separation and cannot be forced apart. The spikelets were held at room temperature and fruit were shed at the mesocarp–pedicel junction between 24–48 h.

4.2. Preparation for light and electron microscopy

Thin sections (1–2 mm) were cut longitudinally through the base of the mesocarp, the abscission zone and the adjoining pedicel and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 100 mM phosphate buffer pH 7.3 for 18 h at room temperature on a rotator, polymerised in Lowicryl HM20, sectioned (2 μm) and stained with toluidene blue for light microscopy. For electron microscopy the material was stained in uranyl acetate and lead citrate, and blocks were sectioned at 70 nm.

4.3. Visualisation of lignin by phloroglucinol/HCl staining

Longitudinal sections (300–600 μm thick) through intact abscission zones of ripe fruit were hand cut from

the fresh tissue and fixed in ethanol:glacial acetic acid (3:1) with several changes of fixative until the orange carotenoids were removed. After washing in deionised water, each individual section was transferred to 500 μl of a saturated solution of phloroglucinol in ethanol for 5 min and then a drop of 35% HCl was added. The xylem vascular elements and fibres in the section at once stain a deep red. Photomicrographs were taken using a Wild Heerbrug Macroskop 420. Percentages of phloroglucinol-stained tissue and non-stained tissue were obtained by computing the area from the micrographs (Fig. 4).

4.4. Visualisation of polygalacturonates in pectin

The method was essentially as described by Varner and Taylor (1989). Longitudinal sections (300–600 μm thick) through intact abscission zone tissue of ripening and ripe fruit were hand-cut from fresh tissue. When sections were transferred to a 5-fold diluted solution of domestic bleach for 30 min before exposure to the nickel solution, the methylesters of pectin are hydrolysed and the whole section becomes black. In order to visualize the unesterified pectin present at the abscission zone, the bleach stage was omitted. A similar use of the method has demonstrated the high levels of polygalacturonate in tomato fruits (Tieman and Handa, 1994).

4.5. Preparation for solid-state ^{13}C CP/MAS NMR

Mesocarp tissue above the abscission zone, the pedicel fibres below the abscission zone and the freshly scraped cells from newly abscinded zones were dried in an oven at 40°C for 24 h. Each tissue was then ground to a fine powder in a mortar and pestle. Standards for polygalacturonic acid and pectins with a known degree of methylesterification were: (i) acid-washed lime pectins, one with a high degree of methylesterification (DM) of 70.8% (M_r 546,000), the other with a low DM of 31.8% (M_r 425,000) (gifts from Citrus Colloids Ltd, Hereford), (ii) polygalacturonic acid potassium salt from orange (Sigma) with a DM of $<1\%$ was washed with 80% ethanol and air-dried.

^{13}C CP/MAS NMR spectra were obtained using a single contact spin-lock CP sequence (Pines et al., 1973) with alternate cycle, spin temperature, inversion (Stejskal and Schaefer, 1975) and with flip-back of ^{1}H magnetization (Tegenfeldt and Haeberlen, 1979) on a Bruker MSL 200 NMR spectrometer at a Larmor frequency for ^{13}C of 50.32 MHz (Edwards et al., 1995). Chemical shifts were referenced to an external secondary standard (admantane upfield resonance = 29.5 ppm, Earl and VanderHart, 1982). The dried, ground samples (200–300 mg) (7 replicates of zone, 3 of mesocarp and 2 of pedicel tissue) were packed in 7 mm zirconia rotors with Kel-F caps. Spectra accumulated for up to ~3,000-6,000 transients with a 4s recycle delay between transients. MAS rates of 3 to 5 kHz were used, and the 5 kHz rate spectrum is presented in each case. Dry nitrogen gas was used for MAS. Dipolar-dephased spectra were recorded with a 50μs delay for dephasing (Alemany et al., 1983). ^{13}C CP/MAS NMR spectral analyses were performed on an abscinded abscission zone tissue sample with a range of cross polarisation contact times from 0.5 to 10 ms. An optimum time of 2 ms was chosen as offering the best compromise between the short (0.75 ms) optimum contact time of the rigid carbohydrate resonances and the long (5–10 ms) values for mobile and non-protonated carbons, and was used for all subsequent spectra. The ^{1}H-pulse effective 90° length was 4 μs ($\omega_{1\mu} = 62.5$ kHz). FIDs were zero-filled to 16 k points prior to Fourier transformation, and no apodization functions were used.

4.6. Preparation for uronic acid estimation

After the ^{13}C CP/MAS NMR spectra were obtained, the powdered samples were first extracted (3×) with chloroform-methanol (1:1), and then with acetone (3×) to remove carotenoids and non-polar compounds. A 5 mg sample of the solvent-extracted, air-dried tissue was added slowly with stirring to 2 ml of concentrated sulphuric acid in a beaker on ice. Half a millilitre of deionised water was added slowly dropwise; after 5 min another 0.5 ml deionised water was added dropwise. The dissolved sample was transferred to a 10 ml volumetric flask and made to volume with repeated washings of the beaker (Ahmed and Labavitch, 1977).

4.7. Uronic acid estimation

The tissue samples (0.4 ml) and galacturonic acid standards (0.4 ml, 1–200 nmol) were dispensed into glass tubes, and 40 μl of 4 M sulphamic acid-potassium sulphamate (pH 1.6) followed by 2.4 ml of concentrated sulphuric acid containing 75 mM sodium tetraborate were added. The solutions were vortexed, the tubes capped with marbles were heated in a boiling water bath for 20 min. They were cooled immediately and 80 μl of 0.15% (w/v) *m*-hydroxydiphenyl in 0.5% sodium hydroxide was added with stirring. A blank containing 80 μl of 0.5% sodium hydroxide without *m*-hydroxydiphenyl was prepared for each sample. The absorbance was read after 15 min at 525 nm (Filisetti-Cozzi and Carpita, 1991).

4.8. Preparation of enzyme extracts

Abscission zone tissue (200 mg) from ripe, abscinded oil palm fruit and the petiole dome tissue from abscinded bean leaf-pulvinus explants (McManus and Osborne, 1990) were each extracted in 400 μl, 50 mM acetate pH 5.3 containing 200mM NaCl with a little acid-washed sand, and the homogenate centrifuged

(Beckman Microfuge, 9000g, 2min). Extracts were used directly for polygalacturonase and cellulase assays.

4.9. Assay for polygalacturonase

Activity was measured by the cyanoacetamide method for reducing groups (Gross, 1982). Enzyme extract (20 μl) was added to (50 μl) polygalacturonic acid (10 mg ml^{-1}, Sigma) in 50 mM acetate buffer pH 5.5 (130 μl). Galacturonic acid (0–100 nmol) was used as a standard.

4.10. Assay for cellulase

Activity was measured by a decrease in viscosity of a solution of carboxymethyl cellulose (CMC, Hercules, USA, Type 7HF). Enzyme extract (100 μl) was added to 400 μl of a 2% aqueous solution of CMC with 50 μl 1 M sodium acetate pH 5.8. Viscosity readings were started after a 5 min equilibration period in the water bath (30°C) and taken thereafter at regular intervals.

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Biotransformation of diterpenes and diterpene derivatives by *Beauveria bassiana* ATCC 7159

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Dedicated to Dr Earle V. Roberts in celebration of his service of over 30 years to the Chemistry Department, University of the West Indies, Mona Campus.

Abstract

The biohydroxylation of stemodin and stemodinone by *Beauveria bassiana* ATCC 7159 gave exclusively 2α,13,18-trihydroxystemodane and 13,18-dihydroxystemodan-2-one respectively. Stemarin was converted to the novel 1β,13,19-trihydroxystemarane and 13-hydroxystemarane-19-carboxylic acid. The synthesis and biotransformation of various derivatives of stemodin have also been studied. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Beauveria bassiana* ATCC 7159; Stemodin; Stemodane; Stemarin; Diterpene; Biotransformation; Hydroxylation

1. Introduction

Stemodia maritima L., known colloquially in Jamaica as ''poor man's strength'', is a member of the Scrophulariaceae family. This plant has yielded several diterpenes and diterpene glycosides (Manchand et al., 1973; Manchand and Blount, 1975, 1976; Hufford et al., 1976, 1992) which bear close resemblance to aphidicolin, a known antiviral and cytotoxic agent isolated from cultures of the fungus *Cephalosporium aphidicola* (Danziel et al., 1973; Ackland et al., 1984). Several hydroxylated analogues of stemodin and stemodinone have been produced by microbial biotransformation, some of which have shown mild antiviral activity. These results have been summarised (Azerad, 2000).

In an ongoing effort to produce novel compounds, unavailable from chemical synthesis and with enhanced biological activity (Hanson et al., 1994; Buchanan et al., 2000), the fungal transformation of *S. maritima* terpenes: stemodin, stemodinone and stemarin was examined. There are no previous reports of microbial transformation of stemarin. *Beauveria bassiana* ATCC 7159 (also known as *B. sulfurescens* or *Sporotrichum sulfurescens*), a microorganism that has gained popularity as a microbial oxidant, was employed to effect the biotransformation reactions (Grogan and Holland, 2000). In addition the syntheses and bioconversion of several derivatives of stemodin were studied. The derivates prepared were the propionate, isobutyrate, ethylcarbonate, diphenylcarbonate, dimethylcarbamate, phenyl carbamate and diphenylcarbamate. Also synthesised were the diethyl- and diphenyl-phosphates as well as 2α-hydroxystemod-12-ene. These derivates were chosen because of their relatively nonpolar nature, as well as the presence of an electronegative atom. Nonpolar groups will facilitate stronger interactions between the substrate and the hydrophobic amino acids that are believed to constitute the enzyme's active site (Vigne et al., 1991). Furthermore it is proposed that an electronegative oxygen atom would help to anchor the substrate there. Carbamate derivatives of various substrates have been used successfully to improve the bioconversion yields as well as varying the sites at which hydroxylation occurs (Vigne et al., 1968, 1991).

2. Results and discussion

Stemodin and stemarin were isolated from *S. maritima* using the protocol previously described (Hufford et al., 1992). Stemodinone was obtained in quantitative

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yield by the oxidation of stemodin with Jones Reagent (Shiddiqui et al., 1988). Although ^{1}H and ^{13}C NMR signals for stemodin have been unambiguously assigned (Hufford, 1988), no ^{13}C NMR assignments have been reported previously in the literature for stemarin (Manchand et al., 1973). However, complete assignments of carbon resonances were essential for the characterisation of any microbial metabolites of stemarin produced. Using 2D NMR experiments, unequivocal assignments of all carbon signals of the monoacetylated stemarin, and thus stermarin, were achieved.

Incubation of stemodin (**1**) with a growing culture of *B. bassiana* ATCC 7159 resulted in the preparation of a single product. The partially purified metabolite was acetylated using standard conditions to afford two products. The most polar metabolite, 2α,18-diacetoxy-13-hydroxystemodane (**5**), was obtained in 53% yield. The electrospray mass spectrum showed an $[M+Na]^+$ peak at m/z 471.3. In the IR spectrum an absorption characteristic of an ester carbonyl was evident at 1737 cm^{-1}. Also present were hydroxyl stretches at 3486 and 3421 cm^{-1} and C–O absorptions at 1248 and 1029 cm^{-1}. In the ^{1}H NMR spectrum there were only three methyl singlets associated with the stemodane skeleton. However, a two proton doublet corresponding to a methylene bonded to an oxygen atom was seen at 3.91 ppm. The loss of a methyl resonating at δ 34.7 (CH_3-18) as well as the appearance of a methylene signal at 72.8 ppm (CH_2–O–) were evident in the ^{13}C NMR spectrum. It was therefore clear that hydroxylation had occurred at C-18 and that only two of the three hydroxyl groups were acetylated. Insertion of a hydroxyl at C-18 had been previously achieved (Azerad, 2000), however, with different fungi and in poorer yield. The second compound isolated was 2α,13,18-triacetoxystemodane. (**4**).

The bioconversion of stemodinone (**2**) afforded a single metabolite. The IR spectrum showed the presence of two hydroxyl stretches at 3422 and 3281 cm^{-1} as well as a carbonyl absorption at 1683 cm^{-1}. In the ^{1}H NMR data shifts indicating the presence of a CH_2OH moiety were seen at 3.02 and 3.53 ppm, each appearing as a doublet. The ^{13}C NMR spectrum showed a methylene bearing oxygen at 70.3 ppm and the disappearance of a methyl signal at 34.3 ppm. It was concluded that the fungus had inserted a hydroxyl at C-18. This compound was previously isolated from the fermentation of stemodinone with *Cephalosporium aphidicola* (Hanson et al., 1994) as a gum. In this instance 13,18-dihydroxystemodan-2-one (**6**) crystallised from Me_2CO as needles. The overall yield was 25.8% and this is similar to that seen in the literature. The yield was approximately half of that obtained when stemodin was the substrate. This could imply that the hydroxyl group binds more strongly in the active site of the enzyme than the carbonyl functionality.

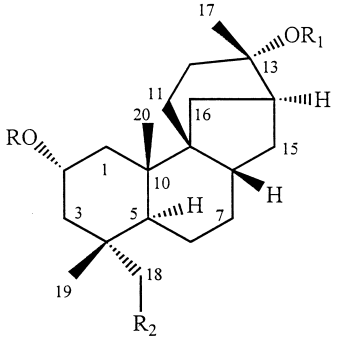
Incubation of stemarin (**3**) with the fungus resulted in the formation of several products, however, only two were available in sufficient quantities to be characterised. The harvesting of the fungus was somewhat different from those previously performed, in that the aqueous phase was acidified to pH 2 before extraction. This was due to the fact that the primary alcohol of stemarin was oxidisable to the carboxyl group under the fermentation conditions. The least polar metabolite, 1β,13,19-trihydroxystemarane had a hydroxyl stretch at 3407 cm^{-1}. The ^{1}H NMR spectrum showed a 1H multiplet at 3.81 ppm corresponding to a CHOH group. In the ^{13}C NMR spectrum the disappearance of a methylene signal at 31.8 ppm and the appearance of a methine bearing oxygen at 75.2 ppm was evident. Downfield shifts of 11.4 and 5.2 ppm were seen for carbons 2 and 10 respectively. Also evident was that carbons 3 and 20 had been shifted upfield by 1.8 and 5.4 ppm respectively. The electrospray mass spectrum of this compound indicated a peak at m/z 365.2 which was assigned to $[M+Na]^+$. It was thus clear that hydroxylation had occurred at C-1 from the β face to produce the hitherto unreported 1β,13,19-trihydroxystemarane (**7**).

The more polar compound was identified as the novel 13-hydroxystemarane-19-carboxylic acid (**8**) on the basis of an $[M+Na]^+$ peak at m/z 365.2. The IR spectrum of **8** showed peaks characteristic of a carboxylic acid. The resonances of the $C\underline{H}_2OH$ moiety of stemarin at 2.86 and 3.43 ppm were absent from the ^{1}H NMR spectrum. The loss of a methylene resonating at δ 70.8 as well as the appearance of a carbonyl signal at 183.8 ppm in the ^{13}C NMR data revealed that the primary hydroxyl group of stemarin had been oxidised to a carboxyl group.

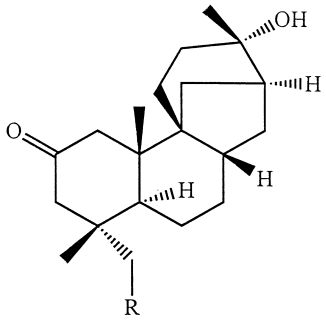
Six novel stemodane derivatives were chosen for the indirect mapping of the cytochrome P-450 enzymes found in *B. bassiana*, namely: the ethyl- and phenyl-carbonates; dimethyl-, phenyl- and diphenyl-carbamates; and diethyl- and diphenyl-phosphates.

Treatment of **1** with ethyl chloroformate (Chheda and Hong, 1971; Satchell and Satchell, 1975) gave 2α-ethylcarbonyloxy-13-hydroxystemodane (**10**) (91.3% yield). Compound **10** was identified by the presence of an $[M]^+$ peak at m/z 373.3 in the chemical ionisation mass spectrum (CIMS), in addition to a peak at m/z 360.2657 in the HREIMS assigned to $[M\text{-}H_2O]^+$ ($C_{23}H_{36}O_3$). 2α-Phenylcarbonyloxystemod-12-ene (**11**) and 13-hydroxy-2α-phenylcarbonyloxystemodane (**12**) (25 and 49.1% yield respectively) were obtained when stemodin (**1**) was treated with phenyl chloroformate. The major product, **12**, showed a peak at m/z 395.4 $[M+NH_4]^+$ in the chemical ionisation mass spectrum.

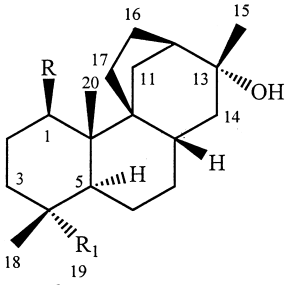
Stemodin (**1**) in pyridine reacted with dimethylcarbamyl chloride (Jager et al., 1968; Agarwal and Khorana, 1972) to produce the mono- and di-protected compounds. The compound of interest, 2α-dimethylcarbamoxy-13-hydroxystemodane (**13**), was identified by the



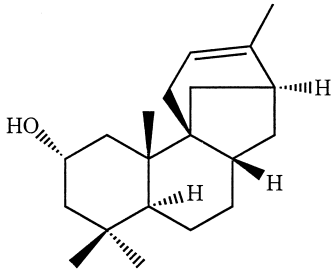
1 R = R_1 = R_2 = H
4 R = R_1 = Ac, R_2 = OAc
5 R = Ac, R_1 = H, R_2 = OAc



2 R = H
6 R = OH



3 R = H, R_1 = CH_2OH
7 R = OH, R_1 = CH_2OH
8 R = H, R_1 = COOH



9

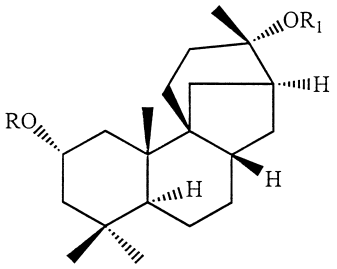
presence of an $[M+NH_4]^+$ peak at *m/z* 395.4 in the chemical ionisation mass spectrum (CIMS), in addition to a peak at *m/z* 359.2816 in the HREIMS assigned to $[M\text{-}H_2O]^+$ ($C_{23}H_{37}NO_2$).

The major product of the reaction of **1** with phenyl isocyanate (Jager et al., 1968; Agarwal and Khorana, 1972) was 13-hydroxy-2α-phenylcarbamoxystemodane (**17**) (63.9% yield). The HREIMS indicated a formula of $C_{27}H_{39}NO_3$ (M^+ = 425.2935). 2α-Diphenylcarbamyloxy-13-hydroxystemodane (**19**) was the only product isolated when **1** was treated with diphenylcarbamyl chloride. The mass spectrum of **19** indicated an $[M]^+$ peak at *m/z* 501.3241.

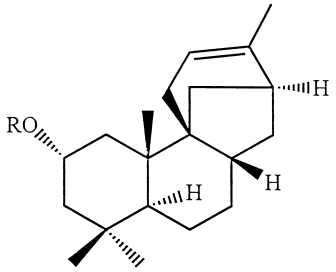
2α-Diethylphosphatostemod-12-ene (**20**) (41%) was prepared from diethyl chlorophosphate (Welsh and Walters, 1978). The HREIMS had an $[M]^+$ peak at *m/z* 424.2744 ($C_{24}H_{38}O_4P$). It was concluded that stemodin had been derivatised at C-2 and that the tertiary hydroxyl had been elminated. Stemodin was reacted with diphenyl chlorophosphate to produce 2α-diphenyl phosphatostemod-12-ene (**21**) and 2α-diphenylphosphato-13-hydroxystemodane (**22**), in yields of 20.2 and 49.8%. Compound **22** could be obtained exclusively if three equivalents of the reagent were used. On the other hand the elimination product (**21**) could be had in quantitative yield by the addition of nine equivalents of diphenyl chlorophosphate. It was clear that elimination of the tertiary alcohol had occurred in addition to the derivatisation of the secondary alcohol.

2α-Diphenylphosphatostemod-12-ene (**21**) and 2α-diphenylphosphato-13-hydroxystemodane (**22**) were very similar, however, no olefinic proton was seen for the latter. Similarly in the carbon spectrum the two olefinic carbon resonances were absent. This suggested that the secondary alcohol had been protected as the diphenylphosphate. Stemodin was stirred in collidine to afford 2α-hydroxystemod-12-ene (**9**) in a modest yield of 58.7%. A peak at *m/z* 288.2461 in the mass spectrum was assigned to $C_{20}H_{30}O$ $[M]^+$. It was concluded that the tertiary alcohol had been eliminated.

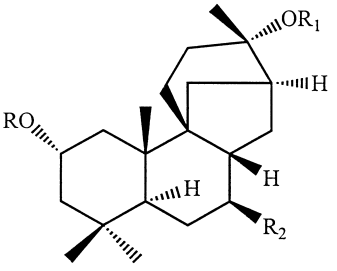
All the major derivatives of stemodin synthesised were incubated with cultures of *B. bassiana*, however, only five of the nine compounds were transformed. The *N, N*-dimethylcarbamate, **13**, was transformed to a single



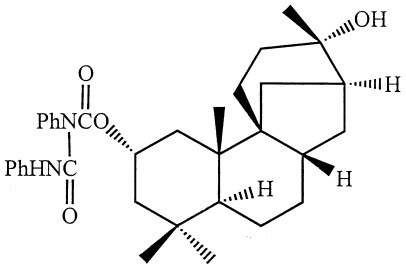
10 R = CH_3CH_2OCO, R_1 = H
12 R = PhOCO, R_1 = H



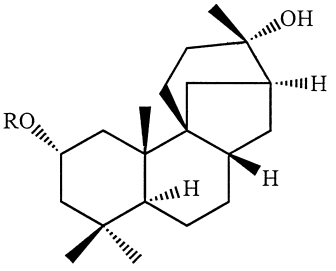
11 R = PhOCO
20 R = $(CH_3CH_2O)_2PO$
21 R = $(PhO)_2PO$



13 R = $(CH_3)_2NCO$, $R_1 = R_2$ = H
14 R = R_2 = H, $R_1 = (CH_3)_2NCO$
15 R = $(CH_3)_2NCO$, R_1 = H, R_2 = OH
16 R = R_1 = PhNHCO, R_2 = H
17 R = PhNHCO, $R_1 = R_2$ = H
19 R = Ph_2NCO, $R_1 = R_2$ = H
22 R = $(PhO)_2PO$, $R_1 = R_2$ = H



18



23 R = CH_3CH_2CO
24 R = $(CH_3)_2CHCO$

product. Its structure has been determined to be that of **15**. The CIMS spectrum showed an $[M+Na]^+$ peak at m/z 416.3. Evidence for the presence of an additional hydroxyl in the molecule was provided by the presence of a multiplet at δ 3.90 (1H).This was supported by the appearance of a methane carbon resonating at 68.6 ppm in the carbon NMR spectrum. It was clear that **15** was monohydroxylated, due to a peak seen at m/z 375.2780 assigned to $[M\text{-}H_2O]$ ($C_{23}H_{36}NO_3$) in the high resolution mass spectrum. Comparative analyses of the spectral data for **15** and the fed compound **13** revealed that the hydroxyl had been inserted on the β face at C-7.

Carbonates **10** and **12** were both hydrolysed by the fungus to give small amounts of stemodin. The *N*-phenyl- and *N*,*N*-diphenylcarbamates were not hydroxylated. This may be as a result of steric factors, since phenylcarbamates of cyclohexane and other smaller molecules had been transformed previously with *B. bassiana* (Vigne et al., 1986). The phosphates **20**, **21** and **22** were also not transformed. Furthermore, only very small quantities of the fed substrates were recovered. Enzymes of the fungus may have hydrolysed the ethyl and phenyl portions of the xenobiotes resulting in the formation of doubly charged stemodylphosphate anions which would have been very soluble in water. 2α-Hydroxystemod-12-ene was also not transformed by the fungus.

Since only one of the stemodane derivatives was hydroxylated by *B. bassiana*, i.e. 2α-dimethylcarbamoxy-13-hydroxystemodane (**13**), ester derivatives of similar size to the aforementioned compound were prepared. Simple esters such as acetates are generally believed to be hydrolysed by esterases present in the fungal cells. Despite this fact other esters (propionate and isobutyrate) were prepared for investigation. 13-Hydroxy-

2α-propionyloxystemodane (**23**) was prepared from the terpene (**1**) which was treated with propionic acid and *p*-toluenesulfonyl chloride (Brewster and Ciotti, 1955). Spectral data for the compound was in accord with its proposed structure. The downfield shift of the C-2 resonance indicated that the reaction had occurred at the secondary hydroxyl group. The isobutyrate (**24**) was prepared from **1** in an overall yield of 65%. Esters **23** and **24** were both partially hydrolysed to stemodin (**1**) under the fermentation conditions. The resulting terpene was then hydroxylated at C-18 as was seen previously.

It would appear that there is more than one cytochrome P-450 enzyme present in *B. bassiana* ATCC 7159 responsible for hydroxylating **1** and related compounds. Recently published results (Holland et al., 1999) suggest that the fungus exhibits up to four distinct hydroxylase enzyme activities. The same enzyme appears to hydroxylate stemodin and stemodinone. The presence of the hydroxyl group at C-13 in stemodin may be necessary to effect biocatalysis, since the Δ^{12} compound (**9**) was not transformed. Stemarin may have been biotransformed by the same enzyme as preliminary studies using Chem3D software suggest that the distance between the two potential binding oxygen atoms in **1**, **2** and **3** is 6.7–7.0 Å. The enzyme responsible for the production of **15** from *N, N*-dimethylcarbamate **13** may be related to those which effect hydroxylations on nitrogen containing compounds. Studies by Furstoss (Archelas et al., 1984) and Griengl (Braunegg et al., 1999) indicate that certain fungal enzymes only hydroxylate substrates containing an amide moiety. The site of hydroxylation was approximately 5.5 Å away from the binding (electronegative) group and this is in agreement with an early model of the enzyme active site (Johnson et al., 1968).

In summary we report the first microbial transformation of stemarin. Two novel metabolites were isolated. The ^{13}C NMR resonances of **3** have been assigned for the first time using one and two dimensional techniques. Stemodin and stemodinone were hydroxylated at the C-18 position by *B. bassiana*. Compound **1** was chemically converted to 14 derivatives, 13 of which were new. Nine of these were fed to growing cultures of the microorganism and five were transformed.

3. Experimental

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Optical rotations were taken on a Perkin–Elmer 241 MC polarimeter. IR spectra were recorded on a Perkin–Elmer 735B spectrometer as KBr pellets. ^{1}H and ^{13}C NMR spectra were determined at 200 and 50 MHz respectively on a Bruker AC200 spectrometer. Samples were run in deuterated chloroform with tetramethylsilane as the internal standard. HRMS (EI) analyses were performed on a Kratos MS50 direct probe instrument with an ionising energy of 70 eV. CIMS (NH_3 reagent gas) were recorded on a VG 7070E spectrometer with an ionising energy of 300 eV. Column chromatography utilised silica gel (230–400 mesh). Thin layer chromatography plates were visualised under ultraviolet light and were sprayed with dodecaphosphomolybdic acid/ceric sulfate or ammonium molybdate–sulfuric acid spray before heating. ^{13}C NMR assignments are reported in Table 1.

3.1. Fermentation conditions

Beauveria bassiana ATCC 7159 was obtained from American Type Culture Collection, Rockville, MD, USA. The fungus was grown on potato dextrose agar slants. The liquid growth medium contained glucose (10 g/l) and corn steep solids (10 g/l) and was adjusted to pH 4.60 using 2 M aq. sodium hydoxide. One 14 day old slant was used to inoculate two 500 ml conical flasks each containing 250 ml liquid medium. The flasks were incubated at 200 rpm at 27°C and the xenobiotes were pulse fed to the culture (as single feeding 3 days after inoculation did not lead to transformation). Thus a solution containing 10% of the total mass of the substrate was fed 24 h after inoculation. The remaining 20, 30 and 40% portions of the substrate were fed at 36, 48 and 60 h after inoculation respectively. The fermentation was allowed to proceed for 14 days. The pH was measured and the mycelium was filtered from the broth. Broth extraction utilised EtOAc (2×750 ml). The mycelium was homogenised in EtOAc. The extracts were dried with sodium sulfate, concentrated in vacuo and analysed by thin layer chromatography. The three terpenes in EtOH (125 mg/ml) were fed to the fungus growing in 20 flasks, while the derivatives in EtOAc (125 mg/ml) were incubated over four flasks unless otherwise stated.

3.2. Incubation of *1*

The terpene (1.0 g) was fed to the fungus in 5 1 of medium. Harvesting after 14 days afforded a combined organic extract of 1.155 g (broth and mycelial). Column chromatography with 25% EtOAc in petrol afforded unchanged stemodin (250 mg). Further elution with 30 and 40% EtOAc in petrol gave a mixture (567 mg) which was acetylated by stirring overnight in pyridine (12 ml) and acetic anhydride (12 ml). The acetylated products were purified by column chromatography. The fraction which eluted with 15% EtOAc in petrol afforded 2α,13,18-triacetoxystemodane (**4**) (64 mg) as a gum, IR: ν_{max} cm^{-1} 1740, 1722, 1368, 1249, 1026; HRMS (EI): *m*/*z* (rel. int.) 388.2604 [M-AcOH]$^+$ (3.0),

Table 1
^{13}C NMR chemical shift assignments for stemodane and stemarane compounds (**1**–**24**)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
C-1	45.7	51.4	31.8	40.9	41.3	51.0	75.2	31.2	44.8	41.6	40.6	40.0	42.0	45.2	42.0	41.8	42.2	41.2	41.6	42.7	42.5	43.3	41.6	41.5
C-2	65.1	212.5	18.3	68.6	68.7	214.2	29.7	18.1	65.2	73.2	74.7	74.6	69.8	65.2	69.9	70.2	70.3	73.5	71.5	74.0	76.6	76.5	69.0	68.8
C-3	50.1	55.8	35.3	40.7	40.8	49.8[a]	33.4	37.0	50.9	46.2	46.4	46.0	46.8	50.7	47.0	46.6	46.3	45.8	46.4	48.5	48.2	47.9	46.4	46.4
C-4	34.6	39.1	38.0	37.9	38.0	43.1	38.0	47.5	34.8	34.6	34.8	34.6	34.3	34.7	34.1	34.5	34.5	34.3	34.4	34.9	35.0	34.9	34.6	34.6
C-5	46.6	47.1	40.9	40.1	40.6	39.2	41.3	43.7	47.1	46.5	47.2	46.4	46.4	46.5	41.7	46.5	46.7	46.5	46.6	47.0	46.9	46.2	46.6	46.6
C-6	22.0	22.4	22.3	21.9	22.0	22.3	22.5	25.3	21.9	21.6	21.8	21.7	21.8	22.0	28.9	21.8	21.9	21.9	21.8	21.8	21.8	21.8	21.9	21.6
C-7	36.4	35.9	30.8	35.7	35.9	35.6	31.0	30.8	36.8	36.2	36.8	36.1	36.2	36.3	68.6	36.1	36.3	36.2	36.4	36.8	36.7	36.2	36.3	36.3
C-8	36.9	37.2	38.9	37.0	36.7	37.2	40.7	38.7	39.1	36.7	39.1	36.6	36.6	37.4	38.5	37.1	36.7	36.8	36.8	39.1	39.1	36.6	36.8	36.8
C-9	50.6	50.0	51.6	50.0	50.2	49.9[a]	51.6	51.8	50.0	50.0	50.0	49.9	49.9	49.9	50.7	50.0	50.1	50.1	50.1	50.0	49.9	50.0	50.1	50.1
C-10	40.1	44.7	38.6	39.7	39.9	44.0	43.8	38.2	40.5	40.0	40.7	41.3	39.9	40.1	41.7	40.0	40.1	40.2	40.2	40.6	40.7	40.2	40.0	40.1
C-11	27.8	27.8	29.4	27.6	27.7	27.9	29.5	29.3	33.3	27.6	33.0	27.5	27.5	27.8	28.0	27.7	27.7	27.7	27.7	33.0	32.9	27.5	27.7	27.7
C-12	32.7	32.6	47.9	30.9	32.7	32.5	47.5	48.4	117.5	32.6	117.4	32.4	32.5	31.3	32.4	31.6	32.7	32.7	32.8	117.5	117.4	32.6	32.7	32.7
C-13	72.3	71.9	73.9	84.3	72.3	72.2	73.9	73.9	145.7	72.2	145.7	72.0	72.0	83.3	72.7	84.5	72.5	72.4	72.4	145.7	145.6	72.2	72.3	72.4
C-14	46.1	45.8	39.9	42.7	38.0	45.6	39.8	39.2	42.6	46.0	42.6	45.9	45.8	43.1	45.9	42.7	46.0	46.1	46.1	42.6	42.4	46.0	46.0	46.1
C-15	38.1	38.1	29.4	37.0	37.9	38.0	29.5	29.6	43.8	37.9	43.8	37.8	37.7	37.2	33.0	37.3	38.0	37.9	38.0	43.8	43.8	37.9	37.9	37.9
C-16	30.1	30.3	26.7	30.2	30.0	30.4	26.5	26.7	35.9	29.9	35.7	29.7	29.8	30.3	30.1	30.3	30.0	30.0	30.0	35.8	35.7	29.9	29.9	30.0
C-17	28.0	28.1	27.4	22.6	28.1	28.1	27.4	27.0	17.8	28.0	17.4	27.9	27.9	23.6[a]	28.3	23.0	28.1	28.2	28.2	17.6	17.5	28.0	28.0	28.1
C-18	34.7	34.3	18.1	72.3	72.8	70.3	18.0	16.8	34.5	34.4	34.4	34.3	34.4	34.6	34.2	34.6	34.7	34.7	34.7	34.4	34.3	34.3	34.4	34.4
C-19	23.7	23.8	70.8	21.5	21.5	20.3	70.4	183.8	23.7	23.4	23.4	23.3	23.3	23.3[a]	23.5	23.4	23.5	23.5	23.5	23.5	23.5	23.5	23.4	23.4
C-20	19.6	18.6	16.9	19.1	19.2	19.5	11.5	16.7	21.7	19.2	21.7	19.1	19.1	19.6	19.3	19.2	19.3	19.4	19.3	21.7	21.7	19.3	19.3	19.3
				20.1[b]	20.1[b]					14.2[c]	120.8[c]	120.9[c]	34.4[c]	36.1[d]	34.2[c]	118.3[d]	123.1[c]	120.0[c]	125.8[c]	16.1[c]	120.1[c]	120.1[c]	9.0[c]	18.9[c]
				20.9[c]	21.0[c]					63.4[c]	125.8[c]	125.6[c]	152.6[c]	155.8[d]	156.5[c]	118.6[c]	128.9[c]	123.9[c]	127.0[c]	63.5[c]	125.1[c]	125.1[c]	27.6[c]	19.0[c]
				22.5[d]	170.6[b]					154.6[c]	129.5[c]	129.1[c]				122.9[d]	138.0[c]	128.1[c]	128.8[c]		129.7[c]	129.6[c]	174.0[c]	34.1[c]
				170.5[d]	171.1[c]						151.1[c]	150.9[c]				123.0[c]	153.3[c]	128.7[c]	142.7[c]		150.7[c]	150.5[c]		176.8[c]
				170.5[b]							153.2[c]	153.1[c]				128.8[d]		128.9[c]	154.6[c]					
				171.0[c]												128.9[c]		129.0[c]						
																138.8[c,d]		137.1[c]						
																152.8[d]		137.8[c]						
																153.3[c]		151.8[c]						
																		155.8[c]						

[a] Signals are interchangeable.
[b] Derivative at C-18.
[c] Derivative at C-2.
[d] Derivative at C-13.

328.2401 [M-2AcOH]$^+$ (3.5), 255.2106 [M-2AcOH−CH_2OAc]$^+$ (13.5), 67.8595 (100); ESMS: *m/z* (rel. int.) 471.3 [M + Na]$^+$ (100); ^1H NMR: δ 0.98 (3H, *s*, H-19), 1.09 (3H, *s*, H-20), 1.37 (3H, *s*, H-17), 2.02 (3H, *s*, 17-$OCOCH_3$), 2.05 (3H, *s*, 2-$OCOCH_3$), 2.08 (3H, *s*, 18-OCO-CH_3), 2.79 (1H, *t*, J = 6.6 Hz, H-14), 3.62 (1H, *d*, J = 11.1 Hz, H-18), 3.90 (1H, *d*, J = 11.1 Hz, H-18), 4.95 (1H, *m*, $W_{1/2}$ = 22.9 Hz, H-2). The fraction which eluted with 20% EtOAc in petrol was 2α,18-diacetoxy-13-hydroxystemodane (**5**) (500 mg) as a gum, IR: ν_{max} cm^{-1} 3486, 3421, 1737, 1246; HRMS (EI): *m/z* (rel. int.) 388.2593 [M-H_2O]$^+$ (8.3), 328.2385 [M-AcOH−H_2O]$^+$ (12.2), 273.2208 [M-AcOH−CH_2OAc]$^+$ (78.1), 268.2177 [M-2AcOH−H_2O]$^+$ (47.8), 255.2115 [M-AcOH−CH_2OAc−H_2O]$^+$ (100); ESMS: *m/z* (rel. int.) 429.3 [M + Na]$^+$ (100); ^1H NMR: δ 0.99 (3H, *s*, H-19), 1.11 (3H, *s*, H-20), 1.13 (3H, *s*, H-17), 2.04 (3H, *s*, 18-$OCOCH_3$), 2.09 (3H, *s*, 2-$OCOCH_3$), 3.66 (1H, *d*, J = 11.1 Hz, H-2), 3.93 (2H, *d*, J = 10.8 Hz, H-18), 4.95 (1H, *bs*, H-2).

3.3. Stemodinone (**2**)

A solution of stemodin (1.0 g, 3.265 mmol) in Me_2CO (100 ml) at −10°C (ice salt) was treated with Jones reagent (6 N, 2.0 ml). The mixture was stirred and allowed to attain room temperature over 30 min. The reaction mixture was cooled to 0°C and EtOH (10 ml) was added. The acid was neutralised by $NaHCO_3$ solution and the Me_2CO was removed in vacuo. The mixture was diluted and extracted with EtOAc (2×200 ml) after which the organic layer was washed with water and dried with Na_2SO_4. Concentration of the solvent in vacuo afforded stemodinone (**2**) (996 mg) which crystallised from EtOAc as needles, mp 213–215°C, $[\alpha]_D$ + 10.4°; lit. mp 215–216°C, $[\alpha]_D$ + 14.3° ($CHCl_3$, *c* 0.44) (Manchand et al., 1973); IR: ν_{max} cm^{-1} 3419, 2864, 1690, 1618; ^1H NMR: δ 0.91 (3H, *s*, H-19), 0.95 (3H, *s*, H-20), 1.08 (3H, *s*, H-18), 1.11 (3H, *s*, H-17), 2.16 (1H, *d*, J = 2.53, H-14), 2.36 (2H, *m*, $W_{1/2}$ = 22.77 Hz, H-1).

3.4. Incubation of **2**

Stemodinone (1.0 g) was incubated with the fungus in 5 l medium. The fermentation was terminated after 14 days to afford an organic extract of 1.322 g. The extract was chromatographed on silica gel. Elution with 27.5% EtOAc in petrol yielded stemodinone (607 mg). Further elution with 60% EtOAc in petrol afforded 13,18-dihydroxystemodan-2-one (**6**) (258 mg) which crystallised from Me_2CO as needles, mp 182–185°C, $[\alpha]_D + 31.3°$ ($CHCl_3$, *c* 0.76); IR: ν_{max} cm^{-1} 3422, 3281, 1683, 1056; ^1H NMR: δ 0.84 (3H, *s*, H-20), 1.00 (3H, *s*, H-19), 1.13 (3H, *s*, H-17), 2.24 (1H, *t*, $J = 11.7$ Hz, H-14), 2.40 (2H, *dd*, $J = 1.6$, 7.3 Hz, H-1), 2.71 (1H, *d*, $J = 13.6$ Hz, H-3), 3.02 (1H, *d*, $J = 11.1$ Hz, H-18), 3.53 (1H, *d*, $J = 11.1$ Hz, H-18).

3.5. Incubation of **3**

Stemarin (1.0 g) was incubated for 14 days with *B. bassiana*. The fermentation beer was acidified before extraction. A broth extract of 409.4 mg and a mycelial extract of 384.4 mg were obtained. The broth extract was subjected to column chromatography. The fraction eluting in 35% EtOAc in petrol contained stemarin. Further elution with 60% EtOAc in petrol afforded 1α,13,19-trihydroxystemarane (**7**) (24.5 mg) as a gum, IR: ν_{max} cm^{-1} 3407, 2926, 2871, 1042; HRMS (EI): *m/z* (rel. int.) 320.2346 $[M\text{-}H_2]^+$ (0.5), 304.2404 $[M\text{-}H_2O]^+$ (12.3), 286.2295 $[M\text{-}2H_2O]^+$ (12.7), 273.2218 (38.9, 254.2016 (100); CIMS: *m/z* (rel. int.) 340.3 $[M + NH_4]^+$ (6.8), 322.3 $[M]^+$ (54.6), 305.3 $[M\text{-}OH]^+$ (23.6), 304.4 $[M\text{-}H_2O]^+$ (19.5), 288.2 $[M\text{-}H_2O\text{-}CH_4]^+$ (23.5), 287.2 (100), 255.2 (27.3): ESMS: *m/z* (rel. int.) 345.3 $[M + Na]^+$ (17.5), 301.2 (100); ^1H NMR: δ 0.70 (3H, *s*, H-18), 1.05 (3H, *s*, H-20), 1.14 (3H, *s*, H-15), 2.84 (1H, *d*, $J = 11.7$ Hz, H-19), 3.41 (1H, *d*, $J = 11.7$ Hz, H-19), 3.81 (1H, *m*, $W_{1/2} = 11.9$ Hz, H-1).

The fraction which eluted with Me_2CO was further purified by column chromatography to give 13-hydroxystemarane-19-carboxylic acid (**8**) (14 mg) which crystallised from Me_2CO as needles, mp 210–212°C, $[\alpha]_D + 81.2°$ ($CHCl_3$, *c* 0.09); IR: ν_{max} cm^{-1} 3447 (b), 2921, 2871, 1700, 1384; HRMS: (EI) *m/z* (rel. int.) 320.2349 $[M]^+$ (15.0), 302.2247 $[M\text{-}H_2O]^+$ (100), 187.1486 (20.3), 93.0706 (22.9); CIMS: *m/z* (rel. int.) 338.4 $[M + NH_4]^+$ (0.4), 320.4 $[M]^+$ (1.9), 276.4 $[M\text{-}CO_2]^+$ (2.6), 35.1 (100); ESMS: *m/z* (rel. int.) 343.2 $[M + Na]^+$ (57), 338.0 $[M + NH_4]^+$ (100); ^1H NMR: δ 0.93 (3H, *s*, H-20), 1.11 (3H, *s*, H-19), 1.13 (3H, *s*, H-15).

3.6. 2α-Hydroxystemod-12-ene (**9**)

A solution of stemodin (400 mg, 1.306 mmol) in 2,4,6-collidine (1.0 ml) was stirred at 190°C for 4 days. The reaction mixture was treated with excess saturated copper(II) sulfate solution, extracted with CH_2Cl_2 (3×50 ml), washed with brine, and dried ($MgSO_4$). The organic extract was concentrated in vacuo to give a pale brown gum which was purified by column chromatography. Elution with 5% EtOAc in petrol afforded 2α-hydroxystemod-12-ene (**9**) (221 mg) which crystallised from Me_2CO as needles, mp 128–130°C, $[\alpha]_D + 19.9°$ ($CHCl_3$, *c* 1.08); IR: ν_{max} cm^{-1} 3349, 1452, 1388, 1029; HRMS (EI): *m/z* (rel. int.) 288.2461 $[M]^+$ (40), 255.2126 (43.3), 175.1490 (100), 148.1255 (28.2), 105.0708 (55.3); ^1H NMR: δ 0.92 (3H, *s*, H-19), 0.95 (3H, *s*, H-18), 0.98 (3H, *s*, H-20), 1.64 (3H, *d*, $J = 0.9$ Hz, H-17), 2.37 (1 H, *m*, $W_{1/2} = 18.2$ Hz, H-14), 3.79 (1H, *tt*, $J = 3.9$, 11.4 Hz, H-2), 5.01 (1H, *bs*, H-12).

3.7. 2α-Ethylcarbonyloxy-13-hydroxystemodane (**10**)

A solution of stemodin (300 mg, 0.9796 mmol) in pyridine (2.0 ml) was cooled to 0°C. Ethyl chloroformate (1.12 ml, 11.75 mmol) was added and the mixture was stirred for 1 h. The solution was treated with saturated copper(II) sulfate, extracted with EtOAc (3×50 ml), washed with brine, and dried (Na_2SO_4). Removal of the solvent afforded 2α-ethylcarbonyloxy-13-hydroxystemodane (**10**) (338.2 mg) which crystallised from EtOH as amorphous crystals, mp 158–160°C, $[\alpha]_D$ −31.5° ($CHCl_3$, *c* 0.80); IR: ν_{max} cm^{-1} 3524, 1722, 1274; HRMS (EI): *m/z* (rel. int.) 360.2657 $[M\text{-}H_2O]^+$ (5.2), 288.2850 $[M\text{-}C_3H_6O_3]^+$ (22.6), 270.2349 $[M\text{-}C_3H_6O_3\text{-}H_2O]^+$ (27.4), 217.1951 (100), 133.1013 (40.7); CIMS: *m/z* (rel. int.) 378.3 $[M]^+$ (2.5), 35.3 (100); ESMS: *m/z* (rel. int.) 401.3 $[M + Na]^+$ (93), 365.1 (100); ^1H NMR: δ 0.97 (6H, *s*, H-18, 19), 1.04 (3H, *s*, H-20), 1.12 (3H, *s*, H-17), 1.31 (3H, *t*, $J = 7.0$ Hz, $C\underline{H_3}CH_2O$), 4.16 (2H, *q*, $J = 7.3$ Hz, $CH_3C\underline{H_2}O$), 4.72 (1H, *tt*, $J = 3.8$, 11.70 Hz, H-2).

3.8. Incubation of **10**

2α-Ethylcarbonyloxy-13-hydroxystemodane (247 mg) was fed to *B. bassiana* growing in 1 l of medium. The extract from the fermentation (200 mg) was purified by chromatography. Elution with 10% Me_2CO in petrol afforded unchanged substrate (107.5 mg). Further elution of the column with 27.5% Me_2CO in petrol gave stemodin (9.1 mg).

3.9. 2α-Phenylcarbonyloxystemod-12-ene (**11**) and 13-hydroxy-2α-phenylcarbonyloxystemodane (**12**)

Stemodin (300 mg, 0.98 mmol) was dissolved in pyridine (0.75 ml). Phenyl chloroformate (0.15 ml, 2.35 mmol) was added and the mixture was stirred at room temperature for 5 h. The solution was treated with saturated copper(II) sulfate, extracted with EtOAc

(3×50 ml), washed with brine, and dried (Na_2SO_4). Removal of the solvent gave a solid (403 mg) which was purified by column chromatography. Elution with 2% EtOAc in petrol afforded 2α-phenylcarbonyloxystemod-12-ene (**11**) (100 mg) as a gum. IR: ν_{max} cm^{-1} 1757, 1259, 1238, 1212, 1182; HRMS (EI): *m/z* (rel. int.) 360.2676 (4.7), 288.2458 (21.4), 270.2349 (27.3), 217.1957 (100), 133.1022 (37.4), 93.0706 (30); ^{1}H NMR: δ 0.97 (3H, *s*, H-19), 0.99 (3H, *s*, H-18), 1.05 (3H, *s*, H-20), 1.64 (3H, *s*, H-17), 2.34 (1H, *m*, $W_{1/2}$ = 19.1 Hz, H-14), 4.87 (1H, *tt*, *J* = 3.8, 12.0 Hz, H-2), 5.0 (1H, *s*, H-12), 7.29 (5H, *m*, $W_{1/2}$ = 34.9 Hz, Ar-H).

Further elution with 12.5% EtOAc in petrol yielded 13-hydroxy-2α-phenylcarbonyloxystemodane (**12**) (205 mg) which crystallised from EtOAc as amorphous crystals, mp 163–165°C, $[\alpha]_D$ +49.1° ($CHCl_3$, *c* 0.54); IR: ν_{max} cm^{-1} 3394, 1750, 1251, 1237, 1203; HRMS (EI): *m/z* (rel. int.) 289.2533 [M-$C_7H_5O_3$]$^+$ (11.1), 271.2421 [M-$C_7H_5O_3$−H_2O]$^+$ (53.5), 189.1645 (85.5), 83.0862 (100); CIMS: *m/z* (rel. int.) 444.5 [M + NH_4]$^+$ (13.6), 426.3 [M]$^+$ (20.7), 35.3 (100); ESMS: *m/z* (rel. int.) 449.3 [M + Na]$^+$ (100); ^{1}H NMR: δ 0.97 (3H, *s*, H-19), 0.98 (3H, *s*, H-18), 1.03 (3H, *s*, H-20), 1.10 (3H, *s*, H-17), 2.16 (1H, *d*, *J* = 11.7 Hz, H-14), 4.85 (1H, *tt*, *J* = 3.5, 11.4 Hz, H-2), 7.21 (3H, *m*, $W_{1/2}$ = 12.0 Hz, H-2′,4′,6′), 7.36 (2H, *m*, $W_{1/2}$ = 13.6 Hz, H-3′,5′).

3.10. Incubation of **12**

13 - Hydroxy - 2α - phenylcarbonyloxystemodane (261 mg) was fed to *B. bassiana* growing in 1 1 of medium. The extract from the fermentation (233.3 mg) was purified by chromatography. Elution with 10% Me_2CO in petrol afforded unchanged substrate (161.4 mg). Further elution with 27.5% Me_2CO in petrol gave stemodin (1.3 mg).

3.11. 2α-Dimethylcarbamoxy-13-hydroxystemodane (**13**) and 13-dimethylcarbamoxy-2α-hydroxystemodane (**14**)

To a solution of stemodin (1.0 g, 3.266 mmol) in pyridine (5 ml) was added dimethylcarbamyl chloride (1.76 g, 1.633 mmol) and a catalytic amount of *N, N*-dimethylaminopyridine. The mixture was stirred for 48 h at room temperature. The solution was treated with saturated copper(II) sulfate, extracted with EtOAc (3×50 ml),washed with brine, and dried (Na_2SO_4). The solution was concentrated in vacuo to give 1.131 g of an off-white solid which was chromatographed. Elution with 20% EtOAc in petrol afforded 2α-dimethylcarbamoxy-13-hydroxystemodane (**13**) (300 mg) which crystallised from Me_2CO as needles, mp 150–152°C, $[\alpha]_D$ −3.9° ($CHCl_3$, *c* 0.93); IR: ν_{max} cm^{-1} 3482, 1686, 1195, 1404; HRMS (EI): *m/z* (rel. int.) 359.216 [M-H_2O]$^+$ (1.2), 288.2452 [M-$C_3H_7NO_2$]$^+$ (7.7), 273.2216 (57.7), 270.2346 [M-$C_3H_7NO_2$−H_2O]$^+$ (67.7), 90.0555 (100); CIMS: *m/z* (rel. int.) 395.4 [M + NH_4]$^+$ (18), 271.4 (100); ESMS: *m/z* (rel. int.) 400.3 [M + Na]$^+$ (100); ^{1}H NMR: δ 0.88 (3H, *s*, H-19), 0.89 (3H, *s*, H-18), 0.97 (3H, *s*, H-20), 1.05 (3H, *s*, H-17), 2.81 (6H, *s*, 2CH_3-N), 4.69 (1H, *tt*, *J* = 3.5, 11.7 Hz, H-2). Further elution with 40% EtOAc in petrol afforded 13-dimethylcarbamoxy-2α-hydroxystemodane (**14**) (38.3 mg) which crystallised from Me_2CO as needles, mp 157–160°C, $[\alpha]_D$ +49.2° ($CHCl_3$, *c* 0.12); IR: ν_{max} cm^{-1} 3515, 1691, 1399, 1206; HRMS (EI): *m/z* (rel. int.) 288.2452 [M-$C_3H_7NO_2O$]$^+$ (27.5), 271.2423 [M-$C_3H_7NO_2$−H_2O]$^+$ (100), 257.2261 [M-$C_4H_{10}NO_2$−H_2O]$^+$ (28.4); ESMS: *m/z* (rel. int.) 400.3 [M + Na]$^+$ (100); ^{1}H NMR: δ 0.92 (3H, *s*, H-19), 0.94 (3H, *s*, H-18), 0.98 (3H, *s*, H-20), 1.38 (3H, *s*, H-17), 2.76 (1H, *t*, *J* = 6.5 Hz, H-14), 2.88 (6H, *s*$(CH_3)_2$N), 3.77 (1H, *tt*, *J* = 3.7, 11.70 Hz, H-2). Untransformed stemodin (363 mg) was eluted in EtOAc.

3.12. Incubation of 2α-dimethylcarbamoxy-13-hydroxystemodane (**13**)

13-Hydroxy-2α-dimethylcarbamoxystemodane (246.4 mg) was incubated with *B. bassiana* growing in 1 1 medium for 14 days. The resulting extract (243.5 mg) was subjected to purification by chromatography. Unchanged xenobiote (150.1 mg) was eluted with 10% Me_2CO in petrol. Further elution with 30% Me_2CO in petrol afforded 2α-dimethylcarbamoxy-7β,13-dihydroxystemodane (**15**) (4 mg), which crystallised from acetone as plates, mp 150–152°C, $[\alpha]_D$ −169.7° ($CHCl_3$, *c* 0.07); IR: ν_{max} cm^{-1} 3493, 3376, 1685, 1196; HRMS (EI): *m/z* (rel. int.) 375.2780 [M-H_2O]$^+$ (7.8), 286.2299 [M-$C_3H_7NO_2$−H_2O]$^+$ (67.0), 268.2190 [M-$C_3H_7NO_2$−2H_2O]$^+$ (100), 253.1963 (72.6), 72.0448 (79.9); ESMS; *m/z* (rel. int.) 416.3 [M + Na]$^+$ (100); ^{1}H NMR: δ 0.97 (3H, *s*, H-19), 0.99 (3H, *s*, H-18), 1.05 (3H, *s*, H-20), 1.17 (3H, *s*, H-17), 2.89 (6H, *s*, $(CH_3)_2$N), 3.90 (1H, *m*, $W_{1/2}$ = 7.9 Hz, H-7), 4.80 (1H, *tt*, *J* = 3.8, 11.4 Hz, H-2).

3.13. 2α,13-Bis(phenylcarbamoxy)stemodane (**16**), 13-hydroxy-2α-phenylcarbamoxystemodane (**17**) and 13-hydroxy-2α-(*N*-phenylcarbamoxy-*N*-phenyl)carbamoxystemodane (**18**)

To a solution of stemodin (0.3 g, 0.98 mmol) in pyridine (1 ml) was added phenyl isocyanate (0.234 g, 2.0 mmol) and the mixture was stirred for 1 h at room temperature. The solution was treated with saturated copper(II) sulfate, extracted with CH_2Cl_2 (3×50 ml), washed with brine, and dried ($MgSO_4$). Removal of the solvent in vacuo afforded 0.5513 g of an off-white solid which was purified by chromatography. Elution with 10% EtOAc in petrol afforded 2α,13-bis(phenylcarbamoxy)stemodane (**16**) (111.5 mg) as a gum, IR: ν_{max} cm^{-1} 3338, 1702, 1603, 1542, 1228; HRMS (EI): *m/z* (rel. int.) 544.3302 [M]$^+$ (0.1), 407.2821 [M-$C_7H_5NO_2$]$^+$

(12.1), 270.2348 (28.0), 175.1487 (54.8), 105.0707 (46.9), 93.0582 (100), 83.0860 (61.2); ^{1}H NMR: δ 0.95 (6H, *s*, H-18, 19), 1.05 (3H, *s*, H-20), 1.42 (H, *d*, J=1.9 Hz, H-17), 2.92 (1H, *t*, J=6.0 Hz, H-14), 4.87 (1H, *tt*, J=3.0, 11.7 Hz, H-2), 6.75 (1H, *s*, NH), 6.92 (1H, *d*, J=7.3 Hz, NH), 7.00 (2H, *t*, J=7.1 Hz, H-4′,4″), 7.24 (4H, *tt*, J=1.9, 3.0, 7.4 Hz, H-3′,3″,5′,5″), 7.36 (4H, *dd*, J=2.5, 8.2 Hz, H-2′,2″,6′,6″).

Further elution with 15% EtOAc in petrol gave 13-hydroxy-2α-phenylcarbamoxystemodane (**17**) (266.4 mg) which crystallised from EtOH as amorphous crystals, mp 139–142°C, $[\alpha]_D$ −30.4° ($CHCl_3$, *c* 1.0); IR: ν_{max} cm^{-1} 3437, 3363, 1733, 1525, 1444, 1221; HRMS (EI): *m*/*z* (rel. int.) 425.2935 $[M]^+$ (29.2), 407.2825 $[M-H_2O]^+$ (2.9), 271.2426 (37.2), 217.1954 (51.3), 189.1647 (66.8), 119.0371 (52.3), 93.0579 (100); ^{1}H NMR: δ 0.97 (3H, *s*, H-19), 0.98 (3H, *s*, H-18), 1.06 (3H, *s*, H-20), 1.12 (3H, *s*, H-17), 4.90 (1H, *m*, $W_{1/2}$=19.9 Hz, H-2), 6.72 (1H, *bs*, NH), 7.04 (1H, *t*, J=7.1 Hz, H-4′), 7.24 (2H, *d*, J=7.9 Hz, H-3′,5′), 7.34 (2H, *t*, J=7.9 Hz, H-2′,6′).

Also eluting in 15% EtOAc in petrol was 13-hydroxy-2α-(*N*-phenylcarbamoxy-*N*-phenyl)carbamoxystemodane (**18**) (16.2 mg) as a gum. IR: ν_{max} cm^{-1} 3842, 1735, 1542, 1327; HRMS (EI): *m*/*z* (rel. int.) 544.3319 $[M]^+$ (2.0), 526.3176 $[M-H_2O]^+$ (0.1, 425.2934 (17.1), 271.2422 (26.8), 217.1955 (25.1), 189.1648 (48.4), 119.0372 (100); ^{1}H NMR: δ 0.90 (3H, *s*, H-19), 0.96 (3H, *s*, H-18), 1.06 (3H, *s*, H-20), 1.11 (3H, *s*, H-17), 2.17 (H, *s*, H-14), 4.89 (1H, *m*, $W_{1/2}$=20.9 Hz, H-2), 7.08 (2H, *t*, J=7.4 Hz, H-4′,4″), 7.31 (4H, *m*, $W_{1/2}$=31.5 Hz, H-3′,3″,5′,5″), 7.55 (4H, *d*, J=7.9 Hz, H-2′,2″,6′,6″).

3.14. 2α-Diphenylcarbamyloxy-13-hydroxystemodane (*19*)

To a solution of stemodin (0.4 g, 1.31 mmol) in pyridine (1 ml) was added diphenylcarbamyl chloride (1.52 g, 6.55 mmol). The mixture was stirred for 2 days at room temperature. The solution was treated with saturated copper(II) sulfate, extracted with CH_2Cl_2 (3×50 ml), washed with brine, and dried ($MgSO_4$). Removal of the solvent in vacuo afforded a burgundy gum (0.6784 g) which was subjected to chromatography. Elution with 15% EtOAc in petrol gave 2α-diphenylcarbamyloxy-13-hydroxystemodane (**19**) (326.4 mg) which crystallised from Me_2CO as amorphous crystals, mp 203–206°C, $[\alpha]_D$ −33.9° ($CHCl_3$, *c* 0.71); IR: ν_{max} cm^{-1} 3441, 1696, 1496, 1347, 1221; HRMS (EI): *m*/*z* (rel. int.) 501.3241 $[M]^+$ (21.1), 483.3131 $[M-H_2O]^+$ (10.0), 271.2421 (29.0), 189.1647 (8.8), 169.0891 (100), 83.0860 (61.8); ^{1}H NMR: δ 0.93 (3H, *s*, H-19), 0.96 (3H, *s*, H-18), 1.05 (3H, *s*, H-20), 1.11 (3H, *s*, H-17), 2.17 (H, *s*, H-14), 4.92 (1H, *tt*, J=3.5, 11.7 Hz, H-2), 7.26 (10H, *m*, $W_{1/2}$=25.9 Hz, Ar-H).

Unreacted stemodin (200 mg) was eluted from the column with EtOAc.

3.15. 2α-Diethylphosphatostemod-12-ene (*20*)

To a solution of stemodin (800 mg, 2.61 mmol) in pyridine (1.0 ml) was added diethyl chlorophosphate (2.24 g, 13.06 mmol). The mixture was stirred at 65°C for 5 h. The solution was treated with excess saturated copper(II) sulfate solution, extracted with CH_2Cl_2 (3×50 ml), washed with brine, and dried ($MgSO_4$). Removal of the solvent in vacuo afforded a brown gum (810 mg) which was purified by chromatography. Elution with 20% EtOAc in petrol afforded 2α-diethylphosphatostemod-12-ene (**20**) (220 mg) as an oil; IR: ν_{max} cm^{-1} 1733, 1266, 1043, 1014; HRMS (EI): *m*/*z* (rel. int.) 424.2744 $[M]^+$ (0.2), 270.2351 (5.0), 255.2117 (5.3), 188.1569 (15.8), 155.0473 (100); ^{1}H NMR: δ 0.94 (3H, *s*, H-19), 0.97 (3H, *s*, H-18), 1.01 (3H, *s*, H-20), 1.33 (6H, *t*, J=7.0 Hz, $(C\underline{H}_3CH_2O)_2P$), 1.64 (3H, *s*, H-17), 4.09 (4H, quintet, J=7.0 Hz, $(CH_3C\underline{H}_2O)_2P$), 4.45 (1H, *m*, $W_{1/2}$=21.8 Hz, H-2), 5.00 (1H, *s*, H-12).

Unchanged stemodin (273 mg) was eluted with EtOAc.

3.16. 2α-Diphenylphosphatostemod-12-ene (*21*) and 2α-diphenylphosphato-13-hydroxystemodane (*22*)

To a solution of stemodin (800 mg, 2.61 mmol) in pyridine (1.0 ml) was added diphenyl chlorophosphate (3.52 g, 13.06 mmol). The mixture was stirred at room temperature for 2 h. The solution was treated with excess saturated copper(II) sulfate solution, extracted with CH_2Cl_2 (3×50 ml), washed with brine, and dried ($MgSO_4$). Removal of the solvent in vacuo afforded a brown gum (1182 mg) which was subjected to chromatography. Elution with 20% EtOAc in petrol yielded 2α-diphenylphosphatostemod-12-ene (**21**) as a gum (275 mg), IR: ν_{max} cm^{-1} 1498, 1281, 1021, 947; HREIMS: *m*/*z* (rel. int.) 396.3392 $[M-124]^+$ (32.1), 396.3400 (100), 363.3065 (69.2), 337.2896 (29.2); ESMS: *m*/*z* (rel. int.) 368 (5), 359 (47), 349 (22), 327 (100), 271 (31); ^{1}H NMR: δ 0.92 (3H, *s*, H-20), 0.94 (3H, *s*, H-19), 0.97 (3H, *s*, H-18), 1.64 (3H, *s*, H-17), 4.70 (1H, *m*, $W_{1/2}$=23.2 Hz, H-2), 4.98 (1H, *bs*, H-12), 7.25 (10H, *m*, $W_{1/2}$=32.6 Hz, Ar-H).

Also eluting in 20% EtOAc in petrol was 2α-diphenylphosphato-13-hydroxystemodane (**22**) (700 mg) which crystallised from Me_2CO as needles, mp 120–122°C, $[\alpha]_D$−15.8° ($CHCl_3$, *c* 1.42); IR: ν_{max} cm^{-1} 3471, 1774, 1597, 1498; HRMS (EI): *m*/*z* (rel. int.) 538.2846 $[M]^+$ (0.6), 468.2434 (3.3), 270.2349 (22.2), 251.0482 (100); ^{1}H NMR: δ 0.90 (3H, *s*, H-20), 0.92 (3H, *s*, H-19), 0.96 (3H, *s*, H-18), 1.10 (3H, *s*, H-17), 4.64 (1H, *m*, $W_{1/2}$=20.9 Hz, H-2), 7.25 (10H, *m*, $W_{1/2}$ =37.5 Hz, Ar-H).

3.17. 13-Hydroxy-2α-propionoxystemodane (**23**)

Propionic acid (0.44 ml, 5.88 mmol) and *p*-toluenesulfonyl chloride (0.448 g, 2.35 mmol) were stirred in pyridine (0.75 ml) for 1 h. Stemodin (300 mg, 0.98 mmol) was then added and the reaction mixture was stirred for an additional 48 h. The solution was treated with saturated copper(II) sulfate, extracted with EtOAc (3×50 ml), washed with brine, and dried (Na_2SO_4). The solvent was removed in vacuo to afford a gum which was purified by column chromatography. Elution with 10% EtOAc in petrol afforded 13-hydroxy-2α-propionoxystemodane (**23**) (261.5 mg) which crystallised from Me_2CO as plates, mp 114–116°C, $[\alpha]_D$ −32.7° ($CHCl_3$, *c* 1.17); IR: ν_{max} cm^{-1} 3487, 1713, 1462, 1344, 1219; HRMS (EI): *m/z* (rel. int.) 362.2814 $[M]^+$ (0.2), 288.2449 $[M\text{-}C_3H_6O_2]^+$ (64.1), 273.2217 $[M\text{-}C_3H_6O_2\text{-}CH_3]^+$ (50.0), 233.1902 (42.9), 217.1592 (100), 133.1014 (54.8), 121.1015 (41.6), 57.0340 $[C_3H_5O]^+$ (56.4); ^1H NMR: δ 0.95 (6H, *s*, H-18, 19), 1.05 (3H, *s*, H-20), 1.12 (3H, *s*, H-17), 1.24 (3H, *t*, *J* = 6.2 Hz, $C\underline{H}_3CH_2CO$), 2.28 (2H, *q*, *J* = 7.7 Hz, $CH_3C\underline{H}_2CO$-), 4.91 (1H, *m*, $W_{1/2}$ = 23.4 Hz, H-2).

Further elution with 50% EtOAc in petrol afforded unchanged stemodin (**1**) (70 mg).

3.18. Incubation of 13-hydroxy-2α-propionyloxy stemodane (**23**)

The xenobiote (177.5 mg) was fed to the fungus growing in 750 ml medium. The extract (147.7 mg) was chromatographed on a silica gel. Elution with 10% EtOAc in petrol afforded fed substrate **23** (5.4 mg). Further elution of the column with 50% EtOAc in petrol gave stemodin (31 mg). The fraction eluting in 70% EtOAc in petrol afforded a mixture which was acetylated and chromatographed. Elution with 30% EtOAc in petrol yielded 2α,18-diacetoxy-13-hydroxystemodane (**5**) (46.9 mg) as a gum.

3.19. 13-Hydroxy-2α-isobutyroxystemodane (**24**)

Isobutyric acid (0.55 ml, 5.89 mmol) and *p*-toluenesulfonyl chloride (0.448 g, 2.35 mmol) were stirred in pyridine (0.75 ml) for 1 h. Stemodin (300 mg, 0.98 mml) was added and the mixture was stirred for 48 h. The solution was treated with saturated copper(II) sulfate, extracted with EtOAc (3×50 ml), washed with brine, and dried (Na_2SO_4). The solvent was removed in vacuo to afford a gum which was purified by column chromatography. Elution with 10% EtOAc in petrol afforded 13-hydroxy-2α-isobutyroxystemodane (**24**) (238.7 mg) which crystallised from Me_2CO as plates, mp 121–124°C, $[\alpha]_D$ −25.5° ($CHCl_3$, *c* 1.2); IR: ν_{max} cm^{-1} 3506, 1712, 1450, 1357, 1206, 1162; HRMS (EI): *m/z* (rel. int.) 361.2735 $[M\text{-}CH_3]^+$ (2.5), 358.2867 $[M\text{-}H_2O]^+$ (3.9), 288.2451 $[M\text{-}C_4H_8O_2]^+$ (51.8), 273.2219 $[M\text{-}C_4H_8O_2\text{-}CH_3]^+$ (67.7), 233.1901 (58.1), 217.1951 (100), 121.1016 (50.7),CIMS: *m/z* (rel. int.) 394.3 $[M+NH_4]^+$ (100), 376.4 $[M]^+$ (15.2), 288.3 (43.0), 271.2 (86.2), 217.2 (36.3); ^1H NMR: δ 0.95 (3H, *s*, H-19), 0.96 (3H, *s*, H-18), 1.04 (3H, *s*, H-20), 1.12 (3H, *s*, H-17), 1.15 (6H, *d*, *J* = 7.0 Hz, $[C\underline{H}_3]_2CHCO_2$), 2.48 (1H, *m*, $W_{1/2}$ = 20.5 Hz, $[CH_3]_2C\underline{H}CO_2$), 4.88 (1H, *tt*, *J* = 3.8, 11.7 Hz, H-2).

Further elution with 50% EtOAc in petrol afforded unchanged stemodin (**1**) (70.4 mg).

3.20. Incubation of **24**

The substrate (184 mg) was fed to *B. bassiana* growing in 750 ml medium. The extract (145.8 mg) was chromatographed. Elution with 10% EtOAc in petrol afforded the fed substrate (**24**) (37.8 mg). Further elution of the column with 50% EtOAc in petrol gave stemodin (64.5 mg). The fraction eluting in 70% EtOAc in petrol afforded a mixture which was acetylated. The product was chromatographed. Eluting in 30% EtOAc in petrol was 2α,18-diacetoxy-13-hydroxystemodane (**5**) (39 mg) as a gum.

Acknowledgements

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The ABC-like vacuolar transporter for rye mesophyll flavone glucuronides is not species-specific

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Abstract

In many cases, the vacuolar uptake of secondary metabolites has been demonstrated to be strictly specific for a given compound and plant species. While most plants contain glycosylated secondary substances, few cases are known where flavonoids may also carry negative charges, e.g. as glucuronide conjugates. Vacuolar transport of glucosylated phenylpropanoid derivatives has been shown to occur by proton–substrate antiport mechanisms (Klein, M., Weissenböck, G., Dufaud, A., Gaillard, C., Kreuz, K., Martinoia, E., 1996. Different energization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. J. Biol. Chem. 271, 29 666–29 671). In contrast, flavone glucuronides appearing specifically in rye mesophyll vacuoles are taken up by direct energisation utilising MgATP, strongly arguing for the presence of an ATP-binding cassette (ABC) transporter belonging to the subfamily of multidrug resistance-associated proteins (MRP) on the rye vacuolar membrane (Klein, M., Martinoia, E., Hoffmann-Thoma, G., Weissenböck, G., 2000. A membrane-potential dependent, ubiquitous ABC-like transporter mediates the vacuolar uptake of rye flavone glucuronides — regulation of glucuronide uptake by glutathione and its conjugates. Plant Journal 21, 289–304). MRPs are known to transport negatively charged organic anions. Results presented here suggest that the vacuolar directly energised MRP-like glucuronate pump for plant-specific flavone glucuronides is ubiquitously present in diverse plant species since rye flavone glucuronides are taken up into vacuoles isolated from the barley mesophyll or from the broccoli stalk parenchyma representing two species which do not synthesise glucuronidated secondary compounds. According to the transport characteristics and inhibition profile observed we propose the existence of a high-capacity, uncoupler-insensitive vacuolar ABC transporter for flavone glucuronides and possibly other negatively charged organic compounds — plant-born or xenobiotic — irrespective of the plant's capability to endogenously produce glucuronidated compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Secale cereale* L. (Poaceae); *Hordeum vulgare* L. (Poaceae); *Brassica oleracea* L. (Brassicaceae); ABC (ATP-binding cassette) transporter; Multidrug resistance-associated proteins; Flavonoids; Secondary plant product transport; Vacuoles

1. Introduction

The giant diversity of secondary metabolites in the plant kingdom results from a large variety of possible modifications like hydroxylation, glycosylation, methylation or acylation reactions sequentially performed on rather few key intermediates. In most cases these different modifications lead to species- or even tissue- or cell-specific substances which are serving mainly constitutive protective functions or which are induced after exposure to different stresses (Dixon and Paiva, 1995; Wink, 1997). However, in order to fulfil these mainly dose-dependent functions, the plant cell faces the dilemma of synthesizing high amounts of a substance that may be potentially toxic to the cytosol (Matile, 1984; Martinoia et al., 2000). Thus, an efficient elimination of secondary products from the cytosol is a crucial step and involves either transport into the apoplastic space ('excretion') where further turnover reactions like polymerisation may occur, or compartmentation into the large central vacuole. For substances transported into the vacuole, a largely metabolically inactive compartment, much less is known about further modifications of secondary products (Sharma and Strack, 1985; Martinoia et al., 2000). However, the nature

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of most of the transport steps — either via transporters localised on the corresponding membranes or via fusion of ER-derived vesicles filled with secondary compounds synthesised by a channeling multienzyme complex (Rasmussen and Dixon, 1999; Winkel-Shirley, 1999) — has remained obscure.

Since the central vacuole may occupy more than 90% of the volume of a differentiated plant cell it represents a large storage compartment for secondary metabolites. Currently, two mechanisms driving the vacuolar accumulation of solutes have been described: (i) a vacuolar H^+-ATPase and a H^+-PPiase act as proton pumps generating an electrochemical gradient which can be used by secondary energised transporters. With regard to secondary plant products we have demonstrated recently that isovitexin, a minor barley flavone C-glucoside occurring in leaf cells is transported across the tonoplast of barley mesophyll vacuoles via a H^+-antiport mechanism (Klein et al., 1996). (ii) In the last years accumulating evidence suggests that members of the widely distributed family of ATP-binding cassette (ABC) proteins occur on plant vacuolar membranes (for recent reviews see Rea et al., 1998; Rea, 1999; Martinoia et al., 2000). Typically, ABC transporters act as pumps for a large variety of structurally diverse substrates. They are directly energised by ATP, sensitive towards the P-type ATPase inhibitor vanadate and, in contrast to transport systems dependent on the transtonoplast electrochemical gradient, they are insensitive towards perturbation of either membrane potential or pH-gradient. Plant transporters with ABC-like properties (Higgins, 1992) have been shown for a range of different substances mainly of non-plant origin (Martinoia et al., 2000). Due to functional and structural considerations, proposed physiological roles of vacuolar ABC transporters belonging to the subfamily of multidrug resistance-associated proteins (MRP) include anthocyanin and isoflavonoid transport in the form of glutathione *S*-conjugates (Marrs et al., 1995; Li et al., 1997) as well as roles in leaf senescence, antioxidative stress response and xenobiotic detoxification. However, the presence of endogenous glutathione *S*-conjugates with products of the phenylpropanoid pathway remains questionable (for review see Martinoia et al., 2000).

The flavone glucuronides luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide (R1) and luteolin 7-*O*-diglucuronide (R2) accumulate specifically in rye mesophyll cells of primary leaves with a developmentally regulated pattern (Strack et al., 1982; Schulz et al., 1985; Schulz and Weissenböck, 1988). In a preceding paper we provided evidence that these flavone glucuronides are substrates of a constitutive MRP-like directly energised transporter on rye vacuolar membranes (Klein et al., 2000) which may also transport other non-plant glucuronides like β-estradiol 17-(β-D-glucuronide) ($E_2$17G) (Klein et al., 1998). These results demonstrate that the transport of negatively charged plant-specific compounds may be a physiological function of MRP-like transporters in rye and that glutathionation is not an essential step prior to vacuolar deposition of these flavonoids.

In several cases it has been described that vacuolar transport of secondary plant products is very specific for a given compound and species (Martinoia et al., 2000). For phenylpropanes, Hopp and Seitz (1987) and Matern et al. (1986) have shown that acylation with sinapic acid is a prerequisite for the species-specific vacuolar uptake of the *Daucus carota* anthocyanin and that acylation of apigenin 7-glucoside with malonic acid is essential for similar uptake into parsley vacuoles. On the other hand it is known that ABC-type transporters often exhibit a rather broad substrate specificity (König et al., 1999) and it can be assumed that plants like animals contain a quite comparable set of ABC transporters. Therefore we were interested in investigating whether the rye-specific flavone glucuronides are recognised by vacuolar transporters of plants which do not endogenously synthesize these compounds.

2. Results and discussion

In a previous publication we have shown that luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide (R1) and luteolin 7-*O*-diglucuronide (R2), two flavone glucuronides appearing specifically in the mesophyll cell layers of rye primary leaves, are taken up into rye vacuoles by a directly energised, ATP-dependent transporter, suggesting that an ABC transporter is involved in the uptake of these compounds (Klein et al., 2000). In order to elucidate whether this uptake system is species-specific, we investigated the transport of these compounds into vacuoles isolated from barley mesophyll and broccoli stalk parenchyma. Both plants are not known to contain any glucuronidated secondary plant products. Barley primary leaves have been shown to contain flavone glucosides with saponarin, a diglucosylated apigenin derivative, as the major compound (Blume et al., 1979; Reuber et al., 1996) and it was proposed that the vacuolar accumulation of flavone C-glucosides is via a H^+-antiporter (Klein et al., 1996).

The uptake of [^{3}H]-R1 into vacuoles isolated from 8 day old pimary leaves of barley was strictly dependent on the presence of MgATP (Fig. 1). In the absence of ATP, no uptake was detectable. In addition, the non-hydrolysable ATP-analogue 5′-adenylyl-β,γ-imidodiphosphate (AMP-PNP) could not substitute for ATP suggesting that the transport energisation was dependent on the hydrolysis of the triphosphate (Table 1). Furthermore, the ATP-dependent uptake was linear for at least 1 h (not shown). After 60 min uptake the vacuolar concentration of R1 in barley vacuoles that were incubated with 20 nM [^{3}H]-R1 and MgATP rose to values between 30 and 50 nM. This result demonstrated uptake against the concentration gradient and thus net accumulation of

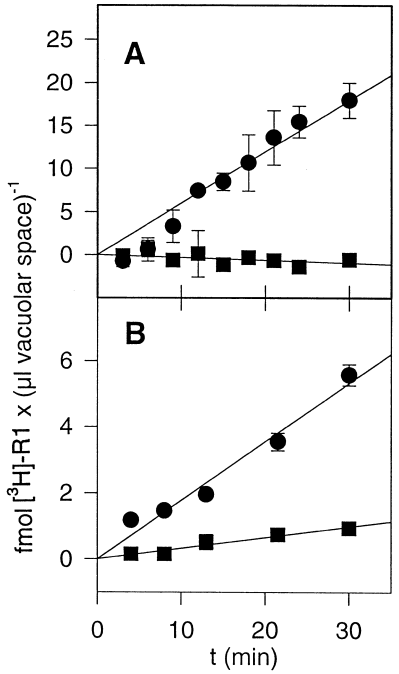


Fig. 1. Time-dependent uptake of luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide ([^{3}H]-R1) into vacuoles isolated from the barley mesophyll (A) and from broccoli stalk parenchyma (B). Isolated vacuoles of barley and broccoli were incubated with 20 nM of [^{3}H]-R1 in the presence (●) or absence (■) of 3 mM MgATP. A representative experiment with four replicates per timepoint is illustrated. Vacuolar contents of [^{3}H]-R1 were corrected for the contamination of the vacuoles after their separation from the surrounding medium.

this flavone glucuronide. Using rye mesophyll vacuoles, we had shown vacuolar accumulation of both glucuronides (Klein et al., 2000). The maximal accumulation ratio achieved for R1 was 2.5-fold after 2 h of uptake in this case. At a fixed substrate concentration of 20 nM the uptake rates for both substrates, [^{3}H]-R1 and [^{3}H]-R2, observed within the linear phase of transport were remarkably similar when vacuoles from the specific species (rye) were compared to barley vacuoles (Table 2). Both vacuolar uptake systems exhibited rates of about 15–20 pmol× (l vacuolar space×s)$^{-1}$. Since the K_M-values reported for flavone glucuronide transport into rye vacuoles were about 100 µM for R1 and R2 (Klein et al., 2000), it could be assumed that the substrate concentrations chosen in these experiments were not rate-limiting for the transport velocity.

The uptake of [^{3}H]-R1 into vacuoles isolated from the broccoli stalk parenchyma which represented a dicotyledonous species and a different tissue, was also strongly stimulated by MgATP. However, in the absence of ATP a low but consistent uptake activity for R1 could be observed (12% of the activity measured in the presence of MgATP, Fig. 1, Table 2). Transport of [^{3}H]-R1 into broccoli vacuoles in the presence of ATP was linear with time for at least 30 min (Fig. 1). For broccoli vacuoles the uptake rate measured within the linear phase of

Table 1
Effect of ATP, 5′-adenylyl-β,γ-imidodiphosphate (AMP-PNP) and different inhibitors on the uptake of [^{3}H]-luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide ([^{3}H]-R1) into mesophyll vacuoles of barley and vacuoles isolated from the broccoli stalk parenchyma[a]

Condition		Uptake into vacuoles	
		Barley	Broccoli
		% of MgATP-stimulated control	
−ATP		0.2±0.1	12.2±3.1
+ATP	(control)	100	100
+AMP-PNP		1.3±1.5	n.d.
+ATP	+vanadate (1 mM)	2.6±2.5	5.6±6.4
+ATP	+bafilomycin A1 (0.1 µM)	96.9±5.1	136.5±19.9
+ATP	+CCCP (5 µM)	81.5±12.7	117.1±32.0
+ATP	+$(NH_4)SO_4$ (5 mM)	97.5±4.5	n.d.
+ATP	+valinomycin (5 µM)	56.1±6.6	n.d.
+ATP	+NaN_3 (1 mM)	90.8±4.1	n.d.

[a] Barley or broccoli vacuoles were incubated in the presence of 20 or 40 nM of [^{3}H]-R1, respectively, and further compounds as indicated for each condition. Uptake rates corresponding to 100% (control in the presence of ATP) were 18.2±2.2 and 4.2±0.5 pmol×(l vacuolar volume×s)$^{-1}$ for barley and broccoli vacuoles, respectively. All experiments in the presence of ATP contained 4 mM $MgSO_4$ and 3 mM ATP. The conditions without ATP were supplied with 1 mM $MgSO_4$. Values are means of three to six independent experiments±SD. Each single experiment consisted of six replicas per condition for mesophyll vacuoles and four replicas for broccoli vacuoles. For barley vacuoles, uptake rates of both substrates were calculated by subtracting the radioactivity determined after 2 min from corresponding 30-min values. In experiments with broccoli vacuoles, uptake rates were calculated by linear regression of three datapoints within the first 20 min of uptake. n.d., not determined.

uptake and in the presence of 20 nM [^{3}H]-R1 and MgATP was clearly lower than the values observed for rye and barley [2.3 pmol×(l vacuolar space×s)$^{-1}$, Table 2, Fig. 1]. However, a lower uptake activity had also been observed in the case of glutathione conjugates for several dicotyledonous species compared to barley (E. Martinoia et al., 1999, unpublished). Using rye vacuoles we investigated via HPLC-separation of vacuolar supernatants that the molecular species present in the vacuoles after uptake still corresponded to [^{3}H]-R1 and [^{3}H]-R2, respectively. Thus, modifications of the two flavone glucuronides during or after the transport step could be excluded (Klein et al., 2000).

The characteristics of the energisation mechanism responsible for the vacuolar uptake of the flavone glucuronides were investigated by analysing the effects of a set of inhibitors and ionophores on [^{3}H]-R1 transport (Table 1). Vanadate, a typical inhibitor of P-ATPases acting as a phosphate analogue, almost completely inhibited the transport of [^{3}H]-R1 in the presence of MgATP into barley mesophyll as well as broccoli parenchyma vacuoles. In contrast, the macrolide antibiotic bafilomycin A1 which had been described as a specific inhibitor of the vacuolar H^+-ATPase (Dröse et al.,

Table 2
Transport rates for the uptake of 20 nM [^{3}H]-luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide ([^{3}H]-R1) and [^{3}H]-luteolin 7-*O*-diglucuronide ([^{3}H]-R2) into rye and barley mesophyll vacuoles and into broccoli stalk parenchyma vacuoles in the presence of MgATP[a]

Vacuoles isolated from	Uptake rate pmol×(l vacuolar volume×s)$^{-1}$	
	[^{3}H]-R1	[^{3}H]-R2
Rye, mesophyll	14.5±5.9 (13)	19.0±4.7 (5)
Barley, mesophyll	18.0±2.2 (4)	20.4±3.4 (3)
Broccoli, stalk parenchyma	2.3±0.2 (3)	n.d.

[a] Isolated vacuoles were incubated in the presence of 20 nM [^{3}H]-R1 or [^{3}H]-R2, 4 mM $MgSO_4$ and 3 mM ATP (added as the sodium salt and buffered with 0.1 M BTP to a final pH of 7.5). At different time points within the linear phase of uptake, the incubation was stopped by silicone oil centrifugation and the radioactivity in the aqueous supernatant was determined. Transport rates were calculated by linear regression of these datapoints. Vacuolar volume was determined by addition of ^3H_2O which rapidly equilibrates across the membrane in separate tubes. In brackets the number of independent determinations±SD is given. Each experiment consisted of three (broccoli) or six (rye, barley) replicates per condition. n.d., not determined.

1993), had no effect on flavone glucuronide uptake into the vacuoles isolated from the two plant species. Neither carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) nor $(NH_4)_2SO_4$ reduced the transport rate for [^{3}H]-R1 into barley and broccoli vacuoles in the presence of MgATP (Table 1). Interestingly, the potassium ionophore valinomycin which abolishes the membrane potential across the tonoplast caused a nearly 50% inhibition of R1-uptake into barley vacuoles. A similar result had been observed for rye vacuoles: using isolated tonoplast vesicles with artificially imposed K^+-gradients we had demonstrated that the ATP-dependent vacuolar glucuronide conjugate transporter is modulated by the transmembrane electrical potential (Klein et al., 2000). Due to the fact that all (rye and barley) or the major portion (broccoli) of the vacuolar R1-uptake was directly linked to ATP hydrolysis, it must be concluded that the vacuolar R1 transporter is ATP-dependent. Thus, the valinomycin inhibition shown here together with the rye tonoplast vesicle experiments argue for an ATP-dependent flavone glucuronide transport system that is able to sense the membrane potential. Since R1 is a negatively charged compound under cytosolic pH conditions, these results provide preliminary evidence that the ATP-dependent flavone glucuronide transport into the vacuole could be electrogenic due to charge movement across the tonoplast. Data presented here and by Klein et al. (2000) exclude at least for barley and rye the presence of an additional secondary energized, e.g. $\Delta\Psi$-dependent transport system. However, since a weak ATP-independent R1-uptake was observed for broccoli vacuoles, it cannot be excluded for this species that another, e.g. secondary energised component is additionally present in the tonoplast (Fig. 1).

Flavone glucuronides and the non-plant glucuronide $E_2$17G exhibited a complex interaction pattern with glutathione or glutathione conjugates where the addition of these substances resulted in stimulation of glucuronide import or inhibition depending on the substrate and competitor chosen (Klein et al., 1998, 2000). Glutathione conjugates as well as glucuronides represent typical substrates for MRP-type transporters (Leier et al., 1994; Loe et al., 1996; König et al., 1999) and it was shown that all MRPs from *Arabidopsis thaliana* characterised to date were functional glutathione conjugate pumps (Lu et al., 1997, 1998; Tommasini et al., 1998). When $E_2$17G is used as a substrate we also demonstrated that stimulation by glutathione conjugates and oxidised glutathione was not restricted to vacuoles from a species synthesising glucuronidated substances but that it also appeared with barley vacuoles (Klein et al., 1998). However, the molecular nature of the interaction between glucuronide uptake and the stimulation or inhibition by glutathione or its conjugates has not been investigated in detail. Therefore, the stimulation of flavone glucuronide uptake by 2,4-dinitrobenzene-glutathione (DNB-GS) was used as the simplest and most common feature to analyse species-specificity of vacuolar R1-uptake. Barley as well as broccoli vacuoles exhibited stimulation of ATP-dependent [^{3}H]-R1 transport by DNB-GS (Fig. 2). However, while the stimulation of R1-uptake into barley vacuoles by DNB-GS was almost 5-fold and therefore comparable to rye vacuoles (3- to 4-fold stimulation; Klein et al., 2000), the effect of DNB-GS was clearly smaller for broccoli-vacuoles (about 1.8-fold;

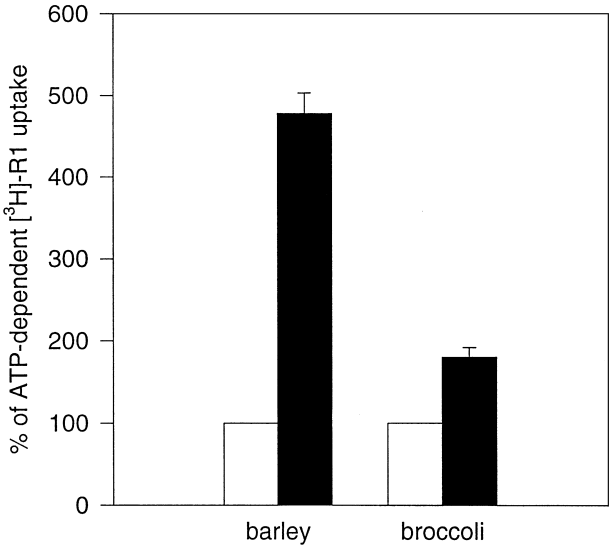


Fig. 2. Effect of 2,4-dinitrobenzene-glutathione (DNB-GS) on the uptake of luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide ([^{3}H]-R1) into vacuoles from barley mesophyll or broccoli stalk parenchyma. Isolated vacuoles were incubated with 20 nM of [^{3}H]-R1 and 3 mM MgATP in the presence (filled) or absence (blank) of 0.2 mM DNB-GS. Transport rates corresponding to 100% are given in Table 2.

Fig. 2). If the stimulation by DNB-GS was taken into account, a 4- to 7-fold vacuolar accumulation of [^{3}H]-R1 against the concentration gradient could be observed only after 30 min in barley vacuoles. For rye vacuoles we had confirmed that DNB-GS accelerated flavone glucuronide transport but did not modify the substrate chemically (Klein et al., 2000).

3. Conclusion and outlook

Since neither protonophores nor specific inhibitors of the proton pumping ATPase inhibited the uptake of [^{3}H]-R1 into vacuoles of the two heterologous species, it could be argued that the pH-gradient across the tonoplast generated by the vacuolar proton pumps was not responsible for MgATP-dependent flavone glucuronide transport and thus that no secondary energised H^+-antiport system was involved (Table 1). Using rye vacuoles we had demonstrated that the vacuolar uptake system for [^{3}H]-R2 exhibited the same characteristics (Klein et al., 2000).

Taken together, the transport of rye flavone glucuronides into vacuoles isolated from other species met the known criteria for the characterisation of ABC-type transporters since: (i) uptake was strictly ATP-dependent and not sensitive towards AMP-PNP; (ii) vanadate strongly inhibited R1-uptake; (iii) an influence of the pH-gradient could be excluded and (iv) flavone glucuronides represented organic anions due to the negative charges supplied by glucuronate residues suggesting that a MRP-like ABC transporter was involved. Our observation that the transport characteristics were essentially the same for vacuoles from plants lacking flavone glucuronides as for rye vacuoles containing R1 and R2 (Klein et al., 2000) indicated that an ABC-type glucuronate transporter could be ubiquitously present on vacuolar membranes irrespective of the composition and conjugation pattern (e.g. glucosylated vs glucuronidated substances) of secondary metabolites.

Most examples described in the scientific literature argue for species- and substrate-specific vacuolar uptake systems for secondary plant products, and a number of different transport and trapping mechanisms have been discussed (Martinoia et al., 2000). Considering the fact that rye flavone glucuronides, like the non-plant $E_2$17G (Klein et al., 1998), were also transported into vacuoles of far related species that do not exhibit synthesis of known glucuronidated compounds raises two questions: Does the directly energised flavone glucuronide transporter in rye mesophyll vacuolar membranes represent an unspecific uptake system catalysing the efficient vacuolar deposition of a variety of conjugated organic anions besides the transport of flavones? Or are there ubiquitous but yet unknown pathways present in plant cells leading to presently not identified glucuronidated compounds that have to be stored in the vacuole in a way comparable to the detoxification of xenobiotic compounds as glucuronates in the animal liver?

4. Experimental

4.1. Reagents

If not stated otherwise, all chemicals were of highest purity available and supplied by Sigma (Deisenhofen, Germany) or Fluka (Buchs, Switzerland). Percoll and Ficoll 400 were obtained from Pharmacia (Freiburg, Germany). Cell wall degrading enzymes were purchased from Seishin Corp. (Japan; cellulase Y-C and pectolyase Y-23) and Roehm and Haas (Germany, Rohament CT). Silicone oils AR 20 and AR 200 were supplied by Wacker Chemie (F.R.G.). The flavonoid glucuronides luteolin 7-*O*-diglucuronide (R2) and luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide (R1) were isolated from *Secale cereale* L. cv. Kustro primary leaves (see Schulz et al., 1985; Klein et al., 1998). Custom tritiation of R1 and R2 was performed by SibTech Inc. (Newington, USA). Purification and analysis of radiochemical purity was determined according to Klein et al. (2000). DNB-GS was prepared as described before (Klein et al., 1998). The vacuolar H^+-ATPase inhibitor bafilomycin A1 (Dröse et al., 1993) was kindly provided by Dr K. Altendorf (University of Osnabrück, Germany).

4.2. Plant material and growth conditions

Rye (*Secale cereale* L. cv. Kustro; Lochow-Petkus, Bergen, Germany) and barley (*Hordeum vulgare* L. cv. Bakara; kindly provided by the Institute of Plant Sciences, ETH Zürich, Switzerland) was grown on vermiculite for eight days in a growth cabinet with 13 h of fluorescent light (approx. 100 $\mu mol \times m^{-2}\ s^{-1}$) at 20°C and 70% relative humidity. Plants were watered daily with Hoagland's solution. Broccoli (*Brassica oleracea* cv. Italica) was bought fresh from local markets.

4.3. Vacuole isolation

Barley vacuoles were isolated as described (Rentsch and Martinoia, 1991). Contamination of barley vacuoles with other cell constitutents was less than 3% as measured by marker-enzyme activities (Rentsch and Martinoia, 1991). For the isolation of broccoli stalk vacuoles, inflorescence and nodes were removed and the green and vascular bundle-rich peripheral part of the internodes was peeled off. The remaining tissue was cut into 2-mm thick slices directly into medium A (700 mM mannitol, 10 mM $CaCl_2$ and 10 mM Mes/KOH pH 5.5). After tissue equilibration (30 min at room temperature), medium A was replaced by the digestion medium, consisting of medium A plus 2% (w/v) cellulase Rohament

CT, 0.2% (w/v) pectolyase Y-23 and 0.3% (w/v) BSA. Digestion of cell walls was performed overnight (16 h) at 25°C. The resulting suspension was filtered over four layers of cheesecloth and two nylon nets (200 μm mesh size). Protoplasts were concentrated by centrifugation for 10 min at 200 ***g***, resulting in a dense band of clean protoplasts and a pellet containing debris of undigested tissue parts that was discarded. Protoplasts were washed twice by sedimentation (5 min, 120 ***g***, 20°C) in medium B (700 mM glycine betaine, 0.2% (w/v) BSA and 5 mM Mes/KOH pH 5.5). To obtain the vacuolar fraction, protoplasts were transfered into lysis medium (430 mM glycine betaine, 7.5 mM EGTA, 5 mM DTT, 0.5% (w/v) BSA, 10 mM Hepes/KOH pH 8.25). Lysis took about 10 min and was monitored by light-microscopical examination. Vacuoles were concentrated by sedimentation (10 min, 120 ***g***, 20°C) in a medium consisting of lysis medium plus 1.5% Ficoll, leaving most of the emerged cytoplasts in the discarded supernatant and producing a soft pellet of clean vacuoles. Before the uptake experiments, vacuoles were suspended twice in freshly prepared wash medium (430 mM glycine betaine, 10 mM Hepes/KOH pH 7.2, 3% (w/v) Ficoll 400 and 0.2% [w/v] BSA). About 7×10^6 protoplasts were recovered per gram fresh weight of broccoli tissue. The yield of vacuoles was about 25%. A detailed analysis of different marker enzyme activities revealed a contamination of the vacuolar fraction with protoplasts of about 4%. Broccoli vacuoles were able to establish and maintain an ATP- or pyrophosphate-driven pH-gradient across the tonoplast which was recorded as quenching of the fluorescence of quinacrine (G. Hoffmann-Thoma, 2000, unpublished).

4.4. Uptake experiments

Transport studies with barley or rye mesophyll vacuoles were performed as described (Klein et al., 1998, 2000) using the silicone oil centrifugation technique (Werdan and Heldt, 1972) by the addition of 30 μl densely packed vacuoles to 70 μl 20 nM [^{3}H]-luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide or [^{3}H]-luteolin 7-*O*-diglucuronide (both 20–30 Ci×mmol^{-1}), 23% (v/v) Percoll, 0.4 M sorbitol, 30 mM KCl, 20 mM Mes/Btp pH 7.2, 0.12% (w/v) bovine serum albumin, 1 mM DTT, and further compounds as given in tables and figures. For broccoli vacuoles, three 100-μl-aliquots of transport mix consisting of vacuole suspension, radioactive flavone glucuronides (20 or 40 nM) and the additives of the separate conditions were pipetted into microcentrifugation tubes prefilled with 100 μl of lipophilic phase consisting of a mixture of silicone oil AR 20 and dibutylphthalate (1:2) on a cushion of 25 ml 1 M aqueous sorbitol. Vacuoles were separated from the uptake medium by sedimentation (1 min, 10 000 ***g***, Beckman microfuge 11). Microcentrifugation tubes were deep-frozen in liquid nitrogen; tube sections containing the vacuolar fraction were excised and each transferred into 200 μl of 1% aqueous sodium dodecyl sulfate. After vigorous mixing and centrifugation (3 min, 10 000 ***g***), the tube sections were removed and 180 μl of the aqueous supernatant were transferred for scintillation counting. The vacuolar volume was calculated in separate tubes by the addition of 3.7 kBq of ^3H_2O which equilibrates rapidly between the medium and the vacuolar lumen. In all cases, vacuolar fractions were suspended into 3 ml of scintillation cocktail (Readysafe, Beckman). Radioactivity was determined by liquid scintillation spectrophotometry. All counts were corrected for background and quenching. Experiments in the presence of glutathione conjugates were performed without DTT which had no effect on the uptake of either substrate. Unless stated otherwise, uptake rates of [^{3}H]-R1 and [^{3}H]-R2 were calculated by subtracting the radioactivity measured after 2 min of incubation from corresponding 30-min values. All experiments were repeated with at least three independent vacuole isolations, each condition consisting of four (broccoli) or six (barley, rye) replicates.

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In vitro inhibition of *Sphaeropsis sapinea* by natural stilbenes

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Abstract

The effects of pinosylvin, pinosylvin monomethyl ether, pinosylvin dimethyl ether, and resveratrol on the fungal shoot blight and canker pathogen of conifers *Sphaeropsis sapinea* were examined in vitro. Effects of compounds, isolates, and concentrations on both conidial germination and mycelial growth were significant (values of $P < 0.001$), indicating inhibitory activity of these compounds.
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Keywords: *Sphaeropsis sapinea*; Conidial germination; Mycelial growth; Natural stilbenes; Antifungal

1. Introduction

Stilbenes are among the biologically active secondary metabolites of plants, including the leaves, bark, and wood of many forest trees. Produced largely during the transition of sapwood to heartwood, accumulation of stilbenes in heartwood has often been suggested as a mechanism of decay resistance. These compounds may also be present before, and produced in response to, injury or colonization by microbial pathogens. Accumulation in pine species in response to invasion by a variety of fungi, especially at the interface between dead, colonized tissue and healthy living tissue, has led to the consideration of a potential role for stilbenes in resistance to disease.

The plant pathogenic fungus *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton [syn. *Diplodia pinea* (Desmaz.) J. Kickx fil.] causes extensive losses of native and exotic conifers throughout the world. Hosts include species in at least eight coniferous genera, including many species of pines (Gibson, 1979; Farr et al., 1989). Damage occurs in nurseries, Christmas tree and ornamental plantings, and forest stands. *S. sapinea* can be an aggressive pathogen that causes shoot blight, cankers, crown wilt, collar rot, and root disease (Gibson, 1979; Chou, 1987; Nicholls and Ostry, 1990; Stanosz and Cummings Carlson, 1996; Swart and Wingfield, 1991; Waterman, 1943). Dark blue to black staining also results from colonization of sapwood, either in standing trees or recently harvested logs.

Although there has been interest in the biological activity of stilbenes toward wood decay fungi for a long time (Hart and Shrimpton, 1979), there is little published information describing the effects of these compounds on shoot blight and canker pathogens that can infect foliage and succulent shoots and colonize living stem tissues. To determine whether stilbenes influence the interaction between *S. sapinea* and red pine, we examined the in vitro effects of pinosylvin (PS), pinosylvin monomethyl ether (PSM), pinosylvin dimethyl ether (PSD), and resveratrol (RES) on germination of asexual spores (conidia) and mycelial growth of *S. sapinea* in similar dose–response experiments. Isolates of two distinct groups of *S. sapinea* that are found in the northcentral US and that vary in their aggressiveness on their red pine host (Blodgett and Stanosz, 1997a, 1999; Stanosz et al., 1999) were studied.

2. Experimental

2.1. Sources of compounds

PS, PSM, and PSD were isolated from bark, cones, and/or wood from *Pinus banksiana* and *P. resinosa* (Celimene et al., 1999). RES (99%) was obtained from Sigma Chemical Company (St Louis, MO).

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2.2. Identification of PS, PSM, and PSD

NMR spectra were measured in $(CD_3)_2CO$ employing a Bruker AC + 300 (1H: 300 MHz; 13C: 75 MHz) spectrometer. Mass spectra were obtained by GC/MS on an HP-5972 instrument (EI, 70 eV). HPLC analysis and purification were carried out on a Beckman instrument; peaks were detected by UV at 280 and 305 nm and the column (250×4.6 mm) was an Altex ODS Serial no. 2UE271. Column chromatography was performed on silica gel (70–230 mesh); TLC was performed on silica gel (Kieselgel, 60F254) and the spots were visualized by UV (254 nm) and by exposure to a solution of phosphomolybdic acid (5% in ethanol).

2.3. Fungal isolates

Two isolates of each of the two RAPD marker groups of *S. sapinea* found in the northcentral US were used for the investigation, and had been collected by M. Palmer, US Forest Service. Palmer's collection numbers, host, and location of collection are as follows: A1 = 411, *P. resinosa*, Clearwater Co., MN; A2 = 128, *P. resinosa*, Grant Co., WI; B1 = 124, *P. banksiana*, Jackson Co., WI; B2 = 215, *P. resinosa*, Douglas Co., WI.

2.4. Spore germination

Stilbenes PS, PSM, PSD, and RES were dissolved in acetone grade 99.5% (Aldrich Chemical Company, Milwaukee, WI) and added to 2% water agar (WA; Difco Laboratories, Detroit), after autoclaving, to achieve concentrations of 0.01, 0.1 and 1.0 mg/ml. Control medium was amended with acetone only. Media were added to 24-well tissue-culture plates (1.0 ml/well; Becton Dickinson & Company, Lincoln Park, NJ). Conidial suspensions (80 μl/well) of respective isolates were added to the surface of the medium in each well. Conidia had been produced on autoclaved pine needles on WA using procedures similar to those described previously (Blodgett and Stanosz, 1997b). Each multiwell plate was sealed with parafilm and incubated at 25°C for 12 h in the dark. A drop of thimerosol solution (0.1 mg/ml) then was added to each well to stop germination and multiwell plates were stored at 4°C until examination. Percentage germination was determined from examination of approximately 50 conidia per replicate. Three replicates (wells) for each combination of compound, isolate, and concentration were used.

Effects of amendments on spore germination were determined by comparison of the frequency of germination in each well with amended medium to that in the respective control well. Germination was expressed relative to that of the control (germination percentage on amended medium/germination percentage on control medium ×100). For statistical analyses, data were expressed as proportions (0.1 was subtracted to correct for proportions exceeding 1.0) and transformed by calculating the arc sine of the square root of the proportion. A three-way analysis of variance was performed to test for effects of compound, isolate, and concentration, and for interactions using the general linear model procedure of Minitab for Windows, release 12.2 (Minitab, Inc., State College, PA).

2.5. Growth of mycelium

Stilbenes PS, PSM, PSD and RES were dissolved in acetone grade 99.5% and added to 2% WA after autoclaving to achieve concentrations of 0.01, 0.1 and 1.0 mg/ml. Control medium was amended with acetone only. Media were added to 9-cm-diameter plastic petri plates (20 ml/plate). Inoculum plugs of the respective *S. sapinea* isolates were cut from margins of actively growing colonies on WA and placed mycelium side down at the edge of the agar in the petri plates. Each plate was individually sealed with parafilm and incubated at ambient laboratory temperature (approx. 23°C) in the dark for 10 days. The radius of each resulting fungal colony was measured and recorded after subtracting the diameter of the inoculum plug. Three replicates (plates) were used for each combination of compound, isolate, and concentration.

Effects of amendments on radial growth of mycelium were determined by comparison of colony radius on each plate with amended medium to that on the respective control plate. Growth was expressed as a percentage of the control (amended/control ×100). For statistical analyses, data were expressed as proportions (0.1 was subtracted to correct for proportions exceeding 1.0) and transformed by calculating the arc sine of the square root of the proportion. A three-way analysis of variance was performed to test for effects of compound, isolate, and concentration, and for interactions using the general linear model procedure of Minitab for Windows, release 12.2 (Minitab, Inc., State College, PA).

3. Results and discussion

Results of these experiments confirm the in vitro activity of PS against *S. sapinea*, and place PSM, PSD, and RES among conifer host compounds to which both conidial germination and mycelial growth of the pathogen can be sensitive. Three-way analysis of variance of conidial germination data indicated effects of compound, isolate, and concentration (values of P each < 0.001), and compound–concentration interaction ($P < 0.001$) (Table 1). Three-way analysis of variance of mycelial growth data indicated effects of compound, isolate, and concentration (values of P each < 0.001), and both compound–isolate and compound–concentra-

Table 1
Frequency of germination of conidia of the fungus *Sphaeropsis sapinea* (expressed as a percentage of the control) on a water agar medium amended with four stilbenes at each of three concentrations

Compound	Isolate	Germination[a] (s.e.) at concentrations (mg/ml)		
		0.01	0.10	1.0
Pinosylvin	A1	81 (3)	44 (2)	29 (5)
	A2	88 (1)	47 (3)	25 (4)
	B1	79 (2)	49 (1)	32 (2)
	B2	77 (1)	46 (1)	33 (3)
Pinosylvin monomethyl ether	A1	62 (2)	59 (0)	46 (1)
	A2	67 (3)	60 (1)	44 (3)
	B1	62 (2)	56 (2)	51 (3)
	B2	57 (4)	54 (3)	52 (3)
Pinosylvin dimethyl ether	A1	92 (4)	92 (2)	91 (2)
	A2	98 (2)	96 (3)	94 (4)
	B1	101 (3)	100 (3)	98 (1)
	B2	99 (2)	99 (2)	92 (1)
Resveratrol	A1	91 (4)	77 (3)	81 (2)
	A2	96 (1)	97 (3)	81 (4)
	B1	104 (3)	91 (2)	83 (3)
	B2	101 (3)	91 (3)	83 (1)

[a] Each value is a mean for three replicates, with approximately 50 spores on each of two media (control and amended) for each replicate. Three-way analysis of variance indicated effects of compound, isolate, and concentration (values of $P < 0.001$), and compound–concentration interaction ($P < 0.001$).

Table 2
Radial growth of mycelium of the fungus *Sphaeropsis sapinea* (expressed as a percentage of the control) on a water agar medium amended with four stilbenes at each of three concentrations

Compound	Isolate	Colony radius[a] (s.e.) at concentrations		
		0.01	0.10	1.0
Pinosylvin	A1	95 (4)	71 (3)	49 (4)
	A2	89 (1)	73 (1)	52 (5)
	B1	90 (3)	69 (5)	59 (2)
	B2	93 (2)	74 (6)	55 (1)
Pinosylvin monomethyl ether	A1	83 (0)	64 (3)	48 (3)
	A2	98 (1)	71 (1)	54 (2)
	B1	99 (4)	81 (2)	69 (5)
	B2	93 (2)	75 (2)	61 (4)
Pinosylvin dimethyl ether	A1	91 (0)	77 (3)	65 (2)
	A2	100 (3)	82 (1)	70 (4)
	B1	97 (2)	88 (1)	81 (0)
	B2	95 (3)	84 (3)	74 (4)
Resveratrol	A1	100 (2)	72 (2)	57 (2)
	A2	96 (3)	74 (4)	61 (1)
	B1	96 (4)	73 (2)	57 (1)
	B2	95 (1)	78 (3)	52 (1)

[a] Each value is a mean for three replicates, for each of two media (control and amended). Three-way analysis of variance indicated effects of compound, isolate, and concentration (values of $P < 0.001$), and both compound–isolate and compound–concentration interaction (values of $P < 0.001$).

tion interaction (values of P each < 0.001) (Table 2). No other interactions in effects on either conidial germination or mycelial growth were significant (values of P all > 0.05).

Differences among compounds in effects on conidial germination and mycelial growth are indicated by comparing responses of *S. sapinea* to increasing concentrations of PS, PSM, and PSD (Tables 1 and 2). PSD had relatively little effect on conidial germination (expressed as a percentage of the control) at any concentration. Conidial germination on medium amended with PSM ranged from 62–67 and 44–52%, respectively, at the lowest (0.01 mg/ml) and highest (1.0 mg/ml) concentrations. Increasing concentrations of PS, however, had the greatest effect on conidial germination. At the lowest and highest concentrations of PS, respectively, conidial germination ranged from 77–88 and 25–33%. Radial mycelial growth (expressed as a percentage of the control) was less at each successively higher concentration of PS, PSM, and PSD. Mycelial growth at the highest concentration of compounds ranged from 49–59% with PS, 48–69% with PSM, and 65–81% with PSD. The relative activities of these three compounds in these experiments (PS > PSM > PSD) is consistent with conclusions from previous in vitro studies of stilbene effects on wood decay fungi growth as reviewed by Hart and Shrimpton (1979). The ability of *S. sapinea* to grow on WA with 1.0 mg/ml of PS, however, seems inconsistent with the statement of Hart and Shrimpton that, generally, PS concentrations of 50 μg/ml (0.05 mg/ml) resulted in total fungistasis (of several wood decay fungi). Although *S. sapinea* might be more tolerant of PS than fungi previously studied, exposure during germination or growth on a solid medium (in the current experiments) might be substantially less than that achieved during growth through a liquid medium with similar concentrations of a compound.

Effects of RES varied between assays and with concentration (Tables 1 and 2). Germination of conidia was relatively unaffected by RES, even at the highest concentration (1.0 mg/ml). Radial mycelial growth was influenced little at the lowest concentration (0.01 mg/ml) of RES, but ranged from 52–61% at the highest concentration (1.0 mg/ml) of this compound. Hart and Hillis (1974) previously noted the lethality of RES to wood decay fungi in a nutrient solution at 400 μg/ml (0.4 mg/ml). As noted above for PS, however, exposure during germination or growth on a solid medium (in the current experiments) might be substantially less than that achieved during growth through a liquid medium with similar concentrations of a compound.

Explanations for the relative tolerance of *S. sapinea* to PS and RES in our assays, compared to wood decay fungi as indicated by Hart and Hillis (1974) and Hart

and Shrimpton (1979), might be found in the fundamental differences between these microorganisms. These fungi are found in distinct taxonomic groups, with most wood decay fungi classified among the Basidiomycota and most shoot blight and canker fungi, including *S. sapinea*, among the Ascomycota or Fungi Imperfecti. In addition, as fungi that successfully infect, colonize, and kill living, responsive host tissues, shoot blight and canker pathogens might tolerate much greater concentrations of particular secondary metabolites, than some wood decay fungi, especially those which are entirely saprophytic. Hart (1981) reported that, compared to several basidiomycete wood decay fungi, *Ceratocystis montia* (an insect vectored, sapwood colonizing blue-stain fungus in the Ascomycota) exhibited greater in vitro tolerance of pinosylvin. Bois et al. (1999), however, observed inhibition of the insect-associated, ascomycete, sapwood staining fungus *Leptographium wingfieldii* in liquid culture concentrations much lower than those at which both spore germination and mycelial growth occurred in our assays. The potential for tolerance to stilbenes and other secondary metabolites to vary both by taxonomic grouping and the fundamental nature of the fungus–host interaction suggests hypotheses for testing in comparative research involving a wider diversity of fungi than those upon which individual studies have focused in the past. Future studies should also test the potential for conidial germination, although initially inhibited by stilbenes, to proceed during a longer incubation period, and should consider effects of stilbenes on fungal biomass as well as radial mycelial growth.

Results of these experiments are not consistent with the earlier report of substantial differences in responses of the same A and B group isolates of *S. sapinea* to PS (Blodgett and Stanosz, 1997b). In the current study, the frequency of conidial germination of A group isolates tended to be slightly less than that of B group isolates at the highest doses of PS and PSM (and the effect of the isolate was statistically significant), but these differences were relatively small. Similarly in the current study, although radial mycelial growth of A group isolates tended to be less than that of B group isolates at the highest doses of PS, PSM, and PSD (and the effect of the isolate was statistically significant), these differences were also relatively small. In earlier work (Blodgett and Stanosz, 1997b), PS applied to the surface of a 1.5% WA medium at a rate of 88 $\mu g/mm^2$ almost completely inhibited the germination of both A and B group conidia. In the same experiment, germination of conidia of both groups was less inhibited by PS applied at one-tenth that rate to the surface of the medium, but the frequency of germination of A group isolates at this lower rate was more than double that of B group isolates. Similarly, mycelial growth of A group isolates was less inhibited by each of these rates of surface-applied PS than that of B group isolates. Differences in procedures again may explain the different results obtained in these two studies. Concentrations to which *S. sapinea* was exposed in the current experiment, in which the PS was incorporated into the medium, may have been insufficient to produce the differential response seen previously.

These experiments justify continued examination of the influence of host compounds on the conifer host–*S. sapinea* interaction. As suggested by Hart and Shrimpton (1979), these in vitro assays were useful to confirm the potential of PS, PSM, PSD, and RES to inhibit spore germination and/or mycelial growth, and a relationship between concentration of the compounds and the degree of inhibition. A potential role in inhibition of spore germination, a process prerequisite for infection and establishment of disease, is of particular interest. As pointed out in previous reviews, however, further investigation must be pursued with care and caution (Hart and Shrimpton, 1979; Kemp and Burden, 1986). Results of in vitro assays may or may not be indicative of those that would be obtained on different substrates, especially living plants, and the concentrations of these compounds localized during colonization of red pine by *S. sapinea* has not yet been determined. Even if accumulated in vivo in sufficient quantity and at the proper location and time to have some role in resistance to pathogen invasion, however, compounds are not likely to be acting in isolation. Rather the potential role of these compounds must be examined in the context of a cascade of complex interactions influenced by a myriad of host, pathogen, and environmental factors.

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Bioactive humulene derivatives from *Asteriscus vogelii*

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Abstract

The bioactive fractions obtained from the extract of *Asteriscus vogelii* afforded a new humulene derivative, the 8-oxo-6,7,9,10-tetrahydrohumulen-1,12-olide (**1**), in addition to the known asteriscunolides A (**2**), C (**3**) and D (**4**), and the acids 8-oxo-α-humula-6*Z*,9*Z*-dien-12-oic acid (**5**), 8-oxo-α-humula-6*E*,9*Z*-dien-12-oic acid (**6**) and 8-oxo-α-humula-6*E*,9*E*-dien-12-oic acid (**7**), characterized as their methyl esters. Evaluation of their phytotoxic and cytotoxic activities was accomplished. Compounds **3** and **4** gave the highest inhibition of plant cell cultures and of the plant *Lemna paucicostata*. Compound **4** was also the most active against P-338 lymphoma in mice, A-549 carcinoma of human lung, HT-29 carcinoma of human colon and MEL-28 human melanoma. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Asteriscus vogelii*; Compositae; Humulenes; Phytotoxicity; Cytotoxicity

1. Introduction

Asteriscunolides A (**2**), C (**3**) and D (**4**) were previously isolated from the aerial parts of *Asteriscus aquaticus* (San Feliciano et al., 1982, 1984, 1985a), *Asteriscus graveolens* (El-Dahmy et al., 1985) and *Asteriscus sericeus* (Jakupovic et al., 1987). Other sesquiterpene lactones such as asteriscanolide and aquatolide were obtained from the extract of *Asteriscus aquaticus* (San Feliciano et al., 1985b, 1989). Flavonoids (Ahmed et al., 1991; Youssef et al., 1995), bisabolone hydroperoxides (Sarg et al., 1994), as well as farnesol and thymol derivatives (Ahmed, 1992), were also described as constituents of extracts from plants of the *Asteriscus* genus. The extract of *Asteriscus graveolens* also afforded the acids **5–7** (El-Dahmy et al., 1985) and its antimicrobial and hypoglycemic activities were reported (Sarg et al., 1994). Asteriscunolides and their corresponding free hydroxyacids were also obtained from the extract of *Nauplius intermedius* (Bohlmann and Pauls, 1992). We have now carried out the isolation of the major chemical constituents of the bioactive fractions, obtained from the crude extract of the aerial parts of *Asteriscus vogelii*. Investigation of their phytotoxic and cytotoxic activities has also been accomplished.

2. Results and discussion

The chloroform crude extract obtained from the aerial parts of *Asteriscus vogelii* was fractionated by CC, affording 30 fractions, 14 of which were obtained in sufficient amount to be evaluated for their phytotoxic, insecticidal and antifungal activities. Only five of those fractions showed significant toxicity against the plant species *Lemna paucicostata* and *Azolla filiculoides* (Table 1); four of these were also toxic against the alga *Scenedesmus acutus*. The investigation of the bioactive fractions led to the isolation of a new humulene derivative, the 8-oxo-6,7,9,10-tetrahydrohumulen-1,12-olide (**1**) as well as the asteriscunolides A (**2**), C (**3**) and D (**4**) and the acids 8-oxo-α-humula-6*E*,9*Z*-dien-12-oic acid (**5**), 8-oxo-α-humula-6*E*,9*Z*-dien-12-oic acid (**6**) and 8-oxo-α-humula-6*E*,9*E*-dien-12-oic acid (**7**).

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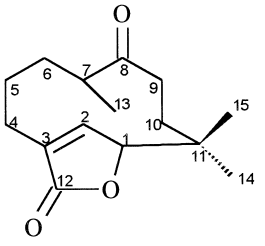
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Table 1
Phytotoxic activity of fractions obtained from *Asteriscus vogelii*[a]

Fractions[b]	Plant species		
	Senedesmus acutus	*Azolla filiculoides*	*Lemna paucicostata*
1	26	0	30
2	1	0	26
3	7	0	24
4	25	0	11
5	26	0	40
6	79	90	37
7	65	80	28
8	0	98	50
9	73	100	58
10	91	100	83
11	0	0	31
12	17	0	35
13	0	0	20
14	0	0	3

[a] Results expressed as % of mortality.
[b] Concentration of all compounds was 6 mg/ml.

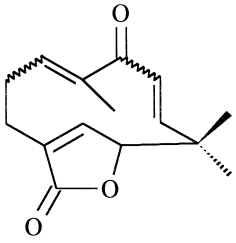


1

Compound **1**, in its HRMS, displayed the molecular ion at *m/z* 250.16138, analyzing for $C_{15}H_{22}O_3$ and its IR spectrum exhibited strong absorptions at 1749, 1702 and 1370 cm^{-1}, corresponding to the carbonyl group of the α,β-unsaturated-γ-lactone, to the carbonyl group at C-8 and to the geminal dimethyl group, respectively.

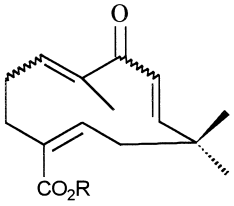
In the ^1H NMR spectrum the singlets at δ 7.08 and δ 4.55 were assigned to the vinyl proton H-2 and to H-1, respectively. The geminal methyl groups appeared each as a singlet at δ 1.21 and δ 1.11. The signal corresponding to the methyl group Me-13 was observed at δ 1.03 as a doublet, exhibiting a coupling constant of $J_{7,13} = 7$ Hz. This signal, together with the absence of those for the olefinic protons, characteristic of the Δ^6 and Δ^9 double bonds of asteriscunolides A, C and D, confirm the skeleton structure of 6,7,9,10-tetrahydrohumulen-1,12-olide for compound **1**.

The ^{13}C NMR spectrum was also in agreement with the proposed structure and the signals were assigned based on heteronuclear correlation ^{13}C-^1H (HMQC) and DEPT experiments. Signals for C-8 and C-12 appeared at δ 213.5 and δ 173.5, which are chemical shifts characteristic for carbonyl groups of a ketone and a lactone, respectively. The double bond $\Delta^{2,3}$ gave the signals at δ 147.5 and δ 134.3 corresponding to C-2 and C-3, respectively. No other olefinic signals were observed in the spectrum. On the other hand, five methylene groups were detected by the presence of the signals at δ 35.0 (C-9), δ 32.0 (C-6), δ 28.4 (C-10), δ 25.2 (C-4) and δ 23.5 (C-5). The chemical shifts at δ 25.2 and δ 35.0 were assigned to C-4 and C-9, respectively, by means of the HMQC spectrum, since these signals correlate with the corresponding protons appearing at δ 2.45–2.31 (H-9α, and H-9β and one of the H-4 protons) and at δ 2.18 (the other H-4 proton). H-7 was included in the multiplet at δ 2.45–2.31 and C-7 appeared at δ 44.9. The presence of three methyl groups was confirmed by the signals at δ 18.5 (C-13), δ 23.8 and δ 29.3 (C-15 and C-14, respectively).



2 6Z,9Z
3 6Z,9*E*
4 6*E*,9*E*

^1H NMR data of astericunolides A (**2**), C (**3**) and D (**4**) are in perfect agreement with those described in the literature (San Feliciano et al., 1984) as well as the ^{13}C NMR spectra of compounds **8** and **9** (El-Dahmy et al., 1985).



5 R = H 6Z,9*E*
6 R = H 6*E*,9Z
7 R = H 6*E*,9*E*
8 R = Me 6Z,9*E*
9 R = Me 6*E*,9Z
10 R = Me 6*E*,9*E*

The 6*E*,9*E* configuration of compound **10** was confirmed by its ^{13}C NMR spectrum since the signals at δ 148.2 for C-6, δ 25.3 for C-5, δ 160.1 for C-10 and δ 11.9 for C-13 are in the range of the chemical shifts characteristic for these carbon atoms in related compounds, possessing the *E*,*E* configuration. COSY, HMQC and DEPT experiments also confirmed these assignments and those for ^1H NMR data.

The results of phytotoxicity and cytotoxicity evaluations of compounds **1–4** and **8–10** are shown in Table 2. Asteriscunolides A, C and D (**2**, **3** and **4**) were shown to be the most potent phytotoxic agents since its activity was >90% against *Lemna paucicostata* and the plant cell cultures. Asteriscunolide A was found to be the most toxic compound for *Scenedesmus acutus* culture. The cytotoxic activity values of pure compounds are shown in Table 2. Considering the IC_{50} values obtained for compounds **2–4**, they were regarded as being significantly active since their cytotoxicity levels were in the same rank, even higher, than those of some known antitumor agents such as Cisplatinum (Table 2). Methyl esters **8–10** were, in general, considered as weak phytotoxic and cytotoxic agents. Compound **8**, however, presented significant growth inhibition of *Lemna paucicostata* and of lymphoma in mice, human lung carcinoma and human melanoma.

Although antitumor activities of similar structures have not yet been described, it seems that the cytotoxic activity found may be related to the presence of the α,β-unsaturated carbonyl moieties, which probably act as Michael acceptors, such as the α,β-unsaturated lactone units in a great diversity of natural sesquiterpene lactones. The results obtained suggest that the bioactivity of the asteriscunolides is possibly due to the presence of the double bonds at C-6 and C-9 together with the lactone moiety, since compound **1** is much less active in spite of the presence of the lactone ring.

3. Experimental

3.1. General

Mps: uncorr.; IR: Hitachi 270-50 ($CHCl_3$); ^1H NMR (ppm, *J* Hz, $CDCl_3$, TMS as an internal standard), Bruker AC 200 (200 MHz), Bruker AC 250 P (250 MHz), Bruker AMX 400 (400 MHz); ^{13}C NMR: Bruker AC 250 ($CDCl_3$, TMS as internal standard); low resolution MS: Hewlett–Packard 5930; high resolution MS: Micromass VG ZAB-2F; ultraviolet (EtOH): Perkin–Elmer 402;

CC was carried out with silica gel 230–400 mesh; prep. TLC was accomplished with silica gel plates PF_{254} (0.25 mm thick) and the compounds were recovered from the adsorbent with acetone.

3.2. Plant material

The aerial parts of *Asteriscus vogelii* were collected in the northern coast of Santiago Island, in Cabo Verde. A voucher specimen has been deposited at the Instituto de Investigação Agrária, Praia, República de Cabo Verde.

3.3. Extraction and isolation

The dried plant material (1 kg) was extracted with $CHCl_3$, at room temperature, and the extract was concentrated at 40°, under reduced pressure. The residue (37 g) was submitted to CC and eluted with stepwise gradients of EtOAc–hexane, starting with the proportion 1:8. Fractions 6 and 7 had the same major compounds, therefore they were joined together and chromatographed on a silica gel column, eluting with EtOAc–hexane (1:10). After final purification by prep. TLC, asteriscunolide A (131 mg) was isolated.

Fractions 9 and 10 also had similar major compounds, so they were subjected together to CC, eluting with Et_2O–petroleum ether (2:1), giving 19 fractions. The joined fractions 13–19 were chromatographed with Et_2O–petroleum ether (2:1) to give asteriscunolides C (104 mg) and D (70 mg). Fractions 8–12 were methylated and submitted to prep. TLC using EtOAc–hexane (1:4), affording compounds **8** (5 mg) and **9** (7 mg). Another fraction, not tested, also contained compounds

Table 2
Phytotoxic[a,b] and cytotoxic[c] activities of pure compounds

	Plant species			Neoplasic cell line[d]			
Compound	*S. acutus*	*L. paucicostata*	Cell culture	P-388	A-549	HT-29	MEL-28
1	3	50	68	40	>40	>40	>40
2	75	90	96	4	4	10	4
3	62	94	100	4	4	10	4
4	49	100	100	1	1	2	1
8	21	89	7	10	10	20	10
9	25	41	0	20	20	>40	20
10	11	61	0	40	40	>40	>40
Cisplatinum	–	–	–	8	8	16	8

[a] Results expressed as % of mortality.
[b] Concentration of all compounds was 6 mg/ml.
[c] Results are expressed as IC_{50} values (μM).
[d] Key: P388 = lymphoma in mice; A-549 = human lung carcinoma; HT-29 = human colon carcinoma; MEL-28 = human melanoma.

8 and **9**, therefore it was selected to be chromatographed in order to isolate further amounts of those compounds. This fraction was separated by CC, eluting with Et_2O–petroleum ether (3:1), giving three main fractions. After methylation, compounds **8** (4 mg), **9** (3 mg), and **10** (11 mg) were isolated by prep. TLC.

3.4. Assays for phytotoxic activity

3.4.1. Azolla filiculoides

400 mg of plant and 1.5 ml of methanol solution of the fractions (6 mg/ml) were added to 150 ml of culture medium. The results were determined after incubation in a green house, at 25°, for 15 days. Control test was carried out using culture medium and plant.

3.4.2. Lemna paucicostata

The plant and 250 μl of methanol solution of fractions or pure compounds (6 mg/ml) were added to 25 ml of culture medium. Incubation was carried out for 2 days, at 27°, under 5000 lx. Control test was performed with *Lemna paucicostata* cultivated in culture medium with methanol.

3.4.3. Scenedesmus acutus

Ten microlitres of methanol solution of the fractions or pure compounds (6 mg/ml) were added to a mixture of 1 ml of culture medium and 1 ml of cell suspension of algae (1×10^3 cells/ml); 500 μl of buffer Na_2CO_3 and $NaHCO_3$ was also added to provide CO_2. Incubation was performed for 24 h, at 23°, under 10 000 lx. Control test was carried out with algae cultivated in culture medium.

3.5. Assays for cytotoxic activity

A modification of the method described by Shroeder et al. (1981) and Bergeron et al. (1984), has been employed for cytotoxicity assays using P-388 (ATCC CCL 46), a suspension cell culture of a lymphoid neoplasm from a DBA/2 mouse, A-549 (ATCC CCL 185), monolayer culture of a human lung carcinoma, HT-29 (ATCC HTB-38), monolayer culture of a human colon carcinoma and MEL-28 (ATCC HTB-72), monolayer culture of a human melanoma.

Cells were maintained, in logarithmic phase of growth in Eagle's Minimum Essential Medium, Earle's Balanced Salt, with non-essential amino acids, with 2 mM L-glutamine, without sodium bicarbonate, (EMEM/neaa), supplemented with 5% Fetal Calf Serum (FCS), 10^{-2} M sodium bicarbonate and 0.1 g/l penicillin G + 0.1 g/l streptomycin sulfate. Cells were seeded into 16 mm diameter wells at $1–2\times10^4$ cells per well in 1 ml aliquots of EMEM 5% FCS containing different concentrations of the sample to be tested. A separate set of cultures without drug was seeded as control of growth, to ensure that cells remained in exponential phase of growth. All determinations were carried out in duplicate. After 3 days of incubation at 37°C, 10% of CO_2 in 98% humid atmosphere, cells in monolayer culture were fixed with 4% formaline and stained with 1% crystal violet. An approximately IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control (Faircloth et al., 1988). The results obtained are shown in Table 2.

3.6. 8-Oxo-6,7,9,10-tetrahydrohumulen-1,12-olide (*1*)

Crystal, mp 112–115° UV λ_{max}^{EtOH} nm (log ε): 206 (3.032), 222 (3.252), 236 (3.507), 276 (3.934); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750 (lactone), 1702 (ketone), 1370, 1326 (geminal dimethyl); ^1H NMR (250 MHz, $CDCl_3$): δ 7.08 (1 H, s, H-2), 4.55 (1 H, *s*, H-1), 2.45–2.31 (4 H, *m*, H-4α, H-7, H-9α, H-9β), 2.18 (1 H, *br t*, $J_{4\beta,9}=13$ Hz, H-4β), 1.88–1.35 (6 H, *m*, H-5α, H-5β, H-6α, H-6β, H-10α, H-10β), 1.21 (3 H, *s*, CH_3-14), 1.11 (3 H, s, CH_3-15), 1.03 (3 H, *d*, $J_{7,13}=7$ Hz, CH_3-13); ^{13}C NMR (250 MHz, $CDCl_3$): δ 213.5 (C-8), 173.5 (C-12), 147.5 (C-2), 134.3 (C-3), 87.1 (C-1), 44.9 (C-7), 38.2 (C-11), 35.0 (C-9), 32.0 (C-6), 29.4 (CH_3-15), 28.4 (C-10), 25.2 (C-4), 23.8 (CH_3-14), 23.5 (C-5), 18.5 (CH_3-13); EIMS (70 eV) *m*/*z* (rel. int.): 250 $[M]^+$ (100), 235 $[M\text{-}Me]^+$ (2), 222 (4), 207 (3), 165 (22), 152 (40), 139 (28), 123 (30), 107 (12), 81 (9), 69 (39); HRMS *m*/*z*: 250.16138 (calcd. for $C_{15}H_{22}O_3$).

3.7. Compounds *2–4* and *8–10*

Asteriscunolide A (**2**) (crystal, mp 155°, lit. 158°), asteriscunolide C (**3**) (crystal, mp 165°, 164° lit.), asteriscunolide D (**4**) (crystal, mp 148°, 145° lit.), methyl 8-oxo-α-humula-6*Z*,9*E*-dien-12-oate (**8**), methyl 8-oxo-α-humula-6*E*,9*Z*-dien-12-oate (**9**) and methyl 8-oxo-α-humula-6*E*,9*E*-diene-12-oate (**10**) were identified by comparison with reported data (San Feliciano et al., 1984; El-Dahmy et al., 1985). Compound **10** was isolated as a colourless oil; ^{13}C NMR (250 MHz, $CDCl_3$): δ 203.9 (C-8), 167.7 (C-12), 160.1 (C-10), 148.2 (C-6), 141.3 (C-2), 138.9 (C-3), 132.7 (C-7), 127.2 (C-9), 51.3 (OMe), 42.9 (C-1), 37.3 (C-11), 35.2 (C-4), 29.6 (CH_3-14 and CH_3-15), 25.3 (C-5), 11.9 (CH_3-13); EIMS (70 eV) *m*/*z* (rel. int.): 262 $[M]^+$ (16), 230 $[M\text{-}MeOH]^+$ (3), 203 $[M\text{-}Me\text{-}CO_2]^+$ (9), 150 (100), 135 (94), 107 (50), 96 (91).

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A new triterpenoid from the fern *Adiantum lunulatum* and evaluation of antibacterial activity

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Abstract

A new triterpenoid, 22,29ξ-epoxy-30-norhopane-13β-ol (**1**) was isolated together with six known compounds viz., fern-9(11)-en-6α-ol, fern-9(11)-ene, fern-9(11)-en-25-oic acid, fern-9(11)-en-28-ol, filicenol-B, adiantone and oxidation product of fern-9(11)-en-6α-ol obtained as 6-oxofern-9(11)-ene from the whole plant of *Adiantum lunulatum*, and their structures were elucidated by means of spectroscopic analysis and antibacterial evaluation of these compounds were conducted. © 2001 Published by Elsevier Science Ltd.

Keywords: Fern; *Adiantum lunulatum*; Adiantaceae; Triterpenoids; 22,29ξ-epoxy-30-norhopane-13β-ol; Antibacterial activity

1. Introduction

Adiantum lunulatum L. (Adiantaceae) is a common fern and used as an ornamental plant and widely distributed in India. The dried whole plant has been used as a medicine for bronchitis and cough (Hashimoto and Nishimoto, 1996). In the course of chemotaxonomic studies on medicinal plants of Indian System of Medicine (ISM), a detailed chemical investigation of the fern *A. lunulatum* was carried out and including the antibacterial evaluation of the isolated triterpenoids.

2. Results and discussion

The freshly collected shade dried and powdered fern *A. lunulatum* was extracted with hexane and CH_2Cl_2–MeOH (1:1). These extracts were subjected to various chromatographic techniques (see Section 4) and afforded the new compound **1** together with six known compounds, Fern-9(11)-en-6α-ol (Sunder et al., 1976) was further confirmed by PCC oxidation to yield 6-oxofern-9(11)-ene (Sunder et al., 1976), fern-9(11)-ene (Agata et al., 1994), fern-9(11)-en-25-oic acid (Shiojima and Agata, 1994), fern-9(11)-en-28-ol (Nakane et al., 1999), filicenol-B (Shiojima et al., 1993), and adiantone (Shiojima and Agata, 1994).

Compound **1** was obtained as a solid, m.p. 264–266°C, $[\alpha]_D^{25}$ +15.1° (*c* 0.25, $CHCl_3$). The IR spectrum showed the bands at 3456 and 1060 cm^{-1} for the presence of a hydroxyl group and an oxirane ring. Its HRMS showed the molecular ion at *m*/*z* 428.3634 (calcd. 428.3654) suggesting the molecular formula to be $C_{29}H_{48}O_2$ that requires six degrees of unsaturation. The EIMS showed diagnostic base peak at *m*/*z* 191 indicated that compound **1** is a hopane derivative (Shiojima et al., 1992). The ^{1}H NMR spectrum displayed signals (see Section 4) for the presence of six tertiary methyls and with conspicuous absence of isopropyl methyl doublets between δ 0.89 and 0.83 (Agata et al., 1994). Further its ^{1}H NMR spectrum showed signals for the presence of a monosubstituted oxirane ring at δ 2.72 (1H, ddd, *J* = 6.12, 6.12, 6.12 Hz), 2.36 (1H, ddd, *J* = 2.56, 4.14, 12.05 Hz), and 2.05 (1H, ddd, *J* = 2.56, 6.12,10.63 Hz) and a D_2O exchangeable hydroxy proton at δ 3.92 (1H, s). The absence of a hydroxyl bearing methine/methylene proton in its ^{1}H NMR spectrum and resistance to form acetate derivative, suggested that the hydroxyl must be a tertiary hydroxyl group. The presence of oxirane and tertiary hydroxyl group was further supported by its ^{13}C NMR spectrum signals at δ 58.78 (*d*), 57.74 (*t*), and 77.34 (*s*). Considering that compound **1** is a hopane

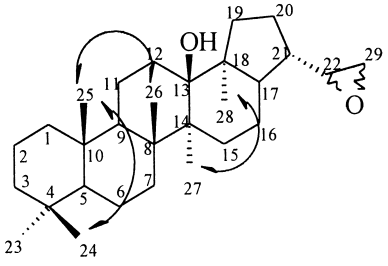
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derivative, the tertiary hydroxyl group could be placed at either C-5, or C-9, or C-13 or C-17. A careful study of ^{1}H NMR chemical shifts of tertiary methyls revealed that C-23, C-24, C-25, and C-27 methyls are closely related to 30-norhopane, (Uddin and Attar-ur-Rahman, 1994), except for the two methyls at δ 1.06 (C-26) and 1.01 (C-28), which are deshielded by approximately 0.37 and 0.15 ppm, respectively. The down field shift of C-26 could be attributed due to 1,3-diaxial interactions of the tertiary hydroxyl group present at C-13 position. The down field shift of C-28 due to the presence of oxirane ring in the same plane of C-28 methyl in the side chain of the molecule. The relative stereochemistry at most of the chiral centers of **1** was established by NOESY spectral correlation, which showed that C-25 methyl at δ 0.80 showed correlation with two methyls at δ 0.79 (C-24) and at δ 1.06 (C-26), respectively. Further the C-28 methyl at δ 1.01 showed correlation with C-27 at δ 0.94. From the foregoing spectral data the structure of compound **1** was established as 22,29ξ-epoxy-30-norhopane-13β-ol and was corroborated by its ^{1}H - ^{1}H COSY, ROESY spectral correlations (Fig. 1).

3. Antibacterial activity

The antibacterial activity was assayed by disk susceptibility tests according to the NCCLS (Wayne, 1997). Inocula were adjusted to 0.5 McFarland turbidity. Excess moisture was allowed to absorb for 10 min before applying dried disks containing the compound. The compound was dissolved in DMSO, which did not influence the growth of bacteria. Kanamycin was also performed as a positive control according to the standard method. The test plates were incubated at 37°C and zones of inhibition were recorded after 24 h. All the compounds were tested (Table 1) against gram negative bacteria *Escherichia coli* (ATCC ♯ 25922), *Pseudomonas aeruginosa* (ATCC ♯ 25619), *Salmonella typhi* (ATCC ♯ 23564) and gram positive bacteria *Bacillus sphaericus* (ATCC ♯ 14577), *Bacillus subtilis* (ATCC ♯ 6051), *Staphylococcus aureus* (ATCC ♯ 9144). Compounds fern-9(11)-ene, fern-9(11)-en-25-oic acid and adiantone were highly active against *S. typhi* and moderately active against *P. aeruginosa*, whereas compound **1** showed moderate activity against *S. typhi*. Remaining compounds did not show considerable activity against tested bacterial strains. Interestingly gram negative bacteria except *E. coli* were highly susceptible for compounds fern-9(11)-ene, fern-9(11)-en-25-oic acid and adiantone and were comparable with positive control kanamycin.



1

Fig. 1. NOESY and ROESY spectra correlations.

4. Experimental

4.1. General

Optical rotations were measured with a JASCO DIP-370 polarimeter. ^{1}H (200 and 400 MHz) and ^{13}C (50 MHz) NMR spectra were recorded using TMS as internal standard. Mass spectra were recorded on a VG Auto Spec-M instrument condition; 70 EV; (HRMS, EIMS). Medium Pressure Liquid Chromatography (MPLC) was performed using silica gel (100–200 mesh Acme's) column, Al_2O_3 (Qualigens, Neutral, grade 1) and silica gel (100–200 mesh, Acme's) was used for column chromatography (CC). Normal-phase thin-layer chromatography (TLC) was performed on silica gel G_{254} (Merck, Germany) and spots were detected by spraying with concentrated H_2SO_4 followed by heating at 100°C.

4.2. Plant material

The plant material *Adiantum lunulatum* was collected from Vijayawada, Andhra Pradesh, India and identified by Prof. M. Prabhakar Rao, Department of Botany Osmania University, Hyderabad, India.

4.3. Extraction and isolation

The shade dried and powdered of whole plant (1.25 kg.) *A. lunulatum* was extracted with hexane (3×5 l), followed by CH_2Cl_2–MeOH (1:1) (3×5 l) respectively, at room temperature. The combined hexane extract was filtered and concentrated under pressure to obtained greenish gummy residue (16.41 g). Silica gel column chromatography of hexane extract by gradient elution using hexane through hexane/benzene mixtures to benzene afforded compound fern-9(11)-en-6α-ol (120 mg), fern-9(11)-ene (950 mg) and fern-9(11)-en-25-oic acid (30 mg). Similarly the combined CH_2Cl_2:MeOH extract was filtered and concentrated under reduced pressure to obtained greenish gummy residue (39.29 g). The concentrated crude extract was subjected to MPLC using silica gel column eluting with hexane through hexane/EtOAc mixtures to

Table 1
Antibacterial activity of compounds (100 μg/disk)[a]

Compound	Gram negative			Gram positive		
	E. coli	*P. aeruginosa*	*S. typhi*	*B. sphaericus*	*B. subtilis*	*S. aureus*
1	–	–	9	–	8	–
Fern-9(11)-en-6α-ol	–	–	–	10	–	–
Fern-9(11)-ene	–	9	13	–	–	–
Fern-9(11)-en-25-oic acid	–	9	14	–	–	–
Fern-9(11)-en-28-ol	–	–	–	–	–	–
Filicenol-B	8	–	–	–	–	–
Adiantone	–	10	17	–	8	–
6-Oxofern-9(11)-ene	–	–	–	–	–	–
Kanamycin[b]	21	15	14	25	31	20

[a] Values are shown in zone of inhibition in mm/dia.
[b] Positive control kanamycin (30 μg/disk).

EtOAc afforded compound **1** (20 mg), fern-9(11)-en-28-ol (25 mg), filicenol-B (25 mg) and adiantone (18 mg).

4.4. 22,29ξ-Epoxy-30-norhopane-13β-ol (*1*)

Colourless solid (20 mg), m.p. 264–266°C, $[\alpha]_D^{25}$ +15.10° (*c* 0.25, $CHCl_3$). IR (KBr) ν_{max} 3456, 2924, 1460, 1376, 1200, 1060, 976 cm^{-1}. ^1H NMR ($CDCl_3$ 400 MHz, δ) 0.84 (3H, *s*, C-23), 0.79 (3H, *s*, C-24), 0.80 (3H, *s*, C-25), 1.06 (3H, *s*, C-26), 0.94 (3H, *s*, c-27), 1.01 (3H, *s*, C-28), 2.05 (1H, *ddd*, *J* = 2.56, 6.12,10.63 Hz), 2.36 (1H, *ddd*, *J* = 2.56, 4.14,12.05 Hz), 2.72 (1H, *ddd*, *J* = 6.12, 6.12, 6.12 Hz), 3.92 (1H, *s*, OH-13). ^{13}C NMR ($CDCl_3$ 50 MHz, δ): 40.35 (C-1), 18.67 (C-2), 42.11 (C-3), 33.39 (C-4), 56.15 (C-5), 18.27 (C-6), 33.04 (C-7), 41.86 (C-8), 50.32 (C-9), 33.39 (C-10), 20.97 (C-11), 21.71 (C-12), 77.34 (C-13), 37.36 (C-14), 33.76 (C-15), 26.73 (C-16), 49.75 (C-17), 37.10 (C-18), 42.11 (C-19), 30.06 (C-20), 42.11 (C-21), 58.87 (C-22), 33.24 (C-23), 21.57 (C-24), 14.76 (C-25), 15.92 (C-26), 14.76 (C-27), 16.50 (C-28), 57.74 (C-29). HRMS, obsd. *m/z* 428.3634 $C_{29}H_{48}O_2$ requires. *m/z* 428.3654 [M+]. EIMS *m/z* 428, [M+ (21)], 398 (10), 191 (100).

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PHYTOCHEMISTRY

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Diterpenoids from *Azorella yareta* and their trichomonicidal activities

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Abstract

Diterpenoids with trichomonicidal activity were isolated from the aerial parts of *Azorella yareta* Hauman. One was 13β-hydroxyazorellane, together with the known constituents mulinolic acid, mulin-11,13-dien-20-oic acid, azorellanol and 13α-hydroxyazorellane. Their structures were determined by spectroscopic and chemical methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Azorella yareta* Hauman; Umbelliferae; Diterpenoid; Mulinane and azorellane derivatives; Mulinolic acid; Mulin-11,13-dien-20-oic acid; Azorellanol; 13α-Hydroxyazorellane; 13β-Hydroxyazorellane; Trichomonicidal activity; *Trichomona vaginalis*

1. Introduction

It has been previously reported that *Azorella compacta* Phil., a yellow–green compact resinous cushion shrub growing in the high Andes of southern Peru and Bolivia, northeastern Chile and northwestern Argentina, contained mulinane and azorellane diterpenoids (Loyola et al., 1997a,b, 1998a,b; Watcher et al., 1998). Those results aroused our phytochemical interest in the constituents of *A. yareta* Hauman, native of northeastern Chile and northwestern Argentina. This paper describes the isolation and structural elucidation of a new diterpenoid, whose structure was established by spectroscopic analysis and on chemical grounds as 9,12-cyclomulin-13-ol or 13β-hydroxyazorellane (**1**), and the isolation and identification of 13α-hydroxyazorellane (**2**), azorellanol (**3**), mulinolic acid (**4**) and mulin-11,13-dien-20-oic acid (**5**). We have examined the anti *Trichomona vaginalis* effect of these diterpenoids using trophozoites of the Ant-1 strain. *T. vaginalis* is a parasitic protozoan that causes trichomoniasis, a sexually transmitted disease of worldwide importance. Recent data have shown that the annual incidence of trichomoniasis is more than 170 million cases worldwide (Meri et al., 2000). We have found that compounds **1**–**5** have trichomonicidal activity.

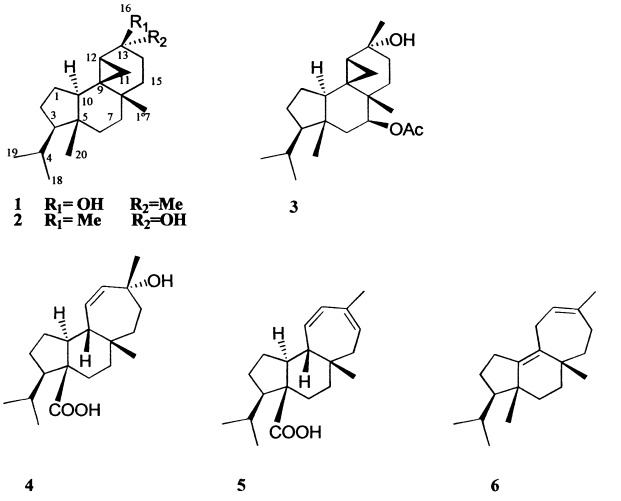
2. Results and discussion

The petrol extract of *A. yareta* Hauman was subjected to silica gel column chromatography and yielded a new diterpenoid (**1**) and the known diterpenoids, 13α-hydroxyazorellane (**2**) (Wächter et al., 1998), azorellanol (**3**) (Loyola et al., 1998a), mulinolic acid (**4**) (Loyola et al., 1996) and mulin-11,13-dien-20-oic acid (**5**) (Loyola et al., 1997a).

The molecular formula of compound **1** was determined as $C_{20}H_{34}O$ (*m*/*z* 290.2606) by HREI-mass spectrometry. The ^{1}H-decoupled ^{13}C NMR spectrum of **1** showed 20 carbon resonances; DEPT analysis, using an angle of 90°, indicated four saturated methines, at δ 59.1, 48.2, 31.5, and 26.3. The DEPT 135° spectrum showed seven methylenes and five methyl carbons indicating, after comparison with a decoupled spectrum (Table 1), that the carbons at δ 70.8, 41.7, 30.7, and 29.3 were not attached to hydrogens. The IR, ^{1}H NMR and ^{13}C NMR spectral data for **1** suggested the presence of a tertiary hydroxyl group (3.335 cm^{-1} *br*, δ 70.8 *s*).

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The absence of olefinic and carbonyl resonances in the ^{13}C NMR spectrum of compound **1** (Table 1) indicated that rings had to account for the remaining four sites of unsaturation in the molecule. The ^{1}H and ^{13}C NMR spectra of compound **1** (Table 1), together with ^{1}H COSY and HMQC and HMBC experiments, revealed the presence of an isopropyl group [δ_C 31.5 (CH), 22.6 (CH_3) and 22.5 (CH_3); δ_H 1.13 overlapped signal, 0.79 d and 0.89 d ($J=6.5$ Hz in both signals)], two tertiary methyl groups (δ_C 25.0 (CH_3), 17.8 (CH_3); δ_H 0.97 *s*, 0.81 *s*], another tertiary methyl group attached to the hydroxyl carbon [δ_C 29.5 (CH_3), 70.8 (C); δ_H 1.35 *s*] and three shielded proton resonances at δ_H 0.36 *t* ($J=5.2$ Hz), 0.62 *dd* ($J=9.2$ and 5.2 Hz) and 0.69 *dd* ($J=9.2$ and 5.2 Hz), which were assigned to the protons of a cyclopropane ring (methylene carbon at δ_C 9.9, methine carbon at δ_C 26.3 and quaternary carbon at δ_C 29.3).

All the above data could be accommodated in an azorellane carbon skeleton (Loyola et al., 1998a; Wächter et al., 1998) as depicted in formula **1**. Most of the structural fragments for compound **1** were identified by ^{1}H COSY, HMQC and HMBC spectra (Table 1).

The signal at δ 0.69 (1H, *dd*, $J=9.2$ and 5.2) was assigned to H-12, the signals at δ 0.36 (1H, *t*, $J=5.2$) and 0.62 (1H, *dd*, $J=9.2$ and 5.2) were attributed to C-11 protons, and the signal at δ 1.35 (3H, *s*) was assigned to the protons of Me-16. The long range ^{1}H–^{13}C correlations of the proton H-11 at δ 0.36 with C-8, C-9, C-10, C-12 and C-13, and the protons H-16 with C-12, C-13, and C-14 supported the notion that the three-membered ring had to be located next to the methyl and hydroxy substituted carbon C-13, as depicted in formula **1**. The signal at δ 2.21 (1H, *dd*, $J=12.7$ and 7.2) was assigned to H-10 and the signal at δ 0.97 (3H, *s*) was assigned to the protons of Me-17. The long range ^{1}H–^{13}C correlations of the proton H-10 at δ 2.21 with C-9, C11 and C-20, and the protons H-17 with C-7, C-8, C-9 and C-15 were in agreement with structure **1**. Thus, we concluded that **1** was an azorellane having a hydroxyl group in 13-position.

Table 1
^{1}H and ^{13}C NMR spectral data for compounds **1** and **2** ($CDCl_3$, 500 MHz, 125 MHz) (J in Hz)[a]

13α-Hydroxyazorellane (**2**)			13β-Hydroxyazorellane (**1**)	
Position	δ_H HMQC	δ_C	δ_H HMQC	δ_C
1	0.81[b]	20.5 *t*	0.84 *m*	20.5 *t*
	1.24[b]		1.13[b]	
2	1.12[b]	27.8 *t*	1.79[b]	27.7 *t*
	1.78[b]			
3	1.12[b]	59.1 *d*	1.13[b]	59.1 *d*
4	1.43[b]	31.5 *d*	1.43 *m*	31.5 *d*
5		42.0 *s*		41.7 *s*
6	1.82[b]	33.9 *t*	1.80[b]	33.9 *t*
	1.59 *dd*		1.56 *dd* (13.5, 5.6)	
7	1.23 *s*	37.6 *t*	1.24[b]	33.5 *t*
	1.82[b]		1.69 *dd* (13.3, 6.6)	
8		30.5 *s*		30.7 *s*
9		26.1 *s*		29.3 *s*
10	2.26 *dd*	48.3 *d*	2.21 *dd* (12.7, 7.2)	48.2 *d*
11	β 0.04 *dd* (5.4, 5.4)	10.5 *t*	0.36 *t* (5.2)	9.9 *t*
	α 0.68 *dd* (5.4, 9.8)		0.69 *dd* (9.2, 5.2)	
12	0.59 *ddd* (9.8, 5.4, 1.2)	25.3 *d*	0.62 *dd* (9.2, 5.2)	26.3 *d*
13		70.1 *s*		70.8 *s*
14	1.25[b]	31.2 *t*	1.22[b]	33.4 *t*
	1.08[b]		1.35[b]	
15	0.91[b]	35.3 *t*	1.1[b]	39.4 *t*
	1.43[b]		1.1[b]	
16	1.23 *s*	30.1 *q*	1.35 *s*	29.5 *q*
17	0.93 *s*	25.1 *q*	0.97 *s*	25.0 *q*
18	0.75 *d*	22.6 *q*	0.79 *d* (6.5)	22.6 *q*
19	0.86 *d*	22.5 *q*	0.89 *d* (6.5)	22.5 *q*
20	0.77 *s*	17.8 *q*	0.81 *s*	17.8 *q*

[a] At 125/500 MHz in $CDCl_3$. Chemical shifts are relative to TMS.
[b] Overlapped signals; the chemical shift of these protons was measured using HMQC spectra.

The ^{1}H NMR and ^{13}C NMR data for **1** are almost similar to that of **2** (Wächter et al., 1998) (Table 1). In fact, the main differences between the ^{1}H NMR spectra of **1** and **2** were in the chemical shifts corresponding to the CH_3-16, H-11β, H-14 and H-15. Moreover, a comparison between the ^{13}C NMR spectra of **1** and **2** (Table 1) showed a significant difference in the C-9 ($\Delta\delta+3.2$), C-11 ($\Delta\delta-0.6$), C-12 ($\Delta\delta+1.0$), C-13 ($\Delta\delta+0.7$), C-14 ($\Delta\delta+2.2$), C-15 ($\Delta\delta+4.1$) and C-16 ($\Delta\delta-0.6$) chemical shifts. Furthermore, the optical rotations of the two compounds are different. Until this moment, all known compounds with an azorellane skeleton showed a very similar chemical shift of the cyclopropane protons (Loyola et al., 1998a; Wächter et al., 1998). This supported the fact that the stereochemistry of C-13 is the same in all cases. In contrast, the signal of H-11β in compound **1** showed a significant downfield shift. In compound **1**, the deshielding shown by carbons C-9 and C-15, with respect to compound **2**, is in agreement with *γ–trans* arrangement between those carbons and C-13 hydroxyl group (Eggert et al., 1976) These data could be attributed to an opposite stereochemistry at C-13 of **1** in

comparison with **2**, in which the hydroxyl group at C-13 was α-orientated (Wächter et al., 1998).

The formation of the dehydration product **6** (see experimental) (Wächter et al., 1998), which was obtained by treatment of **1** and/or **2** with *p*-toluensulfonic acid, supported the structure proposed for **1** as a 13β-hydroxyazorellane.

From a parasitological point of view, different studies have shown that at least 5% of clinical isolates of *T. vaginalis* are resistant to metronidazole chemotherapy (Narcisi and Secor, 1996). Clearly, new antitrichomonal agents are needed to treat resistant organisms (Petrin et al., 1998). A potential source of new antitrichomonal compounds could be plants. However, this possibility has not been explored systematically and only a single paper has shown the activities of water soluble extracts obtained from *Aloe barbadensis* (Rojas et al., 1995).

Under the conditions used, the LD_{50} range of compounds tested in this paper was 40–120 μM, which is nearly ten times higher than the metronidazole LD_{50} (6.6 μM). Among the azorellane diterpenoids tested, compounds **1** and **2** had the lowest trichomonicidal activity, with LD_{50} values of 100 and 119 μM, respectively. This lower activity can be attributed to the absence of an acetic acid moiety esterified at C-7. Azorellanol **3** has an acetate group on C-7 and displayed a LD_{50} of 40.5 μM. On the other hand, diterpenoids such as mulinane type **4** and **5** displayed a better activity, but mulinolic acid **4** was more active than its dehydratation product **5**. The enhanced activity of mulinane **4** could be due to the presence of a OH group on its C-13.

Even though, the trichomonicidal activity of the isolated compounds from *A. yareta* did not reach our expectations, it is important to keep the search of new compounds and to assess their cytotoxic effects in comparison to metronidazole and to consider their trichomonicidal activity againts metroimidazol-resistant *T. vaginalis* strains.

3. Experimental

3.1. General

Mp. uncorr. Plant material, collected in "Quebrada de las Llaretas", Vallenar, Chile, in January 1999, was identified by Professor C. Marticorena and voucher specimens were deposited in the Herbarium of Universidad de Concepción, Concepción, Chile.

3.2. Extraction and isolation of diterpenoids

Dried and finely powdered whole plant of *A. yareta* Hauman (3.0 kg) was extracted with petrol at room temp. After filtration, the solvent was evapd. under red. pres. and low temp. yielding a gum (220 g). The concd. petrol ext. was adsorbed on silica gel (300 g) and slurried onto the top of a column containing silica gel (2.0 kg) in petrol and eluted with petrol–EtOAc gradient with increasing amounts of EtOAc to produce six fractions. Fraction 2 (100 g) eluted with PE/EtOAc (18:2) was further separated and purified by silica gel column chromatography (PE/EtOAc, 19:1) to yield: mulin-11,13-dien-20-oic acid (**5**) (1.5 g), 13α-hydroxyazorellane (**2**) (1.6 g), mulinolic acid (**4**) (1.2 g) and 13β-hydroxy azorellane (**1**) (980 mg).

Fraction 4 eluted with petrol–EtOAc (14:6) (60 g) was further separated and purified by silica gel column chromatography (EP/EtOAc, 15:5) to yield azorellanol (**3**) (9.3 g).

13β-Hydroxyazorellane (**1**): Needles, mp 95–96° (petrol–EtOAc); $[\alpha]_{24}^{D} = +17.2$ ($CHCl_3$; *c* 0.23). IR ν_{max}^{KBr} cm^{-1}: 3335 (OH). ^1H NMR and ^{13}C NMR (see Table 1). HREI–MS: found 290.2606, (calc. 290.2610 for $C_{20}H_{34}O$).

3.3. Dehydration of 13α-hydroxyazorellane (*2*) and 13β-hydroxyazorellane (*1*)

Compound **1** (35 mg) was dissolved in C_6H_6 (5 ml) and treated with *p*-TsOH for 1 h at room temp. The reaction mixt. was evaporated in vacuo and partitioned between EtOAc and H_2O. The EtOAc layer was concd. to give a residue, which was further purified by Sephadex LH-20 chromatography (MeOH) to afford (**6**) (23 mg). Dehydration of compound **2** (32 mg) using the same conditions furnished a compound identical to **6** (21 mg) (R_f, ^1H NMR).

3.4. Assay of trichomonicidal activity

T. vaginalis trophozoites (Ant-1 strain) were cultured on Diamond's medium supplemented with 10% horse serum. Trichomonicidal activities of the different natural products were evaluated according to González et al. (1991) with slight modifications. Parasite suspension (5×10^5 organisms per ml) was prepared and 100 μl was placed in each well of a 96-well microplate and then mixed with two-fold dilutions of each compound in a range from 0–500 μM. Metronidazole (Sigma Chemical Co.) was used as control. The assays were incubated for 18 h at 37°C, in a 5% CO_2 atmosphere. Then, aliquots of each assay mixture were evaluated under a light microscope and the percentage of live parasites was recorded. Experiments were performed in triplicate and represent the average of at least two independent experiments performed under identical conditions using the same protocols.

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Further glucosides of lichens' acids from Central Asian lichens

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Abstract

Eight compounds isolated from an extract of Central Asian lichens comprised new glucosides having murolic, protoconstipatic and allo-murolic acids as the aglycones and a saccharide moiety linked at C-18 made up of four glucoses. The structures were elucidated using extensive spectroscopic analysis (1D and 2D NMR, MS, IR, UV and CD) and by chemical methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Glucosides; Lichens' acids; Lichens; Central Asia

1. Introduction

Lichens are symbiotic associations comprising fungal (mycobiont) and algal or cyanobacterial (photobiont) partners. They occur in a great variety of different habitats, and can survive severe environmental conditions such as drought, low temperatures, continuous light, or prolonged darkness. It has been suggested that in response to these stresses, natural selection has favoured species producing high concentrations of secondary metabolites (Ravinskaja, 1984).

Since ancient times some lichens have been used medicinally (Ichonose et al., 1994). Chemical and pharmacological investigations have shown that lichen secondary metabolites exhibit interesting biological activities and can be useful as antibiotics, UV absorbents, and antioxidants (Yamamoto et al., 1993).

As a continuation of our search for unusual bioactive compounds from Central Asian lichens (Řezanka and Dembitsky, 1998, 1999; Řezanka and Guschina, 1999, 2000), we examined the following nine lichen species: *Acarospora gobiensis* H. Magn.; *Cladonia furcata* (Huds.) Schrad., *Lecanora fructulosa* (Dicks.) Ach.; *Leptogium saturninum* (Dicks.) Nyl.; *Peltigera canina* (L.) Willd.; *Rhizoplaca peltata* Ram.; *Parmelia camtschadalis* Ach., *Parmelia tinctina* Mah. et Gill. and *Xanthoria elegans* (Link.) Th. Fr. These lichens were collected in the foothills of the Kungey-Alatau mountain range, Tien Shan Mountains, Issyk-Kul region (Republic of Kirghizstan). Prolonged periods of drought, high temperatures, and high levels of UV radiation characterize this region.

Although thousands of plant glycosides have been discovered in nature (Ikan, 1999), a much smaller number have been identified in lichens e.g. 1-(*O*-α-D-glucopyranosyl)-3 *S*,25*R*-hexacosanediol from *Solorina crocea* (Huneck and Yoshimura, 1996), and galapagin, lobodirin, mollin and roccellin (Huneck et al., 1992). The present study reports the structural elucidation of eight new glycosides (**1–8**), having murolic, protoconstipatic and allo-murolic acids as the aglycones and an oligosaccharide moiety linked at C-18 made up of two or three or four glucoses.

2. Results and discussion

In a recent paper (Řezanka and Guschina, 2000), we reported the isolation of several organic acids in lichens, murolic, protoconstipatic, allo-murolic acids and (18*R*)-18-*O*-β-D-glucopyranoside of murolic acid, and eight new glycosides from the water–methanolic extract of the Central Asian lichens collected in July 1996 along the shores of lake Issyk Kul, Tian-Shan mountain. Further fractionation of this extract by Sephadex LH-20 chromatography, followed by reversed-phase preparative HPLC was used to separate the new glucosides (**1–8**), together with the above four known compounds (Table 1). The structures of compounds were determined

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Table 1
Occurrence of lichens' acids and their glucosides from lichens of Tian Shan Mountains

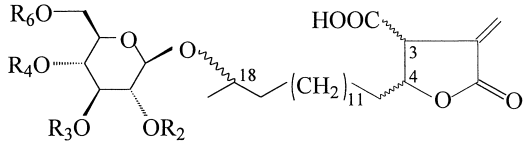
Name of lichen	Name of compounds; mg/100 g of dry weight												
	A	**B**	**C**	**D**	**E**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Acarospora gobiensis	22.5	–[a]	–	8.7	–	–	–	–	–	–	–	–	3.2
Cladonia furcata	–	24.3	–	–	11.4	0.5	–	–	2.9	0.8	–	7.8	–
Lecanora fructulosa	–	13.5	–	–	–	3.8	–	–	3.4	1.9	–	1.5	–
Leptogium saturninum	–	–	19.8	–	–	–	6.8	7.1	–	–	3.9	–	–
Parmelia camtschadalis	–	–	25.4	–	–	–	0.9	0.9	–	–	1.4	–	–
P. tinctina	–	19.1	–	–	12.0	4.2	–	–	6.3	1.4	–	4.3	–
Peltigera canina	–	22.7	–	–	–	0.2	–	–	5.7	3.1	–	0.7	–
Rhizoplaca peltata	13.8	–	–	1.8	–	–	–	–	–	–	–	–	6.1
Xanthoria elegans	–	–	5.4	–	–	–	2.9	6.3	–	–	4.1	–	–

[a] Less than 0.1 mg/100 g of dry wt.
A = 18-hydroxy-murolic acid, B = 18-hydroxy-protoconstipatic acid, C = 18-hydroxy-allo-murolic acid, D = (18*R*)-18-*O*-*β*-D-glucopyranoside of murolic acid, E = (18*S*)-18-*O*-*β*-D-glucopyranoside of protoconstipatic acid.

unambiguously using both 1D and 2D ^{1}H and ^{13}C NMR experiments in conjunction with the analysis of mass spectral and other spectroscopic data (UV, CD and IR).

Compound **1** was obtained after HPLC as a white amorphous powder with $[\alpha]_D^{23} -14^{\circ}$ but without an exact melting point as the glycoside decomposed. The molecular weight determined by positive HRFABMS (high resolution fast atom bombardment mass spectrometry) was 693.8068 $(M+H)^+$, which corresponds to a molecular formula of $C_{33}H_{56}O_{15}$, and negative FABMS gave two ions at *m/z* 529 $(M\text{-}H\text{-}162)^-$ and *m/z* 367 $(M\text{-}H\text{-}2\times162)^-$. The exact disposition of the two-monosaccharide units and the position the interglycosidic linkage in compound **1** was determined using 2D NMR spectroscopy. HOHAHA (homonuclear Hartman–Hahn) experiments allowed resolution of the overlapped spectral region of the disaccharide moiety of **1** into a subset of individual monosaccharide spectra; 2D-COSY (correlated spectroscopy) experiments established the proton sequence with each sugar fragment starting from the well-resolved anomeric proton signals of a *β*-D-glucopyranoses (*δ* 4.71, *d*, *J* = 7.0 Hz and *δ* 5.45, *d*, *J* = 7.9 Hz) (Table 3). Chemical shifts, multiplicity of the proton signals, values of coupling constants, and chemical shifts of carbons indicated that the sugars must be in the *β*-D-glucopyranosyl forms (Tables 2 and 3) (Kasai et al., 1977).

In compound **1** a glycosidation shift (Tori et al., 1977) at C-2′ (ca +8.5 ppm) and the chemical shifts of H-1′ (*d* 4.71)



1 3S, 4S, 18R, R_2 = Glc, $R_3 = R_4 = R_6$ = H
2 3S, 4R, 18R, R_3 = Glc, $R_2 = R_4 = R_6$ = H
3 3R, 4S, 18S, R_6 = Glc, $R_2 = R_3 = R_4$ = H
4 3S, 4S, 18R, $R_2 = R_3$ = Glc, $R_4 = R_6$ = H
5 3S, 4S, 18R, $R_2 = R_6$ = Glc, $R_3 = R_4$ = H
6 3R, 4S, 18S, R_2 = Glc′′2-1Glc′′′, $R_3 = R_4 = R_6$ = H
7 3S, 4R, 18R, $R_3 = R_6$ = Glc, $R_2 = R_4$ = H
8 3R, 4S, 18S, R_2 = Glc′′6-1Glc′′′′, R_3 = Glc, $R_4 = R_6$ = H

Fig. 1. The structures of glucosides (**1–8**) from lichens.

Table 2
^{13}C NMR spectra of glucosides (**1–8**)

No. of atom	Compound (*δ* relative to TMS)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1′	101.4	103.8	104.6	104.4	104.3	104.8	106.3	105.2
2′	**82.0**	73.5	73.7	**81.1**	**82.3**	**82.4**	73.4	**81.8**
3′	77.9	**87.6**	76.7	**89.0**	77.4	77.2	**88.2**	**87.8**
4′	71.1	70.1	70.5	69.9	71.2	72.3	69.3	71.3
5′	77.4	78.2	75.4	78.0	77.2	77.4	76.7	75.5
6′	63.1	62.3	**68.5**	62.1	**68.3**	62.4	**68.9**	62.2
1″	106.8	104.9	103.4	104.0	103.5	103.5	105.6	104.4
2″	76.0	75.2	73.1	76.1	76.1	**85.9**	75.8	75.3
3″	78.5	78.4	77.5	78.9	78.5	77.9	78.0	77.8
4″	71.9	70.5	69.2	71.2	72.8	71.7	71.4	71.6
5″	76.9	78.0	77.0	77.7	78.3	79.4	78.8	77.3
6″	62.6	62.6	61.1	62.5	63.7	62.6	62.4	**70.3**
1‴				105.2	105.0	106.9	103.2	104.8
2‴				75.3	75.5	77.0	74.9	75.9
3‴				78.7	78.9	77.7	77.8	77.6
4‴				71.5	71.8	69.5	71.0	71.2
5‴				78.3	78.0	78.3	78.3	78.5
6‴				61.8	63.0	61.1	62.1	62.4
1⁗								104.1
2⁗								76.9
3⁗								78.1
4⁗								70.9
5⁗								78.0
6⁗								62.7

Table 3
^{1}H NMR spectra of glucosides (**1–3**)

No. of atom	Compound (δ; relative to TMS)		
	1	**2**	**3**
1′	5.07 (1H, *d*, $J=7.0$)	5.01 (1H, *d*, $J=7.0$)	5.12 (1H, *d*, $J=7.5$)
2′	3.98 (1H, *dd*, $J=7.0$; 9.0)	4.12 (1H, *dd*, $J=7.0$, 9.0)	4.24 (1H, *dd*, $J=7.8$, 8.8)
3′	3.94 (1H, *t*, $J=9.0$)	4.18 (1H, *dd*, $J=9.0$, 9.0)	4.32 (1H, *t*, $J=8.0$)
4′	3.64 (1H, *dd*, $J=9.3$; 9.3)	4.23 (1H, *dd*, $J=9.0$, 9.0)	4.30 (1H, *t*, $J=7.4$)
5′	3.68 (1H, *m*)	4.03 (1H, *m*)	4.15 (1H, *m*)
6′	4.09 (1H, *dd*, $J=12.0$, 5.0)	4.21 (1H, *dd*, $J=12.1$, 2.5)	4.38 (1H, *dd*, $J=11.1$, 7.0)
	4.38 (1H, *dd*, $J=12.0$, 1.4)	4.41 (1H, *dd*, $J=12.1$, 5.7)	4.72 (1H, *dd*, $J=11.1$, 3.0)
1″	5.45 (1H, *d*, $J=7.9$)	5.25 (1H, *d*, $J=7.5$)	5.16 (1H, *d*, $J=6.9$)
2″	4.01 (1H, *dd*, $J=8.7$, 7.9)	4.05 (1H, *dd*, $J=7.5$, 7.5)	3.95 (1H, *dd*, $J=8.2$, 7.4)
3″	4.15 (1H, *dd*, $J=8.7$, 8.7)	4.14 (1H, *dd*, $J=7.5$, 8.5)	4.07 (1H, *dd*, $J=7.4$, 9.0)
4″	4.11 (1H, *dd*, $J=8.8$, 8.7)	4.10 (1H, *dd*, $J=8.5$, 8.0)	4.01 (1H, *t*, $J=9.0$, 8.0)
5″	4.03 (1H, *m*)	4.00 (1H, *ddd*, $J=8.0$, 4.8, 2.1)	3.82 (1H, *dq*, $J=9.4$, 6.2)
6″	4.23 (1H, *dd*, $J=10.8$, 5.5)	4.25 (1H, *dd*, $J=12.3$, 4.8)	4.17 (1H, *dd*, $J=11.2$, 5.8)
	4.35 (1H, *dd*, $J=10.8$, 1.7)	4.50 (1H, *dd*, $J=12.3$, 2.1)	4.26 (1H, *dd*, $J=11.2$, 1.2)

and C-1′ (δ 101.4) of glucose indicated this monosaccharide was glycosidated at C-2 and linked at the aglycon. The one signal due to the anomeric proton of glucose (δ 5.45, *d*, $J=7.9$ Hz), correlating to the C-1″ resonance at δ 106.8 by HETCOR indicated that the glucose 2 unit was linked to a secondary alcoholic carbon (C-2′ of the glucose 1). By HOHAHA, this proton showed connectivities with a signal δ 4.01 (*dd*, $J=7.9$, 8.7 Hz, H-2″ by COSY), which was correlated by HETCOR (heteronuclear correlation) to a carbon resonance at δ 82.0 (C-2′) showing glycosylation at position 2′. The glucose 2 was determined to be terminal by the absence of a glycosylation shift. These deductions were confirmed by a COLOC (correlation via long range couplings) spectrum, which showed some diagnostic long-range correlations between H-1′ of glucose (δ 4.71) and C-18 (δ 61.0) of the aglycone (Rieser et al., 1992; Seo et al., 1978), between H-1″ (δ 5.45) of the glucose 2 unit and C-2′ (δ 82.0) of glucose 1, (see Tables 2 and 3). The structure of glucoside **1** was determined as the (18*R*)-18-*O*-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside of allo-murolic acid.

Acid methanolysis of **2** gave only glucose. Positive HRFABMS of **2** give a pseudomolecular ion at *m*/*z* 693.8064 $(M+H)^+$, ($C_{33}H_{56}O_{15}$) and showed the negative FABMS $(M-H)^-$ ion at *m*/*z* 691 with prominent fragments at *m*/*z* 529 $(M-H-162)^-$ and 513 $(M-H-178)^-$ (cleavage of a hexose unit with or without the glycosidic oxygen) and at *m*/*z* 367 $(M-H-162-162)^-$ due to the subsequent loss of two hexose units. The DEPT (distortionless enhancement by polarization transfer) ^{13}C NMR spectrum showed 33 signals, of which 12 were assigned to the saccharide portion and 21 to the aglycone moiety. The oligosaccharide structure was determined by 2D NMR. Even at high field (500 MHz) the 1D sugar spectral region of **2** was complex as most of the shifts were found between δ 4.00 and 5.25 and were overlapped by the aglycone signals. 1D and 2D TOCSY (total correlated spectroscopy) spectroscopy experiments allowed resolution of the overlapped spectrum of oligosaccharide into a subset of individual monosaccharide spectra. In the 1D and 2D TOCSY spectrum of **2** the anomeric proton signal ascribable to a β-D-glucopyranose (H-1′, δ 5.01, *d*, $J=7.0$ Hz) showed connectivities to four methine groups. This together with the 2D DQF-COSY (double quantum coherence-correlated spectroscopy) spectrum established the proton sequence within this sugar fragment as H-1′ (δ 5.01), H-2′ (δ 4.12), H-3′ (δ 4.18), H-4′ (δ 4.23) and H-5′ (δ 4.03), H-6a′ (δ 4.21), H-6b′ (δ 4.41) (Table 3).

Similar observations of the TOCSY and COSY experiments for second sugar residue (Tables 2 and 3) allowed complete sequential assignments for all proton resonances starting from the anomeric proton signal. HSQC experiments which correlated all proton resonances with those of each corresponding carbon (Tables 2 and 3) permitted assignments of the interglycosidic linkages by comparison of the ^{13}C shifts with those of the corresponding pyranosides, taking into account the known effects of glycosidation. The absence of any ^{13}C glycosidation shift for second glucopyranosyl residue suggested that this sugar was a terminal unit, while the glycosidation shift on C-3′ ($\delta+9.7$ ppm) of the glucopyranosyl unit linked at C-18 by a glycosidic bond allowed us to establish the presence of two glucopyranosyl residues at C-18. The position of each sugar unit was deduced from an HMBC experiment. The ^{1}H NMR and ^{13}C NMR data indicated the β configuration at the monomeric positions for glucopyranosyl units (Table 3). Therefore the structure (18*R*)-*O*-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of murolic acid was assigned to **2**.

Compound **3**, obtained as an amorphous white powder, presents in its positive HRFABMS spectrum a pseudomolecular peak $(M+H)^+$ at *m*/*z* 693.8066, corresponding to the molecular formula $C_{33}H_{56}O_{15}$. In the

negative FABMS spectrum there is, besides the molecular peak $(M\text{-}H)^-$ at m/z 691, a peak due to the loss of the glucose $(M\text{-}H\text{-}162)^-$ at m/z 529 and of gentiobiose $(M\text{-}H\text{-}324)^-$ at m/z 367. Its ^{13}C NMR spectrum shows 33 resonances, sorted by DEPT experiments in to 1 CH_3, 14 CH_2, 3 CH, and 3 quaternary C. The remaining 12 resonances of the ^{13}C NMR spectrum of **3** are generated by two hexoses, and indicate exactly two β-D-glucopyranoses 1–6 linked to form the disaccharide gentiobiose, as demonstrated by the downfield shift of the inner sugar methylene (C-6′). The ^1H NMR spectrum pattern of the aglycone moiety is very similar to that of protoconstipatic acid. Similar to (18 *S*)-18-*O*-β-D-glucopyranoside of protoconstipatic acid (Řezanka and Guschina, 2000), the aglycone was determined as protoconstipatic acid, and the sugar moiety as gentiobiose with the site of glycosylation at the 18-OH. So compound **3** can be identified as the (18 *S*)-18-*O*-β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside of protoconstipatic acid.

Acid hydrolysis of **4** afforded only D-glucose, confirmed by specific rotation $[\alpha]_D^{23}-52.7$. The white powder of glucoside **4** gave positive HRFABMS m/z 855.9503 $(M+H)^+$, corresponding to formula $C_{39}H_{66}O_{20}$; in negative FABMS ions at m/z 691 $(M\text{-}H\text{-}162)^-$, m/z 529 $(M\text{-}H\text{-}2\times162)^-$ and 367 $(M\text{-}H\text{-}3\times162)^-$ occurred. In the ^1H NMR spectrum of **4**, three anomeric proton signals appeared at δ 5.64, 5.60, and 5.34, indicating that each a glucose has a β-configuration. The corresponding three-anomeric carbons were observed at δ 104.4, 104.0, and 105.2. Also, the downfield shifted ^{13}C NMR resonances among the sugar units were observed at δ 81.1 and 89.0, indicating the probable points of glycosidic linkage in the oligosaccharide to be at C-2′ and C-3′. ^1H-^1H COSY and HMQC experiments revealed the glycosidic attachments at C-2′ (δ 81.1) and C-3′ (δ 89.0) for glucose (glucose 1). Further, the HMBC spectrum showed connectivities between the H-1′ proton of glucose 1 and C-18 of the aglycone, the H-1″ proton of glucose 2 and C-2′ of glucose 1, and the H-1‴ proton of glucose 3 and C-3′ of glucose 1. Thus, **4** was formulated as (18*R*)-18-*O*-β-D-glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-2)]-β-D-glucopyranoside of allo-murolic acid.

FABMS in the negative-ion mode were run for compound **5**, which gave $(M\text{-}H)^-$ ions at m/z 853, and in positive HRFABMS the corresponding ion at 855.9509 $(M+H)^+$ gave the molecular formula $C_{39}H_{66}O_{20}$. The ^1H NMR spectrum of **5** was typical for a glycoside, with the sugar region showing three anomeric proton doublets, with coupling constants characteristic of β-anomers ($J_{1,2}$ ca 8 Hz). The glycosidic protons were resolved well enough to allow most of the coupling constants to be measured. Assignment of the different resonances to each one of the three residues was achieved through COSY and TOCSY experiments. The large coupling constants found for protons H-2, H-3, and H-4 of the spin-systems of each residue were consistent with glucose moieties (see Table 4). The ROESY (rotating frame nuclear Overhauser effect spectroscopy) spectrum of **5**, in addition to the expected intraresidue signals, gave the cross peaks H-1″−H-2′, H-1‴−H-6a′, and H-1‴−H-6b′, suggesting that the glucose-2 and glucose-3 units are linked to glucose-1 at C-2′ and C-6′, respectively.

The ^{13}C NMR spectrum of **5** gave 39 carbons and supported the deductions derived from the interpretation of the ^1H NMR spectrum. Compound **5** showed one carbonyl peak, i.e. δ 173.8 (carboxylic). The rest of the ^{13}C NMR chemical shifts were assigned from the HMQC spectrum (see Table 2). The values for the aglycone moiety agreed with those published for similar aglycones (Řezanka and Guschina, 2000), and the value of the anomeric carbon for glucose-1 δ 104.3 demonstrated that it was linked as an acetal to the C-18 hydroxyl group of the aglycon. In addition, C-2′ and C-6′ of the same unit gave values shifted downfield from those expected for unsubstituted glucopyranose, which indicated that glucose-1 was glycosylated at these positions by the other two glucose units (glucose-2 and glucose-3), thus supporting the ROESY results. Unequivocal demonstration of the type of glycosidic linkages was obtained from a HMBC experiment, which, in addition to expected intraresidue peaks for all different moieties (the aglycon and the three sugar residues), gave the cross peaks H-1″−C-2′, H-1‴−C-6′ and H-18−H-1′. Accordingly, glycoside **5** was assigned as the (18*R*)-18-*O*-β-D-glucopyranosyl-(1-2)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of allo-murolic acid.

Compound **6** was obtained as an amorphous powder. Acid hydrolysis of **6** afforded only protoconstipatic acid as the aglycon and D-glucose as the sugar components. The positive FAB mass spectrum of **6** showed a protonated molecular ion at m/z 855.9503 $(M+H)^+$ as a base peak (corresponding to formula $C_{39}H_{66}O_{20}$) together with a fragment ion at m/z 529 $(M\text{-}H\text{-}2(\text{hexose})^-$ (negative FABMS). The ^{13}C and DEPT NMR spectra of **6** showed 39 signals, of which 18 were assigned to the saccharide portion and 21 to the aglycon moiety. The ^1H NMR spectrum showed three anomeric proton signals at δ 5.05 (1 H, *d*, $J=7.7$ Hz), δ 5.58 (1 H, *d*, $J=8.4$ Hz), and δ 5.28 (1H, *d*, $J=8.2$ Hz). The ^{13}C signals of **6** were superimposable onto those of glycosides **4** and **5**, except for the signals due to the sugar moiety. In the ^{13}C NMR spectrum the presence of three anomeric signals at δ 104.8, 103.5, and 106.9 confirmed the three sugar residues. Assignments of the sugar proton resonances were achieved by a ^1H-^1H COSY spectrum, and the ^{13}C data were assigned by a HMQC (heteronuclear multiple quantum coherence) spectrum. Interglycosidic linkages were established by HMBC techniques. The HMBC spectrum of **6** showed cross peaks between the signals at δ 5.05 (glucose H-1′) and δ 61.0 (C-18 of the

Table 4
^{1}H NMR spectra of glucosides (**4–7**)

	Compound (δ relative to TMS)			
No. of atom	**4**	**5**	**6**	**7**
1′	5.64 (1H, *d*, J=8.0)	5.61 (1H, *d*, J=8.2)	5.05 (1H, *d*, J=7.7)	5.18 1H, *d*, J=7.9)
2′	4.56 (1H, *t*, J=8.0)	4.06 (1H, *dd*, J=8.2; 9.2)	4.31 (1H, *dd*, J=7.7, 8.7)	4.30 (1H, *dd*, J=7.9, 8.9)
3′	4.36 (1H, *dd*, J=9.0, 9.0)	3.97 (1H, *dd*, J=9.2, 9.7)	4.70 (1H, *dd*, J=9.1, 8.7)	4.25 (1H, *dd*, J=9.0, 8.9)
4′	4.16 (1H, *dd*, J=9.0, 9.0)	3.84 (1H, *dd*, J=9.7; 9.2)	4.01 (1H, *dd*, J=9.1, 10.0)	4.33 (1H, *dd*, J=9.0, 9.4)
5′	4.05 (1H, *ddd*, J=9.0, 5.0, 2.0)	3.74 (1H, *ddd*, J=9.2, 4.9, 2.0)	3.77 (1H, *td*, J=10.0, 8.1)	4.10 (1H, *ddd*, J=9.4, 4.5, 1.8)
6′	4.29 (1H, *dd*, J=12.0, 2.0)	4.17 (1H, *dd*, J=11.6, 4.9)	4.08 (1H, *dd*, J=11.3, 6.9)	4.28 (1H, *dd*, J=9.4, 4.5)
	4.58 (1H, *dd*, J=12.0, 5.0)	4.27 (1H, *dd*, J=11.6, 2.2)	4.37 (1H, *dd*, J=11.3, 4.1)	4.51 (1H, *dd*, J=9.4, 4.5)
1″	5.60 (1H, *d*, J=8.0)	5.48 (1H, *d*, J=7.7)	5.58 (1H, *d*, J=8.4)	5.35 (1H, *d*, J=8.2)
2″	4.10 (1H, *dd*, J=8.5, 8.0)	3.92 (1H, *dd*, J=7.7, 8.7)	4.76 (1H, *dd*, J=8.4, 8.7)	4.08 (1H, *dd*, 8.2, 8.7)
3″	4.27 (1H, *dd*, J=8.5, 8.5)	4.10 (1H, *dd*, J=8.7, 8.8)	3.94 (1H, *dd*, J=8.7, 8.8)	4.17 (1H, *dd*, J=8.7, 8.6)
4″	4.31 (1H, *dd*, J=9.5, 8.5)	4.19 (1H, *dd*, J=8.8, 10.8)	4.39 (1H, *dd*, J=8.8, 9.3)	4.12 (1H, *dd*, J=8.6, 8.3)
5″	3.85 (1H, *ddd*, J=9.0, 4.0, 2.5)	4.08 (1H, *ddd*, J=1.6, 7.0, 10.8)	3.97 (1H, *m*)	3.98 (1H, *ddd*, J=8.3, 4.1, 1.9)
6″	3.99 (1H, *dd*, J=12.5, 2.5)	4.12 (1H, *dd*, J=11.8, 7.0)	4.35 (1H, *dd*, J=9.0)	4.20 (1H, *dd*, J=8.3, 4.1)
	4.21 (1H, *dd*, J=12.5, 4.0)	4.32 (1H, *dd*, J=11.8, 1.6)	4.67 (1H, *dd*, J=9.0)	4.38 (1H, *dd*, J= 8.3, 1.9)
1‴	5.34 (1H, *d*, J=8.0)	5.30 (1H, *d*, J=7.6)	5.28 (1H, *d*, J=8.2)	5.12 (1H, *d*, J=7.7)
2‴	4.08 (1H, *dd*, J=8.0, 8.0)	4.03 (1H, *dd*, J=7.6, 9.1)	4.05 (1H, *dd*, J=8.2, 8.0)	4.05 (1H, *dd*, J=7.7, 8.4)
3‴	4.25 (1H, *dd*, J=8.0, 8.0)	4.24 (1H, *dd*, J=9.1, 9.2)	4.22 (1H, *dd*, J=8.0, 8.1)	4.23 (1H, *dd*, J=8.4, 8.3)
4‴	4.13 (1H, *dd*, J=8.1, 8.0)	4.15 (1H, *dd*, J=9.2, 9.4)	4.10 (1H, *dd*, J=8.1, 9.5)	4.14 (1H, *dd*, J=8.3, 10.2)
5‴	3.95 (1H, *ddd*, J=8.1, 5.2, 2.3)	4.00 (1H, *ddd*, J=9.4, 4.9, 2.0)	3.74 (1H, *ddd*, J=9.5, 5.7, 2.4)	3.92 (1H, *ddd*, J=10.2, 4.2, 2.1)
6‴	4.19 (1H, *dd*, J=12.0, 5.0)	4.29 (1H, *dd*, J=2.2, 4.9)	3.74 (1H, *dd*, J=10.7, 5.7)	4.01 (1H, *dd*, J=10.2, 2.1)
	4.41 (1H, *dd*, J=12.0, 2.5)	4.48 (1H, *dd*, J=12.2, 4.0)	4.28 (1H, *dd*, J=10.7, 2.4)	4.35 (1H, *dd*, J=10.2, 4.2)

aglycon), 5.58 (inner glucose H-1″) and 82.4 (glucose C-2′), and 5.28 (terminal glucose H-1‴) and 85.9 (inner glucose C-2″). The anomeric configurations of the glucoses were determined as β based on the large coupling constants ($J_{1,2}$~8 Hz) in the ^{1}H NMR spectrum. Furthermore, acid hydrolysis of **6** gave protoconstipatic acid and D-glucose. Thus, the structure of glycoside **6** was determined to be protoconstipatic acid (18 *S*)-18-*O*-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside.

Glycoside **7** had the same molecular formula $C_{39}H_{66}O_{20}$ as **4–6** from its positive HRFABMS (m/z 855.9508; i.e. $[M+H]^+$), negative FABMS (give ion at m/z 853 $(M\text{-}H)^-$ and from ^{13}C DEPT NMR data. Its ^{1}H and ^{13}C NMR spectra indicated that **7** possessed the same aglycon as **5** but differed in the sugar (Tables 2 and 4). Three sugar units in **7** were indicated, as there were three anomeric protons and carbons (Tables 2 and 4). It was apparent that the three sugars were present in one saccharide unit attached to C-18. The overall structure assignment was accomplished using the same protocol as for the above-mentioned glucosides, which permitted the full assignment of the protons and carbons, and the sugar components were identified as glucoses. The linkage positions for the sugar units were established using the HMBC and NOESY correlations. Compound **7** had nearly the same sugar arrangement as **5** except that the terminal glucose at the C-2′ in **5** was replaced by glucose and linked to the C-3′ instead. The linkage was also supported from fragmentation patterns observed in the FABMS experiment. FABMS analysis of the deprotonated molecular ion $(M\text{-}H)^-$ at m/z 853 gave the splitter ions at m/z 691 $(M\text{-}H\text{-}162)^-$ and m/z 673 $(M\text{-}H\text{-}H_2O\text{-}162)^-$ by the loss of one of the terminal hexose units. Furthermore, subjected to MS analysis, the prominent fragment at m/z 367 is formed by loss of the trisaccharide chain linked to C-18 of glycoside. The β configuration for the sugars was determined from their $^3J_{1,2}$ (Table 4). Based on the above information, glycoside **7** is the (18 *R*)-18-*O*-β-D-glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of murolic acid.

An elemental formula of $C_{45}H_{76}O_{25}$ was suggested for **8** from its FABMS (both positive- and negative-ion) and ^{13}C NMR data. In the ^{13}C NMR spectrum, olefinic carbons at δ 126.1 and 133.7 were observed and gave evidence for the presence of one double bond. A characteristic CH_2 doublet of protons in the ^{1}H NMR spectrum at δ 6.02 and δ 6.37 (J=3.0 Hz), indicated that the aglycon included a methylene group and hence might be γ-lactonic acid, as was seen for the methylene group in protoconstipatic acid. The saccharide composition of **8** was identified by GC and the sugars were identified as glucose. In the ^{1}H NMR spectrum of **8**, four anomeric proton signals at δ 4.93, 5.58, 5.27, and 5.14 were observed, corresponding to signals at δ 105.2, 104.4, 104.8, 104.1, respectively, and indicating that, **8** possesses four sugar units. All four anomeric protons showed β glycosidic linkages according to the coupling constants of their anomeric protons (J= 7.6–7.8 Hz). From the ^{1}H-^{1}H COSY and TOCSY spectra, all proton signals belonging to each sugar moiety in **8** were identi-

Table 5
^{1}H NMR spectra of glucoside (**8**)

No. of atom	Compound (δ relative to TMS) **8**
1′	4.93 (1H, *d*, J=7.6)
2′	4.65 (1H, *dd*, J=7.7, 8.7)
3′	4.20 (1H, *dd*, J=9.1, 8.7)
4′	3.78 (1H, *dd*, J=9.1, 10.0)
5′	4.04 (1H, *dd*, J=10.0, 8.1)
6′	4.57 (1H, *dd*, J=11.3, 6.9)
	4.22 (1H, *dd*, J=11.3, 1.4)
1″	5.58 (1H, *d*, J=7.7)
2″	4.12 (1H, *dd*, J=8.4, 8.7)
3″	4.32 (1H, *dd*, J=8.7, 8.8)
4″	4.28 (1H, *dd*, J=8.8, 9.3)
5″	3.99 (1H, *ddd*, J=2.0 7.0, 10.8)
6″	4.62 (1H, *dd*, J=11.8, 7.0)
	4.73 (1H, *dd*, J=11.8, 1.6)
1‴	5.27 (1H, *d* , J=7.8)
2‴	4.05 (1H, *dd*, J=7.6, 9.1)
3‴	4.53 (1H, *dd*, J=9.1, 9.2)
4‴	4.17 (1H, *dd*, J=9.2, 9.4)
5‴	4.07 (1H, *dd*, J=9.4, 4.9, 2.0)
6‴	4.41 (1H, *dd*, J=12.2, 4.9)
	4.30 (1H, *dd*, J=12.2, 2.0)
1⁗	5.14 (1H, *d*, J=7.8)
2⁗	4.02 (1H, *dd*, J=8.2, 9.2)
3⁗	4.25 (1H, *dd*, J=9.2, 9.7)
4⁗	4.26 (1H, *dd*, J=9.7, 9.2)
5⁗	4.09 (1H, *ddd*, J=9.2, 4.9, 2.0)
6⁗	4.24 (1H, *dd*, J=11.6, 4.9)
	4.38 (1H, *dd*, J=11.6, 2.2)

fied, starting from the anomeric protons. All sugar connectivities were established using NOESY and HMBC experiments. In the NOESY spectrum cross-peak signals were observed between H-1′ and H-18, H-1″ and H-2′, H-1‴ and H-3′, H-1⁗ and H-6″. The HMBC experiment showed long range correlations between H-1′ and C-18, H-1″ and C-2′, H-1‴ and C-3′, H-1⁗ and C-6″. Thus, the structure of **8** was assigned as the (18 *S*)-18-*O*-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of protoconstipatic acid.

To summarize the present study, the chemical structures and structural relationships among the 8 component glucosides produced by lichens from Central Asia are shown in Fig. 1.

We have isolated new glucosides containing from one to four glucose units. The above-mentioned glycosides were identified in lichens for the first time.

In contrast to glucosidic compounds from higher plants, the first glucosides identified from lichens (with different amount of glucoses) exhibit more variability of aglycones and saccharides. This may be caused by the very stressful environment, in which lichens grow, namely temperatures as high as 80°C, desiccation by wind and saline substrates.

3. Experimental

3.1. General experimental procedures

UV spectra were measured in heptane within the range of 200–350 nm by a Cary 118 (Varian) apparatus. Circular dichroism (CD) measurement was carried out under a dry N_2 on a Jasco-500A spectropolarimeter at 24°C. A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT, USA) IR spectrophotometer was used for scanning IR spectroscopy of acids and glycosides as neat films. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C). High- and also low-resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HRFABMS (positive and/or negative ion mode) were obtained with a PEG-400 matrix.

3.2. Plant material

The specimens of *Acarospora gobiensis* H. Magn., *Cladonia furcata* (Huds.) Schrad, *Lecanora fructulosa* (Dicks.) Ach., *Leptogium saturninum* (Dicks.) Nyl., *Peltigera canina* (Neck.) Hoffm., *Rhizoplaca peltata* Ram., *Parmelia camtschadalis* Ach., *P. tinctina* Mah. et Gill., and *Xanthoria elegans* (Link.) Th. Fr. were collected in July 1996 along the shore of Lake Issyk Kul, Tian-Shan mountain.

3.3. Extraction and isolation

The aqueous-MeOH layer obtained from the Blight and Dyer (1959) method of lipid extracts from 100 g of air-dried lichens, (see Řezanka and Guschina, 2000) was separated on a Sephadex LH-20 column eluting with MeOH–H_2O (9:1) yielding three fractions. Fraction A was further fractionated by RP-HPLC on a C18-Bondapak column (30 cm×7.8 mm, flow rate 2.0 ml/min) with ACN–H_2O (1:2) to yield compounds **1–3**. Fraction B was separated by RP-HPLC with ACN–H_2O (2:5) to yield the compounds **4–7**. Fraction C using ACN–H_2O (1:3), yielded only compound **8**.

3.4. Acid hydrolysis of glycosides

Glycosides (~0.5 mg) were refluxed in 2 N HCl (0.5 ml) for 2 h. Aglycone were extracted three times with EtOAc (10 ml). After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$ and lyophilized.

The identification and the D or L configuration of sugars was determined using gas chromatography according to the method of Gerwig et al. (1978), with some modifications, as previously described (Řezanka and Guschina, 2000).

3.5. Enzymatic hydrolysis and determination of the glycosides

A solution of glycoside (2 mg) in acetate buffer (pH 4.4, 10 ml) was treated with β-glucosidase (20 mg) for 48 h at 37°C. The reaction solution was evaporated to dryness, and the residue was chromatographed on a column of silica gel (10 g), using CH_2Cl_2–MeOH–H_2O (90:10:1) to provide lichen acids for ^{1}H NMR and CD analysis.

3.6. (S)-MTPA and (R)-MTPA esters

(S)-MTPA ester of aglycones from glucosides **1–8** (Ohtani et al., 1991a,b). To a CH_2Cl_2 solution (100 μl) of aglycone (0.3 mg), DMAP (1.0 mg), and Et_3N (2 μl) was added (R)-(−)-MTPACl (2.0 mg) at room temperature, and stirring was continued for 3 h. After evaporation of solvent, the residue was purified by Si gel TLC (hexane–AcOEt, 2:1) to provide the (S)-MTPA ester as a colorless oil.

(R)-MTPA Ester of aglycones from glucosides **1–8**. Each aglycone (0.3 mg) was treated with (S)-(+)-MTPACl (2.0 mg) by the same procedure as described above to provide the (R)-MTPA ester as colorless oil.

Murolic acid, colorless plates, m.p. 112.5°C, $[\alpha]_D^{24}+11.3$ (*c* 0.15, $CHCl_3$), UV, CD, and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

Protoconstipatic acid, white flakes, m.p. 104°C, $[\alpha]_D^{24}-12.8$ (*c* 0.16, $CHCl_3$), UV, CD and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

allo-Murolic acid, white plates, m.p. 89.5°C, $[\alpha]_D^{24}-15.9$ (*c* 0.11, $CHCl_3$), UV, CD and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*R*)-18-*O*-β-D-Glucopyranoside of murolic acid, colorless powder, mp 146.5°C, $[\alpha]_D^{24}-188°$ (*c* 0.15, MeOH); UV 219 nm (log ε 3.88); HRFABMS *m/z* 531.6645 $[MH]^+$ and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*S*)-18-*O*-β-D-Glucopyranoside of protoconstipatic acid, colorless powder, mp 157°C, $[\alpha]_D^{24}-179°$ (*c* 0.14, MeOH); UV 218 nm (log ε 3.82); HRFABMS *m/z* 531.6648 $(MH)^+$, and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-2)-β-D-glucopyranoside of allo-murolic acid (**1**), white amorphous powder, $[\alpha]_D^{23}-14°$; HRFABMS *m/z* 693.8068 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8063; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$ and 367 $(M-H-162-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*R*)-*O*-β-D-Glucopyranosyl-(1-3)-β-D-glucopyranoside of murolic acid (**2**), white powder, $[\alpha]_D^{23}-35°$; HRFABMS *m/z* 693.8064 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8062; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$, 513 $(M-H-178)^-$, 367 $M-H-162-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*S*)-18-*O*-β-D-Glucopyranosyl-(1-6)-β-D-glucopyranoside of protoconstipatic acid (**3**), white powder, $[\alpha]_D^{23}-18°$; HRFABMS *m/z* 693.8066 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8070; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$, 367 $(M-H-162-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-2)]-β-D-glucopyranoside of allo-murolic acid (**4**), white powder, $[\alpha]_D^{23}-52.7°$; HRFABMS *m/z* 855.9503 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9499; negative FABMS *m/z* 691 $(M-H-162)^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-2)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of allo-murolic acid (**5**), colorless powder, $[\alpha]_D^{23}-42.0°$; HRFABMS *m/z* 855.9509 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9504; negative FABMS *m/z* 853 $(M-H)^-$, 691 $(M-H-162)^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*S*)-18-*O*-β-D-Glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside of protoconstipatic acid (**6**), colorless powder, $[\alpha]_D^{23}-62.0°$; HRFABMS *m/z* 855.9503 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9500; negative FABMS *m/z* 529 $(M-H-162-162)^-$ and 367 $(M-H-3\times-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of murolic acid (**7**), colorless powder, $[\alpha]_D^{23}-49.0°$; HRFABMS *m/z* $C_{39}H_{66}O_{20}$ $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9508; negative FABMS *m/z* 853 $(M-H)^-$, 691 $(M-H-162)^-$, 673 $(M-H-H2O-162)^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*S*)-18-*O*-[β-D-Glucopyranosyl-(1-6)]-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of protoconstipatic acid (**8**), white powder, $[\alpha]_D^{23}-91.0°$; HRFABMS *m/z* $C_{45}H_{76}O_{25}$ $(M+H)^+$, calculated for $[C_{45}H_{76}O_{25}+H]^+$ 1018.0914; negative FABMS *m/z* 1015 $(M-H)^-$, 853 $(M-H-162)^-$ and 691 $(M-H-2\times162)^-$; 529 $(M-H-3\times162)^-$; 367 $(M-H-4\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 5.

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Polyphenolic compounds from *Geranium pratense* and their free radical scavenging activities

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Abstract

Two new polyphenolic compounds, myricetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside and (-)-6-chloroepicatechin, were isolated from the aerial parts of *Geranium pratense* subsp. *finitimum* (Woronow) Knuth, along with three known polyphenolic compounds [quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside, quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside, methyl gallate] and tryptophan. Quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-galactopyranoside, quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside and quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside were found to be effective against free radical induced impairment of endothelium-dependent relaxation in isolated rat aorta. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Geranium pratense* subsp. *finitimum* (Woronow) Knuth; Geraniaceae; Flavonoid; Myricetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside; (-)-6-chloroepicatechin; Free radical scavenger

1. Introduction

Geranium (cranesbill) species (Geraniaceae) are known as "Turnagagası" in Turkish folk medicine and aerial parts of the plant are used as antidiarrheic, diuretic, tonic, hemostatic, stomachic and antidiabetic drug (Baytop, 1984). *Geranium pratense* subsp. *finitimum* (Woronow) Knuth is widely distributed in Turkey (Davis, 1966). In our previous investigation, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-galactopyranoside, quercetin 3-*O*-α-L-arabinopyranoside, kaempferol 3-*O*-β-D-glucopyranoside and kaempferol 3-*O*-β-D-galactopyranoside were isolated from the title plant (Akdemir et al., 2000).

In this paper, we report the isolation and the structure elucidation of two new polyphenolic compounds, myricetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside (**1**) and (-)-6-chloroepicatechin (**2**) (Structure scheme 1), along with three known polyphenolic compounds, quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside (**3**), quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside (**4**), methyl gallate (**5**) and the amino acid, tryptophan (**6**).

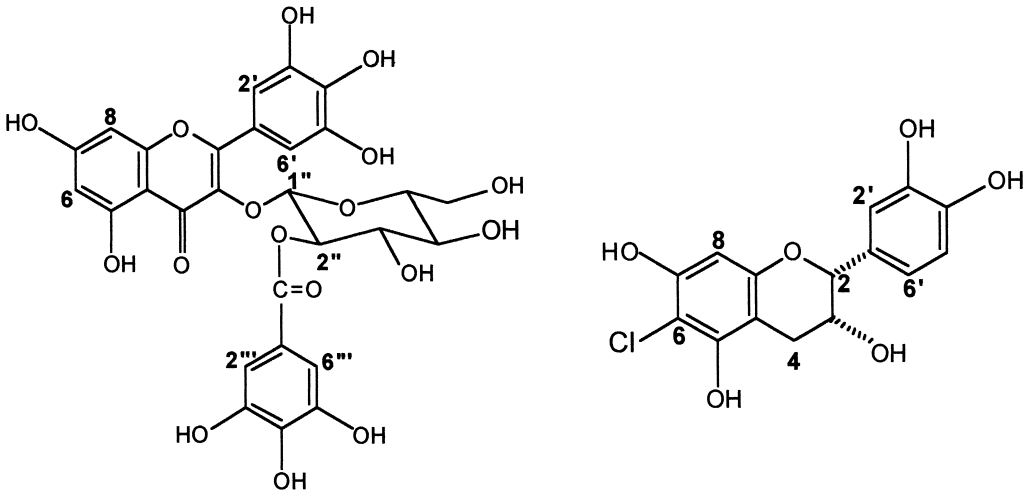
The free radical scavenging activity of the flavonoid fractions (fraction C and E) and flavonoids (quercetin 3-*O*-α-L-arabinopyranoside, kaempferol 3-*O*-β-D-galactopyranoside, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-galactopyranoside, kaempferol 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside and quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside) isolated from *Geranium pratense* subsp. *finitimum* in pure form or as mixture were also determined. For this purpose, the effects of the aqueous phase after $CHCl_3$ partitioning, flavonoid fractions and the major components of these fractions against free radical-induced impairment of endothelium-dependent relaxation in isolated rat thoracic aorta were studied. Free radicals were generated by the electrolysis of the physiological buffer in which the tissues were bathed (Gümüşel et al., 1996; Jackson et al., 1986a,b; Kelicen et al., 1997).

2. Results and discussion

Compounds **1–6** were isolated from the aerial parts of *Geranium pratense* subsp. *finitimum* by chromatographic methods.

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Compound 1 **Compound 2**

Structure scheme 1.

Compound **1** was pale yellow at 254 nm and purple at 366 nm and yellow under UV/NH_3 on a TLC plate indicating a flavonoid. In the UV spectrum, maxima occurred at 267, 361 nm and a shoulder at 293 nm, characteristic of a flavonol system. The IR spectrum suggested the presence of hydroxy, carbonyl and aromatic moieties.

The ^{1}H NMR spectrum showed aromatic protons [δ 6.30 (1H, *d, J*= 2.1 Hz, H-8), 6.13 (1H, *d, J*= 2.1 Hz, H-6), 7.20 (2H, *s*, H-2′/6′)] due to the myricetin skeleton (Harborne, 1988), together with an anomeric proton (δ 5.76 (1H, *d, J*=7.9 Hz)] indicating a sugar moiety. The configuration of the anomeric proton of the sugar was proposed to be β on the basis of the coupling constant (7.9 Hz). The sugar was found to be a β-D-glucopyranose according to ^{1}H NMR data and by comparison with the published data (Agrawal, 1992). Furthermore an additional signal was observed as singlet in the aromatic field [δ 7.12 (2H, *s*)], which indicated the presence of the second tetrasubstituted aromatic ring.

In the ^{13}C NMR and DEPT spectra of compound **1**, 24 carbon peaks were shown, including 14 quaternary, 9 methine and one methylene carbon peaks as well as two quaternary and two methine signals with double intensities, indicating the presence of 28 carbon atoms in the structure. Of the two carbonyl signals at δ 178.94 and 168.09, the first one attributed to myricetin, however the second carbonyl signal (168.09 ppm) together with the ^{1}H NMR data of tetrasubstituted aromatic ring and a direct comparison with literature (Sakar et al., 1993), showed the presence of a gallic acid moiety. Additionally, ^{13}C NMR spectrum showed four chemically equivalent signals at 109.89 ppm (C-2′/6′), 146.38 ppm (C-3′/5′), 110.64 ppm (C-2‴/6‴) and 146.46 ppm (C-3‴/5‴). In the ^{13}C NMR spectrum of **1**, 15 carbon signals were assigned to myricetin, so 6 carbon signals were assigned to glucose moiety and the remaining 7 carbon signals were assigned to gallic acid. The proton signal of H-2″ appeared to be shifted down field approximately +1.5 ppm suggesting that a substituent was present at this site. This observation was also supported by the down field shifted C-2″ signal (76.21 ppm). The observed down field shifts of H-2″ and C-2″ clearly proved that the gallic acid moiety was linked to the sugar moiety at C-2″. On the other hand the HMBC spectrum also led us to establish the locations to be connected among the myricetin, sugar and galloyl moieties of compound **1** on the basis of the cross peaks; one due to the coupling between H-1″ and C-3 and the other due to the coupling between H-2″ and C-7‴. The assigment of the ^{1}H and ^{13}C NMR chemical shift values on the myricetin ring of compound **1** was also supported by HMQC and HMBC correlations. The correlations on both the glucose and gallic acid moieties on the HMBC and HMQC spectra of compound **1** were consistent with the data from 2D ^{1}H COSY. From the above experimental results, the chemical structure of compound **1** was proposed as myricetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside.

Compound **2** was purple at 254 nm and dark gray at 366 nm and it reacted positively on TLC when sprayed with vanilin–H_2SO_4 (pink–red) indicating a phloroglucinol derivative. The UV spectrum in methanol displayed λ_{max} at 213, 281 nm and a shoulder at 236 nm. The IR spectrum suggested the presence of a hydroxy and aromatic groups. The ^{1}H NMR spectrum of compound **2** showed aromatic proton signals as a singlet at δ 5.98, two doublets at δ 6.72 and 6.66 as well as a doublet

of doublet at δ 6.60. However, the additional signals appeared at δ 4.49 (1H, *s*), 3.92 (1H, *m*), 2.67 (1H, *dd*, $J=16.1/7.3$) and 2.45 (1H, *dd*, $J=16.1/5.2$) were in good agreement for the signals of H-2, H-3, H-4α and H-4β of an epicatechin moiety, respectively, by comparing with published data (Sakar et al., 1993). The assignments of H-4α were facilitated by the observation of a large coupling constant ($J=16.1/7.3$) between H-3 and H-4α. The resonances were observed as an ABX system at δ6.72 (*d*, $J=2.1$ Hz), 6.66 (*d*, $J=8.2$ Hz) and 6.60 (*dd*, $J=8.2/2.1$ Hz) indicated the presence of a trisubstituted B ring as in epicatechin. In general, the proton assignments made in this manner were in agreement with the published ^{1}H NMR data for epicatechin except for the absence of the signal for H-6. The singlet signal at δ 5.98 also showed that A ring was pentasubstituted and this signal could be attributed to H-8. However, the EI-MS showed two peaks at *m*/*z* 326 and 324 (relative intensities 1:3.75) clearly indicating that there is a chlorine atom in the molecule. Therefore, by the above mentioned discussion, the chemical structure of compound **2** was proposed as (-)-6-chloroepicatechin.

Compound **1** and **2** were isolated from nature for the first time in this study.

In addition, the ^{1}H and ^{13}C NMR spectral data of compounds **3–6** were compared with spectral values in literatures (Pakulski and Budzianowski, 1996; Kuroyanagi et al., 1982; Bedir, 1998; Brückner et al., 1988) and found to be identical with quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside, quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside, methyl gallate and tryptophan, respectively. This is the first report for the isolation of compounds **3–6** from genus *Geranium*.

The polyphenolic compounds of *Geranium* species such as flavonoids and tannins have been shown to inhibit lipid peroxidation of the biological membranes and possess free radical scavenging/antioxidant activity both in vivo and in vitro (Lamaison et al., 1993; Saija et al., 1995). Free radicals are highly reactive species, which play an important role in pathological states including various vascular diseases (Kennedy et al., 1989; Opie, 1989; Lawson et al., 1990). In the present study, we have evaluated the free radical scavenging activity of polyphenolic compounds: the mixture of quercetin 3-*O*-(2″-*O*-galloyl)-β-D-glucopyranoside and quercetin 3-*O*-(2″-*O*-galloyl)-β-D-galactopyranoside (GP-4) isolated in this study together with the mixture of quercetin 3-*O*-α-L-arabinopyranoside and kaempferol 3-*O*-β-D-galactopyranosid(GP-1); the mixture of quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-galactopyranoside (GP-2) as well as a pure compound, kaempferol 3-*O*-β-D-glucopyranoside (GP-3) which were all isolated from *Geranium pratense* subsp. *finitimum* in our previous study (Akdemir et al., 2000). GP-1, GP-2 and GP-4 were tested as mixtures due to the difficulties in separation from similar chemical structures. The effect of these compounds were tested against electrolysis-induced impairment of endothelium-dependent relaxation in aortic rings isolated from rats. Electrolysis of the physiological solutions has been shown to generate free radicals such as hydrogen peroxide, superoxide and hydroxyl radicals. We have previously reported the functional endothelial injury which can be prevented by free radical scavengers in aortic rings subjected to electrolysis (Gümüşel et al., 1996; Kelicen et al., 1997).

The present experiments demonstrated that in the aortic rings precontracted with phenylephrine, acetylcholine (10^{-8}–10^{-5} M) induced concentration-dependent relaxation. This relaxation was endothelium-dependent, since it was abolished after removal of the endothelium. In aortic rings subjected to electrolysis, endothelium-dependent relaxation response to acetylcholine was significantly inhibited (Fig. 1).

Incubation of the aortic rings with the aqueous phase after $CHCl_3$ partitioning (500 μg/ml) and fraction C (100 μg/ml) did not alter the inhibition of acetylcholine response after electrolysis, but fraction E (100 μg/ml) provided protection against electrolysis-induced inhibition of the relaxation response elicited by acetylcholine.

Incubation of the aortic rings with the major components of fraction E (GP-2 and GP-4) provided protection at 10^{-4} M concentration against the electrolysis-induced inhibition of acetylcholine response. The protection offered by incubation with either fraction E or GP-2 was partial, as acetylcholine-induced relaxation was still significantly less than the control response. However, the protection offered by GP-4 was found to

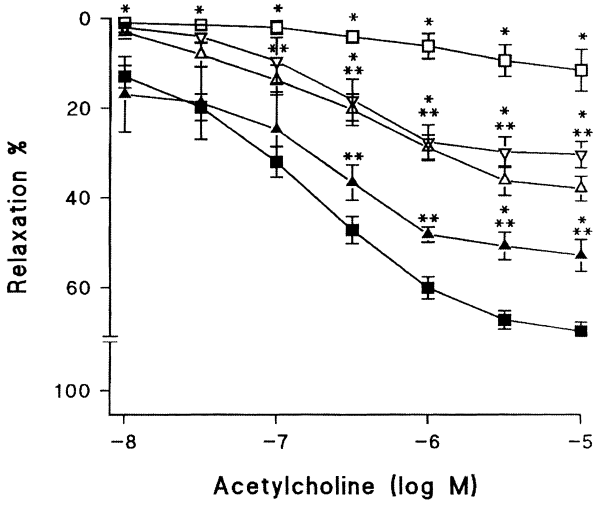


Fig. 1. Acetylcholine induced relaxation response in precontracted aortic rings before (■) and after electrolysis (□), and the effect of incubation with Fraction E (▽) (100 μg/ml), GP-2 (△) (10^{-4} M) and GP-4 (▲) (10^{-4} M) before electrolysis. The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as mean±SEM significantly different from acetylcholine response * before, and ** after electrolysis ($p<0.05$).

be more pronounced than the same concentration of GP-2. Incubation with 10^{-4} M concentration of GP-4 completely inhibited the electrolysis-induced impairment of the relaxation response.

Major components of fraction C (GP-1 and GP-3) were also tested at 10^{-4} M concentration and were found to be ineffective.

In conclusion, the results of the present experiments show that Fraction E and its polyphenolic compounds GP-2 and GP-4 were effective against free radical-induced impairment of the endothelium-dependent relaxation response in rat aortic rings. These findings indicate that GP-2 and GP-4, but not GP-1 and GP-3 possess free radical scavenging activity, and thus protective activity against endothelial injury by free radicals.

Flavonoids have been shown the possess several biological properties (including hepatoprotective, antithrombotic, antiinflammatory and antiviral activities), many of which may be related, partially at least, to their antioxidant and free radical scavenging activity (Saija et al., 1995).

Definite structural features are important determinants for radical scavenging activity and/or antioxidant potential of flavonoids: 1. Antioxidant activity is expected for flavonoids having more than four hydroxyl groups (Faure et al., 1990). 2. The *ortho*-dihydroxy (catechol) structure of the B ring of flavonoids, which confers higher stability to the radical form and participates in electron delocalization. 3. The 2,3-double bond in conjugation with a 4-oxo-function in the C ring is responsible for electron delocalization from the B ring. The antioxidant potency is dependent from the presence of the aromatic nuclei. When these compounds react with free radicals, the phenoxyl radicals produced are stabilized by resonance effect of the aromatic nucleus. 4. The presence of both 7- and 5- and additionally 3-hydroxyl groups in A and C rings are required for maximum radical scavenging potential (Saija et al., 1995; Cotelle et al., 1996). Since GP-2 and GP-4 were owing to the same structural properties, they showed good free radical scavenging activity. However, GP-4 was found to be more active than GP-2 due to the presence of a gallic acid moiety.

3. Experimental

3.1. General

UV spectra: on a Shimadzu UV-160A (MeOH). IR spectra: on a Perkin–Elmer 1720X FT-IR Spectrophotometer (KBr disc). Optical rotation: Rudolph Autopol IV polarimeter (10 cm cell, CH_3OH). ^1H NMR, ^{13}C NMR, COSY, HMQC and HMBC experiments were recorded on a JEOL JNM-A 500 spectrometer. EI-MS: Finnigan TSQ 7000.

3.2. Plant material

The aerial parts of *Geranium pratense* subsp. *finitimum* were collected in Rize, Turkey, in August, 1997. A voucher specimen has been deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 97-009).

3.3. Extraction and isolation

The air-dried overground parts of *Geranium pratense* subsp. *finitimum* (1.09 kg) were extracted with MeOH. After evaporation under vacuum the residue (77.94 g) was partitioned between $CHCl_3$ and H_2O. The aqueous phase was lyophilized (67.41 g) and subjected to polyamide CC, eluting with H_2O and mixtures of H_2O–MeOH of increasing polarity, afforded six fractions (Fr. A–F). Fr. E (0.87 g) was further fractioned by MPLC using 25–60% MeOH to give fractions E-I–III. Fr. E-II was chromatographed on polyamide eluting with $CHCl_3$–MeOH–EtCOMe–$(Me)_2CO$ (3:2:0.5:0.5) to give fraction E-IIa–c. Fr. E-IIc was then chromatographed on Sephadex LH-20 (MeOH) to give compound **1** (3.45 mg). Fr. E-I was chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80:20:2) to give compound **2** (1.52 mg). Fr.E-III was fractioned by MPLC using CH_3CN:*iso*-PrO: H_2O (20:5:75) to yield fractions E-IIIa–b. Fr. E-IIIa was subjected to polyamide CC, eluting with $CHCl_3$–MeOH–EtCOMe–$(Me)_2CO$ (3:3:1:1) to yield a mixt. of **3** and **4** (43.6 mg). Separation of Fr.D (3.15 g) by MPLC (30–40% MeOH) followed by silica gel CC using $CHCl_3$–MeOH–H_2O (50:50:5) and then Sephadex LH-20 CC (MeOH) afforded **5** (12.1 mg). Fr.A (0.74 g) was applied to MPLC (15–55% MeOH) and then subjected to silica gel CC using $CHCl_3$–MeOH–H_2O (70:30:3) to give compound **6** (6.69 mg).

3.4. Myricetin 3-O-(2″-O-galloyl)-β-D-glucopyranoside (*1*)

$[\alpha]_D^{25}$-65 (MeOH, c 0.17). UV λ_{max} (MeOH) nm: 267, 293 (sh), 361. IR ν_{max} (KBr) cm^{-1}: 3735 (OH), 1745 (C=O), 1609 (C=C). ^1H NMR (CD_3OD): δ 7.20 (2H, *s*, H-2′/6′), 7.12 (2H, *s*, H-2‴/6‴), 6.30 (1H, *d*, J=2.1 Hz, H-8), 6.13 (1H, *d*, J=2.1 Hz, H-6), 5.76 (1H, *d*, J=7.9 Hz, H-1″), 5.15 (1H, *dd*, J=9.4/7.9 Hz, H-2″), 3.80 (1H, *t*, J=9.7 Hz, H-6″b), 3.65 (1H, H-3″), 3.64 (1H, *dd*, J=11.9/6.1 Hz, H-6″a), 3.46 (1H, *t*, J=9.4 Hz, H-4″), 3.29 (1H, H-5″). ^{13}C NMR (CD_3OD): δ 178.94 (C-4), 168.09 (C=O), 166.33 (C-7), 163.13 (C-5), 158.35 (C-9), 158.22 (C-2), 146.46 (C-3‴/5‴), 146.38 (C-3′/5′), 140.03 (C-4‴), 137.95 (C-4′), 135.13 (C-3), 122.17 (C-1′), 121.44 (C-1‴), 110.64 (C-2‴/6‴), 109.89 (C-2′/6′), 105.77 (C-10), 100.69 (C1″), 99.89 (C-6), 94.65 (C-8), 78.72 (C-5″), 76.57 (C-3″), 76.21 (C-2″), 71.53 (C-4″), 62.50 (C-6″).

3.5. (-)-6-Chloroepicatechin (2)

$[\alpha]_D^{25}$-84 (MeOH, c 0.18). UV λ_{max} (MeOH) nm: 213, 236 (sh), 281. IR ν_{max} (KBr) cm^{-1}: 3421 (OH), 1623 (C=C). EI-MS (*m/z*): 326 $[M+2]^+$, 324 $[M]^+$. 1H NMR (CD_3OD): δ 6.72 (1H, *d*, $J=2.1$ Hz, H-2′), 6.66 (1H, *d*, $J=8.2$ Hz, H-5′), 6.60 (1H, *dd*, $J=8.2/2.1$ Hz, H-6′), 5.98 (1H, *s*, H-8), 4.49 (1H, *s*, H-2), 3.92 (1H, *m*, H-3), 2.67 (1H, *dd*, $J=16.1/7.3$ Hz, H-4α), 2.45 (1H, *dd*, $J=16.1/5.2$ Hz, H-4β).

3.6. Pharmacological studies

3.6.1. Preparation of isolated rat aorta

The aortic rings isolated from rats (male, 150–200 g) were mounted in organ baths filled with Krebs–Henseleit solution (95% O_2–5% CO_2, 37°C) (Gümüşel et al., 1996; Kelicen et al., 1997).

A resting tension of 2 g was applied and the rings were equilibrated for 90 min before experiments. Isometric tension was recorded with an 'isometric force transducer' and 'transducer data acquisition system' (MAY 95, Commat Ltd, Turkey) on an IBM-compatible personal computer.

3.6.2. Experimental protocol

The aortic rings were contracted to 80% of the maximum contraction to phenylephrine (10^{-6} M) and the relaxation response to acetylcholine (10^{-8}–10^{-5} M) were measured. In some experiments, acetylcholine response was also elicited in endothelium-denuded aortic rings.

The aortic rings were exposed to free radicals generated by the electrolysis of the bathing solution using two platinum electrodes (d.c. current, 20 mA, 5 min). The electrodes were placed 1 cm apart from the tissue so that the aortic rings were not exposed to field stimulation. After the electrolysis, the baths were washed out and the tissues were allowed to equilibrate for 5 min before acetylcholine response was repeated using the same protocol.

In a group of experiments aortic rings were incubated for 30 min before electrolysis with each aqueous extract (500 μg/ml), fraction C (100 μg/ml), fraction E (100 μg/ml), GP-1, GP-2, GP-3 and GP-4 (10^{-4} M). Then the concentration–response curve to acetylcholine was obtained as described above.

3.6.3. Statistical analysis

Relaxations were expressed as the percentage of papaverine (10^{-4} M) induced relaxation. Papaverine was added to the organ bath after the maximum response to acetylcholine was obtained. Results are expressed as the mean±SEM. The comparison was performed using Mann–Whitney U-test and values of $p<0.05$ were considered to be significantly different.

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Synthesis, characterisation and water sorption properties of pectin-dehydrogenation polymer (lignin model compound) complex

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Abstract

Coniferyl alcohol was polymerised in the presence of pectin and a pectin–DHP complex was isolated. Characterisation of the complex has demonstrated that DHP (20% wt) was covalently linked by ester bonds to the pectin. The water sorption behaviour of the pectin–DHP complex was determined at several relative vapour pressures and compared with those obtained on pectin, DHP and a blend of both polymers in the same proportion as in the complex. The complex exhibited a lower hydrophilicity resulting from three associated phenomena: grafting, pectin–DHP interactions and the formation of a chemical network. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Dehydrogenation polymer (DHP); Pectin; Lignin carbohydrate complex (LCC); Water sorption

1. Introduction

In plant cell walls, lignins are deposited after the polysaccharides and consequently polymerisation of lignols occurs in a swollen gel (Terashima et al., 1993). In woody xylem, lignification is an uneven process moving from the outer part to the inner part of the cell wall (Takabe et al., 1981). The first lignified region is the primary wall which contains a large amount of pectins (Westermark et al., 1986). These polysaccharides are mainly composed of uronic acid, which can react with quinone methides formed during the polymerisation pathway of lignin, to form a covalent bond (Sipilä, 1990; Tanaka and Nakatsubo, 1976). The occurrence of such lignin carbohydrate complexes (LCC) is now well established (Das Nath et al., 1981; Takahashi and Koshijima, 1988); however, their influence on the architecture of the cell wall is not yet fully understood. Simultaneously with lignin deposition, water is removed resulting in the shrinkage of the cell wall. This phenomenon has been observed by electron microscopy of slowly frozen, partly lignified cell walls. Crystalline ice was observed only in the unlignified part demonstrating the removal of some of the water from the lignified part (Terashima et al., 1993). The chemical process in charge of such physiological events has not been investigated to our knowledge. The decrease of water content may have a large effect on enzyme accessibility and on the mechanical and permeability properties of the cell wall. The understanding of the underlying phenomena could be beneficial for studies of delignification reactions during pulping and biodegradation processes, and for comprehension of cell wall formation.

In the present study, coniferyl alcohol was polymerised in the presence of pectin and a pectin–DHP complex was isolated and characterised by chemical analysis. Then in order to evaluate the influence of a lignin-like structure on hydratation properties, the water sorption behaviour of the complex was determined and compared with those of pure DHP, pectin and a blend containing the same proportion of both polymers.

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2. Results and discussion

2.1. DHP synthesis

All of the reactions were run according to the classical ''Zutropfverfahren'' method consisting of a slow and continuous addition of coniferyl alcohol and hydrogen peroxide to a solution of peroxidase (Freudenberg and Neish, 1968). The usual way to collect DHP at the end of the reaction is to centrifuge the suspension and collect the insoluble part. Surprisingly, in the presence of pectin, no precipitate was formed and only a colloidal opacity of the solutions was noted. The increase of the solubility of the DHP in the pectin mixture has to be related with the ''sugar in'' effect observed by Shigematsu et al. (1994a) who have demonstrated that monolignol solubilities are increased in sugar solutions. The same effect may be responsible for the high DHP solubilities. As a consequence, samples were first concentrated under vacuum and then freeze-dried. The same work up was applied to the reference DHP explaining the quite unusual yield for DHP synthesis (100%). After freeze drying, in the case of the experiment in presence of pectin, the unbound DHP were removed by washing the solid material in dioxane/water solution to afford two fractions: pectin–DHP soluble in dioxane/water (PDHPS) and pectin–DHP insoluble in dioxane/water (PDHPI). PDHPS yielded 22% expressed as the weight percentage of the total starting materials (coniferyl alcohol and pectin). The insoluble fraction represented 77% of the total material whereas pectin made up only 66% of the total starting material, that is to say 117% of the starting pectin. This weight increase may indicate that some DHP remains with the pectin and further analyses were performed to determine the composition of both fractions.

2.2. Determination of the DHP content of the fractions

Acetyl bromide assays were performed without the addition of perchloric acid according to Hatfield et al. (1999) and the standard deviation was less than 4% between triplicates. The pectin were found to be nearly free of phenol structures. The very low value observed ($<2\%$) is under the precision limit of the method and can be attributed to a small amount of phenols or, more probably, to artefact occurring during the determinations. The PDHPS contained 95% of DHP indicating that some polysaccharides (bound or unbound) were extracted by dioxane/water. The DHP content of the insoluble part (PDHPI) was found to be 22%. This value is in a good agreement with the conclusion based on the yield of the fractions. Moreover, this result was confirmed with the overall recovery obtained in sugar analysis of the PDHPI which is 73% whereas the recovery of the crude pectin is 90%. The difference corresponds to 24% of the total mass which confirms the DHP content of the fraction. The sugar composition of the PDHPI is identical to that of the initial pectin showing that this fraction is representative of all the pectin at least in its monomeric composition.

2.3. DHPs characterisation

DHP fractions were characterised by nitrobenzene oxidation and thioacidolysis. Both of these degradation methods provide information on the relative amounts of uncondensed and condensed guaiacyl propane bonds. Thioacidolysis is a highly specific method which cleaves the alkyl aryl ether bonds (8-O-4) whereas nitrobenzene oxidation can, in addition, degrade other structures such as some carbon-carbon intermonomeric bonds (8-1 for instance) (Chang and Allan, 1971) resulting in a higher degradation yield. Usually, this increase ranges between 15 and 30% (Lapierre, 1986). Therefore, another reactivity difference must be mentioned. The first step of the thioacidolysis degradation pathway is the substitution of the hydroxyl group of the carbon 7 position by a thioethyl group. The cleavage process cannot take place when this position is substituted by an acetyl group as demonstrated by model compound studies (Lapierre, 1986). In contrast ester groups are readily cleaved by nitrobenzene oxidation which occurs under alkaline conditions at higher temperature. During the polymerisation process, radicals exist as four mesomeric forms (Fig. 1); coupling of the radicals located on phenolic oxygen and the carbon 8 result in the 8-O-4 quinone methide. This electrophilic compound can react very efficiently with nucleophilic species and especially with the free carboxylic groups of the pectin leading to ester linkages between pectin and DHP on the C7 position of the guaiacyl monomer (Sipilä, 1990; Tanaka and Nakatsubo, 1976). Analysis of the degradation results (Table 1) demonstrated that the thioacidolysis degradation yields of the reference DHP and PDHPS are 22 and 27% lower than their nitrobenzene oxidation yields. These values are in good agreement with those reported in the literature as previously discussed. In contrast, for PDHPI the thioacidolysis yield was strongly decreased in comparison to nitrobenzene oxidation (nitrobenzene oxidation: 787 μmol/g, thioacidolysis: 30 μmol/g). This cannot be explained by the presence of pectin because thioacidolysis of a mixture of pectin and standard DHP led to the same result as the DHP alone. The combination of nitrobenzene oxidation and thioacidolysis supports strongly the hypothesis of the formation of ester bonds between pectin and DHP. Such linkage was already evidenced in natural lignin carbohydrate complex of beech (Takahashi and Koshijima, 1988) and jute fiber (Das Nath et al., 1981).

Moreover, another result should be mentioned in Table 1. The yields of vanillin and thioethylated monomers were lower in the case of PDHPS than for

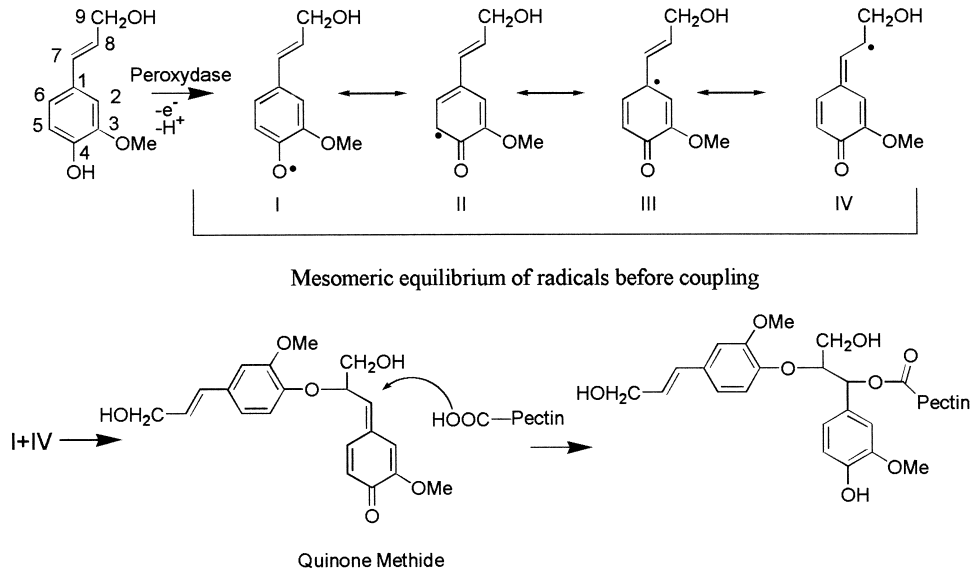


Fig. 1. Radical mesomeric forms of coniferyl alcohol and coupling of pectin on quinone methide during the reaction process.

reference DHP. Since the standard deviations between triplicates, for both methods, are less than 5% and the thioacidolysis yield is consistent with those reported in the literature, this difference is significant (Jacquet et al., 1997; Cathala et al., 1998). On the contrary, Terashima et al. (1996) reported that the soluble part of DHP synthesised in the presence of pectin contains a higher amount of 8-O-4 linkages than a DHP obtained in absence of pectin. However, the synthesis was not conducted in the same manner: concentrations of pectin and coniferyl alcohol were not identical and, moreover, hydrogen peroxide was supplied in larger amounts in our experiment (3 equivalents vs 1 in Terashima's study). As previously discussed, pectin increases the solubility of the DHP and the excess of hydrogen peroxide can generate more frequent reactions between oligomers to produce a more "bulk type polymer". Recovery yield for the PDHPI was nearly identical suggesting that soluble and insoluble DHP have similar structure.

In order to confirm the occurrence of ester bonds between pectin and DHP, the complex was submitted to alkaline and acidic degradationtable. After neutralisation and freeze drying the degraded samples were extracted by dioxane/water (9/1, v/v) solution. The ratio of the UV absorbance of these solutions to solutions of reference DHP (submitted to the same procedure) allows the determination of the amount of phenolic structures liberated by both degradation methods. Under alkaline condition 50% of the DHP was recovered, whereas only 5% was extracted after the acidic hydrolysis;

Table 1
Chemical analysis of pectin, of reference DHP and of pectin–DHP soluble (PDHPS) and insoluble (PDHPI) in dioxane/water fractions

	DHP content (wt%)	Sugar analysis monomeric composition (%) *Yield of degradation*	Nitrobenzene oxidation (μmol of vanilin per g)	Thioacidolysis yield (μmol of thioethylated monomer per g)
Reference DHP	100	Rha- Gal- GalA-	950	687
PDHPS	95	Rha- Gal- GalA-	754	590
PDHPI	22	Rha 1.5 Gal 5.87 GalA 92.5 *73%*	787	30
Pectin	<2% (see text for details)	Rha 2.4 Gal 6.2 GalA 91.2 *90%*	0	0

this support strongly the fact that the DHP are covalently linked to the pectin by ester bonds, even though alkali treatment can cause side effects (β-elimination of pectin for instance), which might solubilise some DHPs.

Both extracted fractions were then submitted to acetylation and analysed by steric exclusion chromatography (SEC) together with the undegraded samples (Table 2). Comparison of the reference DHP and the PDHPS indicates that both have the same M_w (weight average molecular weight) but not the same M_n (number average molecular weight) implying that the distributions are different. The increase of the solubility of the DHP in the pectin solution allows more frequent coupling between oligomers whereas higher molecular weight DHPs precipitate in the reference experiment limiting their reactivity and so low molecular weight molecules are the favoured substrates until the precipitation limit is reached. This could explain the less broad distribution of the reference DHP compared to the more polydisperse PDHPS.

Both degradation methods reduce the molecular weight of the DHP. Thus, the bound DHPs have M_w higher than 1400 g/mol. Although, no reasonable assumption can be made as to the real M_w of this fraction without further analysis, it is clear that the pectin linked DHPs have a polymeric structure. Thus the probability for the same DHP molecule to be linked with two chains of pectin, increases. Such a bridge pattern can lead to the formation of a three-dimensional network explaining the colloidal suspension observed during the polymerisation and the insolubility of the product.

2.4. Water sorption properties

In Fig. 2 the moisture sorptions of PDHPI, pectin, reference DHP and a physical blend of pectin and DHP (PB, pectin/DHP, 80/20, wt/wt) are plotted against relative humidity. Pectin, which contains more sorption sites, which are moreover ionic, sorbed most moisture, whereas DHP, which is a neutral polymer, sorbed the least. Both of these curves are in good agreement with those previously reported (Berthold et al., 1996; Fringant et al., 1996b). The other samples (PDHPI, PB), both consisting of a mixture of charged and neutral polymers, present an intermediate level of moisture sorption. The physical blend contained 80% pectin and 20% DHP and had been prepared in the same way as the other samples (pH 6, one night of stirring, concentration and freeze-drying) except the dioxane/water extraction. Although both PB and PDHPI have the same DHP content, a significant difference between these products is noted. Since the quinone methide reacts with carboxylate groups, grafting results in the disappearance of sorption sites (COO^-Na^+). In order to evaluate this effect, the two curves were compared to theoretical isotherms (Fig. 3) calculated as followed:

$$\%\ \text{sorbed water}_{RHi} = (\%\ \text{water in DHP}_{RHi} \times \%\ \text{DHP}) + (\%\ \text{water in Pectin}_{RHi} \times \%\ \text{Pectin})$$

PB was perfectly fitted by the calculated curve for the same proportion of the two polymers. On the other hand PDHPI can be fitted at low relative humidity to the theoritical curve for a 63/37 pectin/DHP (wt/wt) blend (whereas the PDHPI contains only 20% DHP). Then, the amount of DHP required to fit the first part ($rh < 0.76$) of the experimental isotherm is quite surprising and it becomes important to estimate the percentage of galacturonic units esterified. An overestimation of this value can be calculated assuming that the difference observed between nitrobenzene oxidation and thioacidolysis yields corresponds to the maximum of the ester bonds formed. The result of this estimation corresponds to 15% of the DHP units involved in such linkage (molecular weight of one DHP monomer is assumed to be equal to 200 g/mol, 5×10^{-3} mole of monomer/g of DHP) and consequently to roughly 4% of the galacturonic units (1 DHP monomer/25 pectin monomers). This amount is quite low for the decrease of moisture uptake observed. For instance, at the relative humidity of 76%, the weight increase of PDHPI is 12% lower than for the PB. Moreover, Fringant et al. (1996a) reported that the hydrophilicity of acetylated starch was strongly decreased only after the limit of one acetyl group per unit. Below this value, the water sorption was not significantly changed. Consequently, it appears that the sorption decrease observed cannot be attributed solely to the grafting. Several authors have already

Table 2
HPSEC analysis of degraded and undegraded DHP fractions

Fraction	Direct acetylation			Acetylation after NaOH degradation			Acetylation after HCl degradation		
	M_w (g/mol)	M_n (g/mol)	M_w/M_n	M_w (g/mol)	M_n (g/mol)	M_w/M_n	M_w (g/mol)	M_n (g/mol)	M_w/M_n
Reference DHP	4900	2700	1.8	2400	1300	1.85	3000	1300	2.3
PDHPs	4900	1800	2.7	nd[a]	nd	nd	nd	nd	nd
PDHPI	–	–	–	1400	700	2.0	1150	550	2.1

[a] nd = not determined.

demonstrated the importance of non-covalent interactions between lignins and polysaccharides by molecular modelling (Houtman and Atalla, 1995), model polymerisation (Tanahashi and Higuchi, 1990) or physical–chemistry properties (Shigematsu et al., 1994a,b, 1995) and so one hypothesis could be that the DHP develop significant interactions with the polar groups of the pectin such as hydrogen bonds, which can compete with water/pectin interactions. Since the DHPs are closely wrapped around the pectin to which they are grafted, non-covalent bonding can be plausibly be proposed as the basis for the difference from the PB where the two polymers behave separately. At higher vapour pressure (relative humidity >0.76), the calculated curve does not fit any more with the experimental data suggesting that DHP and pectin chains are interconnected to form a

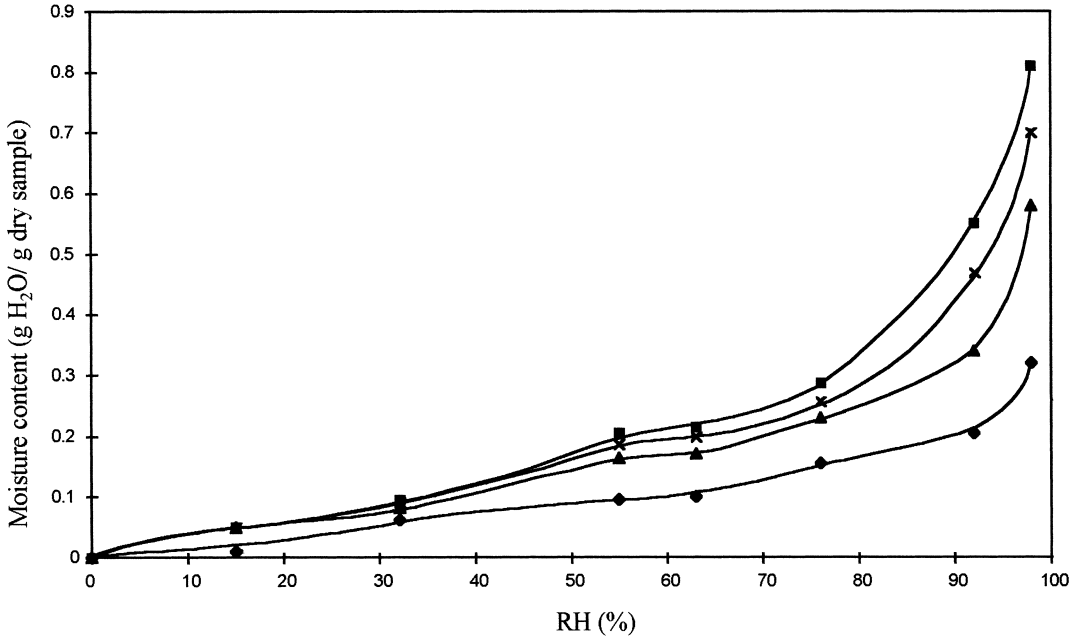


Fig. 2. Experimental isotherms of water sorption for Pectin (■), PDHPI (▲), DHP (◆) and PB [physical blend Pectin/DHP (80/20, wt/wt)] (×) expressed as the moisture content versus relative humidity.

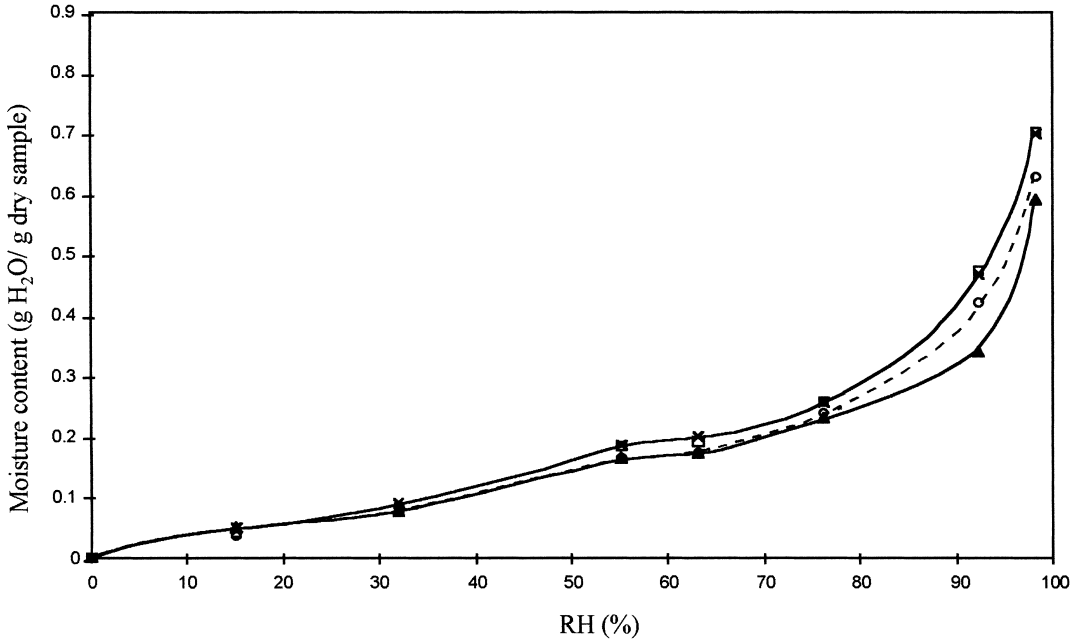


Fig. 3. Experimental isotherms of water sorption for PDHPI (▲) and physical blend pectin/DHP (80/20, wt/wt) (×) expressed as the moisture content versus relative humidity, compared to the calculated isotherms for blend containing Pectin/DHP in 80/20 (□) and 63/37 (○) proportions.

three-dimensional network. A chemical network can interfere with water sorption by limiting the swelling capacity of the polymers, as it was reported concerning the water sorption of dextran gels with different densities of crosslinking (Ruike et al., 1999). From these results it can be hypothesised that the water sorption phenomenon proceeds in two steps: first, under low relative vapour pressure (<0.76), water molecules interact with sorption sites (water–sites interaction) and can be limited by a decrease in the number of sites (owing to grafting) and by competition with the pre-existing hydrogen bond network (DHP/Pectin non covalent interaction). Secondly, at higher relative humidity (>0.76), water clusters could be formed (water/water interactions) and consequent "liquid" water is observed if extensive swelling is not hindered by chemical crosslinking points (chemical network).

3. Conclusion

Polymerisation of coniferyl alcohol in presence of pectin results in an interconnected pectin–DHP complex. Similar network formation was also demonstrated with feruloyl polysaccharides (Oosterveld et al., 1997; Ishii, 1997) suggesting that dehydrodiferulate formation could be a possible polysaccharide crosslinking mechanism in growing cells. In our study, crosslinking is only due to the polymerisation of coniferyl alcohol suggesting that the reaction of the quinone methide could be a complementary pathway for the formation of a chemical network in situ and especially in plants which contains low amounts of hydroxycinnamic acids (Hartley and Harris, 1981).

In plant cell walls, the removal of water resulting in shrinkage could be an effect of grafting, interactions and networking between polysaccharides and lignins. Moreover, previous model studies (Cathala et al., 2000) at a hydrophobic/hydrophilic interface (air/water) have demonstrated that DHP layers form denser structures when the DHP concentration increases implying that lignin-like structures can remove water from their environment probably by preferential self-association due to hydrogen bonding and/or a hydrophobic effect. This effect may also be complementary to those observed in this study.

4. Experimental

4.1. DHP synthesis

Three solns were prepared as follows. Soln 1: 20 mg of peroxidase (HRP type VI, 250–330 unit/mg, purchased from Sigma) was dissolved in 500 ml of H_2O for reference DHP and in 500 ml of 1 g/l soln of pectin (pH = 6) for the pectin experiment. Soln 2: 250 mg of coniferyl alcohol was dissolved in 25 ml of Me_2CO and 225 ml of H_2O. Soln 3: 0.5 ml of hydrogen peroxide (30% wt in H_2O) was mixed with 250 ml H_2O. Solns 2 and 3 were added to soln 1 during 12 h (20.8 ml/h). Then the mixture was allowed to react for an additional 4 h. During all the reactions the pH was kept constant (pH = 6) by controlled addition by an automatic system of a NaOH soln (2.5×10^{-3} M). H_2O was partly removed under vacuum and the residual suspension (roughly 40 ml) was freeze dried. The pectin–DHP mixture was extracted with dioxane/H_2O soln (9/1) to afford soluble pectin–DHP (PDHPS) and insoluble pectin–DHP (PDHPI).

4.2. Acetyl bromide assay

The DHP content was determined by the method proposed by Hatfield et al. (1999) without the addition of $HclO_4$. For all the determinations the acetyl bromide concentration was 25% (wt/wt) in glacial HOAc. The detection was done at 280 nm (Lambda 14, Perkin–Elmer) after diluting 1 ml of the reaction mixture in NaOH (5 ml, 2 M) and 14 ml of glacial HOAc.

4.3. Carbohydrate analysis

All the samples (1–3 mg) with fucose as internal standard were degraded by methanolysis 2 M HCl/MeOH (16 h, 80°C). Samples were dried under N_2 and hydrolysed by TFA 2 M (1 h, 121°C) as proposed by De Ruiter et al. (1992). After reaction, samples were diluted 15–25-fold, filtered and finally analysed by HPAEC-PAD using a PA1 anion-exchange column (4×250 mm, Dionex). Neutral monosaccharides were separated in 4 mM NaOH for 12 min and for a further 7 min with a linear gradient up to 100 mM. Elution of acidic monosaccharides was achieved using a linear gradient up to 300 mM NaOAc in 100 mM NaOH for 30 min. The flow rate was 1 ml/min and detection was performed by pulsed amperometry (PAD 2, Dionex) after mixing of the eluent with 300 mM NaOH (0.7 ml min^{-1}).

4.4. Nitrobenzene oxidation

The procedure was adapted from those described by Billa et al. (1996). NaOH (5 ml, 2 M) and nitrobenzene (0.5 ml) were added to the samples (10–20 mg) in Teflon vials enclosed within a stainless autoclave. The reaction mixture was then heated at 160°C for 3 h. After cooling the internal standard was added (3-ethoxy-4-hydroxybenzaldehyde) then the mixture was extracted with CH_2Cl_2 (3×20 ml) to remove nitrobenzene degradation products. The aq layer was then acidified with HCl 6 M to pH ca 1 and extracted with CH_2Cl_2 (3×20 ml). The organic layers were combined, and dried over Na_2SO_4

and the solvent was removed under vacuum. The oxidation products were analysed by HPLC (Waters Spherisorb ODS2 column, 4.6×250 mm) using a linear gradient starting from 100% of H_2O (1/1000, v/v, phosphoric acid) to 60% of MeOH. Vanillin and internal standard were both detected at 313 nm. All the experiments were run as triplicates and the standard deviation was less than 5%.

4.5. Thioacidolysis

Thioacidolysis was performed according to Lapierre's procedure (Lapierre et al., 1986). The main degradation products were analysed by GC as TMSi derivatives using a J&W Scientific column (DB 1, 30 m×0.25 mm, 0.25 μm film). Detection was by FID using tetracosane as internal standard. All the experiments were run as triplicates and the standard deviation was less than 3%.

4.6. Alkaline and acidic degradation

20 mg of PDHPI and 4 mg of reference DHP were stirred for 5 h in 1 M HCl or 1 M NaOH (2 ml) at 25°C. After neutralisation, the samples were freeze-dried and extracted with dioxane/H_2O 9/1, v/v, 10 ml). The degradation yield was determined as the ratio of the UV absorbance of the PDHPI and reference DHP solutions for each method. NaOH yield = 50%. HCl yield = 5%.

4.7. Steric exclusion chromatography

Before injection, samples were submitted to acetylation by a mixture of Ac_2O and pyridine (1/1, v/v) for 24 h at room temp. The reactions were then poured on to ice and extracted with CH_2Cl_2. The organic layer was washed with NaCl sat soln. Two Polymer Laboratories (Mixed D 300×7.5 mm) columns were coupled and THF was used as solvent at the flow rate of 1 ml/min. 100 μl of 1 mg/ml solutions were injected through the columns at 40°C. Detection was done at 280 nm. M_w, M_n were determined using the relative calibration method based on the elution of ten polystyrene standards.

4.8. H_2O sorption

The different moisture levels used were obtained by keeping the samples enclosed in glass vessels with P_2O_5 or saturated salt solns at 20°C. Salt solns used were (relative humidity): P_2O_5 (0%), LiCl (15%), $CaCl_2$ (32%), $Ca(NO_3)_2$ (55%), NH_4NO_3 (63%), NaCl (76%), Na_2CO_3 (92%), $CuSO_4$ (98%) (Weast and Astle, 1979). Samples were weighed every two days until equilibrium was reached.

Acknowledgements

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Terpenoids from *Guarea rhophalocarpa*

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Abstract

Four new terpenes including, two sandaracopimaradiene diterpenoids, ent-8(14),15-sandaracopimaradiene-2α,18-diol, and ent-8(14),15-sandaracopimaradiene-2β,18-diol, and two lanostane triterpenoids, 23-hydroxy-5α-lanosta 7,9(11),24-triene-3-one, and 5α-lanosta-7,9(11),24-triene-3α,23-diol, were isolated from the methanolic extract prepared from the leaves of *G. rhopalocarpa* together with the known steroid stigmasterol and the coumarin, scopoletin. The isolates showed weak antiprotozoal activity against *Leishmania donovani* promastigotes, and *Trypanosoma brucei brucei* blood stream trypomastigotes, and were devoid of interesting activity towards *Plasmodium falciparum*. The isolates did not show significant cytotoxic activity against KB cells. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Guarea rhopalocarpa*; Meliaceae; Leaves; ent-8(14),15-Sandaracopimaradiene-2α,18-diol; ent-8(14),15-Sandacopimaradiene-2β,18-diol; 23-Hydroxy-5α-lanosta-7,9(11),24-triene-3-one, and 5α-lanosta-7,9(11),24-triene-3α,23-diol; *Leishmania donovani*; *Trypanosoma brucei brucei*; *Plasmodium falciparum*; KB cells

1. Introduction

The genus *Guarea* has 150 species of trees and shrubs in tropical America and 20 in Africa (Schultes and Raffouf, 1990); 8 of them occur in Panama (D'Arcy, 1987). Eight species of *Guarea* have been investigated chemically, and a number of secondary metabolites have been isolated including, sesquiterpenes, limonoids, and coumarins (Garcez et al., 1998; Jimenez et al., 1998), diterpenoids (Pereira et al., 1990; Furlan et al., 1996), tetranortriterpenoids (Akinniyi et al., 1980), triterpenes (Moutoo et al., 1992; Furlan et al., 1993), and steroids (Zelnik and Rosito, 1971). A number of *Guarea* species have been used in traditional medicine for the treatment of different diseases and their effects has been demonstrated experimentally. For example, *G. guidonia* used for rheumatism in Brasil showed antiinflammatory activity in rats (Oga et al., 1981) and antiviral effects in vitro (Simoni et al., 1996); *G. sepium* used as an antihelmintic drug in Brazil was active against the helminths, *Strongyloides stercoralis* and *Ancylostoma caninum* (Gilbert et al., 1972), and *G. trichilioides* used for the treatment of cancer in French Guiana, showed cytotoxic activity against P388 murine cell line (Lukacova et al., 1982). Furthermore, some limonoids have shown antileukemic (Pettit et al., 1983), and allelophathic (Cespedes et al., 1999) activities.

Guarea rhopalocarpa (syn. *Guarea tuisiana*) Radlkofer (Meliaceae) is a tree of lowland tropical rain forest and montane rain forest in Costa Rica and West and Central Panama. No phytochemical studies have been described for *G. rhopalocarpa*. In the search for antiprotozoal drugs from plants it was found that the methanolic extract of *G. rhopalocarpa* displayed activity on *Leishmania donovani* promastigote [$IC_{50} = 62.5$ μg/ml (IC95% = 70.7, 54.3)], *Trypanosoma brucei brucei* blood stream trypomastigote forms [$IC_{50} = 16$ μg/ml (IC95% = 27, 5)], *Plasmodium falciparum* [$IC_{50} = 89$ μg/ml (IC95% = 103, 75)], and KB cells [$IC_{50} = 32.9$ μg/ml (IC95% = 41.3, 24.1)], thus this species was studied further. This paper described the isolation and structural elucidation

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of the major active constituents of *G. rhopalocarpa*. The activity of isolates against *L. donovani*, *T. b. brucei* blood stream trypomastigote forms, *Plasmodium falciparum*, and KB cells was also determined.

2. Results and discussion

Air-dried powdered leaves of *G. rhopalocarpa* were extracted sequentially with methanol and water at room temperature. The concentrated methanolic extract was diluted with water and partitioned with $CHCl_3$ and *n*-BuOH. Each fraction was dried in vacuo, and tested for activity against *L. donovani* promastigotes. The activity of tested samples was recorded as the inhibitory concentration (IC_{50}) value, which is the concentration required of drug to cause 50% inhibition of parasite or cell growth compared with the untreated control. The chloroformic fraction showed the highest activity [IC_{50} = 45 μg/ml (IC95% = 50.8, 39.2)], whereas the methanolic [IC_{50} = 62.5 μg/ml (IC95% = 73.8, 51.2)], and the butanolic [IC_{50} = 300 μg/ml (IC95% = 311, 289)] fractions were less active, and the aqueous fraction was inactive [IC_{50} = 500 μg/ml (IC95% = 507, 493)] against *L. donovani* promatigotes. Further column chromatography of the active $CHCl_3$ fraction (8 g) led to the isolation of four novel active terpenoids (Fig. 1), including two diterpendiol compounds, ent-8(14),15-sandaracopimaradiene-2α,18-diol (**1**), and ent-8(14), 15-sandaracopimaradiene-2β,18-diol (**2**), and two lanostane triterpenoids, 23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one (**3**), and 5-lanosta-7,9(11),24-triene-3α,23-diol (**4**), together with the known steroid, stigmasterol (**5**, Rubinstein et al., 1976), and the coumarin, scopoletin (**6**, Murray et al., 1982). The identification of the novel compounds was achieved by NMR techniques using 1D and 2D experiments. The known substances were identified by comparison of their spectroscopic (^1H, ^{13}C, and EIMS) and physical (mp) data with literature.

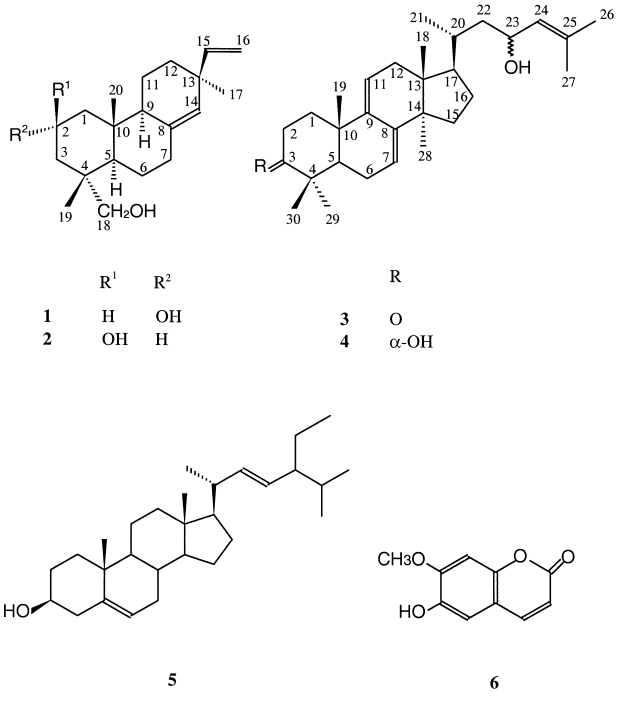


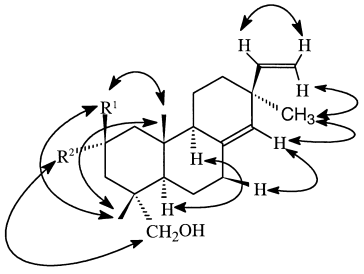
Fig. 1. Structures of compounds **1–6** isolated from *G. rhopalocarpa*.

Compound **1** was assigned the molecular formula $C_{20}H_{32}O_2$ (M^+, *m/z* 304) by a combination of high resolution MS and quantification of the number of methyl, methylene, methine, and quaternary carbon atoms revealed in the ^{13}C NMR and DEPT spectra (Table 1). The IR spectrum showed the presence of alcohol (3360–3340 cm^{-1}) and olefin groups (1640 cm^{-1}). The 1H NMR spectrum (Table 1) revealed the presence of three tertiary methyl groups (δ 1.04, 0.85, and 0.83 each, *s*, 3H), one AB system (δ 3.35 and δ 3.20, each, 1H, *d*, *J* = 11 Hz) of a primary alcohol group, one proton geminal (δ 3.86, *tt*, *J* = 11, 3.8 Hz) to a secondary alcohol, and four olefinic protons. The olefinic protons were attributed to a monosubstituted olefin, constituting one ABX system (δ 4.91 *dd*, H-16A; 4.87 *dd*, H-16B; and 5.77 *dd*, H-15X), and one signal to a trisubstituted double bond (δ 5.25, *brs*, H-14). The values at δ 129.35, 149 and 110.16 were assigned to C-14, C-15 and C-16, respectively in the ^{13}C NMR and HMQC spectra (Table 2). The location of the vinylic group at C-13 in the skeleton was determined by the HMBC spectrum. The above information was in agreement with a sandaracopimaradiene diterpenediol. Long-range heterocorrelations were observed for the following resonances: CH_3-17 vs C-14, C-15, C-13. Furthermore, the orientation of the substitutents at C-13 was determined by the NOESY spectrum. Cross-peaks observed for the following signals: H-15/H-12βec/H-16A, and CH_3-17/H-16B/H-14, clearly indicated one α-equatorial methyl group and one β-axial vinylic group at C-13 (Chamy et al., 1998). The position of the second double bond was determined by the vicinal coupling (*J* = 7 Hz) along the H-9 to H-11 bond observed in the ^1H NMR which was consistent with a non-chair C-ring containing the double bond between C-8 and C-14 (Van Puyvelde et al., 1987). This was confirmed by long-heterocorrelations observed for the CH_3-17 and C-14 in the HMQC spectrum, and the interactions observed for the following resonances: H-14/CH_3-17/H-7δ in the NOESY spectrum. The chair form of the B-ring with an exocyclic double bond was indicated by the vicinal axial-equatorial coupling *J* (ae) of 4 Hz and *J* (ee) of 2 Hz along the H-6/H-7 bond, and confirmed by NOESY spectrum (Fig. 2), which showed resonances for the following protons: H-5αax/H-9αax. The coupling constants for the proton geminal to the secondary alcohol group (δ 3.86, *tt*, *J* = 11, 3.8 Hz) at C-2; the existence of the fragment: –CH_2-CH(OH)–CH_2–, connected to quaternary

Table 1
^{1}H NMR, HMQC and HMBC data for compounds **1** and **2**[a]

	1				**2**			
Position	H	δ_H	HMBC	HMQC (H to C)	H	δ_H	HMQC	HMBC (H to C)
1	1-ax	0.98 *t* (11.8)	47.84	C-20	1-ax	1.55 *m*	45.10	C-20
	1-ec	2.06 *td* (11, 7.1)			1-ec	1.80 *m*		
2	2-ax	3.86 *tt* (11, 3.8)	65.05		2-ec	4.23 *tt* (8,4)	67.86	
3	3-ax	1.39 *dd* (11, 4)	44.20		2-ax	1.52 *m*	40.59	C-19
	-ec	1.6 *dd* (11, 4)			2-ec	1.74 *m*		
4	–	–	39.40		–	–	37.83	
5	5-ax	1.37 *m*	46.89		5-ax	1.38 *m*	46.69	C-19
6	6-ec	1.26 *m*	22.10		6-ax	1.38 *m*	22.92	
	6-ec	1.48 *m*			6-ec	1.55 *m*		
7	7-ax	2.11 *dd* (4, 2)	35.51		7-ax	2.11 *dd* (4.2)	36.04	
	7β-ec	2.24 *dd* (4, 2)			7β-ec	2.24 *dd* (4, 2)		
8	–	–	136.39		–	–	137.02	
9	9-ax	1.83 *t* (7.7)	50.51		9-ax	1.77 *m*	51.29	C-20
10	–	–	39.62		–	–	38.27	
11	11-ax	1.52 *m*	18.78		11β-ax	1.50 *m*	19.19	
	11-ec	1.63 *m*			11–ec	1.62 *m*		
12	12-ax	1.34 *m*	34.46		12-ax	1.36 *m*	34.84	C-17
	12-ec	1.49 *m*			12-ec	1.50 *m*		
13	–	–	37.45		–	–	37.59	C-17
14	14	5.25 brs	129.35		14	5.20 *brs*	129.49	C-17
15	15X	5.77 *dd* (17.48, 10.6)	149.00		15X	5.77 *dd* (17,10)	149.16	C-17
16	16B	4.87 *dd* (10, 1.48)	110.16		16B	4.87 *dd* (11, 1.48)	110.62	
	16A	4.91	dd (17, 1.48)		16A	4.91 *dd* (17, 1.48)		
17-CH_3	17-ec	1.04 *s*	25.92	C-13, C-14, C-15	17-ec	1.04 *s*	26.56	C-13, C-14, C15
18	18B	3.20 *d* (11)	71.28		18B	3.10	d (11) 71.61	C-19
	18A	3.35 *d* (11)			18A	3.30 d (11)		
19-CH_3	19-ax	0.85 *s*	16.50	C-3, C-4, C-5, C-18	19-ax	1.00 *s*	20.50	C-3, C-4, C-18
20-CH_3	20-ax	0.83 *s*	18.95	C-1, C-5, C-9, C-10	20-ax	1.07 *s*	18.88	C-1, C-9, C-10

[a] Coupling constants (*J* in Hz). Assignments were based on DEPT, ^{1}H–^{1}H COSY, HMQC, HMB, and NOESY experiments. TMS was used as internal standard.



	R^1	R^2
1	H	OH
2	OH	H

Fig. 2. NOESY correlations for compounds **1** and **2**.

carbon atoms at both ends in the ^{1}H–^{1}H COSY spectrum; together with the interactions observed for the proton geminal to the secondary alcohol group with CH_3-19 and CH_3-20 in the NOESY spectrum were consistent with an α-equatorial hydroxyl group located at C-2 in a chair-ring A. The couple of doublets of the AB system (δ 3.35 and 3.20, each, 1 H, *d*, $J = 11$ Hz) in the ^{1}H NMR spectrum, and the long-range heteronuclear correlations displayed for the following signals: CH_3-19 vs C-4, C-3, C-5, C-18; CH_3-20 vs C-1, C-5, C-9, C-10 in the HMBC spectrum were consistent with an α-equatorial C-4 hydroxymethyl group (Chamy et al., 1998). ^{1}H and ^{13}C NMR chemical shifts of compound **1**, fitted extremely well with those of 8(14),15-sandaracopimaradiene-2α,18-diol (Van Puyvelde et al., 1987), mp 203–204°C, $[\alpha]_D$-21.7, except for optical rotation, although of similar magnitude, were of opposite sign, in **1**, mp. 156°C, $[\alpha]_D + 11.36$. On the basis of these observations, the compound **1** was identified as ent-8(14),15-sandaracopimaradiene,2α,18-diol.

The compound **2** had a molecular formula of $C_{20}H_{32}O_2$ (M^+ 304 *m*/*z*, HRMS) the same as the compound **1**. Its 1D and 2D NMR spectral features were almost identical to **1** (Table 1) except for the chemical shift and coupling constants observed for the proton geminal to the secondary alcohol group (δ 4.23, *tt*, $J = 8,4$ Hz, in **2**; δ 3.86, *tt*, $J = 11$, 3.8 Hz, in **1**) at C-2 and the chemical shifts of the tertiary methyl groups CH_3-19 (δ 1.0 in **2**, δ 0.85 in **1**), and CH_3-20 (1.07 in **2**, in 0.83 **1**) in the ^{1}H NMR spectrum. Furthermore, differences were also observed in the chemical shifts of carbons C-1 to C-5, CH_3-19 and CH_3-20 in the ^{13}C NMR spectra. The NOESY spectrum (Fig. 2) showed interactions for the

Table 2
^{1}H- and ^{13}C NMR data in $CDCl_3$ of **3** and **4**[a]

Position	**3** δ_H	**4** δ_H	**3** δ_c	**4** δ_c
1	–	–	34.24	34.80
2	2.71 td (16.7)	1.9, 1.06 each, *m*	38.87	34.83
3	–	3.46 *brs*	217.33	76.30
4	–	–	51.39	50.36
5	2.36 *m*	2.36 *m*	50.96	48.43
6	1.80 *m*	1.55–2.0 *m*	24.77	23.91
7	5.28 *m*	5.25 *m*	118.49	118.13
8	–	–	146.21	145.98
9	–	–	146.08	145.85
10	–	–	35.31	34.80
11	5.32 *d* (3)	5.28 *d* (3)	101.82	101.44
12	2.0 *m*	2.20 *m*	35.50	34.83
13	–	–	44.10	43.67
14	–	–	48.27	48.43
15	–	–	31,95	33.78
16	–	–	27.78	27.34
17	–	–	52.76	50.56
18-CH_3	0.80 *s*	0.91 *s*	24.94	23.00
19-CH_3	1.04 *s*	0.85 *s*	25.02	25.00
20	–	–	27.68	27.32
21-CH_3	1.02 *d* (6.4)	0.98 *d* (6.4)	48.07	45.14
22	2.15 *m*	2.15 *m*	40.04	44.62
23*	4.87 *m*	4.78 *m*	74.37	73.90
	4.92 *m*	4.82 *m*	76.14	75.66
24	5.14 *brd* (7)	5.13 *brd* (7)	128.29	128.06
25	–	–	137.36	136.78
26-CH_3	1.70 *d* (0.6)	1.72 *d* (0.6)	26.92	25.84
27-CH_3	1.68 *d* (0.6)	1.70 *d* (0.6)	18.20	18.32
28-CH_3	1.00 *s*	0.77 *s*	13.15	12.92
29-CH_3	1.10 *s*	0.91 *s*	21.97	21.77
30-CH_3	0.90 *s*	0.92 *s*	22.99	27.77

[a] Coupling constants (*J* in Hz). Assignments were based on DEPT, ^{1}H–^{1}H COSY, and HMQC experiments. TMS was used as internal standard. *C-23 epimers.

following signals: H-2αec/CH_2OH-18, which were consistent with an β-axial configuration of the hydroxyl group at C-2. On the basis of the spectral evidence the compound **2** was identified as ent-8(14),15-sandaracopimaradiene-2β,18-diol.

Compound **3** gave a molecular peak at *m*/*z* 438 ($C_{30}H_{46}O_2$), and showed absorptions in the UV spectra at 250, 242, 235 nm (MeOH) characteristic of the presence of a transoid heteroannular diene moiety. The IR spectrum showed absorptions typical of alcohol (3400 cm^{-1}), and keto (1700 cm^{-1}) groups. Analysis of NMR data (Table 2), indicated that **3** was a lanostane type triterpenoid (De Pascual et al., 1987; Shiao, et al., 1988; Gewali et al., 1990; Furlan et al., 1993), possessing a heteroannular diene moiety, one allylic alcohol group and one keto group. The ^{1}H and ^{13}C NMR spectra showed the presence of five tertiary methyl [δ_H 0.80, 0.90, 1.0, 1.10, and 1.04 (each, *s*, 3H)], one secondary methyl (δ_H 1.02, *d*, $J = 6.4$ Hz), and two olefinic methyl (δ_H 1.70, 1.68) groups. Furthermore, one triplet of doublets (δ_H 2.71; δ_c 38.87) characteristic of the axial H-2 proton, when a keto group is present at C-3 (δ_c 217.33); and three olefinic (δ_H 5.14, 5.28, and 5.32; δ_c 128.29, 118.48, and 101.82) protons, were also observed in the NMR spectra. The signals due to one proton geminal to an allyl secondary hydroxyl group were observed and these were duplicated (δ_H 4.87, 4.92; δ_c 74.37,76.14), indicating that **3** was a mixture of C-23 epimers. This was confirmed by GC–EIMS analysis which showed a mixture of two epimers at a ratio 8:2. The EIMS of **3** exhibited two abundant mass fragments, one at *m*/*z* 311 $[M\text{-}C_8H_{15}O]^+$ due to loss of the side chain, and another fragment at *m*/*z* 297, which represents further loss of 14 amu due to the loss of one tertiary methyl group. Cross-peaks were observed for 26-CH_3/27-CH_3/H-24 in the ^{1}H–^{1}H COSY spectrum. Futhermore, H-24 was in turn coupled to the secondary alcohol at C-23 (δ_H 4.87, 4.92), which in turn was coupled to the H-22 (δ 2.15 m). This indicated that the isolated double bond in the side chain was at C-24 position. On basis of the above information **3** was identified as the lanostane triterpenenoid 23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one.

The compound **4** (*m*/*z* 440, M^+, $C_{30}H_{48}O_2$), showed UV absorptions at 235, 243, 251 nm, of a heteroannular diene moiety similar to compound **3**. The IR spectrum showed alcohol (3400 cm^{-1}), and olefinic groups (1620, 1640 cm^{-1}). Comparison of NMR spectra of **4** with **3** showed that the chemical shifts and coupling patterns for the olefinic protons, H-7, H-11, and H-24, were almost identical in both terpenes. However, the ^{1}H NMR spectrum of **4** showed a broad singlet at δ_H 3.46 (δ_c 76.30) of one α-hydroxyl group at C-3 instead of the carbonyl signal (δ_c 217.33) observed in **3**. Furthermore, in the ^{1}H–^{1}H COSY spectrum was observed cross-peaks for the following signals: H-2/H-3, H-6/H-7, H-11/H-12; and H-23/H-24. The duplicity of the signals for the proton geminal to a secondary hydroxyl group C-23 (δ_H 4.78, 4.82; δ_c 73.90, 75.66), indicated that **4** was a mixture of C-23 epimers (1:1 ratio), this was confirmed with a GC–EIMS analysis. On the basis of the above information the compound **4** was identified as the lanostane triterpenoid, 5α-lanosta-7,9(11),24-triene-3α,23-diol.

Table 3 shows the in vitro antiprotozoal activity and cytotoxic activities exhibited by the compounds **1–6** isolated from *G. rhopalocarpa*. The activity observed for the terpenoids **1–4** against *L. donovani* promastigotes, indicates that these compounds are partially responsible for the activity exhibited by the active $CHCl_3$ fraction ($IC_{50} = 45$ μg/ml). Although the antiprotozoal activity of tested compounds was no comparable to the standard drugs. It is important to point out that the stereochemistry of the hydroxyl group at C-2 have influence in the antileishmanial activity of sandaracopimaradiene diterpenediols **1** and **2**, with **1** being 3.3 times more active than **2** against *L. donovani* promastigotes. However,

Table 3
Antiprotozoal and cytotoxic activities of **1–6**

Samples	IC_{50} μM (IC95%)[a]					Selectivity index			
	L. donovani		*T. b. brucei*	*P. falciparum* K1	KB cells	*L. donovani*		*T. b. brucei*	*P. falciparum*
	Promastigotes	Amastigotes	Blood stream trypomastigotes			Promastigotes	Amastigotes		
1	16.8 (23.8, 9.8)	> 90	> 30	166 (177.8, 155.4)	48 (53.4, 42.6)	1.12	–	–	0.29
2	49.7 (56.2, 43.2)	> 90	> 30	104 (114.9, 93.5)	75.8 (82, 68.8)	1.52	–	–	0.73
3	7.2 (10.3, 4.1)	74.4 (87.4, 61.4)	5 (8.9, 1.1)	95 (103.2, 86.8)	30.2 (26, 34.2)	4.19	0.40	6.04	0.32
4	20 (25.1, 19.9)	20 (22.3, 17.7)	1.75 (1.88, 1.62)	69.6 (76.7, 62.5)	21.2 (25.4, 17)	1.06	1.06	12.11	0.30
5	> 1209	> 90	> 30	> 1209	> 1272	–	–	–	–
6	374 (385, 331)	> 90	> 30	ND	130.2 (139.5, 121)	0.35	–	–	–
Standard drugs									
Pentamidine	0.40 (0.52, 0.28)	ND	0.00034 (0.0004, 0.00028)	ND	0.17 (0.186, 0.154)	0.42	00	500	–
Pentostam	500 (519, 481)[b]	9.75 (15.87, 3.63)[b]	ND	ND	332 (351.6, 313.6)	0.66	34.90	–	–
Chloroquine	ND	ND	ND	0.59 (0.668, 0.492)	160 (177.8, 143.4)	–	–	–	272.20
Podophyllotoxin	ND	ND	ND	ND	0.015 (0.0159, 0.0141)	–	–	–	–

[a] The IC_{50} values and IC95% for each sample were calculated by linear regression analysis using x/fit, MS Excel statistical program. Each sample was tested three times in different occasions.
[b] Values are given in μg/ml.
ND : not determined.

both compounds **1** and **2** were inactive towards *L. donovani* amastigotes and *T. b. brucei* blood stream trypomastigotes. On the other hand, comparison of the lanostane triterpenoids **3** and **4** showed that the presence of a ketone group at C-3 enhances the activity against *L. donovani* promastigotes, with **3** being 2.3 times more active than **4** which possess an hydroxyl group at C-3. However, the presence of a hydroxyl group at C-3 enhances the activity against *L. donovani* amastigotes and *T. b. brucei* blood stream trypomastigotes, with **4** being more active than **3**. To our knowledge there is no record in the literature of antiprotozoal activity of *Guarea* sp., sandaracopimaradiene diterpenediol and lanostane triterpenoids. The antimalarial, antileishmanial and Chagas' disease activities for the coumarin, scopoletin (**6**) have been reported recently and are in agreement with our results (Bravo et al., 1999).

The cytotoxicity of compounds **1–6** against KB (human epidermoid carcinoma of the nasopharynx) cells was also determined to estimate the selectivity of these compounds to the parasites versus mammalian cells. KB cells are known to exhibit an intermediate sensitivity to a large number of cytotoxic agents compared with other tumor cell lines (Angerhofer et al., 1999). Table 3, shows that the selectivity index (SI) values of pentostam (34.9), pentamidine (500), and chloroquine (272.2) against *L. donovani*, T. *b. brucei* and *P. falciparum* were more favourable than the values calculated for compounds **1–6** (0.29–12.11). However, the SI of pentamidine (0.42) was less favourable to the values calculated (1.06–4.19) for compounds **1–4** against *L. donovani* promastigotes. It is possible that these compounds could represent templates, for the producing of semisynthetic derivatives with higher selectivity towards *L. donovani* amastigotes and *T. brucei* blood stream trypomastigotes. These studies may also provide valuable insight into new mechanism of antileishmanial and antitrypanosomal action or structure-activity relationships. Finally, it is possible that other active constituents could be present in lower concentration in the plant material which could contribute with the antiprotozoal activity of this plant, and there may be some synergistic effects exerted by compounds **1–6** when present in the plant extract.

3. Experimental

3.1. Plant material

Leaves of *Guarea rhopalocarpa* were collected from Fortuna, Chiriqui Panama, Panama, in February 1991. Prof. Mireya Correa, from the Department of Botany, Faculty of Natural Science identified the material. A Voucher specimen (CIFLORPAN 763 PMA) has been deposited in the Herbarium of the University of Panama, Panama City, Republica de Panama.

3.2. Extraction and isolation

Air-dried and powdered leaves (650 g) of *G. rhopalocarpa* were extracted sequentially with methanol and water at room temperature. The H_2O extract was lyophilized and the MeOH extract was concentrated to dryness under reduced pressure. The concentrated methanolic (30 g, 4%) extract was diluted with water and partitionated sequentially with $CHCl_3$ and *n*-BuOH. Each fraction was concentrated in vacuo, and evaluated for in vitro activity against *L. donovani* promastigotes. The chloroformic fraction (8 g) showed the strongest activity ($IC_{50}=45$ μg/ml) and was subsequently fractionated in column chromatography over silica gel (70–230 mesh, 200 g) eluted with a gradient of hexane-acetone (from 100:0 to 20:80). A total of 190 fractions (100 ml each) were collected and analysed by TLC. The fractions were combined according to their TLC profiles, dried in vacuo and evaluated for antileishmanial activity. The most active fractions (F: 7-15) were further chromatographed separately on silica gel column chromatography eluted with a gradient of hexane-acetone (from 100:0 to 20:80). Six compounds were obtained the terpenoids, ent-8(14),15-sandaracopimaradiene-2α,18-diol (**1**, 45 mg, 0.0069%), ent-8(14),15-sandaracopimaradiene-2β,18-diol (**2**, 35 mg, 0.0053%), 23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one (**3**, 63 mg, 0.0096%), 5α-lanosta-7,9(11),24-triene-3α,23-diol (**4**, 100 mg, 0.015%), the steroid, stigmasterol (**5**, 22.6 mg, 0.0034%), and the coumarin, scopoletin (**6**, 25.12 mg, 0.0038%). Final purification of the individual constituents was achieved by Sephadex LH-20 column chromatography eluted with acetone. TLC plates were performed on precoated aluminium sheets (silica gel 60 F_{254}, 0.25 mm). Developed TLC plates were observed under UV light (254/365 nm) and then sprayed with 1% vanillin–5% H_2SO_4 reagent followed by heating at 100°C.

3.3. Spectroscopy

^1H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT, ^1H–^1H COSY, NOESY, HMQC, and HMBC spectra were recorded in a Bruker AMX-400 instrument. EIMS was measured on a VG-Analytical ZAB IF instrument. GC–MS spectra: samples were dissolved in $CHCl_3$, and injected into the column GC1 (Poly diethyl silane) in the following conditions: Length 25 m; internal diameter 0.25 mm, injector temp. 250°C; interface temp. 310°C; carrier gas helium; flow rate 1 cm^3 min^{-1}; head pressure 10 lbs $sqin^{-1}$, initial temp. 125°C; 2nd temp. 175°C; 3rd temp. 220°C; final temperature 300°C. IR spectra were determined on a Perkin Elmer 841 spectrometer. UV spectra were obtained on a Perkin Elmer 402 Ultraviolet-Visible Spectrophotometer using spectroscopic grade methanol. The optical rotations were determined on a Bellinghmam and Standley P 506 polarimeter at $\lambda=584$ nm and 26.6°C. Melting points were determined in Stuart scientific SMPI melting point apparatus and are uncorrected.

3.3.1. Ent-8(14),15-sandaracopimaradiene-2α,18-diol (*1*)

Colourless crystals (methanol), mp. 182°C. $[\alpha]_D+8.77$ ($CDCl_3$, *c* 0.001). IR *v* max (KBr) cm^{-1}: 3360–3340, 2980, 1640, 1450, 1380, 1090. HRMS: 304.243200 (calcd. 304.240231 $C_{20}H_{32}O_2$). EIMS *m/z* (rel. int.): 304 $[M]^+$ (4), 286 (33), 271 (16), 255 $[M\text{-}H_2O\text{-}CH_2OH]^+$ (36), 241 (16), 227 (3), 213 (5), 199 (7), 187 (100), 173 (12), 159 (16), 145 (21), 133 (37), 121 (83), 105 (64), 91 (72), 79 (44), 67 (27), 55 (63). NMR data see Table 1.

3.3.2. Ent-8(14),15-sandaracopimaradine-2β,18-diol (*2*)

Colourless crystals (methanol), mp. 156°C. $[\alpha]_D+11.36$ ($CDCl_3$, *c* 0.001). IR *v* max (KBr) cm^{-1}: 3360–3340, 2989, 1640, 1450, 1390, 1100. HRMS: 304.243200 (calcd. 304.240231 $C_{20}H_{32}O_2$). EIMS *m/z* (rel. int.): 304 $[M]^+$ (43), 289 (14), 286 (10), 273 (100), 255 $[M\text{-}H_2O\text{-}CH_2OH]^+$ (86), 187 (34), 175 (26), 159 (17), 148 (26), 133 (46), 121 (87), 105 (72), 91 (96), 79 (56), 67 (39), 55 (98). NMR data see Table 1.

3.3.3. 23-Hydroxy-5α-lanosta-7,9(11),24-triene-3-one (*3*)

Colourless needles (methanol), mp. 95°C. $[\alpha]_D$ −32.25 ($CHCl_3$, *c* 0.01). UV λ max nm: 250, 242, 235 (MeOH). IR *v* max (KBr) cm^{-1}: 3400, 2980, 1700, 1450, 1380, 1050, 760. HRMS: 438.337000 (calcd 438.334131 $C_{30}H_{46}O_2$). EIMS *m/z* (rel. int.): 438 $[M]^+$ (5), 436 (55), 421 (100), 393 (34), 355 (7), 341 (32), 311 (18), 297 (40), 273 (18), 253 (16), 245 (11), 227 (12), 201 (12), 199 (10), 189 (6), 187 (17), 163 (22), 159 (16), 150 (62), 148 (42), 135 (15), 123 (29), 105 (28), 95 (45), 91 (12), 81 (41), 67 (15), 55 (11), 41 (18). NMR data see Table 2.

3.3.4. 5α-Lanosta-7,9(11),24-triene-3α,23-diol (*4*)

Colourless needles (MeOH), mp. 105°C. $[\alpha]_D$ −20 ($CHCl_3$, *c* 0.01). UV λ max nm: 235, 243, 251. IR *v* max (KBr) cm^{-1}: 3400, 2980, 1620, 1640, 1440, 1380, 1220, 1060, 1000, 750. HRMS: 440.347000 (calcd 440.349781 $C_{30}H_{48}O_2$). EIMS *m/z* (rel.int.): 440 $[M]^+$ (25), 438 (76), 423 (28), 405 (30), 387 (6), 351 (3), 338 (4), 323 (6), 313 (16), 299 (10), 281 (9), 255 (14), 229 (7), 213 (7), 187 (10), 175 (10), 150 (100), 148 (38), 107 (41), 95 (48), 81 (18), 55 (14), 43 (12). NMR data see Table 2.

3.4. Biological assays

3.4.1. Antiprotozoal activity

L. donovani (Strain MHOM/ET/67L82) promastigotes were cultured in RPMI 1640 and 10% heat inactivated foetal calf serum, at 26°C. Each compound was

diluted in a two-fold dilution in a 96-well microtitre plate in triplicate at each concentration. Each well contained 100 μl of the diluted sample and 2×10^5 promastigotes (100 μl). After 72 h the number of live parasites in each well was counted on a Neubauer haemocytometer and compared with untreated controls. Pentamidine was used as standard drug. The test against *L. donovani* amastigotes test was carried out as previously described (Yardley et al., 1996). Pentostam was used as standard drug. *Trypanosoma brucei brucei* (S427) blood stream trypomastigote forms were maintained axenically in HMI-18 medium with 20% heat inactivated foetal calf serum at 37°C in 5% CO_2 air mixture. The samples were diluted in a 96-well microplate in a two fold dilution series with three replicates for each concentration. Each well received 100 μl of diluted sample and 2×10^4 trypanosomes (100 μl). The plates were covered and incubated at 37°C in 5% CO_2-air mixture. After 72 h live parasites were counted on a haemocytometer and compared with untreated control. Pentamidine was used as standard drug. The antiplasmodial activity was determined against the multidrug resistant strain K1 of *P. falciparum* (K1) using the methodology described previously (Ekong et al., 1990). Chloroquine diphosphate was used as a standard drug.

3.4.2. Cytotoxic activity

The KB cell assay was based on the method previously reported by Anderson et al. (1991). Podophyllotoxin was used as drug standard.

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Phytochemistry 56 (2001) 211–213

PHYTOCHEMISTRY

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Book reviews

Secondary Plant Products. Antinutritional and Beneficial Actions in Animal Feeding

John C. Caygill, Irene Mueller-Harvey (Eds.), Nottingham University Press, 1999, ISBN: 1-897676-28-X, £25

On the first of September in 1998 a meeting was held at the University of Reading to consider secondary compounds and their possible effects on animal nutrition. Obviously this was (and still is) a timely issue as farmers move back to feeding plant products to livestock.

The chapters in the book fall into two major categories, the first of which considers individual classes of compound and their biological significance. Therefore review articles cover tannins (Irene Muellar-Harvey), tannins and effects on non-ruminants (Vernon) and effects of dietary tannins on ruminant livestock (Butter, Dawson and Buttery). Plant protease inhibitors and plant lectins are both comprehensively reviewed by Grant. These chapters are a particularly nice synthesis of work in these clearly defined areas and in many cases give a good animal perspective on plant chemistry. Review chapters are well referenced and offer students and scientists a convenient route into an understanding of research in these complex disciplines.

The other chapters in the book deal with wider issues such as an overview of antinutritional factors in higher plants (Harborne) and an interesting chapter by Fox and Domoney entitled 'Plant Breeding: what is being, can be and should be done'. If I were to have a criticism of this book it is that much descriptive work is included but it is difficult to get a view as to future research directions in what is clearly an important discipline in agricultural research. One need only consider the current programmes of work on analysing the effects of secondary products, functional foods and human nutrition to realise the level of interest in plant chemistry that is developing within the scientific community as a whole.

This book does not cover generic topics such as how should we define an individual plant component as beneficial, deleterious or neutral for particular nutritional applications; what level of evidence is required for defining optimal levels of end products in animal feedseeds or forages; and how do we structure multidisciplinary research to answer these critically important questions? Nevertheless I can warmly recommend this reasonably priced text as an introduction to secondary metabolism and animal feeding and it will certainly be used as a source text by myself and by members of my laboratory.

Mark P. Robbins
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Arabidopsis, A Practical Approach

Edited by Z.A. Wilson, Practical Approach Series, Oxford University Press, Oxford, UK, 2000, 296 pp., ISBN 0-19-963564-1, £32.50

The "tentacles" of Arabidopsis are spreading throughout all areas of plant science. While some researchers have been reluctant to assimilate what appeared to them as an invasive young newcomer, most have agreed that the little crucifer brings to plant biology the power of genetics in a most accessible way. Phytochemistry is not an exception to this. The discovery that auxin biosynthesis can take place without its once thought-off key precursor, tryptophan, was due to the analysis of an Arabidopsis mutant. Similarly, brassinosteroids, now regarded as important plant growth regulators, lingered at the edges of mainstream research, until Arabidopsis cabbage-type mutants showed the dramatic consequences of their deficiency. These are just examples of the prospects opened by Arabidopsis classical genetics. And in reality there is today much more to the little thale cress than just classical genetics. "Metabolome" analysis is just one of the new words and areas starting to be developed in the genome era.

The present volume, put together by Dr. Zoe Wilson, at the University of Nottingham, in the well known "Practical Approach" series, is therefore a most timely resource for those trying to incorporate this model species into their work, as well as for those already using it.

The volume is composed of ten chapters, written by British specialists of the highest international standing in each of the areas, plus a complement of other European and American groups. The first two chapters are written by the curators of the two international Arabidopsis stock centres, and provide detailed information on the handling of both live plant and nucleic acid resources, available from them. The two following chapters deal with the analysis of mutants, from visible to gene-expression phenotype, and the mapping of both identified genes (mutants in the region can give a clue to function) and unknown genes identified by mutation. Cytogenetics and Arabidopsis transformation, both transient and stable, are covered in the following chapters, which also deal with the use of protoplasts and reporter genes. The next two chapters introduce the identification of genes from mutants. They also explain the use of collections of mutagenised plants to identify knock-out plants in genes/pathways of interest, what is known as reverse genetics, from gene to phenotype. These "tag"-mutagenised plants can also be used in classical, forward genetics, allowing a much quicker route from phenotype to gene. The final two chapters describe key concepts and the current state of genome projects, as well how to fully exploit them using internet resources.

The book, in the "Practical Approach" tradition, is full of simple, but descriptive protocols which, where examined in some detail, I could find easy to follow on the bench. There have been, to my knowledge, two previous Arabidopsis "methods" volumes. This one obviously provides the most up-to-date information, but in a compact format. It is also in some respects complementary: the authors' interests inevitably transpire, and the chapters on mutant characterisation, the genome initiative, or the use of web-based resources, are highly original. Inevitably, some of them will be extremely difficult to keep up to date for long, and in fact many of the internet resources have since become accessible through a single, new US-based entry point, www.arabidopsis.org (how did nobody think of this before?).

This excellent team effort should help make Arabidopsis an even more accessible resource for researchers of all walks of modern plant science.

Enrique López-Juez
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Medicine Quest: In Search of Nature's Healing Secrets
By Mark J. Plotkin, Published by Penguin Group, ISBN 0-670-86937-6, price $22.95

I have not had the pleasure of reading Mark Plotkin's earlier book, *Tales of Shaman's Apprentice*. As a scientist, had I seen this title on the shelves I would probably have passed it by but now, having read *Medical Quest*, I think this would have been a mistake.

The authors of most science texts write with a specific readership in mind. Dr Plotkin, however, has produced a book which will provide most readers with something of significant interest.

Yes, an initial scan of the Index, which goes from "ABT-594" to " zoopharmacognosy" via "iso-hydrohistrionicotoxin", is guaranteed to frighten all but the most dedicated 'boffins'. However, the curious should read further and establish that there is a wealth of fascinating, anecdotic, 'connective tissue' which links the various facets of the serious comment that the author makes about the treasures and potential treasures of the natural world.

The essential case for protecting biodiversity for medical purposes is made obvious and early in the text. However, it is doubtful whether most readers will fully appreciate the full complexity of the biosphere and the massive range of chemical structures that its occupants have produced, mainly for their own protection. Dr Plotkin does this. The problem, now, is to be able to exploit these materials for the benefit of the developed and developing worlds with the minimum of environmental damage. The battle against age-old diseases and future surprises will not go away, indeed the capacity for microbial counter attacks against an ever advancing medical front are probably without end. We need new items for our chemical armoury and the plant and animal worlds will provide us with compounds and ideas that we have not dreamed of — amazing products of evolution which have allowed living organisms to survive in unsatisfactory environments for many millennia.

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PHYTOCHEMISTRY

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The reader is led gently into each chapter with entertaining, largely factual material but, be warned, the topics that follow may not be for those with a nervous disposition. This reviewer (perhaps in second childhood) particularly enjoyed the chapters entitled "The Fungus Among Us" and "Hideous Healers"- with "*Cordyceps* (lying menacingly) quiescent on the forest floor ..." and descriptions of modern developments in leech and maggot therapy.

This is a well-written book of the right length with a serious message It contains a wealth of information, backed by an excellent bibliography, which will refresh scientific thought and entertain those who tend to avoid it. For the latter, perhaps a glossary would have been helpful and edifying?

Definitely a book for the coffee table or the library stacks.

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PERGAMON

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PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Announcement

Plant Biochemistry Summer Course 2001

The Institute of Biological Chemistry will conduct an advanced training course in plant biochemistry at Washington State University from July 8–21, 2001. The summer course, which has been offered every two years since 1995, is supported by grants funds provided by the National Science Foundation and the Department of Energy. The course is designed to teach fundamental principles and recent advances in areas including structure-function of plant organelles; ion transport; photosynthesis; carbohydrate and lipid biosynthesis and metabolism; nitrogen fixation and amino acid metabolism; synthesis, catabolism, physiology and mechanisms of plant hormones; synthesis and catabolism of unique plant products such as terpenoids, alkaloids, cyanoglucosides; phenylpropanoids; signaling mechanisms in plant defense responses; metabolic regulation; protein synthesis and intracellular targeting of macromolecules. The course is intended for graduate students, postdoctorals, faculty, and industrial scientists, and will include instruction by approximately 40 internationally-recognized plant scientists. Funds have been allocated to partially offset travel expenses for up to 65 participants. For further information and an application form, contact Ms. Karen Maertens, Institute of Biological Chemistry, 285 Clark Hall, PO Box 646340, Washington State University, Pullman, WA 99164-6340, USA. Tel.: +1-509-335-5496; fax: 509-335-7643; e-mail: maertens@wsu.edu.

Plant Biochemistry Summer Course 2001
8–21 July
Institute of Biological Chemistry
Washington State University
Pullman, WA 99164, USA
Contact: Karen Maertens (maertens@wsu.edu) or visit the website at
HTTP://www.ibc.wsu.edu/events/2001_course.html

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Appreciation and communications in honour of the retirement of Jeffrey Harborne FRS as Editor of *Phytochemistry*



Jeffrey Harborne (third from left, front row) with friends and colleagues, Kew 22 July 1999.

This issue is dedicated to the occasion of the retirement of the founding Editor of *Phytochemistry*, Jeffrey Harborne FRS. Jeffrey remains as Honorary Editor, and has provided a section on the history of *Phytochemistry*. His historical review is followed by four appreciations of his contributions to natural product chemistry and by three major reviews written in his honour. These communications were presented at a festschrift held in July 1999 at Kew Gardens, London. This was a fitting venue for such a celebration by Jeffrey's many friends and colleagues.

The Editors and members of the Editorial Board also wish to show their appreciation as follows:

Neil Towers, long serving member of the Editorial Board writes, "Jeffrey Harborne's infectious bonhomie and natural ebullience do not suggest a person totally committed to the science of plants. His name is synonymous with phytochemistry and particularly the documentation of the tremendous advances in phytochemistry over the last 40 or 50 years. Phytochemistry or the chemistry of plants is a term coined by him and by the late Tony Swain, an equally erudite colleague whose untimely death left a gap in the ranks of scholars seeking to understand the wealth of organic chemistry presented by plants. I first met these two wonderfully enthusiastic phytochemists and Bate-Smith on a bed (the rooms were sparsely furnished), imbibing adult beverages, in one of the chambers at Cambridge University during the first meeting of the Plant Phenolics Group in 1957. The mysteries of many biochemical pathways, particularly the phenylpropanoid pathway, were unraveling and there was an air of great excitement surrounding the biosynthesis of coumarins, isoflavonoids, lignans etc. Jeffrey had already accumulated a storehouse of knowledge in phytochemistry and was absolutely familiar with details of plant biochemistry and the chemical interrelationships of plants with the animal and fungal kingdoms. And he started writing soon after. His knowledge of the cinnamate pathway, especially as it related to flavonoids, and the significance of these compounds in taxonomic relationships of plants was of great interest to him and he has made and

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continues to make very important contributions to this subject. In fact Jeffrey Harborne could be named Monarch of the Flavonoids! His clear and precise style of scientific writing as well as his vast accumulation of knowledge has made him one of the most important and interesting of human data bases, available to students and advanced scholars, of phytochemistry and chemical ecology. His very many books in these areas including chemical ecology in relation to plants are still tremendously important and popular because they present information absolutely free of jargon and eminently readable.''

Norman Lewis: ''Although I had been very aware of Jeffrey Harborne and his enormous scientific contributions for many years, I did not actually meet him until the Groupe Polyphénol meeting that was held in Peterborough, Ontario, Canada, in 1988. I have wonderful memories of both that meeting and of Jeffrey in particular. Every morning both Jeffrey and myself essentially showed up first for breakfast, and I quickly learned from those morning conversations the depths of Jeffrey's alertness and recall. Jeffrey was able to recollect, in quite extraordinary detail and accuracy almost every aspect of the meeting, the discussions of the participants, and their activities as the conference progressed. It was most impressive. Over the years that followed, it has also never ceased to amaze me how many scientific projects Jeffrey was involved in whether as a scientist or in an Editorial capacity''.

Dieter Strack says ''Jeffrey was the one who inspired my interest in phytochemistry and biochemical ecology. His reviews and papers in *Phytochemistry* were my prime reading as a PhD student and I vividly remember the day in the early 80s when I received the first letter from Jeffrey on acceptance of a manuscript which I had submitted to *Phytochemistry*. I must admit it made me proud that Jeffrey accepted my work. Twenty years later, being one of the Regional Editors of Phytochemistry, I feel obliged to do the best I can to keep the high standard of 'Jeffrey's journal'.''

Paul Bolwell says ''I had already heard about Jeffrey as an undergraduate in both my plant biochemistry and chemotaxonomy courses for my botany degree which I took in 1969. My mentor, Vernon Butt, known to many of you, constantly referred to his work and so indirectly Jeffrey stimulated me to think of a career in 'green medicine' at that time. Eventually my career took a different path into biochemistry and molecular biology. Nevertheless, it was fascinating to learn at a lunch recently the revelation from Vernon and Jack Pridham, who was my previous head of department at Royal Holloway, that they were all near contemporaries with Jeffrey in the same laboratory in Bristol. Thus there is a continuity of sorts that adds to the privilege to follow on from Jeffrey as the UK-based Editor and eventually do some green medicine in a hands-off way!''

Tom Mabry adds *with great admiration* ''Jeffrey's phytochemical expertise, especially in chemical, evolutionary and ecological aspects of flavonoids and other phenolics, has fascinated me for over 35 years, and I have had the pleasure of having Jeffrey with me in Texas as a Visiting Professor''.

Jonathan Gershenzon also adds ''All of us at the newly-established Max Planck Institute for Chemical Ecology are aware of the great debt we owe to Jeffrey Harborne. Without his enormous contributions as a researcher, teacher, author, and Editor who championed the idea that plant secondary metabolites do have important functions in the natural world, our institute would simply not exist in its present form.''

Toni Kutchan endorses this, ''Jeffrey was one of the early pioneers of chemical ecology. He was a believer in a biological role for plant secondary metabolites at a time when this class of compounds was largely viewed as ''plays of nature'' and waste products. By co-founding *Phytochemistry*, he provided an internationally recognized vehicle for publication of experimental results obtained in the area of plant natural product research. He effectively paved the way for those of us who have followed in this field and time has shown his early views to be absolutely correct. Jeffrey has our admiration and respect''.

Ulrich Matern wishes to say that ''In the beginning of my career Jeffrey Harborne appeared to me as a ''jack-in-the-box'': whenever I tried to screen the literature for particular phenylpropanoids, his name sprang out sooner or later. This situation has basically remained unchanged, but after having met him personally years ago I am also grateful for his tremendous expertise and personal advice. Much later, I learned to appreciate additional features of his personality, among others the extraordinary talent to honour and summarize the merit of colleagues, including my PhD supervisor Hans Grisebach, who had passed away. This gift of Professor Harborne is probably as great as his professional achievements''.

G. Paul Bolwell
Norman G. Lewis
Dieter Strack
Phytochemistry Regional Editors

PERGAMON

Phytochemistry 56 (2001) 219–221

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

A brief history of the journal *Phytochemistry* 1961–1999

1. Founding the journal

The main reason for the founding of a new journal for plant biochemistry around 1960 was the considerable advances that were being made in this subject at that time. With the advantage of paper chromatography and UV spectroscopy, it was possible to recognise the widespread occurrence of many plant molecules, thought to be rare before. Furthermore, the radiocarbon techniques developed by Melvin Calvin, allowed us for the first time to solve the mysteries of the biosynthesis of polyphenols and other secondary metabolites. The formation of the Plant Phenolics Group in 1957 (eventually to become the Phytochemical Society of Europe), with Tony Swain as Secretary and E.C. Bate-Smith as Chairman was another key development. It became clear at meetings of this Group that a new journal would eventually be needed to cater for all the scientists attracted to this area of research. '*The Biochemical Journal*', the only other reasonable place for publication of plant biochemical papers, was obviously dominated by manuscripts on clinical and animal biochemistry.

The critical event — the founding of the journal — occurred during a short meeting in an office in Fitzroy Square, London between Robert Maxwell, an up-and-coming Science Publisher, his managing director, Gilbert Richards and Tony Swain. There was an immediate go-ahead for this venture, with Pergamon Press as publishers and Tony Swain in the Editorial Chair. A distinguished cast of scientists, including Sir Robert Robinson, OM, FRS, Nobel Laureate, were recruited to the Editorial Board. The first issues came out in 1961–1962 with four parts making up 277 pages. Scientists contributing to those first four issues included such well known figures as A.C. Neish, J.W. Fairbairn, T.W. Goodwin, D.P. Hackett, J. Friend, E. von Rudloff, A. Kjaer, A.M. Mayer and Leo Marion. Publication has continued to develop and expand ever since (Table 1). In 1999, '*Phytochemistry*' appeared in 24 issues, making up three volumes 50–52 and containing over 4000 pages.

2. My early publications

My first encounter with '*Phytochemistry*' was as an author. In 1958, I began to write a series of papers on 'Plant Polyphenols' describing my research results. These first papers appeared in the '*Biochemical Journal*', but as soon as the new journal was announced, I transferred the series to this new venture. I published two papers in volume 1, two in volume 2 and three each in volumes 3 and 4. One of the early papers that I was quite proud of, was one on the glycosidic pattern of anthocyanin pigments (1963, Vol. 2, p. 85) which described a completely new series of sugar derivatives that had not been recognised before by Sir Robert Robinson, one of the early pioneers of anthocyanin chemistry.

My own researches on plant phenolics were carried out at the John Innes Institute, then established in a Georgian mansion close to Hertford, north of London. Although I had an enzyme biochemist to work with, I was mainly surrounded by geneticists, mycologists, physiologists and plant breeders. I needed some contact with other plant biochemists. Where better than at Cambridge, in the Low Temperature Station, which was only a short car journey away. Here Bate-Smith, although director of the station, was carrying out a mammoth survey of the plant kingdom for tannins and other phenolic constituents. He was ably supported by Tony Swain, whose main interests at the time were in phenolic biosynthesis and phenolic production in tissue culture. We joined forces for regular discussions of progress in the plant polyphenol field. Lunches over a cheese roll and a pint or two of best bitter at the nearby pub cemented our relationship.

In 1965, our meetings were somewhat disrupted by my leaving the John Innes Institute and taking up a Science Research Council research post at the University of Liverpool to work principally on the family Umbelliferae. At the same time, Tony was recruited by Lord Zuckerman, chief scientific adviser to the British government to serve in the Cabinet Office in London as scientific adviser. In spite of the great demands on his time, Tony continued to edit the journal, travelling back

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Table 1
Some landmarks in the production of the journal *Phytochemistry*

Year	Volume	Issue	Occasion
1966	5	–	First volume with 1000 pages
1968	7	–	First volume with 2000 pages
1971	10	–	First volume with 3000 pages
1985	24	–	First volume with 4000 pages
1986	25	7	Twenty-fifth anniversary issue
1988	27	8	Tony Swain Memorial issue (1922–1987)
1993	34	1	65th Birthday of Editor-in-Chief
1995	40	6	In honour of Professor A.G. Gonzalez
1998	47	4	65th Birthday of Professor C.A. Ryan
1998	47	5	65th Birthday of Professor A. Cavé
1998	49	2	75th Birthday of Professor G.H.N. Towers
1998	49	5	'Ten Years On' a further memorial meeting for the founding editor
1999	50	–	50th volume of the journal

to Cambridge each weekend to deal with the papers and to direct his research group. He did, however, introduce me to some of the tasks of scientific editor and I began to become involved with him in the editorial processes.

3. Taking over the helm

The real crunch for me came in 1968. Tony had completed his stint in the Cabinet Office in Whitehall and he decided that he needed a couple of years of sabbatical leave in Harvard and Yale in order to return to academia as a research scientist. Someone was desperately needed to carry on the journal editing in the UK and that someone was me. I was immediately established as an Associate Editor, with a part-time secretary, so that I could run the show, deferring when necessary to Tony. At this time, Tony was getting deep into hallucinogenic plants and travelling up to Amazon in the 'Alpha Helix' with Richard Schultes.

The editing task returned to normal in 1970, when Tony returned to England to set up an AFRC-funded research unit at the Royal Botanic Gardens, Kew. Now, with myself at Reading University, only a 40 minute drive away from Kew, it became possible to have regular monthly meetings to sort out the 'difficult' papers. We developed a happy partnership in an editorial team of three (including the production controller at Pergamon) which eventually involved several others. In recognition of my contribution to the journal, I was appointed an executive editor in 1972 and continued so until 1986, when I became Editor-in-Chief.

4. Pergamon Press at Oxford

Editing '*Phytochemistry*' at this period required regular trips to the Pergamon Press empire situated in the grounds of Robert Maxwell's mansion at Headington Hill Hall, Oxford. Robert Maxwell was a 'larger than life' character, oozing charm one minute and laying down the law the next. We always got on better when Maxwell was away and we dealt directly with Gilbert Richards. Pergamon Press went through several production crises at this time, all of which were capably solved by Gilbert. I remember once travelling down to Exeter to visit our printers in order to oversee the typesetting of a particular issue. The typesetters were busy working on the weekly magazine 'Radio Times' and did not really seem to know much chemistry! One of the pleasing aspects of being a Pergamon Editor was the opportunity of being able to oversee the whole process of publication from submitted manuscript to the printed article.

During our visits to Oxford, we also kept in touch with a member of staff in the production department of Pergamon who was responsible for our journal. I remember one girl in particular, a delightful lass with auburn hair named Jane Morris, after the pre-Raphaelite beauty. She was able to put away a pint of ale in one minute flat. She knew all the rowing lads and took me to the river to watch the annual College bumps.

5. Editorial Board meetings

Annual Editorial Board Meetings were a regular feature and most British and European Board members were usually able to attend. Policy matters were discussed and any changes in policy were put to the Board. One notable meeting was held in Caius College, Cambridge at the invitation of Tom Ap Rees, when we helped to celebrate Bate-Smith's 80th birthday and his retirement from the Board. Another special occasion was a meeting in Lausanne, organised by Kurt Hostettmann, during which the Editorial Board (Table 2) went for a mountain walk at Champex. More recently, a board meeting organised by Meinhart Zenk in Munich included a celebration and a mini symposium to mark the 80th birthday of Trevor Goodwin, FRS, a founder member of the Board.

6. Associate Editors

By this time, over a thousand manuscripts were being submitted annually to the editors. The need for further editors became apparent. Tony Swain moved to Boston University in 1975 and was able to handle North American manuscripts directly. Subsequently, Geoffrey Cordell took on this responsibility. In the UK, a team of other editors developed to deal particularly with the correction of scientific details and errors in style. John Goad, for example, was a valuable additional editor, since he was able to provide his expertise on steroid nomenclature. Others who joined the team and provided their own special accomplishments included Peter Lea,

Table 2
Editorial Board members who attended the special anniversary meeting at Lausanne to celebrate the first 30 years of *Phytochemistry*

Professor Y. Asakawa, Tokushima
Professor E.A. Bell, CB, Kew
Professor E.G. Brown, Swansea
Sir Leslie Fowden, FRS, Rothamsted
Professor T.W. Goodwin, FRS, Liverpool
Professor O.R. Gottlieb, Rio de Janeiro
Professor V. Herout, Prague
Professor K. Hostettmann, Lausanne
Professor E. Leete, Minneapolis
Professor T.J. Mabry, Austin
Professor P. Mazliak, Paris
Professor G. Ourisson, Strasbourg
Professor J.D. Phillipson, London
Professor T. Ap Rees, Cambridge
Professor G.H.N. Towers, Vancouver
Professor H. Wagner, Munich
Professor P.G. Waterman, Glasgow
Professor M.H. Zenk, Munich

David Threlfall, Terry Smith, Peter Holloway and Christine Williams. These busy scientists over many years provided me with essential back-up in the editorial task.

7. Authors and referees

So far, I have not said anything about the authors and referees, without which there would be no journal. All I can say is that during my many years of editorship, I have been blessed with a team of hard-working and long-suffering referees and with a host of polite and helpful authors. A significant amount of the refereeing has been generously carried out by members of the Editorial Board. Their expertise has been essential to maintain a good standard of excellent science.

Table 3
First ten winners of the Pergamon *Phytochemistry* Prize

Awarded annually to recognise sustaining and outstanding contributions to the subject of phytochemistry. Prize includes a cheque for $5500 and the presentation of a framed certificate	
1990	Edward Leete
1991	Meinhart Zenk
1992	Otto Gottlieb
1993	Jeffrey Harborne
1994	Eric Conn
1995	Jake Macmillan
1996	David Phillipson
1997	Yoshinori Asakawa
1998	Tom Mabry
1999	Ken Markham

What about the authors? All I can say is that I have rarely received a paper for the journal which has not taught me something. And some papers have been a joy to read and to publish. To mention authors by name may appear invidious, but I would particularly remember handling the papers of that select band of phytochemists (Table 3) whose excellence has led them to the award of the Pergamon Phytochemistry Prize. It is, of course, the publication of the key papers in a particular subject which most pleases the heart of an editor. I would conclude with a thank you to all the many authors over the years who have sent me their papers for publication in our special journal. I wish the new team of regional editors every success in their future endeavours.

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Ecological biochemistry and its development

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Abstract

The concept that chemistry and biochemistry may play significant roles in ecological relationships is not a new one. It is only within the last quarter of a century, however, that this concept has gained general acceptance and ecological biochemistry has become a well defined interdisciplinary subject in its own right. Examples are given of different types of biochemical relationships involving plants and animals and reference to other areas of the subject are made. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Danaus plexippus*; *Asclepias* species; Cardenolides; Pyrrolizidine alkaloids; Flavonol glycosides; 3-Hydroxy-2-butanone; *Caryedes brasiliensis*; *Dioclea megacarpa*; Canavanine; *Eumaeus atala florida*; *Zamia floridana*; Cycasin; Feeding deterrents; Polymorphism in plants; Cyanogenic glycosides; Polymorphism in herbivores; Adaptation to monofluoracetate

1. Introduction (with acknowledgement to the monarch butterfly)

As previously pointed out (Rothschild, 1972) it is more than a century since early pioneers in the field of insect mimicry, such as Bates, Muller and Wallace, postulated that certain aposematic insects may be protected from potential predators by sequestered toxins obtained directly from their host plants. Haase indeed claimed specifically that brilliantly coloured danaid butterflies whose larvae feed on poisonous *Asclepias* species make use of the plant toxins in their own defense. More than 50 years were to pass, however before it was shown (Reichstein et al., 1968) that cardiac glycosides (cardenolides) synthesized by milkweeds (*Asclepias* species) on which the larvae of the monarch butterfly *Danaus plexippus* feed could also be detected in the insect. This important confirmation that insects can sequester toxins originating in their food plants was obtained through the joint efforts of chemists and biologists and such collaboration must be seen as the sine qua non of ecological biochemistry. The confirmation also made the monarch butterfly something of an ecological celebrity and a brief account of further studies relating to this insect will serve in this introduction to illustrate some of the various, complex and subtle ways to which chemistry can be put by even a single organism. Evidence that the sequestered toxins do actually deter potential avian predators was obtained by Brower et al. (1968). They demonstrated that toxin-free monarch butterflies whose larvae had been raised on a cardenolide free diet were eaten with impunity by the inexperienced young of the blue jay *Cyanocitta cristata*. In contrast butterflies containing cardenolides whose larvae had fed on *Asclepias* species, their usual host plants, caused the jays to vomit and avoid future encounters with butterflies of similar appearance.

In addition to its need for cardenolides as defense compounds the monarch butterfly has a requirement for pyrrolizidine alkaloids from which it synthesizes sex pheromones. These compounds, which are liberated from hair pencils on the wings of the males during courtship, encourage the female to mate. Unlike the cardenolides, the pyrrolizidine alkaloids are not ingested from food plants by the larvae of the butterfly but are obtained by the adult male which visits species of the Boraginaceae and Compositae containing these alkaloids. The insect typically sucks in alkaloid-containing exudates, from sources such as withered leaves, through its proboscis. The more recent identification of flavonol glycosides as oviposition stimulants in the leaves of *Asclepias curassavica* (Haribal and Renwick, 1996) emphasizes again the importance to this butterfly of plant secondary compounds.

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Not all of the monarch's requirements for ecochemicals are met by plants however, and its ability to synthesize its own defense compounds is seen in the response of its larvae to the potential threat posed by other insects. In a fascinating paper, Rothschild and Bergstrom (1997) have described the vigorous waving induced in the larvae of this butterfly by the buzzing of bees and wasps in their vicinity and the even greater activity induced by low flying planes of the Royal Air Force. This physical response to the proximity of wasps (or war planes) is also accompanied by a chemical response. The larvae liberate a mixture of over 16 volatile compounds into the atmosphere immediately surrounding them. The compound occurring in highest concentration (ca 30% of the mixture) is 3-hydroxy-2-butanone [**1**], a ketone which did not occur in the *Asclepias* species on which the larvae had fed.

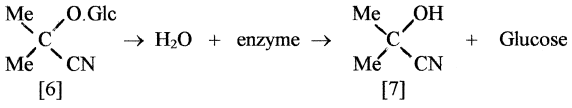
$CH_3.CO.CH(OH).CH_3$
[1]

$H_2N.(:NH).NH.O.CH_2.CH_2.CH(NH_2).CO_2H$
[2]

$H_2N.(:NH).NH.\ CH_2.CH_2.CH_2.CH(NH_2).CO_2H$
[3]

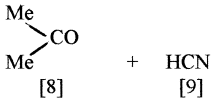
$Glc.O.CH_2.N{:}N.CH_3$
$\downarrow$
O
[4]

$H_2N.CH_2.CH_2.CH(NH_2).CO_2H$
[5]



[6] [7]

$\downarrow$



[8] [9]

$CH_2Fl.CO_2^-$
[10]

A final reference to the monarch butterfly is anecdotal, but may serve to identify a problem worthy of solution and possibly a novel way of solving it. Some years ago the butterfly house in Syon Park lost a number of monarch butterflies. Subsequently, eggs and larvae of the same species were found on *Asclepias* plants in the Royal Botanic Gardens Kew rather more than a mile away across the river Thames. Assuming that the eggs were laid by some of the Syon Park escapees, we are faced with the intriguing problem as to how these exotic insects found their equally exotic food supply (and possibly pyrrolizidine containing plants) while at large in an alien environment. Did they see them? Did they detect volatile compounds liberated by the plants? and if so what were the compounds? Perhaps the controlled release of exotic insects (or other animals) in botanic gardens might provide new insights into the ways that animals perceive plants and the chemicals that they contain.

In this short presentation an arbitrary selection of subjects has had to be made. The examples chosen are concerned with plant and animal interrelationships but it is hoped nevertheless that they will illustrate something of the diversity and interest of the subject and provide an insight into the ways in which it has developed.

2. Beetles and seeds

Seeds are important sources of food to animals large and small. Plants cannot afford to have all their seeds eaten, however, and adaptations such as hard seed coats and dispersal by dehiscence can reduce the chances of this happening. The presence of secondary compounds that are toxic to, or deter feeding in, potential predators can serve the same purpose.

The seeds of species belonging to the less advanced tribes of the Papilionoideae (Leguminosae) characteristically contain quinolizidine alkaloids. These alkaloids are toxic even at low concentrations to the larvae of the cow pea weevil *Callosobruchus maculatus*. Seeds from species of the more advanced tribes lack these alkaloids but frequently contain major concentrations (ca 1–10%) of non-protein amino acids such as canavanine [**2**] which is a close analogue of the protein amino acid arginine [**3**]. Canavanine, though less toxic than the alkaloids, is nevertheless toxic to the larvae of *C. maculatus* at the concentrations in which it occurs in most legume seeds. It was suggested, therefore, that canavanine might serve the seed both as a nitrogen store (it contains twice as much nitrogen as storage protein) and a defense against seed beetles (Bell, 1981). The apparent freedom of most canavanine-containing seeds from beetle infestation supported this hypothesis but one notable exception was known. The seeds of *Dioclea megacarpa,* which may contain over 10% of canavanine, provide food for the larvae of *Caryedes brasiliensis* a seed beetle found in Costa Rica. Biochemical studies (Rosenthal et al., 1976) showed, however, that *C. brasiliensis*

possesses an arginyl *t*RNA synthetase that is able to discriminate against canavanine and prevent the toxic effects which its incorporation into insect protein in place of arginine would otherwise cause. This finding has a wider significance, however. It suggests that one of the most convincing pieces of evidence for the defensive role of a secondary compound may be evidence that a successful predator is specifically adapted to circumvent the potentially adverse effects of that compound.

3. Caterpillars and leaves

Nicotine has a long history of use as an insecticide yet the larvae of the tobacco hornworm *Manduca sexta* feed unharmed on the leaves of the tobacco plant *Nicotiana tabacum*. This insect is not poisoned by the alkaloid because it has evolved a highly efficient means of excreting it (Self et al., 1964). The monarch butterfly, as mentioned previously, benefits rather than otherwise from the cardenolides ingested by its larvae when feeding on *Asclepias* species. Two other species of the Lepidoptera which make use of a host plant's chemical defenses, but in rather a different way, are the tiger moth *Seirarctia echo* and the lycaenid butterfly *Eumaeus atala florida* whose larvae feed on the young leaves of the cycad *Zamia floridana*. The Cycadales characteristically synthesize and store glycosides of the highly toxic carcinogen methylazoxymethanol (MAM). When ingested by animals the glycosides are hydrolysed by β-glucosidase enzymes of the gut and free MAM is liberated. Although β-glucosidase is present in the gut of *S. echo*, the insect is protected from the toxic effects of liberated MAM by its ability to glycosylate that compound (Teas, 1976). Cycasin [**4**], the non-toxic β-D-glucoside so formed, is stored in the moth and also in the butterfly, which presumably detoxifies MAM in the same way. The brilliantly coloured adults of *E. atala* have been found to contain as much as 1.8% (dry weight) of cycasin while the young leaves on which their larvae feed contain only 0.2% (Rothschild et al., 1986). Like the monarch butterfly, *S. echo* and *E. atala* have the warning colouration of aposematic species. The presence of cycasin in dried museum specimens of cycad-feeding aposematic species of other genera and its absence from a cryptic species whose larvae feed on the same plants is a further indication of the compound's defensive role (Nash et al., 1992).

A compound acting as a feeding deterrent may be as important as a toxin in protecting a plant from predation. The effectiveness of a feeding deterrent, as of a toxin, will however be related to its concentration within the plant. This is well illustrated by the reaction of the larvae of the African leaf worm *Spodopteris littoralis* to the non-protein amino acid L-2,4-diaminobutanoic acid [**5**] which occurs in the leaves of *Lathyrus latifolius*. At low concentrations the amino acid acts as a phagostimulant and at high concentrations as a feeding deterrent (Bell et al., 1996). The insect's response to L-2,4-diaminobutanoic acid cannot but remind us of our own to mustard or curry powder.

4. Biochemical polymorphism in plants

Individual plants within a population may differ from one another in their biochemical characteristics. Clover *Trifolium repens* and birdsfoot trefoil *Lotus corniculatus* both exists in two forms, one of which liberates hydrogen cyanide when crushed and the other which does not. The cyanogenic forms contain both cyanogenic glycosides, and a hydrolytic enzyme which catalyses the breakdown of the glycosides when brought into contact with them in the damaged tissue. For example, linamarin [**6**] which occurs in both species is hydrolysed enzymatically to give acetone cyanohydrin [**7**] which in turn breaks down spontaneously to acetone [**8**] and hydrogen cyanide [**9**]. The acyanogenic forms of the plants may lack either the cyanogenic glycosides, the enzyme, or both. In Europe, a remarkable correlation was found to exist between the frequency of occurrence of the cyanogenic form of *T. repens* and the mean January temperature (Daday, 1954). The significance of this became apparent when Jones (1972) demonstrated that slugs and snails preferred the acyanogenic forms of both species. In regions such as the British Isles and the Iberian peninsular where winter temperatures are relatively mild and the molluscs continue to be active, cyanogenic forms dominate. In Scandinavia and Russia, lower winter temperatures ensure that the plants are able to establish themselves before they are eaten by slugs or snails and here the acyanogenic forms predominate. Another example of polymorphism of this type is seen in bracken *Pteridium aquilinum* where the acyanogenic form is grazed by sheep and deer while the cyanogenic form is avoided (Cooper-Driver and Swain, 1976).

5. Biochemical polymorphism in herbivores

Many indigenous Australian plants are toxic to domestic animals introduced from Europe. These plants include species of the legume genera *Gastrolobium* and *Oxylobium* that grow in Western Australia. The toxin that they contain is monofluoroacetate [**10**] which is highly poisonous to many mammals including man. After ingestion the compound is converted to fluorocitrate which inhibits the enzyme aconitate hydratase, blocks the tricarboxylic acid cycle and causes respiratory failure.

Of the native fauna, the western grey kangaroo *Macropus fuliginosus,* has evolved a tolerance to this compound. This tolerance is found in animals from

both southwestern and southeastern Australia. Similar tolerance is found in the rat kangaroo *Bettongia penicillata* and the bush rat *Rattus fuscipes* but only in those members of the two species living in southwestern Australia where plants containing monofluoroacetate grow. These findings suggest that the rat kangaroo and the bush rat originated in eastern Australia and only when members of the species moved west and encountered plants containing the toxin was their tolerance to monofluoroacetate developed. In contrast the western grey kangaroo probably originated in the west and tolerance was retained in populations as they spread eastward (Mead et al., 1985).

6. Induced response to herbivory

Plants are not only able to accumulate defense compounds prior to attack by a potential predator, they can also respond to such an attack. Insect feeding, or indeed mechanical damage, may induce increased synthesis of pre-existing defense compounds such as alkaloids, terpenoids, phenolics, or proteinase inhibitors. Alternatively it may activate entirely new biosynthetic pathways. Paré and Tumlinson (1997) have shown for example that the beet army worm *Spodoptera exigua* feeding on the cotton plant *Gossium hirsutum* activates pathways leading to the de novo synthesis of a number of volatile terpenes and indole. The activation of these pathways involves both plant and insect, the trigger being an insect modified fatty acid of plant origin. The liberated volatiles serve to attract parasitoids, usually parasitic wasps, that prey on the herbivorous caterpillars. It was subsequently shown (De Moraes et al., 1998) that the composition of the volatile "distress signal" mixture liberated by the plant under attack is strictly related to the identity of the attacker. Tobacco, cotton and maize produce different volatile blends depending on whether they are being eaten by *Heliothis virescens* or *Heliocoverpa zea*. The specialist wasp *Cardiochiles nigriceps* is able to recognise these differences and seek out cotton or tobacco plants suffering infestation by its host *H. zea*.

7. Conclusion

Examples of plant and animal interactions at the chemical and biochemical level have been given. Enough, it is hoped, to indicate how subtle and complex they can be. Time has not allowed mention of other important areas such as the biochemical adaptations of plants and animals to their physical environments, of higher plants to each other, of higher plants to lower plants and micro-organisms, or of animals to each other. As with plant and animal interrelationships our knowledge of these other areas has greatly increased in the last quarter of a century due primarily to the skill and dedication of a small number of enthusiasts. Not least of these is Jeffrey Harborne who is now retiring as Editor of *Phytochemistry*. He and his colleagues have made many original contributions in studies ranging from the response of plants to fungal attack (Harborne and Ingham, 1978) to the role of sesquiterpene lactones as feeding deterrents in insects (Rees and Harborne, 1985). As Editor of *Phytochemistry* he has welcomed and encouraged the submission of original interdisciplinary papers. He has contributed to a great many symposia and edited proceedings volumes such as *Phytochemical Ecology* (see Jones, 1972) and *Biochemical Aspects of Plant and Animal Coevolution* (Harborne and Ingham, 1978). He has kept us abreast of current developments with excellent literature reviews; notably the *Recent Advances in Chemical Ecology* series (Harborne, 1986, 1989, 1993a, 1997, 1999). His most influential written contribution may, however, have been *Introduction to Ecological Biochemistry* (Harborne, 1993b). This small volume, which is now in its fourth edition, has awakened the interest of generations of students (and others) to a fascinating subject and encouraged them to enter a field where the increasingly sophisticated analytical techniques of the physical sciences can be applied to the solution of biological problems.

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E. Arthur Bell, after a first degree in chemistry at Kings's College Newcastle (University of Durham), spent a short period with Imperial Chemical Industries at Billingham. A research award then took him to Trinity College Dublin where he worked for his PhD on the mode of action of the sulphonamide drugs, under the direction of Professors Cocker and O'Meara. An assistant lectureship in biochemistry at TCD and access to Trinity College Botanic Garden were keys to a growing interest in phytochemistry.

From Dublin he moved to King's College London to become Lecturer and then Reader in Biochemistry. Here, he continued his research on non-protein amino acids and related compounds, encouraged by Leslie (now Sir Leslie) Fowden, who was at that time a neighbour in University College. While at King's, he was recruited by Jeffrey Harborne as part of a plan to widen the interests of the old Plant Phenolics Group and create a society concerned with all aspects of plant chemistry and biochemistry. This was the Phytochemical Society of which he was later to become chairman.

In 1968, he was appointed Professor of Botany in the University of Texas at Austin, working with Tom Mabry and Billie Turner. He returned to King's College London in 1972 as Professor of Biology and Head of the Department of Plant Sciences.

In 1981, he became Director of the Royal Botanic Gardens Kew and retired in 1988. For 25 years, he served on the Editorial Board of Phytochemistry. In retirement, he is Visiting Professor at King's College London and Adjunct Professor at the University of Texas at Austin. CB (1988); FKC; Hon. FTCD; Hon. Mem. Phytochemical Society of Europe.

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Contributions of Jeffrey Harborne and co-workers to the study of anthocyanins

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Abstract

Jeffrey Harborne and his co-workers have played a unique role in the over-all study of plant pigments and of anthocyanins in particular through their many publications and through Jeffrey's editorial work with *Phytochemistry*. Jeffrey has made important contributions to our understanding of the separation and structural identification of anthocyanins; to co-pigmentation; and to the role of anthocyanins in systematics and ecology in both reproductive and vegetative tissues. This work has had considerable influence on much of the current research on the genetics and regulation of anthocyanin biosynthesis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: J.B. Harborne; Anthocyanins; Phytochemistry

1. Introduction

It was a great pleasure to be present at a meeting held at the Royal Botanic Gardens, Kew in July 1999 in honor of the retirement of Professor Jeffrey Harborne FRS (University of Reading) as Executive Editor of *Phytochemistry*. Throughout his working life, Jeffrey Harborne has made invaluable contributions to the field of phytochemistry focusing mainly on flavonoid compounds. Of special interest is his work on plant pigments and in particular his work on the anthocyanins.

Floral and fruit colors derive from a small group of pigments principally carotenoids, betacyanins (Centrospermae), anthocyanins and other flavonoids (flavones and flavonols). Anthocyanins are responsible for the wide range of colors in the petals of flowering plants and they may vary the color from salmon- pink, through scarlet, magenta and violet to deep blue (Haslam, 1995). They are present in flower petals to attract pollinators and in fruits and seeds as attractants for seed dispersers (Willson and Whelen, 1990). Structurally anthocyanins are glycosylated polyhydroxy/methoxy derivatives of 2-phenyl benzopyrilium (flavylium) salts. Six aglycones (anthocyanidins) dominate natural anthocyanin structures (Fig. 1) and most are substituted at the 3- and/or 5-hydroxy positions. The three main chromophores, pelargonidin, cyanidin and delphinidin occur usually singly or occasionally as mixtures and essentially all pink, scarlet and orange-red flowers contain pelargonidin, all crimson and magenta flowers cyanidin and all mauve and blue flowers delphinidin. Anthocyanins are hydrophilic and generally present in the plant cell vacuoles. Anthocyanins are also present in vegetative tissues, in leaves, stems and roots and in emergent seedlings. They are visible in the leaves of fall foliage, in rapidly developing shoots and on the undersurface of leaves of many tropical understory herbs.

In every aspect of current anthocyanin research, Jeffrey Harborne has acted as a pioneer — in the separation and structural identification of anthocyanins; in genetics and the inheritance of flower color; in plant systematics; in pollination ecology and in focusing attention on the ecological role of anthocyanins in vegetative tissues. This paper briefly discusses some of the work of Jeffrey Harborne and his many collaborators and serves to illustrate the influence that this research has had and is having on current research on anthocyanin pigments.

2. Chemical basis of flower color variation

2.1. Chemical identification

Some of the earliest work on anthocyanins, concerning the chemical and genetic basis of flower color variation, was carried out by Robert Robinson and his wife Gertrude,

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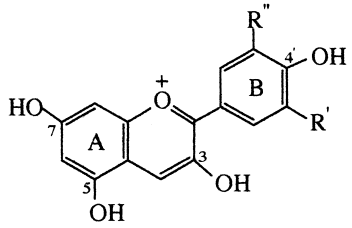


Fig. 1. Structure of the aglycone, anthocyanidin. The six most common anthocyanidins found in natural pigments are: pelargonidin R′ = H, R″ = H; cyanidin R′ = OH, R″ = H; peonidin R′ = OMe, R″ = H; delphinidin R′ = OH, R″ = OH; petunidin R′ = OMe, R″ = OH; and malvidin R′ = OMe, R″ = OMe.

at Oxford, and by Scott-Moncrieff at the John Innes Institute, England (Scott-Moncrieff, 1981). It was at this latter Institute that Jeffrey Harborne began his professional scientific career in 1955. Harborne did much of the early work on chemical identification of anthocyanins using both spectral (Harborne et al., 1953; Harborne, 1957a) and chromatographic methods (Harborne, 1959). Based on the chromatographic methods, first reported by Bate-Smith (1948) and Bate-Smith et al. (1953), Harborne developed suitable solvents for separating pigments with different glycosylation patterns (Harborne, 1957b). Thus, for the first time it became possible to identify plant anthocyanins and their sugar components on a micro-scale combining both spectral and chromatographic techniques (Harborne, 1962, 1963a, 1964a, 1965a,b).

In the early 1960's it was also shown that in many cases the sugars attached to the anthocyanidins are acylated with hydroxycinnamic or hydroxybenzoic acids (Harborne, 1964b). In addition to aromatic acylation, in the 1980's, new mild extraction techniques demonstrated the widespread occurrence of acylation with aliphatic dicarboxylic acids and anthocyanins occurring as zwitterions (Harborne and Boardley, 1985; Harborne, 1986a,b; Harborne et al., 1986a,b; Harborne and Self, 1987; Harborne et al., 1987; Harborne, 1988a; Harborne et al., 1989a; Harborne, 1990; Harborne et al., 1993). When plants in the Compositae (Asteraceae) which had earlier been screened for anthocyanins were reinvestigated, it was found that practically all species had malonyl or succinyl groups attached to the floral anthocyanins (Takeda et al., 1986). In angiosperms, malonylated residues linked to the glucosyl units are present in at least a third of all plant species analyzed.

2.2. Co-pigmentation

The observation that the color of isolated anthocyanins could be varied by the presence of other substances, such as "tannin", was first made by Willstatter and Zollinger (1916) and Robinson and Robinson (1931) (in Haslam, 1993). Jeffrey Harborne's work and those of many others have clearly shown how anthocyanins with the same chromophoric structure can give rise to many different colors (Goodwin, 1988; Harborne, 1993).

Co-pigments are important as under very weakly acidic conditions, pH 4–6, as is typical in cell vacuoles, and in the absence of other substances, most anthocyanins exist substantially in stable colorless forms. Between pH 4 and 6, four anthocyanin structural types exist in equilibrium, namely the flavylium cation, the quinonoidal anhydro base, the colorless carbinol bases and the pale yellow "reversed" chalcones. Equilibration between the quinonoidal and carbinol bases occurs via the flavylium cation:

$$\text{quinonoidal anhydro base} \rightleftarrows \text{flavylium cation} \rightleftarrows \text{carbinol bases}$$

As the pH is raised, increasing amounts of the anhydrobase are formed. At neutral pH, the anionic form of the quinonoidal base (blue) is formed; in more acidic conditions the flavylium ion (red) predominates (Haslam, 1993, 1998).

Co-pigments may act either inter- or intramolecularly (Figueiredo et al., 1996). They have little or no color by themselves but probably stabilize and enhance the color of the anthocyanins by intermolecular hydrophobically reinforced π–π stacking of their aromatic nuclei with those of the pigment (Brouillard et al., 1991; Mistry et al., 1991). Such π–π interactions are thought to stabilize the flavylium-cation/anhydro-base forms which prevents the usual reaction with water to form the colorless carbinol-base forms.

Various molecules have been identified as good natural intermolecular co-pigments including the hydroxycinnamoyl esters (mentioned above) such as chlorogenic acid, galloyl esters (tannins) and flavone and flavonol glycosides (Figueiredo et al., 1996). When caffeine was tested, it was found to preferentially stabilize the quinonoidal base (Haslam, 1993). An anthocyanin in which the anhydro-base form is stabilized is the 'heavenly blue anthocyanin' from the blue petals of the Morning Glory (*Ipomoea tricolor*) in which two of the caffeyl ester groups are thought to be stacked intramolecularly with the anthocyanin nucleus (Mistry et al., 1991). Sugar attachment and methylation are also both probably important for pigment stability and generally have little effect on flower color per se. Methylation of one or more of the free hydroxyl groups also improves the stability of the anthocyanidin chromophore and is relatively common in the more highly specialized plant families (Harborne, 1993).

Also associated with co-pigmentation is the presence of metal ions (Goto et al., 1986). The presence of metal ions or metallo anthocyanins with magnesium and iron have been isolated in the blue pigment, commelinin, from the petals of *Commelina communis* and also from the cornflower, *Centaurea cyanus* while in *Hydrangea* flowers the metal present is aluminium.

2.3. Other factors affecting pigmentation

Additional factors which affect flower color are vacuolar pH. Both environment and genetic factors appear to be involved in the control of vacuolar pH. The pH of the vacuole generally varies between pH 4.5 and 5.5 but can range from pH 2.8 measured in a Begonia cultivar to pH 7.5 found in Morning Glory cv Heavenly Blue. The pH in epidermal cells from different parts of the same flower may also vary (Haslam, 1995). On aging, there is often an increase in the pH of the plant cell vacuole leading to an increase in the quinonoidal base which causes the aging or bluing effect. In petunia, seven genetic loci have been defined (pH 1–7) that, when mutated cause bluing of the flower. These mutations do not alter the anthocyanin composition but do increase the pH of the petal extracts (Mol et al., 1998). Mol et al. (1998) have recently emphasized that the shape of the epidermal cells is also an important factor in pigmentation. For example, whether the cell is conical or flat, influences the optical properties of anthocyanin and thereby the color that is perceived (Mol et al., 1998). Factors known to influence floral pigmentation are summarized in Table 1.

3. Distribution of anthocyanins

For more than 30 years, Jeffrey Harborne and coworkers have been working on the systematics of flower color (Harborne, 1963b, 1988b; Harborne and Turner, 1984). As anthocyanins are almost universal in flowering plants they are chemical compounds that are useful for systematic purposes both at the family and genus level. Systematic studies have been made using the distribution of anthocyanins present in the floral petals of many major plant groups including Poaceae (Harborne and Clifford, 1967); Umbelliferae (Harborne, 1976); Onagraceae (Harborne et al., 1976); Commelinaceae (Harborne and Stirton, 1980); the Araceae (Harborne et al., 1981); Lamiales (Harborne, 1992a); Leguminosae (Fabaceae) (Harborne et al., 1970), and the Compositae (Asteraceae) (Harborne, 1996), and at genus level in *Collomia* (Harborne et al., 1982) and *Patersonia* (Harborne et al., 1989b). Many family or genera have characteristic patterns- for example, substitution at the 3-,7- and 3′-positions is a characteristic feature in anthocyanins isolated from orchid flowers (Harborne and Williams, 1998) while anthocyanins acylated with *p*-hydroxybenzoic acid appear to occur characteristically in two unrelated plant families, the Campanulaceae and the Ranunculaceae (Harborne and Williams, 1995).

Table 1
Modification of floral pigmentation

1. Variation in pigment class
2. Concentration of individual pigments
3. Structural variations within a pigment class
4. Modifications of anthocyanidin 3-glycosides:
(a) Further glycosylation
(b) Methylation
(c) Acylation with aromatic compounds (hydroxy cinnamoyl esters)
(d) Acylation with aliphatic dicarboxylic acids (malonate)
(e) Presence of flavonol or flavone co-pigment
(f) Metal chelation (magnesium and iron)
5. Changes in vacuolar pH
6. Cell shape

Although anthocyanins are almost universal in the roots, stems, leaves and flowers of angiosperms and are also common in both the vegetative and reproductive structures of many gymnosperms (Anderson, 1992; David Lee, personal communication), they are of sporadic occurrence in other groups of plants. Anthocyanin-like pigments have been detected in liverworts and mosses (Markham, 1988, 1990) and pelargonidin and cyanidin derivatives in the fern *Davallia divaricata* (Harborne, 1965c).

4. Ecological roles of anthocyanins

4.1. Role of anthocyanins in reproductive tissues

Anthocyanins play a vital role in reproductive tissues (Harborne, 1980, 1992b). Jeffrey Harborne was one of the first scientists to establish by chemical experimentation the direct relationship between anthocyanin type (flower color) and pollination ecology. He provided clear evidence that natural selection for particular colors in different environments depends upon the most active pollinators present. (Harborne, 1993). Harborne demonstrated how selection has worked in two directions: from cyanidin as the most basic or most primitive type of plant pigment, to flowers with scarlet orange colors (pelargonidin) arising by loss mutations. In the second direction, gain mutations have occurred in temperate climates producing the delphinidin colors common in bee pollinated families, such as the Primulaceae, Polemoniaceae, Hydrophyllaceae and Boraginaceae. The further loss mutation from pelargonidin to give the 3-desoxyanthocyanidin- luteolinidin and apigeninidin, only appears in very advanced tropical angiosperm families such as the Gesneriaceae and Bignoniaceae. Analysis of the anthocyanins present in 18 representative members of the Polemoniaceae showed a clear cut correlation between flower color, anthocyanidin type and pollinator (Harborne and Smith, 1978). A more extensive survey also demonstrated the correlation between anthocyanin type, pollinator and flower color in the Lamiaceae (Saito and Harborne, 1992).

Evolutionary changes in flower color can also be observed at the species level, and any change in color may have important evolutionary consequences (Weiss,

1991). Mutational inactivation of only a few structural genes may be sufficient to change the type of pollinators which in turn may result in genetic isolation and possibly speciation (Vickery, 1995). For example, species of *Mimulus* (monkey flower) are reproductively isolated owing to *M. lewisii* pollination preference by bumblebees and *M. cardinalis* by hummingbirds (Bradshaw et al., 1995).

4.2. Role of anthocyanins in vegetative tissues- protection from photoinhibition and photo-oxidation

The roles of anthocyanins in vegetative tissues have been less well studied. Jeffrey Harborne regards their function in vegetative organs as "an unraveling mystery" (D. Lee, personal communication). In vegetative tissues, anthocyanins are not present in the epidermis but in the vacuoles of cells in the spongy mesophyll. Young leaves of tropical plants, young, rapidly growing plants, autumnal leaves (Haslam, 1993) and leaves of plants under stress are often red in color.

Leaf undersurfaces of many tropical rainforest understory herbs are red because of the presence of anthocyanins (Benzing and Friedman, 1981). Their presence on the undersurface of the leaves is assumed to be selectively advantageous to plants growing in extreme shade perhaps because the presence of anthocyanins increases the amount of available light for photosynthesis (Lee et al., 1979). However, no mechanism has yet been discovered by which anthocyanins could backscatter radiation and increase light-capturing efficiency in the red wavelength. Gould et al. (1995) suggest an alternative role for anthocyanins, namely reducing susceptibility to photoinhibition. In the understory, plants can be subject to photodamage by brief flecks of sunlight or intermittent light. Anthocyanins absorb at the same wavelength as chlorophyll *b* in the 520/530 nm wavelength range. Comparing leaves with the red or green undersurface in two taxa native to rainforest understory shade, *Begonia pavonina* Ridl. (Begoniaceae), from Bukit Lanjang Forest Reserve, Malaysia and *Triolena hirsuta* Triana (Melastomaceae) from La Selva Research Station in Costa Rica, Gould et al. (1995) found that when anthocyanins are present not only is photoinhibition reduced, but photosynthesis is increased with higher chlorophyll concentrations. Anthocyanins reduce the effects of high light intensity by absorption (Gould et al., 1995).

Leaves of young, rapidly growing plants are often red in color. Under conditions of rapid cell wall elongation (growth) there is the potential for excess peroxide to diffuse into the vacuole and hydrogen peroxide and other oxygen radicals are also produced in times of plant stress. A function for anthocyanins in vegetative tissues is as antioxidants by reacting with free radicals and thus interrupting the propagation of new free radical species or by chelating metal ions such as Fe^{2+}. (Cooper-Driver and Bhattacharya, 1998). The production of anthocyanins- peroxidase system in the vacuoles may contribute to the overall mechanisms for protecting plants from oxidative damage (Yamasaki, 1997).

Many phenolic compounds are being sought for their antioxidant and antithrombotic properties, as potential agents against cancer and cardiac diseases. Antioxidant supplements can significantly improve certain immune responses. For example, the antioxidant and antiinflammatory activity of glycosides of cyanidin from tart cherries has recently been confirmed and these are being incorporated into meat products for improved nutritional qualities (Wang et al., 1999).

5. Genetic manipulation of flower color

Early in Jeffrey Harborne's career, he expressed an interest in the breeding of blue roses (Harborne and Rowley, 1958) but producing a blue rose has eluded traditional breeding techniques. However, an understanding of the biosynthetic pathway of anthocyanins and the enzymes involved, coupled with new DNA recombinant techniques has opened up a whole new chapter in manipulation of flower color.

5.1. Biosynthesis of anthocyanins

The synthesis of anthocyanins involves the enzyme, *chalcone synthase* (CS) which catalyzes the stepwise condensation of three malonyl-CoA units with *p*-coumaroyl-CoA to yield tetrahydroxychalcone (naringenin chalcone) (see Fig. 2). *Chalcone isomerase* (CI) then catalyzes the stereospecific isomerization of the yellow-colored tetrahydroxychalcone to the colorless flavanone, naringenin. Flavanones act as intermediates for the synthesis of flavones, isoflavones and 3-OH flavanones (dihydroflavonols). Narigenin is converted to dihydrokaempferol (DHK) or to a 3-0H flavanone by *flavanone 3-hydroxylase* (F3H).

The next stage is the conversion of the dihydroflavonol to the anthocyanin (see Fig. 3). DHK can be hydroxylated by the microsomal cytochrome P450 enzymes, *flavonoid 3′-hydroxylase* (F3′H) to produce dihydroquercetin (DHQ) or by *flavonoid 3′,5′-hydroxylase* (F3′5′H) to produce dihydromyricetin (DHM) (Holton and Cornish, 1995). The precise number of enzymes and their sequence of operation from the dihydroflavonol stage to the anthocyanin is still not entirely clear but it appears that three or more enzymes are involved. During the first stage, the dihydroflavonols are reduced to flavan-3,4-*cis*-diols (leucoanthocyanidins) by *dihydroflavonol 4-reductase* (DFR). Further oxidation, dehydration and glycosylation of the different leucoanthocyanidins by *anthocyanidin synthase* and *anthocyanin glucosyltransferase* produce the corresponding brick-red pelargonidin, red cyanidin and blue delphinidin glucosides.

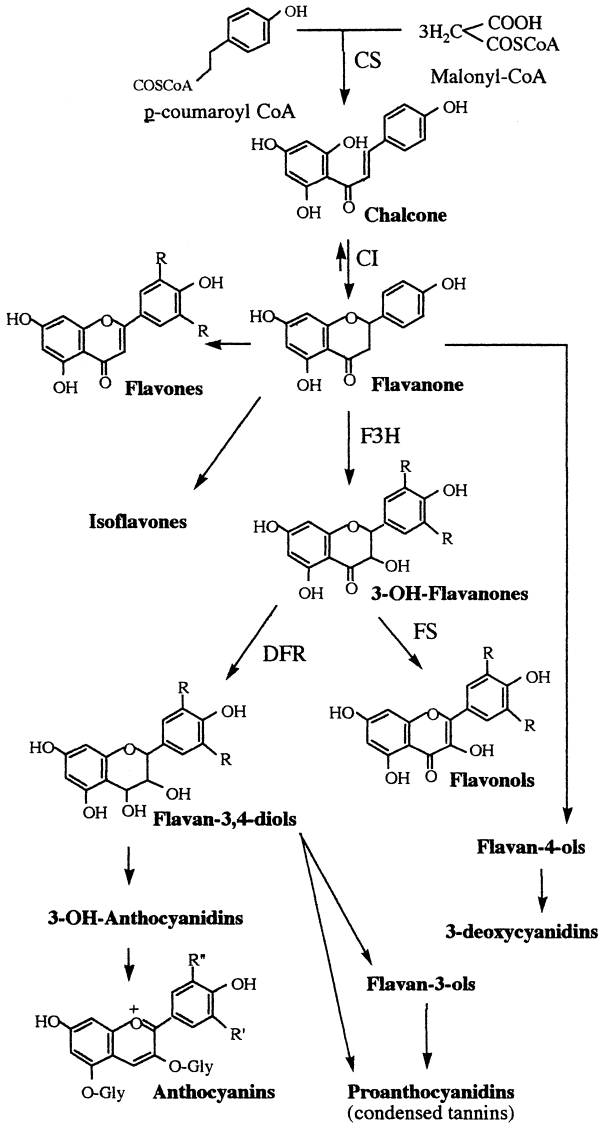


Fig. 2. Overall scheme for the biosynthesis of phenolic compounds. Abbreviations: enzymes CS: chalcone synthase; CI: chalcone isomerase; F3H: flavanone 3-hydroxylase; DRF: dihydroflavonol reductase: FS: flavonol synthase. Adapted from Cooper-Driver and Bhattercharya (1998).

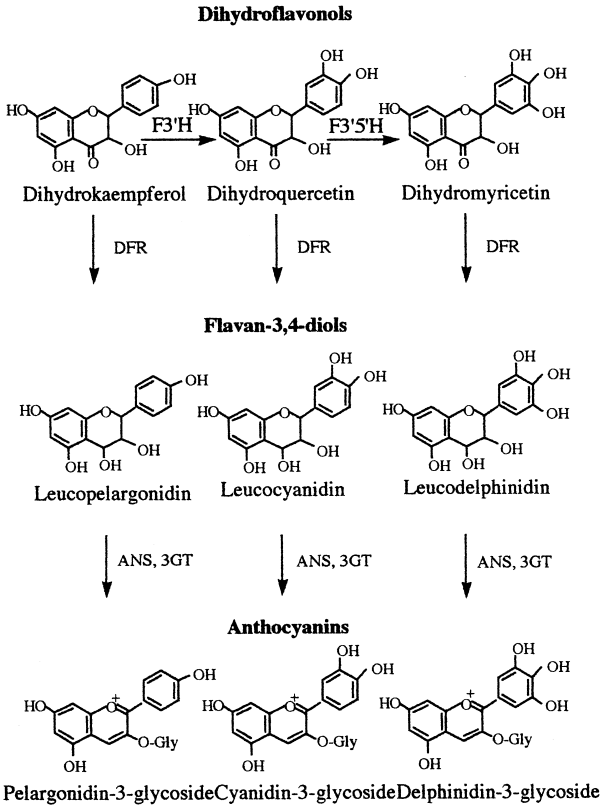


Fig. 3. Biosynthetic conversion of dihydroflavonols to anthocyanins. Abbreviations: enzymes F3′H: flavonoid 3′-hydroxylase; F3′5′H: flavonoid 3′,5′-hydroxylase; DFR: dihydroflavonol reductase: ANS: anthocyanidin synthase; 3GT: anthocyanin glucosyltransferase. Adapted from Holton and Cornish (1995).

Now that many of the genes encoding anthocyanin biosynthetic enzymes have been characterized and cloned from maize, snapdragon and petunia (i.e. chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavanoid 3′- hydroxylase, flavonoid 3′5′-hydroxylase, dihydroflavanol 4- reductase, anthocyanidin synthase, 3-glucosyltransferase, anthocyanin glucosyltransferases, and methyl transferases) transgenic approaches are now possible (Mol et al., 1998). This has allowed flower color to be altered in a highly directed fashion.

5.2. Creation of novel flower colors

The creation of novel flower colors in a number of commercially important species has been attempted by producing flowers with reduced pigmentation or conversely by increasing anthocyanin accumulation; introducing genes encoding novel enzyme activities or by the inactivation of endogenous genes.

5.2.1. Reduced pigmentation — antisense or co-suppression of CS genes

The key enzyme in the biosynthetic pathway that initiates the diversion of metabolic intermediates into pigment biosynthesis is chalcone synthase (CS) which catalyses the synthesis of narigenin from which all other flavonoids are derived. Antisense' or co-suppression of the CS genes have been described in petunia and in chrysanthemum (Courtney-Gutterson et al., 1994) resulting in flowers with reduced pigmentation.

5.2.2. Increased anthocyanin accumulation — co-suppression of an FS gene

As dihydroflavonols are the precursors of both anthocyanins and flavonols, the dihydroflavonols are potential substrates for the enzymes DFR, F3′H and F3′5′H, leading to pigment production, and/or for flavonol synthase (FS) leading to flavonol synthesis. Therefore competition between flavonoid-metabolizing

enzymes, FS and DFR for common dihydroflavonol substrates can shift the flavonol:anthocyanin ratio (Holton and Cornish, 1995). Co-suppression of the FS gene leads to increased anthocyanin accumulation.

5.2.3. New pigments — adding enzymes

In Petunias no pelargonidin derivatives are formed. This is due to the substrate specificity of the petunia dihydroflavonol 4-reductase (DFR) which is able to reduce dihydroquercetin and dihydromyricetin but is unable to reduce dihydrokaempferol. The same enzyme in *Zea mays* (maize) has a different substrate specificity since it is able to reduce the dihydrokaempferol substrate. A gene encoding the DFR from maize has been introduced into the petunia mutant — resulting in a change from white to strong brick-red coloration, due to the pelargonidin that is now being produced.

There is still a strong commercial interest in breeding stable blue flowers. Blue flowers are missing from a number of important ornamental plants, including carnations, chrysanthemums and roses (Holton and Tanaka, 1994). None of these plants is capable of synthesizing blue delphinidin pigments, presumably due to the absence of a gene encoding F3′5′H (see Fig. 4). A

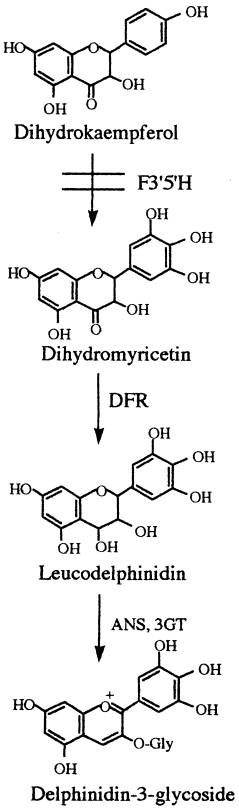


Fig. 4. Several commercially important plants lack the ability to synthesize delphinidin presumably due to an inability to convert dihydrokaempferol to dihydromyricetin. Abbreviations: enzymes as in Fig. 3.

possible novel way of breeding blue roses is suggested by Haslam (1993). As caffeine preferentially stabilizes the quinonoidal base form of anthocyanins, introducing the gene for caffeine synthesis clearly opens up another avenue for developing a blue rose and other blue flowers (Haslam, 1993). Blue flowers are of considerable interest as the presence of this color invariably involves some sort of unusual anthocyanin-copigment interaction and in several cases additional metallo-complexation (Bloor, 1999).

6. Concluding remarks

Currently there is a tremendous resurgence of interest in flavonoids generally and in anthocyanins in particular. Due to improved analytical techniques, namely HPLC, NMR and MS, many new anthocyanin structures are presently being recorded (Harborne and Williams, 1998). The majority of the new structures have both aliphatic (especially malonyl and acetyl) and aromatic (*p*-coumaroyl, feruloyl, caffeoyl and sinapoyl) substitution. A significant number of 3,7-disubstituted and 3,7,3′ trisubstituted pigments have also been described. The first macrocyclic anthocyanin has been isolated from flowers of two carnation cultivars (Bloor, 1998) a malic acid ester of cyanidin 3,5-diglucoside in which the malyl group is linked to both sugars through the 6-hydroxy position.

On the molecular side, current work is focusing on the genetics and regulation of anthocyanin biosynthesis, vacuolar transport mechanisms, the mechanisms by which regulatory genes determine tissue-specific patterns of pigmentation (Dooner et al., 1991), and ways to control vacuolar pH and co-pigmentation (Mol et al., 1998). Ecologically, we are only just beginning to recognize the important role anthocyanins play in growth, stress, and senescence.

Since the early 1950's, Jeffrey Harborne has made outstanding contributions to the study of anthocyanins (see selected references in this paper). Many of these papers have been published in the journal "*Phytochemistry*", thereby encouraging and shaping the work of future generations of phytochemists. We owe Jeffrey Harborne and all the other members of the Editorial Board of "*Phytochemistry*" a great debt for all the work they have done over the years in stimulating our interest in plants and their secondary metabolites. Perhaps for Jeffrey one day the dream of creating a blue rose may finally come true.

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Gillian Cooper-Driver graduated from University College London in 1955 with a BSc Honours in botany and subsidiary chemistry. She subsequently completed an MSc in microbiology and moved to the USA to work with F.C. Steward at Cornell University, New York State, USA on carrot tissue culture. After a period spent raising a family, Gillian returned to botanical work with the Phytochemical Unit, Agricultural Research Council, at the Royal Botanic Gardens, Kew headed by the phytochemist, Tony Swain. While at Kew, she completed her PhD, University of London, working on the chemotaxonomy of ferns and the chemical ecology of bracken, focusing on phenolic compounds. During her time at Kew, she developed and consequently maintained close ties with Jeffrey Harborne and Christine Williams at Reading University. In 1976, Gillian moved to the USA to take up an academic position at Boston University. For the next 15 years, she continued to study the chemical ecology of ferns in nearby Natick, Massachusetts and in the White Mountains of New Hampshire funded by the National Science Foundation and by Earthwatch. Her research focused on environmental influences on chemical defense strategies in ferns against insect herbivores and fungal pathogens. She also continued to have a strong interest in the biochemical evolution of phenolic compounds. In 1992, Gillian was at last able to fulfill her life long ambition to spend some time in southern Africa and through Fulbright Fellowships was able to work both as a Visiting Professor at the National University of Lesotho and later at the National Botanical Research Institute in Namibia. During this period her research interests became focused on factors affecting fungal infection of the seeds of the unique gymnosperm, *Welwitschia mirabilis,* in the Namib desert. She is currently on a 2 year contract in southern Africa at the University of Lesotho and plans to spend summer and winter breaks continuing her *Welwitschia* research.

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Phytochemistry and medicinal plants

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Abstract

A truncated history of the contribution of plants to medicine is given with reference to some of the less well known ancestors of the Harborne family. Six of the top 20 prescriptions dispensed in 1996 were natural products and the clinical use of drugs such as artemisinin, etoposide and taxol has once more focussed attention on plants as sources of novel drug entities. High through-put robotic screens have been developed by industry and it is possible to carry out 50,000 tests per day in the search for compounds which have specificity of action against a key enzyme or a subset of receptors. Bioassay-guided fractionation of plant extracts linked to chromatographic separation techniques leads to the isolation of biologically active molecules whose chemical structures can readily be determined by modern spectroscopic methods. The role of academics in the search for new drugs is discussed by reference to some of our research into natural products with activity on the central nervous system, on pain receptors, the malaria parasite *Plasmodium falciparum,* the wound healing properties of the sap of species of *Croton* (Dragon's blood), and a traditional Chinese medicine used to treat eczema. Expertise in phytochemistry has been essential for this research and the strong lead shown by Professor Jeffrey Harborne is gratefully acknowledged. © 2001 Published by Elsevier Science Ltd.

Keywords: J.B. Harborne; Medicinal plants; Phytochemistry; Academics; New drugs; Central nervous system; Eczema; Malaria; Pain; Wound healing

1. Introduction

The use of plants as medicines goes back to early man. Certainly the great civilisations of the ancient Chinese, Indians, and North Africans provided written evidence of man's ingenuity in utilising plants for the treatment of a wide variety of diseases. In ancient Greece, for example, scholars classified plants and gave descriptions of them thus aiding the identification process. Theophrastus has been described by some as the father of botany (Fig. 1) but little, if anything, has been recorded on his distant relative J.B. Theophrastus[1] who extolled the virtues of medicinal plants and forecast the possibility of discovering flavonoids. As Europe entered the dark ages much of this information would have been lost had it not been for the monasteries that acted as centres for the production of medicinal plants which were used to heal the suffering of mankind. There is still much we can learn from investigating the old herbals, particularly those less well known such as the one attributed to the monk J.B. Harbonus[1].

It was not until the 19th century that man began to isolate the active principles of medicinal plants and one particular landmark was the discovery of quinine from *Cinchona* bark by the French scientists Caventou and Pelletier (Fig. 2). Much less is known about the isolation of quinine by J.B. Caventou[1] and J.B. Pelletier[1]. Such discoveries led to an interest in plants from the New World and expeditions scoured the almost impenetrable jungles and forests in the quest for new medicines (Fig. 3). One of the lesser known intrepid explorers was J.B. van Harbon[1] who was never happier than when he was able to hatchet his way through the jungle stripping off the barks from every tree in sight. Such expeditions would last for years and it was not until the plants arrived at a well equipped phytochemical laboratory that the real discoveries could be made (Fig. 4). Laboratories such as those of Professor J.B. de Harbonney[1] became centres for the isolation of the active principles of medicinal plants from around the globe. Years of toil would be rewarded by the isolation of numerous flavonoids which were welcomed by the cognoscenti as well as the rapidly expanding pharmaceutical companies.

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[1] The lecture presented made reference to imaginary forefathers of Jeffrey B. Harbone.

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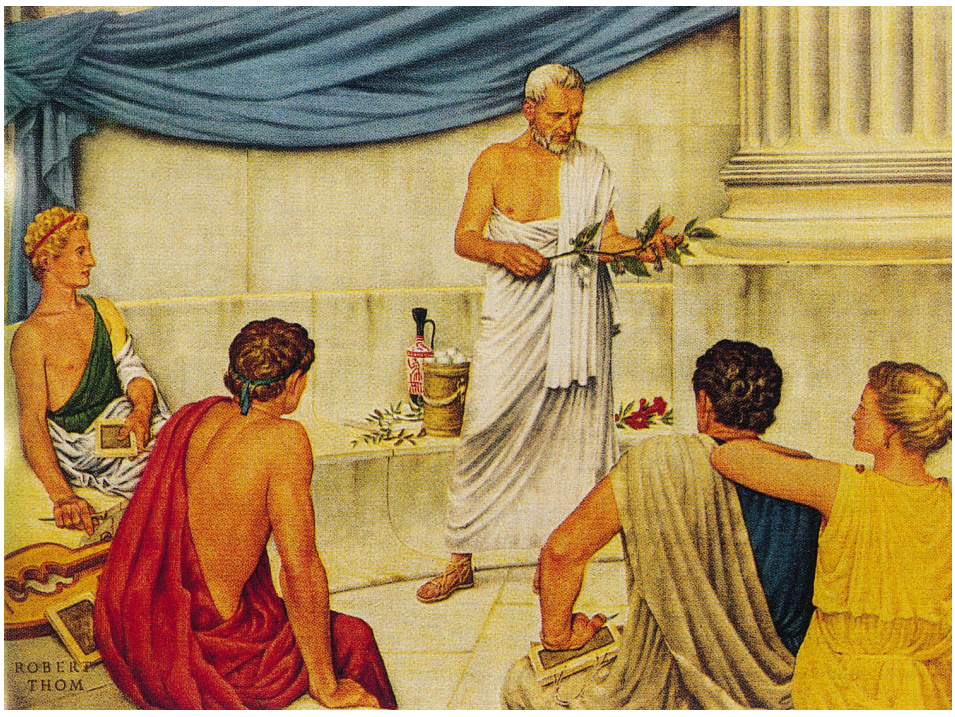


Fig. 1. Theophrastus — father of botany.



Fig. 2. First of the alkaloid chemists; Caventou, Pelletier and Quinine.

2. New drugs from nature

Prior to World War 2, a series of natural products isolated from higher plants became clinical agents and a number are still in use today. Quinine from *Cinchona* bark, morphine and codeine from the latex of the opium poppy, digoxin from *Digitalis* leaves, atropine (derived from (−)-hyoscyamine) and hyoscine from species of the Solanaceae continue to be in clinical use. The antibiotic era dawned during and after World War 2 due to



Fig. 3. Wresting the Jungle's secrets.

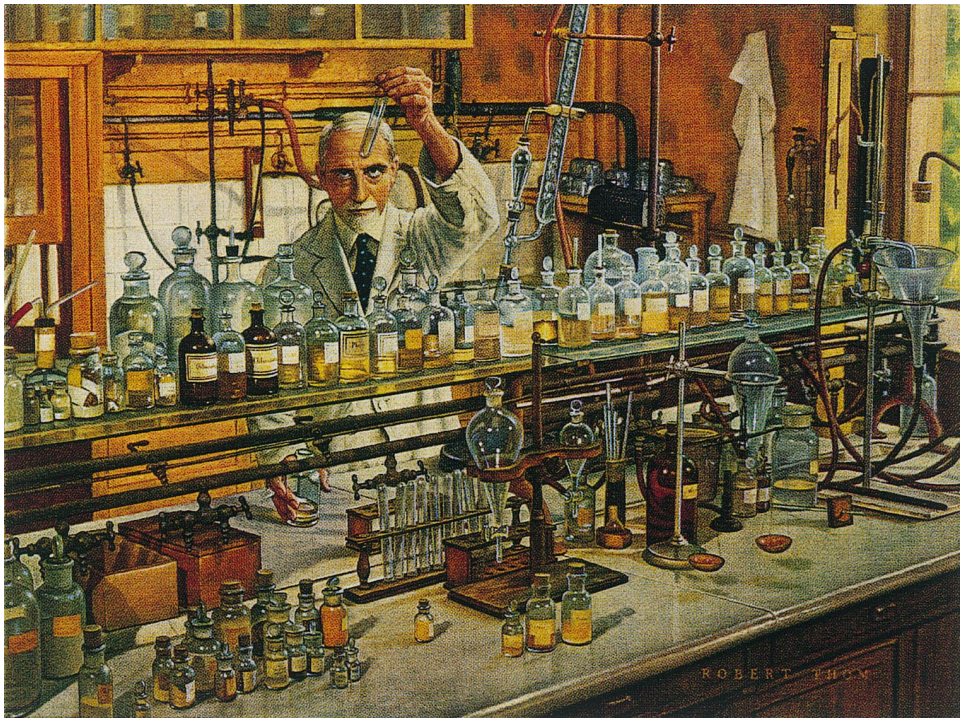


Fig. 4. The development of chemotherapy.

the antibacterial effects of a whole series of natural products isolated from species of *Penicillium*, *Cephalosporium*, and *Streptomyces*. In the post-war years there were relatively few discoveries of new drugs from higher plants with the notable exception of reserpine from the *Rauwolfia* species heralding the age of the tranquillisers and also vinblastine and vincristine from *Catharanthus roseus* which were effective in cancer chemotherapy.

Despite these discoveries the impact of phytochemistry on new drug development waned and inevitably the innovative pharmaceutical industry turned to synthetic chemicals. Successful clinical agents emerged from multidisciplinary research teams in which pharmacologists and synthetic chemists collaborated, e.g. atenolol (beta-blocker) and captopril (ACE-inhibitor) for treatment of hypertension, salbutamol (adrenoceptor stimulant) for asthma and the benzodiazepines (hypnotics and anxiolytics) for insomnia and anxiety attacks.

During recent years, the attention of the pharmaceutical industry has switched once more to the natural world and this may be illustrated by reference to three clinical drugs, taxol, etoposide and artemisinin (Phillipson, 1999a). Taxol is obtained from the bark of the Western Pacific Yew, *Taxus brevifolia*. The isolation and structure determination of taxol followed on from experiments that showed that a crude extract was active against cancer cells in laboratory tests. Although this activity was discovered in the early 1960's, it was not until 1971 that the structure elucidation of this complex diterpene was determined. In 1979 it was reported that the mode of action was through promotion of the assembly of tubulin into microtubules. Clinical trials did not take place until the early 1980's and it was not until the 1990's that taxol and its semisynthetic derivative taxotere were shown to be clinically effective against breast and ovarian cancers. The long period for the development of taxol as a clinical agent, its difficulty in procurement as a natural product and the complexity of its chemical structure all attest to the difficulties faced by the pharmaceutical industry in developing clinical agents from natural sources.

The resin podophyllin obtained from the root of the mayapple, *Podophyllum peltatum*, is toxic and is used clinically to remove warts. The major constituent of the resin is the lignan podophyllotoxin which inhibits cell division. Because of its toxic properties it would seem to be not worthwhile pursuing any medicinal activities even though its effects on cell division would indicate potential use in cancer chemotherapy. However, a semisynthetic modified glucoside, etoposide, which has a different mode of action inhibiting topoisomerase II, has found clinical application in the treatment of lung and testicular cancers.

Artemisinin is an unusual sesquiterpene endoperoxide that has been isolated as the active principle of the Chinese antimalarial herb *Artemisia annua*. Clinical trials have demonstrated that artemisinin is an effective antimalarial and can be used to treat infections of multidrug resistant strains of *Plasmodium falciparum* the cause of human malignant cerebral malaria. Semi-synthetic derivatives including artemether (the methyl ether of dihydroartemisinin) have improved pharmacokinetic properties and are also of current clinical use. The active moiety of artemisinin is 1,2,4-trioxane and a series of synthetic analogues show remarkable activity against *Plasmodium* species in vitro and in vivo. Whether or not these will prove to be effective clinical agents or will lead to new clinical drugs is a matter for future research.

The prospect of new drugs and medicines from plant sources is discussed further with reference to some of our research investigations (Phillipson, 1995, 1999a,b).

3. Will further new drugs be developed from natural product research?

The clinical applications of taxol, etoposide and artemisinin have helped to revive an interest in higher plants as sources of new drugs (Phillipson, 1999a). Despite the belief that the majority of clinical drugs are synthetic in origin, it is interesting to note that 6 out of the top 20 pharmaceutical prescription drugs dispensed in 1996 were natural products and that over 50% of the top 20 drugs could be linked to natural product research. In recent years the development of sensitive biological testing systems, mainly by industry, has led to the procedure of high through-put screening. Such screens are carried out robotically and it is possible for a pharmaceutical or biotechnological company to run 50,000 biological tests per day. The test screens are based on specific enzymes within an animal or microbial biosynthetic pathway or on receptors or subsets of receptors. New screens are continually being introduced and batteries of compounds, synthetic and natural, are tested as screens come on line. Hence, banks of compounds or extracts are needed for industrial biological tests. It is estimated that there are some 250,000 species of higher plants and the majority of these have not been examined in detail for their pharmacological activities. Specific plants may have been subjected to particular tests, e.g. for cardiac activity, but they have not been examined for any other type of activity. The major screens for biological activities of plant extracts have been carried out in the search for new anticancer, antiviral and antifertility drugs. The development of the rapid screening tests now in use in industry has meant that many more plants can be evaluated for a wide range of biological activities. Unfortunately the results of such tests do not necessarily reach the public domain and are kept in locked industrial files.

There still remains an urgent need to develop new clinical drugs and this can be exemplified by the numerous diseases which result from the malfunction of the central nervous system (CNS), e.g. Alzheimers and Parkinsons disease, epilepsy, migraine, pain, schizophrenia, sleeping disorders. Natural products already have a proven track record for CNS activities, e.g. caffeine, codeine, morphine, nicotine, reserpine and it is possible that there are further such drugs still to be found from nature (Phillipson, 1999b).

With this in mind, we collaborated with two major international pharmaceutical companies, Glaxo (now Glaxo–Wellcome) and Pfizer. In one investigation, some 10 Chinese plants were assessed for their activities against 18 radioligand-receptor binding assays which are implicated with CNS. The other investigation was concerned with pain and some 600 species of plant were tested. The pain receptors used were bradykinin II, neurokinin I, and a calcitonin gene related peptide. Half of the plants were selected from the ethnobotanical literature as being used for the treatment of pain and the other half were from a random sample. The results showed that there were more positive hits for activity in the biological screens for the selected group of plants (Phillipson, 1999b).

4. Malaria

In 1996 it was reported that there were between 1.5 and 2.7 million deaths annually and that the majority of these were children. There are in the order of 500 million new incidences of malaria annually. It is without doubt one of the major threats to mankind and chemotherapy is hindered by the increase in drug resistant strains, particularly of *Plasmodium falciparum*. In the mid 1980's we posed the question " Are new antimalarial drugs awaiting discovery from plants?" Quinine, the first effective antimalarial drug is still in clinical use and the more recently discovered artemisinin has proved to be an incentive for further research into plants. The only major scientific paper of antimalarial testing of plant extracts by the mid 1980's dated back to 1947 when it was reported that some 600 species of higher plant representing some 126 families had been tested against avian malarias. Several plants were active but the research pointed to two particular plant families, Simaroubaceae and Amaryllidaceae which had numerous active species. It is pertinent to ask why it took more than 30 years for this research to be followed through. The answer probably lies in the techniques which were available for carrying out this type of research. Avian malarias were used because they were the only tests for activity against *Plasmodium* species apart from those using monkeys. The avian tests which used live chickens and ducklings were notoriously difficult to carry out and were not thought to be necessarily predictive of activity against *Plasmodium* species which affected humans. These tests were not suitable for bioassay-guided fractionation of plant extracts. Furthermore, the chemical techniques available were also not suitable for this type of research. In the 1940's and 1950's there were not the sophisticated chromatographic separation techniques which are available today. Even if an active principle were isolated there were none of the spectroscopic techniques available for structure determination such as nuclear magnetic resonance spectroscopy, mass spectrometry or X-ray crystallography. By the mid 1980's not only were these chemical techniques available but also it was possible to test for activity against *P. falciparum in vitro* and there was a reliable test in mice against *P. berghei* (Phillipson, 1995).

Following the lead from the 1947 paper, we tested activities of 5 species of Simaroubaceae against *P. falciparum* in vitro and utilised bioassay-guided fractionation techniques to isolate a series of active terpenoids (quassinoids). Some 40 quassinoids became available for structure–activity studies and this led to the preparation of semi-synthetic and synthetic analogues. Despite considerable research effort, no new clinical drug was developed from this work. Investigation of a range of plants used in traditional medicine for the treatment of malaria led to the isolation of a series of other compounds with activity against *P. falciparum* including isoquinoline and indole alkaloids, flavonoids, mono-, di- and sesquiterpenoids. These results provided some scientific evidence which helped towards the justification of claims for the use of a number of traditional medicines and they also provided template molecules for synthetic approaches to new antimalarial drugs. Widening the range of biological tests to include other species of protozoa demonstrated activity of natural products against other tropical diseases which affect mankind, e.g. trypanosomiasis and leishmaniasis (Phillipson, 1995 and 1999a).

5. Do traditional medicines necessarily contain a single active ingredient?

The isolation and use of natural products such as digoxin, morphine and quinine has resulted in replacing the plant extracts used with single chemical entities. There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle which can completely replace the plant extract. Three examples from our research have shown that this may not necessarily be true (Phillipson, 1995).

Artemisinin is without doubt the potent antimalarial active principle of *Artemisia annua*. Crude extracts of *A. artemisia* contain a plethora of other compounds including a series of flavonoids and some of these enhance the activity of artemisinin against *P. falciparum* in vitro. Whether these findings have clinical relevance has not been determined but they do lend support to the view that there may be some advantages to the medical use of extracts as opposed to isolated single entities.

Dragon's blood is a term used for the blood red sap obtained from the bark of a number of S. American *Croton* species which are used for the treatment of wounds. The major constituents of the sap are polymeric anthocyanidins which co-occur with many minor constituents including diterpenes and simple phenols. Chemical and biological investigation of the properties

of Dragon's blood led us to conclude that there is not one single wound healing principle. When the sap is used to cover a wound it forms a protective occlusive layer whilst some of the simple phenols act as potent antimicrobial agents and other compounds exert anti-inflammatory effects.

In the 1980's it was noted by clinical dermatologists at Great Ormond Street Hospital for Sick Children in London that some of their young patients with severe atopic eczema were showing signs of improvement in their disease state. These improvements were not due to hospital therapy but to the co-administration of a traditional Chinese medicine (TCM). The patients had visited a TCM practitioner in central London and had been prescribed a multi-herbal prescription from which an aqueous extract was prepared for oral use. In 1992, it was reported that a double blind placebo controlled clinical trial of a ten herb mixture for oral use in children with non-exudative eczema confirmed substantial clinical benefit as assessed by currently accepted Western orthodox medical practitioners. Our scientific investigations utilising an anti-inflammatory/analgesic test with mice showed that four of the ten herbs possessed significant activity in the mice but they proved to be inactive clinically in children. After some considerable investigation we concluded that not only was there no single active ingredient but also that it required all ten herbs to be present for clinical effectiveness. There are more than 12 different biological activities from the herbs in this TCM prescription including anti-inflammatory, immuno-modulatory, anti-allergic, sedative and anti-pruritic. The chemical composition of the 10 herbs is a complex mixture of natural product molecules.

6. Conclusions

Plants continue to be used world-wide for the treatment of disease and novel drug entities continue to be developed through research into their constituents. In the developed countries, high-throughput screening tests are used for bioassay-guided fractionation leading to the isolation of active principles that may be developed into clinical agents either as the natural product or a synthetic modification or a synthesised analogue with enhanced clinical action or reduced adverse side effects. Despite the massive arsenal of clinical agents developed by the pharmaceutical industry there has been an aversion by many members of the public and herbal remedies have proved to be popular as alternative or complementary treatments of disease. There is a need to evaluate herbal treatments by clinical trials using currently accepted protocols. In the developing countries large numbers of the World's population are unable to afford pharmaceutical drugs and they continue to use their own systems of indigenous medicine that are mainly plant based. There is a great need to harness scientific and clinical research in order to investigate the quality, safety and efficacy of these herbal therapies.

The aim of the pharmaceutical industry is to develop novel drug entities for the treatment of disease. Such drugs require specificity of action and are, for e.g. aimed at a particular subset of receptor. Although natural products continue to supply banks of compounds for new screens, the focus of industry is currently on combinatorial synthesis for new drug development. It must not be forgotten that natural products which result from millennia of biosynthetic pathways modified by evolution have a well established track record as medicinal agents and present a wide range of structural diversity. Drug development through natural product research is not without its problems and there is, for e.g. a need to eliminate common natural products such as saponins, tannins, etc. from plant extracts prior to testing by biological screening procedures. Academics can play a useful role in this area of research. They cannot match industry in the wide range of screens but they can use selective targets and collaborate with industry. This type of research needs a multi-disciplinary approach and this includes expertise in phytochemistry.

It is a pleasure and an honour to present this lecture and to acknowledge the lead which Professor Jeffrey Harborne has given to Phytochemistry over so many years. I am one of those who owe a great debt to him and to the example which he has set. My own specialisation of Pharmacognosy was virtually wiped out from Pharmacy undergraduate curricula and for many years has been considered to be an outmoded area of research. Techniques in Phytochemistry have revolutionised our ability to investigate the medicinal agents present in plants and this is acknowledged by the industrial interest in plants over recent years. Thanks to the hard work and tenacity of Jeffrey Harborne we have been able to publish research articles in *Phytochemistry* and to continue working on the wealth of chemical diversity that exists in the plant kingdom.

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Importance of flavonoids in insect–plant interactions: feeding and oviposition

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Abstract

Jeffrey Harborne and colleagues have been responsible for collating the majority of data on the role of flavonoids in insect–plant interactions. This article examines some of this information and assesses our knowledge about the role flavonoids play in insect feeding and oviposition behaviour. It is clear that insects can discriminate among flavonoids and that these compounds can modulate the feeding and oviposition behaviour of insects, but further work is required to understand the neural mechanisms associated with these behavioural responses. Despite the wealth of data about the diversity of flavonoids in plants, very few of these compounds have been tested against insects and their role in the evolution of host range in insect–plant interactions has yet to be determined. © 2001 Published by Elsevier Science Ltd.

1. Introduction

The majority of phytophagous insects are monophagous or oligophagous, feeding on a very narrow range of plant species or families. Their host range is limited, in part, by the chemical complexity of the plants they encounter: host-plants being selected because they either contain stimulants or lack deterrents. Given that plant compounds play such a major role in the selection behaviour of insects, the ability of insects to perceive and discriminate among compounds must be profound. The nature of plant-derived allelochemicals or secondary metabolites involved in the different stages of insect–plant interactions, from habitat selection to host acceptance, is very varied. In the last decade our ability to isolate these plant–derived compounds and then determine their structures has improved with the advances in analytical phytochemical techniques. Consequently, the opportunities to assess the role of these compounds in insect-plant interactions using behavioural and electrophysiological bioassays has improved. Jeffrey Harborne and the many collaborators that have worked with him and Christine Williams at the University of Reading, UK have played a major role in investigating as well as collating information from the literature about the distribution of secondary metabolites in plants, especially flavonoids (Harborne et al., 1975; Harborne and Mabry, 1982; Harborne, 1988, 1994), and their role in the ecology of insect–plant interactions (Harborne and Grayer, 1994). Their work has resulted in a better understanding of the distribution of flavonoids in the flowering plants and the role these compounds can have in insect–plant interactions. This paper provides a brief overview of our knowledge about how flavonoids influence insect feeding and oviposition behaviour and is based on information collated by Harborne and Grayer (1994) on flavonoid–insect interactions.

Most plants contain an array of flavonoids and although there are examples of chemical convergence when two or more unrelated families contain similar flavonoids, the flavonoid profiles often differ among families, genera and species (Harborne and Turner, 1984). The fact that phytophagous insects usually differentiate among families suggests that flavonoids play a role in host selection. However, it is still unclear whether it is the overall profile of flavonoids and other compounds that determines host acceptance, or the presence or absence of specific compounds.

Some flavonoids accumulate in plants and it is the study of the accumulation of these flavonoids in a species that has been shown to be the most appropriate way

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to use flavonoid data as characters in chemosystematics, rather than recording the presence or absence of flavonoids (Harborne and Turner, 1984). Thus, should entomologists evaluate the role of flavonoids in host selection by testing the insect responses to the flavonoids that accumulate in host and non-host plants? If yes, then there is very little literature to support this approach, especially with respect to flavonoids that occur on the plant surface and could modulate host acceptance or rejection. Many entomologists have undertaken activity-guided fractionation studies of extracts from plants and have isolated flavonoids that stimulate host-selection behaviour (Feeny et al., 1988; Honda, 1990). However, they do not often provide evidence that the active flavonoids are missing from non-host plants. Other entomologists have taken a more reductionist approach by looking at the structure–activity relationships of a range of flavonoids without taking into account whether the flavonoids occur in host or non-host plants (Simmonds et al., 1990). This latter approach may provide us with information about the types of flavonoids that could modify insect feeding and development but it does not provide any direct evidence about the role of flavonoids in a specific host–plant interaction. Despite the interest in host-selection behaviour and the information available about the distribution and diversity of flavonoids in plants, the proportion of flavonoids investigated for their role in host selection is small (Harborne and Grayer, 1994).

2. Feeding

Rutin (quercetin-3-*O*-rhamnosyl [1→6] glucoside), one of the most widely studied flavonol glycosides, is a phagostimulant to many polyphagous insects including the locust *Schistocerca americana* (Bernays et al., 1991) and the caterpillar *Heliothis virescens* (Blaney and Simmonds, 1983). The behavioural response of insects to rutin can vary depending on the concentration tested and the age of the insect tested. For example, rutin at concentrations greater than 10^{-3} M deterred final stadium *Heliothis zea* and *Heliocoverpa armigera* larvae from feeding, but at concentrations less than 10^{-4}M it stimulated feeding in final stadium larvae (Blaney and Simmonds, 1983). Rutin also deters second stadium *H. zea* larvae from feeding (Isman and Duffey, 1982). Once rutin is ingested the glycosidic bond is hydrolysed to release the aglycone quercetin. Quercetin can inhibit mitochondrial ATPase (Lang and Racker, 1974) and cytochrome P450-dependent mixed function oxidases (MFO) (Mitchell et al., 1993). This inhibitory activity could explain why rutin has detrimental effects on the development of early stadia larvae of *H. zea* (Isman and Duffey, 1982), *Spodoptera litura* (Stevenson, 1992) and gypsy moth, *Lymantria dispar,* (Beninger and Abou-Zaid, 1997), whereas the later and larger stadia of these species consume rutin-enriched diets with no adverse effects. Levels of MFO increase as the larvae mature so that fifth stadium larvae could have enough MFO to detoxify quercitin. In addition, larvae gain some protection from the intake of flavonoids. *Schistocerca americana* nymphs fed rutin had lower mortality than those without rutin (Bernays et al., 1991) and *H. zea* was protected from viral infections when feeding on rutin-rich tomato plants (Felton et al., 1987). Some of the rutin ingested by *S. americana* is metabolised to a β-3-*O*-glucoside and sequestered into the exocuticle (Bernays et al., 1991). It was thought that this might provide the grasshopper with some protection against infection by fungi but Bernays and Chapman (2000) report that there was no protection against infection by either *Beauveria brassiana* or *Entomophaga* spp.

Barbosa et al. (1983, 1986) showed that early stadium larvae of the gypsy moth do not survive well on pine trees, such as the white pine, *Pinus strobus* L., but the later stadia do survive and may get some benefits from including pine in their diet. Beninger and Abou-Zaid (1997) tested whether flavonoids could attribute to this toxicity of pine to the gypsy moth. They showed that the growth of larvae of the gypsy moth could be inhibited by flavonol glycosides from pine needles. Rutin, quercetin 3-*O*-glucoside and quercetin reduced the growth of second stadium gypsy moth larvae when tested at 0.05 and 0.1%. These flavonoids also reduce growth and survivorship of the European corn borer, *Ostrina nubilalis* (Abou-Zaid et al., 1993).

Harborne and Grayer (1994) provide an overview of flavonoids that stimulate feeding including the flavonol isoquercitrin (quercetin 3-*O*-glucoside), one of the first flavonoids to be shown to influence insect feeding behaviour. This flavonoid is an important stimulant for silk worm larvae as it promotes the biting and consumption of leaves. Flavonoids also influence the feeding behaviour of the flea beetle *Phyllotreta armoraciae*, which uses both glucosinolates and flavonoids in the selection of its host plant, the horseradish *Armoracia rusticana* (Nielson et al., 1979).

Quercetin and quercetin-derivatives do not always influence insect behaviour. For example, Lindroth and Peterson (1988) found that quercetin and rhamnetin (7-methoxy-quercetin) did not influence the growth of *Spodoptera eridania*. Faini et al. (1997) reported that the feeding behaviour of *Spodoptera littoralis* was not affected by quercetin 7-methyl ether, isolated from *Flourensia thurifera,* when applied to lettuce disks at 20 μg per disc.

Apigenin-*C*-glycosides, such as schaftoside, which occurs in plant phloem, modify the feeding behaviour of the brown planthopper, *Nilaparvata lugens* (Grayer et al., 1994). When the planthoppers were exposed for a 25-hour period to rice stems with high levels of flavonoids, their weight decreased and those on rice varieties with

lower levels of flavonoids gained weight (Stevenson et al., 1996). Furthermore when nymphs were exposed to an artificial diet containing schaftoside at either 250 or 500 μg/ml the nymphs were dead after 10 and 5 days, respectively, whereas 20% of the nymphs on a control diet lacking schaftoside were still alive after 11 days (Stevenson et al., 1996). This suggests that schaftoside could play an important role in the resistance of rice to planthoppers. However, further experiments are needed to evaluate whether the resistance to planthoppers of varieties of rice with high levels of flavonoids can be attributed to the level of schaftoside or one of the other apigenin-*C*-glycosides in rice.

The fact that the feeding behaviour of insects can be modulated by flavonoids suggests that insects are able to perceive the compounds. For example, lepidopteran larvae have sensory receptors on the neurones in their maxillary stylcononic sensilla that respond to a range of phagostimulants and deterrents, such as sugars, amino acids, alkaloids and diterpenoids (Schoonhoven, 1982). There is evidence that some species of grasshoppers (Bernays and Chapman, 2000) and Lepidoptera (Simmonds et al., 1990) have taste receptors that respond to phenolics. However, we know very little about the specificity of these receptors and whether they differentiate among different groups of flavonoids such as chalcones, flavones, flavonols and isoflavones.

The neural mechanism associated with the perception of flavonoids appears to be complex. Neurones in the lateral and medial styloconic sensilla on the maxilla of *Yponomenta* larvae did not respond to rutin, although it occurs in many plants eaten by *Yponomenta* larvae (Van Drongelen, 1979). The lack of a neural response from these sensilla to rutin does not mean that the insect cannot perceive rutin. It could be that other gustatory sensory sensilla are used to perceive the compound or that rutin might not stimulate a neural response when tested by itself, but if tested in combination with other compounds it could modulate the response to mixtures (Schoonhoven, 1982). As yet, electrophysiological experiments using mixtures with and without rutin have not been undertaken. Kaempferol and quercetin occur in species of *Brassica* but they did not evoke any responses from neurones in the medial and lateral maxillary styloconic sensilla of larvae of either the small, *Pieris rapae,* or large, *P. brassicae,* cabbage butterflies (Van Loon, 1988), whereas a neurone in the lateral sensilla of both species did respond to catechin, another flavonoid that occurs in *Brassica*.

Host selection is not a static process as the needs of the insect will vary throughout its life cycle and the suitability of the plant to the insect will vary with time. To date we have no detailed information on whether insect damage to a plant during feeding results in changes in the flavonoid profile of the leaf being eaten or in nearby leaves and whether the palatability of that leaf would alter if the profile of flavonoids did change. After a leaf has been damaged by an insect a cascade of enzyme reactions occur in a plant that could alter flavonoid profiles and thus host selection. For example, in the tobacco plant, insect damage induces a ''wound response pathway'' which involves the release of systemin, a mobile 18 amino acid peptide that activates a signal pathway resulting in the stimulation of the fatty acid-derived jasmonic acid. This results in the induction of proteinase inhibitors, phenolics and polyphenyl oxidases (Baldwin, 1999). Systematic acquired resistance is another defence mechanism that occurs after pathogen or insect damage and may involve the activation of phenylalanine ammonia-lyase (PAL). This enzyme acts as a catalyst in phenylpropanoid biosynthesis and the production of the phenylpropanoid-derived metabolite salicylic acid which mediates the levels of phenylpropanoid by-products such as chlorogenic acid (Felton et al., 1999). The by-products of these reactions alter the palatability and suitability of the plant to an insect. Results from work on tobacco plants have shown that there are complex interactions between the jasmonic acid and salicylic acid pathways. For example, plants in which PAL has been suppressed have low levels of salicylic acid but can have an effective grazing-induced resistance to *Heliothis virescens*, whereas in those plants in which PAL has been over expressed there is a decrease in grazing-induced resistance (Felton et al., 1999). It appears that high levels of salicylic acid block the biosynthesis of jasmonic acid and ability of the plant to respond to wound-induced signalling, whereas jasmonc acid can block the ability of salicylic acid to produce pathogen-induced proteins. Modulating these biosynthetic pathways is likely to influence the levels of flavonoids in plants and could thus alter the suitability of a plant as a host.

If damage to the plant results in an increase of flavonoids that do not deter insects from feeding and are not toxic, then the insect might benefit from consuming these flavonoids. Harborne and Grayer (1994) showed that there is some evidence in the literature to suggest that the fitness of insects can be improved if they are able to sequester flavonoids in their bodies or wings.

3. Oviposition

Flavonoids have been implicated as being important in host recognition and host acceptance by adult insects. Once an adult female butterfly or moth has landed on a plant she will often walk over the leaf or stem surface. The duration of this walk and the commencement of the next behaviour may depend on what stimuli she has received from the compounds on the plant surface. Some species will commence drumming with their forelegs on contact with a potential host, others will lower their

antennae and probe the leaf surface. These behavioural traits result in the contact sensilla on the tarsi and antennae being brought into contact with compounds on the surface of the plant. Contact chemoreceptors on the tarsi of adult Lepidoptera have been shown to respond to a range of extracts that contain flavonoids and the same extracts stimulate oviposition in behavioural tests. In many cases the stimulatory activity of the extracts cannot be attributed to one compound alone but involves synergistic interactions among the compounds (Feeny et al., 1988). For example, the black swallowtail butterfly (*Papilio polyxenes*) oviposits on wild carrots, *Daucas carota* (Umbelliferae), and the main stimulants in this plant include the acylated flavone glycoside, luteolin 7-*O*-(6-*O*-malonyl)-β-D-glucoside (LMG) and *trans*-chlorogenic acid which Roessingh et al. (1991) have shown stimulate receptors in tarsal sensilla. The composition of substitutions on luteolin are important for this synergistic action as the luteolin glycoside without malonyl was inactive as a synergist (Feeny et al., 1988). Recent experiments with *P. polyxenes* ovipositing on another host, wild parsnip, have challenged the importance of LMG as a synergist. When fractions containing the flavonoid glycoside were added to other fractions from wild parsnip containing chlorogenic acid and the amine, tyramine, there was no further increase in oviposition responses of *P. polyxenes*. However, the level of the LMG in the fraction of wild parsnip was 53% lower than occurred in wild carrot and the parsnip extract did contain other flavonoid glycosides, which have not been identified (Carter et al., 1998). It is possible that one or more of the other flavonoids in the carrot fraction could have had mild deterrent activity which could have masked the stimulatory activity of LMG. Another species of *Papilio*, *P. xuthus* can be deterred from ovipositing by the flavonoid glycoside quercetin 3-*O*-(2-β-D-xylopyranosylrutinoside). Nishida et al. (1990) isolated this flavonoid from *Orixa japonica,* a rutaceous plant, which is not a host of *P. xuthus*. Other flavonoid glycosides have been reported to stimulate oviposition (Ohsugi et al., 1985). These include, vicenin 2 (apigenin 6,8-di-C-glucoside), narirutin (naringenin 7-*O*-β-rutinoside), hesperidin (hesperetin 7-*O*-β-rutinoside) and rutin. *Papilio protenor* uses the flavonoid glycosides naringin and hesperidin to assist it select citrus fruit (Honda, 1990). It is clear from these studies that at least three species of *Papilio, P. xuthus*, *P. protenor* and *P. polyxenes,* all use flavonoid glycosides in their host selection behaviour.

The female monarch butterfly, *Danaus plexippus,* also use flavonoids in its host selection behaviour. Haribal and Renwick (1996) identified three quercetin glycosides (quercetin 3-*O*-β-D-glucopyranosyl (1-6)-β-D-galactopyranoside, quercetin 3-*O*-(2'',6''-α-L-dirhamnopryranosyl) -β-D-galactopyranoside and rutin) on the leaves of the host plant *Asclepias curassavica* that stimulated oviposition. Each flavonoid was able to stimulate oviposition when tested on its own. Electrophysiological experiments have shown that the taste sensilla on the fore-tarsi of the monarch butterfly do not respond to these flavonoids, although sensilla on the mid-tarsi and antennae do respond (Baur et al., 1998). The lack of neural response from the sensilla on the fore-tarsi indicates that these sensilla are not directly involved in the host acceptance behaviour. Although the role of fore-tarsi drumming has been thought to be an important feature of host selection behaviour, it is known not to occur on all plants (Baur et al., 1998). It would appear that receptors in the sensilla on the mid-legs of monarch butterflies provide the adult with sensory information about flavonoids. Host discrimination by other butterflies, such as *Pieris brassicae*, also involve the sensilla on the tarsi of the mid-legs (Ma and Schoonhoven, 1973). The neural response to flavonoids of receptors in sensilla on the antennae of the monarch butterfly, indicate that they could also play a key role in host recognition. However, the response of these sensilla to the flavonoids varied and the response of many was masked by poor signal-to-noise ratio. As yet there is very little information in the literature about the neural specificity of the sensilla on the antennae of butterflies and moths to non-volatile compounds. Their importance in the host recognition behaviour of adults will become clearer when more is known about the specificity of the receptors in these sensilla.

A technical problem encountered with testing some of the flavonoids in the electrophysiological experiments relates to the solubility of the compounds. For example, most aglycones are less polar than the glycosides and it is not easy to get them to dissolve directly in an electrolyte. However, if the less polar flavonoids are initially taken up in acetone or ethanol and then diluted to the required concentration with an electrolyte, such as a potassium chloride solution, they will usually remain in solution if tested at concentrations less than 10^{-3} M.

4. Structure–activity relationships

At least two factors support the claim that flavonoids play a role in insect–plant interactions. Firstly, flavonoids can modulate the feeding and oviposition behaviour of insects and secondly that the distribution of flavonoids varies among plant species. However, we still need to establish whether the distribution of specific flavonoids overlaps with the presence or absence of these flavonoids in host-and/or non-host plants. This information can be obtained by screening plants for the presence or absence of specific flavonoids, but before doing this we need to know more about how specific the responses of an insect are to flavonoids. Structure–activity type studies can provide us with this information.

They can assist us to establish the types of flavonoids that modulate insect behaviour and whether changes in the composition of substitutions on a flavonoid molecule will alter the responses of an insect to that compound. This type of study can assist us to establish which of the many categories of flavonoids have the most influence on insect behaviour. To date there are very few structure–activity studies with flavonoids, and both the insects and the flavonoids tested vary from one study to another.

Overall, methoxylation of the hydroxy groups on flavonoids resulted in a decrease in activity (Norris, 1977; Elliger et al., 1980; Lane et al., 1985). Elliger et al. (1980) screened a wide range of flavonoids and showed that the toxicity of flavonoids against *Heliothis zea* decreased with increased methoxylation. Similarly, the responsiveness of gustatory neurones of *Pieris* species to flavonoids decreased with increased methoxylation (Van Loon, 1988). In contrast, kaemperol and quercetin flavonoids that have an oxidised C-ring, inhibited feeding in *Scolytus multistriatus* (Norris, 1977) but they were inactive against species of *Pieris* (Van Loon, 1988). Other studies have shown that differences in the composition of the sugar residues on the flavonoid glycosides influence the responses of chrysomelid beetles (Matsuda, 1978). de Boer and Hanson (1987) showed that the quercetin glycoside, rutin, is more active as a phagostimulant for the tobacco hornworm, *Manduca sexta*, than a mixture of the aglycone quercetin and the sugar rutinose. They also showed that *Manduca sexta* can differentiate between the phagostimulant rutin and the antifeedant quercetin-3-rhamnoside, which lacks the glucose present in rutin. *Bombyx mori* can differentiate among glycosides with different sugar moieties; quercetin-3-*O*-glucoside (isoquercitrin) is a feeding stimulant, the 3-*O*-rutinoside was inactive and the 3-*O*-rhamnoside was a deterrent (Hamamura et al., 1962).

Simmonds et al. (1990) used behavioural and electrophysiological bioassays to compare the responses of the oligophagous larvae, *Spodoptera exempta*, with those of the polyphagous larvae, *Spodoptera littoralis*, to a range of flavonoids. The compounds tested were either synthesised or isolated from species of *Lonchocarpus* and *Tephrosia* (Leguminosae), and included 12 flavanones, 2 methoxyflavones and 7 chalcones. In a binary choice test using glass-fibre discs treated with sucrose, 14 of the 19 compounds tested at 100 ppm elicited an antifeedant response from *S. exempta*, whereas only 7 of the 21 compounds tested against *S. littoralis* showed significant antifeedant activity. Only one of the flavonoids tested, the flavanone, 3-hydroxyisolonchocarpin (**12**), was a potent phagostimulant for *S. littoralis* but, whether the flavanone would have stimulated *S. exempta* to feed is not known because of lack of material available for testing (Fig. 1). A comparison of the activity of isolonchocarpin (**9**), which had potent antifeedant activity with the phagostimulant 3-hydroxyisolonchocarpin (**12**), indicates that the addition of a hydroxy group at the 3-position of isolonchocarpin can alter the activity of the compound (Fig. 1). The methoxyflavones and a high proportion of the flavanones were more active as antifeedants against *S. exempta* than *S. littoralis*. Some of the flavonoids were retested using leaf material as a substrate and their antifeedant activity was retained. However, when some compounds were tested in a no-choice test with glass-fibre discs, the antifeedant activity of a few compounds was lost. The differences in behavioural responses to a compound recorded in these bioassays reflect in part the potency of the compound. For example, a compound showing antifeedant activity in all bioassays is very potent. However, if a compound has activity in a choice bioassays but the activity is lost in a no-choice bioassay, this indicates that the insect can perceive the compound but adapts to it during the course of the experiment. Thus the effectiveness of that compound as an antifeedant decreases with time.

Choice bioassays can be used in structure–activity studies to monitor how small changes in parts of a compound can alter its activity. They were used by Simmonds et al. (1990) to detect differences in the palatability of chalcones and flavanones. For example, chalcone **4** which is the methylated derivative of chalcones **3** was a more potent antifeedant against both species of *Spodoptera*, whereas flavanones with either the hydroxy and methoxy substitution were active antifeedants against *S. exempta,* but the methylated flavanone was more active against *S. littoralis* than the hydroxy flavanone. The presence (**5, 9**) or absence (**6**, **11**) of a double bond in the dimethyl chromene ring of the chalcones and flavanones did not alter the potency of the compounds against *S. exempta*, whereas the chalcone and flavanone with the double bond were active against *S. littoralis*.

When the lateral and medial maxillary styloconic sensilla on the larvae of both species were stimulated with the compounds, responses were recorded from both sensilla, but the responses were greater from the medial sensilla than the lateral (Simmonds et al., 1990). In *S. exempta*, the flavonoids stimulated one of the four taste neurones in the medial sensilla. The firing rate of this neurone was negatively correlated to the amount of glass-fibre disc eaten in a no-choice bioassay: the response of the neurone was greatest when the larvae did not eat much of the flavonoid-treated disc. This neurone is often called the "deterrent neurone" as any compound that elicits a response that is greater than about 30 impulses per second will usually deter feeding. In *S. littoralis*, the flavonoids stimulated one to three of the four taste neurones in the medial sensilla and the neural response was positively correlated to the amount eaten: the rate of firing of the neurones increased as the amount eaten increased. Thus the gustatory neural code

associated with the perception and resulting behavioural response to the flavonoids differed between the two species.

A comparison of the electrophysiological responses and behavioural responses of the two species of *Spodoptera* to a selection of chalcone/flavanone pairs of compounds shows that in the majority of cases the flavanones were more potent antifeedants than the chalcones (Simmonds et al., 1990). It is clear from these experiments that the neural responses or "gustatory code" associated with the perception of these compounds and the associated effect on feeding behaviour in a no-choice bioassay of the two species of *Spodoptera* differed. For example, a comparison of the activity of compound **1** with that of **8**, **2** with **9**, **3** with **10** and **7** with **13** shows differences in the responses of species of *Spodoptera* (Figs. 1 and 2). With *S. exempta* the chalcones **1**, **2**, **3**, and **7** showed no antifeedant activity in a no-choice test and stimulated a low neural response from the neurone in the medial sensilla, whereas the flavanones **8**, **9** and **10** all stimulated a high neural response from the medial sensilla and very little disc material was eaten. However, the receptors on this neurone did not differentiate between the behaviourally inactive chalcone **7** and flavanone **13**. In contrast, *S. littoralis* did differentiate between these compounds; the chalcone **7** deterred feeding and elicited a low neural response, whereas discs treated with **13** were eaten and the compound elicited a high neural response. With *S. littoralis,* the chalcones **1** and **2** deterred feeding and had a low neural response, whereas the chalcone **3** and the flavanones **8**, **9**, **10** and **14** did not deter feeding but they all stimulated a high neural response. These results show that changes to the substitutions on the flavonoid can alter the ability of the compound to stimulate taste receptors on neurones in the styloconica sensilla of *Spodoptera* larvae.

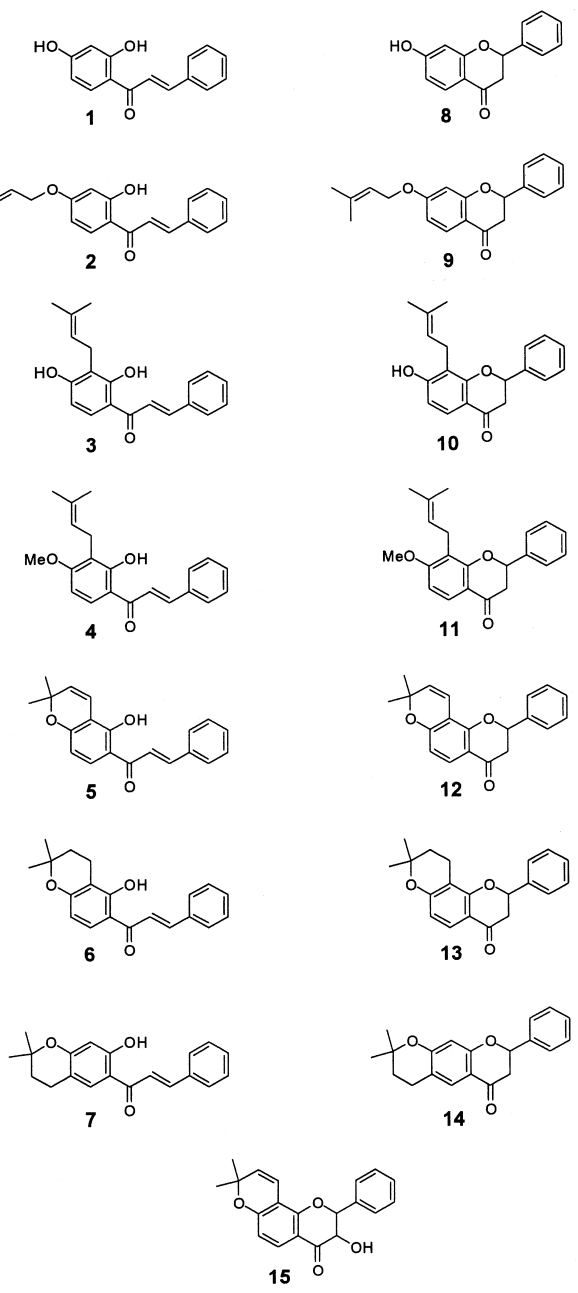


Fig. 1. Structures of the flavonoids (based on Simmonds et al., 1990: Fig. 1) Compounds **1** = 2′,4′-dihydroxychalcone; **2** = 4′-*O*-prenyl-chalcone; **3** = 3′ prenyl-chalcone; **4** = derricin; **5** = lonchocarpin; **6** = 6-cinnamoyl-5-hydroxy-2, 2-dimethylchroman; **7** = 6-cinnamoyl-7-hydroxy-2-dimethylchroman; **8** = 7-hydroxyflavonone; **9** = 7-*O*-prenyl-flavonone; **10** = 8-prenyl-flavanone; **11** = 7-*O*-methyl-8-prenyl-flavanone; **12** = isolonchocarpin; **13** = dihydroisolonchocarpin; **14** = 6″,6″-dimethyl-2*H*-pyrano (2″,3″,7,6)-flavanone; **15** = 3-hydroxyisolonchocarpin.

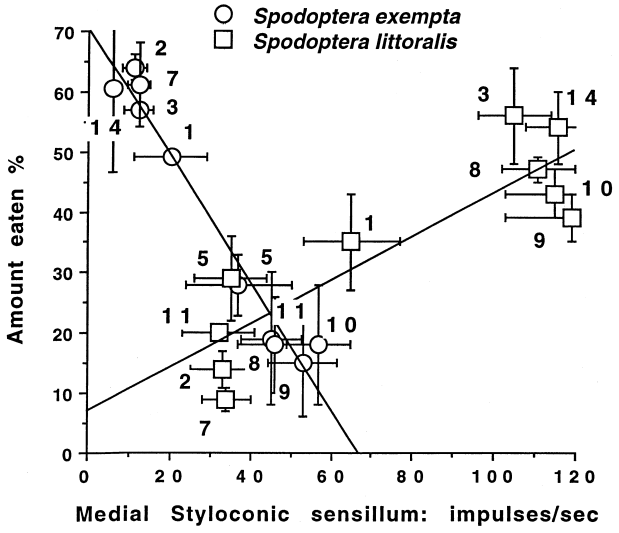


Fig. 2. Relationship between the neural response from the medial styloconic sensilla and the amount eaten in a no-choice bioassay using plant material treated with 30 μl aliquots of one of the flavonoids at 100 ppm (based on data in Simmonds et al., 1990; Figs. 2 and 3). *Spodoptera exempta*: neural response relates to neurone A in the medial sensilla and wheat discs were used in the behavioural tests. *Spodoptera littoralis*: neural response relates to the total response from 3 neurones in the medial sensilla and cotton discs were used in the behavioural tests. Numbers refer to the compounds in Fig. 1; range bars = S.E.M.; replications = 10-15 larvae per compound in the behavioural bioassay and 3–7 larvae in the electrophysiological bioassay.

Although these species can differentiate among flavonoids, we have very little information about whether this ability is used in their host-selection behaviour. *S. exempta* is an oligophagous species feeding mostly on Poaceae, whereas *S. littoralis* is a polyphagous species feeding on a wide range of plants including Poaceae. If flavonoids are involved in host selection, are *S. exempta* larvae stimulated to feed by flavonoids on grasses or are they deterred from feeding on non-host plants by flavonoids on the surface of these plants? To date, we do not have the appropriate information to answer this question. The fact that *S. exempta* was deterred by a greater number of flavonoids than *S. littoralis* (Simmonds et al., 1990) would suggest that they would be deterred by a greater range of flavonoids in plants and this could, in part, explain why they have a narrower host range than *S. littoralis*. However, more experimental evidence which includes data from Poaceae-derived flavonoids is needed to support this theory.

Plants contain a great diversity of flavonoids and the proportion studied in any form of biological system is low and as yet it is difficult to predict from their structures whether they might or might not influence insect behaviour. Further experiments are needed to increase our knowledge about how insects not only perceive these flavonoids as adults and as larvae but also how they utilise them in their diet. This would assist us understand more fully the role they play in plant-insect interactions and the evolution of host specificity. For example, do adults and larvae respond in the same way to flavonoids present in host and non-host plants? If flavonoids on a leaf deter an adult from laying an egg on that leaf would these flavonoids be toxic to the larval stage?

Jeffrey Harborne has made a significant contribution to our knowledge about the distribution of flavonoids in flowering plants and a start has been made into understanding their role in insect-plant interactions. Collaborations involving natural product chemists, entomologists, molecular biologists, mycologists, botanists and ecologists will be essential if we are to advance our knowledge about the role of flavonoids in the evolution of insect–plant relationships and in the ecology of tri-trophic interactions.

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Monique Simmonds first started her interest in plant-animal interactions when at University in Leeds and later in Germany when she studied the role of insects as vectors of disease. At Birkbeck College she worked with Wally Blaney, Reg Chapman and Elizabeth Bernays on the development of insect assays to assess the role of plant-derived compounds in insect-plant interactions. It was during these studies that she first started to work on flavonoids and met Jeffrey Harborne. Monique Simmonds then moved to Royal Botanic Gardens, Kew and worked with Linda Fellows on the biological activity of a range of plant and fungal compounds. In 1994, she became Head of a newly created Section in the Jodrell Laboratory at Kew, Biological Interactions. This Section now contains a group of experienced natural product chemists, Drs Renee Grayer, Geoffrey Kite, Nigel Veitch, Phillip Stevenson and Tetsuo Kokubun. They all received their training in flavonoid chemistry from Jeffrey Harborne and his colleagues at Reading. This Section at Kew is now investigating the uses of chemical characters in the new phylogeny of the flowering plants as well as the biological activity of plant and fungal compounds.

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Review

Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants

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Dedicated to Jeffrey Harborne on his retirement as editor of *Phytochemistry*

Abstract

A brief review is given of some biological, chemical and chemotaxonomic aspects of phytoalexin research. Emphasis is placed on the search for antifungal compounds in the plant families Leguminosae and Rosaceae, and in rice, *Oryza sativa*. The possible role of phytoalexins in the resistance of rice plants against the fungus *Pyricularia oryzae* (= *Magnaporthe grisea*) is discussed, and the future prospects of phytoalexin research are outlined. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Leguminosae; Rosaceae; Gramineae; Rice; *Oryza sativa*; Plant–fungal interactions; Phytoalexins; Preformed antifungal compounds

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1. Introduction

In 1968 Jeffrey Harborne founded the Phytochemical Unit at the Department of Botany, University of Reading, with the support of the Science Research Council (UK), and the authors of this paper had the privilege to be among the numerous students, postdoctoral fellows and visitors from all five continents to work in Jeffrey's laboratory in the last 30 years. Countless novel flavonoids, anthocyanins and other compounds were isolated and identified in the Unit during this time, the chemotaxonomy of many plant genera and families were studied, and research was carried out on the chemical aspects of plant–insect and plant–fungal interactions. Indeed, this laboratory has been one of the leading centres for the study of induced antifungal compounds, or phytoalexins. Therefore, in recognition of Jeffrey's contribution to this area of study, we will give an overview of the research on phytoalexins and also constitutive antifungal compounds that took place in the laboratory at Reading from the 1970s to the present day. Some emphasis will be placed on the research projects in which we were involved ourselves, the chemical basis of resistance of rice plants against the rice blast pathogen, *Pyricularia oryzae*, and the antifungal compounds produced by the Rosaceae.

2. Plant–fungal interactions

It is estimated that there are about 250,000 species of higher plants, but six times as many (1.5 million) species of fungi. Fungi are, ultimately, all dependent on plants for their carbon and energy source, like most other organisms that are not able to photosynthesise. Fortunately for plants, the relationship between them and fungi is usually a mutually beneficial one. The great majority of fungi are saprophytic, i.e. they live on dead plant material, breaking this down and so recycling the nutrients to become available again for living plants. During the course of evolution, some fungi have started to interact actively with living plants. Most of these interactions are advantageous to plants, e.g. for their growth and development, as in the cases of mycorrhizae and endophytes. A small minority of fungal species has developed further and broken the fine balance of mutual benefit to become plant pathogens. However, in most plant populations there are individuals that are resistant to fungal infection.

The interaction between plants and their pathogens is complex and may be very specific to a given combination of the plant and the fungus. The defence strategies of plants against their pathogens are manifold and include the use of antifungal chemicals. On the other hand, pathogens have evolved mechanisms to evade these chemicals. The chemical aspects of the warfare between plants and fungi are discussed below.

3. Constitutive and induced antifungal compounds

When a fungal spore comes in contact with a plant surface, the microclimate (temperature, humidity, light conditions, etc.) has to be right before it can germinate. Then it has to break several lines of defence set up by the plant before reaching a living cell. These include mechanical barriers such as a thick cuticle, and chemical ones such as exudate compounds which inhibit spore germination and germ tube elongation. These constituents are part of the arsenal of constitutive (or preformed) antifungal compounds produced by plants, also called preinfectional metabolites, prohibitins or phytoanticipins. If all these plant weapons are not sufficient to stop germination of the fungal spore and penetration of the hyphae through the epidermis, the plant usually responds by blocking or delaying the advancement of the invader. Reactive oxygen species (ROS) are often generated as warning signals within the cell or to the neighbouring cells, triggering off various reactions (Lamb and Dixon, 1997; Wojtaszek, 1997). These include the structural reinforcement of the cell wall, the hypersensitive response (a programmed cell death), development of systemic acquired resistance (SAR) and the accumulation of newly produced antifungal chemicals which are called phytoalexins. The term phytoalexin is usually restricted to antibiotic compounds which require de novo expression of the enzymes involved in their biosynthetic pathway (Anderson, 1991). This is a very economical way to counteract pathogens, because the carbon and energy resources are diverted to phytoalexin synthesis only at the early period of infection, and only at its site. Unchallenged plants can use these resources for more basic processes of life such as development of flowers and production of seeds, or accumulation of reserve carbohydrates in their storage organs. Some plants do not produce phytoalexins when challenged by pathogens, but release toxins that are normally stored as less toxic glycosides in the vacuoles of their cells, e.g. phenolic and iridoid glycosides, glucosinolates and saponins (Osbourn, 1996). If the integrity of the cell is broken when penetrated by fungal hyphae, the glycoside comes into contact with hydrolysing enzymes present in other compartments of that cell, releasing the toxic aglycone. Although this aglycone released after fungal attack is not present in the intact plant and is newly produced, it is strictly speaking not a phytoalexin, because the enzymes involved (glycosidases) were already present in the healthy plant and were not formed de novo.

4. Brief history of phytoalexins

As long ago as 1911 the French botanist Noel Bernard discovered that plants can produce antifungal substances which are specifically formed when the plant

is attacked by fungi. He found that the tubers of two orchid species, *Orchis morio* and *Loroglossum hircinum* (= *Himantoglossum hircinum*) became resistant to further fungal attack after they had been infected by the fungus *Rhizoctonia repens* (see Stoessl and Arditti, 1984). By placing infected tuber tissues on agar and introducing fungi onto the medium, Bernard found that the fungus-infected tissue produced a diffusible inhibitor of fungal growth, but the compounds involved were not identified until many decades later. Müller and Börger (1940) observed the same phenomenon in potato tubers infected by *Phytophthora infestans*, and they called these induced substances 'phytoalexins' (Greek φυτον = plant; αλεξειν = to defend). Müller and Börger defined phytoalexins as "chemical compounds produced as a result of invasion of living cells by a parasite". This definition has been modified frequently whenever new evidence revised earlier concepts. For instance, it soon became clear that phytoalexins were not only formed in plants after exposure to fungi, but also by various non-biological stress factors such as irradiation with short-wavelength UV light or treatment with heavy metal ions such as copper or mercury salts. For this reason Ingham (1973) redefined phytoalexins as "antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors". Others called compounds induced by environmental factors 'stress compounds'. Furthermore, the term phytoalexin is generally limited to secondary metabolites of low molecular weight, usually below 1000, so that it does not apply to antifungal peptides and proteins produced by plants.

It is often difficult to determine whether a compound is constitutive or induced, for the compound may normally be present in hardly detectable quantities, but dramatically increase in concentration after infection. Strictly speaking this is not a phytoalexin because it is not synthesised de novo. Therefore, Stoessl (1980) proposed a more pragmatical definition of phytoalexins: "Products of higher plant metabolism, absent from healthy tissues or present only in negligible traces, which accumulate in significant amounts in response to fungal or bacterial challenge". In 1994 the new term "phytoanticipin" was proposed to replace and include all types of low molecular weight antifungal metabolites other than phytoalexins that are supposed to play a role in disease resistance of plants (Van Etten et al., 1994). Yet another point of confusion between constitutive and induced antifungal compounds is that the same compound may be a preformed antifungal substance in one species and a phytoalexin in another. For instance, the flavanone sakuranetin is a constitutive antifungal compound in the leaves of blackcurrant (*Ribes nigra*, Grossulariaceae) (Atkinson and Blakeman, 1982) and of *Hebe cupressoides* (Scrophulariaceae) (Perry and Foster, 1994), but induced in the leaves of rice (*Oryza sativa*, Gramineae) (Kodama et al., 1992a). Moreover, some compounds may be a phytoalexin in one organ and constitutive in another part of the same plant, such as momilactone A, which occurs constitutively in rice husks (Kato et al., 1973), and rice stems (Lee et al., 1999), but is a phytoalexin in rice leaves (Cartwright et al., 1981). Thus, phytoalexins are defined by the dynamics of biosynthesis and the functions, not by the chemical structural class they belong to, or the biosynthetic pathway through which they were formed.

Phytoalexins have been found in both Gymnospermae and Angiospermae, and within the latter group in both Monocotyledoneae and Dicotyledoneae (Harborne, 1999). All the major structural classes such as phenolics, terpenoids and alkaloids, are represented, and the structures are often unique at the family level. For instance, the majority of phytoalexins produced by members of the family Leguminosae are isoflavonoids, whereas sulfur-containing indoles are found uniquely in the family Cruciferae. However, in some other families, a variety of phytoalexins belonging to different compound groups are produced, e.g. in the Gramineae, Compositae and Moraceae (Grayer and Harborne, 1994; Harborne, 1999). The role that phytoalexins may play in disease resistance of plants is discussed later in this paper.

5. Phytoalexin research in Reading

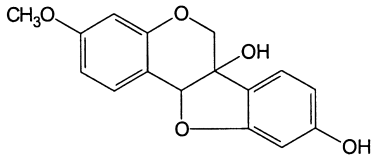
Jeffrey Harborne's first PhD student, and later post-doctoral fellow, in the area of phytoalexin research, was John Ingham, who induced, isolated and identified more than a hundred different phytoalexins from over 500 species of the Leguminosae subfamily Papilionoideae in the 1970s and 1980s. Owing to his contribution, the chemistry of phytoalexins in this group is probably better known than that of any other plant family. Ingham isolated and identified no less than 53 novel isoflavonoid structures from legumes, most of which were phytoalexins: 6 isoflavones, 5 isoflavanones, 1 rotenoid, 29 pterocarpans, 8 isoflavans, 3 coumestans and 1 coumaranochromone (see Harborne and Baxter, 1999). An example is 6a-hydroxyisomedicarpin (**1**), a phytoalexin from the leaves of *Melilotus alba* (Ingham, 1976a). Some species of Leguminosae produced different types of phytoalexins, e.g. stilbenoids, as in the case of *Arachis hypogaea* hypocotyls (Ingham, 1976b). Harborne and Ingham were probably the first scientists to realise the chemotaxonomic potential of phytoalexins. Good chemotaxonomic results were obtained at various levels of classification, and they published a paper in *Nature* on the phytoalexin variation in *Trigonella* species (Ingham and Harborne, 1976). This laid the foundation for further chemosystematic studies on phytoalexins and preformed antifungal substances in the Leguminosae by several generations of students and also by visitors, resulting in many publications. For instance, Dave Robeson studied the phytoalexin

response in 60 species of the genera *Lathyrus, Lens, Pisum* and *Vicia*, tribe Vicieae, of the Leguminosae. *Lathyrus* and *Vicia* are similar morphologically and had always been considered closely related, whereas *Pisum* and *Lens* were thought to be more distantly related. However, *Vicia* and *Lens* both appeared to produce furanoacetylene phytoalexins such as wyerone (**2**), whereas these were absent from *Lathyrus* and *Pisum*. Species of the latter genera produce pisatin (**3**) and related pterocarpans instead, which in turn are uncommon in *Vicia* and *Lens*. This may mean that *Vicia* and *Lens* are closely related, and *Lathyrus* and *Pisum* are close relatives, whereas *Lathyrus* and *Vicia* may be more distant than previously considered (Robeson and Harborne, 1980). More than a decade later Indrani Seneviratne did similar studies on the genus *Vigna* (Seneviratne and Harborne, 1992). Satoshi Tahara, a post-doctoral visitor, worked on the constitutive antifungal compounds from the genus *Lupinus*, because this taxon did not appear to produce phytoalexins. More than thirty novel isoflavonoids were isolated and identified (see Harborne and Baxter, 1999), e.g. lupinisoflavone A (**4**) from the roots of *Lupinus albus* (Tahara et al., 1984).

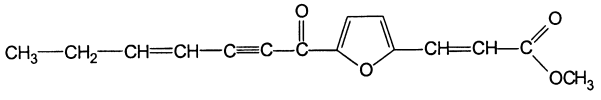
But not only the phytoalexins of the Leguminosae were studied in Reading; other students from undergraduates to PhDs have investigated the antifungal compounds in various families including the Compositae, Rosaceae and Umbelliferae. Furthermore, Jeffrey Harborne's interest also extended to their potential in plant protection, either as novel fungicides or as resistance factors of crop plants against pathogenic fungi. For instance, Peter Marshall studied phytoalexins in the Compositae and also worked on plant protection. He induced phytoalexins in excised leaf or stem segments by inoculating the fungus *Botrytis cinerea*. The compounds involved appeared to be unstable polyacetylenes, which had to be treated with uttermost care (low temperatures and shielded from daylight). Two novel compounds were isolated from *Coleostephus myconis*, and these were named (*E*)- and (*Z*)-myconisols (**5**) (Marshall et al., 1987). The Leguminosae also yielded a potentially useful crop protection agent, a fungitoxic chromone, which was obtained as a phytoalexin from *Lathyrus odoratus* (Robeson et al., 1980).

6. Phytoalexins and constitutive antifungal compounds in the Rosaceae

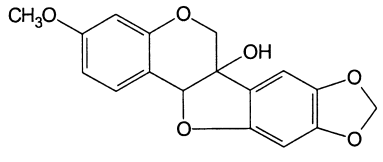
Apart from the Leguminosae, a plant family that was surveyed thoroughly in Reading for its phytoalexin response was the Rosaceae. More than 130 species were studied (Kokubun and Harborne, 1994, 1995). In all over 20 antifungal compounds were isolated and identified by activity-guided fractionation, combined with a simple



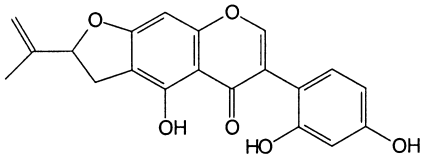
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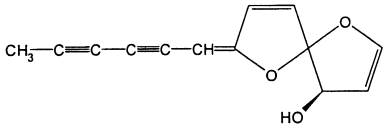
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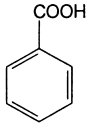
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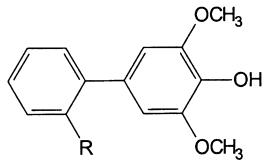


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and rapid detection method (TLC-direct bioautography) (Homans and Fuchs, 1970).

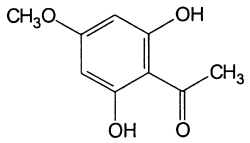
The family Rosaceae was chosen primarily because of its enormous economic importance, both in ornamental and in culinary terms (many fruit trees and shrubs). The reported occurrence of isoflavonoids, although sporadic and without taxonomic value, in *Prunus* and *Cotoneaster* (see Ingham, 1983) suggested that isoflavonoids might be distributed widely as the phytoalexins in the family. Since ten or so phytoalexins had been isolated from the family before the start of the project, it was known that phytoalexin synthesis was a feature of the family. Besides, benzoic acid (**6**), a phytoalexin found in the apple Bramley's Seedling, was the simplest of all phytoalexins known at the time (Brown and Swinburne, 1971), and it was expected that equally simple phytoalexins might be uncovered if the family was surveyed systematically. The Harris Garden, the botanic garden attached to the Plant Science Laboratories in Reading, contained numerous shrubby and woody species of Rosaceae, which meant that living plant material was amply available.

The dynamic response of the plants, in terms of phytoalexin production, was most pronounced in the sapwood tissue of the subfamily Maloideae, where a number of variously substituted biphenyls and dibenzofurans were produced (Kokubun and Harborne, 1995). On the other hand, the wood tissue of the other three subfamilies, namely, Rosoideae, Spiraeoideae and Prunoideae, showed no sign of phytoalexin production at all. Instead, several constitutive antifungal metabolites were detected, but they were not isolated/identified; that was out of the scope of the project. Also, their identity was somewhat predictable, i.e. flavanones and isoflavones in the wood of *Prunus* species, tulipaline B in *Spiraea* (Okuma et al., 1975), and catechins and proanthocyanidins that are ubiquitous in the family, but the antifungal activity of these is only weak.

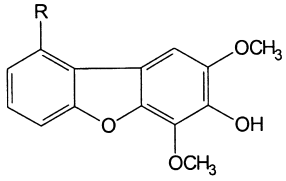


7 R = H

10 R = O-glucose



8



9 R = H

11 R = O-glucose

Apart from the wood tissues, there were only two cases of positive phytoalexin production: in the leaves of *Sorbus aucuparia*, subfam. Maloideae (Kokubun and Harborne, 1994) and the roots of *Sanguisorba minor*, subfam. Rosoideae (Kokubun et al., 1994), where aucuparin (**7**) and 2′,6′-dihydroxy-4′-methoxyacetophenone (**8**), respectively, were identified. The latter case is the only positive result in the whole of the subfamily Rosoideae, despite a wide-ranging search for phytoalexins in naturally diseased tissue, and by artificial inoculation of many tissues, including leaves and fruits of strawberries and raspberries, hips and petals of roses, etc. Nevertheless, this case of positive phytoalexin synthesis may warrant a re-investigation on the alleged phytoalexin in the root of strawberry *Fragaria* cv. SteleMaster (Mussel and Staples, 1971), the chemical structure of which has never been worked out.

The majority of the phytoalexins when they are formed appear to be biphenyls or dibenzofurans. Although they share the same C_6–C_6 carbon skeleton, these two types have never been observed to co-occur in the same tissue. This apparent dichotomy was most striking in the case of *Eriobotrya japonica*; the leaves gave the dibenzofuran eriobofuran (**9**) whereas the wood synthesized biphenyls such as aucuparin (**7**). These antifungal compounds could not be detected after acid hydrolysis of the corresponding healthy tissue, thereby confirming their phytoalexin status. These observations could, however, well be questioned following the work carried out on cell suspension cultures of a scab-resistant apple *Malus*×*domestica* cv. Liberty (Hrazdina et al., 1997; Borejsza-Wysocki et al., 1999). These workers did not only find both biphenyls and dibenzofuran in a single treatment (yeast extract), but they also isolated glycosides as well (**10**, **11**). It would be very desirable to see whether this is also the case for differentiated tissues, since undifferentiated tissues display a somewhat unique profile of secondary metabolites (e.g. Monir and Proksch, 1989; Banthorpe and Brown, 1989) and may possess a glycosidation metabolism that is absent from the parent plant (e.g. Kokubo et al., 1991).

In terms of chemical structures, biphenyls and dibenzofurans are rarely encountered in nature. The C_6–C_6 structure without alkyl substitution has been recorded so far in Umbelliferae (Williams and Harborne, 1972), Salicaceae (Malterud and Sandanger Dugstad, 1985),

Leguminosae (Ghosal et al., 1988), Guttiferae (Cardona et al., 1990; Garcia Cortez et al., 1998), Podostemaceae (Burkhardt et al., 1992) and Verbenaceae (Cambie et al., 1997). It is interesting to note that *Calophyllum pandiflorum*, a member of the Guttiferae, also produces dibenzofurans (Ito et al., 1996). As these families are taxonomically unrelated to each other, it may be that their biosynthetic pathway has evolved independently. The biosynthetic pathway leading to this structure has not been elucidated, although two different pathways are possible regarding the substitution pattern on the two aromatic rings: peroxidase-mediated radical coupling of simple phenols (Kobayashi et al., 1994), or the shikimate-acetate/malonate pathway via stilbene synthase-like enzymes (Cotterill et al., 1974).

Two recent reports on disease resistance factors of the Rosaceae may be mentioned here, although this research was not carried out at Reading. Firstly, 3,5-di-*O*-caffeoylquinic acid was found to be the factor inhibiting the formation of the lesions in *Alternaria*-infected *Pyrus* leaves. Although this constitutive compound is not very potent as a fungicide, it is nevertheless thought to play a role in the resistance mechanism by inhibiting the formation of infection hyphae (Kodoma et al., 1998). At Reading, hydroquinone, present in pear leaves as the glycoside arbutin, was identified as the principal fungicidal compound in this fruit tree (Kokubun and Harborne, 1994). This highlights further the complexity of a disease situation; a phytoalexin alone is not the sole determinant of resistance. Secondly, very recently it has been reported that unripe nectarine fruits (*Prunus persica* cv. Fantasia) produced seven triterpenoid antifungal compounds following mechanical damage and fungal infection (El Lahlou et al., 1999).

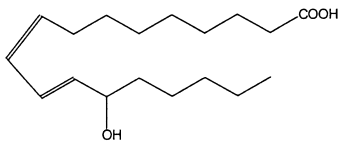
7. Phytoalexins in rice plants and their possible role in the resistance of rice against the rice blast pathogen, *Pyricularia oryzae*

Rice (*Oryza sativa*, Gramineae) is among the world's most important staple foods. Serious diseases such as rice blast, caused by the fungus *Pyricularia oryzae* (syn. *Magnaporthe grisea*), can cause considerable losses in yield, leading to severe hardship and famine in developing countries. One way to combat these problems is to breed and grow blast-resistant rice cultivars, which is now done extensively. However, the blast resistance of a rice cultivar hardly ever lasts more than a few years, since the pathogen mutates very quickly to outwit the plant's resistance. Therefore, plant breeders continuously have to develop new resistant rice cultivars, which are selected by means of extensive and time-consuming field trials. It would be much quicker and cheaper if there were simple chemical tests to find out whether a new rice cultivar is resistant, e.g. determination of the phytoalexin response of newly developed genotypes. One of the aims of our research was to see whether this is feasible, i.e. whether there is a positive correlation between phytoalexin production and resistance of a rice cultivar to the blast pathogen.

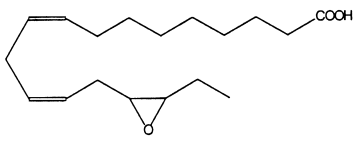
The fact that phytoalexins play an important role in the disease resistance of many crop plants has now been firmly established (Kuć, 1991; Hain et al., 1993; Paxton, 1994). A number of criteria have been proposed to examine whether a phytoalexin can be considered to play a role in plant defence (see Subba Rao and Strange, 1994): (1) the compound must accumulate in response to infection; (2) the compound must be inhibitory to the invading organism; (3) the compound must accumulate to inhibitory concentrations in the vicinity of the parasite at the time the parasite ceases growing; (4) varying the rate of accumulation of the phytoalexin should cause a corresponding variation in the resistance of the plant; (5) varying the sensitivity of the invading organism should cause a corresponding variation in its virulence.

However, there are several aspects of plant resistance or susceptibility that cannot be explained by production of phytoalexins or other antifungal compounds alone. During the course of the coevolution between plant host and pathogens, fungi have developed their own biochemical strategies for survival. Some pathogens have evolved ways to detoxify phytoalexins by means of enzyme systems which convert these compounds to less toxic derivatives (Van Etten et al., 1989). Others have developed mechanisms to suppress the defence response of the host plant (Oku and Shiraishi, 1994), making the plant unable to perceive the pathogen entering it. This leads to failure of the hypersensitive response that is often considered to be a prerequisite for induced disease resistance including the production of phytoalexin (Dangl et al., 1996). In this case infection happens even if the plant involved is capable producing sufficient amounts of phytoalexin to inhibit hyphal growth of the fungus.

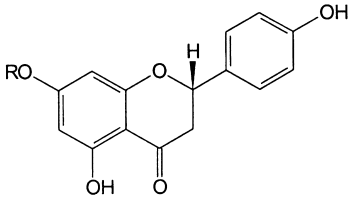
Leaves of rice plants produce a wide variety of both preformed and induced antifungal compounds. The preformed or constitutive antifungals include silicate (Wadham and Wynn Parry, 1990), some twelve C_{18} hydroxy and epoxy fatty acids based on linolenic acid, e.g. **12** and **13** (Kato et al., 1993b), and an alkylresorcinol (Susuki et al., 1996). Rice leaf phytoalexins comprise both flavonoid and diterpenoid constituents. Only two flavonoid rice phytoalexins are known, the flavanones sakuranetin (**14**, Kodama et al., 1992a) and naringenin (**15**, Grayer et al., 1996). Although naringenin accumulates in elicited rice leaves, strictly speaking it may not be a phytoalexin, as this compound is not only the precursor of sakuranetin, but also of most constitutive leaf flavonoids and therefore is likely to be a constitutive compound itself in rice leaves. Very recently a phytoalexin-specific *O*-methyltransferase was purified from jasmonic acid and $CuCl_2$-treated rice leaves which converts naringenin to the much more antifungal sakuranetin (Rakwal et al.,



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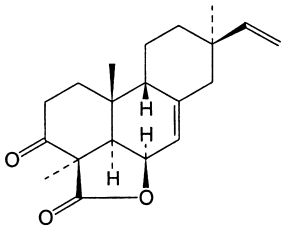


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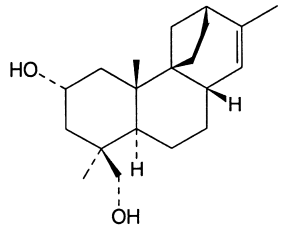


14 R = CH_3

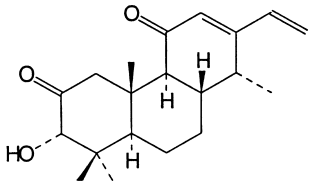
15 R = H



16



17



18

2000). The first diterpenoid phytoalexins discovered in rice were momilactones A (**16**) and B (Cartwright et al., 1981), followed by the oryzalexins A-F and S (**17**) (Akatsuka et al., 1983, 1985; Kono et al., 1984; Sekido et al., 1986; Kodama et al., 1992b; Kato et al., 1993a; Tamogami et al., 1993) and four phytocassanes (e.g. phytocassane D, **18**) (Koga et al., 1995).

Many genes involved in rice resistance to the blast fungus have been identified, and it is very likely that at least some of these genes code for a quick response by the plant's defence system and the production of high concentrations of phytoalexins. Some of the criteria proposed by Subba Rao and Strange (1994) cited above were tested to see whether phytoalexins play a role in the resistance of rice plants to the blast pathogen, using the highly resistant rice cultivar Tetep. Leaves were punch-inoculated with an avirulent strain of *Pyricularia oryzae*. After fixed periods of 3–5.8 days the concentrations of the rice phytoalexins sakuranetin, oryzalexins D, E and S, and momilactone A were determined, because standard compounds were available for these (Dillon et al., 1997). Three days after inoculation, the first three criteria were met by at least two of the phytoalexins produced, sakuranetin and momilactone A. To see whether higher quantities of phytoalexins are accumulated by rice genotypes with a high degree of resistance than by genotypes with a low resistance (criterion 4), a number of rice genotypes of different susceptibility to *Pyricularia oryzae* were tested. These were obtained from the International Rice Research Institute, The Philippines, and from the Central Rice Breeding Station, Sri Lanka. Phytoalexins were induced by means of short-wave UV irradiation of the leaves, because this method was quick and gave the most consistent results between replicates (Grayer et al., 1996). The experiments showed that there are significant and consistent differences among rice cultivars in their phytoalexin response to UV irradiation. The phytoalexins that have the highest antifungal activity (sakuranetin, momilactone A and oryzalexin S) were produced in very much higher quantities in the resistant cultivars Tetep and Ta-poo-cho-z than in the susceptible IR 50 and B 40. Indeed, B 40 did not produce any sakuranetin or oryzalexin S (Dillon et al., 1997). Of course, the phytoalexin response of rice plants to a fungus may be completely different from their response to UV irradiation. However, although the results of phytoalexin production by Tetep after inoculation with *P. oryzae* and after UV irradiation were not completely identical, they showed a similar trend. These results, and those obtained for resistant genotype Ta-poo-cho-z and for susceptible IR 50 and B 40 after UV irradiation, strongly suggest that phytoalexin production could be

an important factor in the resistance of rice against the rice blast pathogen. The resistant Carreon, however, failed to produce high concentrations of phytoalexins. This cultivar showed hardly any necrosis after UV irradiation, in contrast to the other four cultivars. This may explain its low phytoalexin production. It is possible that infection with a pathogen is a more effective way of inducing phytoalexins in Carreon, but it is also possible that blast resistance in Carreon is based on different factors, e.g. a very impenetrable epidermis or multifunctional exudate chemicals possessing both antimicrobial activity and UV-filtering functions. This would be worth investigating further. The results obtained with Carreon mean that some resistant rice genotypes may be overlooked in a screening programme based phytoalexin production alone, even if a pathogen is used for elicitation rather than an abiotic elicitor. Furthermore, if an abiotic elicitor such as UV-irradiation is employed, there is no guarantee that this always will result in the induction of phytoalexins, even if a biotic elicitor would have done so. Nevertheless, a biochemical approach could complement field trials and offer a number of advantages.

8. Prospects of phytoalexin research

Because induction of phytoalexins is now recognised as an important mechanism of plants to defend themselves against fungal pathogens, plant pathologists should be able to exploit this ability of plants to combat plant diseases. When phytoalexin research was in its infancy, there were great expectations that phytoalexins could be utilised as novel natural fungicides. So far this prospect has proved to be rather disappointing. The toxicity of many phytoalexins appears to be rather unspecific, so that they often are as toxic to other organisms including humans as they are to fungi. Furthermore, their fungicidal activity is generally much lower than that of commercial fungicides already on the market, and they are generally very expensive to isolate or synthesise (Kuć, 1991). More highly active analogues may be produced, but these have the same environmental disadvantages as synthetic fungicides currently in use.

As mentioned before, phytoalexins are not only induced or elicited in plants by microorganisms, but also by UV irradiation and a range of chemicals, including heavy metal salts and certain cell wall components of fungi including chitin (all agents that can induce phytoalexins are called 'elicitors'). Therefore, an alternative to protect crop plants would be to spray them with environmentally harmless phytoalexin elicitors. Sinha (1994) tested a large variety of different elicitors to see what their effect was on the prevention of various fungal diseases in a number of crop plants and achieved good results. However, according to Kuć (1991), the repeated application of elicitors of phytoalexin accumulation can cause a marked diversion of energy and carbon resources from vital processes, resulting in resistant but stunted plants. Therefore crop yields may be less than if the plants were infected!

Plant resistance can also be enhanced by incorporation of genes coding for phytoalexin synthesis. For instance, the gene for stilbene synthase has been successfully transferred from the grape vine, *Vitis vinifera*, to tobacco plants, so that the latter produced the stilbene phytoalexin resveratrol, and acquired improved resistance to infection by *Botrytis cinerea* (Hain et al., 1993). However, it is relatively easy to modify plants genetically to produce resveratrol, as this only needs one enzyme (the other enzymes needed for its biosynthesis are present in most plants). It is not so easy to do the same for many other phytoalexins. For instance, for non-leguminous plants many new enzymes would be required to produce pterocarpan phytoalexins, so that many genes would have to be transferred. Furthermore, the enhancement of plant resistance by gene transfer has several undesirable aspects. If the genes coding for the production of a particular powerful phytoalexin were to be transferred to many crop plants, pathogens could develop that could break that resistance factor, e.g. by evolving enzymes to detoxify that particular phytoalexin. These could become 'superbugs' and wipe out not only all the genetically modified crops, but also any wild plant species that were dependent on that phytoalexin for their defence.

The transfer of genes to produce a particular phytoalexin may not render a plant resistant to diseases, because it is not usually the inability of a plant to produce these compounds that makes it susceptible. The speed and magnitude of the gene expression, i.e. to produce the phytoalexins rapidly and in high enough concentrations, may be a more important factor (Kuć, 1991). For instance, the susceptible rice genotype B 40 does not lack the ability to synthesise phytoalexins, but produces them in much lower concentrations than the resistant genotypes Tetep and Ta-poo-cho-z (Dillon et al., 1997). Therefore, a better way of utilising the knowledge of phytoalexin production in plants for crop protection is to select genotypes for their yield of induced antifungal compounds and their speed of phytoalexin response. Another way would be to stimulate the plants (even susceptible genotypes) to produce their own phytoalexins rapidly and in large quantities. This can be done by means of immunisation, i.e. by inoculation with a non-pathogenic microorganism or an incompatible strain of a pathogen. This causes a systemic resistance: after any subsequent infection with a pathogen the plant responds rapidly to accumulate phytoalexins at the site of infection and produces lignin and other products to halt the growth of the pathogen (Kuć, 1991). For example, systemic resistance to *Pyricularia oryzae* in rice was induced by inoculation of the first leaf with the bacterium *Pseudomonas syringae* pv. *syringae*, which

causes a hypersensitive response in rice. Lesions caused by a subsequent inoculation with *Pyricularia oryzae* were dramatically decreased in number and size (Smith and Métraux, 1991). This resistance may have been the result of a rapid production of phytoalexins, although this was not investigated.

Apart from having various different potentials for agriculture, phytoalexin production is also a treasure chest for other fields of science, including natural product chemistry, pharmacy, ecology, biochemistry and molecular biology. For the chemist phytoalexins present ample opportunities for elucidating novel structures. Because these compounds are not usually present in healthy plants, they are not often found during standard plant screening programmes. Moreover, only some 40 out of the ca. 150 plant families of Angiospermae and Gymnospermae have been surveyed yet for their phytoalexin response (Harborne, 1999), so many new discoveries could still be made. For the pharmacist phytoalexins have the potential to show pharmacological activities, as these compounds are by definition antimicrobial. Interesting areas of research for biochemists are the biosynthesis and enzymes involved in the production of the myriad of phytoalexins found so far. The *de novo* synthesis of phytoalexins provides molecular biologists with a very convenient source for studying gene regulation mechanisms, because new expressions of genes take place in a very short time. This is a rapidly expanding area of phytoalexin research at the moment. Finally, the plant's strategy of producing phytoalexins is a worthwhile object of study for ecologists, because this has contributed to the survival of all extant plant species on which all other organisms ultimately depend.

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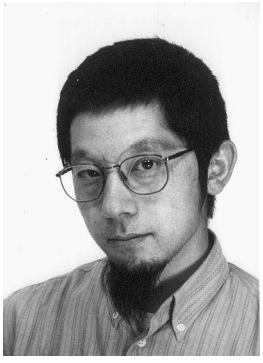
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Renée Grayer studied biology with chemistry at the University of Leiden, The Netherlands. She attained a PhD at the same university on the chemotaxonomy of the genus *Veronica* (Scrophulariaceae), supervised by Prof. Robert Hegnauer. In 1971 she spent 3 months in Prof. Jeffrey Harborne's phytochemical unit at the University of Reading to learn techniques to separate and identify flavonoids, and subsequently she worked in that laboratory from 1976 to 1994 on a variety of projects. These included the chemotaxonomy of the Leguminosae, flavonoid sweeteners from plants, and the chemical basis of resistance of crop plants to insect pests and fungal diseases. In 1994 she took up a position at the Royal Botanic Gardens, Kew (Biological Interactions Section, Jodrell Laboratory), where she mainly works on the chemotaxonomy of the Lamiaceae. Together with Jeffrey she wrote a number of research papers, review articles and chapters of books, and she also wrote many entries for the Phytochemical Dictionary and Handbook of Natural Flavonoids, both edited by Jeffrey Harborne and Herbert Baxter. Since 1987 she has compiled the Index to the journal Phytochemistry, taking over from Tony Swain.



Tetsuo Kokubun left his native Japan in the summer of 1991 to do a PhD with Jeffrey Harborne at the University of Reading, UK. The aim of his study was to find phytoalexins in the Rosaceae. The Botanical Garden at the university supplied the living plant material for this study, and Jeffrey's enthusiasm the inspiration. This work resulted in seven papers, mostly published in this journal. In 1997, after postdoctoral positions in Auckland, New Zealand, and in Reading, he moved to the Biological Interactions Section, Jodrell Laboratory, Royal Botanic Gardens, Kew, where he now works on bioactive fungal metabolites and antimicrobial compounds from higher plants. However, he has always stayed in touch with Jeffrey, and recently assisted him in the compilation and editing of the Handbook of Natural Flavonoids — the latest compendium in the field.

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Review

Polyhydroxylated alkaloids — natural occurrence and therapeutic applications

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Abstract

Over one hundred polyhydroxylated alkaloids have been isolated from plants and micro-organisms. These alkaloids can be potent and highly selective glycosidase inhibitors and are arousing great interest as tools to study cellular recognition and as potential therapeutic agents. However, only three of the natural products so far have been widely studied for therapeutic potential due largely to the limited commercial availability of the other compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Glycosidase; Inhibitors; Pyrrolidines; Piperidines; Pyrrolizidines; Indolizidines; *Nor*tropanes; N-Containing sugars

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1. Introduction

Drug discovery programmes have typically used solvents such as methanol, chloroform or hexane for extraction. However, most preparations used in traditional medicines are formulated in water and carbohydrates and glycosides which are sparingly soluble in dry methanol and less polar solvents are important biomolecules. It is becoming apparent that the water soluble fractions of medicinal plants and microbial cultures contain many interesting novel structures including carbohydrate analogues. Such analogues include a rapidly growing number of reported polyhydroxylated alkaloids with molecular weights below 250 Da which show great stereoselectivity in biological activity. Such selectivity is not surprising when the chemical and biological diversity in terms of structural information of small sugars is considered. For instance, there are 2^9 stereoisomers of sucrose alone and even glucose has 2×2^5 possible isomers in the pyranose and furanose forms. This amazing diversity in such small molecules displays a remarkable economy in structural information in nature, completely surpassing in molecular weight terms anything achieved by the amino acids. The polyhydroxylated alkaloids found in plants and micro-organisms are arousing considerable interest as potential therapeutic agents and as tools used to understand biological recognition processes and so this paper will review their distribution and therapeutic activities known to date.

The first natural polyhydroxylated alkaloid to be detected was the piperidine alkaloid nojirimycin (Fig. 1a) which was isolated from a *Streptomyces* filtrate in 1966 by Inouye et al., but most have been discovered since 1983. These alkaloids can be considered as analogues of monosaccharides in which the ring oxygen has been replaced by nitrogen. They are monocyclic and bicyclic polyhydroxylated derivatives of the following ring systems: pyrrolidine, piperidine, pyrrolizidine (two fused pyrrolidines with N at the bridgehead), octahydroindolizine or indolizidine (fused piperidine and pyrrolidine) and *nor*tropane. Systematically, these alkaloids have been described in the literature as derivatives of the parent heterocyclic compounds or sugars. 1-Deoxynojirimycin (2*S*-hydroxymethyl-3*R*,4*R*,5*S*-trihydroxy-piperidine or 1,5-dideoxy-1,5-imino-D-glucitol; Fig. 1b) was originally

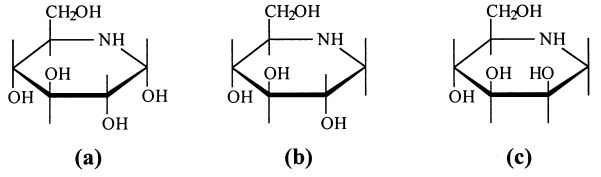


Fig. 1. Diagram illustrating the common nomenclature for polyhydroxylated piperidine alkaloids: (a) 5-amino-5-deoxy-D-glucopyranose has the trivial name nojirimycin; (b) 1-deoxynojirimycin (DNJ) is the trivial name for 1,5-dideoxy-1,5-imino-D-glucitol; and similarly (c) 1-deoxymannojirimycin (DMJ) is the common name for 1,5-dideoxy-1,5-imino-D-mannitol.

synthesised by removing the anomeric hydroxyl group of nojirimycin (Inouye et al., 1968) but it was later isolated from plant sources and bacterial cultures (Yagi et al., 1976; Murao and Miyata, 1980). This derivative is more stable than nojirimycin and has become a model compound in this area of research, giving rise to a trivial nomenclature for 1-deoxy analogues of other alkaloids of this type. Thus, the 1-deoxy piperidine analogue of mannose has been given the trivial name of 1-deoxymannojirimycin (DMJ) (Fig. 1c). The nomenclature used most frequently for particular compounds and their common abbreviations will be used in this review.

2. The distribution of polyhydroxylated alkaloids

Table 1 lists the micro-organisms and plants reported to produce polyhydroxylated alkaloids which have a structural resemblance to carbohydrates. Some of the alkaloids such as 2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (DMDP) and 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) (Fig. 2) would appear to be fairly widespread secondary metabolites as they have been reported from species of both tropical and temperate plants from quite unrelated families and DMDP is also produced by a species of *Streptomyces* (Watanabe et al., 1995). Others, such as the indolizidine alkaloid castanospermine (Fig. 6), have only been found in two closely related legume genera. The polyhydroxylated pyrrolizidine alkaloids (Fig. 5) appeared to be restricted to the Leguminosae until their discovery in 1994 in a member of the Casuarinaceae and then in the closely related Myrtaceae. Recently they have also been found in abundance in members of the Hyacinthaceae (Kato et al., 1999; Asano et al., 2000a). The polyhydroxylated *nor*tropane alkaloids (Fig. 7) seem to be largely limited to the closely related families Solanaceae and Convolvulaceae, where they co-occur with tropane alkaloids. However, they have also been found in species of *Morus* (Moraceae) (Asano et al., 1994a,b) which is a family not noted for production of tropane alkaloids. [For descriptions of the chemotaxonomic distribution of calystegines in the Solanaceae and Convolulaceae, refer to Griffiths (1998) and Schimming et al. (1998), respectively.]

While there may be phylogenetic reasons for particular distributions of the polyhydroxylated alkaloids in plants, caution should nevertheless be exercised in using the presence of these compounds as taxonomic markers. One reason is that these alkaloids can be released into the soil by producer plants and micro-organisms from whence some such as DMDP and castanospermine can be readily taken up and accumulated in plant tissues of completely unrelated neighbouring species (Nash et al., 1996). It may also be the case that micro-organisms (*Rhizobium*, other rhizosphere organisms, or endophytes) closely associated with specific plants are capable of

Table 1
The naturally-occurring polyhydroxylated alkaloids

Alkaloid	Source and reference
Pyrroline (Fig. 2)	
3,4-Dihydroxy-5-hydroxymethyl-1-pyrroline (Nectrisine or FR-900483)	*Nectria lucida* F-4490 (ATCC 20722) (Ascomycetes) (Shibata et al., 1988)
Pyrrolidines (Figs. 2 and 3)	
2*R*-Hydroxymethyl-3*S*-hydroxypyrrolidine (CYB-3)	*Castanospermum australe* (Leguminosae) seeds/leaves (Nash et al., 1985)
N-Hydroxyethyl-2-hydroxymethyl-3-hydroxypyrrolidine	*Castanospermum australe* (Leguminosae) seeds (Molyneux et al., 1991)
1,4-Dideoxy-1,4-imino-D-arabinitol (D-AB1)	*Angylocalyx* spp. (Leguminosae) seeds/leaves/bark (Jones et al., 1985) *Arachniodes standishii* (Polypodiaceae) leaves (Furukawa et al., 1985; Jones et al., 1985) *Morus bombycis* (Moraceae) leaves (Asano et al., 1994a) *Eugenia* spp. (Myrtaceae) leaves/bark (Nash et al., 1996) *Hyacinthoides non-scripta* (Hyacinthaceae) bulb/leaves (Watson et al., 1997) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999) *Adenophora triphylla* var. *japonica* (Campanulaceae) whole plant (Asano et al., 2000b)
1,4-Dideoxy-1,4-imino-(2-*O*-β-D-glucopyranosyl)-D-arabinitol	*Morus bombycis* (Moraceae) leaves (Asano et al., 1994a]
	Morus alba (Moraceae) roots (Asano et al., 1994b)
1,4-Dideoxy-1,4-imino-D-ribitol	*Morus alba* (Moraceae) roots (Asano et al., 1994b)
2*R*,5*R*-Dihydroxymethyl-3*R*,4*R*-dihydroxy-pyrrolidine (DMDP)	*Derris elliptica* (Leguminosae) leaves (Welter et al., 1976]
	Lonchocarpus spp (Leguminosae) seeds/leaves (Evans et al., 1985a) *Endospermum* sp. (Euphorbiaceae) leaves (Horn et al., 1987) *Omphalea diandra* (Euphorbiaceae) leaves (Kite et al., 1990) *Streptomyces* sp. KSC-5791 (Actinomycetes) (Watanabe et al., 1995) *Nephthytis poissoni* (Araceae) fruit/leaves (Dring et al., 1995; Nash et al., 1996) *Aglaonema* spp (Araceae) leaves (Dring et al., 1995; Asano et al., 1997a) *Hyacinthoides non-scripta* (Hyacinthaceae) bulb/leaves (Watson et al., 1997) *Campanula rotundifolia* (Campanulaceae) leaves (Nash et al., 1997) *Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999) *Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
6-Deoxy-DMDP	*Angylocalyx* spp. (Leguminosae) seeds/leaves/bark (Molyneux et al., 1993a)
6-*C*-Butyl-DMDP	*Adenophora triphylla* var. *japonica* (Campanulaceae) whole plant (Asano et al., 2000b)
6-Deoxy-6-*C*-(2,5-dihydroxyhexyl)-DMDP	*Hyacinthoides non-scripta* (Hyacinthaceae) fruits/stalks (Kato et al., 1999]
	Scilla campanulata (Hyacinthaceae) bulb (Kato et al., 1999)
2,5-Imino-2,5,6-trideoxy-D-*gulo*-heptitol	*Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a)
2,5-Dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homo-DMDP)	*Hyacinthoides non-scripta* (Hyacinthaceae) (Watson et al., 1997) *Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999) *Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)

(*continued on next page*)

Table 1 (*continued*)

Alkaloid	Source and reference
Homo-DMDP-7-*O*-apioside	*Hyacinthoides non-scripta* (Hyacinthaceae)) bulb/leaves (Watson et al., 1997) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999) *Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
Homo-DMDP-7-*O*-β-D-xylopyranoside	*Hyacinthoides non-scripta* (Hyacinthaceae) fruits/stalks (Kato et al., 1999) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999) *Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
6-Deoxy-homo-DMDP	*Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a) *Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
Gualamycin	*Streptomyces* sp. NK11687 (Tsuchiya et al., 1995)
Broussonetinines A and B, Broussonetines A, B, C, D, E, F, G, H, I, J, K and L	*Broussonetia kazinoki* (Moraceae) branches (Shibano et al., 1997a,b, 1998a,b, 1999a,b)
Piperidines (Fig. 4)	
Nojirimycin	*Streptomyces roseochromogenes* R-468 (Inouye et al., 1966, 1968; Ishida et al., 1967) *Streptomyces lavandulae* SF-425 (Inouye et al., 1966, 1968; Ishida et al., 1967) *Streptomyces nojiriencis* SF-426 (Inouye et al., 1966, 1968; Ishida et al., 1967)
1-Deoxynojirimycin (DNJ)	*Morus* sp. (Moraceae) roots (Yagi et al., 1976) *Bacillus amyloliquefaciens, B. polymyxa, B. subtilis* (Schmidt et al., 1979) *Streptomyces lavandulae* subsp. *trehalostaticus* no. 2882 (Murao and Miyata, 1980) *Omphalea queenslandiae* (Euphorbiaceae) leaves (Kite et al., 1991) *Endospermum medullosum* (Euphorbiaceae) leaves (Kite et al., 1991) *Morus bombycis* (Moraceae) leaves (Asano et al., 1994a) *Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a) *Adenophora triphylla* var. *japonica* (Campanulaceae) whole plant (Asano et al., 2000b)
1-Deoxynojirimycin-2-*O*-, 3-*O*-, 4-*O*-* α-D-glucopyranosides and 2-*O*-, 6-*O*-α-D-galactopyranoside and 2-*O*-, 3-*O*-, 4-*O*-, 6-*O*-β-D-glucopyranoside	*Morus alba* (Moraceae) roots (Asano et al., 1994b) **Streptomyces lavandulae* GC-148 (Ezure, 1985)
N-Methyl-1-deoxynojirimycin	*Morus alba* (Moraceae) roots (Asano et al., 1994b)
α-Homonojirimycin (HNJ)	*Omphalea diandra* (Euphorbiaceae) leaves (Kite et al., 1988) *Endospermum medullosum* (Euphorbiaceae) leaves (Kite et al., 1991) *Nephthytis poissoni* (Araceae) leaves (Nash et al., 1996) *Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a) *Hyacinthus orientalis* (Hyacinthaceae) bulb (Asano et al., 1998a)
α-Homonojirimycin-7-*O*-β-D-glucopyranoside (MDL 25,637)	*Omphalea diandra* (Euphorbiaceae) leaves (tentative) (Kite et al., 1990) *Nephthytis poissoni* (Araceae) leaves (Nash et al., 1996) *Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a)

(*continued on next page*)

Table 1 (*continued*)

Alkaloid	Source and reference
	Hyacinthus orientalis (Hyacinthaceae) bulb (Asano et al., 1998a)
	Lobelia sessilifolia (Campanulaceae) whole plant (Ikeda et al., 2000)
α-Homonojirimycin-5-*O*-α-D-galactopyranoside	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a)
α-4-*Epi*homonojirimycin	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a; Martin et al., 1999)
α-1-Deoxy-1-*C*-methyl-homonojirimycin (Adenophorine)	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
Adenophorine-1-*O*-β-D-glucopyranoside	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
1-Deoxyadenophorine	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
5-Deoxyadenophorine	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
5-Deoxyadenophorine-1-*O*-β-D-glucopyranoside	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
β-1-*C*-butyl-deoxygalactonojirimycin	*Adenophora* spp. (Campanulaceae) roots (Ikeda et al., 2000)
β-Homonojirimycin	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a)
	Hyacinthus orientalis (Hyacinthaceae) bulb (Asano et al., 1998a)
β-4,5-Di*epi*homonojirimycin	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1998b)
Nojirimycin B (Mannojirimycin)	*Streptomyces lavandulae* SF-425 (Niwa et al., 1984)
1-Deoxymannojirimycin (DMJ)	*Lonchocarpus sericeus* (Leguminosae) seeds/leaves (Fellows et al., 1979)
	Streptomyces lavandulae GC-148 (Ezure et al., 1988]
	Omphalea diandra (Euphorbiaceae) leaves (Kite et al., 1988)
	Endospermum medullosum (Euphorbiaceae) leaves (Kite et al., 1991)
	Derris malaccensis (Leguminosae) (Asano et al., 1994c)
	Angylocalyx spp. (Leguminosae) seeds/leaves/bark (Nash et al., 1996)
	Hyacinthus orientalis (Hyacinthaceae) bulb (Asano et al., 1998a)
	Adenophora triphylla var. *japonica* (Campanulaceae) whole plant (Asano et al., 2000b)
α-Homomannojirimycin	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a)
	Hyacinthus orientalis (Hyacinthaceae) bulb (Asano et al., 1998a)
β-Homomannojirimycin	*Aglaonema treubii* (Araceae) leaves/roots (Asano et al., 1997a)
	Hyacinthus orientalis (Hyacinthaceae) bulb (Asano et al., 1998a)
Galactostatin	*Streptomyces lydicus* PA-5726 (Miyake and Ebata, 1988)
Fagomine	*Fagopyrum esculentum* (Polygonaceae) seeds (Koyama and Sakamura, 1974)
	Xanthocercis zambesiaca (Leguminosae) seeds (Evans et al., 1985b), leaves/roots [Kato et al., 1997a)
	Morus bombycis (Moraceae) leaves (Asano et al., 1994a)
	Morus alba (Moraceae) roots (Asano et al., 1994b)
	Lycium chinense (Solanaceae) roots (Asano et al., 1997b)
3-*Epi*fagomine	*Morus alba* (Moraceae) roots (Asano et al., 1994b)
	Xanthocercis zambesiaca (Leguminosae) leaves/ roots (Kato et al., 1997a)
3,4-Di*epi*fagomine	*Xanthocercis zambesiaca* (Leguminosae) leaves/roots (Kato et al., 1997a)
Fagomine-4-*O*-β-D-glucopyranoside	*Xanthocercis zambesiaca* (Leguminosae) seeds (Evans et al., 1985b) leaves/roots (Kato et al., 1997a)
Fagomine-3-*O*-β-D-glucopyranoside	*Xanthocercis zambesiaca* (Leguminosae) leaves/roots (Kato et al., 1997a)

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Table 1 (*continued*)

Alkaloid	Source and reference
6-Deoxy-fagomine	*Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
α-1-*C*-Ethyl-fagomine	*Adenophora triphylla* var. *japonica* (Campanulaceae) whole plant (Asano et al., 2000b)
Pyrrolizidines (Fig. 5)	
Alexine	*Alexa* spp. (Leguminosae) seeds/leaves (Nash et al., 1988a)
3,7a-Di*epi*alexine	*Castanospermum australe* (Leguminosae) seeds/leaves (Nash et al., 1988b)
7a-*Epi*alexine (Australine)	*Castanospermum australe* (Leguminosae) seeds/leaves (Molyneux et al., 1988)
1,7a-Di*epi*alexine	*Alexa* spp. and *Castanospermum australe* (Leguminosae) seeds/leaves (Nash et al., 1990a)
7a-*Epi*alexaflorine	*Alexa grandiflora* (Leguminosae) leaves (de S. Pereira et al., 1991)
Casuarine	*Casuarina equisetifolia* (Casuarinaceae) bark (Nash et al., 1994) *Eugenia jambolana* (Myrtaceae) leaves (Wormald et al., 1996)
Casuarine-6-*O*-α-D-glucopyranoside	*Casuarina equisetifolia* (Casuarinaceae) bark (Nash et al., 1994) *Eugenia jambolana* (Myrtaceae) leaves (Wormald et al., 1996)
(1*S*, 2*R*, 3*R*, 7a*R*)-1,2-Dihydroxy-3-hydroxymethylpyrrolizidine (Hyacinthacine A_1)	*Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)]
(1*R*, 2*R*, 3*R*, 7a*R*)-1,2-Dihydroxy-3-hydroxymethylpyrrolizidine (Hyacinthacine A_2)	*Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
(1*R*, 2*R*, 3*R*, 5*R*, 7a*R*)- 1,2-Dihydroxy-3-hydroxymethyl-5-methylpyrrolizidine (Hyacinthacine A_3)	*Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
(1*S*, 2*R*, 3*R*, 5*R*, 7a*R*)-1,2-Dihydroxy-3,5-dihydroxy-methylpyrrolizidine (Hyacinthacine B_1)	*Hyacinthoides non-scripta* (Hyacinthaceae) fruits/stalks (Kato et al., 1999) *Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999)
(1*S*, 2*R*, 3*R*, 5*S*, 7a*R*)-1,2-Dihydroxy-3,5-dihydroxy-methylpyrrolizidine (Hyacinthacine B_2)	*Scilla campanulata* (Hyacinthaceae) bulb (Kato et al., 1999)
(1*S*, 2*R*, 3*R*, 5*R*, 7*R*, 7a*R*)-3-Hydroxymethyl-5-methyl-1,2,7-trihydroxypyrrolizidine (Hyacinthacine B_3)	*Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
(1*S*, 2*R*, 3*R*, 5*R*, 6*R*, 7*R*, 7a*R*)-3-Hydroxymethyl-5-methyl-1,2,6,7-tetrahydroxypyrrolizidine (Hyacinthacine C_1)	*Hyacinthoides non-scripta* (Hyacinthaceae) fruits/stalks (Kato et al., 1999) *Muscari armeniacum* (Hyacinthaceae) bulbs (Asano et al., 2000a)
(1*R*, 2*R*, 3*R*, 5S, 8*R*)-1,2-Dihydroxy-3-hydroxymethyl-5-[(1*R*)-1,10-dihydroxy-6-oxo-decyl]-pyrrolizidine (Broussonetine N)	*Broussonetia kazinoki* (Moraceae) branches (Shibano et al., 1999b)
Indolizidines (Fig. 6)	
Swainsonine	*Swainsona canescens* (Leguminosae) leaves (Colegate et al., 1979) *Astragalus* spp. (Leguminosae) leaves/stems (Molyneux and James, 1982) *Oxytropis* spp. (Leguminosae) leaves/stems (Molyneux and James, 1982) *Rhizoctonia leguminicola* (Basidiomycetes) (Schneider et al., 1983) *Metarhizium anisopliae* (Deuteromycetes) (Hino et al., 1985) *Ipomoea* sp. aff. *calobra* (Convolvulaceae) seeds (Molyneux et al., 1995) *Ipomoea carnea* (Convolvulaceae) leaves/stems (de Balogh et al., 1999)
Swainsonine *N*-oxide	*Astragalus lentiginosus* (Leguminosae) (Molyneux and James, 1982)

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Table 1 (*continued*)

Alkaloid	Source and reference
Lentiginosine	*Astragalus lentiginosus* (Leguminosae) leaves (Pastuszak et al., 1990)
2-*Epi*lentiginosine	*Rhizoctonia leguminicola* (Basidiomycetes) (Harris et al., 1987) *Astragalus lentiginosus* (Leguminosae) leaves (Pastuszak et al., 1990)
Castanospermine	*Castanospermum australe* (Leguminosae) seeds/leaves/bark (Hohenschutz et al., 1981) *Alexa* spp. (Leguminosae) seeds/leaves/bark (Nash et al., 1988c)
6-*Epi*castanospermine	*Castanospermum australe* (Leguminosae) seeds/leaves/bark (Molyneux et al., 1986; Nash et al., 1990b)
6,7-Di*epi*castanospermine	*Castanospermum australe* (Leguminosae) seeds (Molyneux et al., 1991)
7-Deoxy-6-*epi*castanospermine	*Castanospermum australe* (Leguminosae) seeds (Molyneux et al., 1990)
Nortropanes (Fig. 7)	
Calystegine A_3 and Calystegine B_2	*Calystegia sepium* (Convolvulaceae) leaves/roots (Tepfer et al., 1988; Goldmann et al., 1990) *Convolvulus arvensis* (Convolvulaceae) leaves/roots (Tepfer et al., 1988; Molyneux et al., 1993b) *Atropa belladonna* (Solanaceae) leaves/roots (Tepfer et al., 1988; Goldmann et al., 1990; Dräger et al., 1995) *Solanum* spp. (Solanaceae) tubers/leaves (Nash et al., 1993) *Ipomoea batatus* (Convolvulaceae) leaves/roots (Nash et al., 1996) *Datura wrightii* (Solanaceae) leaves (Nash et al., 1993) *Physalis alkekengi* var. *francheti* (Solanaceae) roots (Asano et al., 1995a) *Hyoscyamus niger* (Solanaceae) leaves/roots (Dräger et al., 1995) *Mandragora officinarum* (Solanaceae) leaves/roots/fruits (Dräger et al., 1995) *Scopolia* spp. (Solanaceae) leaves/roots (Dräger et al., 1995; Asano et al., 1996a) *Ipomoea* sp. *aff. calobra* (Convolvulaceae) seeds (Molyneux et al., 1995) *Calystegia japonica* (Convolvulaceae) roots (Nash et al., 1996) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b) B_2 only in *Morus alba* (Moraceae) fruits (Asano et al., 1997c) *Ipomoea carnea* (Convolvulaceae) leaves/stems (de Balogh et al., 1999)
N-Methyl-calystegine B_2	*Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine A_5	*Physalis alkekengi* var. *francheti* (Solanaceae) (Asano et al., 1995a) *Scopolia japonica* (Solanaceae) roots (Asano et al., 1996a) *Hyoscyamus niger* (Solanaceae) leaves/roots (Asano et al., 1996b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine A_6	*Hyoscyamus niger* (Solanaceae) leaves/roots (Asano et al., 1996b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine A_7	*Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine B_1	*Convolvulus arvensis* (Convolvulaceae) leaves/roots (Tepfer et al., 1988; Molyneux et al., 1993b) *Calystegia sepium* (Convolvulaceae) leaves/roots (Goldmann et al., 1990)

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Table 1 (*continued*)

Alkaloid	Source and reference
	Physalis alkekengi (Solanaceae) roots (Asano et al., 1995a) *Hyoscyamus niger* (Solanaceae) leaves/roots (Dräger et al., 1995) *Mandragora officinarum* (Solanaceae) leaves/roots/fruits (Dräger et al., 1995) *Scopolia* spp. (Solanaceae) leaves/roots (Dräger et al., 1995; Asano et al., 1996a) *Ipomoea batatus* (Convolvulaceae) leaves/roots (Nash et al., 1996; Asano et al., 1997c) *Duboisia leichhardtii* (Solanaceae) leaves (Kato et al., 1997b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine B_1-3-*O*-β-D-glucopyranoside	*Nicandra physalodes* (Solanaceae) fruits (Griffiths et al., 1996)
Calystegine B_3	*Physalis alkekengi* (Solanaceae) roots (Asano et al., 1995a) *Scopolia japonica* (Solanaceae) roots (Asano et al., 1996a) *Hyoscyamus niger* (Solanaceae) leaves/roots (Asano et al., 1996b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine B_4	*Scopolia japonica* (Solanaceae) roots (Asano et al., 1996a) *Duboisia leichhardtii* (Solanaceae) leaves (Kato et al., 1997b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine B_5	*Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine C_1	*Morus alba* (Moraceae) roots (Asano et al., 1994b) *Ipomoea batatus* (Convolvulaceae) roots (Nash et al., 1996) *Scopolia japonica* (Solanaceae) roots (Asano et al., 1996a) *Duboisia leichhardtii* (Solanaceae) leaves (Kato et al., 1997b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b) *Ipomoea carnea* (Convolvulaceae) leaves/stems (de Balogh et al., 1999)
N-Methyl-calystegine C_1	*Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)
Calystegine C_2	*Duboisia leichhardtii* (Solanaceae) leaves (Kato et al., 1997b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b]
Calystegine N_1	*Hyoscyamus niger* (Solanaceae) leaves/roots (Asano et al., 1996b) *Lycium chinense* (Solanaceae) roots (Asano et al., 1997b)

producing polyhydroxylated alkaloids which could then be mistakenly considered of plant origin.

From Table 1, it can be seen that the majority of naturally-occurring polyhydroxylated alkaloids have been isolated from plants. Although the first alkaloid of this type to be discovered (nojirimycin) was isolated from the fermentation broth of a species of *Streptomyces*, only a limited number of bacteria (principally Actinomycetes) have subsequently been found that produce polyhydroxylated alkaloids. Also, only three of the compounds listed in Table 1 are known to be produced by fungi (nectrisine, swainsonine and 2-*epi*lentiginosine). However, the apparent restricted distribution of polyhydroxylated alkaloids amongst micro-organisms may be misleading as the techniques used to screen the cultures are frequently inappropriate for detecting such highly polar compounds. However, there are actually quite a range of microbial products that can be described as carbohydrate analogues and some of these with therapeutic potential are discussed in Section 5.1.

The recent increase in the rate at which novel water-soluble alkaloids are reported would suggest that many more await discovery from diverse sources, including plants and micro-organisms in many taxa not previously considered to be alkaloid producers. Several hundred related alkaloids have also been synthesised which have allowed investigations of structure-activity relationships, but it is now becoming apparent that many of these synthetic structures also occur naturally. For example, the 7-*O*-β-D-glucopyranosyl derivative of α-homonojirimycin was designed to be a transition state analogue of sucrose (Liu, 1987) before it and its aglycone were discovered as

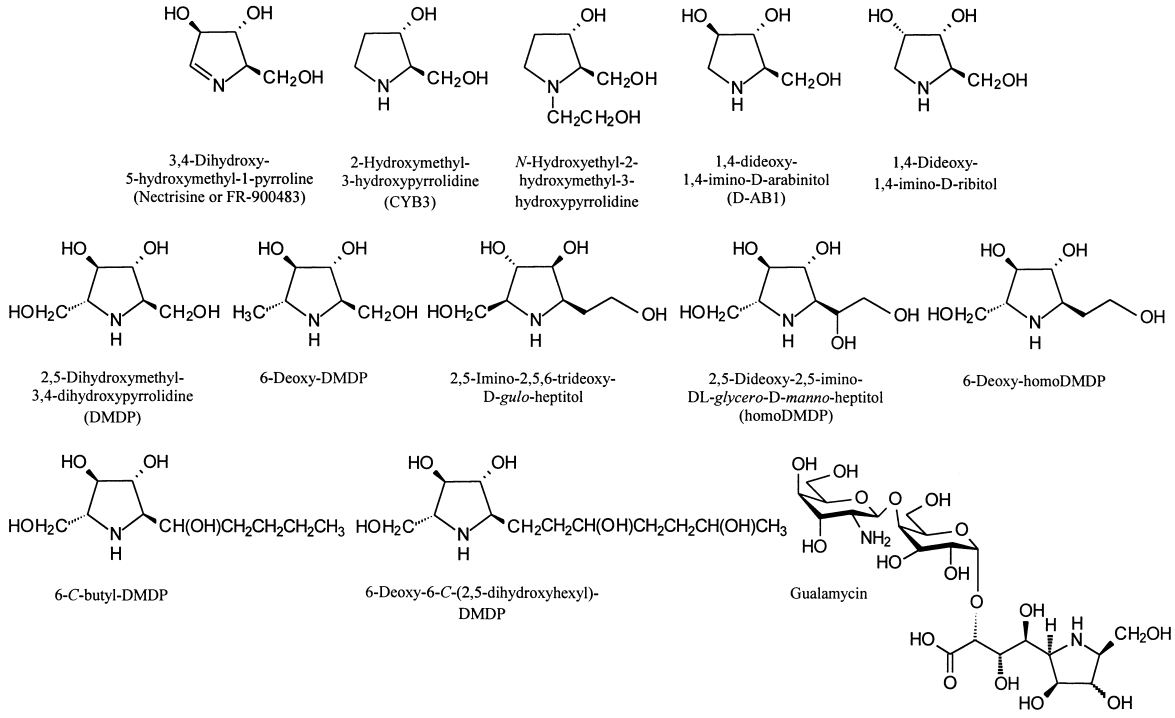


Fig. 2. Some naturally-occurring polyhydroxylated pyrroline and pyrrolidine alkaloids.

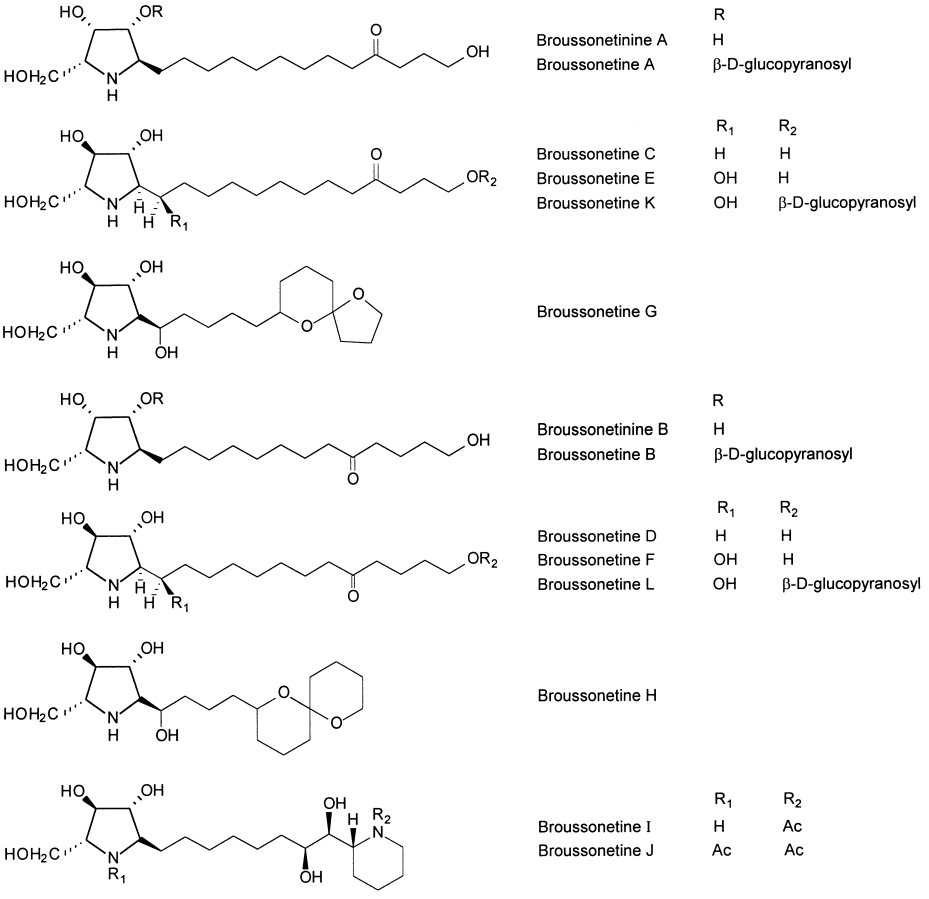


Fig. 3. Polyhydroxylated pyrrolidine alkaloids and derivatives isolated from *Broussonetia kazinoki*.

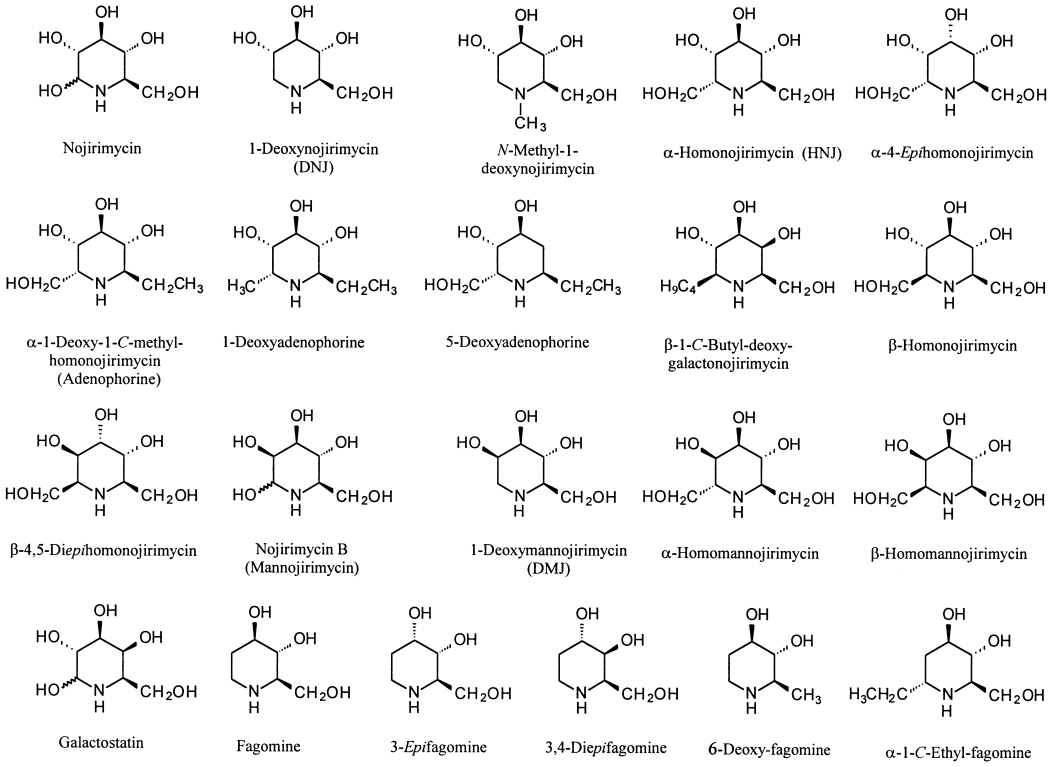


Fig. 4. Some naturally-occurring polyhydroxylated piperidine alkaloids.

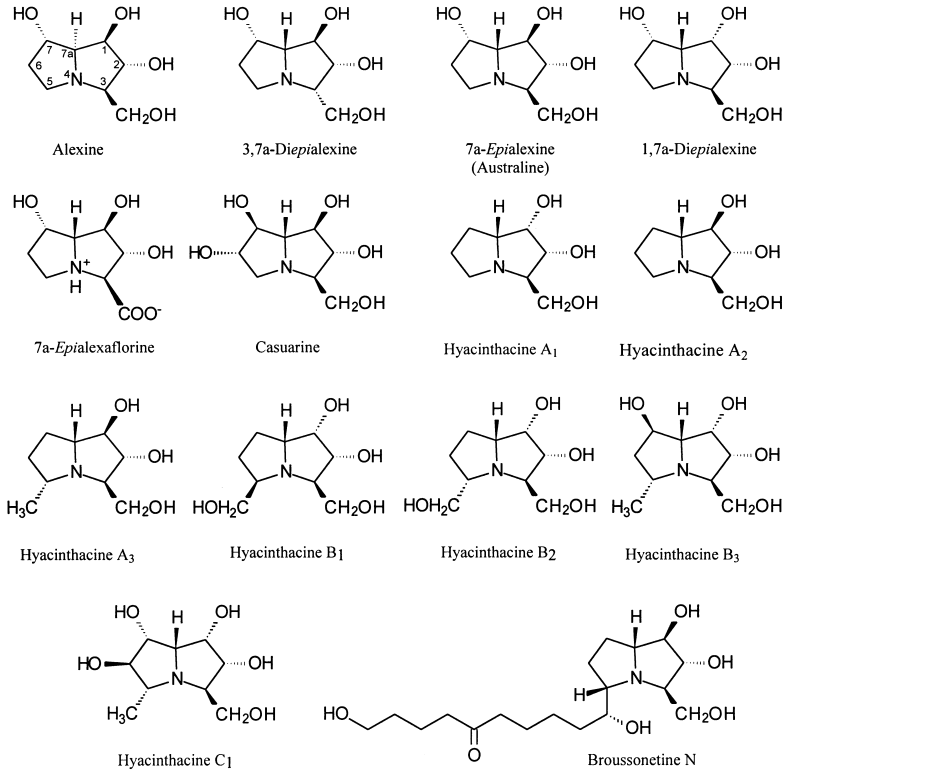


Fig. 5. Some naturally-occurring polyhydroxylated pyrrolizidine alkaloids [recently, Wormald et al. (1998) reported that 7,7a-di*epi*alexine, originally identified as a natural product from *Castanospermum australe* (Nash et al., 1990a) was in fact incorrectly described in the original paper as identification was based on a comparison with erroneous NMR data published for 7a-*epi*alexine by Molyneux et al. (1988). Therefore, 7,7a-di*epi*alexine can no longer be regarded as a natural product and so it is not depicted here].

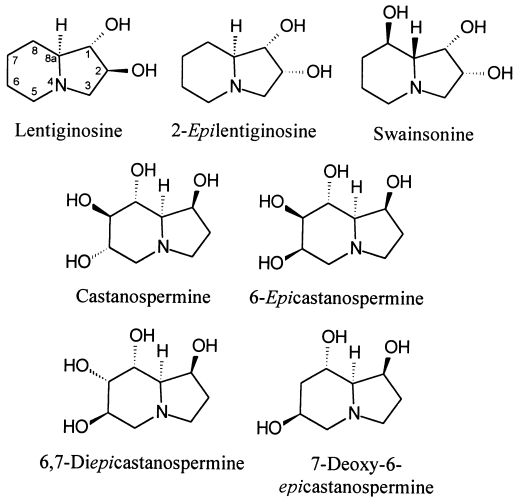


Fig. 6. Some naturally-occurring polyhydroxylated indolizidine alkaloids.

natural products (Kite et al., 1990). Similarly, the synthetic piperidine alkaloids β-homonojirimycin (Holt et al., 1994; Martin and Saavedra, 1995), α-homomannojirimycin (Bruce et al., 1992) and β-homomannojirimycin (Holt et al., 1994) were all recently isolated from *Aglaonema treubii* (Araceae) (Asano et al., 1997a).

3. Polyhydroxylated alkaloids as glycosidase inhibitors

Most of the polyhydroxylated alkaloids listed in Section 2 that have been studied in detail have been shown to inhibit glycosidases in a reversible and competitive manner (Legler, 1990; Asano et al., 1995b). Glycosidases are enzymes that catalyse the hydrolysis of the glycosidic bonds in complex carbohydrates and glycoconjugates. The wide variety of functions in which glycosidases are involved makes them essential for the survival and existence of all living organisms. For example, digestive glycosidases break down large sugar-containing molecules to release monosaccharides which can be more easily taken up and used metabolically by the organism; lysosomal glycosidases catabolise glycoconjugates intracellularly; and a wide range of glycosidases are involved in the biosynthesis of the oligosaccharide portions of glycoproteins and glycolipids which play vital roles in mammalian cellular structure and function. Thus, membrane glycoproteins include receptors for biologically important molecules such as hormones, low-density lipoprotein or acetylcholine whilst others are involved in cell–cell adhesion. The oligosaccharide chains play an important role in the correct functioning of these proteins by stabilising them and ensuring that they have the correct conformation and they may also be involved in the targeting mechanism of certain proteins (Elbein, 1989).

Since the mode of action of glycosidases involves the cleavage of glycosidic bonds between sugar molecules, individual glycosidases show specificity for certain sugar molecules and for a specific anomeric configuration of that sugar. Polyhydroxylated alkaloids can be extremely potent and specific inhibitors of glycosidases by mimicking the pyranosyl or furanosyl moiety of their natural substrates. Therefore, the number, position and configuration of the hydroxyl groups of each alkaloid dictate the type of glycosidases which are inhibited. For example, the configuration of the hydroxyl substituents of the glucosidase inhibitor nojirimycin correspond to those of glucose in the pyranose configuration. Inouye et al. (1968) reported that nojirimycin exists in aqueous

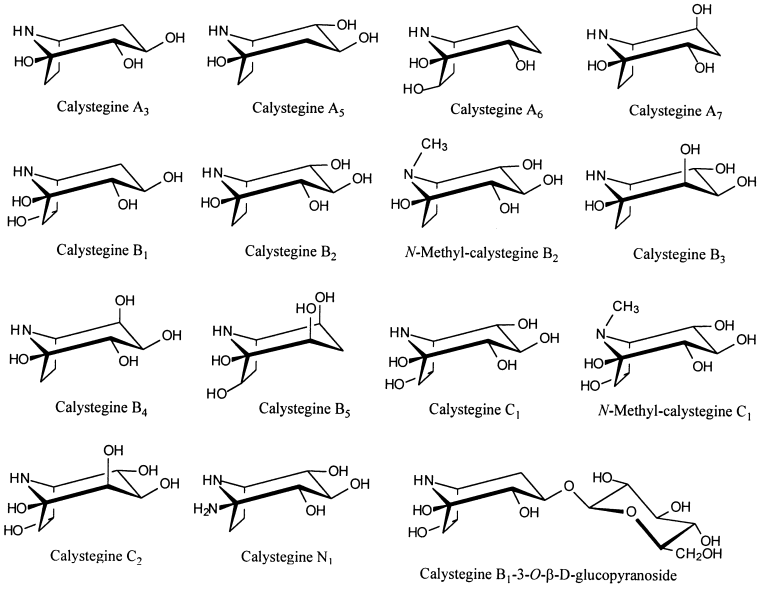


Fig. 7. Some naturally-occurring polyhydroxylated *nor*tropane alkaloids.

solution in both α- and β- forms with an equilibrium of 60% of the former and 40% of the latter and each of these forms are responsible for the inhibition of α- and β-glucosidases, respectively. Although the spatial arrangement of the hydroxyl groups of polyhydroxylated alkaloids serves as a means of recognition by specific glycosidases, it is the influence of the endocyclic nitrogen atom on the conformation and electrostatic properties of the molecule that is important for inhibition of enzyme activity. It has been suggested that when a polyhydroxylated alkaloid binds to the active site of a glycosidase, protonation of the compound leads to the formation of an ion pair between the inhibitor and a carboxylate anion in the active site of the enzyme (Legler, 1990). The protonated inhibitor closely resembles the transition state of the natural substrate and hence the enzyme has a high affinity for the molecule. However, the strength of the binding and hence the effectiveness of the inhibition depends to a degree on the p*K*a of the inhibitor and the pH optimum of the enzyme (Lalegerie et al., 1982).

Although the nature of the glycosidases that will be inhibited by certain polyhydroxylated alkaloids can be predicted, to some extent, from the number, position and configuration of the hydroxyl groups, there can be marked differences in the inhibition of isoenzymes of a given glycosidase in different species (Scofield et al., 1995a,b) and even within the same cell. For example, in human liver cells there are multiple forms of α-mannosidase which are structurally, functionally and genetically quite distinct (Winchester, 1984). They also have different subcellular locations. The lysosomal α-mannosidase, which catabolises glycoconjugates, has an acidic pH optimum (around 4.0) whereas the cytosolic α-mannosidase functions best at near neutral pH (6.5). There are also two forms of α-mannosidase in the Golgi (α-mannosidase I and II, respectively) which are involved in glycoprotein processing, as is another α-mannosidase which is associated with the endoplasmic reticulum. The lysosomal, Golgi α-mannosidase II and neutral α-mannosidases are all inhibited by polyhydroxylated alkaloids with the same substituents and chirality as mannofuranose (e.g. swainsonine) whereas Golgi α-mannosidase I is inhibited by mannopyranose analogues such as DMJ (Winchester et al., 1993).

There is now a vast amount of literature on the inhibition of glycosidases by particular polyhydroxylated alkaloids and related compounds (both natural and synthetic) and an overview of this area is given in some reviews (Legler, 1990; Winchester and Fleet, 1992; Asano et al., 1995b). It should be noted, however, that the polyhydroxylated alkaloids discussed above are all inhibitors of exoglycosidases, which cleave the glycosidic linkage at the non-reducing terminus of the saccharide chain, liberating monosaccharide units. However, certain animal cells also possess endoglycosidases which act on glycosidic linkages within saccharide chains giving rise to smaller oligosaccharide units. Endoglycosidases are not generally inhibited by polyhydroxylated alkaloids. A large amount of research has been conducted on the inhibition of exoglycosidases involved in glycoprotein formation and catalysis which has been shown to be important in both the toxicity and the potential therapeutic uses of the polyhydroxylated alkaloids (Sections 4 and 5). Table 2 lists some of the exoglycosidases that the polyhydroxylated alkaloids are reported to inhibit. This is not a comprehensive list, as not all naturally-occurring polyhydroxylated alkaloids have been tested on a wide range of glycosidases. It should also be remembered that due to the variations in specificity of isoenzymes, both between species and within the same cell, it is difficult to predict, for example, that a potent inhibitor of certain α-D-glucosidases will inhibit all α-D-glucosidases as strongly, if at all.

4. Mammalian toxicity of polyhydroxylated alkaloids

As might be expected for a group of compounds that affect carbohydrate metabolism, the polyhydroxylated alkaloids have been reported to have a wide range of effects on a number of organisms and some cause serious livestock poisonings. In fact, it was the toxicity to livestock of the legumes *Swainsona canescens* and *Castanospermum australe* in Australia that first led to the isolation of the toxic principles swainsonine (Colegate et al., 1979) and castanospermine (Hohenschutz et al., 1981).

Cattle eating *Swainsona* species developed a syndrome called "pea struck" which is due to decreased α-mannosidase activity resulting in accumulation of mannose-rich oligosaccharides in lysosomes. This results in neuronal vacuolation, axonal dystrophy, loss of cellular function and ultimately death (Dorling et al., 1978). The disorder "locoism" in the western United States was also found to be due to swainsonine in locoweeds (*Astragalus* and *Oxytropis* species), (Molyneux and James, 1982; Molyneux et al., 1985). The clinical symptoms of swainsonine poisoning in livestock on ingestion of plants containing this compound include depression, tremors, nervousness, emaciation, gastrointestinal malfunction and reproductive alterations such as abortion and birth defects (Stegelmeier et al., 1995a). Poisoning of animals in China by *Oxytropis ochrocephala* and *Oxytropis kansuensis* has also been shown to be due to the presence of swainsonine (Cao et al., 1992).

The concentration of swainsonine detected in all plants implicated in poisonings is extremely low. For example, Molyneux and James (1982) isolated swainsonine from the vegetative parts of *Astragalus lentiginosus* in a yield of 0.007% (dry weight) and the levels detected in *O. ochrocephala* and *O. kansuensis* were 0.012 and 0.021% (dry weights), respectively (Cao et al., 1992).

Table 2
A list of some of the exoglycosidases that have been found to be inhibited by naturally-occurring polyhydroxylated alkaloids

Alkaloid	Glycosidases inhibited
Pyrroline	
Nectrisine (FR-900483)	α-Glucosidase, α-mannosidase (Shibata et al., 1988; Kayakiri et al., 1991)
Pyrrolidines	
2*R*-Hydroxymethyl-3*S*-hydroxypyrrolidine (CYB-3)	Weak inhibitor of isomaltase (Scofield et al., 1986)
1,4-Dideoxy-1,4-imino-D-arabinitol (D-AB1)	Potent inhibitor of α-glucosidases (including maltase, isomaltase, sucrase and trehalase, Glucosidase II), α-D-arabinosidase, β-glucosidases (including lactase and cellobiase) and α-mannosidases (Mannosidase II and lysosomal). (Fleet et al., 1985; Scofield et al., 1986; Axamawaty et al., 1990; Asano et al., 1995b)
1,4-Dideoxy-1,4-imino-(2-*O*-β-D-glucopyranosyl)-D-arabinitol	Weak inhibitor of α- and β-glucosidases (Asano et al., 1994b)
1,4-Dideoxy-1,4-imino-D-ribitol	Weak inhibitor of α-glucosidase (Winchester and Fleet, 1992)
2*R*,5*R*-Dihydroxymethyl-3*R*,4*R*-dihydroxy-pyrrolidine (DMDP)	β-Glucosidases (including cellobiase) and α-glucosidases (including invertase, sucrase, isomaltase, trehalase), human lysosomal β-mannosidase, β-galactosidase (including lactase) and β-xylosidase (Evans et al., 1985a; Fellows et al., 1986; Scofield et al., 1995a and b; Asano et al., 1995b)
6-Deoxy-DMDP	β-Mannosidase, β-galactosidase and α-fucosidase (Molyneux et al., 1993a)
6-*C*-Butyl-DMDP	Good β-glucosidase inhibitor and poor inhibitor of β-galactosidase (Asano et al., 2000b)
6-Deoxy-6-*C*-(2,5-dihydroxyhexyl)-DMDP	Moderate inhibitor of amyloglucosidase and weak inhibitor of α-fucosidase only (Kato et al., 1999)
2,5-Imino-2,5,6-trideoxy-D-*gulo*-heptitol	Moderate inhibitor of α-fucosidase (Asano et al., 1998a)
2,5-Dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homo-DMDP)	Potent inhibitor of β-glucosidases and β-galactosidases (including lactase) and trehalase, moderate inhibitor of other α-glucosidases (including maltase) and α- and β-mannosidases (Watson et al., 1997; Kato et al., 1999)
Homo-DMDP-7-*O*-apioside	Good inhibitor of β-galactosidases (including lactase) and amyloglucosidase (Kato et al., 1999)
Homo-DMDP-7-*O*-β-D-xylopyranoside	Potent inhibitor of β-glucosidases and β-galactosidases (including lactase) and moderate inhibitor of amyloglucosidase and β-mannosidase (Kato et al., 1999)
6-Deoxy-homoDMDP	Potent inhibitor of α-glucosidases (including maltase) (Asano et al., 1998a)
Broussonetines A and B	Not inhibitory (Shibano et al., 1997b)
Broussonetinines A and B	Potent inhibitors of β-galactosidase and α-mannosidase (Shibano et al., 1997b)
Broussonetines C and D	Potent inhibitors of β-galactosidase and β-mannosidase (Shibano et al., 1997a)
Broussonetines E and F	Potent inhibitors of β-glucosidase, β-galactosidase and β-mannosidase and good inhibitors of α-glucosidase (Shibano et al., 1997b)
Broussonetines G, H, K and L	Potent inhibitors of β-glucosidase, β-galactosidase and β-mannosidase (Shibano et al., 1998a, 1999a)
Piperidines	
Nojirimycin	β-Glucosidases and α-glucosidases (including sucrase, maltase, isomaltase and amylase), β-galactosidases and *N*-acetyl-β-D-glucosaminidases (Fuhrmann et al., 1985; Legler, 1990)
1-Deoxynojirimycin (DNJ)	Potent inhibitor of α-glucosidases (including trehalase, invertase, sucrase, maltase, isomaltase), more weakly inhibitory to Glucosidase I and II, and β-glucosidases, also α-mannosidases, α-fucosidase, α- and β-galactosidases (including lactase) (Evans et al., 1985a; Fellows et al., 1986; Legler, 1990; Asano et al., 1995b)
1-Deoxynojirimycin-2-*O*-, 3-*O*-, 4-*O*-α-D-glucopyranosides and 2-*O*-, 6-*O*-α-D-galactopyranoside and 2-*O*-, 3-*O*-, 4-*O*-, 6-*O*-β-D-glucopyranoside	Varying levels of inhibition of α-glucosidases (Asano et al., 1994b)
N-Methyl-1-deoxynojirimycin	More potent inhibitor of α-glucosidases than DNJ (Asano et al., 1995b)

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Table 2 (*continued*)

Alkaloid	Glycosidases inhibited
α-Homonojirimycin (HNJ)	α-Glucosidases (including maltase, sucrase and trehalase), β-glucosidases, lactase and α-galactosidases (Kite et al., 1988, 1990) and moderate inhibitor of Glucosidase I and II (Zeng et al., 1997)
α-Homonojirimycin-7-*O*-β-D-glucopyranoside (MDL 25,637)	α-Glucosidases (including Glucosidase II and trehalase) (Kyosseva et al., 1995)
α-4-*Epi*homonojirimycin	Poor inhibitor of α- and β-galactosidases (Asano et al., 1998b; Martin et al., 1999)
α-1-Deoxy-1-*C*-methyl-homonojirimycin (Adenophorine)	Good α-galactosidase inhibitor and moderate to poor inhibitor of α-glucosidases (Ikeda et al., 2000)
Adenophorine-1-*O*-β-D-glucopyranoside	Good trehalase inhibitor but moderate to poor inhibitor of other α-glucosidases (Ikeda et al., 2000)
1-Deoxyadenophorine	Good α-galactosidase inhibitor and moderate to poor inhibitor of α-glucosidases (Ikeda et al., 2000)
5-Deoxyadenophorine	Good α- and β-galactosidase inhibitor and poor trehalase inhibitor (Ikeda et al., 2000)
5-Deoxyadenophorine-1-*O*-β-D-glucopyranoside	Potent inhibitor of α-glucosidases and α-galactosidases (Ikeda et al., 2000)
β-1-*C*-Butyl-deoxygalactonojirimycin	Potent α-galactosidase inhibitor only (Ikeda et al., 2000)
β-Homonojirimycin	Weak inhibitor of β-glucosidase (Asano et al., 1997a)
β-4,5-Di*epi*homonojirimycin	Potent inhibitor of α-glucosidases (including maltase and sucrase) and α-galactosidase, moderate inhibitor of α-fucosidase (Asano et al., 1998b)
Nojirimycin B (Mannojirimycin)	α-mannosidases (Niwa et al., 1984)
1-Deoxymannojirimycin (DMJ)	α-Mannosidases (Mannosidase I) and α-fucosidase (Ishida et al., 1967; Bischoff and Kornfeld, 1984; Evans et al., 1985a)
α-Homomannojirimycin	Weak inhibitor of human liver α-mannosidases (Asano et al., 1997a)
β-Homomannojirimycin	β-Mannosidase (Asano et al., 1997a)
Galactostatin	β-Galactosidases (Miyake and Ebata, 1988)
Fagomine	Weak inhibitor of α-glucosidases (including isomaltase and sucrase) (Kato et al., 1997a)
3-E*pi*fagomine	Isomaltase, β-galactosidases and lactase (Kato et al., 1997a)
3,4-Di*epi*fagomine	Not inhibitory (Kato et al., 1997a)
Fagomine-4-*O*-β-D-glucopyranoside	Weak inhibitor of β-galactosidase (Scofield et al., 1986; Kato et al., 1997a)
Fagomine-3-*O*-β-D-glucopyranoside	Not inhibitory (Kato et al., 1997a)
α-1-*C*-Ethyl-fagomine	Good β-galactosidase inhibitor and poor inhibitor of α-glucosidases (Asano et al., 2000b)
Pyrrolizidines	
Alexine	Disaccharidase-type α-glucosidases (trehalase, amyloglucosidase) and β-galactosidase (Nash et al., 1988a, 1990a)
3,7a-Di*epi*alexine	Disaccharidase-type α-glucosidases (amyloglucosidase and sucrase) (Nash et al., 1990a)
7a-*Epi*alexine (Australine)	Disaccharidase-type α-glucosidases (amyloglucosidase, sucrase, maltase) and Glucosidase I, β-glucosidase, β-galactosidase (Molyneux et al., 1988; Zeng et al., 1997)
1,7a-Di*epi*alexine	α-glucosidases (amyloglucosidase), weakly inhibitory to β-glucosidases (Nash et al., 1990a)
7a-*Epi*alexaflorine	Weak inhibitor of amyloglucosidase and sucrase (de S. Pereira et al., 1991)
Casuarine	α-Glucosidases (including trehalase, amyloglucosidase and glucosidase I) (Bell et al., 1997)
Hyacinthacine A_1	Potent inhibitor of lactase, moderate inhibitor of amyloglucosidase and α-fucosidase and a poor inhibitor of α- and β-glucosidases and β-mannosidase (Asano et al., 2000a)
Hyacinthacine A_2	Potent inhibitor of amyloglucosidase, good inhibitor of lactase, moderate inhibitor of β-glucosidase and trehalase and weak inhibitor of β-mannosidase (Asano et al., 2000a)
Hyacinthacine A_3	Good inhibitor of amyloglucosidase and moderate inhibitor of lactase (Asano et al., 2000a)

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Table 2 (*continued*)

Alkaloid	Glycosidases inhibited
Hyacinthacine B_1	Moderate to poor inhibitor of β-glucosidases and β-galactosidases (Kato et al., 1999)
Hyacinthacine B_2	Moderate to good inhibitor of β-glucosidases and β-galactosidases (including potent inhibition of lactase) (Kato et al., 1999)
Hyacinthacine B_3	Good inhibitor of lactase and amyloglucosidase and poor inhibitor of α-fucosidase (Asano et al., 2000a)
Hyacinthacine C_1	Moderate inhibitor of amyloglucosidase (Kato et al., 1999)
Broussonetine N	Good inhibitor of β-glucosidase, β-galactosidase and β-mannosidase (Shibano et al., 1999b)
Indolizidines	
Swainsonine	Potent inhibitor of α-mannosidases (Tulsiani et al., 1982; Cenci di Bello et al., 1989a,b)
Lentiginosine	Amyloglucosidase (Pastuszak et al., 1990)
2-*Epi*lentiginosine	Not inhibitory (Pastuszak et al., 1990)
Castanospermine	α-Glucosidases (including amyloglucosidase, sucrase, maltase, isomaltase, trehalase, amylase, Glucosidase I and II) and β-glucosidases (including lactase and cellobiase), β-glucocerebrosidase and β-xylosidase (Saul et al., 1983; Scofield et al., 1995a,b)
6-*Epi*castanospermine	Amyloglucosidase, neutral α-mannosidase (Molyneux et al., 1986; Winchester et al., 1990)
6,7-Di*epi*castanospermine	Amyloglucosidase and fungal β-glucosidase (Molyneux et al., 1991)
7-Deoxy-6-*epi*-castanospermine	Amyloglucosidase and yeast α-glucosidase (Molyneux et al., 1990)
Nortropanes	
Calystegine A_3	Potent inhibitor of β-glucosidases, α- and β-galactosidases (including lactase) and trehalase (Asano et al., 1995a)
Calystegine A_5	Not inhibitory (Asano et al., 1995a)
Calystegine A_6	Moderate inhibitor of trehalase only (Asano et al., 1996b)
Calystegine A_7	Moderate inhibitor of trehalase only (Asano et al., 1997b)
Calystegine B_1	Potent inhibitor of β-glucosidases (including cellobiase) and β-galactosidase (including lactase) and a weak inhibitor of trehalase (Asano et al., 1995a)
Calystegine B_1-3-*O*-β-D-glucopyranoside	Potent inhibitor of rice α-glucosidase and weak inhibitor of β-glucosidases (Asano et al., 1997d)
Calystegine B_2	Potent inhibitor of β-glucosidases, disaccharidase-type α-glucosidases (including maltase, sucrase and trehalase) and α- and β-galactosidase (including lactase) (Asano et al., 1996a)
N-Methyl-calystegine B_2	Potent inhibitor of α-galactosidases and a moderate inhibitor of trehalase (Asano et al., 1997b)
Calystegine B_3	Weak inhibitor of β-glucosidases, α-galactosidases and trehalase (Asano et al., 1995a; Kato et al., 1997b)
Calystegine B_4	Moderate inhibitor of α-galactosidases and trehalase, but weak inhibitor of β-glucosidases (including cellobiase) and lactase (Asano et al., 1996a)
Calystegine C_1	Potent inhibitor of disaccharidase-type α-glucosidases and β-glucosidases, moderate inhibitor of α- and β-galactosidases (Asano et al., 1995a, 1996a; Kato et al., 1997b)
N-Methyl-calystegine C_1	Weak inhibitor of some α-galactosidases (Asano et al., 1997b)
Calystegine C_2	Moderate inhibitor of β-glucosidases, α-mannosidases and trehalase (Kato et al., 1997b)
Calystegine N_1	Weak inhibitor of disaccharidase-type α-glucosidases and potent inhibitor of β-glucosidases (including cellobiase), lactase and α-galactosidase (Asano et al., 1996b)

However, swainsonine can accumulate within the tissues of the body as it has the ability to permeate the plasma membrane freely but once inside lysosomes it becomes protonated due to the low pH and so becomes concentrated (Chotai et al., 1983). Therefore, poisoning by swainsonine generally takes several weeks of ingestion before it becomes apparent. If affected animals are denied access to plants containing swainsonine early enough, lysosomal function will return to normal as the alkaloid is excreted from the body via the urine (Daniel et al., 1984). Although many of the lesions will disappear, some long-term effects may result in reduced animal

performance (Stegelmeier et al., 1995a). A threshold of toxicity is difficult to establish for swainsonine, but Molyneux and co-workers (1994) suggested that a concentration in the diet of as little as 0.001% should be of concern.

Even before swainsonine was identified as the toxic principle in *Swainsona* species, Dorling et al. (1978) recognised that the lysosomal storage disorder induced in animals grazing these plants was biochemically and morphologically similar to the rare genetically-determined mannosidosis that occurs in humans (Ockerman, 1973) and Angus cattle (Hocking et al., 1972). [N.B. Mannosidosis has subsequently also been found to occur naturally in cats (Burditt et al., 1980) and guinea pigs (Crawley et al., 1999).] This disease is characterised by accumulation in cells and excretion in the urine of mannose-rich oligosaccharide which results from a deficiency in all tissues of lysosomal α-D-mannosidase (Jolly et al., 1981). The condition is ultimately fatal. In infants the clinical syndrome may include mental retardation, central nervous system derangement, abnormal musculoskeletal development, locomotor incoordination, abnormal facial appearance and an increased susceptibility to recurrent infections (Ockerman, 1973). However, swainsonine-induced mannosidosis is not an exact model of genetic mannosidosis since this compound inhibits α-mannosidase II in addition to the lysosomal α-mannosidase. Nevertheless, swainsonine has proved a valuable tool in the study of this lysosomal storage disease since it has provided the means of inducing a reversible phenocopy of the genetic disorder in animals and in tissue culture (Cenci di Bello et al., 1983). The use of such models could allow investigation of the viability of enzyme replacement therapy in the treatment of this disease (Winchester, 1992).

Comparison of the sequences of the major oligosaccharides which accumulate and are excreted in the urine of humans suffering from mannosidosis with those excreted in the swainsonine-induced disease has allowed the intracellular substrate specificity of lysosomal α-mannosidase to be defined (Daniel et al., 1992). In 1983, Cenci di Bello and co-workers first realised that the human lysosomal α-mannosidase actually consists of two enzymes which are specific for different mannose linkages. The major enzyme present catalyses α-1,3-linkages but the other is specific for α-1,6-linkages (de Gasperi et al., 1992). In human mannosidosis the α-1,3 activity is absent but the α-1,6 activity is unaffected. Swainsonine inhibits the activity of both enzymes. However, cattle do not possess the α-1,6 form of lysosomal α-mannosidase and so the storage products in the bovine form of genetic mannosidosis bear a greater similarity to those seen in the form of the disease induced by swainsonine (de Gasperi et al., 1992).

Castanospermum australe contains the potent α- and β-glucosidase inhibitor castanospermine at a concentration of approximately 0.3% of the dry weight of the seed (Hohenschutz et al., 1981). Pigs, cattle and horses have been reported to be poisoned when they have consumed the seeds of *C. australe*. The chief symptom is gastroenteritis, owing to inhibition of the activity of intestinal brush border digestive disaccharidases (e.g. sucrase, maltase and trehalase) (McKenzie et al., 1988). The upset can be so severe that the animals can die (Pan et al., 1993). Other symptoms include myocardial degeneration and nephrosis (McKenzie et al., 1988). Several cases of human poisoning have also occurred (Everist, 1974). Histologic changes observed in poisoned livestock and rodent feeding trials include degenerative vacuolation of hepatocytes and skeletal myocytes (McKenzie et al., 1988; Stegelmeier et al., 1995a,b). Castanospermine inhibits the activity of lysosomal α-glucosidase which leads to the accumulation of glycogen within the lysosomes (Saul et al., 1985). This mimics the situation in the genetically-determined storage disorder glycogenosis type II (Pompe's disease) which is caused by a deficiency of lysosomal α-glucosidase (Glew et al., 1985). However, castanospermine also inhibits the activity of lysosomal β-glucosidase and this affects the catabolism of glycosphingolipids (Cenci di Bello et al., 1988).

Recently, swainsonine has been detected in species of the Convolvulaceae from Australia and Africa (Molyneux et al., 1995; de Balogh et al., 1999). These plants are reported to produce neurological disorders in livestock with clinical symptoms similar to those caused by the swainsonine-containing members of the Leguminosae. However, these species also contain the *nor*tropane alkaloids calystegines which inhibit α- and β-glucosidases and α-galactosidase and so there may be a combination of toxic effects. The calystegines are also common in the Solanaceae and there are several *Solanum* species that can poison livestock. Symptomatically, these cases resemble "locoism" (Molyneux et al., 1996). For example, *Solanum dimidiatum* causes "Crazy Cow Syndrome" in Texas (Menzies et al., 1979) and *Solanum kwebense* causes "Maldronksiekte" in South Africa (Pienaar et al., 1976). Both disorders are characterised by neurological signs of cerebellar dysfunction, including staggering and incoordination, with severe cellular vacuolation and degeneration of Purkinje cells in the brain. Both plants contain a range of calystegines, including calystegine B_2 (Nash et al., 1993). Therefore, it seems likely that these syndromes are also lysosomal storage disorders caused by glycosidase inhibition produced by the calystegines (Nash et al., 1993).

5. Therapeutic potential of polyhydroxylated alkaloids

Very few polyhydroxylated alkaloids are available commercially. Those which are available (principally DNJ, DMJ, castanospermine and swainsonine) have become standard reagents used to investigate the

potential therapeutic and biochemical applications of this class of glycosidase inhibitor. Therefore, most of the following discussion of the therapeutic applications of polyhydroxylated alkaloids is based on a limited number of compounds that have been tested thoroughly, simply because these are the only ones which are readily available. It should also be noted that the doses required for beneficial effects in human disease states are generally below those causing the toxicities described in Section 4.

5.1. Anti-cancer agents

Both catabolic and glycoprotein processing glycosidases are involved in the transformation of normal cells to cancer cells and in tumour cell invasion and migration. Many tumour cells display aberrant glycosylation due to an altered expression of glycosyltransferases (Hakomori, 1985) and it has been known for a long time that the levels of glycosidases are elevated in the sera of many patients with different tumours (Woollen and Turner, 1965). Secreted glycosidases may be involved in the degradation of the extracellular matrix in tumour cell invasion (Bernacki et al., 1985). Furthermore, the lysosomal system is highly active in transformed cells, presumably reflecting enhanced turnover of glycoproteins and other macromolecules, and possibly increased exocytosis of lysosomal hydrolases (Olden et al., 1991). The use of polyhydroxylated alkaloids to prevent the formation of aberrant asparagine-linked oligosaccharides during glycoprotein processing and to inhibit catabolic glycosidases is being actively pursued as a therapeutic strategy for cancer.

Although a number of the polyhydroxylated alkaloids have been reported to show anti-cancer activity, research has concentrated on developing swainsonine as a drug candidate for the management of human malignancies. Swainsonine has a complex mode of action in the whole animal. It inhibits the growth of tumour cells and prevents the dissemination of malignant cells from the primary tumour to secondary sites (a process known as metastasis). However, swainsonine also has a direct stimulatory effect on the immune system. There is a wealth of literature available on the anti-cancer properties of swainsonine, probing various aspects of its activity using a multitude of different rodent and human cell lines and animal models. A summary of the essential findings from this work follows.

The action of swainsonine to prevent tumour cell proliferation is related to its inhibition of the processing of their asparagine-linked oligosaccharides. Neoplastic transformation in both human and rodent tumour cells is often accompanied by increased expression of β-1,6-branched oligosaccharides in complex-type *N*-linked glycans, which are apparently required for efficient tumour cell metastasis in vivo (Dennis et al., 1990; Baptista et al., 1994). Swainsonine blocks expression of complex-type oligosaccharides in malignant cells. It would seem that loss or truncation of β-1,6-branched oligosaccharides in metastatic tumour cells has the effect of reducing cell motility by increasing cellular adhesion, it reduces their capacity to invade other tissues and it also reduces solid tumour growth, possibly by decreasing the cellular response to autocrine growth stimulation (Dennis et al., 1990; Olden et al., 1991). However, since all cellular glycoproteins which normally express complex-type oligosaccharides are affected by swainsonine treatment, investigations were carried out to determine whether the effects of swainsonine on cellular proliferation could also be widespread amongst non-transformed tissues in the body. These studies showed that the effects of this compound would appear to be cell-specific, since it does not affect the processing of all glycoproteins equally (Olden et al., 1991; Spearman et al., 1991; Korczak and Dennis, 1993).

There is considerable evidence that swainsonine enhances the natural anti-tumour defences of the body (Kino et al., 1985; Humphries et al., 1988). In animal models, it was observed that the reduction in metastasis of tumour cells induced by swainsonine administration continued for a number of days after the drug was withdrawn (Olden et al., 1989). It was discovered that this results from the ability of swainsonine to activate immune effector cells such as natural killer cells (which are peripheral blood lymphocytes), T-lymphocytes and macrophages (White et al., 1991; Das et al., 1995). These phagocytic cells have diverse functions that include antigen presentation, cytokine production (e.g. interleukins), immune surveillance and cytocidal activity against tumour cells. However, many of the properties of these cells are apparent only after they have been activated (Das et al., 1995). Swainsonine stimulates proliferation of spleen cells, thereby increasing the numbers of natural killer cells. It also increases lymphocyte sensitivity to interleukin-2 (IL-2) and other cytokines (Yagita and Saksela, 1990; Colombo et al., 1992). Studies showed that the extent of activation by swainsonine of peritoneal macrophages to cytotoxicity against tumour cells was comparable to that obtained with known macrophage-activating agents such as interferon and bacterial lipopolysaccharide. It was associated with increased secretion of interleukin-1 (IL-1) by the macrophages, induction of protein kinase C activity and enhanced secretion of the major histocompatibility antigen on the cell surfaces (Grzegorzewski et al., 1989). Swainsonine can also induce tumouricidal activity in resident tissue-specific macrophages of both the lung and spleen (Das et al., 1995), with activation being both time- and dose-dependent. This is relevant to the clinical management of metastatic diseases since visceral organs are common sites for metastasis formation.

Before swainsonine could be used clinically, the possible adverse effects of this agent had to be evaluated.

As previously discussed, the systemic administration of high doses of swainsonine to sheep has been reported to induce a neural lysosomal mannoside storage disease (Huxtable and Dorling, 1982). However, no evidence of an overt toxic reaction was observed when swainsonine was administered orally to rodents (Huxtable and Dorling 1985) so it was considered that the mannosidosis induced by swainsonine could be a species- or tissue-specific phenomenon (Tulsiani and Touster, 1987). Swainsonine is both water and lipid soluble and therefore, it diffuses efficiently into tissues. In tissue culture swainsonine has been shown to attain concentrations inside cells similar to that in the culture medium within minutes (Chotai et al., 1983). In 1989, Mohla and co-workers compared the fates of swainsonine administered to mice by different routes (orally, intravenously, intraperitoneally and subcutaneously). They found that in all cases the compound was cleared rapidly from the blood. Bowen et al. (1993) published a preliminary pharmacokinetic evaluation of swainsonine in mice after intravenous administration. They injected one dose of 3 μg/ml which was rapidly cleared from the blood with a half-life of 31.6 min. The swainsonine rapidly entered various body organs and tissues from the blood. The highest levels were found in the bladder (owing to urinary excretion of the compound), kidney and thymus, but the lowest levels were found in the brain, which suggested that toxicity to the central nervous system may not be a problem at swainsonine levels which are sufficient to prevent metastasis. In a follow-up study (Bowen et al., 1997) swainsonine levels in various tissues were compared for up to three days after discontinuing oral administration of this compound to mice in their drinking water. These researchers found that swainsonine was predominantly retained for at least 72 h in lymphoid tissue (spleen and thymus) which was consistent with the sustained immunomodulatory and anti-metastatic properties of this compound.

The results from the tissue culture and animal models were so encouraging that the efficacy of swainsonine was examined in phase I clinical trials in humans with advanced malignancies (Baptista et al., 1994; Goss et al., 1994, 1997). In the first study, the subjects were nineteen terminally ill cancer patients with either leukemia or breast, colon, lung, pancreatic, or head and neck cancers. Swainsonine was administered by continuous intravenous infusion over 5 days in the dose range 50–550 μg/kg body weight/day. The serum concentrations of this drug reached 3–11.8 mg/l which is 100–400 times greater than the 50% inhibitory concentration for Golgi α-mannosidase II and lysosomal α-mannosidases (Baptista et al., 1994). The patients had elevated levels of the liver enzyme aspartate aminotransferase in the serum that was indicative of hepatocyte damage and they also suffered from pulmonary and peripheral oedema (Goss et al., 1994). However, the patient with head and neck cancer had over 50% tumour remission and two others showed symptomatic improvement. A second clinical trial was then undertaken by the same researchers to evaluate swainsonine by oral administration biweekly (dose range 50–600 μg/kg body weight) to 16 patients with advanced malignancies (Goss et al., 1997). Serum drug levels peaked 3–4 h following a single oral dose. The maximum tolerated dose was found to be 150 μg/kg body weight/day due to increased levels of aspartate aminotransferase in the serum and oedema, similar to that seen in the previous trial. Other adverse effects included fatigue, anorexia, abdominal pain and neurological symptoms. It was also found in both studies that the immune system was depressed rather than stimulated and it was thought that this was due to the high doses administered. Interestingly, recent observations in cases of induced chronic locoweed poisoning in cattle similarly indicate that the immune response is depressed by prolonged exposure to high doses of swainsonine (Stegelmeier et al., 1998a). Further clinical trials were suggested by Goss et al. (1997) to investigate alternative dosing schedules with lower starting doses in order to determine whether the toxic effects of swainsonine can be overcome. Indeed, swainsonine is currently undergoing phase II clinical trials in Canada with apparently very promising results (Dr. Jeremy Carver, CEO of GLYCODesign, pers. commun.).

In 1993, Dennis and co-workers synthesised derivatives of swainsonine with lipophilic groups attached to the hydroxyls at either position 2 or 8. The 2-carbonoyloxy esters of swainsonine were relatively poor inhibitors of α-mannosidases in vitro but entered cells at a rate comparable to swainsonine where they were converted to swainsonine by intracellular esterases. In vivo, the analogues were found to have comparable activities to swainsonine as stimulators of bone marrow cell proliferation. This has led to the suggestion that these or similar analogues could be useful as pro-drugs which could be preferentially hydrolysed to release swainsonine only once they were inside tumour and/or lymphoid cells. As such they could be expected to have improved pharmacological properties and reduced side-effects (Dennis et al., 1993).

In addition to swainsonine, some of the other naturally-occurring polyhydroxylated alkaloids listed in Table 1 also show various anti-cancer properties. However, none of these compounds has been exploited to nearly the same extent as swainsonine. For example, the potent α-glucosidase inhibitor castanospermine (also an indolizidine alkaloid) has been reported to inhibit experimental metastasis in mice (Ostrander et al., 1988).

Various isoenzymes of the catabolic exoglycosidase β-*N*-acetylglucosaminidase have also been investigated as potential targets for cancer therapy due to their altered expression in a range of human cancer cell types and their potential involvement in the degradation of the

extracellular matrix in tumour cell invasion (Woynarowska et al., 1992; Martino et al., 1997). β-*N*-Acetylglucosaminidases release *N*-acetyl-D-glucosamine from glycoproteins and a number of inhibitors of these enzymes have been chemically synthesised, such as 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol (Fleet et al., 1986) and a synthetic derivative of castanospermine (6-acetamido-6-deoxycastanospermine) (Liu et al., 1991). However, potent natural product inhibitors of β-*N*-acetylglucosaminidase have also been isolated from microbial sources. These include nagstatin from *Streptomyces amakusaensis*, strain MG846-fF3 (Aoyagi et al., 1992) which is a nitrogenous *N*-acetyl-D-glucosamine analogue fused with an imidazole ring. Synthetic derivatives of nagstatin have subsequently been produced with enhanced activity (Tatsuta et al., 1995). Other inhibitors of β-*N*-acetylglucosaminidase are Pyrostatins A and B (4-hydroxy-2-imino-1-methylpyrrolidine-5-carboxylic acid and 2-imino-1-methylpyrrolidine-5-carboxylic acid, respectively) which were isolated from filtrates of *Streptomyces* sp. SA-3501 (Aoyama et al., 1995).

5.2. Immune stimulants

The effect of swainsonine on bone marrow proliferation was initially investigated because its potential use in cancer therapy required that it have minimal toxicity to bone marrow and other normal tissues. In fact, a frequent limitation of the suitability of drug candidates as chemotherapeutic agents is their toxicity to normal tissues, especially bone marrow (Frei and Cannellos, 1980; Hryniuk and Levine, 1986). It was found that swainsonine stimulated cell proliferation of murine bone marrow following haematological injuries although it did not stimulate healthy bone marrow progenitor cells in vivo when administered alone (Olden et al., 1991; White et al., 1991; Klein et al., 1999). The possibility that swainsonine could confer protection against the cytotoxic effects of both cell cycle-specific and -non-specific cytotoxic anticancer agents was first examined in a murine model system by Oredipe et al. (1991). The results indicated that the intraperitoneal administration of swainsonine decreased the lethality of methotrexate, 5-fluorouracil, cyclophosphamide and doxorubicin in non-tumour-bearing mice. However, these responses were critically dependent on the dose, sequence and timing of swainsonine administration. More recently, Klein et al. (1999) reported that swainsonine protected the bone marrow cells of mice from the toxic effects of cyclophosphamide without interfering with the drug's ability to inhibit tumour growth. Also, swainsonine did not stimulate proliferation of haematopoietic tumour cells which would suggest that this drug could still be used in patients with this type of cancer.

Klein et al. (1999) also assessed the protective properties of swainsonine in vivo with the myelosuppressive agent 3′-azido-3′-deoxythymidine (known as Zidovudine or AZT), which is often used in therapy for the acquired immune deficiency syndrome (AIDS). Swainsonine administered by intraperitoneal injection increased both total bone marrow cellularity and the number of circulating white blood cells in mice treated with doses of AZT that typically lead to severe myelosuppression, which is the major dose-limiting feature in chemotherapeutic regimens for AIDS using this drug (McLeod and Hammer, 1992). In addition, swainsonine protected human myeloid progenitor cells from AZT toxicity in vitro (Klein et al., 1999).

Therefore, the possibility exists that swainsonine could be used to accelerate the recovery of bone marrow cellularity and competence following high-dose chemotherapy or autologous bone marrow transplantation, or used as an adjuvant during AZT treatment. Thus, the complications and increased risk of opportunistic infections associated with the prolonged immune-deficient state in these clinical conditions could be minimised and the cure rates of cytoreductive treatment could be significantly improved. This promising therapeutic application of swainsonine is currently being pursued in the USA in phase I and II clinical trials by the Canadian company GLYCODesign Inc., under the product code GD0039.

Finally, it is worth mentioning that the pyrroline alkaloid nectrisine (Fig. 2), which inhibits α-glucosidases in addition to α-mannosidases, has been reported to restore the immune response of immunosuppressed mice (Kayakiri et al., 1991).

5.3. Anti-diabetic agents

It can be seen from Table 2 that a large number of the naturally-occurring polyhydroxylated alkaloids are potent inhibitors of the various α-glucosidase-specific disaccharidases involved in mammalian digestion (e.g. sucrase, maltase, isomaltase, etc.). These enzymes are expressed at the surface of the epithelial cells of the brush border in the small intestine. In the late 1970s, it was realised that inhibitors of these enzymes, such as DNJ, could be used therapeutically in the oral treatment of the non-insulin-dependent (type II) diabetes mellitus (Yagi et al., 1976; Schmidt et al., 1979).

It was found that the activity of 1-deoxynojirimycin (DNJ) in vivo against intestinal sucrase was lower than that seen in vitro and this initiated a synthetic programme to produce derivatives with enhanced activity. The *N*-alkyl derivatives were most effective and this led to the development of *N*-hydroxyethyl-deoxynojirimycin (known as Miglitol or BAY m-1099) as a drug candidate (for a review, see Junge et al., 1996). This derivative has improved retention in the small intestine which increases its potential in vivo antidiabetic activity. Miglitol does not have hypoglycemic activity like insulin and the

sulfonylureas. Instead, by inhibiting the breakdown of complex carbohydrates in the gut it reduces glucose uptake and so reduces the postprandial rise in blood glucose which is characteristic of diabetes (Jacob, 1995). However, Miglitol is absorbed appreciably from the gut into the bloodstream and additional effects of this compound have been observed. It has been reported to reduce postprandial insulin secretion, lessen diabetic glucosuria and reduce the carbohydrate-driven synthesis of very low density lipoproteins (Müller, 1989). The 7-*O*-β-D-glucopyranosyl derivative of α-homonojirimycin (MDL 25637) is another drug candidate which acts in the same manner as Miglitol (Liu, 1987; Rhinehart et al., 1987). This was synthesised prior to its discovery as a natural product (Kite et al., 1990).

A naturally-occurring glycoside of DNJ (2-*O*-α-D-galactopyranosyl-deoxynojirimycin) and fagomine (which can be considered to be 2-deoxy-DNJ) have both recently been shown to have potent anti-hyperglycemic effects in isolated perfused pancreases from streptozotocin-induced diabetic mice (Kimura et al., 1995; Nojima et al., 1998). Taniguchi et al. (1998) reported that fagomine has the ability to potentiate glucose-induced insulin secretion from isolated rat pancreatic islets. Although the exact mechanism of action of this compound has not yet been elucidated, it would appear that fagomine does not affect the enzymes in the citric acid cycle, but rather it accelerates the steps in the glycolytic pathway that occur after the formation of glyceraldehyde 3-phosphate (Taniguchi et al., 1998).

Castanospermine can be regarded as a bicyclic derivative of DNJ, with an ethylene bridge between the hydroxymethyl group and the ring nitrogen. However, owing to its toxicity, this compound was considered unsuitable for therapeutic use in diabetes mellitus. Unsuccessful attempts were made to synthesise derivatives of castanospermine that were better tolerated (Junge et al., 1996).

5.4. Anti-viral agents

Another therapeutic application of polyhydroxylated alkaloids is as anti-viral agents. Inhibitors of processing α-glucosidases, such as castanospermine and DNJ, have been shown to decrease the infectivity of human immunodeficiency virus (HIV) in vitro at concentrations which are not cytotoxic to lymphocytes, whereas specific inhibitors of processing α-mannosidases (swainsonine and 1-deoxymannojirimycin) have no effect on HIV (Tyms et al., 1987; Fleet et al., 1988; Taylor et al., 1991). Castanospermine and DNJ also reduce the infectivity of other retroviruses such as the feline equivalent of HIV (Stephens et al., 1991) and human cytomegalovirus (CMV) which is an opportunistic pathogen in AIDS which is caused by HIV infection (Taylor et al., 1988).

HIV primarily infects cells of the immune system. Essential to infection is the interaction between the heavily glycosylated viral envelope glycoproteins gp120 and gp41 with the CD4 receptor which is a membrane glycoprotein found on the surface of T-lymphocytes and other cells of the immune system. In the presence of castanospermine and DNJ the glycosylation patterns of the viral coat glycoproteins are altered. Although this does not prevent formation of viral particles, they no longer have the ability to interact correctly with the CD4 receptor and so they are non-infectious (Taylor et al., 1991; Fischer et al., 1995).

Trials were conducted to try to define the structural features necessary in polyhydroxylated alkaloids for antiviral activity (Fleet et al., 1988; Karpas et al., 1988). It was discovered that *N*-substituted derivatives of DNJ were more potent in vivo than the natural product. In particular *N*-butyl-DNJ (SC-48334) had enhanced anti-HIV activity (possibly because the aliphatic chain increased uptake by the cells) and this compound was developed as a drug candidate and was evaluated in phase II clinical trials. In combination with dideoxynucleoside derivatives which target the viral reverse transcriptase activity (e.g. AZT), *N*-butyl-DNJ caused diarrhoea, abdominal pain and weight loss in human patients after oral administration (Fischl et al., 1994). Chemical modifications of *N*-butyl-DNJ were undertaken to try to eliminate the gastrointestinal toxicity. A perbutyrylated ester (SC-49483) was reported by Jacob (1995) to be undergoing a phase II clinical trial. This is effectively a pro-drug as it was shown to be absorbed from the gut and metabolised in the gastrointestinal mucosa to release the active agent *N*-butyl-DNJ into the plasma. Although the anti-viral efficacy of this compound in humans has not been reported, it apparently did not cause diarrhoea in Rhesus monkeys (Jacob, 1995).

The mechanism of action of *N*-butyl-DNJ at the molecular level appears to involve the inhibition of the host cell enzymes α-glucosidases I and II. This arrests *N*-glycan processing such that immature glucosylated *N*-glycans are present on the HIV envelope glycoproteins gp120 and gp41. Conformational changes occur in the V1 and V2 loops of gp120 when it folds in the presence of these glucosylated *N*-glycans. As a consequence, the virus can still bind to its cellular receptor CD4 but gp120 is unable to undergo the post-CD4-binding conformational change required to expose the fusion peptide of gp41 that normally facilitates entry of the virus into the host cell (Fischer et al., 1995, 1996a,b). However, because glucosidases I and II are located in the lumen of the endoplasmic reticulum, it was found that very high extracellular concentrations of *N*-butyl-DNJ were required to achieve enzyme inhibition. Karlsson et al. (1993) reported that although *N*-butyl-DNJ had a K_i of 0.2 μM against purified glucosidase I in vitro, extracellular concentrations of 0.5 mM were required to achieve inhibition of glucosidase I in intact cells in tissue

culture. When *N*-butyl-DNJ was evaluated in HIV patients, the serum levels achieved were only in the range of 10–50 μM which could explain the lower than anticipated antiviral efficacy of the drug that was observed during the clinical trials (Fischl et al., 1994; Platt and Butters, 1998). Recently, the pharmacokinetic behaviour of a more lipophilic derivative of DNJ (*N*-benzyl-DNJ) has been studied in rats (Faber et al., 1998). The plasma half life and tissue residency of this derivative were reported to be greater than those of the *N*-alkyl derivatives.

Similarly, synthetic modification of castanospermine showed that the lipophilic 6-*O*-acyl derivatives were more potent inhibitors of HIV than the natural product (Sunkara et al., 1989; Anderson et al., 1990). In particular, the 6-*O*-butanoyl derivative (MDL 28,574) was approximately twenty times more active than castanospermine and 50 times more active than *N*-butyl-DNJ (Taylor et al., 1991). However, once inside cells, 6-*O*-butanoylcastanospermine appeared to be hydrolysed to release castanospermine and hence this compound can also be considered to be a pro-drug (Bridges et al., 1995; Kang, 1996). Recently, this compound was reported to be tolerated well by patients during a phase II clinical trial (Richmond et al., 1996). Studies in vitro have shown synergistic activity against HIV type 1 and 2 replication when castanospermine (and its 6-*O*-butanoyl derivative) are combined with AZT and other similar dideoxynucleoside drugs (Johnson et al., 1989; Taylor et al., 1995).

Nectrisine (an inhibitor of both α-glucosidases and α-mannosidases) has also been reported to have anti-viral activity. It has been shown to inhibit the retrovirus Friend leukaemia virus in vivo in mice and it too can potentiate the activity of AZT (Tatatsuki et al., 1990).

5.5. Treatment of glycosphingolipid lysosomal storage diseases

The glycosphingolipid (GLS) lysosomal storage diseases result from mutations in the genes that encode the enzymes required for GLS catabolism within lysosomes. They are a relatively rare group of human disorders that can give rise to progressive neurodegeneration due to lysosomal storage within cells of the central nervous system. In the most severe forms of these diseases, death occurs in early infancy (Platt and Butters, 1998). An enzyme replacement therapy is currently available for type 1 Gaucher disease (caused by a defect in β-glucocerebrosidase) (Cox, 1994), but there are diseases associated with virtually every enzyme in the glycosphingolipid degradation pathway. However, a relatively new approach to the generic treatment of this family of disorders relies on the fact that the mutations giving rise to the enzyme defects generally do not totally eliminate the catalytic activity of the enzymes. Therefore, drugs could be used to regulate the rate of biosynthesis of the glycosphingolipids so that the amount of substrate the defective enzyme has to catabolise is reduced to a level that matches the residual enzyme activity, thus balancing synthesis with degradation and preventing storage. This type of treatment is termed "substrate deprivation".

The first step in GLS biosynthesis is the transfer of glucose from UDP-glucose to ceramide which is catalysed by the enzyme UDP-glucose: *N*-acetylsphingosine D-glucosyltransferase (EC 2.4.1.80). In 1994, Platt and co-workers discovered that this enzyme is inhibited by the α-glucosidase inhibitor *N*-butyl-DNJ (K_i 7.4μM Platt et al., 1994a). Subsequently, a galactose analogue of *N*-butyl-DNJ (*N*-butyl-deoxy-galactonojirimycin or *N*-butyl-DGJ) was found to be a more selective inhibitor of this process in vitro (Platt et al., 1994b). These compounds act as competitive inhibitors for ceramide in the glucosyltransferase assay and as non-competitive inhibitors for UDP-glucose, indicating that inhibitory activity is by ceramide mimicry. Therefore, the potential GSL lysosomal storage diseases that could be targets for treatment using *N*-butyl-DNJ and *N*-butyl-DGJ are Gaucher, Fabry, Tay-Sachs, Sandhoff, ceramide lactoside lipidosis and fucosidosis (Platt and Butters, 1998). In 1997, Platt et al. reported successfully preventing GSL storage in a mouse model of Tay-Sachs disease using *N*-butyl-DNJ.

When considering the potential clinical application of *N*-butyl-DNJ for the treatment of GLS lysosomal storage diseases, it is worth considering the findings of the clinical trials of this compound as an anti-HIV agent. In Section 5.4, it was reported that the serum levels of the compound attained after oral dosing were insufficient for inhibition of glucosidase I because the drug could not reach its target due to the location of the catalytic site of the enzyme within the lumen of the endoplasmic reticulum. However, the glucosyltransferase inhibited by *N*-butyl-DNJ is known to have its catalytic site orientated towards the cytosolic face of an early Golgi compartment which would make this enzyme target more accessible to the drug (Platt and Butters, 1995). Also, Platt and Butters (1998) considered that the serum levels reported for *N*-butyl-DNJ from the clinical trials would be sufficient to inhibit GLS biosynthesis. Since *N*-butyl-DNJ was reasonably well tolerated during the HIV clinical trials, the clinical evaluation of this compound for the treatment of GLS lysosomal storage diseases is planned (Platt and Butters, 1998).

An alternative approach involving the treatment of Fabry disease with 1-deoxy-galactonojirimycin (DGJ) itself has recently been proposed by Fan et al. (1999). Fabry disease is characterised by a deficiency in lysosomal α-galactosidase A. In some forms of the disease, this deficiency arises from a genetic mutation that affects the folding of the enzyme, although the catalytic site is unaffected. Incomplete folding is recognised by

the "quality control" system that operates in the endoplasmic reticulum resulting in abortive maturation of the mutant enzyme (Ishii et al., 1996). DGJ is a potent competitive inhibitor of α-galactosidase A. However, when lymphoblasts from Fabry patients were exposed to this compound at concentrations below those normally required for intracellular inhibition of α-galactosidase A, DGJ seemed to accelerate maturation of the mutant enzyme which was successfully transported from the endoplasmic reticulum to the Golgi apparatus and then correctly targeted to the lysosome, resulting in an elevated expression of enzyme activity. This phenomenon was also seen after oral administration of DGJ to transgenic mice that over-expressed the same mutant form of α-galactosidase A. Fan and co-workers (1999) proposed that this may be caused by DGJ occupying the catalytic site of the defective enzyme, thereby stabilising its conformation which might, in turn, control the flexibility of folding of the protein, thus allowing resumption of the glycoprotein processing and leading to maturation of the enzyme. Therefore, DGJ acts as a "chemical chaperon" to force the mutant enzyme to assume the correct conformation in order to complete its biosynthesis. Fan et al. (1999) also suggested that the binding of DGJ to α-galactosidase A could be pH-dependent such that the formation of the enzyme-inhibitor complex is favoured by the near neutral pH of the endoplasmic reticulum and dissociation occurs in the acidic environment of the lysosome. Fan and co-workers concluded that it may be possible to use other competitive inhibitors as "chemical chaperons" for the defective enzymes involved in other types of GSL lysosomal storage disorders.

5.6. Treatment of infectious agents and associated complications

One promising new area of research is the potential use of synthetic derivatives of polyhydroxylated alkaloids to specifically target the enzymes involved in the biosynthesis of the cell walls of human-pathogenic micro-organisms. For example, recent studies on the structure of mycobacterial cell walls have identified a disaccharide linker between the arabinogalactan polysaccharide and the peptidoglycan that contains L-rhamnopyranose (Besra et al., 1995). This is a common feature amongst the Actinomycetes (Lee et al., 1996). Rhamnose has no role in mammalian metabolism so compounds which interfere specifically with rhamnose metabolism should not have any deleterious effects on the host animals and this could provide a new approach to the treatment of diseases induced by mycobacteria, such as tuberculosis and leprosy, as well as diseases caused by other pathogens which also utilise rhamnose as part of their cell wall structure. The L-rhamnopyranose unit is introduced into the cell wall via the sugar nucleotide deoxythymidine diphosphorhamnose (dTDP-L-rhamnose) and it is possible that a chemotherapeutic approach to the treatment of these diseases could be to find compounds which inhibit either the microbial biosynthesis of dTDP-L-rhamnose from D-glucose-1-phosphate (a multi-enzyme conversion) (Tsukioka et al., 1997) or its subsequent incorporation into the cell wall via rhamnosyl transferases. It is reasonable to suggest that the types of structures which might inhibit these biochemical steps should contain a rhamnose fragment or a rhamnose analogue. Recently, a wide range of synthetic iminosugar derivatives have been evaluated in vitro against the L-rhamnosidase naringinase from *Penicillium decumbens* (Davis et al., 1999; Shilvock et al., 1999) and some have also been shown to inhibit the conversion of dTDP-D-glucose to dTDP-L-rhamnose in cell-free extracts of *Mycobacterium smegmatis* (Shilvock et al., 1999). Initial findings indicate the most promising candidate compounds are piperidine analogues of L-rhamnopyranose bearing amide substituents.

A similar line of investigation involves alkaloidal mimics of galactofuranose. The arabinogalactan polysaccharide in the cell walls of Actinomycetes is composed of the furanose forms of both arabinose and galactose (Lee et al., 1996). Uridine diphosphogalactofuranose (UDP-Gal*f*) is formed by the contraction of UDP-galactopyranose in a reaction catalysed by UDP-galactosyl mutase and the UDP-Gal*f* is then incorporated into the cell wall via a galactosyltransferase. Since galactofuranose has no role in mammalian metabolism, specific inhibition of either UDP-Gal mutase or the UDP-Gal*f* transferases by galactofuranose mimics may well be achieved without any harm to the mammalian hosts. In vitro studies have recently identified pyrrolidine analogues of galactofuranose that inhibit the biosynthesis of mycobacterial cell walls, probably through their action on the UDP-galactosyl mutase (Lee et al., 1997).

Gram-negative bacteria are characterised by a second membrane external to the peptidoglycan matrix. A major constituent of this membrane is lipopolysaccharide. Although lipopolysaccharides differ between bacterial species, they follow the same general format. The "anchor" is a large structure called lipid A, a polyacylated β-1,6 disaccharide of glucosamine 1,4′-bisphosphate. This is elaborated with a "core" polysaccharide and finally capped with a repeating structure called the *O*-antigen, a highly variable and species-specific saccharide (Sears and Wong, 1999). Lipopolysaccharides from many bacterial species are highly toxic to humans. They are commonly known as "endotoxins" and are responsible for septic shock. Unfortunately, treatment of pathogenic Gram-negative bacteria with antibiotics can cause the release of endotoxin from the lysed cells which can produce more acute and lethal symptoms than the original infection. A new therapeutic approach to endotoxemia is based on the generation of catalytic

antibodies capable of hydrolysing the inter-glycosidic bond in lipid A. By targeting this portion of the lipopolysaccharide it is hoped that a generic treatment can be developed for Gram-negative bacteria. Van den Berg et al. (1999) reported successfully raising polyclonal antibodies in mice that act in a manner analagous to β-*N*-acetylglucosaminidases using an immunoconjugate based on a derivative of 2-acylamido-deoxynojirimycin. This compound is a modification of the potent β-*N*-acetylglucosaminidase inhibitor 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol first synthesised by Fleet et al. (1986) with the addition of an acyl chain to give a structure that more closely mimics the native lipid A. Evaluation of the efficacy of derived monoclonal antibodies is apparently on-going (van den Berg et al., 1999).

Synthetic esters of castanospermine have been shown to be useful adjuncts in the treatment of cerebral malaria (Bitonti et al., 1993). Red blood cells which are infected with the parasite *Plasmodium falciparum* become "sticky" and less deformable. This results in a build-up of cells on the vascular wall and creates occlusions to blood flow which are thought to cause the cerebral, renal and liver complications of *P. falciparum* infections. Bitonti et al. (1993) claimed that intravenous administration of various castanospermine esters could potentially reduce the adhesion of infected erythrocytes to the vascular endothelial wall.

5.7. Other therapeutic applications

Castanospermine has been investigated as a potential therapeutic agent in the treatment of autoimmune diseases such as multiple sclerosis and arthritis. Willenborg et al. (1992) found that castanospermine not only inhibited the development of arthritis in mice but it also inhibited the progression of the disease when treatment was commenced after the onset of symptoms. It prevented the passage of leucocytes through vascular subendothelial basement membranes to sites of inflammation by interfering with the biosynthesis of *N*-linked oligosaccharide hydrolases and their subsequent targeting to lysosomes. These enzymes are expressed at the surface of leucocytes and their activity is necessary for cell migration (Bartlett et al., 1995).

Castanospermine may also have a role, one day, in transplant surgery as it can inhibit the rejection of transplanted tissues. Kidney, pancreas and heart allograft survival have all been shown to be prolonged in a dose-dependent manner in the presence of castanospermine (Grochowicz et al., 1992, 1993; Hibberd et al., 1995). The compound appears to modify the expression of adhesion molecules and other cell surface glycoprotein receptors that may be involved in the alloreactive response in allograft recipients. It downregulates the expression of genes encoding the glycoproteins CD54 and CD11a, both of which are considered to have a unique and vital role in the rejection process. This downregulation may act to modulate the rejection process by influencing the interaction between endothelial cells and graft-infiltrating cells, reducing cell migration into the allograft (Grochowicz et al., 1997; Hibberd et al., 1997).

6. Concluding remarks

There is no doubt that there are many more polyhydroxylated alkaloids waiting to be discovered, including glycosides and other derivatives of those known already. Some of these, or semi-synthetic derivatives, are likely also to be inhibitors of endoglycosidases or glycosyltransferases, which opens up a wider range of potential specific applications. When more of the natural compounds become commercially available for screening there is sure to be an even wider range of potentially valuable activities found than shown to date with castanospermine, swainsonine and deoxynojirimycin.

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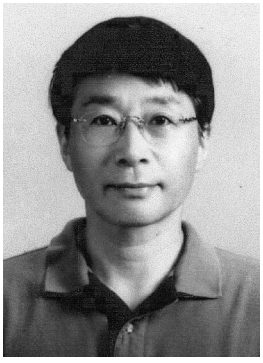
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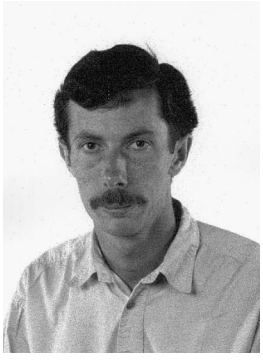
George W.J. Fleet. Having received his PhD at Cambridge University in 1969, Professor George Fleet conducted post-doctoral work with Professor Rodney Porter and Dr. Jeremy Knowles at Oxford on azide affinity labelling, and with Professor E.J. Corey at University of Harvard on prostaglandin synthesis. He is currently working at the Dyson Perrins Laboratory in Oxford. His major interests are in synthesis of carbohydrate mimetics and amino acids derived from carbohydrates likely to generate secondary structure in short sequences. He was awarded Pfizer prizes for organic synthesis in 1984 and 1985. In 1995 he received the RSC Bader Award for Organic Synthesis and was RSC Haworth Lecturer in 1999. Author of over 320 papers and published US patents.



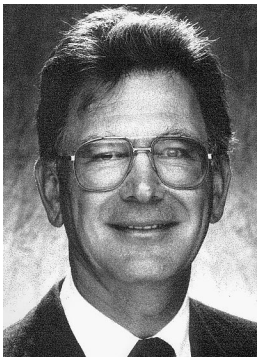
Alison A. Watson. Alison graduated in Biochemistry from King's College, London University in 1987. Her PhD research on optimising swainsonine production from the fungus *Metarhizium anisopliae* was conducted jointly at the Royal Botanic Gardens, Kew, Birkbeck College, London University, and Imperial Biotechnology Ltd at Imperial College. Post-doctoral positions at Kew Gardens involved research on various agrochemical applications of plant compounds. In 1993 she moved to the Chemistry Group at the Institute of Grassland and Environmental Research near Aberystwyth working on a variety of projects including work funded by MAFF on calystegine alkaloids in food plants and developing assays for screening compounds affecting mycobacterial cell wall biosynthesis with the Dyson Perrins Laboratory at Oxford University. Since 1999 she has been Senior Biochemist with the natural products company MolecularNature Limited. Alison has published 34 scientific papers and book chapters.



Naoki Asano. Dr. Asano received his BSc at the Science University of Tokyo in 1974. His PhD at Shizuoka College of Pharmacy led on to post-doctoral research at the Institute for Biomedical Research at the University of Texas at Austin (1986–1987). Dr. Asano is currently Associate Professor at the Faculty of Pharmaceutical Science at Hokuriku University, Japan. His research interests include searching for new structural classes of glycosidase inhibitors and discovering their therapeutic applications. Dr. Asano is author of over 80 scientific publications, book chapters and patents.



Robert J. Nash. Robert Nash graduated in Botany/Zoology (1980) at the University of Reading where Professor Jeffrey Harborne sparked his interest in the activities of natural products. A PhD at King's College London with Professor Arthur Bell on the purification and biological activity of castanospermine and related compounds led to post-doctoral research at the Royal Botanic Gardens Kew with Dr. Linda Fellows on an MRC-funded search for new anti-viral agents from plants. After 11 years at Kew, he joined the Institute of Grassland and Environmental Research (IGER) near Aberystwyth in 1993 as head of the Chemistry Group. In 1999, he became Research Director of the natural products company MolecularNature Limited based at IGER and was appointed as visiting professor of the Department of Pharmaceutical Sciences at the University of Strathclyde in January 2000. He has over 90 scientific publications, patents and book chapters.



Russell J. Molyneux. Russell Molyneux received his BSc (Chemistry) in 1960 and his PhD (Organic Chemistry) in 1963 from the University of Nottingham. Following post-doctoral and faculty appointments at the University of Oregon and Oregon State University, respectively, he joined the Western Regional Research Center (Agricultural Research Service — US Department of Agriculture) in Albany, CA as a Research Chemist, where he is currently a Project Leader. In 1993, he was awarded a Sir Frederick McMaster Fellowship (CSIRO, Australia) and in 1998 was the recipient of the Award for Advancement of Agricultural and Food Chemistry (American

Chemical Society). He was appointed as an Associate Editor of the *Journal of Agricultural and Food Chemistry* in 1999. His research interests have focused on the isolation and identification of natural products belonging to many structural classes, with particular emphasis on natural toxins. His studies of alkaloid constituents of native plants responsible for poisoning of livestock grazing rangelands in the western US have been of particular significance and have been extended to similar problems in many other parts of the world. His research on the locoweed toxin, swainsonine, led to an expansion of interest in structurally related polyhydroxy indolizidine, pyrrolizidine and tropane alkaloids from the genera *Astragalus, Oxytropis, Castanospermum* and *Ipomoea*. At present his research programme deals primarily with mycotoxins contaminating food crops and fungal metabolites responsible for phytotoxicity to grapevines in California. He is the co-editor of two books and has published 196 scientific papers, patents and book chapters.

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Review

Recent advances in phytochemistry of bryophytes-acetogenins, terpenoids and bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts

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Dedicated to Professor Jeffrey B. Harborne on the occasion of his retirement as Editor-in-Chief of *Phytochemistry*

Abstract

Bryophytes contain a large number of terpenoids and phenolic compounds. Recent topics relating to the chemical constituents found in 36 Japanese, 3 New Zealand, 2 European, 1 Argentinean and 1 Taiwanese liverworts and 2 Japanese mosses and their biological activity are discussed. The chemosystematics of some liverworts as well as the chemical relationship between liverworts and mosses, and bryophytes and ferns are also discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bryophytes; Liverworts; Mosses; Terpenoids; Bibenzyls; Bis(bibenzyl)s; Biological activity; Chemosystematics

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1. Introduction

The bryophytes are taxonomically placed between the algae and the pteridophytes and more than 20,000 species are known in the world. They are divided morphologically into three classes, Hepaticae (liverworts 6000 species), Musci (mosses 14,000 species) and Anthocerotae (hornworts 300 species). The phytochemistry of bryophytes has been neglected for a long time because they are morphologically very small and difficult to collect in large amounts as pure samples, their identification is difficult and they are considered to be nutritionally useless to humans. However, the bryophytes have been used as medicinal plants to cure cuts, burns, external wounds, bacteriosis, pulmonary tuberculosis, neurasthenia, fractures, convulsions, scalds, uropathy, pneumonia, neurasthenia etc. It is estimated that 80% of the bryophytes contain a bitter principle, some species present a surprisingly pungent taste and others can induce allergenic contact dermatitis. Almost all species of bryophytes are not damaged by insect larvae, fungi, bacteria, slugs, snails and mammals.

We have focused on the bioactive constituents, their enantioselective total synthesis and chemosystematics of the bryophytes as well as the evolutionary relationships between terrestrial spore-forming green plants and algae using their characteristic chemical indicators (Asakawa, 1982, 1994, 1995, 1997). Among the bryophyte species, the chemical constituents of liverworts have been investigated in most detail because liverworts contain cellular oil bodies, which are composed of lipophilic terpenoids and aromatic compounds, while the other two classes does not contain such oil bodies. At present more than 700 terpenoids and 220 aromatic compounds (excluding flavonoids) have been isolated from or detected in the Hepaticae and their structures elucidated (Asakawa, 1982, 1995). A number of the isolated compounds possess interesting pharmacological activity (Asakawa, 1982, 1988, 1990a, 1993, 1995, 1998, 1999). By contrast, only three monoterpene hydrocarbons have been detected in mosses and only one kaurene-type diterpenoid was isolated from the *Saelania* species of moss (Asakawa, 1995). No sesquiterpenoids have been detected in the Musci although more than 14,000 species are known in nature. Generally the chemical constituents of the Musci, Hepaticae and Anthocerotae differ from each other, and the Hepaticae produce constituents very similar to those found in the Phaeophyceae (brown algae) (Asakawa, 1995). Many reviews have been published concerning the biologically active chemical constituents that occur in bryophytes (Asakawa, 1981, 1982, 1995, 1997; Zinsmeister and Mues, 1990; Zinsmeister et al., 1991; Keseru and Nogradi, 1995).

In this mini-symposium, the new terpenoids and cyclic bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts and mosses (Table 1) that have been isolated during the last 4 years in our laboratory, together with their biological activity, are summarized. The chemosystematics and chemical interrelationship between liverworts and mosses, and bryophytes and ferns is also discussed.

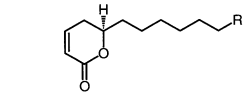
Table 1
Chemically analyzed liverworts and mosses

Liverworts	Mosses
Japanese	
Blasia pusilla	*Floribundaria aurea*
Cavicularia densa	*subsp. nipponica*
Cheilolejeunea imbricata	*Plagiomnium acutum*
Chiloscyphus polyanthos	
Conocephalum conicum	
Dumortiera hirsuta	
Frullania densiloba	
Frullania hamatiloba	
Frullania tamarisci	
subsp. *obscura*	
Herbertus sakuraii	
Jackiella javanica	
Jungermannia exsertifolia	
subsp. *cordifolia*	
Jungermannia hattoriana	
Jungermannia infusca	
Jungermannia rotundata	
Lejeunea aquatica	
Lejeunea flava	
Lejeunea japonica	
Lepidozia vitrea	
Leptolejeunea elliptica	
Marchantia paleacea	
var. *diptera*	
Marchantia polymorpha	
Mastigophora diclados	
Odontoschisma denudatum	
Pallavicinia subciliata	
Pellia endiviifolia	
Plagiochila fruticosa	
Plagiochila ovalifolia	
Porella acutifolia subsp. *tosana*	
Porella japonica	
Porella perrottetiana	
Ptychantus striatus	
Radula perrottetii	
Reboulia hemisphaerica	
Riccardia multifida subsp. *decrescens*	
Scapania undulata	
Taiwanese	
Mylia nuda	
New Zealand	
Archilejeunea olivacea	
Jamesoniella tasmanica	
Plagiochila conjugatus	
Argentinean	
Dumortiera hirsuta	
European	
Barbilophozia barbata	
Barbilophozia hatcheri	

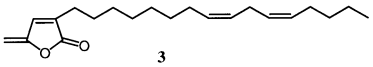
2. Chemical constituents of bryophytes and their biological activity

2.1. Acetogenins

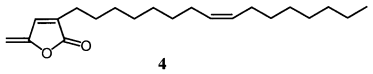
There are ca. 3000 Lejeuneaceae species. Most of them produce many different skeletal sesqui- and diterpenoids (Asakawa, 1982, 1995, 1997). *Cheilolejeunea imbricata* emits a strong milky fragrance resulting from a mixture of (*R*)-dodec-2-en-1, 5-olide (**1**) and (*R*)-tetradec-2-en-5,6-olide (**2**) (Toyota et al., 1997d). This species is chemically very distinct from the other *Lejeunea* species since neither terpenoid nor aromatic compounds have been detected except the above two lactones.



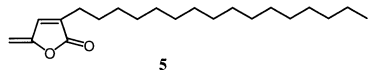
1 R=Me
2 R=$(CH_2)_2Me$



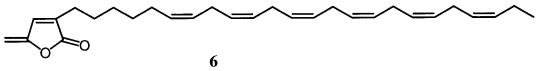
3



4



5



6

Two unstable long-chain lipids possessing a γ-lactone ring were isolated from the methanol extract of the large thalloid liverwort, *Marchantia pleacea* var. *diptera* and shown to be 2-(8′*Z*,11′*Z*-hexadecadienyl)-penta-2,4-dien-4-olide (**3**) and 2-(8′*Z*-hexadecenyl)-penta-2,4-dien-4-olide (**4**), together with (+)-7,13-labdadien-15-ol (Toyota et al., 1997b). The isolation of compounds **3** and **4** from a liverwort for the first time is of phylogenic interest, although the closely related compounds **5** and **6** have already been found in marine gorgonians (Ravi and Wells, 1982; Kikuchi et al., 1983).

2.2. Monoterpenoids

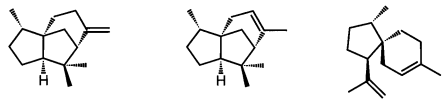
Many liverworts emit a characteristic smell when crushed. Such a fragrance is generally due to monoterpenoids.

Cyclocitral was isolated from *Jungermannia hattoriana* (Nagashima et al., 1997c). This was the first example of the isolation of this aldehyde from a liverwort, although a number of monoterpenoids have been detected not only in leafy-stem but also in thalloid liverworts.

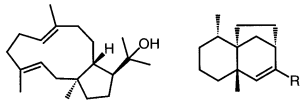
A total of 280 samples of *Conocephalum conicum* a large thalloid liverwort, were analyzed by GC–MS to show the presence of three chemo-types; (−)-sabinene (type I), (+)-bornyl acetate (type II) and methyl cinnamate (type III) (Toyota et al., 1997e). The type III is not only chemically different from the type I and II, but also morphologically different. We proposed that Japanese *C. conicum* should be separated into at least two species (Toyota et al., 1997e).

2.3. Sesquiterpenoids

The Mniaceae mosses show hemostatic activity and induce allergenic contact dermatitis (Asakawa, 1990a,b). In order to isolate the biologically active substances, the Japanese *Plagiomnium acutum* has been analyzed chemically. Surprisingly, this moss biosynthesizes *ent*-sesquiterpene hydrocarbons, *ent*-β-cedrene (**7**) together with α-cedrene (**8**), α-acoradiene (**9**) and (+)-dolabella-3,7-dien-18-ol (**10**) (Toyota, Kimura and Asakawa, 1998a). This is the first isolation of a sesquiterpenoid from mosses. These are previously known compounds except for *ent*-β-cedrene (**7**). However, these results provide important insight into morphological and taxonomical aspects of the Musci. The Hepaticae contain sesquiterpenoids, 80% of which are the enantiomers of those found in higher plants. These terpenoids and their absolute configurations are very valuable markers for the classification of the Hepaticae. *Plagiomnium acutum* is chemically very similar to some liverworts because it produces *ent*-sesquiterpenes and dolabellane diterpenoids, although the two plant groups are morphologically quite different. This evidence shows that some mosses may be evolutionarily related to the Hepaticae.



7 **8** **9**



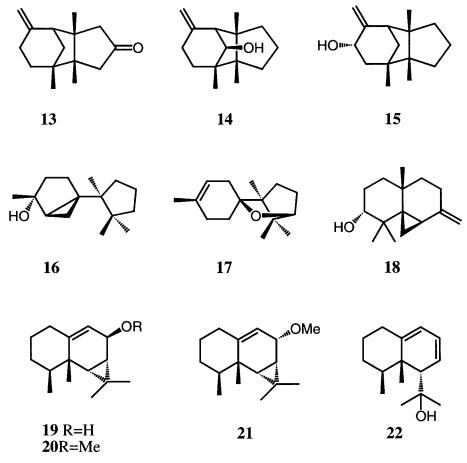
10

11 R=COOH
12 R=CHO

A new tricyclic sesquiterpenoid, nudenoic acid (**11**) has been isolated from *Mylia nuda*, the endemic species in Taiwan and its structure elucidated by X-ray crystallographic analysis. This species also contains the unstable nudenal (**12**) (Liu et al., 1996).

Reboulia hemisphaerica which is widely distributed in Asia and Europe is a rich source of different sesquiterpenoids and bis(bibenzyl)s. It produces a new gymnomitrane-type sesquiterpene ketone (**13**), gymnomitrol

(**14**), (+)-gymnomitr-8(12)-en-9α-ol (**15**), (−)-cyclopropanecuparenol (**16**), 6β,10β-epoxycupar-3-ene (**17**), 3(15)-thujopsen-10α-ol (**18**), *ent*-arist-9-en-8α-ol (**19**), 8β- and 8α-methoxyaristol-9-ene (**20** and **21**), together with the known rebouliadienol (**22**), 1(10),8-aristoladiene (**23**) and three bis(bibenzyls) which are liverwort constituents (Toyota et al. 1999a). *Reboulia hemisphaerica* collected at four different locations all contained at least at ca. 20% of **9** as judged by TLC analysis of their crude ether extract. It was observed that **23** was obtained from **19**, and **20** and **21** from **19** during chromatographic procedures using MeOH-CH_2Cl_2 as solvent (Toyota et al. 1999a). The aristolane-type sesquiterpenoid is one of the most important chemical markers of *Reboulia* species since it is rare in the Hepaticae. Recently, compounds **22** and **23**, whose absolute configurations were the same as those found in the compounds from *R. hemispaerica,* have been isolated from the mushroom *Russula leppida* (Vidari et al., 1998). Rebouliadienol (**22**) might also be biosynthesized from **19** as shown in Fig. 1.



The liverworts, *Chilochyphus polyanthos* and *Lepidozia vitrea* are rich sources of eudesmane-type sesquiterpenoids. The former species produces eudesm-3-en-7α-ol (**24**), together with two similar eudesmane-type sesquiterpene alcohols (**26** and **27**) while the latter species produces *ent*-eudesm-3-en-7β-ol (**24′**), the enatiomer of **24**. Interestingly, however, both liverworts biosynthesize eudesm-4(15)-en-7α-ol (**25**) and its enatiomer (**25′**). Two eudesmanes, eudesm-6β,7α-diol (**28**) and its enantiomer (**28′**) were also isolated from *L. vitrea* (Fig. 2) (Toyota et al., 1999b).

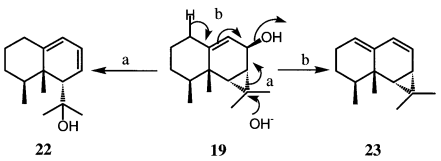


Fig. 1. Formation of rebouliadienol (**22**) and 1(10),8-aristoladiene (**23**) from *ent*-arist-9-en-8α-ol (**19**).

The family of Frullaniaceae comprises more than 500 species several of which cause strong allergenic contact dermatitis (Asakawa, 1982, 1995) and divided into six chemo-types; a sesquiterpene lactone-bibenzyl-type (type I), a sesquiterpene lactone-type (type II), a bibenzyl type (type III), a monoterpene-type (type IV), cyclocolorenone-type (type V) (Asakawa, 1995) and labdane-sesquiterpene lactone type (type VI) (Toyota et al., 1988). *Frullania tamarisci* produces not only monomer eudesmanolides but also a new eudesmanal (**30**) and its dimer (**29**) with 4-epi-arbusculin A (**31**) at the C-4 ether linkage. The dimer might be formed by the pathway shown in Fig. 3. There are two chemical races in this species, one of which contained the dimer (**29**) (14.2% of the total extract) and the other that does not show any evidence for the presence of the dimer (Toyota et al., 1998c). *Frullania densiloba* produces two new eudesmanolides, densilobalides A (**32**) and B (**33**), together with α-cyclodihydrocostunolide (**34**). These lactones are from the *ent*-eudesmane series (Nagashima et al., 1997d). *F. tamarisci* subsp. *obscura* and *F. densiloba* are classified into chemo-type I since they produce sesquiterpene lactones as the major components.

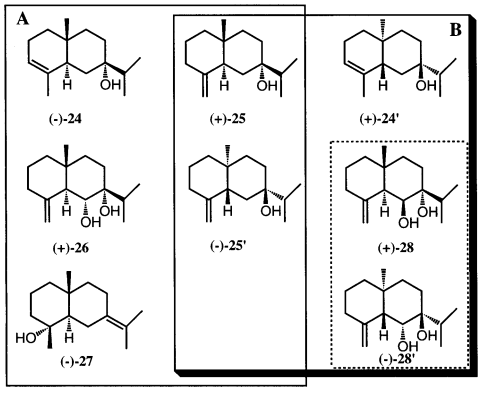


Fig. 2. Eudesmane-type sesquiterpenoids isolated from the liverworts, *Chiloscyphus polyanthos* (**A**) and *Lepidozia vitrea* (**B**).

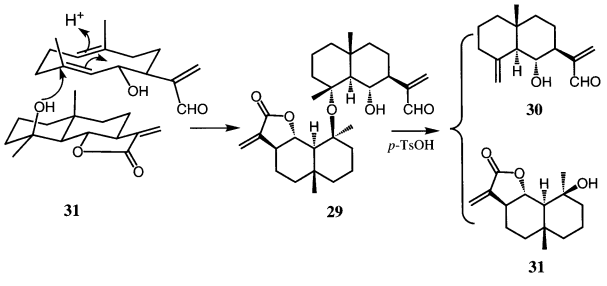
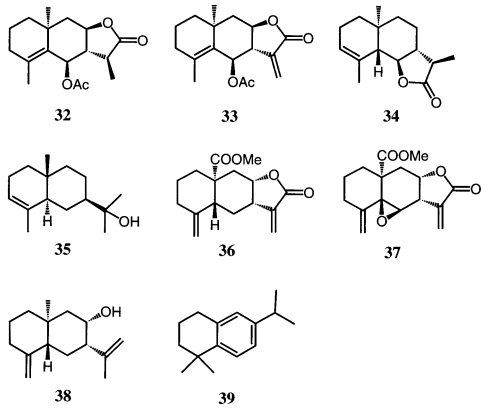


Fig. 3. Possible biosynthetic pathway of dimeric eudesmanolide (**29**).



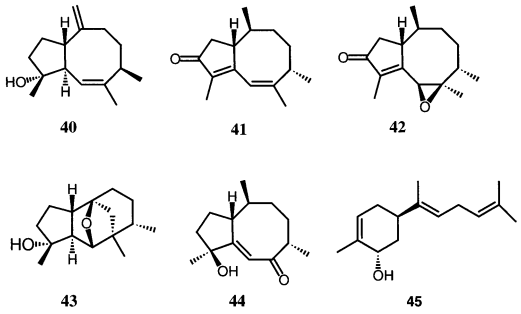
32 33 34 35 36 37 38 39

The specific optical rotation of α-eudesmol (**35**) has been reported to have positive sign between 28 and 30°, respectively. However, a careful chemical examination and redetermination of the absolute configuration of α-eudesmol isolated from the liverwort *Porella perrottetiana* has indicated that the sign and value of the specific rotation is in fact negative and 6.9–8°, respectively (Toyota et al., 1999c).

There are at least 3000 species of the Plagiochilaceae. Of these ca. 60 species have been investigated chemically and classified into eight chemo-types as follows: 2,3-secoaromadendrane (type I), bibenzyl (type II), cuparene-herbertane (type III), bibenzyl-herbertane (type IV), gymnomitrane-bicyclogermcrane (type V), bicyclogermacrane-spathulenol (type VI), pinguisane (type VII) and sesquiterpene lactone-type (VIII). A New Zealand liverwort, *Plagiochila conjugatus* elaborates two unique eudesmanolides (**36** and **37**), together with *ent*-eudesm-4(15),11-dien-8β-ol (**38**). These compounds are all from the *ent*-eudesmane series (Asakawa et al., 1996). Thus *P. conjugatus* is placed in chemo-type VIII.

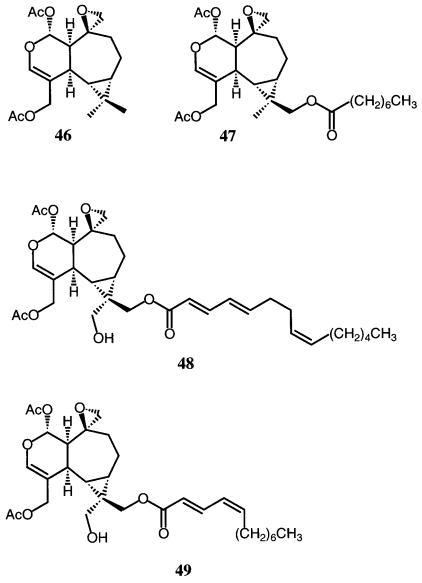
Archilejeunea olivacea which belongs to the subfamily Ptychanthoideae of the Lejeuneaceae and *Leptolejeunea elliptica* to the subfamily Lejeuneoideae are chemically very characteristic since the former species produces olivacene (**39**), a new naturally occurring sesquiterpene hydrocarbon, together with β-monocyclonerolidol and (−)-spathulenol, and the latter species does *p*-ethyl anisol, *p*-ethyl phenol and *p*-ethylphenyl acetate (Toyota et al., 1997c).

Dumortenol (**40**) and the related compounds (**41**) and (**42**), sesquiterpenoids with the novel dumortane skeleton, and rearranged dumortane-type (**43**) and nordumortane-type (**44**) compounds have been isolated from the Argentinean *Dumortiera hirsuta* (Toyota et al., 1997a; Bardon et al., 1999) and their structures elucidated by a combination of 2D NMR data and X-ray crystallographic analyses. However, the Japanese same species does not contain any dumortane-type sesquiterpenoid, but a new bisabolane-type sesquiterpene alcohol, (4*S*),(6*R*)-2,7,10-bisabolatrien-4-ol (**45**) with 13 known compounds (Toyota et al., 1997f). The absolute stereochemistry of **45** has been established by comparing the CD spectral data of 4-keto derivative from **45** with those of *d*- and *l*-carvone. Thus there are at least two chemo-types of *D. hirsuta*.



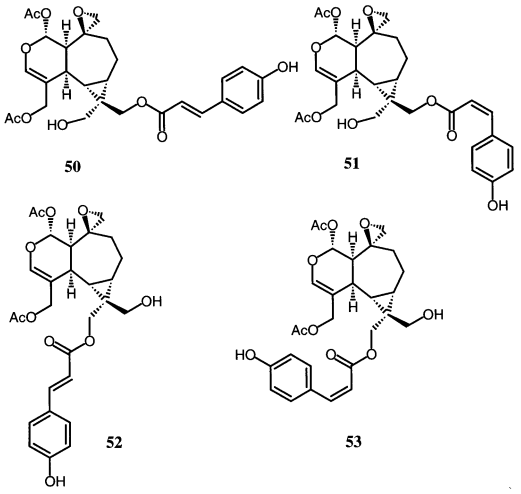
40 41 42 43 44 45

Plagiochila ovalifolia belonging to the chemo-type I of the Plagiochilaceae produces not only plagiochiline A (**46**) but also three new 2,3-secoaromadendrane-type sesquiterpene hemiacetals, plagiochiline esters (**47**–**49**) which show cytotoxic activity (ID_{50} 3.0 μg/ml for **46** and 0.05 μl/ml for **48** and **49**) against P-388 murine leukemia tumor cells (Toyota et al., 1998e). In addition, four new plagiochiline esters (**50**–**53**) have been isolated from the same species (Toyota and Asakawa, unpublished results).

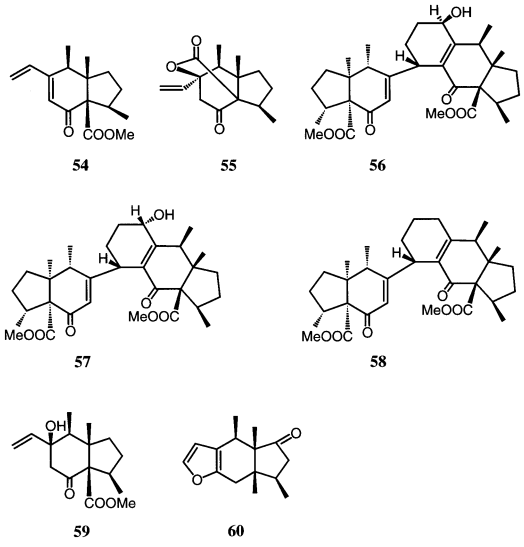


46 47 48 49

The pinguisane-type sesquiterpenoids have been detected in the Hepaticae, especially in the Lejeuneaceae, Trichocoleaceae, Ptilidiaceae, Porellaceae and Riccardiaceae. However, these types of compound have not been found in any other organism. From the ether extract of *Porella acutifolia* subsp. *tosana*, two new pinguisane-type sesquiterpenes, acutifolones A (**54**) and B (**55**) and three novel Diels–Alder reaction type dimeric sesquiterpenoids, bisacutifolones A (**56**), B (**57**) and C (**58**) have been isolated together with the previously known 7-oxopinguisenol-12-methyl ester (**59**) (Hashimoto



et al., 1998a; 1999b). Their absolute stereochemistries were determined by a combination of 2D-NMR and CD spectra, X-ray crystallographic analysis, modified Mosher's method, and chemical degradation.



Pinguisane- and nor-pinguisane-type sesquiterpenoids are biosynthetically quite interesting since their structures are difficult to rationalize in terms of isoprene rule. We proposed the biosynthetic hypothesis of pinguisane-skeleton, which might be derived from *trans*,*cis*-farnesol via bisabolane and acorane (Tori et al., 1993). A biosynthetic study on the formation of pinguisone (**60**) was carried out by feeding [2–^{13}C] acetate to cultured gametophytes of the liverwort *Aneura pinguis* and found that two-methyl migration and C–C bond cleavage of the main chain in farnesyl diphosphate in the formation of pinguisone (route a) as shown in Fig. 4 (Tazaki et al., 1995). According to our proposal, pinguisone must be labeled as **60** as shown in Fig. 4. Thus the route (b) was ruled out.

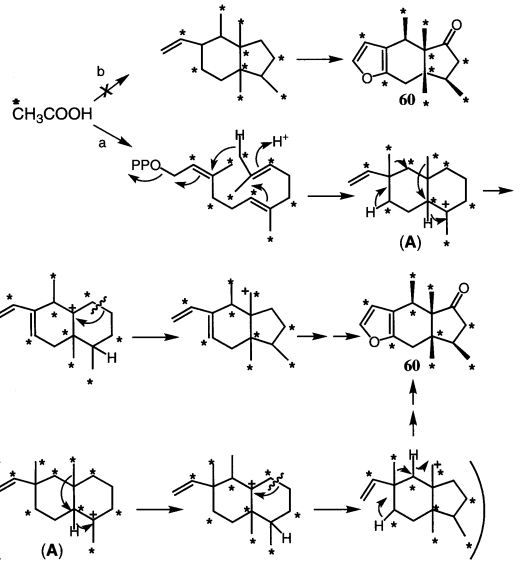
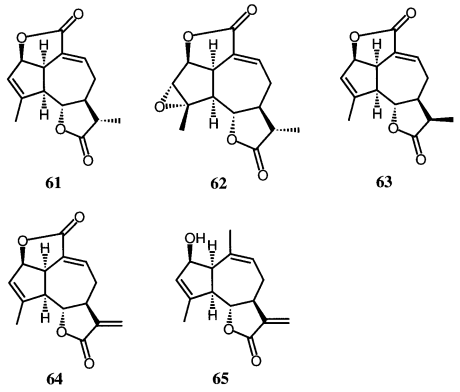


Fig. 4. Incorporation of [2-^{13}C]acetate into pinguisone (**60**).

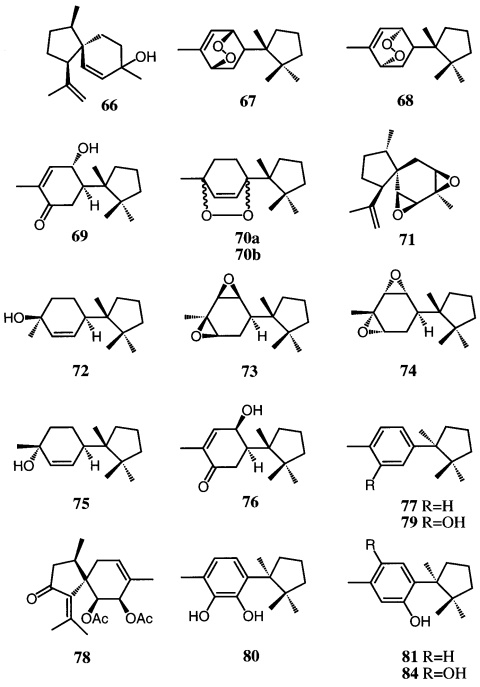
Cathepsin B and cathepsin L are correlated with osteoporosis (Katsunuma, 1997). We are currently searching for enzyme inhibitors from natural products to develop chemopreventive drugs for these diseases. The crude ether extract of *Porella japonica* showed a strong inhibition against cathepsin B and L. Biological activity-guided fractionation of the extract gave porelladiolide (**61**) and its epoxide (**62**) as well as three new guaianolides (**63**–**65**) (Hashimoto et al., 1998b). Only compound **64** showed a weak inhibition against cathepsin B (13.4% at 10^{-5} M) and cathepsin L (24.7% at 10^{-5} M).



Since the *Jungermannia* species are morphologically very small, their classification is rather difficult. This genus contains a large number of taxonomically complex species, which are polymorphic. *Jungermannia infusca,* which has many chemotypes, contains a new acorane- (**66**) and five cuparene-type sesquiterpenoids (**67–70a,b**) the structures of which were elucidated by X-ray crystallographic analysis (Nagashima et al., 1998b, 1999b).

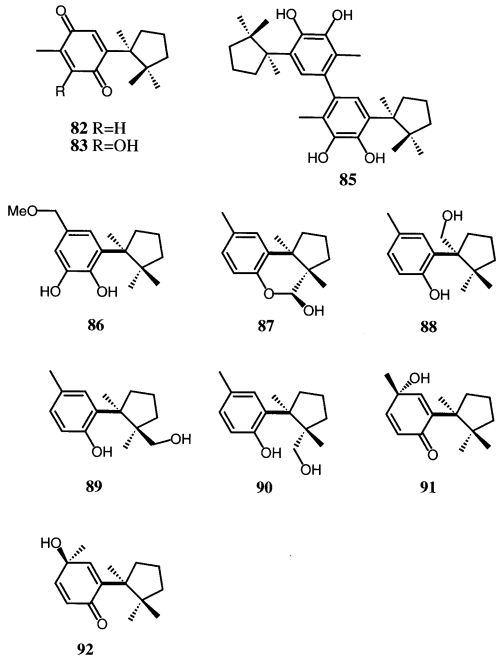
Jungermannia hattoriana elaborates a new acorane-type sesquiterpenoid, acoradiepoxide (**71**), three new cuparene-type sesquiterpenoids, neocuprenenol (**72**), cuparadiepoxide (**32**), epi-cuparadiepoxide (**74**), together with three known related compounds, (+)-cuprenenol (**75**), rosulantol (**76**) and (+)-cuparene (**77**) (Nagashima et al., 1997c). Thus the above two *Jungermannia* species are closely related chemically. The absolute configuration of the isolated cuparenes is the same as that found in corresponding compounds from higher plants.

The Finnish liverwort *Barbilophozia barbata* produces a new acorane-type sesquiterpenoid, barbiacoradienone (**78**) together with the known dolabellane- and fusicoccane-type diterpenoids (Nagashima et al., 1999c).

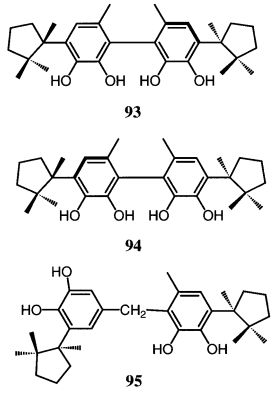


Lejeunea aquatica produces 2-cuparenol (**79**), 1,2-cuparenediol (**80**), 1-cuparenol (**81**), cuparene-1,4-quinone (**82**), deoxyhelicobasidin (**83**) and β-barbatene as well as 1,4-cuparenediol (**84**) and aquaticenol (**85**) which have been recently isolated for the first time from the nature (Toyota et al., 1997c). *Lejeunea flava* and *L. japonica* elaborate compounds **80** and **82**, and **77** and **84**, respectively. Aquaticenol (**85**) was synthesized by the dimerization of 1,2-cuparenediol (**80**) in the presence of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in nitromethane. The compounds described above are all from the *ent*-cuparene series. Thus the present three *Lejeunea* species are chemically very similar.

Herbertus species are rich sources of herbertane-type sesquiterpenoids. Seven new herbertane-type sesquiterpenoids 12-methoxyherbertene-1,2-diol (**86**), herberteneacetal (**87**), 1,13-dihydoxyherbertene (**88**), 1,14-dihydroxy-



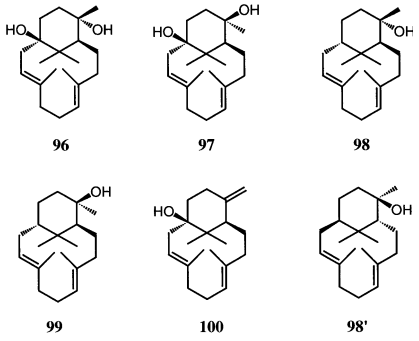
herbertene (**89**), 1,15-dihydroxyherbertene (**90**), herbertenone A (**91**) and herbertenone B (**92**) with three know herbertene dimers, mastigophorenes A-C (**93-95**), which show neurite sprouting activity (Asakawa, 1998, 1999) have been isolated from *Herbertus sakuraii* (Irita et al., 2000).



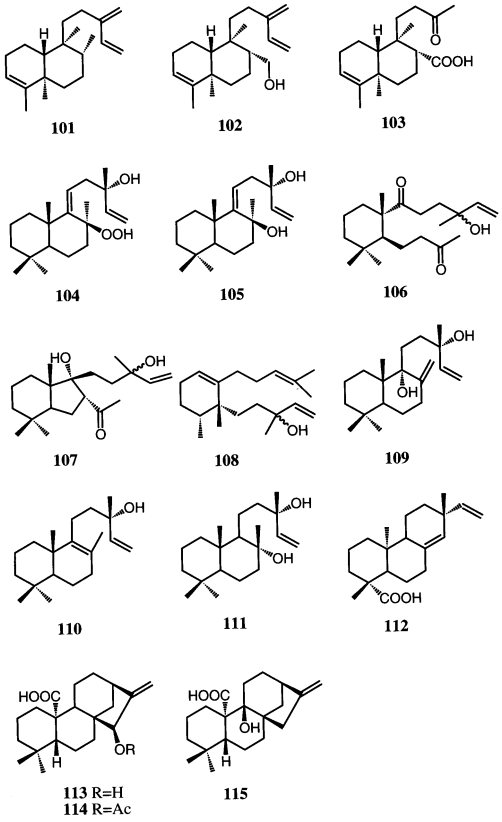
2.4. Diterpenoids

Liverworts are also rich sources of a number of different skeletal diterpenoids. Five *ent*-verticellane-type diterpenoids (**96–100**) have been isolated as the main components from *Jackiella javanica* together with two sesquiterpenoids, *ent*-germacrene-D and *ent*-1(10)-aristolene (Nagashima et al., 1997a). *Ent*-verticellanes are significant chemical markers of *J. javanica* since no verticellanes have been detected in or isolated from any of the 1000 species of liverworts so far chemically examined. It is noteworthy, however, that the enantiomer **98'** of **98** has been isolated from a conifer *Sciadopitys verticillata* (Karlsson,

et al. 1978). Verticellanes are considered to be biosynthetic precursors of taxol (Koepp et al., 1995)



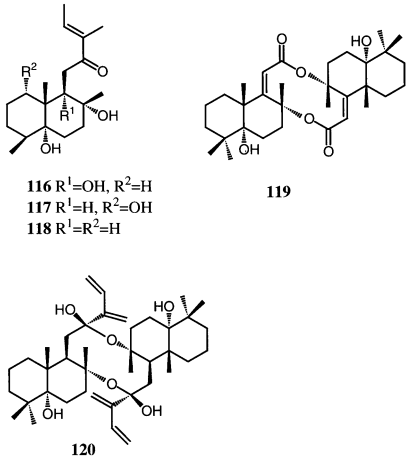
The *Jungermannia* species are rich sources of labdane clerodane- and kaurane-type diterpenoids (Nagashima and Asakawa, 1998a). Two new clerodane-type compounds (**96** and **97**) and a new bis-norclerodane- (**103**), together with 12 known clerodane-, labdane- and *ent*-kaurane-type diterpenoids (Nagashima et al. 1998c), two new labdane- (**104** and **105**) and two new seco-labdane-type diterpenoids (**106** and **107**) (Nagashima et al., 1999b) and a monocyclic diterpene alcohol (**108**) (Nagashima et al., 1997a), have been isolated from the Japanese *Jungermannia infusca*.



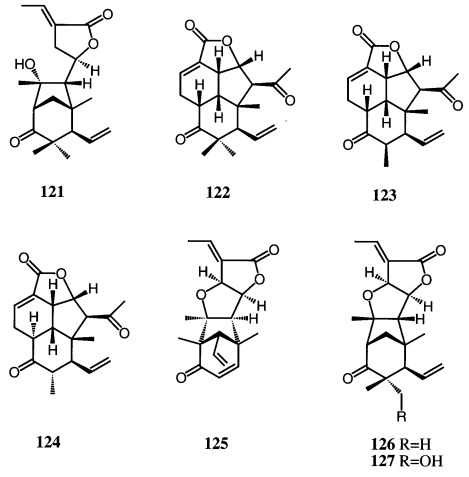
Another new labdane-type diterpenoid, (+)-labda-8(17),14-diene-9*R**,13*S**-diol (**109**) has been isolated from *J. hattoriana* along with two labdanes (**110** and **111**) and a pimarane-type diterpenoid (**112**) (Nagashima et al., 1997c). This is the first isolation of diterpenoids, which have the 10α- and 10β-methyl groups from the same liverwort.

Jungermannia rotundata produces three new kaurane-type diterpenoids, the rotundeic acids A-C (**113**–**115**) (Nagashima et al., 1997b). As far as we are aware, this is the first isolation of *ent*-kaurane-type diterpenoids, with the carboxylic acid at C-10, from bryophytes or higher plants although *ent*-kaurane-10-carboxylic acid is a known oxidation product of *ent*-kaurane-20-ol.

Three new labdane diterpenoids (**116**–**118**) together with two novel dimeric labdane diterpenoids, scapaundulins A (**119**) and B (**120**) have been isolated from *Scapania undulata*. The structures of the dimeric compounds possess a C_2 axis of symmetry (Yoshida et al., 1997a).



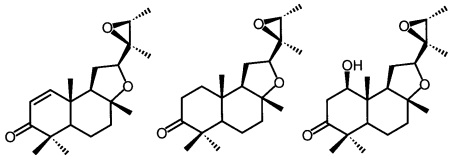
Pallavicinia subciliata, which is a very small thalloid liverwort, elaborates six novel secolabdanoids (**121**–**125**, **127**), together with pallavicinin (**126**) (Wu et al., 1994). The structures **125** and **127** were established by their X-ray crystallographic analysis (Toyota et al. 1998d). This is the first isolation of such complex diterpenoids in nature.



The large leafy liverwort, *Porella perrottetiana* contains a large amount of labda-12,14-dien-7α,8α-diol (**128**),

which is easily converted into the animal perfume, (−)-ambrox (**129**) in good yield *via* six steps (Fig. 5) (Hashimoto et al., 1998d).

Frullania hamatiloba elaborates three characteristic labdane epoxides (**130**–**132**), together with two known fusicoccane-type diterpene epoxides, caryophyllene epoxide and a few flavones (Hashimoto et al., 1998b). The chemical character of *F. hamatiloba* is the presence of the oxirane ring in the sesqui- and diterpenoids.



Dolabellane- and/or fusiccocane-type diterpenoids have been found in the Hepaticae, namely the Cephaloziaceae, Pleuroziaceae, Plagiochilaceae and Lophoziaceae (Asakawa, 1995; Nagashima et al., 1999c). The Japanese moss, *Plagiomnium acutum* elaborates (+)-dolabella-3,7-dien-18-ol (**10**) (Toyota et al., 1998a). This is the first example of the isolation of dolabellane-type diterpenoids from mosses. This result implies a possible chemical correlation between mosses and liverworts.

Five dolabellane-type diterpenoids (**133**–**137**), four of which are new compounds (**133**–**136**), have been isolated from *Odontoschisma denudatum* with anastreptene, an aromadendrane-type sesquiterpene, as a major component (Hashimoto et al., 1998b). Furthermore two new

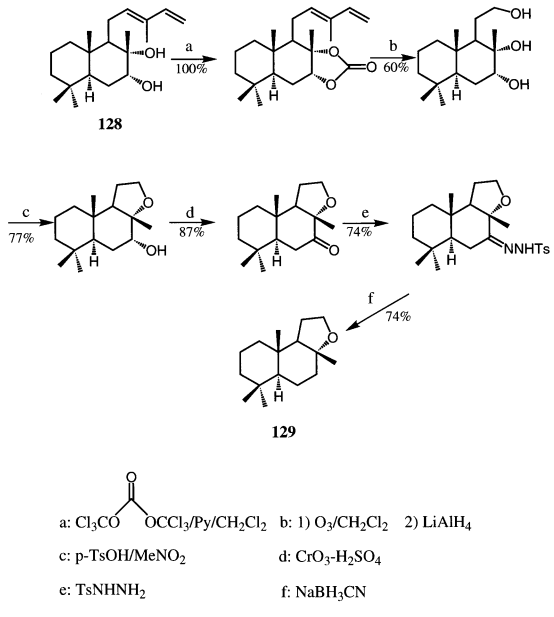
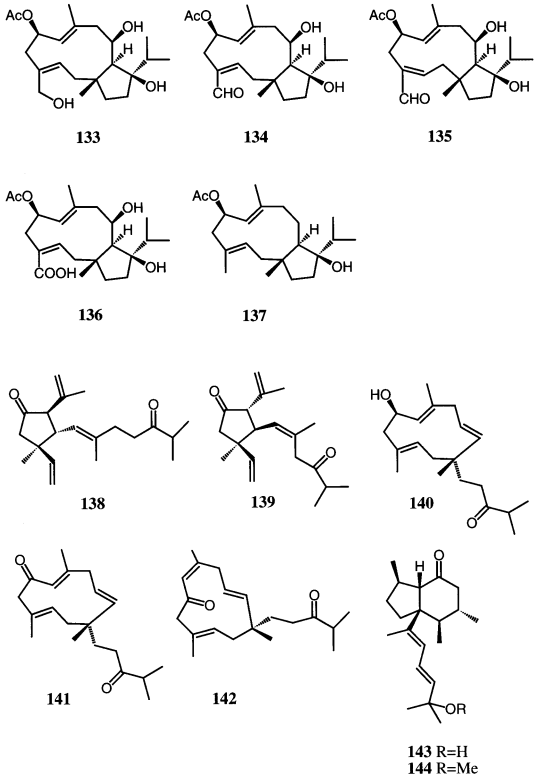


Fig. 5. Preparation of the animal perfume (−)-ambrox (**129**) from labda-12,14-dien-7α,8α-diol (**128**).

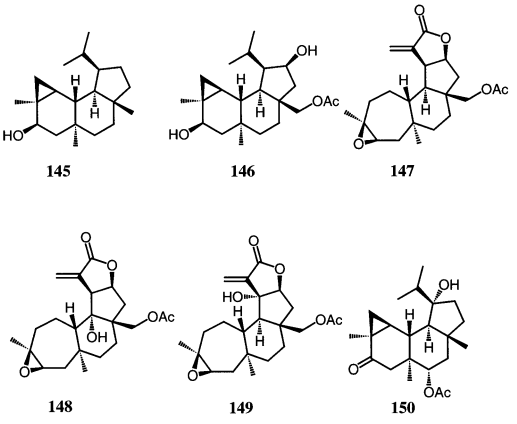
skeletal diterpenoids, neodenudatenones A (**138**) and B (**134**) which are the first examples of such compounds in nature have also been isolated from the same species (Hashimoto et al., 1998c) together with three novel *ent*-vibsane-type diterpenoids named denudatenones A-C (**140**–**142**) (Hashimoto et al., 1998e). Compounds **138** and **139** are enantiomers except for the geometry of the double bond in the side chain. Vibsane-type diterpenoids are very rare compounds in nature (Fukuyama et al., 1996) and the *ent*-vibsane-type diterpenoids from the liverworts were the first examples.



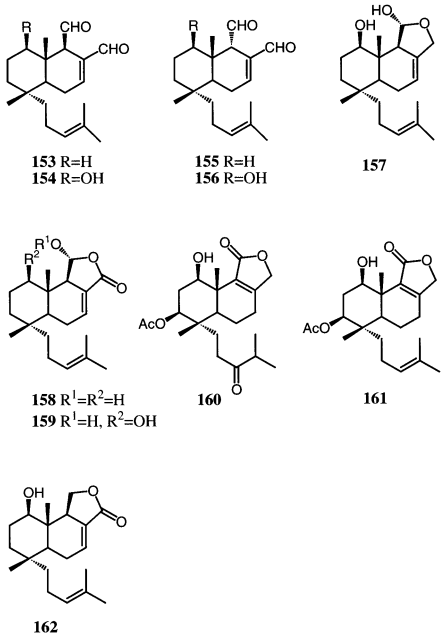
Two novel skeletal diterpenoids, hacherenone (**143**) and its methyl ether (**144**) have been isolated from the German liverwort *Barbilophozia hatcheri* together with the known compounds, barbiacoradienone (**78**) and a carotane-type sesquiterpene, hercynolactone (Nagashima et al., 1999a).

The Jungermanniaceae, Plagiochilaceae, Scapaniaceae and Schistochilaceae families are rich sources of the verrucosane- and homoverrucosane-type diterpenoids (Asakawa, 1995). Five unique diterpenoids have been isolated from the New Zealand liverwort, *Jamesoniella tasmanica*, which belongs to the Lophoziaceae. They were shown to be 13-epi-neoverrucosane- (**145** and **146**) and highly oxygenated 13-epi-neohomoverrucosane-type diterpenoids (**147**–**149**) (Toyota et al., 1996a). We proposed the biogenetic pathway of neoverrucosane- and neohomoverrucosane-type diterpenes in which generation of intermediate (**152**) from intermediate (**151**) was suggested to imply four successive 1,2-hydride shift

(route a) as shown in Fig. 6. (Toyota et al. 1996a). Recently, the biosynthesis of 8α-acetoxy-13α-hydroxy-5-oxo-13-*epi*-neoverrucosane (**150**) in the liverwort *Fossombronia alaskana* was investigated using [1-^{13}C]- and [U-$^{13}C_6$]glucose as precursors (Eisenreich et al., 1999). This feeding experiment showed that the C_5 building blocks isopentenyl pyrophosphate and dimethylallyl pyrophosphate of the neoverrucosane are biosynthesized predominantly *via* the deoxyxylose pathway. The incorporation experiment with [6,6-$^2H_2$]glucose ruled out our previous hypothesis (route a) and an unusual 1,5-hydride shift (route b) from intermediate (**151**) before formation of the cyclopropyl moiety was confirmed in compound **150** as indicated in Fig. 6.



Sacculatane-type of diterpenoid has been known only in the liverworts, Pelliaceae, Porellaceae and Trichocoleacea. Eight new sacculatane-type diterpenoids (**154**, **156**, **157**–**162**) have been isolated from *Pellia endiviifolia*. Compounds **154**, **156**, **157** and **159**–**162** were the first naturally occurring sacculatane-type diterpenoids bearing a 1β-hydroxyl group. Pungent-tasting sacculatal (**153**) and 1β-hydroxysacculatal (**154**) possess potent piscicidal activity against killie-fish, which died within 20 min at a concentration of 1 ppm, indicating that both 8- and 9β-diformyl groups were essential to the piscicidal activity (Hashimoto et al., 1995). Sacculatal (**153**), showed anti-HIV-1 activity (IC_{50} 68.8 μg/ml), together with cytotoxicity against KB cells (ID_{50} 3.2 μg/ml), Lu1 cell (ID_{50} 5.7 μg/ml), LN Cap cells (ID_{50} 7.6 μg/ml) and ZR-75-1 cell (ID_{50} 7.6 μg/ml) and antimicrobial activity against *Streptococcus mutans* (IC_{50} 8 μg/ml) (Asakawa and Cordell et al., unpublished results).



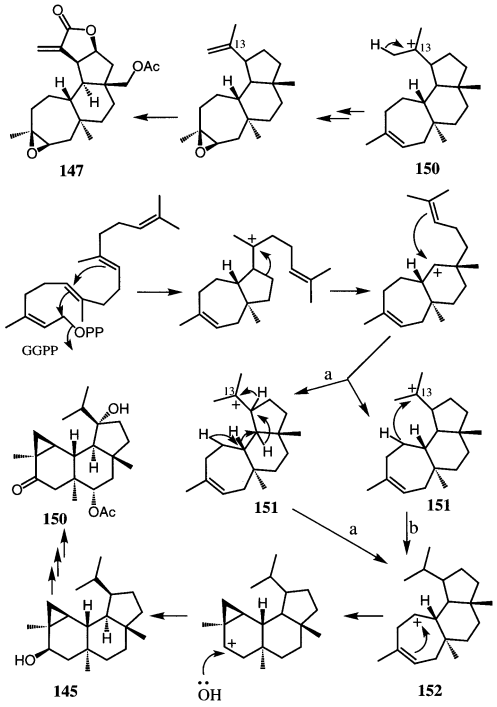
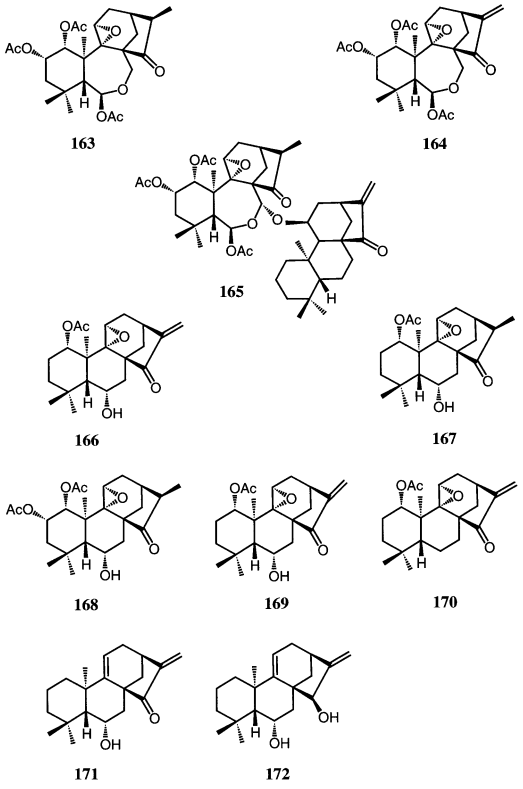


Fig. 6. Biosynthetic pathway of 13-*epi*-neoverrucosane- (**145**, **150**) and 13-*epi*-neohomoverrucosane-type diterpenoids (**147**) from GGPP.

Jungermannia exsertifolia subsp. *cordifolia* is an interesting liverwort because it produces many kaurane-type diterpenoids including those of the 6,7-seco-type. Fractionation of the ether extract gave two new secoexsertifolins A (**163**) and B (**164**), a bis-*ent*-kaurene, exsertifolin A (**165**) and their related compounds, exsertifolins B-H (**166**, **166**–**172**) (Nagashima et al., 1996). This is the first example of the isolation of 6,7-seco-9,11-epoxy-*ent*-kaurane- and a 9,11-epoxy-*ent*-kaurane-type diterpenoids from liverworts.

2.5. Triterpenoids

The mosses contain many triterpenoids (Asakawa, 1995). In recent years no new triterpenoids have been discovered. The fractionation of the crude ether extract of the moss *Floribundaria aurea* subsp. *nipponica*



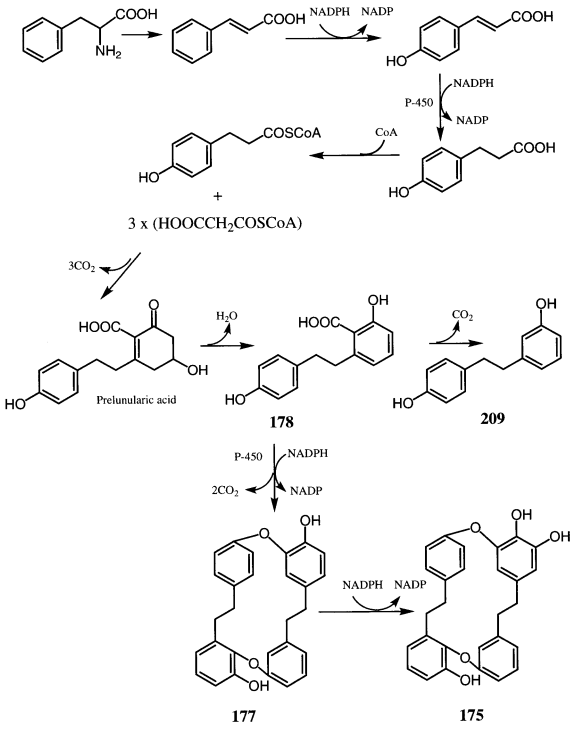
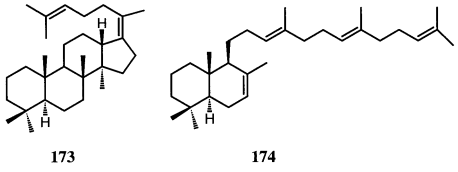


Fig. 7. Biosynthetic pathway of marchantin A (**175**) and marchantin C (**177**).

showed that it elaborates a new triterpene hydrocarbon, dammara-17*Z*,21-diene (**173**), and polypoda-7,13,17,21-tetraene (**174**) which is the first example of their isolation from a moss, although it has been isolated from ferns (Toyota et al., 1998b).

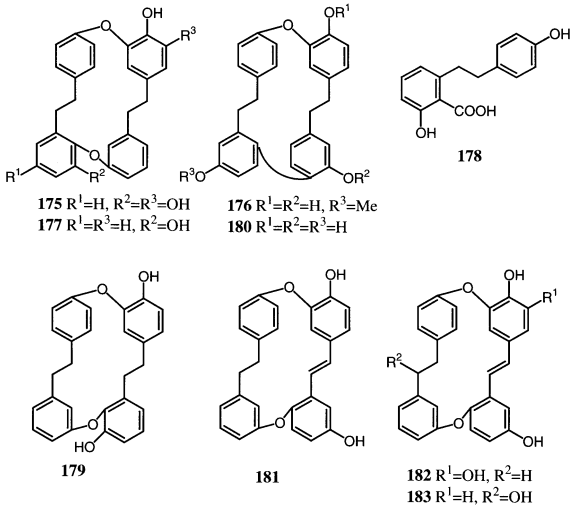


2.6. Bis(bibenzyl)s

2.6.1. Occurrence in nature

Liverworts are rich sources of bis (bibenzyl) compounds. Since we firstly reported the isolation of two cyclic bis(bibenzyl)s, marchantin A (**175**) from *Marchantia* and riccardin A (**176**) from *Riccardia* species (Asakawa, 1982), more than 60 macrocyclic bis(bibenzyls) have been isolated only from liverworts (Anton et al., 1997, 1999; Asakawa, 1995; Asakawa et al., 1987; 1996; Hashimoto et al., 1994a,b, 1996, 1999a, 2000; Kunz and Becker, 1994; Yoshida et al., 1996; 1997b; Martini et al., 1998).

Marchantin A (**175**) is representative of the bis (bibenzyl)s which are widespread in Marchantiales species. It shows antifungal, antimicrobial and muscle relaxing and cytotoxic activities as well as 5-lipoxygenase, cyclooxygenase and calmoduline inhibitory and cardiotonic activities (Asakawa, 1990a,b, 1993, 1995, 1998, 1999; Schwartner et al., 1995). We have reported the biosynthetic pathway of marchantin A (**175**) and C (**177**) found in *Marchantia polymorpha* as shown in Fig. 7 (Friederich et al., 1999a,b). Two specific cytochrome P-450 were detected in cell suspension cultures of *M. polymorpha*, the first catalyzes the coupling of two molecules of lunularic acid (**178**) to form marchantin C (**177**) and CO_2 and the second hydroxylates marchantin C to marchantin A (**175**). The precursor feeding experiments using $^{3}H/^{14}C$ doubly labeled substrates showed that lunularic acid (**178**) is the sole substrate for the coupling reaction to afford marchantin C (**177**).



The tentative structure of isomarchantin C (**179**) from the Indian *Marchantia* species has been proposed (Asakawa et al., 1987). *Dumortiera hirsuta* also produces the same isomarchantin C (**179**), together with marchantin C (**177**) and riccardin C (**180**) and the structure of **179** has now been established by X-ray crystallographic analysis (Toyota et al., 1997f). Isomarchantin C possessed the strong inhibitory activity against cathepsin B (93%) and cathepsin L (95%) at 10^{-5} M (Asakawa, 1999).

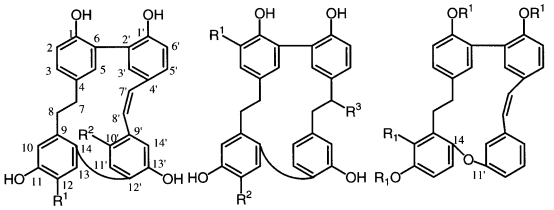
Ptychantols A-C (**181**–**183**), which possess a *trans*-stilbene structure, have been isolated from the leafy-stem liverwort, *Ptychantus striatu*s, which belongs to the subfamily Ptychantoideae of the Lejeuneaceae (Hashimoto et al., 1999a). The stereochemistry of ptychantol A (**181**) has been determined by X-ray crystallographic analysis. The presence of macrocyclic bis(bibenzyls) are very rare in the Lejeuenaceae. At present, only isomarchantin C (**177**) has been found in *Bryopteris firicina* which belongs to the subfamily Bryopteridoideae (Nagashima et al., 1994).

Plagiochila fruticosa contains many 2,3-secoaromadendrane-type sesquiterpenoids (Asakawa, 1995). Further fractionation of the methanol extract gave four unique bis(bibenzyl)s, isoplagiochins C (**184**) and D (**185**) which possess two C_6–$C_{2'}$ and C_{14}–$C_{12'}$ biphenyl bonds (Hashimoto et al., 1996), together with isoplagiochins A (**186**) and B (**187**) that possesses a C_{14}–C-$_{11'}$ ether linkage and a C_6–$C_{2'}$ biphenyl bond (Hashimoto et al., 1994a). This is the first report of the isolation of a macrocyclic bis(bibenzyl)s, possessing two biphenyl linkages between rings A/C and B/D. The isoplagiochin series of compounds might be biosynthesized from isoperrottetin A (**188**) which was isolated from the liverwort *Radula perrottetii* (Toyota et al. 1994). The isoplagiochins C (**184**) and D (**185**) obtained from *P. fruticosa* did not show optical activity, however, the same compounds isolated from *Herbertus sakuraii* displayed optical activity [**184**: $[\alpha]_D$ +74.8; CD 241 nm ($\Delta\epsilon$ +24.4), 213 (−63.2); 185 $[\alpha]_D$ +47.5; CD 252 nm ($\Delta\epsilon$ +2.4), 225 (2.5)] (Hashimoto et al., 2000).

Herbertus sakuraii also elaborates three new dichlorinated macrocyclic bis(bibenzyls), 2,12-dichloroisoplagiochin D (**189**), 12,7′-dichloroisoplagiochin D (**190**) and 12,10′-dichloroisoplagiochin C (**191**) and their structures elucidated by a combination of 2D-NMR spectroscopy and X-ray crystallographic analysis (Hashimoto et al., 2000). The same compound (**189**), and 12-chloroisoplagiochin D (**192**), which have been found in an unidentified *Plagiochila* species (Anton et al., 1997), have been isolated from *Mastigophora diclados*. *H. sakuraii* is closely related chemically to *M. diclados* since they produce the same unique herbertane- and dimeric herbertane-type sesquiterpenoids as well as cyclic bis(bibenzyl)s.

The similar chlorinated bis(bibenzyl)s, bazzanins A-J (**193**–**202**) which are derived from isoplagiochin C (**184**) and isoplagiochin D (**185**) have been isolated from the European liverwort *Bazzania trilobata* (Martini et al., 1998) although *B. trilobata* is morphologically quite different from *Herbertus* and *Mastigophora* species.

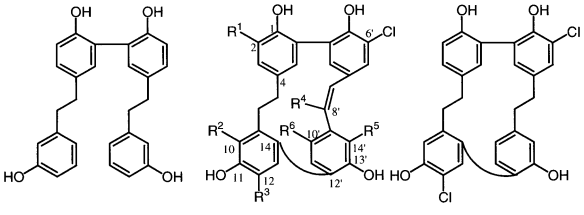
Blasia pusilla, which belongs to the Blasiaceae grows on a wet sand and contains species of the symbiotic blue-green alga genus *Nostoc*. Fractionation of the



184 R^1=R^2=H
191 R^1=R^2=Cl

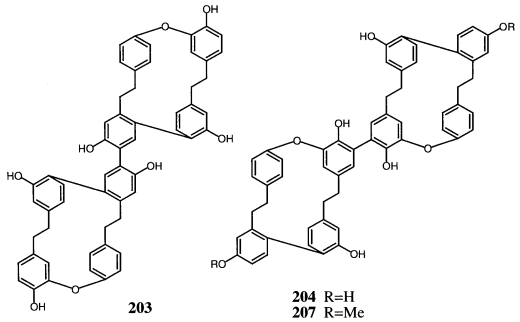
185 R^1=R^2=R^3=H
189 R^1=R^2=Cl, R^3=H
190 R^1=H, R^2=R^3=Cl
192 R^1=R^3=H, R^3=Cl

186 R^1=R^2=H
187 R^1=H, R^2=OH



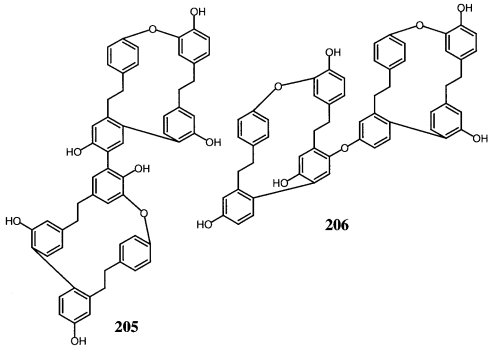
188

193 R^1=R^2=R^3=R^4=R^5=R^6=H
194 R^1=R^2=R^3=R^5=R^6=H, R^4=Cl
195 R^1=R^2=R^5=R^6=H, R^3=R^4=Cl
196 R^1=R^2=R^3=R^6=H, R^4=R^5=Cl
197 R^1=R^2=R^6=H, R^3=R^4=R^5=Cl
198 R^1=R^3=R^5=H, R^2=R^4=R^6=Cl
199 R^1=R^2=R^4=R^5=Cl, R^3=R^6=H
200 R^1=R^3=H, R^2=R^4=R^5=R^6=Cl
201 R^1=R^3=R^4=R^5=R^6=Cl, R^2=H



203

204 R=H
207 R=Me

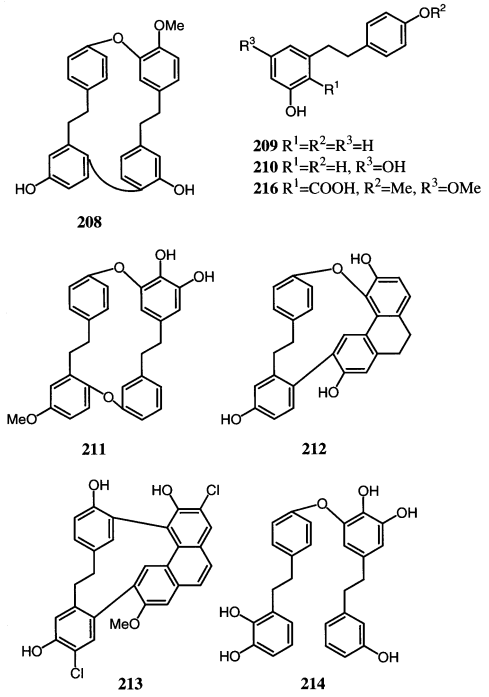


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methanol extract resulted in the isolation of four unique dimeric bis(bibenzyl)s, pusilatins A–D (**203**–**206**) along with riccardin C (**180**) and F (**208**) and shikimic acid, lunularic acid (**178**), lunularin (**209**), dihydroresveratol (**210**) and four known benzoates (Hashimoto et al., 1994b; Yoshida et al., 1996). The structure of pusilatin A (**203**) was characterized by a combination of 2D-NMR spectroscopy and X-ray crystallographic analysis of its hexacetate. Pusilatins B (**204**) and C (**205**) showed DNA polymerase β inhibitory activity (IC_{50} 13.0 and 5.16 μM) and moderate cytotoxicity (Hashimoto et al., 1994b; Yoshida et al., 1996). Pusilatin B (**204**) (6′,6‴-bis-riccardin C) was subsequently isolated from an axenic cultured liverwort *Ricciocarpos natans* (Ricciaceae) shortly after our report (Kunz and Becker, 1994).

Riccardia multifida subsp. *decrescens* (Riccardiaceae) produces pusilatin E (**207**) that is a symmetric dimer of riccardin A (**176**), which has been isolated from the same species together with marchantin I (**211**). Riccardin A (**176**) was treated with manganese triacetate to give pusilatin E (**207**) followed by demethylation with borontribromide to afford pusilatin B (**204**) (Yoshida et al., 1997b). It is noteworthy that the Blasiaceae and Riccardiaceae species belonging to the Metzgeriales and Ricciaceae to the Marchantiales produce the same type of cyclic bis(bibenzyl) dimers.



(+)-Cavicularin (**212**), a unique cyclic bibenzyl-dihydrophenanthrene derivative, has been isolated from *Cavicularia densa* (Blasiaceae) and its structure established by X-ray crystallographic analysis (Toyota et al., 1996b). Compound **212** is optically active ($[\alpha]_D$ +168.2) and possesses both planar and axial chiralities. This is the first isolation of such a compound from nature. However, the dimeric cyclic bis(bibenzyls) found in *B. pusilla* has not been detected in *C. densa*. On contrast, *B. pusilla* does not contain cavicularin. These chemical results are fit to the recent classification of the Blasiaceae in which two subfamilies, Blasioideae and Cavicularioideae have been prepared (Furuki and Mizutani, 1994). The similar bibenzyl-phenanthrene compound **213** with two chlorine atoms has been isolated from *Bazzania trilobata* which is morphologically quite distinct from *C. densa* (Martini et al., 1998).

2.6.2. Role of bis(bibenzyl)s in bryophyte evolution

There are two main hypotheses regarding the evolution of the bryophytes (Fig. 8). The first is the progressive theory, which states that the bryophytes have their origins in the filamentous fresh water green algae or unicellular green algae and that the pteridophytes (ferns) are in turn derived from the bryophytes. The second hypothesis, the reductive theory, regards the green algae as being the ancestors of primitive vascular plants, such as the extinct class Rhyniophytinia, from which both bryophytes and pteridophytes evolved. However, no conclusive evidence for these hypotheses has been obtained from paleontology because of the absence of bryophyte fossils. In order to understand the evolutionary stages between spore-forming terrestrial green plants, we are currently studying the chemical constituents of ferns. The fractionation of the methanol extract of the Japanese fern *Hymenophyllum barbatum* resulted in the isolation of an acyclic bis(bibenzyl) compound, perrottetin H (**214**) (Oiso et al., 1999) which has been isolated from the liverwort, *Jubula japonica* (Toyota and Asakawa, 1993). This is the first isolation of a bis(bibenzyl) compound from the pteridophytes. The occurrence of perrottetin H in *H. barbatum* is significant, since previously, various acyclic and cyclic bis(bibenzyl)s have been isolated only from the Hepaticae and are recognized as one of the most important chemical indicators of this class (Asakawa, 1995). A number of monomeric bibenzyls, such as lunularic acid (**178**) and lunularin (**209**) and prenyl bibenzyl (**215**) are also significant chemical markers of the Hepaticae. It is noteworthy that the bibenzyl (**216**) and prenyl bibenzyl (**217**) which are similar compounds to **178**, **209** and **215**

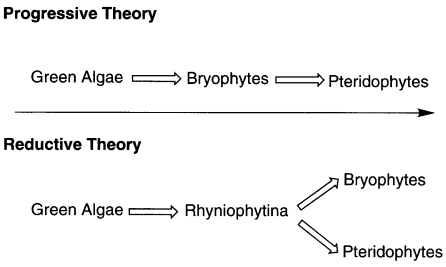
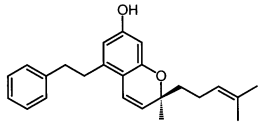
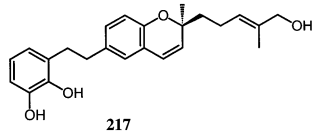


Fig. 8. Evolutionary processes leading to bryophytes and pteridophytes.

have also been isolated from the ferns, *Notholaena dealbata*, *N. limitanea* (Wollenweber and Fovre-Bonvin, 1979) and *Sceptridium ternatum* (Tanaka et al., 1986), respectively. These bibenzyls and bis(bibenzyl)s might be considered to be living fossils that could link the evolutionary stages between the bryophytes and the pteridophytes. It is still difficult to conclude which of the two evolutionary processes depicted in Fig. 8 explain the evolution of the bryophytes, although on the basis of the present results it is clear that there are some significant chemical affinities between the ferns and liverworts that biosynthesize typical bibenzyls and bis(bibenzyl) derivatives.



215



217

2.7. Conclusion

At present only 5% of total bryophytes have been studied chemically. Although the bryophytes are very small terrestrial spore-forming green plants and they are almost useless for use in the human diet, there are a number of new terpenoids and aromatic compounds, including several new carbon skeletons, several of which show interesting biological activity. It is also noteworthy that some species belonging to the same genus produce normal or the enantiomeric terpenoids. Some liverworts elaborate both enantiomers of the same compound.

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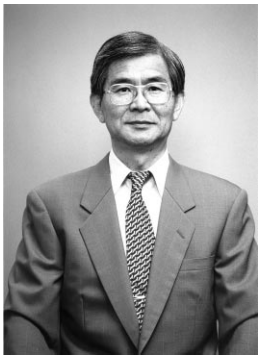
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Yoshinori Asakawa began his studies as a student of biology at Tokushima University in 1960, but turned his attention to organic chemistry. It was in the latter field that he earned his MS and PhD degrees at Hiroshima University, completing his studies in 1972. Yoshinori was an Assistant at Hiroshima University until 1976; however, during that period of time, he spent a 2 years leave at the Universite Louis Pasteur in Strasbourg, France where he worked with Prof. Guy Ourisson. He became Associate Professor in 1976 and full Professor of Faculty of Pharmaceutical Sciences, Tokushima Bunri University in 1981. He was appointed as Dean (1986–1988 and 2000–) of the same Faculty and Director of Institute of Pharmacognosy (1986–) of the same University. He is Editor of *Spectroscopy*, Associate Editor of *Phytomedicine* and serves on the editorial boards of *Phytochemistry, Planta Medica, Fitotherapia, Flavour* and *Fragrance Journal* amongst others. His main research interests are biologically active substances of cryptogams (bryophytes, pteridophytes and mushrooms etc.) and their chemosystematics, and evolutionary and differentiation processes. He has studied the chemistry of approximately 1000 species of bryophytes and isolated and structurally determined several hundred new natural products. Many of these have been tested for their biological activities (pharmacology, cytotoxicity, allergenic properties, tastes, smell) and some have been successfully launched into industrial development. Many interesting new terpenoids structures have been uncovered and unique type of cyclic and acyclic bis(bibenzyls) discovered in Marchantiaceae, Riccardiaceae and Plagiochilaceae. In the field of taxonomy of Hepaticae, he has produced chemical evidence that supports the union of Jungermanniales and Metzgeriales within one subclass. His evolutionary studies support a close relationship between hepatics and the algae on the basis of their chemistry. He also demonstrated that hepatics often regarded as "primitive" plants, are able to produce the same terpenoids as those that occur in higher plants, although often with corresponding but different enantiomeric antipode. He has also made important contributions to organic syntheses, developing the use of m-chloroperbenzoic acid as an oxidizing agent, and he has also contributed to the biotransformation of terpenoids and aromatic compounds by fungal cultures and mammals. He has published 420 papers, half of them in *Phytochemistry* and 25 books including two volumes of *Progress in the Chemistry of Organic Natural Products* (p. 1–285; 1–562). For his outstanding research, the first Hedwig medal from the International Association of Bryologists, Pergamon Phytochemistry Prize and Certificate from Elsevier Science and Tokushima News Paper Prize (Science) were awarded to Professor Asakawa.

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Announcement

2001 Keystone Symposium: Plant Foods for Human Health: Manipulating Plant Metabolism to Enhance Nutritional Quality (E5)

Organizers: Dean DellaPenna and Michael A. Grusak
6–11 April 2001
Beaver Run Resort, Breckenridge, Colorado, USA
Abstract deadline: 6 December 2000. Early registration: 6 February 2001

Plant foods contain a bountiful array of vitamins, minerals, and beneficial roles in human health and well-being. As a means to improve global health, strategies have been undertaken to enhance the nutrient quality and density of plant foods by targeting key enzymes in specific phytochemical pathways. The identification and cloning of specific pathway enzymes have benefited immensely from the ongoing integration of plant molecular genetics, biochemistry and genomics. However, our understanding of plant primary and secondary metabolism is incomplete, especially with respect to the control of global carbon flux to specific end products, and the regulation of central metabolites that are common intermediates of multiple pathways. Understanding the interconnections of these pathways is critical to the success of future nutritional enhancement efforts. This Symposium is intended to foster communication of the most current research.

Meeting schedule, topics and registration details can be found at: http://www.symposia.com/MeetingDetail.cfm?MeetingNumber = E5&Year = 2001

Other inquiries can be directed to Dean DellaPenna at: dellapen@msu.edu.

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Minor diterpenes from *Persea indica*: their antifeedant activity

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Abstract

The new diterpenes anhydrocinnzeylanone, garajonone and 2,3-didehydrocinnzeylanone, and the known anhydrocinnzeylanine, have been isolated from *Persea indica*. The antifeedant activity of these compounds has been evaluated showing the importance of the 11-hemiketal group for the antifeedant effects of ryanodane diterpenes. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Persea indica*; Lauraceae; Diterpenes; Ryanodane; Anhydrocinnzeylanone; Garajonone; 2,3-Didehydrocinnzeylanone; Antifeedant activity

1. Introduction

In a previous work, we have reported on the isolation of the toxic diterpenes ryanodol and cinnzeylanol from the Canarian endemic species *Persea indica* (Lauraceae), their ecological relationship with the wild rats (González-Coloma et al., 1990, 1993) and the insecticidal effects on *Macaronesia fortunata* and *Heliothis armigera* (González-Coloma et al., 1992). We have also communicated the isolation of other diterpenes of this type such as cinnzeylanine, cinnzeylanone, epi-cinnzeylanol and 14-monoacetate of ryanodol (González-Coloma et al., 1996), as well as new substances with the rare isoryanodane skeleton, such as indicol, vignaticol and perseanol (Fraga et al., 1997). Now, following the study of the minor components from this plant, we have isolated another three new diterpenes anhydrocinnzeylanone (**1**), garajonone (**3**) and 2,3-didehydrocinnzeylanone (**4**), in addition to the known anhydrocinnzeylanine (**2**). Finally, we describe the antifeedant activity of these compounds.

2. Results and discussion

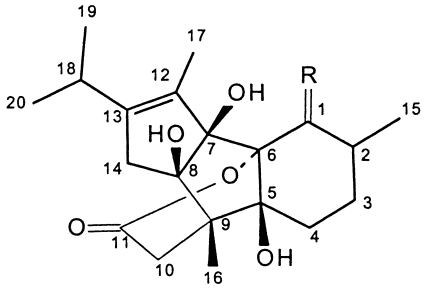
The structure **1** was assigned to one of the compounds isolated from *Persea indica* on the basis of the following considerations: the fragment of higher mass observed in its high resolution MS was that at m/z 346.1795, which is formed from the molecular ion by loss of water. Thus, the molecular formula of this substance was $C_{20}H_{28}O_6$. The ^{13}C NMR was very informative, showing resonances of five methyls, four methylenes, two methines, five tetrasubstituted carbons, a tetrasubstituted double bond (δ 132.3 and 145.7), and two oxo groups at δ 166.3 and 213.4, the first of which must form a part of a lactonic group. Of the five quaternary carbons, four bore an oxygen atom (δ 85.1, 86.8, 87.2 and 97.7), and of these three carried hydroxyl groups. The hydroxylic hydrogens, probably associated by hydrogen bonds, resonate at δ 3.86, 5.15 and 6.88 as broad singlets. The tetrasubstituted double bond was located at C-12,C-13 considering that the signal of a methyl group over a double bond was present in the ^1H NMR spectrum as a singlet at δ 1.64. Thus, we assigned an anhydroryanodane structure with a tetracyclic skeleton to this compound. The resonances of the C-10 and C-14 methylenes were also observed in the ^1H NMR spectrum (see experimental). Finally, the oxo group at δ 213.4 was assigned at C-1, because the carbon resonances of the cyclohexanone ring (Table 1) were very similar to those of cinnzeylanone (**5**). On the other hand, the anhydroryanodane diterpenes can be prepared by acid treatment of the corresponding ryanodane derivative. Anhydrocinnzeylanine (**2**) has thus been obtained from cinnzeylanine (**5**) (Isogai et al., 1976, 1977). In our case, acid treatment of **5** led to anhydrocinnzeylanone (**1**) confirming the structure now given to this compound.

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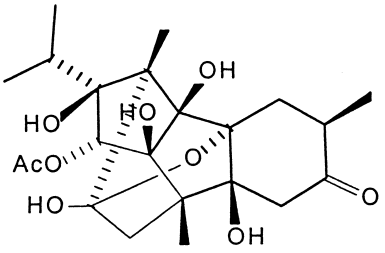
[1] On leave from Centro de Investigaciones de Biología Marina (CIBIMA), Autonomous University of Santo Domingo, Dominican Republic.

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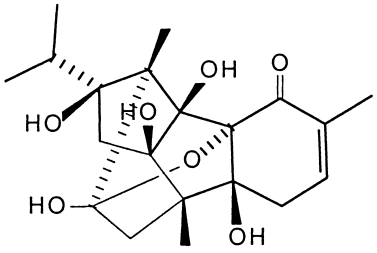


1 R = O

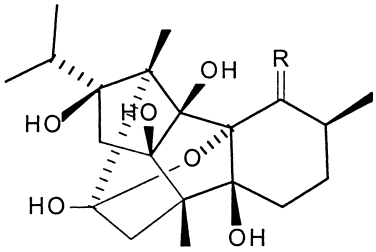
2 R = α-OH,H



3

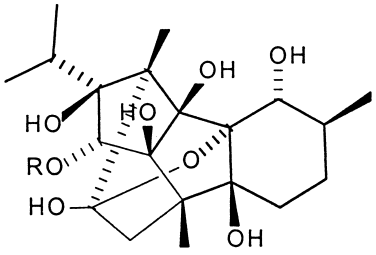


4



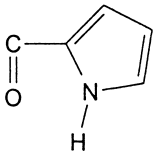
5 R = O

6 R = α-OH,H



7 R = H

8 R = Ac

9 R = 

Another new ryanodane derivative obtained from this plant was named garajonone (**3**). This compound showed a fragment at *m/z* 404.1842 in its HRMS, which is formed from the molecular ion by loss of two molecules of water. In consequence, the molecular formula of this substance must be $C_{22}H_{32}O_9$. Its ^1H NMR spectrum showed signals of two angular methyls, an isopropyl group, a secondary methyl, an acetoxyl group and its geminal proton. This last signal appears as a singlet at δ 5.10, which was similar to that observed for the resonance of H-14 in ryanodol monoacetate (**8**) (δ 5.09, *s*). The ^{13}C NMR spectrum showed resonances of six methyl groups, three methylenes, three methines and six tetra-substituted carbons, five of which bear one oxygen atom (δ 83.2, 85.3, 86.0, 86.7 and 94.5) and one two oxygens (δ 100.7). In this spectrum an oxo group at δ 210.6 was also observed. The high resonance value for C-2 (δ 40.3) indicated that this carbonyl group must be located at C-1 or C-3. The C-1 position was discarded because the cyclohexanone carbon resonances were very different

from those observed in cinnzeylanone (**5**). The C-3 position was also confirmed by the low field shown by the two H-4 (δ 2.28 and 3.12) and the H-2 (δ 2.60). The ^{13}C NMR are presented in Table 1.

The structure of 2,3-didehydrocinnzeylanone (**4**) was assigned to another diterpene, which was isolated in a very small amount. The molecular ion appears at *m*/*z* 380 in its MS. The IR and UV showed absorbances of an α,β-unsaturated oxo group. In its ^{1}H NMR spectrum the resonance appeared of two angular methyls, an isopropyl group, the methyl over the 2,3-double bond (δ 1.85, *br s*), the vinylic proton (δ 6.74, *br d*) and the two H-4 at δ 2.28 (*dd*) and 2.94 (*dt*). Double resonance experiments, irradiating at H-3 and the two H-4, confirmed these assignments. We could run the ^{13}C NMR with difficulties, due to the scarcity of the sample available, assigning it by comparison with that of cinnzeylanone (**5**), and thus confirming the structure given to this compound.

Another substance isolated from this species was identified as anhydrocinnzeylanine (**2**), which had been obtained from *Cinnamomum cassia* (Yagi et al., 1980). The ^{13}C NMR signals have now been assigned and are listed in Table 1.

The biological activities of ryanoid compounds have been studied to some extent. Ryanodine (**9**) (Wiesner et al., 1967) is a very potent nonselective probe for the Ca^{2+} release channels of the housefly, cockroach and mouse muscle membranes and also a nonselective injected toxicant for insects and mammals, as well as an antifeedant against *S. litura* (Waterhouse et al., 1987; Jefferies et al., 1992; González-Coloma et al., 1996). Furthermore, a previous study has shown that ryanodol diterpenes are more effective antifeedants and less toxic than the ryanodine-type ones, with cinnzeylanone (**5**) and cinnzeylanine (**6**) being the most promising molecules against *Spodoptera littoralis* and *Leptinotarsa decemlineata* (Colorado potato beetle, CPB), respectively (González-Coloma et al., 1999).

Continuing with the study of the antifeedant activity of the ryanodane diterpenes from *P. indica*, we have evaluated the biological action of the minor diterpenes isolated from this plant. Table 2 shows the antifeedant effects of garajonone (**3**) and the anhydroryanodane derivatives **1** and **2**. The effects of **5**, **6**, **8** and **9** have been included for comparison purposes. Among the test compounds, only **1** proved to be an effective antifeedant against both insect species, with an activity level ten times lower than the structurally related ryanoid **6** against *S. littoralis*.

To check for sublethal effects of the test compounds on *S. littoralis*, sixth-instar larvae (L6), with and without pre-treatment with the polysubstrate monooxygenase inhibitor PBO (piperonyl butoxide), were injected with 10 μg each in order to identify detoxification-mediated effects on their consumption indices (RCR, Relative Consumption Rate, and RGR, Relative Growth Rate). The effects of the test compounds on *S. littoralis* nutritional indices are presented in Table 3 and expressed as percent of control for comparison purposes. The toxicity of **1** did not show a great variation with PBO pre-treatment, indicating that this compound was not detoxified by *S. littoralis* polysubstrate monooxygenases (PSMOs). None of the test ryanoids was toxic when injected to adult CPB in the absence or presence of PBO (Table 3).

The activity of the test compounds varied with the insect species. Thus, *L. decemlineata* was less sensitive to the antifeedant and toxic actions of the test compounds

Table 1
NMR data of compounds **1**–**5** (50 MHz)

C	1	2	3	4	5
1	213.4	74.8	33.3	n.o.[c]	214.9
2	41.1	32.4	40.3	134.1	41.6
3	30.7	27.2	210.6	140.2	31.0
4	26.1	25.9	45.3	33.1	26.5
5	85.1	84.1	86.0[a]	n.o.	81.3
6	97.7	94.5	85.3	88.1	88.2
7	86.8[a]	88.0	94.5	97.2	97.6
8	87.2[a]	90.5	86.7[a]	88.1	88.2
9	47.1	46.8	46.9	47.4	47.6
10	40.5	39.9	42.2	42.0	41.7
11	166.3	167.5	100.7	101.6	101.7
12	145.7	146.0	63.2	64.7	64.7
13	132.3	132.0	83.2	82.0	81.9
14	37.9	37.6	75.1	50.8	50.5
15	13.3[b]	1.7	14.4	15.1	13.0
16	13.0[b]	13.1	10.5	10.1	10.4
17	10.7	17.7	9.3	9.3	9.5
18	27.0	10.9	32.9	32.9	32.9
19	20.0	20.1	17.4	18.3	18.2
20	20.3	20.3	18.0	18.4	18.3

[a] These values can be interchanged.
[b] These values can be interchanged.
[c] Not observed.

Table 2
Effective antifeedant doses (EC_{50}) of the test compounds **1**–**3** and related ryanoids **4**–**7** against *S. littoralis* larvae and *L. decemlineata* adults in choice assays

Compound	*S. littoralis* EC_{50} (95% CL)[a] (nmol/cm^2)	*L. decemlineata* EC_{50} (95% CL)[a] (nmol/cm^2)
1	>27	>27
2	0.22 (0.04, 1.23)	2.29 (0.83, 6.15)
3	>23	>23
5[b]	1.46 (0.21, 10.21)	0.22 (0.01,2.88)
6[b]	0.01 (3.0×10^{-3}, 7.0×10^{-2})	C[c]
8[b]	0.63 (0.09, 4.41)	0.57 (0.03, 9.77)
9[b]	0.93 (0.42, 10.21)	0.77 (0.25, 3.71)

[a] 95% Confidence limits (lower, upper).
[b] From González-Coloma et al., 1999.
[c] Knock-down-like effects at 10 μg/cm^2.

Table 3
Relative consumption (RCR) and growth (RGR) rates of *S. littoralis* L6 larvae orally injected with 10 μg of the test compounds with or without PBO pre-treatment (5 μg)[a]

	S. littoralis				*L. decemlineata*	
	−PBO		+PBO		Mortality (%)	
Compound	RCR[b]	RGR[c]	RCR[b]	RGR[c]	−PBO	+PBO
1	100	100	93	93	0	0
2	72	77	75	90	0	3
3	55[d]	57[d]	64[d]	64[d]	0	13
5[f]	94	82	100	74	0	24
6[f]	90	80	66	100	0	61[e]
8[f]	74	62	95	100	16	52[e]
9[f]	70	47[d]	69	61[d]	0	13

[a] Data is expressed as % of control. Haemolymph injection (5 μg, $n=20$) effects of the test compounds on *L. decemlineata* adults (% mortality after 48 h) without and with PBO pre-treatment (5 μg).

[b] RCR = $I/(BI)\times T$, I = mg food consumed, T = feeding period (days), BI = initial insect weight (mg).

[c] RGR = $\Delta B/(BI)\times T$, B = change in insect body weight (mg).

[d] Denotes a significant difference from the control, $P<0.05$ LSD test.

[e] Denotes a significant difference from the control; contingency table analysis ($P<0.05$).

[f] From González-Coloma et al., 1999.

than *S. littoralis*, and we did not find any correlation between antifeedant and toxic effects of **2** and **3**, as previously shown for other structurally-related *P. indica* ryanoids (González-Coloma et al., 1999). This lack of a behavioural–toxicity relationship may reflect the polarity requirements for gustatory versus internal target site interactions (Mullin et al., 1997).

The comparative study of their structure-activity relationships showed that changes in the polarity of the cyclohexane ring associated with both C-1 and C-3 substituents determined the loss of the antifeedant effect with an increase in toxicity (**3** versus **8**). The importance of the C-1 substituent and the polarity of the cyclohexane ring have been previously established for this type of activity (González-Coloma et al., 1996, 1999; Fraga et al., 1997). Furthermore, both anhydro-compounds (**1** and **2**) were less potent antifeedants (both insects) or toxicants (only CPB) than the more polar related ones **5** and **6**, respectively, indicating that the presence of the hemiketal group at C-11 is important for the antifeedant effects of the ryanodane diterpenes.

Available evidence suggests that a ligand-protein receptor interaction is mediating insect taste transduction (Mullin et al., 1997). Previous results suggest a common ligand-gated ion channel mediated taste response to non-alkaloidal ryanoids, with the lepidopteran being more sensitive than the chrysomelid beetle, as previously shown for aconitine, a sodium channel antagonist diterpene alkaloid (González-Coloma et al., 1998, 1999). The results presented here for the antifeedant action of **2** further support this hypothesis.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR were taken in $CHCl_3$; MS: direct inlet 70 eV. Column chromatography was performed on silica gel 0.063–0.2 mm. Ryanodine was purchased from Sigma.

3.2. Isolation of the products

P. indica branches were collected at Monte de Las Mercedes, Tenerife, in March. Air-dried, chopped, aerial parts (1.3 kg) were extracted with ethanol in a Soxhlet apparatus. The cold extract was filtered and then concentrated in vacuo to afford a syrupy gum (307 g). This gum was treated with EtOAc and the solution separated by filtration. The solvent was evaporated off and the residue (138 g) chromatographed in silica gel eluting with petrol–EtOAc mixtures. In this way, after several dry column rechromatographies, the following diterpenes were obtained: anhydrocinnzeylanine (**2**) (9 mg), 2,3-didehydrocinnzeylanone (**4**) (1 mg), anhydrocinnzeylanone (**1**) (7 mg) and garajonone (**3**) (4 mg).

3.3. Anhydrocinnzeylanone (*1*)

$[M\text{-}H_2O]^+$ at m/z 346.1795, $C_{20}H_{26}O_5$ requires 346.1780; ^{1}H NMR (400 MHz): δ 0.95 and 1.00 (each 3H, *d*, $J=6$ Hz, H-19 and H-20), 1.09 (3H, *d*, $J=6$ Hz, H-15), 1.15 (3H, *s*, H-16), 1.64 (3H, *s*, H-17), 2.16 (1H, *dd*, J = 18 and 2.5 Hz, H-14), 2.45 and 2.52 (each 1H, *d*, $J=20$ Hz, H-10), 2.64 (1H, *d*, $J=18$ Hz, H-14), 2.67 (1H, *m*, H-18), 3.19 (1H, *m*, H-2), 3.86, 5,15 and 6.88 (each 1H, *br s*,-OH); EIMS m/z (rel. int.): 346 $[M\text{-}H_2O]^+$ (2), 287 (12), 194 (100), 166 (42), 154 (55), 151 (19), 139 (30).

3.4. Anhydrocinnzeylanine (*2*)

^{1}H NMR (200 MHz): δ 0.93 and 0.96 (each 3H, *d*, $J=6$ Hz, H-19 and H-20), 1.09 (3H, *s*, H-16), 1.60 (3H, *br s*, H-17), 2.12 and 2.65 (each 1H, *s*, $J=17$ Hz, H-14), 2.18 (3H, *s*), 2.39 (2H, *s*, H-10), 2.69 (1H, *m*, H-18), 3.98, 4.14 and 4.73 (each 1H, *br s*,-OH), 5.33 (1H, *d*, $J=10$ Hz, H-1); EIMS m/z (rel. int.): 390 $[M\text{-}H_2O]^+$ (5), 331 (8), 237 (21), 177 (100), 154 (27), 149 (11), 139 (16).

3.5. Garajonone (*3*)

$[M\text{-}2H_2O]^+$ at m/z 404.1842, $C_{22}M\text{-}HH_{28}O_7$ require 404.1835. ^{1}H NMR (200 MHz): δ 0.83 (3H, *d*, $J=7$ Hz, H-15), 1.05 and 1.09 (each 3H, *d*, $J=6$ Hz, H-19 and H-20), 1.08 and 1.42 (each 3H, *s*, H-16 and H-17), 1.97 (2H, *s*, H-10), 2.10 (3H, *s*, -OAc), 2.28 (1H, *d*, $J=13$ Hz, H-4), 2.60 (1H, *m*, H-2), 3.12 (1H, *dd*, $J=13$ and 2 Hz, H-4), 2.68, 2.93, 3.22, 3.46 and 4.78 (each 1H, *br s*,-OH),

5.10 (1H, *s*, H-14); EIMS *m/z* (rel. int.): 404 $[M\text{-}2H_2O]^+$ (5), 361 (7), 344 (7), 319 (11), 301 (7), 221 (8), 193 (100), 152 (97).

3.6. 2,3-Didehydrocinnzeylanone (*4*)

IR ν_{max} (film) cm^{-1}: 3400, 2930, 1670, 1600, 1450, 1380, 1090; UV λ_{max} (EtOH) nm: 274 ^1H NMR (400 MHz); δ 1.01 (6H, *d*, $J=6.4$ Hz, H-19 and H-20), 1.06 and 1.35 (each 3H, *s*, H-16 and H-17), 1.85 (3H, *br s*, H-15), 2.28 (1H, *dd*, 14 and 4 Hz, H-4), 1.92 and 2.59 (each 1H, *d*, $J=16$ Hz, H-14), 2.94 (1H, *dt*, $J=14$ and 3, H-4), 6.74 (1H, *br d*, $J=3$ Hz, H-3); EIMS *m/z* (rel. int.): 380 $[M]^+$ (4), 256 (30), 213 (28), 199 (12).

3.7. Acid treatment of cinnzeylanone

Compound **5** (30 mg) was refluxed in 5% HCl–MeOH (1 ml) for 20 min. Evaporation of the solvent and chromatography of the residue, eluting with petrol–EtOAc (3:1), gave anhydrocinnzeylanone (**1**) (22 mg).

3.8. Insect cultures

L. decemlineata and *S. littoralis* colonies were reared on potato foliage and artificial diet, respectively, and maintained at 24±1°C, >70% (relative humidity) with a photoperiod of 16:8 h (L:D) in a growth chamber.

3.9. Insect bioassays

3.9.1. Choice feeding assays (⩽6 h)

These experiments were conducted with adult *L. decemlineata* (Colorado potato beetle, CPB) and newly-emerged fifth-instar *S. littoralis* larvae (González-Coloma et al., 1999). Percent feeding reduction (% FR) was determined for each arena by the equation % FR = [1−(treatment consumption/control consumption)] ×100. Compounds with an FR > 50% were tested in a dose–response experiment to calculate their relative efficacy (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (% FR on log dose).

3.9.2. Oral cannulation

This experiment was performed with pre-weighed newly emerged (24 h) *S. littoralis* L6-larvae in the absence or presence of PBO under the same environmental conditions as above. PBO pre-treated larvae (+PBO) were orally injected with 10 μg of this agent in 2 μl DMSO as previously described (González-Coloma et al., 1999). At the end of the experiments (72 h), the Relative Consumption Rate (RCR) and the Relative Growth Rate (RGR) were calculated on a dry weight basis (Farrar et al., 1989). All dry larval weight measures were log-transformed prior to an analysis of variance (ANOVA) to check for treatment effects. Treatment means differences were checked with least significant difference (LSD) tests.

3.9.3. Hemolymph injection

DMSO solutions of the test alkaloids (10 μg each per insect) were injected as previously described (González-Coloma et al., 1998). Pre-treated insects were injected with 5 μg of PBO 1 h prior to the injection of the test compounds. Toxicity symptoms and mortality were recorded up to 2 days after injection by maintenance of beetles on their respective potato leaf food. Percent mortality was analysed with contingency tables and corrected according to Abbott (1925).

Acknowledgements

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Constituents of the essential oil of *Cyperus alopecuroides*

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Abstract

We have investigated the constituents of the essential oil of *Cyperus alopecuroides*. Three new compounds, the hydrocarbon, (−)-eudesma-2,4(15)-11-triene, the sesquiterpene alcohol (−)-eudesma-3,11-dien-5-ol and the diterpene hydrocarbon (−)-dolabella-3,7,18-triene were isolated and their structure elucidated. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Cyperus alopecuroides*; *Cyperaceae*; Sesquiterpene; Diterpene; (−)-Eudesma-2,4(15),11-triene; (−)-Eudesma-3,11-dien-5-ol; (−)-Dolabella-3,7,18-triene

1. Introduction

The genus *Cyperus* of the familly of the Cyperaceae is well-known for its essential oil (Couchman et al., 1964). Hydrodistillation of the rhizomes of *Cyperus alopecuroides* yields a yellow oil with a pleasant odour. The isolation of the sesquiterpenes, cyprotene (**1**), cypera-2,4-diene (**2**), epoxycyperene (**3**), eudesma-2,4,11-triene (**4**) and eudesma-3,5,11-triene (**5**) (Fig. 1) from this oil has been formerly reported (Mekem et al., 1997). We have completed our investigation of the same essential oil and report in this paper the isolation and characterisation of the two new sesquiterpenoids **6** and **7** and the diterpene **8**.

2. Results and discussion

The investigation of the essential oil of *C. alopecuroides* by chromatographic methods and GC–MS has enabled the identification of many sesquiterpenoids including α-cubebene (**10**) (Joulain and König, 1998), α-copaene (**11**), (Bohlmann and Zdero, 1978), β-caryophyllene (**12**), (Joulain and König, 1998), α-humulene (**13**), (Joulain and König, 1998), cyperene (**14**) (Trivedi et al., 1964), rotundene (**15**), (Panikar and Motl, 1977), α-selinene (**16**), (Klein and Rojahn, 1970), β-selinene (**17**) (Maurer and Grieder, 1977), *trans*-calamenene (**18**) (Joulain and König, 1998), δ-cadinene (Joulain and König, 1998), cyperol (Hikino et al., 1967), isocyperol (Hikino et al., 1967), cyperenal (Takano and Kawaminami, 1988) and eudesma-3,11-dien-2-one (Demole and Enggist, 1983) (Fig. 1). In addition, we could identify two new eudesmane sesquiterpenes **6** and **7** and a dolabellane diterpene hydrocarbon **8**.

The hydrocarbon **6** was isolated from the essential oil of *C. alopecuroides* by preparative GC. This was assigned the molecular formula $C_{15}H_{22}$ ($[M]^+$; $m/z=$ 202). The ^{13}C-NMR and DEPT spectra allow the identification of 15 carbon atoms: two methyl groups, six methylene groups, two of which being olefinic, four methine groups, two of which are vinylic and three quaternary carbon atoms, two being olefinic. The ^{1}H-NMR spectrum shows two protons in the olefinic region (δ 4.83) with a coupling correlation with a vinylic methyl group at δ 1.68 as indicated in the ^{1}H-^{1}H-COSY diagram, which suggests the presence of an isopropenyl substituent in the molecule. Further examination of the ^{1}H-NMR spectrum reveals a methylene group (δ 1.72 and δ 1.88) coupling with an olefinic proton at δ 5.58, itself coupled to another olefinic proton at δ 6.19. Moreover, one can observe in the ^{1}H-^{1}H-COSY diagram coupling correlations of these two olefinic protons with the two methylene protons at δ 4.83. These informations lead to the substructure –CH2–CH=CH–C=CH_2. Coupling correlations are also observed between the tertiary methyl group at δ 0.77 and methylene group (δ 1.09 and δ 1.42), which couples with another methylene group (δ 1.38 and δ 1.50). The latter methylene group further

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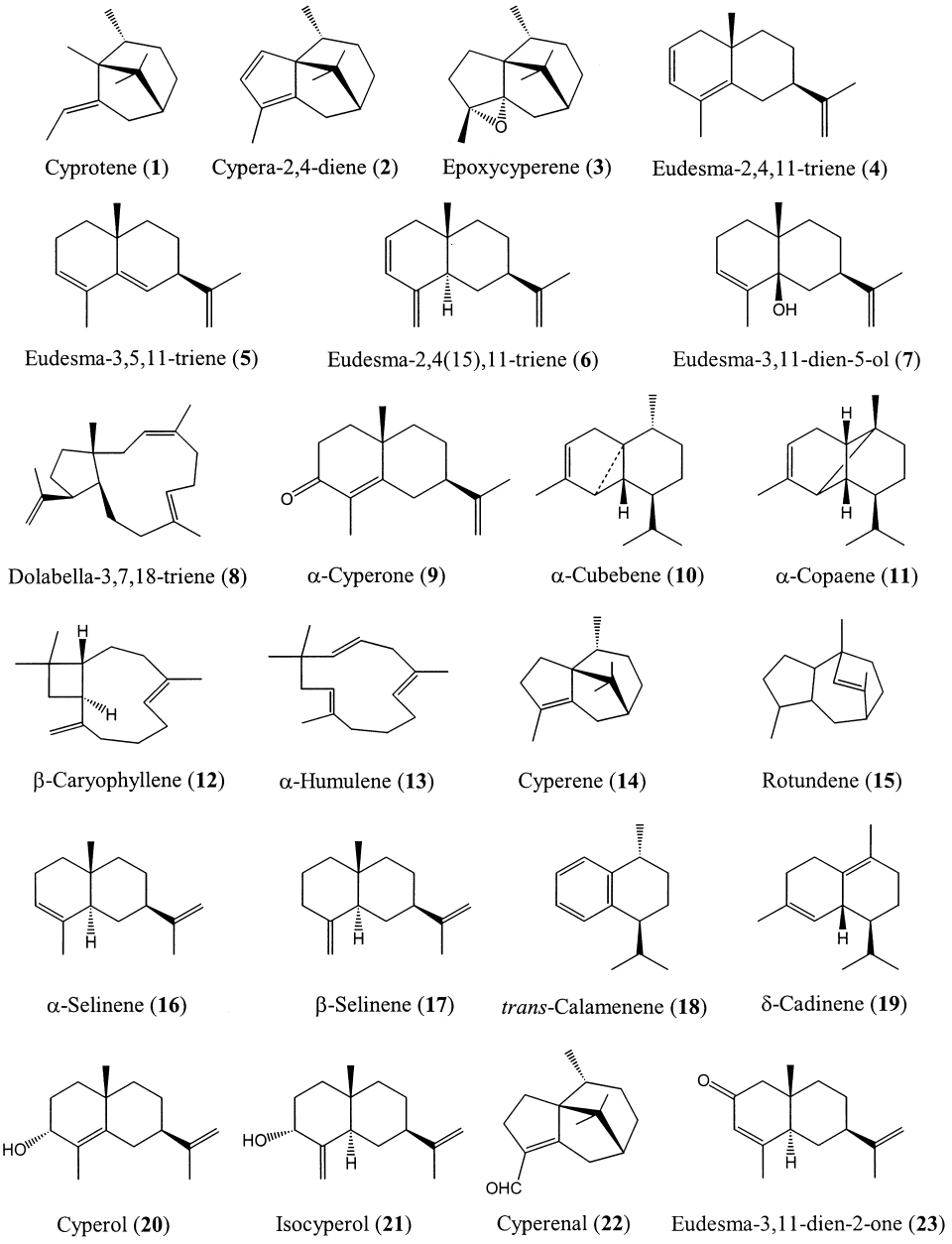


Fig. 1. Terpenoids from *Cyperus alopecuroides*.

couples with a methine proton (δ 2.00), itself coupled to another methylene group (δ 1.29 and δ 1.83) which couples again to a methine proton (δ 1.89). From these data the substructure CH_3–C–CH_2–CH_2–CH–CH_2–CH- is derived. In combining the three substructures, an eudesmane type skeleton is obtained. The relative stereochemistry of the compound follows from the exploitation of the coupling constants in the ^{1}H-NMR-spectrum. The *trans*-configuration of the decalin skeleton results from the antiperiplanar coupling ($J = 12.0$ Hz) between H-5 and H-6α (Fig. 2). The proton H-7 exhibits an axial coupling constant ($J = 12.0$ Hz) indicating that the

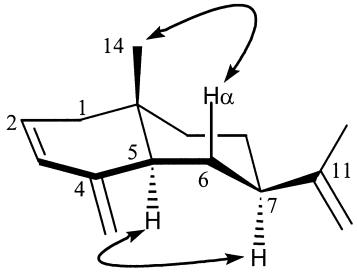


Fig. 2. Important proton–proton spatial correlations from the NOESY diagram of **6**.

isopropenyl group is equatorial. Moreover, NOESY correlations are observed between the methyl group protons H-15 and H-6α and also between H-5 and H-7 (Fig. 2). Compound **6** has the absolute configuration shown in Fig. 2 since it could be identified by GC and GC–MS as a minor product in the formation process of (−)-eudesma-2,4,11-triene and (−)-eudesma-3,5,11-triene from α-cyperone of known absolute configuration (Howe and McQuillin, 1955), which was isolated from the same plant (Mekem et al., 1997).

In order to analyse the low boiling part of the oil, a combination of column chromatograhy at low temperature (−20°C) and gas chromatography was used. From a late hydrocarbon fraction a compound was isolated with a longer retention time compared to sesquiterpene hydrocarbons. The unknown compound was assigned the molecular formula $C_{20}H_{32}$ of a diterpene hydrocarbon in agreement with the molecular ion in its mass spectrum ($[M]^+$, $m/z = 272$). The ^{13}C-NMR spectrum shows twenty carbon signals, six being olefinic. From the DEPT spectrum three methyl groups, eight methylene groups, one being olefinic, four methine carbon signals, two of which are olefinic and four quaternary carbon atoms, three being olefinic, were assigned. The interpretation of the proton NMR spectrum and the ^{1}H-^{1}H-COSY diagram allowed the formation of three substructures. First of all the presence of an isopropenyl group was indicated by the coupling correlations between the two olefinic methylene protons at δ 4.58 and δ 4.76 (CH_2-19, Fig. 3) and the olefinic methyl group CH_3-20 at δ 1.65. The methine proton at δ 1.69 (CH-11) couples with the methylene group CH_2-10 (δ 1.16, δ 1.27) which couples with another methylene group CH_2-9 (δ 1.82 and δ 2.07). The methine proton CH-11 couples with another methine proton CH-12 (δ 2.57), which again couples to the methylene group CH_2-13 (δ 1.50 and δ 1.57), which further couples with a methylene group CH_2-14 (δ 1.36 and δ 1.47) giving the substructure –CH_2–CH_2–CH–CH–CH_2–CH_2–. The olefinic proton CH-7 (δ 4.79) couples with the methylene group CH_2-6 (δ 2.21 and δ 2.90) itself coupled to the methylene group CH_2-5 (δ 2.01 and δ 2.14) to give the partial structure –CH_2–CH_2–CH=C. Finally the methylene group CH_2-2 (δ 1.64 and δ 2.15) couples with the olefinic proton CH-3 (δ 5.07) leading to the substructure –CH_2–CH=C. The combination of the four substructures and subsequent localisation of the methyl groups was achieved with the help of proton-carbon long-range correlations obtained from the HMBC diagram. Four sets of spatial interactions between different protons indicated by the NOESY technique helped to establish the relative configuration of the molecule (Fig. 3). The methyl group CH_3-15 shows spatial interactions with the methyl group CH_3-20 as well as with the two olefinic protons H-7 and H-3. Furthermore, the proton H-11 interacts with the protons H-12 and H-7. The drawn conformation corresponds to that of minimal energy and was calculated by the program MM2 (Fig. 4). In conclusion, compound **8** is a diterpene with a dolabellane skeleton. Its absolute configuration was not investigated. The structure and chemistry of some 140 naturally occuring dolabellane type diterpenes from molluscs, soft corals, liverworts and higher plants have recently been reviewed (Rodriguez et al., 1998). To our knowledge compound **8** was never identified as a natural compound before.

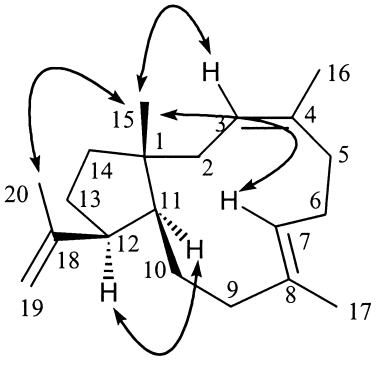


Fig. 3. Relative configuration of **8** with important NOESY correlations.

The alcohol **7** was isolated from the oxygenated fraction of the essential oil of *C. alopecuroides* by combination of preparative GC and thin layer chromatography. The pure compound **7** was colourless and was attributed the molecular formula $C_{15}H_{24}O$ by mass spectrometry ($[M]^+$, $m/z = 220$), which means that the sum of rings and double bonds is 4. The ^{13}C-NMR spectrum reveals two double bonds, one of which is characteristic of an isopropenyl group (δ 108.5, C-12, δ 149.4, C-11). Compound **7** has therefore a bicyclic structure. The constitution of the molecule is derived from the interpretation of the ^{1}H-NMR and ^{1}H-H-COSY spectra. The two olefinic protons at δ 4.64 couple with the olefinic methyl group at δ 1.75, which confirms the presence of an isopropenyl group in the molecule. The methylene group CH_2-1 (δ 1.21 and δ 1.76) couples with another methylene group CH_2-2 (δ 1.96 and δ 2.13), which itself couples with the olefinic proton CH-3 (δ 5.54). The latter further couples to the olefinic methyl group CH_3-15 (δ 1.75), giving the substructure CH_2–CH_2–CH=C–CH_3. The methyl group CH_3-14 shows a long-range coupling with the methylene group CH_2-9 (δ 1.24 and δ 1.64), which couples again with the methylene group CH_2-9 (δ 1.24 and δ 1.64), which couples again with the methylene group CH_2-8 (δ 1.45) itself coupled to the methine proton CH-7 (δ 1.72) which further couples with the methylene group CH_2-6 (δ 1.52 and δ 1.54) to give the substructure CH_3–C-CH_2–CH_2–CH–CH_2–. The alcoholic nature of the compound was deduced from the its mass spectrum which shows a peak for [M]+-H_2O. Furthermore, the ^{13}C-NMR off-reso-

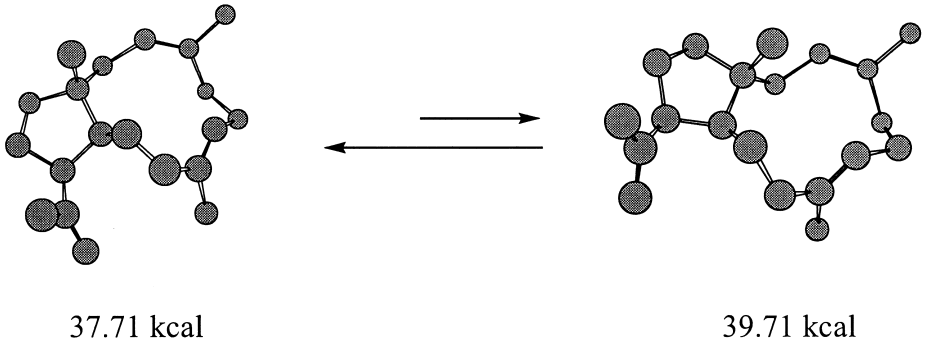


Fig. 4. Low energy conformations of **8** as calculated by MM2.

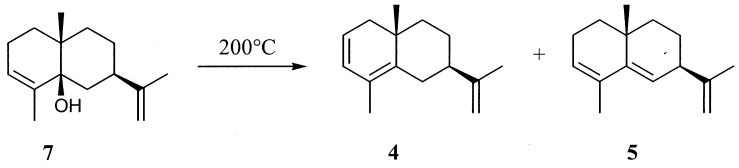


Fig. 5. Deydration of alcohol **7** at higher temperature.

nance spectrum shows a singlet at δ 73.2 indicating a tertiary alcohol. Further proof of the tertiary nature was given by the difficulty to perform a phase-transfer catalysed methylation of the alcohol. The absolute configuration of the molecule was deduced from the following facts: Dehydration of **7** at 200°C yielded the hydrocarbons **4** and **5,** both of kown absolute configuration (Mekem et al., 1997) (Fig. 5) and the two methyl groups CH3-14 and CH3-15 of **7** show a long-range coupling correlation to each other in the ^{1}H-^{1}H-COSY spectrum. This observation and the known requirements for a J^6 coupling through σ-bonds (Schaefer, 1996) is only possible in case of a *cis*-fusion of the decalin system of **7** which corresponds to the absolute configuration of **7**, as drawn in Fig. 6.

3. Experimental

3.1. Plant material

The collection of *C. alopecuroides* and the preparation of the essential oil was described in a previous publication (Mekem et al., 1997).

3.2. NMR spectroscopy

NMR measurements were carried out with a Bruker WM 400 instrument for 1D and ^{1}H-^{1}H-COSY spectra and with Bruker WM 500 instrument for HMBC, HMQC and NOESY spectra. TMS was used as internal standard.

3.3. GC–MS

GC–MS measurements were carried out on a Hewlett Packard HP 5890 gas chromatograph coupled to a VG Analytical 70-250S mass spectrometer.

3.4. Preparative GC

The essential oil of *C. alopecuroides* was preliminary separated by preparative GC, as reported earlier (Mekem et al., 1997), yielding twenty fractions. The treatment of the sixth fraction CA6 by preparative GC gave two subfractions, one containing α-selinene and β-selinene and the other containing **6** as pure compound.

3.5. Polarimetry

Perkin-Elmer 241 polarimeter was used. Only the sense of optical rotation was determined because of the small quantity of isolated material.

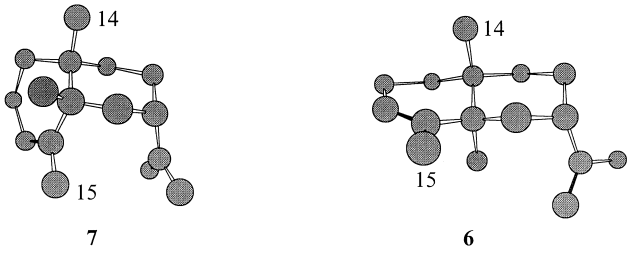


Fig. 6. Two possible isomers of alcohol **7.** Only the left structure displays the geometry which would allow long-range coupling between H-14 and H-15.

3.6. (−)-Eudesma-2,4(15),11-triene (**6**)

^{1}H-NMR (400 MHz, $CDCl_3$): $\delta = 0.77$ (*s*, 3H), 1.09 (*ddd*, 1H, $J_1 = J_2 = 12.6$ Hz, $J_3 = 5.7$ Hz), 1.29 (*q*, 1H, $J_1 = J_2 = J_3 = 12.0$ Hz), 1.38 (*ddd*, 1H, $J_1 = J_2 = 12.6$ Hz, $J_3 = 3.8$ Hz), 1.42 (*dd*, 1H, $J_1 = 12.6$ Hz, $J_2 = 2.80$ Hz), 1.50 (*qt*, 1H, $J_1 = 12.6$ Hz, $J_2 = 5.7$ Hz, $J_3 = J_4 = 2.8$ Hz), 1.72 (*dd*, 1H, $J_1 = 18.3$ Hz, $J_2 = 5.6$ Hz), 1.83 (*dt*, $J_1 = 12.0$ Hz, $J_2 = J_3 = 3.8$ Hz), 1.88 (*d*, 1H, $J = 18.3$ Hz), 1.89 (*dd*, 1H, $J_1 = 12.0$ Hz, $J_2 = 3.8$ Hz), 2.00 (*dq*, 1H, $J_1 = 12.0$ Hz, $J_2 = J_3 = J_4 = 2.8$ Hz), 4.83 (*m*, 2H), 5.58 (*qt*, $J_1 = 9.7$ Hz, $J_2 = 5.6$ Hz, $J_3 = J_4 = 2.0$ Hz), 6.19 (*dd*, 1H, $J_1 = 9.7$ Hz, $J_2 = 3.1$ Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): 17.11 (*q*), 20.98 (*q*), 27.23 (*t*), 29.52 (*t*), 33.73 (*s*), 41.10 (*t*), 43.18 (*t*), 45.24 (*d*), 45.79 (*d*), 108.92 (*t*), 110.03 (*t*), 126.99 (*d*), 130.61 (*d*), 146.50 (*s*), 150.43 (*s*). MS (EI, 70 eV), *m*/*z* (rel. int.): 202(47) $[M]^+$, 187(22), 173(14), 159(30), 145(51), 131(50), 119(53), 105(77), 79(59), 67(36), 53(39), 41(86).

3.7. Isolation of (−)-dolabella-3,7,18-triene (**8**)

In order to investigate the oxygenated part of the essential oil, it was worth beginning with a prefractionation of the raw oil by column chromatography at low temperature (−20° C) with petroleum ether as the eluent. From this first separation process eight fractions were obtained. The four first fractions contained only hydrocarbons. The fifth fraction CA5 also contained only hydrocarbons, but an interesting fact was that one of these hydrocarbons had a very high retention time. GC–MS analysis of the sample showed that it was a diterpene. Its isolation was achieved by preparative GC using a Varian 1400 instrument, equipped with a stainless steel column (1.85 m × 4.3 mm) with 10% polydimethylsiloxane SE 30 on Chromosorb W-HP. Helium was used as carrier gas at a flow rate of 240 ml/min.

3.8. (−)-Dolabella-3,7,18-triene (**8**)

^{1}H-NMR (500 MHz, $CDCl_3$) :1.00 (3H, *s*), 1.16 (1H, *m*), 1.27 (1H, *ddd*, $J_1 = 19.5$ Hz, $J_2 = 10.1$ Hz, $J_3 = 5.0$ Hz), 1.36 (1H, *ddd*, $J_1 = 13.2$ Hz, $J_2 = 9.5$ Hz, $J_3 = 7.5$ Hz), 1.44 (3H, *s*), 1.45 (3H, *s*), 1.47 (1H, *m*), 1.50 (1H, *m*), 1.57 (1H, *m*), 1.64 (3H, *s*), 1.64 (1H, *m*), 199 (1H, *m*), 2.01 (1H, *m*), 2.14 (1H, *m*), 2.15 (1H, *dd*, $J_1 = 13.2$ Hz, $J_2 = 11.3$ Hz), 2.21 (1H, *m*), 4.59 (1H, *m*), 4.76 (1H, *m*), 4.79 (1H, *b(br)*, $J = 10.7$ Hz), 5.07 (1H, *dd*, $J_1 = 11.3$ Hz, $J_2 = 4.4$ Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) : δ 16.00 (*q*), 17.05 (*q*), 23.90 (*q*), 24.74 (*q*), 24.81 (*t*), 28.89 (*t*), 30.08 (*t*), 38.22 (*t*), 40.28 (*t*), 42.13 (*d*), 42.68 (*d*), 43.76 (*d*), 46.88 (*s*), 51.41 (*d*), 111.53 (*t*), 126.17 (*d*), 127.72 (*d*), 134.33 (*s*), 134.88 (*s*), 147.28 (*s*). MS (EI, 70 eV), *m*/*z* (rel. int.) : 272 (22) $[M]^+$, 257 (12), 229 (12), 215 (6), 203 (8), 189 (25), 175 (30), 161(25), 147 (50), 135 (31), 121 (81), 107 (82), 93 (100), 81 (89), 79 (65), 77 (37), 68 (70), 67 (71), 55 (72), 41 (90).

3.9. Isolation of (−)-eudesma-3,11-dien-5-ol (**7**)

The sixth fraction CAT6 contained four major peaks consisting of caryophyllene oxide, cyperotundone, α-cyperone and an unknown alcohol. To isolate the unknown compound, the sample was submitted to preparative GC. The separation which was monitored by analytical GC and GC–MS yielded six other fractions.

The sixth fraction CAT6.6 contained α-cyperone. The fourth fraction CAT6.4 contained the unknown alcohol as major product, but the intention to purify it by preparative GC was unsuccesfull with all the available columns. Finally preparative TLC with the system chloroform–methanol (10:1) as eluent was used and succeeded in eliminating the most disturbing impurities. The major band of the TLC plates was taken up in diethyl ether and after evaporation of the solvent the sample was treated by preparative GC to remove the final impurities and 3 mg of (−)-eudesma-3,11-dien-5-ol were obtained.

3.10. (−)-Eudesma-3,11-dien-5-ol (**7**)

^{1}H-NMR (400 MHz, $CDCl_3$) : 1.08 (3H, *s*), 1.21 (1H, $J_1 = 13.9$ Hz, $J_2 = 6.3$ Hz), 1.24 (1H, *dt*, $J_1 = 13.6$ Hz, $J_2 = J_3 = 3.1$ Hz), 1.45 (1H, *dd*, $J_1 = 12.3$ Hz, $J_2 = 3.8$ Hz), 1.52 (1H, *m*), 1.54 (1H, *t*, $J = 12.9$ Hz), 1.64 (1H, *m*), 1.72 (1H, *m*), 1.75 (6H, *s*), 1.76 (1H, *m*), 1.88 (1H, *dt*, $J_1 = 12.9$ Hz, $J_2 = J_3 = 2.2$ Hz), 1.96 (1H, *ddddd*, $J_1 = 18.8$ Hz, $J_2 = 6.3$ Hz, $J_3 = 5.1$ Hz, $J_4 = J_5 = 1.6$ Hz), 2.13 (1H, *m*), 4.64 (2H, *m*), 5.54 (1H, *m*). ^{13}C-NMR (100 MHz, $CDCl_3$) : 18.85 (*q*), 20.87 (*q*), 22.17 (*q*), 22.28 (*t*), 26.51 (*t*), 31.34 (*t*), 32.78 (*t*), 35.81 (*t*), 36.57 (*s*), 42.85 (*d*), 73.16 (*s*), 108.47 (*t*), 126.47 (*d*), 136.49 (*s*), 149.35 (*s*). MS, (EI, 70 eV), *m*/*z* (rel. int.) : 220 (11) $[M]^+$, 202 (27), 187 (17), 177 (2), 162 (34), 145 (17), 131 (32), 124 (100), 123 (92), 109 (42), 91 (30), 82 (60), 67 (27), 55 (39), 41 (66).

3.11. Dehydration of (−)-eudesma-3,11-dien-5-ol (**7**)

The dehydration of the alcohol **7** occurs at the injection port of the preparative gas chromatograph (200°C). 10% (in g) of the injected product were dehydrated and yielded (−)-eudesma-2,4,11-triene (40%) and (−)-eudesma-3,5, 11-triene (60%). The spectral data of both hydrocarbons were already reported (Mekem et al., 1997).

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Secoiridoid constituents from the fruits of *Ligustrum lucidum*

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Abstract

Two secoiridoid glucosides, lucidumosides A and B, as well as six known glucosides, oleoside dimethyl ester, ligustroside, oleuropein, nuezhenide, isonuezhenide, and neonuezhenide, were isolated from the fruits of *Ligustrum lucidum*. Their structures were elucidated by spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Ligustrum lucidum*; Oleaceae; Secoiridoid glucoside; Lucidumoside A; Lucidumoside B

1. Introduction

The fruits of *Ligustrum lucidum* Ait. (Oleaceae) are known as Nuzhenzi and they are commonly used for their tonic effects in Chinese medicine (Li et al., 1994). Previous studies have found volatile components, triterpenes, flavonoids, secoiridoid glucosides, and phenolic compounds from this plant, and constituents such as phenylethanoids, monoterpenes, and secoiridoid glucosides from other species of the genus *Ligustrum* (Inouye and Nishioka, 1972; Garibodi et al., 1986; He et al., 1992, 1994; Tian et al., 1996; Fukuda et al., 1996). Screening studies in our laboratory have found that the ethanol extract of the fruits of *L. lucidum* showed significant inhibitory effects on free radical induced hemolysis of red blood cells. This paper describes the isolation and structure elucidation of six known glucosides, oleoside dimethyl ester (**1**), ligustroside (**2**), oleuropein (**3**), nuezhenide (**4**), isonuezhenide (**5**), and neonuezhenide (**6**) and two new secoiridoid glucosides, named lucidumosides A (**7**) and B (**8**) from the active fraction of the fruits of *L. lucidum* (Scheme 1).

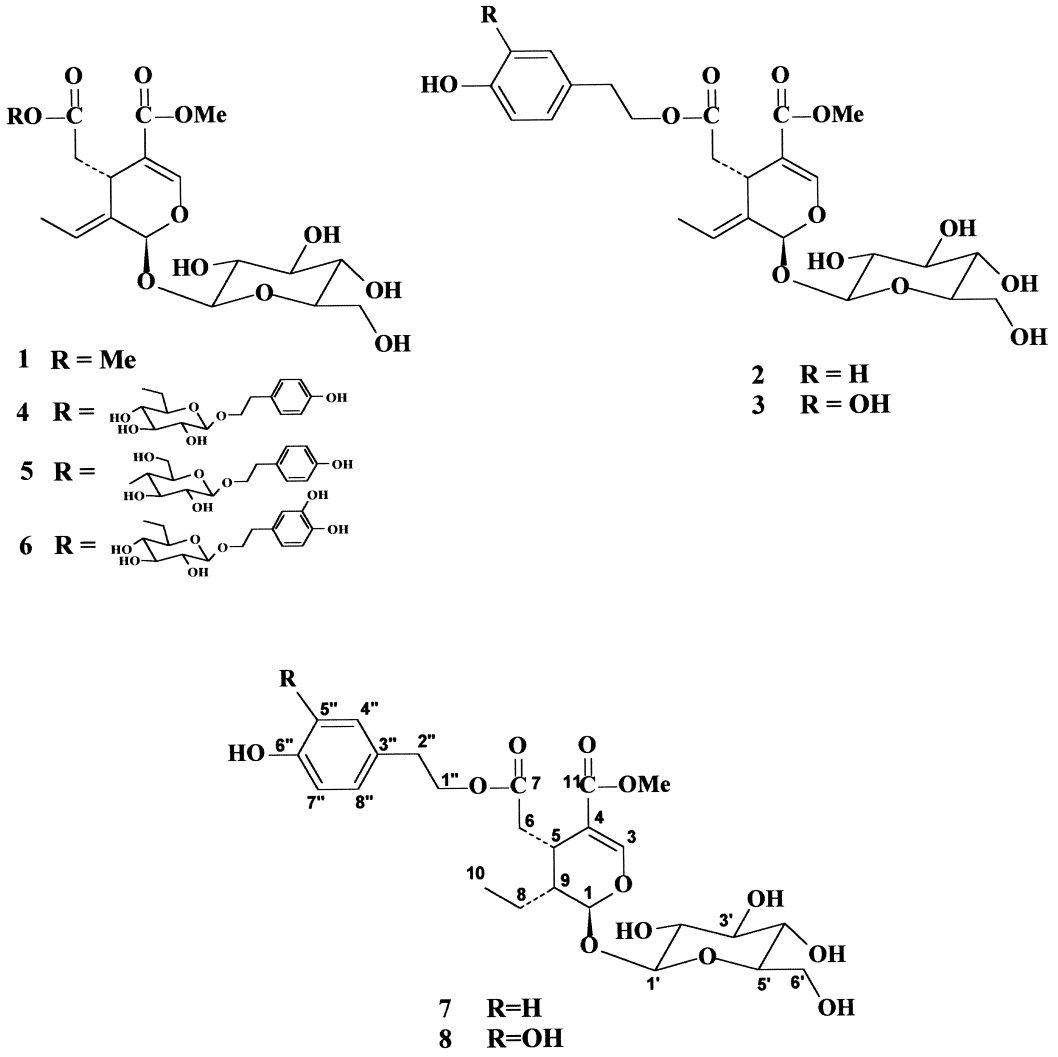
2. Results and discussion

The six known structures, oleoside dimethyl ester (**1**), ligustroside (**2**), oleuropein (**3**), nuezhenide (**4**), isonuezhenide (**5**) and neonuezhenide (**6**), were identified by comparing their ^{1}H and ^{13}C NMR and MS data with those reported in literature (Kikuchi and Yamakuchi, 1985; Garibodi et al., 1986; Fukuyama et al., 1987).

Compound **7** was obtained as a colorless powder. The molecular formula was determined to be $C_{25}H_{34}O_{12}$ ($[M+H]^+$ 527.5455, calc. 527.5457) by positive HRFABMS. The IR spectrum showed the presence of hydroxyl groups (3400 cm^{-1}) and two carbonyl groups (1700 and 1630 cm^{-1}). The ^{1}H and ^{13}C NMR signals of **7** were assigned by using a 2D NMR experiment including ^{1}H-^{1}H and ^{1}H-^{13}C COSY spectral data. In the ^{1}H NMR spectrum, the characteristic signals due to an olefinic proton (H-3) appeared at δ 7.48 as a singlet, an acetalcarbinol proton (H-1) as a doublet at δ 5.42, two methine groups, two methylene groups, one methyl group, and one methoxyl group, were assigned as the secoiridoidic aglycone moiety. The doublet at δ 4.69 was attributed to the anomeric proton of the glucose moiety. In addition, the characteristic aromatic protons of A_2B_2 spin systems at δ 7.06 and δ 6.72 showed with J values of 8.8 Hz together with two methylene groups indicated the presence of a *para*-substituted phenylethyl group (Table 1). The HMBC experiment of **7** showed two ^3J interactions between H-1″ (δ 4.18) and C-7 (δ 174.1) as

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Scheme 1.

well as protons of a methoxyl group (δ 3.64) and C-11 (δ 168.6). The phenylethyl group in compound **7** should be conjugated at C-7. Analysis of the ^{1}H and ^{13}C NMR spectra indicated the aglycone moieties of compounds **7** and **2** were different. The H-8 signals appeared at δ 1.60 and δ 1.75 as two multiplets in compound **7**, whereas the signal at δ 6.08 was a quartet in compound **2**. Futhermore, the ^{1}H NMR spectrum of compound **7** showed its H-9 signal as a multiplet at δ 2.10 and the H-10 signal as a triplet at δ 1.17. This suggested that the bond between C-8 and C-9 in compound **7** was a single bond, which is consistent with the evidence observed in the ^{13}C NMR spectrum (Table 2). In order to define the stereochemistry of compound **7**, a NOESY experiment was performed. The NOESY spectrum of **7** exhibited a NOE enhancement between H-1 (δ 5.42) and H-10 (δ 1.17) as well as H-5 (δ 3.27) and H-9 (δ 2.10) in Fig. 1. This indicates that H-5 and H-9 are on the β-face while H-1 is on the α-face as depicted in compound **7**. Thus **7** was lucidumoside A.

Compound **8** was obtained as a colorless powder. Its molecular formula was established as $C_{25}H_{34}O_{13}$ ($[M+H]^{+}$ 543.5447, calc. 543.5451) by positive HRFABMS. The IR spectrum showed the presence of hydroxyl groups (3400 cm^{-1}) and two carbonyl groups (1700 and 1630 cm^{-1}). The ^{1}H and ^{13}C NMR chemical shift assignments were made by means of ^{1}H-^{1}H and ^{1}H-^{13}C COSY spectra. Analysis of ^{1}H and ^{13}C NMR indicated that the spectral data of compound **8** were similar to those of compound **7** except for the appearance of a set of signals as the aromatic protons with ABX system at δ 6.76 (^{1}H, *s*), δ 6.75 (^{1}H, *d*, $J=8.6$ Hz) and δ 6.57 (^{1}H, *d*, $J=8.6$ Hz). By comparison of the ^{13}C NMR spectrum of compound **8** with those of compound **7**, the chemical shift assignable to C-5″ of 8 shifted downfield at δ 146.8, *ortho* C-4″ and C-6″ shifted upfield at δ 117.0 and δ 144.8, respectively. This information indicated that the phenyl ring in phenylethyl moiety in compound **8** was 3,4-substituted. In HMBC experiments of **8**, characteristic correlation peaks were

Table 1
^{1}H NMR spectral data for compounds **2**, **7** and **8** in CD_3OD[a]

Proton	**2**	**7**	**8**
Aglycone			
1	5.90 (1H, *s*)	5.42 (1H, *d*, 6.0)	5.44 (1H, *d*, 6.1)
3	7.51 (1H, *s*)	7.48 (1H, *s*)	7.46 (1H, *s*)
5	4.12 (1H, *m*)	3.27 (1H, *m*)	3.26 (1H, *m*)
6	2.45 (1H, *dd*, 9.2, 14.1)	2.47 (1H, *dd*, 9.0, 13.2)	2.45 (1H, *dd*, 8.8, 14.2)
	2.80 (1H, *dd*, 4.4, 14.1)	2.65 (1H, *dd*, 3.1, 13.2	2.61 (1H, *dd*, 3.3, 14.2)
8	6.08 (1H, *q*, 7.1)	1.75 (1H, *m*)	1.71 (1H, *m*)
		1.60 (1H, *m*)	1.62 (1H, *m*)
9		2.10 (1H, *m*)	2.09 (1H, *m*)
10	1.65 (3H, *d*, 7.1)	1.17 (3H, *t*, 6.0)	1.16 (3H, *t*, 6.0)
OMe	3.72 (3H, *s*)	3.64 (3H, *s*)	3.61 (3H, *s*)
Glucose			
1′	4.81 (1H, *d*, 7.7)	4.69 (1H, *d*, 7.8)	4.66 (1H, *d*, 7.8)
Phenylethyl			
1″	4.21 (2H, *t*, 7.0)	4.18 (2H, *m*)	4.06 (2H, *m*)
2″	2.82 (2H, *t*, 7.0)	2.83 (2H, *t*, 6.0)	2.86 (2H, *t*, 6.0)
4″	7.05 (1H, *d*, 8.7)	7.06 (1H, *d*, 8.8)	6.76 (1H, *s*)
5″	6.73 (1H, *d*, 8.7)	6.72 (1H, *d*, 8.8)	
7″	6.73 (1H, *d*, 8.7)	6.72 (1H, *d*, 8.8)	6.75 (1H, *d*, 8.6)
8″	7.05 (1H, *d*, 8.7)	7.06 (1H, *d*, 8.8)	6.57 (1H, *d*, 8.6)

[a] Coupling constants (*J* values in Hz) are shown in parentheses.

Table 2
^{13}C NMR spectral data of compounds **2**, **7** and **8** in CD_3OD

Carbon	**2**	**7**	**8**
Aglycone			
1	94.9	98.4	98.8
3	155.0	154.0	154.1
4	109.1	110.4	110.7
5	31.5	30.8	30.9
6	41.1	36.6	38.4
7	173.0	174.1	174.2
8	124.8	35.0	35.1
9	130.0	37.4	37.8
10	13.6	14.3	14.3
11	168.4	168.6	168.9
OMe	51.9	51.8	51.7
Glucose			
1′	100.5	100.6	100.9
2′	74.4	74.7	74.6
3′	77.6	77.9	78.1
4′	71.2	71.5	71.8
5′	78.5	78.3	78.3
6′	62.5	62.8	62.7
Phenylethyl			
1″	66.7	66.9	67.0
2″	34.9	35.1	36.0
3″	129.7	129.9	131.4
4″	130.8	130.8	117.0
5″	116.1	116.2	146.8
6″	156.7	156.9	144.8
7″	116.1	116.2	116.4
8″	130.8	130.8	121.8

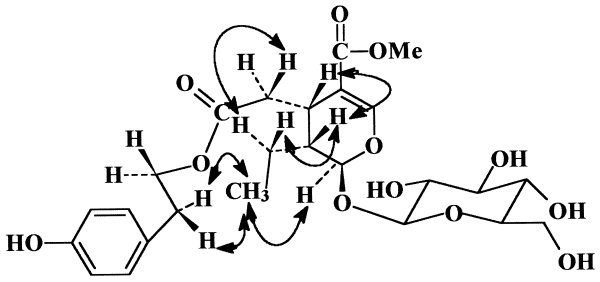


Fig. 1. NOESY experiment of compound **7**.

observed between H-1″ (δ 4.06) and C-7 (δ 174.2) as well as protons of a methoxyl group (δ 3.61) and C-11 (δ 168.9). The 3,4-dihydroxyl phenylethyl group should be connected to the aglycone at C-7. The result of NOESY experiment of compound **8** are identical with those of **7**, suggesting that H-5 and H-9 are on the β-face while H-1 is on the α-face as compound **8**. The structure of compound **8** was characterized as lucidumoside B.

3. Experimental

3.1. General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded using SHIMADZU UV-3100PC spectrophotometer. IR

absorption spectra were obtained with an IR-450 instrument as a film on KBr disk. FABMS were recorded on VG Autospec 3000 system, and ESIMS on Finnigan TSQ 7000. ^{1}H and ^{13}C spectra were obtained with Bruker 400 instrument operating at 400 MHz for ^{1}H, 100 MHz for ^{13}C, respectively. Chemical shifts are reported in parts per million on the δ scale with TMS as the internal standard, and coupling constants are in Hertz. Column chromatographic separations were performed with silica gel (Qingdao Haiyang Chemical Group Co. Ltd, China), Chromatorex ODS (Fuji Silysia Chemical Ltd, Japan), D-101 (Tianjin Agricultural Chemical Co. Ltd, China), and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd). TLC separations were performed on precoated Si gel 60 F_{254} plates (0.2 mm thick, Merck) with $CHCl_3$–MeOH (10:1, 9:1 and 8:2, v/v) and RP-18 F_{254s} (0.2 mm thick, Merck) with MeOH–H_2O (8:2, 7:3, 6:4 and 6:5, v/v), and spots were detected by UV illumination and by spraying 10% ethanolic H_2SO_4 reagent.

3.2. Plant material

Dry fruits of *L. lucidum* was bought from a Chinese medicine shop in Hong Kong. The voucher specimen was deposited in the Museum of Institute of Chinese Medicine, Chinese University of Hong Kong.

3.3. Extraction and isolation

The dried fruits (2.30 kg) of *L. lucidum* were extracted with EtOH (5 l×3). The EtOH extract was concentrated in vacuo to give a residue (775.3 g). The residue was suspended in H_2O (50 l), and divided into aqueous and insoluble parts. The aqueous layer was absorbed on D101 (2 kg) and then eluted with H_2O (20 l), 60% EtOH aqueous (6 l) and EtOH (6 l), to afford three fractions A (170.1 g), B (63.1 g), and C (1.8 g). Fraction B (28.4 g) was applied to a silica gel column (2 kg) eluted with $CHCl_3$–MeOH (8:2) and then the silica gel in the column was divided equally into 18 fractions, each fraction was eluted with MeOH (300 ml) and concentrated. The 18 fractions were combined into six fractions, frs. 1–6, based on silica TLC ($CHCl_3$–MeOH, 8:2) results. Fr. 2 (0.90 g) was purified by Sephadex LH-20 eluting with EtOH–H_2O (6:4) and then by silica gel eluting with $CHCl_3$–MeOH to give oleoside dimethyl ester (**1**) (5.1 mg), lucidumoside A (**7**) (7.8 mg), and lucidumoside B (**8**) (6.5 mg). Fr. 3 (1.08 g) was purified by silica gel ($CHCl_3$–MeOH (9:1) as eluent) and then by ODS [MeOH–H_2O (7:3)] to afford ligustroside (**2**) (190.0 mg) and oleuropein (**3**) (6.5 mg). Fr. 5 (2.1 g) was purified by Sephadex LH-20 [EtOH–H_2O (6:4)], silica gel [$CHCl_3$–MeOH (8:2) and ODS [MeOH–H_2O (6:5)] to give nuezhenide (**4**) (9.1 mg) and isonuezhenide (**5**) (13.2 mg). Fr. 6 (1.5 g) was chromatographed on Sephadex LH-20 [MeOH–H_2O (6:4)], and purified by silica gel [$CHCl_3$–MeOH (8:2)] and RP-18 [MeOH–H_2O (6:5)] to yield neonuezhenide (**6**) (20.1 mg).

3.4. Lucidumoside A (7)

Colorless powder, $[\alpha]_D^{15}$ −90°C (*c* 0.41, MeOH); UV (EtOH) λ_{max} (log ε) 238 (4.00), 279 (3.37) nm; IR (KBr) λ_{max} 3400, 1700, 1630, 1510, 1080, 920, 830 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectra (see Tables 1 and 2); ESIMS *m/z* 525 $[M-H]^-$, 405 $[M-121]^-$, 363 $[M-glc]^-$; HRFAB-MS *m/z*: found 527.5455 $[M+H]^+$ ($C_{25}H_{35}O_{12}$, calc. 527.5457).

3.5. Lucidumoside B (8)

Colorless powder, $[\alpha]_D^{15}$ −103°C (*c* 0.21, MeOH); UV (EtOH) λ_{max} (log ε) 239 (3.91), 280 (3.40) nm; IR (KBr) λ_{max} 3400, 2950, 1700, 1630, 1520, 1070 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectra (see Tables 1 and 2). ESI-MS *m/z*: 541 $[M-H]^-$, 379 $[M-glc]^-$; HRFAB-MS *m/z*: found 543.5447 $[M+H]^+$ ($C_{25}H_{35}O_{13}$, calc. 543.5451).

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Triterpenoids from *Dysoxylum malabaricum*

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Abstract

Two triterpenes 21*R*,23*R*-epoxy-21α-ethoxy-24*S*,25-dihydroxyapotirucall-7-en-3-one and 24*R*-acetoxy-3β,25-dihydroxycycloartane were isolated from the leaves of *Dysoxylum malabaricum* together with eight known compounds lupeone, lupeol, sitosterol, dipterocarpol, cycloart-25-ene-3β,24-diol, 24*R*,25-dihydroxycycloartan-3-one, 3β,24*R*,25-trihydroxycycloartane and ergosta-5,24(24[1])-diene-3β,4β,20*S*-triol. The complete ^{1}H and ^{13}C NMR spectral assignment of the new apotirucallene triterpenoid has been achieved by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Meliaceae; *Dysoxylum malabaricum*; Apotirucallane and cycloartane triterpenes; Structure elucidation

1. Introduction

Dysoxylum malabaricum Bedd (Meliaceae) is a large sized tree common in the western Ghats of Kerala, India. The earlier reports on the leaf constituents of this plant deal with the isolation of three dammarane triterpenes dymalol, ocotillone and shoreic acid (Govindachari et al., 1994), two steroids (ergosta-5,24(24[1])-diene-3β,4β, 20*S* triol, ergosta-5,24(24[1])diene-3β,7α-diol) and 3β, 24*R*,25-trihydroxycycloartane (Govindachari et al., 1997). We have reported the isolation and detailed NMR studies on three dammaranes (cabraleadiol, cabraleone and shoreic acid) from the stem bark of this plant (Hisham et al., 1996). The present study deals with a reinvestigation on the leaves of the plant collected from Kerala, India and reports the isolation of ten triterpenes/sterols of which two were found to be new.

2. Results and discussion

The concentrated EtOAc extract was chromatographed over a silica gel column and eluted with solvents of increased polarity to get ten triterpenoids/sterols (**1**–**10**). Compounds **1**, **2** and **3** were identified as lupeol, lupeone and sitosterol by direct comparison. Compound **4** was identified as dipterocarpol (20*S*-hydroxydammar-24-en-3-one) (Baker et al., 1976), **5** as cycloart-25-ene-3β,24-diol (Greca et al., 1994), **8** as 24*R*,25-dihydroxycycloartan-3-one (Barik et al., 1994) on the basis of NMR data. Compounds **9** and **10** were identified as previously reported 3β,24*R*,25-trihydroxycycloartane (Greca et al., 1994) and ergosta-5,24(24[1])-diene-3β,4β-20*S*-triol (Govindachari et al., 1997) from the title plant.

Compound **6**, a new triterpene, showed the absorption bands for hydroxyl groups (3300 cm^{-1}) and keto group (1700 cm^{-1}) in the IR spectrum. The ^{1}H NMR spectrum showed seven tertiary methyl groups as singlets and a primary methyl group as an unsymmetrical triplet . Further, the ^{1}H NMR spectrum showed a vinyl proton signal at δ 5.32 (*d*) in addition to signals of three oxymethine protons at δ 3.24 (1H, *d*), 4.24 (1H, *ddd*), 4.89 (1H, *d*) and two one-proton signals at δ 3.43 (*dq*) and 3.70 (*dq*). The $\{^{1}H\}$–^{13}C NMR spectrum showed the signals for 32 carbons and an INEPT spectra could distinguish eight CH_3 carbons, nine CH_2 carbons, eight CH carbons and seven quaternary carbons. The EI mass spectrum showed a M^+ ion at *m*/*z* 516 corresponding to the formula of $C_{32}H_{52}O_5$ which was in

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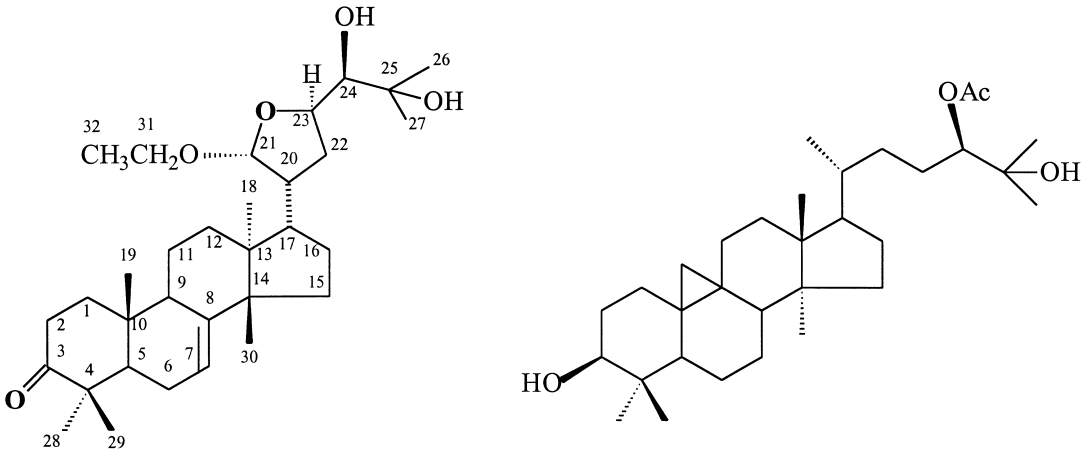
agreement with NMR spectroscopic data. The EI mass spectrum showed prominent ions such as m/z 470 (27%) $(M\text{-}EtOH)^+$, 452 (27%) $(M\text{-}EtOH\text{-}H_2O)^+$ indicating the presence of an ethoxy group and hydroxyl groups in the molecule. The presence of the ethoxy group was further evident in the NMR spectrum from an oxymethylene carbon shift at δ 65.0, which showed correlation with the diastereotopic one-proton multiplets at δ 3.43 and 3.70 in the HMQC spectrum and both protons showed correlation with the methyl proton triplet at δ 1.22 (δ_C 15.2) in the ^1H–^1H COSY spectrum. The presence of a tertiary hydroxyl group was inferred from a quaternary carbon signal at δ 73.1, a secondary hydroxyl group from a CH carbon signal at δ 75.4. In addition, the ^{13}C NMR spectrum showed one more oxymethine carbon signals at δ 76.8 and an acetal carbon at δ 107.8. A 3-oxotirucall-7-ene skeleton was inferred from the familiar ^{13}C NMR signals (Mohamed et al., 1999). The ^1H–^1H COSY, HMQC and HMBC experiments carried out on **6** traced out the proton–proton and proton–carbon connectivities confirming the assignment of a 3-oxo-apotirucalla-7-ene skeleton in the molecule with a 21,23-epoxy-21-ethoxy-24,25-dihydroxy side chain (Table 1). The NMR chemical shifts for the side chain atoms in **6** were essentially identical to those signals in 21*R*,23*R*-epoxy-24*S*,25-dihydroxy-21α-methoxy-side chain present in holstinone C, an apotirucallane triterpene, recently isolated from *Turraea holstii* (Mulholland et al., 1999) except with a difference that the methoxy group in the side chain in holstinone C is replaced by an ethoxy group in **6**. Therefore, the stereochemistry of the chiral centres in the side chain of **6** was assigned as 21*R*,23*R*-epoxy-21α-ethoxy,24*S* in comparison with the ^1H and ^{13}C chemical shifts observed for the side chain in holstinone C thus identifying **6** as 21*R*,23*R*-epoxy-21α-ethoxy-24*S*,25-dihydroxyapotirucall-7-en-3-one (Scheme 1).

Compound **7**, a new cycloartane triterpene, was isolated as a minor constituent. The ^1H NMR spectrum showed the presence of six tertiary methyl groups as

Table 1
1D and 2D NMR experiments (500 MHz, $CDCl_3$) of **6**

Position	δ C	δ H (multiplicity, J in Hz)[a]	HMBC correlations
1	38.5 *t*	1.99 (*m*) H-1a; 1.48 (*m*) H-1b	H-19, 2a
2	34.9 *t*	2.75 (*dt*, 14.7, 5.5) H-2a; 2.25 (*m*) H-2b	H-1a
3	216.9 *s*	–	
4	47.9 *s*	–	H-29, 28
5	52.4 *d*	1.72 (*m*) H-5	H-29,28, 19, 6, 7
6	24.3 *t*	2.10 (*m*) H-6	H-7
7	113.1 *d*	5.32 (*d*, 3.7) H-7	H-6
8	145.1 *s*	–	H-6, 30
9	48.3 *d*	2.32 (*m*) H-9	H-19
10	35.1 *s*	–	H-19
11	17.8 *t*	1.6 (*m*)	
12	31.6 *t*	1.75 (*m*) H-12a; 1.50 (*m*) H-12b	H-18
13	43.7 *s*	–	H-18, 30
14	51.0 *s*	–	H-30, 18, 7
15	33.8 *t*	1.55 (*m*) H-15a; 1.50 (*m*)H-15b	H-30
16	27.5 *t*	1.95 (*m*) H-16a; 1.30 (*m*) H-16b	H-15b
17	50.4 *d*	1.79 (*m*) H-17	H-18,12a, 22b
18	22.3 *q*	0.87 (*s*) H-18	H-12a
19	12.7 *q*	1.02 (*s*) H-19	H-1b
20	47.9 *d*	2.20 (*m*) H-20	H-22b, 17
21	107.8 *d*	4.89 (*d*, 3.7)	
22	34.4 *t*	1.95 (*m*) H-22a; 1.75 (*m*) H-22b	
23	76.8 *d*	4.24 (*ddd*, 10.1, 4.6, 1.8) H-23	H-22b
24	75.4 *d*	3.24 (*d*, 8.3)	H-27
25	73.1 *s*	–	H-27, 26
26	26.4 q	1.27 (*s*)	
27	26.5 q	1.25 (*s*)	
28	21.6 q	1.12 (*s*)	H-29
29	24.5 q	1.05 (*s*)	H-28
30	27.29 q	1.02 (*s*)	H-15b
31	65.0 q	3.70 (*dq*) H-31a; 3.43 (*dq*) H-31b	
32	15.4 *q*	1.22 (*t*, 7.3) H-32	
24-OH		2.56 (*d*,10.1)	
25-OH		2.85 (*br s*)	

[a] Assignments based on HMQC.



6 **7**

Scheme 1.

singlets at δ 1.20 (6H, *s*, 2Me), 0.96 (6H, *s*, 2Me), 0.88, 0.81, a secondary methyl group as a doublet at δ 0.88 (*d*, $J=6.3$ Hz) and an acetyl methyl singlet at δ 2.02. A cyclopropane group was evident from two one-proton broad singlets at δ 0.33 and 0.55 . An acetoxyl group was inferred from an acetyl methyl shift at δ 2.02. Further, the ^{1}H NMR showed a carbinol methine proton signal at δ 3.27 and an acetoxy methine proton signal at δ 4.74 (*d*, $J=6.6$ Hz). The ^{13}C NMR spectrum showed signals of 32 carbon atoms of which two oxymethine carbon signals at δ 78.8 and 80.6 were assigned for the hydroxyl and acetoxyl bearing carbons respectively and the signal of a quaternary carbon at δ 72.5 indicated the presence of a tertiary hydroxyl group. The EI mass spectrum showed a weak molecular ion at *m*/*z* 502 consistent with a molecular formula $C_{32}H_{54}O_4$ in corroboration with the NMR data. The alkaline hydrolysis of **7** gave compound **9** indicating that it is the monoacetyl derivative of **9**. The acetoxylation at C-24 was deduced on the basis of a comparison of ^{13}C NMR data of **7** with those of **9**. Both compounds showed identical shifts for the carbons in the nucleus indicating their identical structural features up to this part which suggested the acetoxyl group is in the side chain ie, at C-24 position. Further evidence for the C-24 acetoxylation came from interpretation of the mass spectral fragment ions at *m*/*z* 315 and 187 formed by C-17/C-20 cleavage . The ion at *m*/*z* 315 represent the nucleus with the 3-hydoxyl group and the ion at *m*/*z* 187 pointed to the nine carbon side chain with the acetoxyl and hydroxyl functionalities thereby characterizing **7** as 24*R*-acetoxy-3β,25-dihydroxycycloartane.

This is the first report of the isolation of the known compounds **1**, **2**, **3**, **4** and **5** from the title plant.

3. Experimental

3.1. General

IR: KBr, ^{1}H NMR: 270, 300 and 500 MHz using TMS as int. std.; ^{13}C NMR: 69.5, 75 and 125 MHz; ^{13}C multiplicities: DEPT or INEPT techniques. EIMS: 70 eV; TLC: Si. gel (250 μm layer thickness). Visualization of spots on thin layer chromatography (TLC) was carried out by spraying with vanilline-H_2SO_4 followed by heating.

3.2. Plant material

Leaves of *D. malabaricum* Bedd. (Meliaceae) were collected from the herbal garden of Kottakkal Arya Vaidyasala, Kottakkal, India and was identified by Dr. Indira Balachandran, Research officer of the herbal garden where a voucher specimen (AH No. DL98) has been deposited.

3.3. Extraction and isolation

The shade dried leaf powder (500 g) was repeatedly extracted four times with EtOAc at room temperature. The combined extract (6 l) was concentrated under reduced pressure to obtain 35 g of viscous gummy material. Fifteen grammes of this was chromatographed over a silica gel column and eluted with hexane, mixtures of hexane–C_6H_6, C_6H_6, mixtures of C_6H_6–$CHCl_3$, $CHCl_3$ and mixtures of $CHCl_3$–EtOAc. Compounds **1** (20 mg), **2** (15 mg), **3** (15 mg) and **4** (10 mg) were obtained from C_6H_6–hexane fractions, **5** (5 mg), **6** (15 mg), **7** (10 mg) and **8** (10 mg) from $CHCl_3$ fractions, **9** (500 mg) and **10** (15 mg) from $CHCl_3$–EtOAc fractions.

3.4. 21R,23R-Epoxy,21α-ethoxy,24 S,25-dihydroxy-apotirucalla-7-en-3-one (**6**)

White crystals. IR ν_{max}^{KBr} cm^{-1}: 3300, 1700; ^1H and ^{13}C NMR see Table 1; EIMS *m/z* (rel. int.) : 516 $[M]^+$ (0.5), 470 (27), 452 (21), 455 (22), 437 (27), 409 (25), 391 (31), 381 (25), 365 (25), 337 (15), 325 (12), 310 (17), 297 (40), 271 (45), 245 (21), 184 (37), 166 (65), 95 (100).

3.5. 24R-Acetoxy-3β,25-dihydroxycycloartane (**7**)

Crystals ($CHCl_3$–hexane) m.p. 160°C; ^1H NMR (300 MHz, $CDCl_3$): δ 4.75 (1H, *d, J* = 6.6 Hz, H-24), 3.27 (1H, *m*, H-3), 2.02 (3H, *s*, Ac Me), 1.20 (6H, *s*, H-26, H-27), 0.96 (6H, *s*, H-18, H-29), 0.88 (3H, *s*, H-28), 0.88 (3H, *d*, H-21), 0.81 (3H, *s*, H-30), 0.55 (1H, *br s*, H-19b), 0.33 (1H, *br s*, H-19a); ^{13}C NMR (75 MHz, $CDCl_3$) (C-1 to C-30): 32.0, 30.4, 78.8, 40.1, 47.1, 21.1, 27.9, 48.0, 20.0, 26.0, 26.0, 35.5, 45.3, 48.8, 32.9, 26.4, 52.1, 18.0, 30.0, 36.3, 18.4, 32.7, 26.4, 80.9, 72.5, 25.0, 26.8, 19.3, 14.0, 25.4, 171.3 ($CH_3\underline{C}O$) , 21.1 (Ac Me); EIMS *m/z* (rel. int.): 502 $[M]^+$ (1), 486 (17), 484 (36), 466 (100), 451 (48), 423 (28), 409 (40), 392 (16), 344 (25), 337 (14), 315 (15), 313 (19), 297 (21), 284 (25), 269 (17), 255 (12), 229 (16), 203 (55), 175 (56), 163 (29).

3.6. Hydrolysis of 7

Ten milligrams of **7** were refluxed with 2 ml methanolic KOH (10%) for 4 h. Usual work up gave 4 mg of **9** and the identity of which was confirmed by m.p., coTLC and ^1H NMR.

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Guaiane dimers from *Xylopia vielana*

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Abstract

From the leaves of *Xylopia vielana* (Annonaceae) the three dimeric guaianes vielanin A–C were isolated and structurally elucidated by mass and NMR spectroscopy as **1–3**. The structure of **1** contains a bridged ring system formed probably via a Diels–Alder reaction of two different guaiane monomers. Compounds **2** and **3** represent symmetric cyclobutanes formally generated from two equal guaiane moieties by [2 + 2] cycloaddition. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Xylopia vielana*; Annonaceae; Vielanins A–C; Sesquiterpenoids; Guaiane dimers; Cyclobutanes

1. Introduction

Xylopia vielana Pierre ex Fin. & Gagn. is a tree growing in Vietnam. In folk medicine the bark serves as an emmenagogue, whereas leaves are used for the treatment of rheumatism and pain. In South Vietnam, the plant is used against malaria (Do, 1991). The genus *Xylopia* is known to contain alkaloids of aporphine and tetrahydroberberine type and diterpenes (Hegnauer, 1964, 1989). In particular, some species contain dimeric guaianes (Martins et al., 1998) and dimeric diterpenes (Vilegas et al., 1991). *X. vielana* has not been phytochemically investigated until now. In continuation of our search for new biologically active compounds from Vietnamese medicinal plants (Porzel et al., 2000) we now report the isolation and structural elucidation of three guaiane dimers from the leaves of this plant.

2. Results and discussion

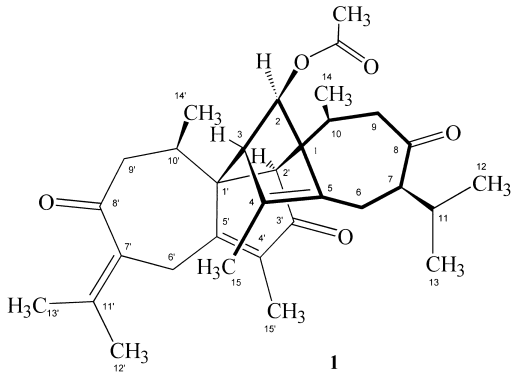
The extract of the leaves (MeOH–H_2O 95:5) of *Xylopia vielana* was partitioned between water and organic solvents of increasing polarity. Five compounds were isolated using normal phase column chromatography on silica gel and reversed phase chromatography. Besides the known flavonoids (−)-epicatechin and quercitrin, three new compounds belonging to the class of dimeric guaianes were obtained.

The molecular mass of 506 for compound **1** was obtained from the negative ESIMS, which showed the $[M–H]^-$ peak at m/z 505. The EIMS gave a very weak molecular ion (0.4%) at m/z 506.3064 leading to the molecular formula $C_{32}H_{42}O_5$. The presence of an acetoxy group was revealed by loss of acetic acid producing the fragment at m/z 446 and by the corresponding signals in the NMR spectra (δ_C 170.7 and 21.19, δ_H 2.02). The remaining 30 carbon NMR signals belonged to two different guaiane moieties. Other functional groups were three carbonyl groups (δ 214.0, 206.9 and 202.9) and three fully substituted double bonds (δ 168.6, 146.2, 140.8, 135.7, 132.5 and 129.4). The location of these functional groups within the two guaiane moieties and the assignment of the NMR signals were achieved by analysis of the C−H long-range correlations from the GHMBC experiment. Some atoms like C-1, C-4, C-5, C-1′, C-4′, C-5′, H-3, H-6^A, H-6′^{B}, and H-15′ showed surprisingly many correlation peaks. This was a first hint to a bridged ring system and also explained the high chemical shifts of the quaternary carbons at δ 64.4 and 59.8. Careful analysis of all C−H long-range correlations led to the depicted structure. Confusing for the structural elucidation was the presence of some $^4J_{CH}$ and $^5J_{CH}$ correlations through double bonds, which were of comparable intensitity with many $^2J_{CH}$ and $^3J_{CH}$ correlations. This applied especially to the $^4J_{CH}$ correlations C-1′/H_3-15′ and C-8′/H_3-12′ and the $^5J_{CH}$ correlations C-5′/H_3-12′, C-7′/H_3-15′ and C-10′/H_3-15′.

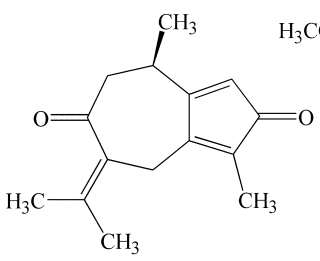
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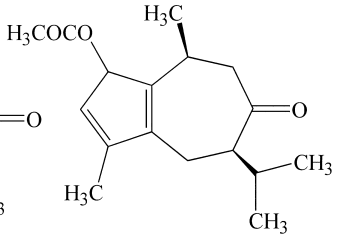
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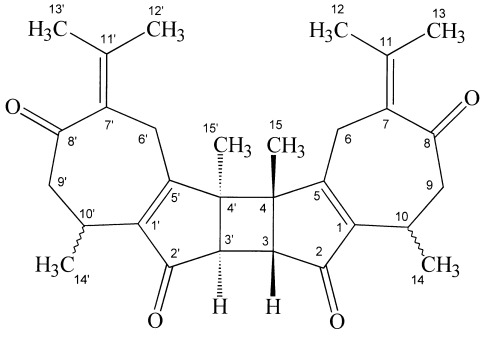
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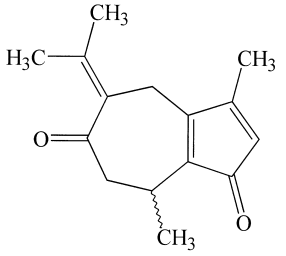
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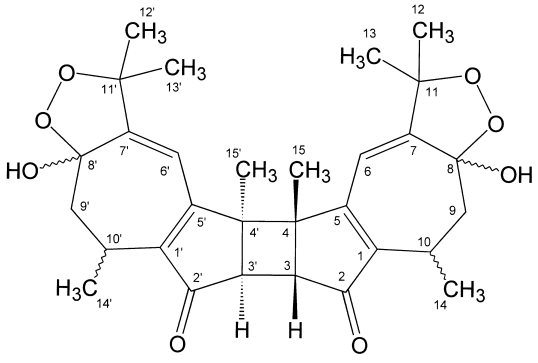


1b



2





3

The resulting structure was confirmed by the MS fragmentation, which was mainly determined by the Retro–Diels–Alder reaction giving monomer **1a** at *m*/*z* 230 (19%) and monomer **1b** at *m*/*z* 276 (6%). The base peak at *m*/*z* 234 resulted from loss of ketene from **1b**, which is typical for unsaturated acetoxy compounds. According to the structure of **1**, the dimer did not loose ketene but acetic acid, which is characteristic for saturated acetoxy compounds, thus proving the formation of a double bond by RDA.

Important information about the configuration was deduced from the NOESY experiment: the configuration of the central bicyclo[2.2.1]heptane moiety with the *endo*-anellation of the five-membered ring at C-1′ and C-2′ was revealed by a significant NOE effect between the methyl protons H_3-15 (δ 1.60) and H-6′^{B} (δ 3.65), which was not possible for the *exo*-isomer. The NOE effects of H-2 (δ 4.87) on the methyl groups H_3-14 (δ 1.08) and H_3-14′ (δ 0.90) and on H-2′ (δ 2.48) indicated the configuration of the acetoxy group at C-2 as *syn* with respect to the Δ^4 double bond and suggested that both methyl groups C-14 and C-14′ were *cis* to the bridge of the norbornene moiety. The NOE effect between H_3-12′ (δ 1.97) and H-6′^{B} (δ 3.65) assigned C-12′ to be the methyl group in *trans*-position to the carbonyl group C-8′. In both seven-membered rings NOE effects across the ring were observed. They were only possible if both involved protons were in axial position and at the same side of the ring. Thus, the seven-membered ring on the left gave rise to NOE effects between H-6′^{A} (δ 2.93) and H-9′^{B} (δ 3.23). In the right-hand seven-membered ring the NOE interactions between H-7 (δ 2.41) and H-10 (δ 2.88) showed that both were in axial position and *cis* so that the substituents at C-7 (isopropyl group) and at C-10 (methyl group) had also to be *cis* resulting in the depicted relative configuration (Table 1).

The new compound **1** was named vielanin A. Its biosynthetic precursors were supposed to be synthons of the instable guaiane-type monomers **1a** and **1b**, occurring also in the mass spectrum of **1**.

The HRMS of vielanin B (**2**) ([M]$^+$ *m*/*z* 460.2596) led to the molecular formula $C_{30}H_{36}O_4$. The ^{13}C NMR showed only 15 carbon signals. Thus, **2** consisted of two equivalent $C_{15}H_{18}O_2$ moieties, which were responsible for the base peak in the EIMS at *m*/*z* 230. The ^{1}H NMR spectrum showed in addition to four methyl groups (3 singlets at δ 2.21, 2.04, 1.88 and one doublet at δ 1.08) only six other signals, which belonged to aliphatic protons. The carbon shifts revealed the presence of two carbonyl groups (δ 203.7 and 205.1) and two double bonds (δ 131.6, 143.8, 146.9 and 171.3). The high

Table 1
NMR data (500/125 MHz) of vielanin A (**1**) in $CDCl_3$

	δ_c	δ_H	C−H long-range correlations	NOE effects[a]
1	64.4	–	H-3 (m)[b], H-6^B (m), H-9^A (w), H-9^B (m), H-14 (*s*), H-15 (vw), H-2′ (s)	
2	86.9	4.87 *d* (1.5)	H-3 (*m*)	H-3, H-14, H-2′, H-14′
3	56.3	3.10 *s*	H-15 (*s*), H-15′ (*w*)	H-2, H-15, H-2′, H-6′^{A}, H-9′^{B}, CO-C$\underline{H_3}$ (vw), H-14′ (w)
4	135.7	–	H-2 (s), H-3 (vw), H-6^A (vw), H-6^B (w), H-15 (s)	
5	132.5	–	H-2 (s), H-3 (s), H-6^B (m), H-7 (m), H-15 (s), H-2′ (s)	
6	25.7	A: 1.85 *ddd* (15.3/4.9/1.8) B: 2.33 *dd* (15.3/7.0)	H-7 (w)	H-7, H-13 (w) H-13, H-15
7	57.0	2.41 *m*	H-6^B (m), H-12 (s), H-13 (s)	H-6^A, H-10, H-12, H-13
8	214.0	–	H-6^A (vw), H-6^B (m), H-7 (w), H-9^A (w), H-9^B (m), H-14 (w)	
9	48.2	A: 2.16 *dd* (13.4/2.1) B: 2.71 *dd* (13.4/12.5)	H-14 (s)	H-10, H-14 H-11 (w), H-12 (w), H-14 (w)
10	28.5	2.88 *dqd* (12.2/6.6/2.5)	H-9^A (m), H-14 (s), H-2′ (s)	H-7, H-9^A, H-14, H-2′
11	27.8	1.98 *m*	H-6^B (w), H-12 (s), H-13 (s)	H-9^B, H-12, H-13
12	21.13	0.82 *d* (6.7)	H-13 (s)	H-7 (w), H-9^B (w), H-11, H-13
13	20.1	0.94 *d* (6.7)	H-12 (s)	H-6^A (w), H-6^B, H-7, H-11, H-12, H-15
14	17.1	1.08 *d* (7.0)	H-9^A (vw)	H-2, H-9^A, H-9^B (w), H-10, H-2′
15	14.3	1.60 *s*	H-3 (w)	H-3, H-6^B, H-13, H-6′^{A}, H-6′^{B} (w)
1′	59.8	–	H-2′ (w), H-6′^{B} (s), H-9′^{A} (m), H-9′^{B} (w), H-14′ (s), H-15′ (m)	
2′	51.8	2.48 *s*	H-3 (s), H-15 (m)	H-2, H-3, H-10, H-14, H-14′
3′	206.9	–	H-2′ (s), H-15′ (s)	
4′	140.8	–	H-2′ (w), H-6′^{A} (w), H-6′^{B} (s), H-15′ (s)	
5′	168.6	–	H-2′ (s), H-6′^{A} (w), H-6′^{B} (s), H-10′ (w), H-12′ (w), H-13′ (w), H-15′ (s)	
6′	30.7	A: 2.93 *br d* (14.0) B: 3.65 *d* (14.0)	–	H-3, H-15, H-9′^{B} H-15 (w), H-12′, H-15′
7′	129.4	–	H-6′^{A} (w), H-6′^{B} (s), H-9′^{A} (w), H-12′ (s), H-13′ (s), H-15′ (m)	
8′	202.9	–	H-6′^{B} (m), H-9′^{A} (m), H-9′^{B} (w), H-10′ (w), H-12′ (m), H-13′ (w)	
9′	47.3	A: 2.57 *dd* (15.3/7.3) B: 3.23 *dd* (15.3/1.5)	H-10′ (w), H-14′ (s)	H-10′, H-14′ (w) H-3, H-10′ (w), H-6′^{A}
10′	34.6	2.46 *m*	H-3 (w), H-2′ (s), H-9′^{A} (m), H-9′^{B} (w), H-14′ (s), H-15′ (w)	H-9′^{A}, H-9′^{B}, H-14′
11′	146.2	–	H-6′^{A} (w), H-6′^{B} (s), H-12′ (s), H-13′ (s)	
12′	23.6	1.97 *s*	H-13′ (s)	H-6′^{B}, H-13′, H-15′
13′	23.7	2.05 *s*	H-12′ (s)	H-12′, H-14′ (w)
14′	16.2	0.90 *d* (7.3)	H-9′^{B} (w), H-10′ (w)	H-2, H-2′, H-9′^{A}, H-13′ (w)
15′	8.6	1.64 *s*	–	H-6′^{B}, H-12′
$\underline{CO}$-CH_3	170.7	–	H-2 (m), CO-C$\underline{H_3}$ (s)	
CO-$\underline{CH_3}$	21.19	2.02 *s*	–	H-3 (vw)

[a] Obtained from the NOESY experiment.
[b] s = strong, m = medium, w = weak, v = very.

chemical shift of the olefinic carbon at δ 171.3 was explained by conjugation to a carbonyl group and ring tension. Analysis of the CH long-range correlations from the GHMBC (Table 2) resulted in a guaiane moiety with two carbonyl groups in positions 2 and 8 and still free valencies at positions 3 and 4. Connecting the free valencies resulted in a dimer with a central cyclobutane ring. This was supported by the fact that C-3 (δ 47.0) showed a $^1J_{C-H}$ and simultaneously a long-range C−H correlation ($^2J_{CH}$ or $^3J_{CH}$) to the protons at δ 2.21 (H-3/H-3′). Because of the equivalence of each two cyclobutane carbons, it was not possible to distinguish between a 3-3′/4-4′ or a 3-4′/4-3′ connection on the basis of the CH long-range correlations. In the case of a 3-3′/4-4′ connection between the two guaiane moieties there would be a $^3J_{HH}$ coupling between H-3 and H-3′ with an expected larger value, whereas in the other case (3-4′/4-3′ connection) a small $^4J_{HH}$ coupling would be observed. The coupling constant between H-3 and H-3′ could not be observed directly because of their equiva-

Table 2
NMR data (500/125 MHz) of vielanin B (**2**) in $CDCl_3$

	δ_c	δ_H	CH long-range correlations	NOE effects[a]
1/1′	146.9	–	H-3/3′ (w)[b], H-6/6′^{A}, H-6/6′^{B}, H-9/9′^{A}, H-9/9′^{B}, H-14	
2/2′	205.1	–	H-3/3′	
3/3′	47.0[c]	2.21 *s*	H-3/3′, H-15/15′	H-14/14′(w), H-15/15′
4/4′	54.5	–	H-3/3′, H-6/6′^{A} (w), H-6/6′^{B}, H-15	
5/5′	171.3	–	H-3/3′, H-6/6′^{A}, H-6/6′^{B}, H-9/9′^{A} (w), H-9/9′^{B} (w), H-12/12′, H-13/13′, H-15/15′	
6/6′	29.2	A: 3.27 *d* (17.3) B: 3.54 *d* (17.3)	H-13/13′ (w)	H-6/6′^{B}, H-9/9′^{B}/H-10/10′, H-15/15′ H-6/6′^{A}, H-9/9′^{A} (w), H-12/12′, H15/15′
7/7′	131.6	–	H-6/6′^{A} (w), H-6/6′^{B}, H-9/9′^{A}, H-12/12′, H-13/13′	
8/8′	203.7	–	H-6/6′^{A} (w), H-6/6′^{B}, H-9/9′^{A}, H-9/9′^{B}, H-12/12′, H-13/13′, H-14/14′	
9/9′	46.9[c]	A: 2.68 *dd* (15.3/7.3) B: 3.02[d] *m*	H-10/10′ (w), H-14/14′	H-6/6′^{B}(w), H-9/9′^{B}, H-14/14′ [d]H-6/6′^{A}, H-9/9′^{A}, H-14/14′
10/10′	27.7	3.01[d] *m*	H-9/9′^{A}, H-9/9′^{B}, H-14/14′	[d]H-6/6′^{A}, H-9/9′^{A}, H-14/14′
11/11′	143.8	–	H-6/6′^{A} (w), H-6/6′^{B}, H-12/12′, H-13/13′	
12/12′	22.5	1.88 *s*	H-13/13′	H-6/6′^{A} (w), H-6/6′^{B}, H-13/13′, H-15/15′
13/13′	23.3	2.04 *s*	H-12/12′	H-12/12′, H-14/14′
14/14′	19.0	1.08 *d* (7.0)	H-9/9′^{A}, H-9/9′^{B}/10/10′	H-9/9′^{A}, H-9/9′^{B}/H-10/10′, H-13/13′
15/15′	19.8	1.25 *s*	H-3/3′	H-3/3′, H-6/6′^{A}, H-6/6′^{B}, H-9/9′10/10′, H-12/12′

[a] Obtained from the NOE difference spectra.
[b] w = weak.
[c] Assignment of 46.9 and 47.0 from APT spectrum, GHMBC correlations not resolved.
[d] Signals of H-9/9′^{B} (3.02) and H-10/10′ (3.01) overlapped.

lence. Thus, the coupling was deduced by analysis of the ^{13}C satellites (Günther, 1992). Those were obtained from the one-dimensional GHSQC experiment showing exclusively the ^{13}C satellites. The protons H-3/H-3′ appeared as doublet of doublets with $^1J_{C-H}$ of 146.9 Hz and $^3J_{HH}$ or $^4J_{HH}$ of 3.3 Hz. A $^4J_{HH}$ coupling for substituted cyclobutanes was expected to range from 0 to 2 Hz and thus was excluded. The $^3J_{HH}$ couplings of cyclobutanes range from 2 to 10 Hz and are normally unreliable for the determination of the relative configuration, because the energy difference between the two puckered conformers is rather low, so that there exists an equilibrium between the conformers with an averaged HH coupling constant (Gaudemer, 1977). In the case of **2** however, the two anellated five-membered rings caused a nearly planar rigid system. A *cisoid*-anellation of the two five-membered rings with *cis*-configuration of H-3 and H-3′ and a dihedral angle of 0–15° would show a HH coupling of about 7 Hz, whereas the measured value of 3.3 Hz supported very well the *transoid*-anellation with *trans*-configuration of H-3 and H-3′ and a dihedral angle of ca 120°.

Very interesting was the observation that in the GHMBC spectrum the correlation peaks between C-4/4′ (δ 54.5) and H_3-15/15′ (δ 1.25) had more than the double intensity than all other correlation peaks originating from methyl groups. This was explained by the fact, that C-4 had one $^2J_{CH}$ and one $^3J_{CH}$ correlation to H_3-15 and H_3-15′ resulting in a strongly increased intensity of the correlation peak. This additionally supported the structure of **2**, whereas an isomeric compound with 3-4′/4-3′ connection should have shown a double intensity of the correlation peak between C-3 and H_3-15/15′.

Analysis of the NOE difference spectra (Table 2) gave some further information: the differentiation between the two methyl groups at C-11/11′ was deduced from the NOE effect between the signal at δ 1.88 (H_3-12/12′) and H-6/6′$^{A/B}$ (δ 3.54 and 3.27). The NOE effects across the seven-membered ring between H-6/6′^{B} and H-9/9′^{A} showed that these protons were on the same side of the ring. Because of the conformational flexibility of the seven-membered ring, the configuration of the methyl group at C-10/10′ could not be determined by NOE effects.

Vielanin B (**2**) can be regarded as [2 + 2] cycloaddition product of two molecules of the instable guaiane monomer **2a** which also appears as fragment in the mass spectrum of **2**.

Like vielanin B (**2**), also vielanin C (**3**) was a symmetric guaiane dimer. The positive ESIMS spectrum showed the $[M + H]^+$ peak at *m*/*z* 525. The corresponding molecular ion with the mass 524 did not appear in the EIMS. The peak with the highest mass was that of the monomeric fragment at *m*/*z* 262, which easily lost water. The high resolution of this fragment at *m*/*z* 244.1104 ($C_{15}H_{16}O_3$) led to the molecular formular $C_{30}H_{36}O_8$ for the dimer containing 13 double bond equivalents.

Table 3
NMR data of vielanin C (**3**) in CD_3OD (500/125 MHz)

	δ_c	δ_H (500 MHz)	CH long-range correlations	NOE effects[a]
1/1′	147.8	–	H-6/6′, H-9/9′^{B}, H-10/10′, H-14/14′	
2/2′	208.8	–	H-3/3′	
3/3′	48.8	2.10 *s*	H-3′/3, H-15/15′	
4/4′	54.5	–	H-3/3′, H-6/6′, H-15/15′	
5/5′	163.9	–	H-3/3′, H-10/10′, H-15/15′	
6/6′	114.5	6.00 *s*	–	H-15/15′, H-12/12′, H-13/13′
7/7′	166.1	–	H-6/6′, H-9/9′^{A} (w)[b], H-9/9′^{B}, H-12/12′, H13/13′	
8/8′	104.2	–	H-6/6′, H-9/9′^{A}, H-9/9′^{B}, H-14/14′ (w)	
9/9′	41.5	A: 1.82 *dd* (13.7 / 13.1) B: 2.21 *dd* (13.7 / 7.0)	H-10/10′, H-14/14′	H-6/6′, H-12/12′ (w), H-14/14′ H-10/10′
10/10′	29.6	3.00 *m*	H-9/9′^{A}, H-9/9′^{B}, H-14/14′	H-9/9′^{B}, H-14/14′
11/11′	86.6	–	H-6/6′, H-12/12′, H-13/13′	
12/12′	27.5	1.49 *s*	H-13/13′	H-6/6′, H-9/9′^{A} (w), H-13/13′
13/13′	23.5	1.56 *s*	H-12/12′	H-6/6′
14/14′	19.2	1.23 *d* (6.7)	H-9/9′^{A}, H-10/10′	H-9/9′^{A}, H-9/9′^{B}, H-10/10′
15/15′	20.4	1.18 *s*	H-3/3′	H-3/3′, H-6/6′

[a] Obtained from the NOE difference spectra.
[b] w = weak.

The ^{13}C spectrum (Table 3) exhibited 15 signals, among them one carbonyl group (δ 208.8) and two double bonds (δ 166.1, 163.9, 147.8 and 114.5) for each monomeric moiety, thus leaving seven double bond equivalents for seven rings. The signals of the two guaiane moieties were assigned by analysis of the C–H long-range correlations from the GHMBC spectrum (Table 3), locating the carbonyl groups at C-2/2′ and the double bonds at positions 1(5)/1′(5′) and 6/6′. The C–H correlations of C-3 with H-3 ($^1J_{CH}$) and H-3′ ($^2J_{CH}$ or $^3J_{CH}$), the highly increased correlation peak between C-4 (δ 54.5) and H_3-15/15′ (δ 1.18) and the similarity of the chemical shifts of the cyclobutane atoms compared with vielanin B (**2**) proposed an analogous structure and relative configuration of the cyclobutane with vielanin C (**3**) containing two additional rings. Because of their chemical shift of δ 86.2 C-11/11′ thus had to bear an oxygen atom. The shift of C-8 (δ 104.2) was that of an acetalic or ketalic group. Connecting C-8/8′ with C-11/11′ via one oxygen atom accounted for the observed chemical shift and produced the two additional oxetane rings. Such an oxetane ring is known as structural element of a guaiane dimer from *Xylopia aromatica* (Martins et al., 1998). Comparing the so deduced structure with the molecular formula from the HRMS resulted in a discrepancy of two oxygen atoms. The chemical shifts of all atoms allowed no insertion of an additional oxygen into the molecule exept the formation of a peroxide resulting in the depicted structure of vielanin C (**3**).

The peroxide group could account for the antimalarial activity of this plant as terpene peroxides like artemisinin are known to possess this activity (Thebtaranonth et al., 1995).

H-9/9′^{A} appeared as *dd* with a geminal coupling of 13.7 Hz with H-9/9′^{B} and a vicinal coupling constant of 13.1 Hz with H-10/10′, thus showing the trans-diaxial conformation of H-9/9′^{A} and H-10/10′. Thus, the methyl group at C-10/10′ was equatorial. As in vielanin B (**2**), the relative configuration could not be determined by the observed NOE effects (Table 3).

3. Experimental

3.1. General experimental procedures

EIMS: AMD 402, 70 eV. ESIMS: Finnigan TSQ 700. NMR: Varian Gemini 300, Unity 500. CC: siliga gel 60, 40–63 μm (Merck), Lichroprep RP-18, 25–40 μm (Merck). Prep. TLC: precoated plates, silica gel 60, F_{254}, thickness 0.5 mm (Merck).

3.2. Plant material

Leaves of *X. vielana* (Lour.) Tan. Pierre ex Fin. & Gagn. were collected in August 1997 in Vinh phuc, North Vietnam, and identified by Ngo Van Trai, Institute of Materia Medica, Hanoi. A voucher specimen is deposited at the Institute of Ecology, National Centre for Natural Science and Technology, Hanoi, Vietnam.

3.3. Extraction and isolation

The plant material was dried (1.54 kg), ground and extracted 4× with MeOH–H_2O (95:5). The organic solvent was evaporated under red. pres. and the aq. residue extracted with *n*-hexane, EtOAc and *n*-BuOH, successively (each 3×) giving 27.4 g *n*-hexane extract, 16.9 g EtOAc extract and 72.9 g *n*-BuOH extract. The EtOAc extract was separated on silica gel (238 g, 63–200 μm)

with solvents of increasing polarity: $CHCl_3$, followed by 10%, 20% and 30% MeOH in $CHCl_3$ and finally $CHCl_3$–MeOH–H_2O (60:35:3). 16 Frs were collected. Fr. 11 (1.2 g, eluted with $CHCl_3$–MeOH 7:3) yielded after fractionation on silica gel with $CHCl_3$–MeOH (85:15) 45 mg (−)-epicatechin. Fr. 6 (1.7 g, eluted with $CHCl_3$–MeOH 8:2) was separated on siliga gel (160 g, 63–200 μm) with *n*-hexane–acetone (8:2) giving 149 fractions. Frs 24–27 (139 mg) were further purified on silica gel using *n*-hexane–acetone (8:2) followed by crystallization to give 20 mg pure vielanin A (**1**) and 33 mg mother liquid. Frs 87–101 (62 mg) were chromatographed on silica gel with *n*-hexane–acetone (7:3) to yield 3 mg of vielanin B (**2**). Vielanin C (**3**, 8 mg) was obtained after reversed-phase chromatography of Frs 142–149 (24 mg) on RP-18 with MeOH–H_20 (6.4). Frs 13–15 of the first column (2.48 g) yielded after chromatography on silica gel and on RP-18 38 mg of quercitrin.

3.3.1. Vielanin A (*1*)

Crystals from MeOH, m.p. 190–191°C. $[\alpha]_D^{27}$ −8° ($CHCl_3$, *c* 0.5). UV λ_{max}^{EtOH} nm (log ϵ): 261 (4.44). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 2964, 2937, 2875, 1741, 1724, 1662, 1635, 1597, 1458, 1440, 1381, 1369, 1333, 1243, 1184, 1073, 1062, 1033, 1007, 991. EIMS *m*/*z* (rel. int.): 506.3064 $[M]^+$, (0.4), ($C_{32}H_{42}O_5$ requires 506.3032), 446.2825 $[M–CH_3COOH]^+$ (11), ($C_{30}H_{38}O_3$ requires 446.2821), 276.1724 [monomer **1b**]$^+$ (6) ($C_{17}H_{24}O_3$ requires 276.1725), 234.1618, [monomer **1b** — C_2H_2O]$^+$ (100), ($C_{15}H_{22}O_2$ requires 234.1620), 230.1322 [monomer **1a**]$^+$ (19), ($C_{15}H_{18}O_2$ requires 230.1307) 216.1492 [monomer **1b** — CH_3COOH]$^+$ (81), ($C_{15}H_{20}O$ requires 216.1514), 174.1412 (15), ($C_{13}H_{18}$ requires 174.1409), 135.0806 (22), ($C_9H_{11}O$ requires 135.0810), 108. 0531 (12) (C_7H_8O requires 108.0575).

3.3.2. Vielanin B (*2*)

m.p. 210–212°C (EtOAc). EIMS *m*/*z* (rel. int.): 460.2596 $[M]^+$ (47) ($C_{30}H_{36}O_4$ requires 460.2614), 244 (25), 231 [Monomer + H]$^+$ (62), 230.1352 [monomer]$^+$ (100), ($C_{15}H_{18}O_2$ requires 230.1307), 229 (54), 215 [monomer — CH_3]$^+$ (30), 204 [monomer — CH_3 −OH]$^+$ (25), 187.1123 [monomer — CH_3−CO]$^+$ (33), ($C_{13}H_{15}O$ requires 187.1123), 173 (17), 159 (20), 145 (13), 105 (13), 91 (14), 69 (17), 55 (13).

3.3.3. Vielanin C (*3*)

m.p. 108–110°C (acetone). $[\alpha]_D^{26}$ −120° ($CHCl_3$, *c* 0.5). UV λ_{max}^{EtOH} nm (log ϵ): 292 (4.22), 204 (4.19). EIMS *m*/*z* (rel. int.): 262 (8) [monomer]$^+$, 244.1104 (59) [monomer — H_2O]$^+$ ($C_{14}H_{13}O_3$ requires 244.1099), 229.0859 (100) [monomer — H_2O−CH_3]$^+$ ($C_{14}H_{13}O_3$ requires 229.0865), 216 (20) [244 — CO]$^+$, 204 (4) [monomer $(CH_3)_2CO$]$^+$, 201 (19) [229 — CO]$^+$, 189 (29) [204 — CH_3]$^+$, 161 (23), 159 (24), 148 (19), 145 (22), 115 (24), 91 (31), 59 (41), 58 (52). Positive ESIMS *m*/*z* (rel. int.): 547 (44) [M + Na]$^+$, 525 (100) [M + H]$^+$.

3.3.4. (−)-Epicatechin

m.p. 233–235°C. $[\alpha]_D^{27}$ −47° (MeOH, *c* 1.0). Negative ESIMS *m*/*z* (rel. int.): 298.0 (100) [M−H]$^-$. The compound was identified by comparison of its ^{13}C NMR data with reference values (Agrawal, 1989).

3.3.5. Quercitrin

Amorphous solid, $[\alpha]_D^{30}$ −110° (MeOH, *c* 1.0). Negative ESI MS *m*/*z* (rel. int.): 447.2 (91) [M−H]−, 119.1 (100). The compound was identified by comparison of its ^{13}C NMR data with reference values (Agrawal, 1989).

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Sesquiterpene polyol esters from *Tripterygium wilfordii*

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Abstract

The extract (T_{II}) of *Tripterygium wilfordii* Hook f. afforded four sesquiterpene esters: 1β,2β,5α,8β,11-pentaacetoxy-4α-hydroxy-3α(2′-methylbutanoyl)-15-nicotinoyl-7-oxo-dihydroagarofuran; 1β,5α,11-triacetoxy-7β-benzoyl-4α-hydroxy-8β-nicotinoyl-dihydroagarofuran; 1β,2β,5α,11-tetraacetoxy-8α-benzoyl-4α-hydroxy-7β-nicotinoyl-dihydroagarofuran; 5α-benzoyl-4α-hydroxy-1β,8α-dinicotinoyl-dihydroagarofuran as well as one other known sesquiterpene ester. Their structures were established on the basis of spectroscopic studies.
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Keywords: *Tripterygium wilfordii*; Celastraceae; T_{II}; Sesquiterpene esters

1. Introduction

The genus *Tripterygium* has been used in traditional Chinese medicine for the treatment of cancer and as an insecticide for hundreds of years. Recently, the extract (T_{II}) of *T. wilfordii* has been employed in the clinical treatment of rheumatoid arthritis, skin disorders, in male-fertility control, and for other inflammatory and autoimmune diseases (Qian, 1987; Matlin et al., 1993; Qian et al., 1995). In the course of our studies on the bioactive metabolites of this genus, we have reported some interleukin-1 inhibitors, triptoquinone A and some analogues (Shishido et al., 1994), 22β-hydroxy-tingenone and celastrol (Takaishi et al., 1997) from *T. wilfordii* var. *regelii*, and also the isolation and structure determination of the HIV inhibitors triptonine B from *T. hypoglaucum* (Duan et al., 1999). As a continuation of previous studies in this area, the triterpene components of the extract (T_{II}) of *T. wilfordii* were examined and four new triterpenes were reported (Duan et al., 2000). This paper deals with the structure determination of four new sesquiterpene esters, along with one known sesquiterpene from the extract of *T. wilfordii*.

2. Results and discussion

The powdered extract (T_{II}) of *T. wilfordii* was subjected to silica gel gelchromatography and the fractions so obtained were further separated chromatographically to afford the sesquiterpene polyol esters **1**–**5**.

Compound **1** had the molecular formula, $C_{36}H_{45}O_{17}N$ (HRFABMS). It showed an ester carbonyl carbon band at 1751 cm^{-1} in the IR spectrum, and the UV spectrum showed the presence of an aromatic moiety (214 and 263 nm). The ^1H NMR spectral data of **1** revealed the presence of five acetyl methyl groups (δ_H 2.16, 2.14, 2.11, 2.02, and 1.91), a nicotinoyl group [δ_H 9.20 (1H, *s*), 8.81 (1H, *br d, J* = 3.8 Hz), 8.28 (1H, *br d, J* = 7.9 Hz), 7.44 (1H, *dd, J* = 7.8, 4.8 Hz)], and two oxygenated methylene groups [δ_H 5.10, 4.45 (each 1H, *d, J* = 11.5 Hz); 4.84, 4.44 (each 1H, *d, J* = 13.1 Hz)], as well as six methine proton signals (δ_H 6.65, 5.65, 5.58, 5.26, 4.79, and 3.14). The ^{13}C NMR spectral data of **1** revealed the presence of eight methyls, two oxygenated methylenes, one ketone, and five oxygenated methine carbons, in addition to one methine, seven ester carbonyl carbons, four quaternary carbons, and one nicotinoyl group [δ_C 165.0 (*s*), 150.8 (*d*), 125.4 (*s*), 137.2 (*d*), 123.7 (*d*), 154.0 (*d*)]. From the above information, the structure of compound **1** was based on a sesquiterpene polyol ester dihydroagarofuran skeleton, this having already been found in the genus of *Tripterygium* and other plants of the Celastraceae family (Takaishi et al., 1991a,b; Gonzalez et

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al., 1988). The ^1H–^1H COSY spectrum of **1** revealed a separated spin-spin system (H-1/H-2/H-3) in the dihydroagarofuran core. The remaining dihydroagarofuran proton signal at δ_H 7.02 (H-5) was correlated with the carbon signals at δ_C 50.3 (C-6), 51.9 (C-9), 94.2 (C-10), and 84.7 (C-13), and the proton signal at δ_H 3.14 (H-6) was correlated with the signals at δ_C 73.9 (C-5), 85.8 (C-13), 196.4 (C-7) and 77.4 (C-8) in the HMBC spectrum.

From the ^1H–^1H, ^{13}C–^1H COSY and HMBC spectra, the presence of a 2-methylbutanoyl group was deduced [$CH_3CH_2(CH)CH_3CO$–]. In the HMBC spectrum, the secondary methyl (δ_H 1.12) functionality of a 2-methylbutanoyl and the methine proton signal (δ_H 4.79, H-3) were correlated with the carbonyl carbon signal at δ_C 174.8, and the proton signal at δ_H 5.10 (H-14a or15a) with the resonance at δ_C 165.0 (nicotinoyl), while the dihydroagarofuran proton signals at δ_H 5.65 (H-1), 5.26 (H-2), 6.65 (H-5), 5.58 (H-8), and 4.84 (H-11a) were correlated with the acetyl carbonyl carbons at δ_C 169.2, 168.5, 169.5, 169.4 and 169.8, respectively. From the above observations, the 2-methylbutanoyl groups were assigned at positions C-3, and the five acetyl groups were assigned at positions C-1,-2,-5,-8, and C-11, respectively. In the NOESY spectrum of **1**, the proton signal at δ_H 5.58 (H-8) correlated with the signals at δ_H 1.60 (H_3-14), and the proton signal at δ_H 4.45 (H-15b) with the signal at δ_H 3.14 (H-6), So the nicotinoyl group was assigned at position C-15. Furthermore, the proton signal at δ_H 4.44 (H-11b) was correlated with the signals at δ_H 6.65 (H-5) and 1.58 (H_3-12), and the signal at δ_H 5.65 (H-1) with the signals at δ_H 5.26 (H-2) and 5.58 (H-8), while the signal at δ_H 4.79 (H-3) was correlated with the signal at δ_H 1.58 (H_3-12). Thus, the relative stereochemistry of the ester groups were elucidated as having 1β, 2β, 3α, 5α and 8β configurations. The ^1H and ^{13}C NMR spectral assignments were obtained by 2D NMR spectra including NOESY. Therefore, compound **1** was determined as shown (Fig. 1).

Compound **2**, $C_{34}H_{39}O_{12}N$, revealed three acetyl groups (δ_H 2.15, 2.08, and 1.49), a benzoyl group [δ_H 7.94 (2H, *d*, J=7.4 Hz), 7.55 (1H, *br t*, J=7.4 Hz), 7.40 (2H, *br t*, J=7.5 Hz)], and a nicotinoyl group [δ_H 9.22 (1H, *s*), 8.79 (1H, *d*, J=3.8 Hz), 8.26 (1H, *d*, J=7.9 Hz), 7.38 (1H, *dd*, J=7.9, 3.8 Hz)], as well as five methine proton signals [δ_H 5.38, 6.92, 2.59, 5.82 (2H)] from its ^1H NMR spectrum. The ^{13}C NMR spectral data were similar to those of **1**, except for the ester groups and the C-2, C-3, C-7 and C-15 carbon signals (Table 2). The isolate was also a dihydroagarofuran polyol ester having three acetyl, one benzoyl and one nicotinoyl group. In the HMBC spectrum of **2**, the proton signals at δ_H 5.38 (H-1), 6.92 (H-5) and 4.82 (H-11) were correlated with the carbon signals at δ_C 170.8, 169.9 and 169.9, respectively. Thus, three acetyl groups were assigned at

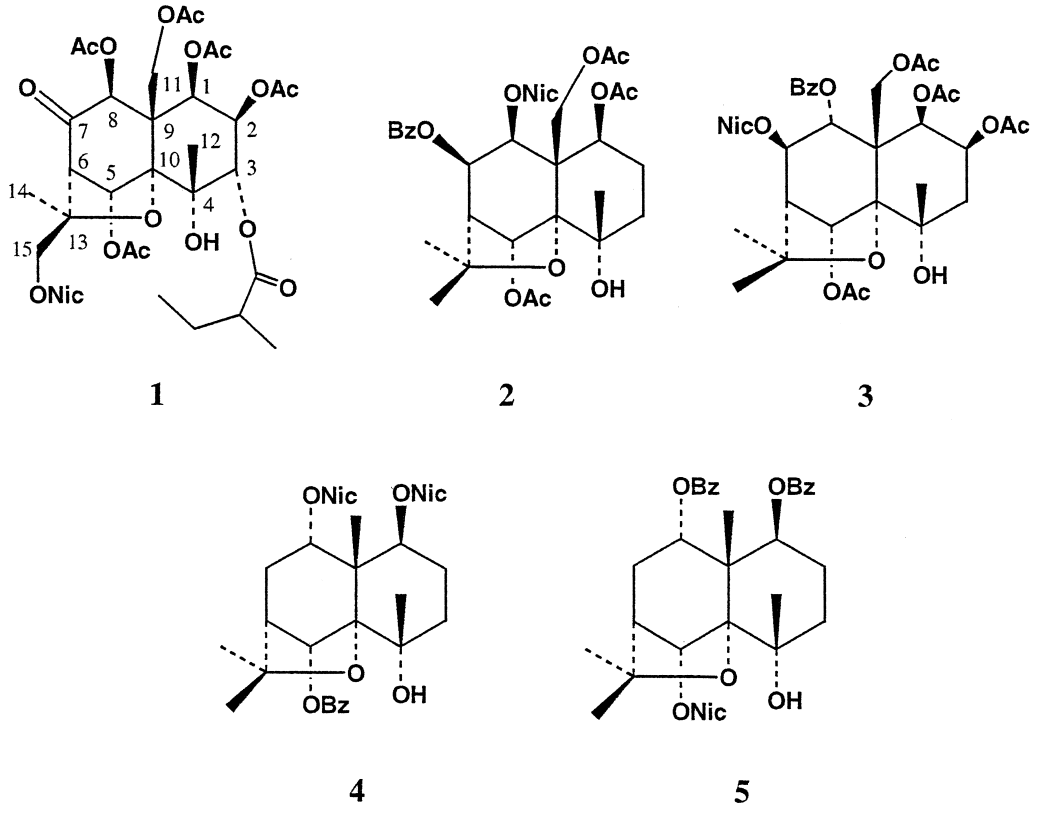


Fig. 1. Structures of compounds **1**–**5**.

positions C-1, C-5 and C-11. However, because the proton signals of H-7 and H-8 had the same chemical shift value at δ_H 5.82, it was impossible to determine the relative positions of the benzoyl and nicotinoyl groups. The ^{1}H NMR spectrum in C_5D_5N showed distinct proton signals at δ_H 6.28 and 6.21. In the HMBC spectrum (C_5D_5N), the proton signals at δ_H 6.21 (H-7) and 6.28 (H-8) were correlated with the carbonyl carbon signals at δ_C 165.4 (benzoyl) and 165.2 (nicotinoyl), respectively. Therefore, the benzoyl and nicotinoyl groups were located at positions C-7 and C-8, respectively. In the NOESY spectrum (C_5D_5N), the proton signal at δ_H 5.25 (H_2-11) correlated with the proton signals at 7.26 (H-5) and 1.61 (H_3-12), and the proton signal at δ_H 6.28 (H-8) with the signals at δ_H 5.86 (H-1) and 1.65 (H_3-14), while the signal at δ_H 6.21 (H-7) correlated with the signal at δ_H 1.65 (H_3-14). In turn, the signal at δ_H 2.67 (H-6) correlated with the signals at δ_H 7.26 (H-5) and 1.63 (H_3-15); therefore, the relative configurations of ester groups of **2** were determined as 1β, 5α, 7β and 8β, respectively (Fig. 1).

Compound **3**, $C_{36}H_{41}O_{14}N$, showed four acetyl, one benzoyl and one nicotinoyl group from its ^{1}H NMR spectrum. It was also a dihydroagarofuran polyol ester by comparison of the ^{13}C NMR spectral data with those of **2** (Table 2). The proton signals of H-2 and H-7 revealed the same chemical shift value (δ_H 5.60) in $CDCl_3$. In the same manner as described above, the four acetyl groups were assigned to positions C-1, C-2, C-5, and C-11, and the nicotinoyl and benzoyl groups were located at C-7 and C-8. In the NOESY spectrum (C_5D_5N), the proton signal at δ_H 5.23 (H-11a) was correlated with the signals at δ_H 6.67 (H-5) and 1.50 (H-12), whereas that of the proton signal at δ_H 5.78 (H-1) was with the signal at δ_H 5.78 (H-2), and the proton signal at δ_H 1.56 (H_3-14) was with that at δ_H 5.73 (H-7). In turn, the proton signal at δ_H 4.82 (H-11$_b$) was correlated with the signal at δ_H 6.06 (H-8). From the above observations, the relative configurations of these ester groups in **3** were elucidated as 1β, 2β, 5α, 7β, and 8α, respectively (Fig. 1).

Compound **4**, $C_{34}H_{36}O_8N$, had two nicotinoyl functionalities and one benzoyl group from the ^{1}H NMR spectrum. By comparing the ^{13}C NMR spectral data with those of regelidine (**5**, Table 2, Hori et al., 1987), it was also deduced to be a dihydroagarofuran polyol ester. In the HMBC spectrum of **4**, the proton signals at δ_H 5.66 (H-1), 5.73 (H-5) and 5.18 (H-8) were correlated with carbon resonances at δ_C 164.3, 165.0 and 165.3, respectively. Thus, the two nicotinoyl groups were assigned at positions C-1 and C-8, and the benzoyl group was located at C-5. In the NOESY spectrum, the proton signal at δ_H 1.62 (H_3-11) correlated with the signals at δ_H 5.73 (H-5), 5.18 (H-8) and 1.41 (H_3-12). In turn, the coupling constant of the signal at δ_H 5.66 (*dd*, 12.2, 3.9, H-1) showed an axial configuration. Thus, the relative configurations of the ester groups were concluded to be 1β, 5α and 8α. The CD spectrum of **4** revealed the first positive Cotton effect at 234 nm and the second negative Cotton effect at 219 nm (Fig. 2), which is attributable to exciton interaction between the nicotinoyl groups at positions C-1 and C-8 (Weigang and Nugent, 1969; Harada and Nakanishi, 1983). Thus, the absolute configurations of **4** were suggested to be 1S and 8S.

Known compound **5** was identified from its spectral data upon comparison with values reported in the literature for regelidine (Hori et al., 1987).

3. Experimental

NMR experiments were run on a Bruker ARX-400 instrument. ^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz, using TMS as int. stand. MS were obtained on a JEOL JMSD-300 instrument. Chromatography column: silica gel 60 (Merck) and Sephadex LH-20 (pharmacia); HPLC: GPC (Asahipak, GS-310 2G, MeOH), silica gel HPLC (Hibar RT 250-25, LiChrosorb Si 60), ODS (INERTSIL PREP ODS, 20.0×250 mm, GL Sciences Inc.). IR spectra were recorded on a 1720 infrared Fourier Transform spectrometer (Perkin–Elmer), UV spectra were obtained using a UV 2100 UV-vis recording spectrometer

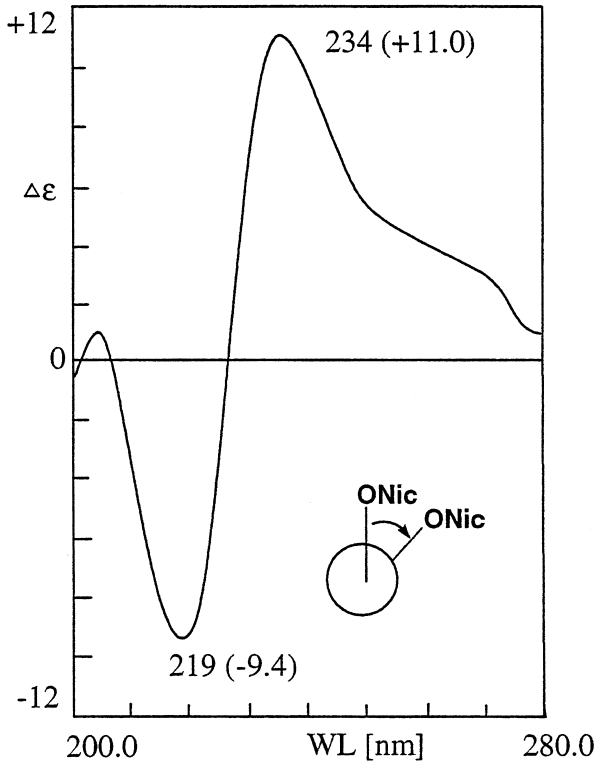


Fig. 2. The CD spectrum of compound **4** in MeOH.

(Shimadzu). Optical rotations were measured with a JASCO DIP-370 digital polarimeter.

3.1. Isolation of compounds 1–5

The powdered extract T_{II} of *T. wilfordii* was purchased in 1997 from the School of Pharmacy, Shanghai Medical University, People's Republic of China. It was extracted from the root xylem with water, then with chloroform and subjected to column chromatographic separation (silica gel, $CHCl_3$–MeOH, 95:5). Samples of T_{II} and the original plant (*T. wilfordii*) are deposited in the Faculty of Pharmaceutical Sciences, University of Tokushima, Japan.

The extract (T_{II}, 54 g) was applied to a silica gel column (1.0 kg, 11×90 cm), this being eluted with solvents of increasing polarity [$CHCl_3$–MeOH (99:1, 95:5, 9:1, MeOH)] to give ten frs (fr. 1–10). Fr. 5 (16.5 g) was subjected to silica gel column (6×80 cm) chromatography, this being eluted with hexane–EtOAc (1:1, 1:2, 1:4) to give 12 frs (fr. 5.1–5.12). Fr. 5.9 (4.8 g) was applied to a Sephadex LH-20 column, to give 5 frs (fr. 5.9.1–5.9.5), whereas Fr. 5.9.1 was subjected to ODS (MeOH–H_2O, 8:2) chromatography to give 7 frs (fr. 5.9.1.1–5.9.1.7). Fr. 5.9.1.4 was separated by ODS (MeOH–H_2O, 7:3) and then Si 60 to give **2** (5 mg) and **5** (17 mg). Fr. 5.9.1.5 was separated by ODS (MeOH–H_2O, 7:3) to give **3** (38 mg) and another three frs. (fr. 5.9.1.5.1-5.9.1.5.3). Fr. 5.9.1.5.2 was further separated by ODS (MeOH-H_2O, 1:1) to give **1** (4 mg). Fr. 5.9.6 was separated by Si 60 ($CHCl_3$–MeOH, 99:1) to give **4** (3.5 mg).

3.2. 1β,2β,5α,8β,11-Pentaacetoxy-4α-hydroxy-3α (2′-methylbutanoyl)-15-nicotinoyl-7-oxo-dihydroagarofuran (1)

Amorphous powder, $[\alpha]_D^{25}+14.1^\circ$ (MeOH, *c* 0.3). UV λ_{max}^{MeOH} nm (log ε): 263 (3.71), 214 (4.31). IR ν_{max}^{KBr} cm^{-1}: 3464, 2965, 2361, 1751, 1649, 1540, 1458, 1421, 1373, 1226, 1090, 1042, 807, 743. ^1H NMR ($CDCl_3$), see Table 1, δ 9.20 (1H, *s*), 8.81 (1H, *br d, J*=3.8 Hz), 7.44 (1H, *dd, J*=4.5, 7.8 Hz), 8.28 (1H, *br d, J*=7.9 Hz) (15-Nic); 1.12 (3H, *d, J*=7.1 Hz)), 2.40 (1H, *q, J*=7.1 Hz), 1.74 (1H, *m*), 1.44 (1H, *m*), 0.87 (3H, *t, J*=7.4 Hz) (3-2-methylbutanoyl). ^{13}C NMR ($CDCl_3$), see Table 2, δ 174.8, 41.0, 25.9, 11.5, 16.4 (3-2-methylbutanoyl); 165.0, 125.4, 137.2, 123.7, 154.0, 150.8 (15-Nic). FAB MS: *m*/*z* 764 $[M+H]^+$. HR FABMS: *m*/*z* 764.2738 $[M+H]^+$, $C_{36}H_{46}O_{17}N$ requires 764.2766.

3.3. 1β,5α,11-Triacetoxy-7β-benzoyl-4α-hydroxy-8β-nicotinoy-dihydroagarofuran (2)

Amorphous powder, $[\alpha]_D^{25}-52.4^\circ$ (MeOH, *c* 0.4). UV λ_{max}^{MeOH} nm (log ε): 253 (3.64), 216 (4.31). IR ν_{max}^{KBr} cm^{-1}: 3648, 3481, 2988, 2361, 1737, 1650, 1541, 1510, 1455, 1421, 1371, 1290, 1229, 1096, 714. ^1H NMR, in $CDCl_3$: see Table 1, δ 7.94 (2H, *d, J*=7.4 Hz), 7.40 (2H, *br t, J*=7.7 Hz), 7.55 (1H, *br t, J*=7.4 Hz) (7-Bz); 9.22 (1H, *s*), 8.79 (1H, *d, J*=3.8 Hz), 7.37 (1H, *dd, J*=3.8, 7.9 Hz), 8.26 (1H, *d, J*=7.9 Hz) (8-Nic); in C_5D_5N: δ 7.26 (1H, *s*, H-5), 6.28 (1H, *d, J*=5.7 Hz, H-8), 6.21 (1H, *dd, J*=5.7, 3.7 Hz, H-7), 5.86 (1H, *br t, J*=7.3 Hz, H-1), 5.25 (2H, *s*, H2-11), 2.67 (1H, *d, J*=3.7 Hz, H-6), 2.32 (1H, m, H-

Table 1
^1H NMR spectral data of compounds **1**–**4**[a]

No.	1	2	3	4
1	5.65 (*d*, 2.9)	5.38 (*dd*, 11.8, 4.4)	5.78 (*d*, 3.7)	5.66 (*dd*, 12.2, 3.9)
2	5.26 (*dd*, 2.8, 2.9)	1.85 (*m*)	5.70 (*m*)	2.12 (*m*)
	–	1.66 (*m*)	–	1.65 (*m*)
3	4.79 (*d*, 2.8)	1.99 (*m*)	2.41 (*dd*, 14.8, 3.9)	2.09 (*m*)
	–	1.74 (*m*)	1.87 (*m*)	1.82 (*m*)
5	6.65 (*s*)	6.92 (*s*)	6.67 (*s*)	5.73 (*s*)
6	3.14 (*s*)	2.59 (*br s*)	2.51 (*d*, 2.7)	2.40 (*br s*)
7	–	5.82 (*br s*)	5.73 (*br d*, 2.8)	2.63 (*m*)
	–	–	–	2.30 (*m*)
8	5.58 (*s*)	5.82 (*br s*)	6.06 (*s*)	5.18 (*d*, 6.9)
11	4.84 (*d*, 13.1)	4.82 (2H, *s*)	5.23 (*d*, 12.9)	1.62 (*s*)
	4.44 (*d*, 13.1)		4.82 (*d*, 12.9)	–
12	1.58 (*s*)	1.36 (*s*)	1.51 (*s*)	1.41 (*s*)
14	1.60 (*s*)	1.60 (*s*)	1.56 (*s*)	1.59 (*s*)
15	5.10 (*d*, 11.5)	1.73 (*s*)	1.52 (*s*)	1.56 (*s*)
	4.45 (*d*, 11.5)	–	–	–
1-Ac	1.91 (*s*)	1.49 (*s*)	1.40 (*s*)	–
2-Ac	2.14 (*s*)	–	1.92 (*s*)	–
5-Ac	2.16 (*s*)	2.15 (*s*)	1.98 (*s*)	–
8-Ac	2.11 (*s*)	–	–	–
11-Ac	2.02 (*s*)	2.08 (*s*)	1.89 (*s*)	–

[a] The signals of other ester groups see Section 3, **1**, **2** and **4**: $CDCl_3$, **3**: C_5D_5N.

Table 2
^{13}C NMR spectral data of compounds **1**–**5**[a] ($CDCl_3$)

Carbon	1	2	3	4	5
1	71.6	78.3	70.8	74.1	73.3
2	68.4	25.0	67.8	23.6	38.9
3	75.2	37.8	42.0	39.0	23.5
4	69.5	70.6	70.0	70.8	70.8
5	73.9	75.3	75.4	80.9	80.9
6	61.9	53.5	53.7	49.1	49.1
7	196.4	71.7	77.8	32.1	31.9
8	77.4	72.8	72.7	73.2	73.3
9	52.2	52.2	54.6	51.9	51.8
10	94.5	91.9	91.5	91.7	91.6
11	60.4	61.1	65.9	20.1	20.1
12	23.8	22.6	24.3	24.1	24.0
13	85.8	82.7	83.3	84.7	84.5
14	19.2	24.5	25.8	25.9	25.9
15	70.1	29.5	29.5	29.8	29.7
1-OAc	20.5	20.8	20.3	–	–
	169.2	169.9	169.3	–	–
2-OAc	21.0	–	21.5	–	–
	168.5	–	169.9	–	–
5-OAc	21.3	21.6	21.1	–	–
	169.5	169.9	169.8	–	–
8-OAc	20.2	–	–	–	–
	169.4	–	–	–	–
11-OAc	20.5	21.4	21.2	–	–
	169.8	170.8	170.5	–	–

[a] The signals of other ester groups see Section 3.

3a), 2.27 (3H, *s*, 11-OAc), 2.17 (3H, *s*, 5-OAc), 1.95 (2H, *m*, H-2), 1.81 (1H, *m*, H-3b), 1.65 (3H, *s*, H-14), 1.63 (3H, *s*, H-15), 1.61 (3H, *s*, H-12), 1.58 (3H, *s*, 1-OAc); 9.71 (1H, *d, J* = 1.5 Hz), 8.90 (1H, *dd, J* = 4.8, 1.5 Hz), 8.50 (1H, *br d, J* = 7.9 Hz), 7.37 (1H, *dd, J* = 7.9, 4.8 Hz) (8-Nic); 8.30 (2H, *d, J* = 7.3 Hz), 7.51 (1H, *br t, J* = 7.3 Hz), 7.39 (2H, *t, J* = 7.3 Hz) (7-Bz). ^{13}C NMR, in $CDCl_3$: see Table 2, δ 164.8, 129.2, 129.6, 128.8, 133.6 (7-Bz); 164.8, 126.0, 137.2, 123.4, 153.9, 151.2 (8-Nic); in C_5D_5N: 92.4 (*s*, C-10), 82.7 (*s*, C-13), 78.8 (*d*, C-1), 75.6 (*d*, C-5), 73.7 (*d*, C-8), 72.1 (*d*, C-7), 70.7 (*s*, C-4), 61.2 (*t*, C-11), 53.8 (*d*, C-6), 52.9 (*s*, C-9), 38.1 (*t*, C-3), 29.1 (*q*, C-15), 25.5 (*t*, C-2), 24.2 (*q*, C-14), 23.5 (*q*, C-12); 165.2 (*s*), 154.3 (*d*), 151.5 (*d*), 137.4 (*d*), 126.5 (*s*), 123.7 (*d*) (8-Nic); 165.4 (*s*), 133.9 (*d*), 130.1 (*s*), 130.0 (*d*), 129.1 (*d*) (7-Bz); 170.7 (*s*), 21.4 (*q*) (11-OAc); 170.4 (*s*), 21.4 (*q*) (5-OAc); 170.0 (*s*), 20.7 (*q*) (1-OAc). EI MS: *m*/*z* (rel. int.) 653 $[M]^+$ (12), 611 (9), 593 (9), 530 (7), 488 (11), 246 (10), 228 (9), 202 (10), 190 (14), 164 (22), 147 (8), 124 (72), 105 (100), 77 (11), 43 (13). HR EIMS: *m*/*z* 653.2498, $C_{34}H_{39}O_{12}N$ requires 653.2472.

3.4. 1β,2β,5α,11-Tetraacetoxy-8α-benzoyl-4α-hydroxy-7β-nicotinoyl-dihydroagarofuran (*3*)

Amorphous powder, $[\alpha]_D^{25}$ −38.4° (MeOH, *c* 1.0). UV λ_{max}^{MeOH} nm (log ε): 263 (3.64), 229 (4.33). IR ν_{max}^{KBr} cm^{-1}: 3468, 2928, 2362, 1744, 1593, 1370, 1232, 1156, 1110, 1047, 1028, 946, 876, 712. ^{1}H NMR (C_5D_5N), see Table 1, δ 9.50 (1H, *s*), 8.67 (1H, *dd, J* = 1.6, 4.8 Hz), 7.21 (1H, *dd, J* = 4.8, 7.9 Hz), 8.37 (1H, d*t, J* = 7.9, 1.7 Hz) (7-Nic); 8.03 (2H, *d, J* = 7.4 Hz), 7.22 (2H, *br t, J* = 7.5 Hz), 7.31 (1H, *br t, J* = 7.3 Hz) (8-Bz). ^{13}C NMR ($CDCl_3$), see Table 2, δ 164.2, 125.8, 137.4, 123.4, 154.0, 151.4 (7-Nic); 164.4, 128.4, 130.3, 128.6, 134.0 (8-Bz). EI MS: *m*/*z* (rel. int.) 711 $[M]^+$ (28), 669 (13), 651 (17), 609 (19), 591 (10), 588 (14), 532 (15), 486 (9), 429 (9), 251 (8), 237 (9), 202 (23), 166 (11), 149 (15), 124 (55), 105 (100), 77 (8), 43 (21). HR EIMS: *m*/*z* 711.2491, $C_{36}H_{41}O_{14}N$ requires 711.2527.

3.5. 5α-Benzoyl-4α-hydroxy-1β,8α-dinicotinoyl-dihydroagarofuran (*4*)

Amorphous powder, $[\alpha]_D^{25}$ +67.7° (MeOH, *c* 0.3). UV λ_{max}^{MeOH} nm (log ε): 263 (3.79), 222 (4.40). IR ν_{max}^{KBr} cm^{-1}: 3650, 3443, 2925, 2854, 2362, 1718, 1650, 1455, 1285, 1455, 1285, 1114, 1025. ^{1}H NMR ($CDCl_3$), see Table 1, δ 8.72 (1H, *s*), 8.66 (1H, *br d, J* = 4.5 Hz), 7.21 (1H, *dd, J* = 4.5, 7.5 Hz), 7.79 (1H, *d, J* = 7.5 Hz) (1-Nic); 7.80 (2H, *d, J* = 7.9 Hz), 7.36 (2H, *br t, J* = 7.9 Hz), 7.53 (1H, *br t, J* = 7.3 Hz) (5-Bz); 9.40 (1H, *s*), 8.82 (1H, *br d, J* = 4.5 Hz), 7.44 (1H, *dd, J* = 4.7, 7.5 Hz), 8.53 (1H, *dd, J* = 1.5, 6.2 Hz) (8-Nic). ^{13}C NMR ($CDCl_3$), see Table 2, δ 164.3, 125.9, 136.5, 123.0, 153.3, 150.5 (1-Nic); 165.0, 129.2, 129.9, 128.3, 133.3 (5-Bz); 165.3, 125.9, 137.7, 123.6, 153.9, 151.7 (8-Nic). EI MS: *m*/*z* (rel. int.) 600 $[M]^+$ (16), 463 (9), 436 (37), 229 (10), 124 (40), 105 (100), 78 (22), 44 (14). HR EIMS: *m*/*z* 600.2448, $C_{34}H_{36}O_8N_2$ requires 600.2472.

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Sesqui- and diterpenoids from two Japanese and three European liverworts

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Dedicated to Professor Otto R. Gottlieb on the occasion of his 80th birthday

Abstract

A new peroxy muurolane-type sesquiterpenoid was isolated from the ether extract of the Belgium liverwort *Scapania undulata*, together with three known *ent*-muurolanes. A new lepidozane-type sesquiterpenoid was isolated from the Japanese *Porella subobtusa* together with a known santalane- and two africane-type sesquiterpenoids. All structures were determined by means of NMR spectroscopic techniques. The chemosystematics of each species are discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Scapania undulata*; *S. nemorea*; *Mylia taylorii*; *Porella subobtusa*; *Jungermannia truncata*; Liverwort; Hepaticae; Muurolane-type; Lepidozane-type; Labdane-type; *seco*-Clerodane-type; Sesquiterpenoid; Diterpenoid; Chemosystematics

1. Introduction

As part of a search for novel and biologically active compounds in the Hepaticae, we are continuing to study the chemical constituents of liverworts (Asakawa, 1982, 1995). Liverworts contain a large amount of mono-, sesqui- and diterpenoids, and aromatic compounds. In particular, liverworts contain pinguisane-type sesquiterpenoids, sacculatane-type diterpenoids and bis(bibenzyl) aromatic compounds which have not been found in higher plants. Chemical differences of the main components in the same liverwort species have been noted from different localities (Asakawa, 1982, 1995; Nagashima and Asakawa, 1998). In this paper, we describe the isolation and characterization of new muurolane- and lepidozane-type sesquiterpenoids from *Scapania undulata* (L.) Dum. and *Porella subobtusa* (Steph.) Hatt., respectively. We report also the distribution of sesqui- and diterpenoids in three European liverworts *S. undulata* (L.) Dum., *Scapnia nemorea* (L.) Dum. and *Mylia taylorii* (Hook.) S. Gray, and two Japanese *P. subobtusa* (Steph.) Hatt. and *Jungermannia truncata* Nees and discuss their chemosystematics.

2. Results and discussion

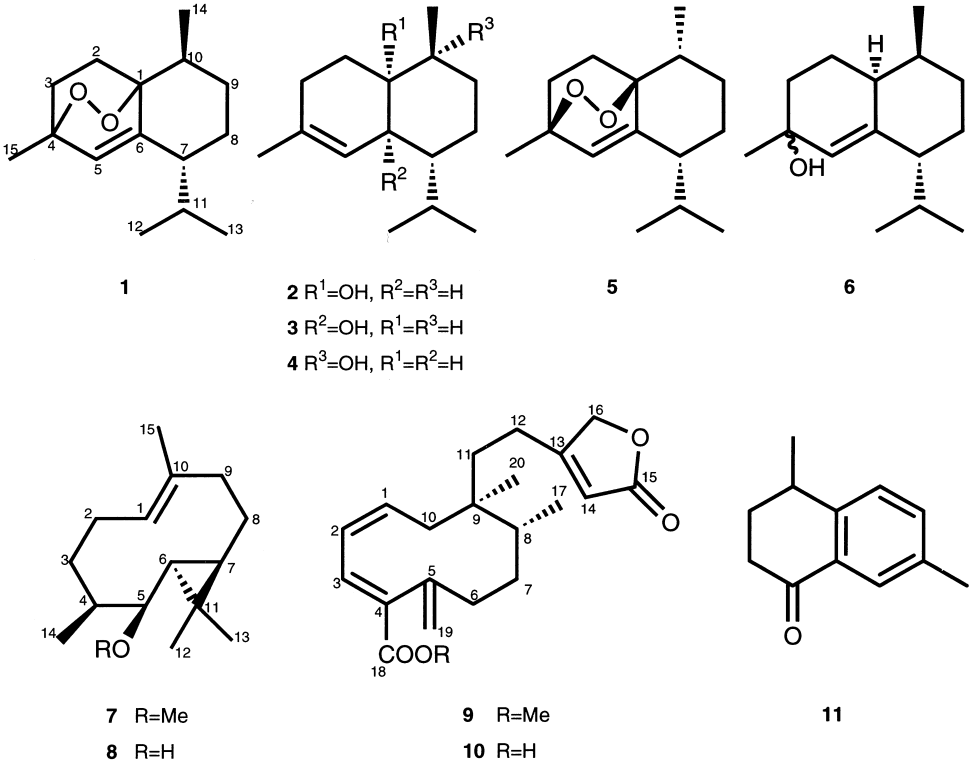
2.1. Scapania undulata

A new peroxy sesquiterpenoid **1** was isolated from the ether extract of Belgian *S. undulata*, together with three previously known muurolanes, (+)-*ent*-epicubenol (Connolly et al., 1982), (+)-4-muurolen-6α-ol and *ent*-T-muurolol (Nagashima et al., 1994).

The EIMS and HR–EIMS spectra of compound **1** produced the molecular formula $C_{15}H_{24}O_2$ (anal. *m*/*z* 236.1763 $[M]^+$) indicating four degrees of unsaturation. The ^1H and ^{13}C NMR spectra of **1** indicated the presence of three secondary methyls, a tertiary methyl, a trisubstituted double bond (δ_C 129.9 *d*, 147.5 *s*) and two quaternary carbons (δ_C 74.9, 77.7) bearing an oxygen atom. The ^1H NMR spectrum of **1** resembled the spectra of muurolanes **2**–**4**. The above spectral data suggested that compound **1** might be a muurolane-type tricyclic sesquiterpenoid. The ^1H–^1H COSY spectrum of **1** indicated the presence of two partial structures A and B (Fig. 1). The HMBC spectrum (Fig. 1) displayed the cross peak between the tertiary methyl proton (H-15) and a methylene carbon (C-3) in the partial structure A, a quaternary carbon at δ 74.9 (C-4), and an olefinic carbon at δ 129.9 (C-5). The olefinic proton (H-5) was correlated with a methine carbon (C-7) in the partial

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structure B and a quaternary carbon at δ 77.7 (C-1), which also correlated with a secondary methyl (H-14) in the partial structure B and a methylene carbon (C-2) in the partial structure A. Additionally, the chemical shifts of two quaternary carbons (δ_C 74.9, 77.7) bearing an oxygen atom of compound **1** appeared lower than that of compounds **2** (δ 72.7) and **6** (δ 67.7). The IR spectrum of **1** showed neither absorption bands of hydroxyl nor carbonyl group. The existence of two oxygen atoms by HR–EIMS and the above spectral data supported the presence of a peroxy group in the structure of **1**. Thus, the structure of **1** was established to be a 1,4-peroxy muurolane-type sesquiterpenoid. This planar structure accorded with **5** isolated from *Bazzania tricrenata* (Buchanan, 1994), *Bazzania trilobata* (Nagashima et al., 1996b) and *Illicium tsangii* (Ngo et al., 1999). However, the ^{1}H and ^{13}C NMR spectra of **1** were not slightly identical with those of **5**, indicating that compound **1** might be a stereoisomer of **5**. In the phase sensitive NOESY spectrum of **1**, the NOEs were observed between (i) H-14 and H-9α, H-9β, and H-2β, (ii) H-10α and H-2α, (iii) H-4β and H-3β, H-6, and (iv) H-8β and H-9β, H-12. The stereochemistry of the secondary methyl (H-14) and isopropyl group at C-7 was revealed to be *trans*, but the stereochemistry of the 1,4-peroxy group remains to be clarified. From the above spectral evidence, the structure of **1** was deduced to be 1,4-peroxy-5-muurolene to take account of the isolation of muurolane-type sesquiterpenoids **2**–**4** from this species.

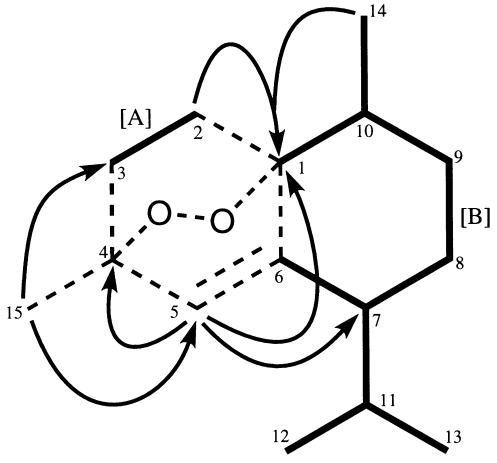


Fig. 1. ^{1}H–^{1}H correlations (bold lines) and HMBC correlations (arrows) of **1**.

2.2. *Porella subobtusa*

A new lepidozane-type sesquiterpenoid **7** was isolated from the ether extract of the Japanese *P. subobtusa*, together with three known sesquiterpenoids, α-santalane-12(*S*),13-diol (Toyota et al., 1992; Nagashima et al., 1996a), caespitenone (Tori et al., 1993) and secoswartzianin B (Tori et al., 1994).

The molecular formula, $C_{16}H_{28}O$ (anal. *m*/*z* 236.2143 $[M]^+$), of **7** was determined by HR–EIMS. The IR spectrum had no absorption for hydroxyl and carbonyl

groups. The ^{1}H NMR spectrum showed the presence of a secondary methyl, an olefinic methyl, two tertiary methyls, a methoxyl group and an olefinic proton, together with two cyclopropane protons (δ 0.15, –0.14 each 1H). The ^{13}C NMR spectrum of **7** contained sixteen carbons and its DEPT spectrum showed the presence of a trisubstituted olefinic carbon and a methine bearing a methoxyl group, together with five methyls, four methylenes, three methines and a quaternary carbon. The ^{1}H and ^{13}C NMR spectra of **7** resembled those of (4*S**, 5*S**, 6*R**, 7*R**)-1(10)*E*-lepidozen-5-ol (**8**) (Tori et al., 1993) except for the presence of a methoxyl group. Accordingly, the structure of **7** was recognized to be a lepidozane-type sesquiterpenoid. The ^{1}H–^{1}H COSY spectrum gave the connectivity of $-CH(1)-CH_2(2)-CH_2(3)-CH(CH_3)(4)-CH(5)-CH(6)-CH(7)-CH_2(8)-CH_2(9)-$. The HMBC spectrum showed the correlation between (i) H-14$_{Me}$ and C-1, C-9, and (ii) H-12$_{Me}$, H-13$_{Me}$ and C-6, C-7, C-11. Thus, the structure of **7** was determined to be a lepidozane-type sesquiterpenoid with a methoxyl group at C-5. The NOESY spectrum showed NOE correlations between (i) H-7 and H-8α, H-13, (ii) H-13 and H-5, (iii) H-6 and H-8β, H-12, H-15, methoxyl group at C-5, and (iv) H-1 and H-4, H-9α. On the basis of the above evidence, the structure of **7** was determined to be (4*S**, 5*S**, 6*R**, 7*R**)-5-methoxy-1(10)*E*-lepidozene.

2.3. *Scapania nemorea, Mylia taylorii and Jungermannia truncata*

ent-Spathulenol (Asakawa et al., 1980) and (−)-diplophyllin (Ohta et al., 1977), and a *seco*-clerodane-type diterpenoid **9** were isolated from the ether extract of the German species *S. nemorea*. The ^{1}H NMR spectrum of **9** was identical with that of the methyl ester derivative of **10** isolated from *Pulicaria angustifolia* (Compositae) (Singh et al., 1985). However, the ^{13}C NMR data of **9** were not reported in the reference, therefore, its NMR assignment was determined by the HMQC and HMBC spectra (Table 2). In addition, compound **9** was isolated for the first time from *S. nemorea* as a natural product.

Trinorsesquiterpene **11** (El-Seedi et al., 1994), (+)-(9*R**, 13*S**)-dihydroxy-8(17),14-labdadiene (Nagashima et al., 1997) and 13-*epi*-sclareol (Bigley et al., 1960) were isolated from the ether extract of the Japanese species *J. truncata*. The ^{1}H NMR spectrum of **11** was identical with the reference data (El-Seedi et al., 1994). Furthermore, chiral GC-MS analysis indicated that **11** was composed of a mixture of both enantiomers (*ca.*1:1).

The ether extract of the Austrian species *M. taylorii* afforded ten sesquiterpenoids, (+)-*ent*-maali-4(15)-en-1β-ol, cyclomyltaylenol, myltaylenol, (+)-globulol, (+)-4(15)-dehydroglobulol, (+)-4(15)-dehydroledol, (−)-myliol, (−)-taylorione, myltaylorione A and myltaylorione B (Matsuo and Takaoka, 1990; Takaoka et al., 1991).

The chemical constituents of *S. undulata* collected in different locations have been analyzed and the muurolane- and longibornane-type sesquiterpenoids or labdane-type diterpenoids isolated as main components (Huneck and Klein, 1967; Matsuo et al., 1973; Connolly et al., 1982; Huneck et al., 1986; Nagashima et al. 1993; Nagashima et al., 1994; Yoshida et al., 1997). The present species produced the muurolane-type sesquiterpenoids as the main components and, therefore, this species is classified into the muurolane-type.

The chemical constituents of *S. nemorea* have already been analyzed. Bibenzyls (Gorham, 1977), aromadendrane- and longifolene-type sesquiterpenoids (Svenson and Bendz, 1972; Andersen et al., 1978) and *seco*-clerodane- and *cis*-clerodane-type diterpenoids (Geis et al., 1999) were isolated. The isolation of an eudesmane-type sesquiterpenoid was the first example from *S. nemorea*.

The labdane-type diterpenoids were obtained from *J. truncata* as main components. In the previous study of the same species (Nagashima et al., 1990; Buchanan et al., 1996; Liu et al., 1997; Nagashima and Asakawa, 1998), the *ent*-kaurane-type diterpenoids were isolated as major components. Thus, the present species is considered to be a new chemo-type of *J. truncata*.

The chemical constituents of the present *P. subobtusa* and *M. taylorii* are no geographical difference in each same species.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR: 400 and 600 MHz (^{1}H NMR) and 100, 150 MHz (^{13}C NMR). Chemical shift values were expressed in δ (ppm) downfield from tetramethylsilane as an internal standard (^{1}H NMR) and δ 77.03 (ppm) from $CHCl_3$ as a standard (^{13}C NMR). TLC: visualized under UV (254 nm) light and by spraying with Godin reagent (Godin, 1954) followed by heating. MeOH–CH_2Cl_2 (1:1) and $CHCl_3$ were used for Sephadex LH-20 and $[\alpha]_D$, respectively.

3.2. Plant material

S. undulata (L.) Dum. (JH3853) was collected in Belgium and identified by Professor S.R. Gradstein (Universität Göttingen, Germany). *S. nemorea* (L.) Dum. (N9607) and *M. taylorii* (Hook.) S. Gray (N9608) were collected in Germany and in Salzburg, Austria, respectively and identified by Dr. S. Huneck (Germany). *P. subobtusa* (Steph.) Hatt. (H9501) and *J. truncata* Nees (N9304) were collected in Tokushima, Japan and identified by Dr. M. Mizutani (Hattori Botanical Lab., Japan). The voucher specimens were deposited at

Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

3.3. GC–mass spectrum

The temperature programming of GC–MS analysis performed from 50°C isothermal for 3 min, then 50–250°C at 5°C min^{-1}, and finally isothermal at 250°C for 15 min and from 80–250°C at 15°C min^{-1}, and finally isothermal at 250°C for 15 min. Injection temp was 250°C. A fused silica column coated with β-DEX 120 (30 m×0.25 mm id, film thickness 0.25 μm) and DB1 (30 m×0.25 mm id, film thickness 0.25 μm) were used.

3.4. Extraction and isolation from Scapania undulata

The dry material (425 g) of *S. undulata* was ground and extracted with Et_2O for 1 month. The crude extract (4.9 g) was divided into 11 fractions by CC on silica gel using *n*-hexane-EtOAc gradient. Fr. 5 was chromatographed on SiO_2 and MPLC (Lobar® column) to give (+)-*ent*-epicubenol (640 mg) and a sesquiterpene mixture, which was rechromatographed on SiO_2 and prep. HPLC (Chemcosorb 5-ODS-H, CH_3CN) to yield compound **1** (5 mg) and (+)-4-muurolen-6α-ol (35 mg). (+)-T-muurolol (16 mg) and (+)-*ent*-epicubenol (116 mg) were isolated by chromatography on Sephadex LH-20 (CH_2Cl_2–MeOH 1:1), SiO_2 and prep. HPLC (Chemcosorb 5-ODS-H, CH_3CN) of Fr. 6. A fatty acid mixture from Fr. 7 was treated with N,O-bis(trimethylsilyl)-acetamide to obtain TMS derivatives which were analyzed by GC–MS to detect palmitic acid, linoleic acid, linolenic acid and octadecanoic acid.

3.5. Compound *1*

Amorphous; $[\alpha]_D$ +41.9° (*c* 2.30); HR–EIMS: found 236.1763 $C_{15}H_{24}O_2$ requires 236.1776; FTIR ν_{max} cm^{-1}: 1458, 1375, 1255, 900, 878, 764; ^1H and ^{13}C NMR: Tables 1 and 2; EIMS *m/z* (rel. int.): 236$[M]^+$(17), 218 (46), 204 (37), 200 (56), 193 (33), 185 (32), 175 (100), 159 (70), 157 (81), 147 (33), 143 (51), 128 (26), 115 (21), 105 (35), 91 (28), 77 (15), 67 (8), 55 (11), 43 (19).

3.6. Extraction and isolation from Porella subobtusa

The ether extract (4.3 g) from dried *P. subobtusa* (93 g) was divided into 10 fractions by CC on silica gel using *n*-hexane–EtOAc gradient. Frs. 5 and 6 were chromatographed on SiO_2, MPLC (Lobar® column, Si 60) and prep. HPLC (Nucleosil 50-5, 10% *n*-Hexane–EtOAc) to give (4*S**, 5*S**, 6*R**, 7*R**)-5-methoxy-1(10)*E*-lepidozene (**7**) (8 mg), caespitenone (57 mg) and secoswartzianin B (11 mg). α-Santalane-12(*S*),13-diol (368 mg) was isolated from Fr. 8 by chromatography on Sephadex LH-20, SiO_2 and MPLC (Lobar® column).

Table 1
^1H NMR data of **1** and **7** (600 MHz, $CDCl_3$)

H	**1**	**7**
1		5.38 *br s*
2	1.27 *ddd* (13.2, 12.1, 4.1), α	1.83 *br q*
	2.35 *ddd* (13.2, 9.6, 3.8), β	2.14 *br s*
3	1.50–1.56 *m*, α	1.10–1.17 *m*
	2.01 *ddd* (13.2, 9.6, 3.8), β	1.70 *br t*
4		1.60 *m*
5	6.09 *d* (1.9)	2.72 *dd* (8.2, 1.9)
6		0.15 *dd* (8.2, 6.0)
7	2.51 *q* like	−0.14 *dd* (10.2, 5.5)
8	1.34 *m*, α	1.10–1.17 *m*
	1.77 *m*, β	1.98 *m*
9	1.50–1.56 *m*, α	1.96 *m*
	1.45 *dddd* (13.2, 13.2, 11.3, 4.7), β	2.30 *br d* (12.9)
10	1.73 *m*	
11	1.90 *oct*. (6.9)	
12	0.82 3H, *d* (6.9)[a]	1.06 3H, *s*
13	0.89 3H, *d* (6.9)[a]	1.00 3H, *s*
14	1.03 3H, *d* (7.1)	1.01 3H, *d* (7.1)
15	1.36 3H, *s*	1.59 3H, *s*
OCH_3		3.29 3H, *s*

[a] May be interchanged in the same column.

3.7. (4S*, 5S*, 6R*, 7R*)-5-Methoxy-1(10)E-lepidozene (7)

$[\alpha]_D$ −75.2° (*c* 2.10); GC–HRMS: found 236.2143 $C_{16}H_{28}O$ requires 236.2141; FTIR ν_{max} cm^{-1}: 1450, 1377, 1190, 1151, 1093, 918; ^1H and ^{13}C NMR: Tables 1 and 2; GCMS *m/z* (rel. int.): 236 $[M]^+$(6), 221 (1), 204 (16), 189 (21), 165 (47), 161 (31), 152 (26), 151 (18), 135 (26), 133 (20), 125 (100), 122 (69), 112 (86), 107 (57), 93 (75), 91 (32), 85 (45), 82 (57), 79 (35), 73 (80), 69 (20), 67 (41), 55 (30), 53 (18), 41 (35).

3.8. Extraction and isolation from Scapanea nemorea

The ether extract (1.5 g) from dried *S. nemorea* (52 g) was chromatographed on silica gel using *n*-hexane-EtOAc gradient to give seven fractions. Fr. 3 was rechromatographed on SiO_2 and prep. HPLC (Nucleosil 50–5, 10% *n*-hexane–EtOAc) to yield *ent*-spathulenol (7 mg) and diplophyllin (9 mg). Fr. 4 was also chromatographed on Sephadex LH-20 and SiO_2 to give diplophyllin (10 mg). 5,10-*seco*-Clerodane-type diterpene **9** (7 mg) was isolated from Fr. 5 by chromatography on Sephadex LH-20 and SiO_2.

3.9. Compound *9*

$[\alpha]_D$ +200.9° (*c* 2.30); HR–EIMS: found 344.1989 $C_{21}H_{28}O_4$ requires 344.1987; FTIR ν_{max} cm^{-1}: 1780, 1750, 1715, 1250; UV λ_{max}nm (log ϵ): 209 (4.22), 242 (3.27, *sh*) (*c* 1.06×10^{-4}, EtOH); ^1H NMR (600 MHz): δ 5.28 (1H, *dddd*, J=12.4, 12.4, 4.1, 1.9 Hz, H-1), 5.96

Table 2
^{13}C NMR data of **1**, **7** and **9** (150 MHz, $CDCl_3$)

C	1	7	9
1	77.7	126.7	126.6
2	27.1	24.8	128.4
3	30.0	30.6	140.7
4	74.8	34.3	136.9
5	129.8	84.7	144.8
6	147.4	34.8	33.8
7	42.9	26.3	29.2
8	22.8	28.0	35.5
9	28.0	39.0	37.8
10	37.2	134.7	35.6
11	32.3	18.6	34.7
12	18.1[a]	21.5	23.6
13	20.6[a]	23.1	170.9
14	14.5	20.1	115.2
15	21.7	17.0	174.0
16			73.0
17			13.8
18			166.6
19			118.1
20			18.5
O$\underline{CH_3}$		56.2	
COO$\underline{CH_3}$			52.1

[a] May be interchanged in the same column.

(1H, *ddd*, $J=11.8$, 2.7, 1.6 Hz, H-2), 7.25 (1H, *dd*, $J=4.7$, 2.5 Hz, H-3), 2.63 (1H, *br d*, H-6α), 2.10 (1H, *ddd*, $J=13.7$, 13.7, 2.5 Hz, H-6β), 0.85 (1H, *m*, H-7α), 1.52 (1H, *m*, H-7β), 1.40 (1H, *m*, H-8), 1.72 (1H, *d quint.*, $J=13.5$, 1.9 Hz, H-10α), 2.29 (1H, *t*, $J=13.5$ Hz, H-10β), 1.48 (1H, *m*, H-11), 1.55 (1H, *m*, H-11), 2.38 (2H, *t* like, J = 7.7 Hz, H-12), 5.86 (1H, *quint.*, $J=1.6$ Hz, H-14), 4.76 (2H, *d*, $J=1.6$ Hz, H-16), 0.78 (3H, *d*, $J=6.6$ Hz, H-17), 4.85 (1H, *br s*, H-19), 5.08 (1H, *t*, $J=1.9$ Hz, H-19), 0.72 (3H, *s*, H-20); ^{13}C NMR: Table 2; EIMS *m/z* (rel. int.): 344[M]$^+$(27), 312 (37), 297 (13), 285 (11), 269 (4), 255 (5), 247 (7), 215 (13), 201 (15), 187 (13), 173(28), 163 (24), 159 (37), 145 (41), 131 (70), 119 (47), 117 (36), 115 (23), 111 (44), 107 (30), 105 (82), 98 (40), 93 (30), 91 (100), 79 (40), 77 (44), 67 (24), 55 (32), 41 (35).

3.10. Extraction and isolation from Jungermannia truncata and Mylia taylorii

The ether extract (1.3 g) of *J. truncata* was chromatographed on silica gel using *n*-hexane–EtOAc gradient to give nine fractions. The GC–MS of Fr. 1 showed the presence of cuparene. Compound **11** ($[\alpha]_D$ +3.3°, *c* 1.28) (3 mg) was purified by chromatography on Sephadex LH-20, SiO_2 and prep. TLC. Fr. 5 was rechromatographed on SiO_2 to give stigmasterol (14 mg) and (+)-(9*R**, 13*S**)-dihydroxy-8(17),14-labdadiene (5 mg). The CC on Sephadex LH-20, SiO_2, MPLC (Lobar® column, Si 60) gave a mixture including 13-*epi*-sclareol which was recrystallized from *n*-hexane–EtOAc to afford the pure compound (96 mg).

The ether extract (2.8 g) from *M. taylorii* (79 g) was divided into 9 fractions by CC on silica gel using *n*-hexane–EtOAc gradient. Fr. 4 was rechromatographed on Sephadex LH-20, SiO_2, MPLC (Lobar® column, Si 60) and prep. HPLC (Nucleosil 50-5, 10% *n*-hexane–EtOAc) to give (−)-taylorione (127.5 mg), myltaylorione A (4 mg) and myltaylorione B (2 mg) were isolated. Fr. 5 was rechromatographed on Sephadex LH-20 to give a mixture including myliol which was recrystallized from *n*-hexane to give (−)-myliol (254 mg) and mother liquid which was rechromatographed on MPLC (Lobar® column, Si 60 or CN) and prep. HPLC (Chemcosorb 5-ODS-H, CH_3CN) to yield (+)-*ent*-maali-4(15)-en-1β-ol (14 mg), cyclomyltaylenol (25 mg), (+)-4(15)-dehydroledol (5 mg) and (−)-taylorione (9 mg). The CC on Sephadex LH-20, SiO_2, MPLC (Lobar® column, Si 60) and prep. HPLC (Chemcosorb 5-ODS-H, CH_3CN) of Fr. 6 gave myltaylenol (10 mg), (+)-globulol (4 mg) and (+)-4(15)-dehydroglobulol (5 mg).

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Flavonoids from *Ochna integerrima*

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Abstract

Two biflavonoids namely, 2″,3″-dihydroochnaflavone and 2″,3″-dihydroochnaflavone 7″-*O*-methyl ether, and a flavonoid glycoside, 6-γ,γ-dimethylallyltaxifolin 7-*O*-β-D-glucoside were isolated from the leaves of *Ochna integerrima*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Ochna integerrima*; Ochnaceae; Biflavonoids; Flavonoid glucoside

1. Introduction

The genus *Ochna* belongs to Ochnaceae of the order Ochnales, with more than 80 species distributed in tropical Asia, Africa and America (Rendle, 1952). Plants in this genus are known to be rich in biflavonoids, anthranoids and isoflavonoids (Kamil et al., 1983, 1987; Messanga et al., 1992, 1994; Mohammad et al., 1982; Okigawa et al., 1976; Rao et al., 1997; Sibanda et al., 1990, 1993). In Thailand, only one species, i.e. *O. integerrima* has been identified (Smitinand and Larsen, 1981). The bark of this plant has been used as a digestive tonic, and the roots as an anthelmintic (Perry, 1980). To our knowledge, neither chemical nor biological work has been done on the constituents of this species. We report herein the isolation and structural elucidation of three new compounds (**1**–**3**) from *O. integerrima*, as part of our continuing chemical investigation of Thai medicinal plants (Likhitwitayawuid et al., 2000).

2. Results and discussion

Compound **1** was analyzed for $C_{30}H_{20}O_{10}$ from its $[M+H]^+$ at m/z 541.1103 in the HR-FABMS. The ^1H NMR spectrum (DMSO-d_6) of **1** (Table 1) showed an olefinic proton signal at δ 6.83 (1H, *s*, H-3), characteristic of a flavone structure, and three aliphatic proton resonances at δ 5.53 (1H, *dd*, $J=12.8$, 3.1 Hz, H-2″), 3.27 (1H, *dd*, $J=12.8$, 17.1 Hz, H_{ax}-3″) and 2.74 (1H, *dd*, $J=17.1$, 3.1, H_{eq}-3″), typical of a flavanone nucleus (Mabry et al., 1970). The ^{13}C NMR and DEPT spectra indicated a flavone–flavanone based biflavonoid structure for **1** with 30 carbon resonances representing two carbonyl, two aliphatic, and 26 olefinic and aromatic carbons. Recently, 2,3-dihydroochnaflavone, a biflavonoid structurally related to ochnaflavone (Okigawa et al., 1976), was described from *O. obtusata* (Rao et al., 1997). Although there were some similarities between the ^1H NMR (acetone-d_6) properties of **1** and those of 2,3-dihydroochnaflavone, careful analysis of the data indicated significant differences. The flavone unit of **1** appeared to be 5,7,3′,4′-tetra-oxygenated (luteolin) as indicated by the proton signals at δ 6.18 (1H, *d*, $J=2.1$ Hz, H-6), 6.47 (1H, *d*, $J=2.1$ Hz, H-8), 7.13 (1H, *d*, $J=8.5$ Hz, H-5′), 7.79 (1H, *d*, $J=2.5$ Hz, H-2′) and 7.85 (1H, *dd*, $J=8.5$, 2.5 Hz, H-6′), whilst the flavanone moiety seemed to be tri-oxygenated with six aromatic protons resonating at δ 5.87 (1H, $J=2.2$ Hz, H-6″), 5.89 (1H, *d*, $J=2.2$ Hz, H-8″), 6.92 (2H, *d*, $J=8.9$ Hz, H-3‴ and H-5‴) and 7.47 (2H, *d*, $J=8.9$ Hz, H-2‴ and H-6‴), reminiscent of 5,7,4′-trihydroxyflavanone (naringenin; Markham and Geiger, 1994). Further supporting evidence (Table 2) came from the ^{13}C NMR spectral data of **1** which showed a close resemblance to those of luteolin and naringenin (Agrawal et al., 1989). At this

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Table 1
^{1}H NMR spectral data for compounds **1** and **2**

Position	**1**[a]	**1**[b]	**2**[b]	Luteolin[b,c]
3	6.71 (*s*)	6.83 (*s*)	6.85 (*s*)	6.69 (*s*)
6	6.27 (*d*, 1.5)[d]	6.18 (*d*, 2.1)	6.19 (*d*, 1.5)	6.22 (*d*, 2.1)
8	6.54 (*d*, 1.5)	6.47 (*d*, 2.1)	6.48 (*d*, 1.5)	6.47 (2.1)
2′	7.81 (*d*, 1.8)	7.79 (*d*, 2.5)	7.81 (*d*, 1.5)	7.43 (*d*, 2.1)
5′	7.24 (*d*, 8.4)	7.13 (*d*, 8.5)	7.15 (*d*, 8.4)	6.92 (*d*, 9.0)
6′	7.89 (*dd*, 8.4, 1.8)	7.85 (*dd*, 8.5, 2.5)	7.85 (*dd*, 8.4, 1.5)	7.44 (*dd*, 9.0, 2.1)
OH-5	12.98 (*s*)	12.90 (*s*)	12.90 (*s*)	13.00 (*s*)
				Naringenin [b,c]
2″	5.58 (*dd*, 12.6, 3.0)	5.53 (*dd*, 12.8, 3.1)	5.58 (*dd*, 12.6, 2.4)	5.43 (*dd*, 12.8, 2.8)
3″	3.22 (*dd*, 17.1, 12.6)	3.27 (*dd*, 17.1, 12.8)	3.33 (*dd*, 16.8, 12.6)	3.26 (*dd*, 17.1, 12.8)
	2.78 (*dd*, 17.1, 3.0)	2.74 (*dd*, 17.1, 3.1)	2.78 (*dd*, 16.8, 2.4)	2.69 (*dd*, 17.1, 2.8)
6″	5.98 (*d*, 1.8)	5.87 (*d*, 2.2)	6.09 (*d*, 1.8)	5.90 (*s*)
8″	6.00 (*d*, 1.8)	5.89 (*d*, 2.2)	6.11 (*d*, 1.8)	5.90 (*s*)
2‴(6‴)	7.58 (*d*, 8.7)	7.47 (*d*, 8.9)	7.49 (*d*, 8.7)	7.32 (*d*, 8.5)
3‴ (5‴)	7.05 (*d*, 8.7)	6.92 (*d*, 8.9)	6.93 (*d*, 8.7)	6.81 (*d*, 8.5)
OH-5″	12.18 (*s*)	12.10 (*s*)	12.10 (*s*)	12.15 (*s*)
CH_3O-7″	–	–	3.79 (*s*)	–

[a] Recorded in acetone-d_6.
[b] In DMSO-d_6.
[c] From Markham and Geiger (1994).
[d] Coupling constant(s) in Hz.

Table 2
^{13}C NMR (DMSO-d_6) spectral data for compounds **1** and **2**

Position	**1**	**2**	Luteolin[a]
2	163.3	163.6	164.5
3	104.0	104.4	103.3
4	182.2	182.6	182.2
4a	104.2	104.6	104.2
5	161.9	162.3	162.1
6	99.4	99.8	99.2
7	164.7	165.1	164.7
8	94.6	94.9	94.2
8a	157.8	158.2	157.9
1′	122.7	123.1	119.3
2′	121.5	121.8	113.8
3′	142.8	143.2	146.2
4′	154.0	154.3	150.1
5′	118.3	118.7	116.4
6′	125.3	125.7	122.1
			Naringenin[a]
2″	78.5	79.1	79.0
3″	42.5	42.9	42.6
4″	196.5	197.5	196.4
4a″	102.2	103.5	102.3
5″	164.0	164.0	163.7
6″	96.4	95.6	96.4
7″	167.2	168.3	166.9
8″	95.5	94.7	95.5
8a″	163.3	163.6	163.3
1‴	132.8	133.0	129.4
2‴	128.8	129.2	128.7
3‴	116.2	116.6	115.7
4‴	158.5	158.9	158.1
5‴	116.2	116.6	115.7
6‴	128.8	129.2	128.7
CH_3O-7″	–	56.8	–

stage, the structure of **1** was proposed to be a biflavonoid consisting of a luteolin unit linked to a naringenin unit through a C–O–C bond. This hypothesis was confirmed by the C–H correlations displayed in the HMBC spectrum (Fig. 1). For example, in the flavone unit, long-range coupling was observed between H-3 and C-2, between H-6′ and C-2, and between H-2′ and C-2, while in the flavanone part an HMBC connectivity was found between H-2″ and C-2‴ (C-6‴). In order to locate the interflavonoid ether bridge, compound **1** was methylated. When treated with CH_3I/Ag_2O, **1** was converted into **1a**, a pentamethylation product. A NOESY experiment on **1a** revealed NOEs between the following pairs of protons: H-6 (δ 6.35, 1H, *d*, $J=2.1$ Hz) and CH_3O-5 (δ 3.97, 3H, *s*); H-6 and CH_3O-7 (δ 3.90, 3H, *s*); H-8 (δ 6.51, 1H, *d*, $J=2.1$ Hz) and CH_3O-7; H-5′ (δ 7.10, *d*, $J=8.7$ Hz) and CH_3O-4′ (δ 3.92, 3H, *s*); H-6″ (δ 6.08, 1H, *d*, $J=2.4$ Hz) and CH_3O-5″ (δ 3.89, 3H, *s*); H-6″ and CH_3O-7″ (δ 3.79, 3H, *s*); H-8″ (δ 6.13, 1H, *d*, $J=2.4$ Hz) and CH_3O-7″, indicating a C–O–C connection between C-3′ and C-4‴. Since **1a** was derived from **1**, it could be inferred that in **1** the C-3′ and C-4‴ carbons were similarly involved in the interflavonoid ether linkage. Hence, from the foregoing spectral and chemical studies, the structure of **1** was deduced to be 2″,3″-dihydroochnaflavone (Scheme 1).

Compound **2** displayed a quasi-molecular ion at *m*/*z* 555.1263 in the HR-FABMS, suggesting the molecular formula $C_{31}H_{22}O_{10}$. The molecular weight of **2**, 14 amu higher than that of **1**, implied that **2** was a methyl derivative of **1**. This was supported by the methyl resonances at δ_H 3.79 (3H, *s*) and δ_C 56.8 in the ^{1}H and ^{13}C

NMR spectra of **2** (Tables 1 and 2). When the ^{13}C NMR spectral data of **2** were compared with those of **1**, it was found that in **2** the signal of C-7″ was downfield shifted (+1.1 ppm) while the C-6″ and C-8″ resonances were upfield shifted (−0.8 ppm). This suggested that the methoxyl group in structure **2** should be located at C-7″. In a NOESY experiment, this methoxyl group showed NOE with H-6″ (δ 6.09, *d*, J=1.8 Hz) and with H-8″ (δ 6.11 *d*, J=1.8 Hz), thereby confirming its location. As expected, **2** furnished **1a** when allowed to react with CH_3I/Ag_2O. Therefore, it was concluded that **2** was 2″,3″-dihydroochnaflavone 7″-*O*-methyl ether. Complete ^1H and ^{13}C NMR spectral assignments of **2** were obtained by examination of the HETCOR and COLOC spectra. It should be noted that in dimers **1** and **2**, a significant downfield shift was observed for both H-2′ and C-2′ as compared with their counterparts in luteolin. This substituent effect may provide a useful guide for the structure determination of new biflavonoids in this series.

The HR-FABMS of compound **3** suggested a molecular formula of $C_{26}H_{30}O_{12}$ with a quasi-molecular ion at *m/z* 535.1820. The UV absorption at 289 nm was typical of a dihydroflavonol chromophore (Mabry et al., 1970). The ^{13}C NMR and DEPT spectra displayed signals for 26 carbons, including 11 sp^3 and 15 sp^2 carbons. From the HMQC spectrum, the correlations at δ_C 83.3/δ_H 5.01 (C-/H-2) and δ_C 71.8/δ_H 4.55 (C-/H-3) gave supporting evidence for a dihydroflavonol structure. In the aromatic region of the ^1H NMR spectrum (Table 3), the resonances at δ 6.88 (1H, *br s*, H-2′), 6.74 (2H, *br s*, H-5′ and H-6′) suggested that ring B of **3** was 3′,4′-dihydroxylated, similar to that of taxifolin (**4**; Markham and Geiger, 1994). Further examination of the ^1H and ^{13}C NMR spectra (Table 3) revealed the presence of two additional substituents: (1) a γ,γ-dimethylallyl group showing signals at δ_C 20.9/δ_H 3.11 and 3.38 (C-/H-9), δ_C 122.5/δ_H 5.17 (C-/H-10), δ_C 130.5 (C-11), δ_C 17.8/δ_H 1.71 (C-/H-12) and δ_C 25.6/δ_H 1.61 (C-/H-13); and (2) a β-D-glucopyranosyl unit with resonances at δ_C 100.1/δ_H 4.91 (C-/H-″); δ_C 73.3/δ_H 3.27 (C-/H-2″), δ_C 76.6/δ_H 3.27 (C-/H-3″); δ_C 69.6/δ_H 3.14 (C-4″); δ_C 77.1/δ_H 3.40 (C-5″) and δ_C 60.6/δ_H 3.40 and 3.43 (C-/H-6″). The isoprene unit was placed at C-6 based on the NOE observed between H-10 and OH-5 (Fig. 2) and the HMBC connectivity between H_2-9 and C-5. Comparison of the chemical shifts of the sugar carbons of **3** with reported values for **5** (Nørbæck et al., 1999) indicated that **3** was a dihydroflavonol with 7-*O*-β-D-glucopyranosyl substitution. This was confirmed by the NOE observed between H-1″ (anomeric proton) and H-8 in the NOESY spectrum, and the long-range coupling of H-1″ with C-7 in the HMBC spectrum. Therefore, **3** was

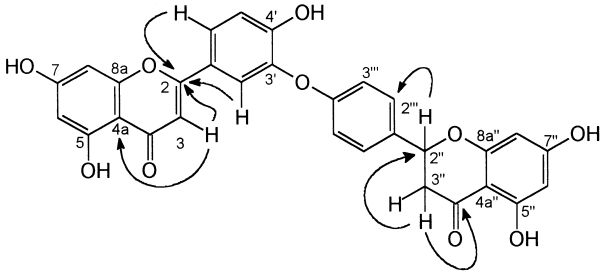
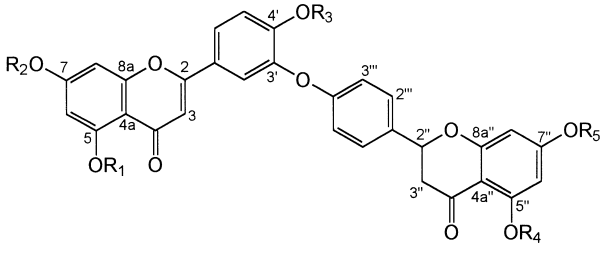


Fig. 1. HMBC correlations for compound **1**.



1 $R_1 = R_2 = R_3 = R_4 = R_5 = H$
1a $R_1 = R_2 = R_3 = R_4 = R_5 = CH_3$
2 $R_1 = R_2 = R_3 = R_4 = H$; $R_5 = CH_3$

Scheme 1.

Table 3
^1H and ^{13}C NMR (DMSO-d_6) spectral data for compounds **3**, **4** and **5**

Position	**3**		**4**	**5**
	^1H	^{13}C	^{13}C[a]	^{13}C[b]
2	5.01 (*d*, 11.0)[c]	83.3	83.1	83.4
3	4.55 (*d*, 11.0)	71.8	71.7	71.9
4		199.0	197.1	198.9
4a		101.7	100.6	102.4
5		159.5	163.3	162.8
6		109.7	96.1	97.1
7		163.1	166.8	165.7
8	6.22 (*s*)	94.0	95.1	97.1
8a		160.5	162.5	158.6
9	3.38 (*m*)	20.9		
	3.11 (*m*)			
10	5.17 (*br dd*, 7.0, 7.0)	122.5		
11		130.5		
12	1.71 (*s*)	17.8		
13	1.61 (*s*)	25.6		
1′		128.0	128.1	127.7
2′	6.88 (*br s*)	115.4	115.3	129.8
3′		145.0	144.9	115.2
4′		145.9	145.7	158.6
5′	6.74 (*br s*)	115.2	115.3	115.2
6′	6.74 (*br s*)	119.5	119.2	129.8
1″	4.91 (*d*, 7.6)	100.1		100.0
2″	3.27 (*m*)	73.3		73.2
3″	3.27 (*m*)	76.6		76.5
4″	3.14 (*m*)	69.6		69.8
5″	3.40 (*m*)	77.1		77.4
6′	3.43 (*m*) 3.65 (*dd*, 11.3, 1.8)	60.6		60.8

[a] From Agrawal et al. (1989).
[b] From Nørbæk et al. (1999).
[c] Coupling constant(s) in Hz.

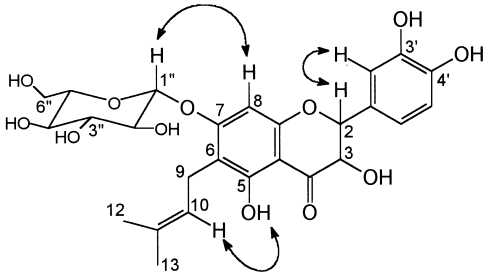


Fig. 2. NOEs observed for compound **3**.

characterized as 6-γ,γ-dimethylallyltaxifolin 7-*O*-β-D-glucoside (Scheme 2).

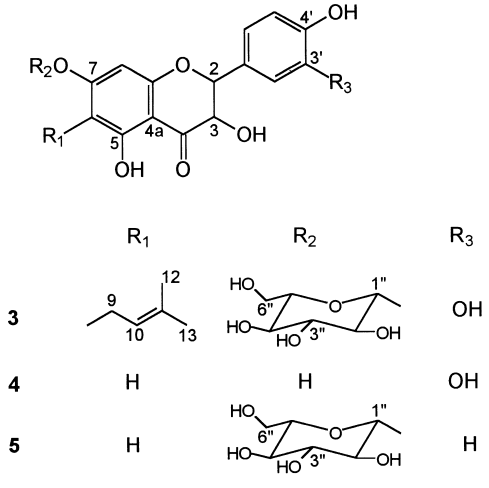
3. Experimental

3.1. Plant material

The leaves of *O. integerrima* (Lour.) Merr. were collected from Sakaeraj Environmental Reserve Station, Nakornratchasima province, Thailand in April 1999. Botanical verification was done by comparison with herbarium specimens at the Royal Forest Department, Bangkok, Thailand. A voucher specimen has been deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.2. Extraction and isolation

The dried leaves of *O. integerrima* (1.8 kg) were extracted with petroleum ether (3×20 l), EtOAc (3×20 l) and MeOH (3×20 l). The EtOAc extract (147 g) was separated by vacuum liquid chromatography (VLC) on SiO_2 with hexane–EtOAc gradient elution to give 15 fractions. Fraction II, after removal of the organic solvent, was fractionated on a silica gel column, eluted with $CHCl_3$–MeOH to give 34 fractions. Fractions 11–14 from this column were combined and evaporated to dryness to give a residue which was further purified by gel filtration (Sephadex LH20, MeOH) to give a yellow powder of **2** (36 mg). Fractions 19 and 20 were combined and dried to compound **1** (27 mg) as a yellow powder. Fraction XIII was further separated on a SiO_2 column with $CHCl_3$–MeOH gradient elution. Fractions 20–23 were combined and dried, and the obtained residue was recrystallized from MeOH to give **3** (413 mg) as yellow needles.



	R_1	R_2	R_3
3			OH
4	H	H	OH
5	H		H

Scheme 2.

3.2.1. Methylation of *1* and *2*

Methylation of **1**: a solution of **1** (2 mg) in MeOH (2 ml) was treated with CH_3I (2 ml) and Ag_2O (20 mg). The reaction mixture was stirred at room temperature for 6 h. After usual workup and purification on a silica gel column, product **1a** (2 mg) was obtained. Methylation of **2**: a solution of **2** (5 mg) in DMF (5 ml) was allowed to react with CH_3I (3 ml) and Ag_2O (40 mg) for 6 h at room temperature. Compound **1a** (2 mg) was obtained after usual workup.

3.2.2. 2″,3″-Dihydroochnaflavone (*1*)

m.p. > 300°C; $[\alpha]_D^{20}$ +6.8°C (*c* 0.2, MeOH); UV λ_{max} MeOH nm (log ε): 334 (4.33), 289 (4.43), 209 (4.78); IR ν_{max} (KBr) cm^{-1}: 3250 (*br*), 1648, 1616, 1500, 1435, 1353, 1304, 1261, 1161, 1089, 1032, 838, 958, 737; ^1H NMR (500 MHz in DMSO-d_6 and 300 MHz in acetone-d_6) see Table 1; ^{13}C NMR (125 MHz, DMSO-d_6) see Table 2; HR-FABMS $[M+H]^+ = m/z$ 541.1103 (calcd. For $C_{30}H_{21}O_{10}$, 541.1135); FABMS *m/z* (rel. int.) 541 (M + H, 9), 309 (12), 155 (61), 135 (42), 119 (100), 103 (46), 85 (57), 77 (10), 57 (14).

3.2.3. 2″,3″-Dihydroochnaflavone pentamethyl ether (*1a*)

EIMS *m/z* (rel. int.) 610 (M^+, 1), 331 (19), 182 (21), 170 (100), 155 (10), 140 (17), 128 (31), 124 (37), 110 (86), 98 (36), 82 (40), 70 (58); ^1H NMR (300 MHz, $CDCl_3$) δ 2.78 (1H, *dd, J* = 16.5, 3.0 Hz, H-3″), 3.02 (1H, *dd, J* = 16.5, 13.2 Hz, H-3″), 3.79 (3H, *s*, CH_3O-7″), 3.88 (3H, *s*, CH_3O-5″), 3.90 (3H, *s*, CH_3O-7), 3.92 (3H, *s*, CH_3O-4′), 3.97 (3H, *s*, CH_3O-5), 5.38 (1H, *dd, J* = 13.2, 3.0 Hz, H-2″), 6.08 (1H, *d, J* = 2.4 Hz, H-6″), 6.13 (1H, *d, J* = 2.4 Hz, H-8″), 6.35 (1H, *d, J* = 2.1 Hz, H-6), 6.51 (1H, *d, J* = 2.1 Hz, H-8), 6.55 (1H, *s*, H-3), 6.98 (2H, *d, J* = 8.7 Hz, H-3‴ and H-5‴), 7.10 (1H, *d, J* = 8.7 Hz, H-5′), 7.41 (2H, *d, J* = 8.7 Hz, H-2‴ and H-6‴), 7.55 (1H, *d, J* = 2.4 Hz, H-2′), 7.70 (1H, *dd, J* = 8.7, 2.4 Hz, H-6′).

3.2.4. 2″,3″-Dihydroochnaflavone 7″-O-methyl ether (*2*)

m.p. 180–182°C; $[\alpha]_D^{20}$ +7.7°C (*c* 0.2, MeOH); UV λ_{max} MeOH nm (log ε): 333 (4.45), 288 (4.56), 211 (4.85); IR ν_{max} (KBr) cm^{-1}: 3401 (*br*), 1651, 1619, 1507, 1437, 1352, 1300, 1161, 1088, 1031, 839, 772, 743;

^{1}H NMR (500 MHz, DMSO-d_6) see Table 1; ^{13}C NMR (125 MHz, DMSO-d_6) see Table 2; HR-FABMS $[M+H]^+ = m/z$ 555.1263 (calcd. for $C_{31}H_{23}O_{10}$, 555.1291); FABMS m/z (rel. int.) 555 (M + H, 8), 309 (18), 155 (67), 135 (35), 119 (100), 103 (42), 85 (56), 77 (10), 57 (13).

*3.2.5. 6-γ,γ-Dimethylallyltaxifolin 7-O-β-D-glucoside (**3**)*
m.p. 170–172°C; $[\alpha]_D^{20}$ −6.6°C (*c* 0.5, MeOH); UV λ_{max} MeOH nm (log ε): 346 (3.57), 289 (4.31), 207 (4.55); IR ν_{max} (KBr) cm^{-1}: 3413 (*br*), 2921, 1636, 1583, 1445, 1286, 1176, 1101, 986,; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) see Table 3; HR-FABMS $[M+H]^+ = m/z$ 535.1820 (calcd. for $C_{26}H_{31}O_{12}$, 535.1815); FABMS m/z (rel. int.) 535 (M + H, 8), 373 (10), 317 (24), 155 (60), 119 (100), 103 (43), 85 (59), 77 (11), 57 (14).

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Anthocyanins from red flower tea (Benibana-cha), *Camellia sinensis*

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Abstract

Three anthocyanins were isolated from the leaves of red flower tea (Benibana-cha), *Camellia sinensis*, and their structures were determined by means of chemical and spectroscopic analyses. Two are the anthocyanins, delphinidin and cyanidin 3-*O*-β-D-galactosides, respectively. Whereas the third, delphinidin 3-*O*-β-D-(6-(*E*)-*p*-coumaryl)galactopyranoside. The anthocyanins were also contained in the flowers of Benibana-cha in different compositions. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Benibana-cha; *Camellia sinensis*; Theaceae; Tea leaf; Delphinidin and cyanidin 3-*O*-β-D-galactosides; Acylated anthocyanin; Delphinidin 3-*O*-β-D-(6-(*E*)-*p*-coumaryl)galactopyranoside; Antioxidative activity

1. Introduction

Benibana-cha, originally grown in China, is a unique tea tree (*Camellia sinensis*) having red flowers and reddish green leaves. The red color is attributed to anthocyanin pigments contained in the plant. Thus the extract of Benibana-cha leaf is expected to contain highly bioactive substances, because it contains antioxidative anthocyanins (Tsuda et al., 1996; Noda et al., 1998; Obi et al., 1998) in addition to multi-biological functional catechins (Guo et al., 1996; Nanjo et al., 1996; Kumamoto and Sonda, 1998; Kondo et al., 1999) which are normal green tea constituents. Since the anthocyanins had not yet been investigated, their structures were investigated using chemical and spectroscopic methods.

2. Results and discussion

From the leaves of Benibana-cha, we extracted the crude pigment which contained twelve or more Benibana-cha leaf anthocyanins (BCL-1 to 12). On purification of the crude extracts through several types of column chromatography, three anthocyanins **1**, **2** and **3** were isolated as powders of trifluoroacetic acid (TFA) salts. On acid hydrolysis, **1** and **3** gave delphinidin (Dp), and **2** gave cyanidin (Cy) as aglycon, and all gave only galactose as the sugar by TLC analysis. On alkaline hydrolysis, **3** gave *p*-coumaric acid as an acyl component and **1** as a deacylated anthocyanin by HPLC analysis, but **1** and **2** were unchanged during similar treatment. In the UV-vis spectra of **1**, **2** and **3**, the values (20–27%) of $E_{440}/E_{\mathrm{vis.max}}$ (a ratio of absorbances at 440 nm and visible maxima) suggested that **1**, **2** and **3** were 3-*O*-substituted (Harborne, 1967). Observation of a bathochromic shift of their $\lambda_{\mathrm{vis.max}}$ on addition of $AlCl_3$ solution suggested that the B-rings of **1**, **2** and **3** were of the catechol- or pyrogallol-type. FABMS and ESI-TOFMS spectra of anthocyanins **1**, **2** and **3** gave clear molecular-ion peaks at m/z 465, 449 and 611 corresponding to the molecular formulae $C_{21}H_{21}O_{12}^{+}$, $C_{21}H_{21}O_{11}^{+}$ and $C_{30}H_{27}O_{14}^{+}$, respectively. Therefore the compositions of **1**, **2** and **3** were deduced as Dp mono-hexoside, Cy mono-hexoside, and Dp-*p*-coumaryl-mono-hexoside, respectively. Anthocyanin **2** was chromatographically identical to cyanidin 3-*O*-β-D-galactoside by comparison with the authentic anthocyanin from berries of *Fatsia japonica* (Terahara et al., 1992). (Fig. 1)

Determination of detailed chemical structures of **1** and **3** were performed through ^{1}H-NMR analyses using

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DQF-COSY and DIFNOE methods as previously reported (Terahara et al., 1998). The assignments are depicted in Table 1. In the low magnetic field (δ_H 6–9 ppm), four singlets of **1** and **3** were assignable to protons of Dp having a symmetric B-ring. In **3**, two pairs of characteristic doublets with large coupling constants ($J = 16$ Hz) were allocated to the olefinic α- and β-protons of the (*E*)-hydroxycinnamoyl moiety. In the high magnetic field (δ_H 3–6 ppm), the assignment of galactosyl protons was facilitated by DQF-COSY and DIFNOE spectra. As the anomeric protons in sugars of **1** and **3** were characteristic doublets shifted to lower field with large coupling constants (7–8 Hz) and the ring protons other than the 4-methine proton had large coupling constants (7–9 Hz), all sugars have the β-D-galactopyranosyl configuration (Terahara et al., 1992). The 5-methine and 6-methylene protons in **3** were observed at lower magnetic field (the chemical shift differences, $\Delta\delta_H$ from $+0.4$ to $+0.5$ ppm) as compared with those of **1**, indicating the 6-OH position of **3** was acylated. Attachment positions of sugars in **1** and **3** were clearly determined by DIFNOE spectra. Cross-peaks of intense NOEs between Dp-4-H and Gal-1-H, proved that Dp-3-OH was glycosylated with Gal. In conclusion, Benibana-cha leaf major anthocyanins **1** and **3** were identified as delphinidin 3-*O*-β-D-galactopyranoside and delphinidin 3-*O*-β-D-(6-*O*-(*E*)-*p*-coumaryl)galactopyranoside, a new anthocyanin, respectively. Pigment **2** was identified as cyanidin 3-*O*-β-D-galactopyranoside.

The crude extract from the leaves of Benibana-cha contains more than twelve anthocyanins, in which anthocyanin **1** is of highest concentration, **2** and **3** followed by those of anthocyanins. These pigments are also contained in the flowers in different composition, in which the order of contents is **2** > **1** > **3**.

So far as we know, tricetinidin has been only found in a manufactured tea (Roberts and Williams, 1958). It is formed as a result of oxidation of (−)-epigallocatechin gallate. Therefore, it is the first time that intact anthocyanins were found in tea leaves.

The leaf extract of Benibana-cha is expected to be used as high functional natural colorants for food or other purposes, because it contains antioxidative anthocyanins (Tsuda et al., 1996; Noda et al., 1998; Obi et al., 1998) in addition to biologically multi-functional catechins (Guo et al., 1996; Nanjo et al., 1996; Kumamoto and Sonda., 1998; Kondo et al., 1999).

3. Experimental

3.1. General experimental procedures

All reagents used were special or HPLC grades purchased from Kishida Chemical Co. and Wako Chemical Ind., Japan. TLC was carried out as previously reported (Terahara et al., 1992). HPLC was done on an L-6200 Intelligent pump system (Hitachi Co., Japan). The analytical HPLC was run on a Luna column (4.5 i.d.×150 mm, Phenomenex Co., USA) at 35°C with monitoring at 520 nm. The flow rate was 1.0 ml min^{-1} with a linear gradient elution for 80 min from 0% to 40% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O, v/v) in solvent A (1.5% H_3PO_4 in H_2O, v/v). Preparative HPLC was performed on an Inertsil ODS 5

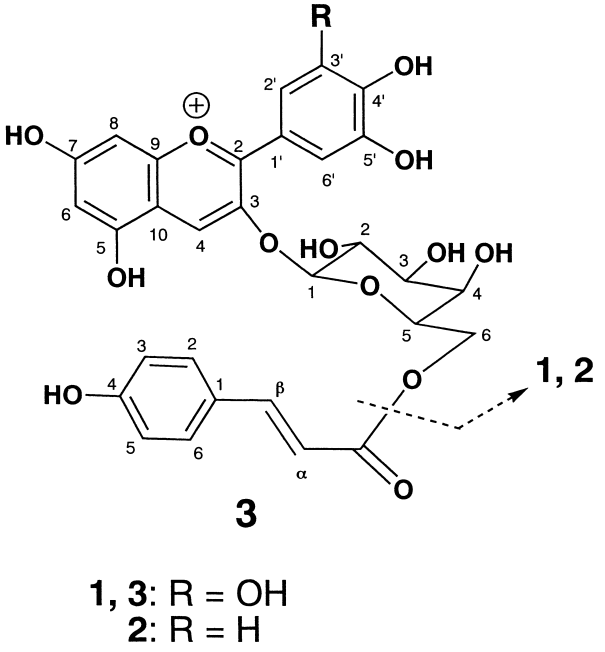


Fig. 1. The structures of anthocyanins **1**, **2** and **3** from leaf of red flower tea (Benibana-cha), *Camellia sinensis*.

Table 1
^{1}H (500 MHz) NMR data of anthocyanins **1** and **3** from red flower tea (Benibana-cha) leaves [δ_H ppm from internal standard $(CH_3)_4Si$ in DMSO-d_6:CF_3COOD (9:1)][a]

		1	**3**	
Assignment		δ_H	δ_H	$\Delta\delta_H$
Delphinidin	4	8.87 *s*	8.74 *s*	−0.13
	6	6.92 *s*	6.82 br*s*	−0.10
	8	6.71 *s*	6.63 br*s*	−0.08
	2′, 6′	7.76 *s*	7.67 *s*	−0.09
Galactosyl	1	5.30 *d* (7.9)	5.32 *d* (7.6)	+0.02
	2	3.92 brt (8.7)	3.83 *t* (8.5)	−0.09
	3	3.60 *dd* (3.3, 8.8)	3.61 *dd* (3.1, 7.9)	+0.01
	4	3.66 *dd* (3.3, 5.5)	3.83 br*d* (3.1)	+0.17
	5	3.62 *m*	4.11 *m*	+0.49
	6a	3.79 *dd* (3.4, 8.2)	4.16 *dd* (2.9, 8.5)	+0.37
	6b	3.86 br*d* (3.4)	4.36 *dd* (2.4, 9.2)	+0.50
p-Coumaryl	2, 6		7.35 *d* (8.2)	
	3, 5		6.76 *d* (8.5)	
	α		6.26 *d* (15.9)	
	β		7.40 *d* (15.9)	

[a] Values in parentheses indicate coupling constants (*J* in Hz). Chemical shift differences: $\Delta\delta_H = (\delta_H$ of **3**$) - (\delta_H$ of **1**$)$.

column (20 i.d.×250 mm, GL Sciences Inc., Japan) at 7 ml min^{-1} by an isocratic elution using mixtures of solvent A (HOAc:H_2O = 15:85, v/v) and solvent B (HOAc:MeCN:H_2O = 15:30:55, v/v), A:B = 75:25 at 520 nm. UV-vis spectra were recorded on MPS-2000 spectrophotometer (Shimadzu Co., Ltd, Japan) in 0.01% HCl–MeOH. FABMS spectra were measured on JMS SX-102 (JEOL Co., Ltd) in MeOH with Magic Bullet or *m*-nitrobenzyl alcohol as a matrix with a positive mode. ESI-TOFMS spectra were measured on Mariner Bio-spectrometry Workstation (PerSeptive Biosystems Inc., USA) in 1%HOAc–50%MeCN with no matrix in the positive mode. ^{1}H (500 MHz) NMR were run on a JMN GX-500 spectrometer (JEOL Co., Ltd) in DMSO-d_6:CF_3COOD = 9:1 with an internal standard $(CH_3)_4Si$.

3.2. Plant materials

Benibana-cha [*Camellia sinensis var. sinensis* (L.) O. Kuntze] was cultivating in farm in Makurazaki, a southern part in Kyusyu, Japan. The leaves were harvested in April 1997, and dried by warm wind overnight. The dried leaves were used for our experiment.

3.3. Isolation and preparation of pigments

The dried leaves (ca. 60 g) of Benibana-cha were immersed in 15% HOAc overnight and filtered. This operation was repeated twice. The combined extract contained twelve or more anthocyanins (BCL) at retention times (contents%); BCL-1 (**1**): 20.0 (4 1.2%), -2 (**2**): 24.1 (14.6%), -3: 28.3 (3.4%), -4: 29.0 (3.0%), -5: 30.1 (0.7%), -6: 32.1 (1.4%), -7: 49.5 (3.2%), -8: 54.1 (0.9%), -9: 55.3 (0.6%), -10 (**3**): 59.0 (14.7%), -11: 65.9 (2.6%) and -12: 69.8 (0.9%) min, respectively, by HPLC analysis. The crude extract was adsorbed on Amberlite XAD-2000 column (60 i.d.×300 mm), washed with 2 liters of 1% HOAc and eluted stepwise with 10, 30, 50, 70% EtOH containing 1% HOAc. The fractions of 30 and 50% EtOH were combined and evaporated under reduced pressure. The pigment fractions were evaporated under reduced pressure. Final purification by prep HPLC of the fractions and precipitation by TFA and ether gave three pure anthocyanins (**1**, 21 mg; **2**, 9 mg; and **3**, 17 mg) as red amorphous powders of TFA salts.

3.4. Chemical analyses

Acid and alkaline hydrolyses of isolated pigments were performed according to previous methods (Terahara et al., 1992).

3.5. Pigment **1** (Delphinidin 3-*O*-β-D-galactopyranoside)

FABMS: m/z 465 [M = $C_{21}H_{21}O_{12}^+$] with Magic Bullet or *m*-nitrobenzyl alcohol as a matrix, 487 [M + Na^+] with Na^+ + Magic Bullet; ESI-TOFMS: m/z 465 [M = $C_{21}H_{21}O_{12}^+$]; UV-vis λ_{max} (0.01% HCl–MeOH) nm:540 (bathochromic shift 15 nm with $AlCl_3$), 277, $E_{440}/E_{vis.max} = E_{440}/E_{540} = 20\%$; ^{1}H NMR data: see Table 1.

3.6. Pigment **2** (Cyanidin 3-*O*-β-D-galactoside)

FABMS: m/z 449 [M = $C_{21}H_{21}O_{11}^+$] with Magic Bullet; ESI-TOFMS: m/z 449 [M = $C_{21}H_{21}O_{11}^+$]; UV-vis λ_{max} (0.01% HCl–MeOH) nm: 529 (bathochromic shift 20 nm with $AlCl_3$), 273, $E_{440}/E_{vis.max} = E_{440}/E_{529} = 25\%$.

3.7. Pigment **3** (Delphinidin 3-*O*-β-D-(6-(*E*)-*p*-coumaryl)galactopyranoside)

FABMS: m/z 611 [M = $C_{30}H_{27}O_{14}^+$]; ESI-TOFMS: m/z 611 [M = $C_{30}H_{27}O_{14}^+$]; UV-vis λ_{max} (0.01% HCl–MeOH) nm: 541 (bathochromic shift 14 nm with $AlCl_3$), 310, 271, $E_{440}/E_{vis.max} = E_{440}/E_{541} = 27\%$, $E_{310}/E_{vis.max} = E_{310}/E_{541} = 176\%$; ^{1}H NMR data: see Table 1.

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Further isoflavonoid metabolites from *Millettia griffoniana* (Bail)☆

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Abstract

Three new isoflavonoids, griffonianone A (**1**), B (**2**) and C (**4**) were isolated from the root bark of *Millettia griffoniana*, along with the known maximaisoflavone G (**5**) and 7-hydroxy-6-methoxy-3′,4′-methylenedioxyisoflavone (**6**). Their structures were assigned on the basis of spectroscopic data and chemical transformations. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Millettia griffoniana*; Leguminosae; Papilionoideae; Isoflavones

1. Introduction

We have previously reported (Yankep et al., 1997, 1998) on the isolation and structural elucidation of a chalcone, a 3-aryl-4-hydroxycoumarin and nine isoflavones from the root bark of *Millettia griffoniana*, Bail (Leguminosae). In continuing the investigation of the $CHCl_3$ extract of the root bark of this species, we now report the isolation and structural elucidation of three other new compounds, griffonianones A (**1**), B (**2**) and C (**4**), along with the known maximaisoflavone G (**5**) and 7-hydroxy-6-methoxy-3′,4′-methylenedioxyisoflavone (**6**).

2. Results and discussion

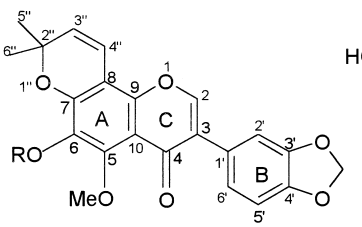
The $CHCl_3$ extract of the ground root bark of *M. griffoniana* was subjected to repeated column chromatography to yield griffonianones A (**1**), B (**2**) and C (**4**) and a solid which was only sparingly soluble in DMSO. The ^1H NMR spectrum (DMSO-d_6) of this solid showed it to be a mixture of two isoflavones (two one proton singlets at δ 8.0 and 8.1 ppm). This solid was acetylated and separated by preparative TLC to afford maximaisoflavone-G acetate (**7**) (Rao et al., 1984) and its isomer (**8**). The structures of the isoflavones were therefore established as the known maximaisoflavone-G (**5**) and its isomer (**6**) (Rao et al 1984; Dagne et al., 1989; Fuendjiep et al., 1998a,b).

Griffonianone A (**1**) gave a negative $FeCl_3$ test and has the molecular formula $C_{28}H_{32}O_7$ determined by HREIMS (m/z 469.2110 $M^{+\cdot}$). The ^{13}C NMR spectrum (Table 1) showed typical 3-hydroxyrotenoid signals (Agrawal and Bansal, 1989) at δ 76.2 ppm (C-2), 67.5 ppm (C-3), 191.3 ppm (C-4), 63.8 ppm (C-11) and 109.1 ppm (C-1′). The presence of this skeleton was also evident from its ^1H NMR spectrum revealing the $-CH_2-CH-$ motive with protons at 4.40 and 4.28 ppm for the methylene protons at C-11 and at 4.38 ppm for the methine protons at C-2. The ^1H NMR spectrum further displayed two three-proton singlets at δ 3.65 and 3.75 ppm assignable to two methoxy groups and a set of peaks characteristic of an *O*-geraniol side chain (Fuendjiep et al., 1998). The aromatic part of the ^1H NMR spectrum exhibited two one-proton singlets at δ 6.52 and 6.46 ppm ascribable to the isolated protons situated at 3′ and 6′ positions, respectively, on ring B bearing oxygen atoms in positions 4′ and 5′. The peak at m/z 208 (**9**) which appeared as the base peak in the EIMS, and which is formed from the molecular ion by a RDA fission of ring C, clearly confirmed that the two methoxy groups were all located on ring B and that

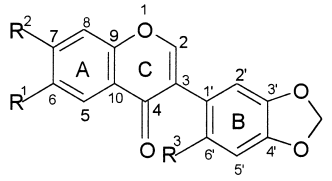
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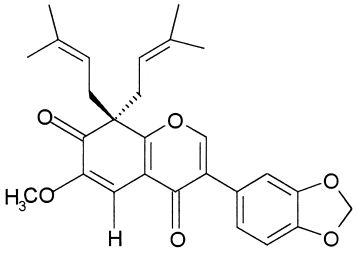
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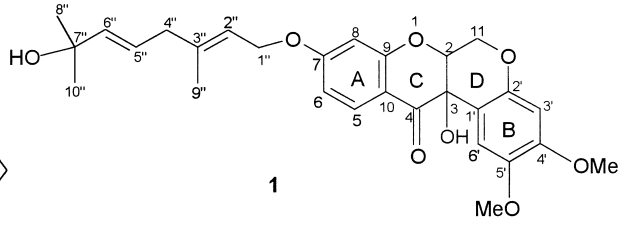
2 : R = H
3 : R = CH_3



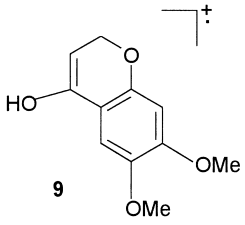
5 : R^1 = H ; R^2 = OH ; R^3 = MeO
6 : R^1 = MeO; R^2 = OH ; R^3 = H
7 : R^1 = H; R^2 = OAc; R^3 = MeO
8 : R^1=MeO; R^2 = OAc; R^3 = H



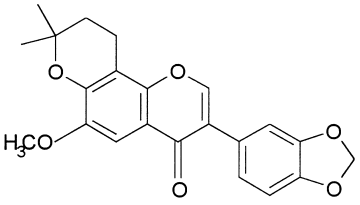
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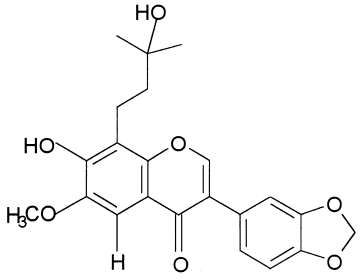
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9



10



11

compound **1** had a 3-hydroxyrotenoid skeleton. The appearance of the H-6 proton at δ 6.52 ppm indicated that this compound had a C/D *cis* ring fusion (Dewick, 1993; Van Puyvelde et al., 1987). Further support in favour of the C/D *cis* configuration came from intramolecular hydrogen bonding (νCO 1674 and νOH 3508 cm^{-1}) shown by the IR spectrum (Ollis et al., 1967). Three aromatic protons in an ABX-system at δ 6.34 ppm (1H, *d*, 2.2 Hz) 6.56 ppm (1H, *dd*, 2.2 and 8.8 Hz) and 7.81 ppm (1H, *d*, 8.8 Hz) were also observed. The deshielded nature of the *ortho* coupled proton of this ABX-system placed it at the 5-position on ring A. The *O*-geranyl side chain was therefore located on the same ring, at the 7-position. From the above spectroscopic evidence, griffonianone A was assigned structure **1** which is 4′,5′-dimethoxy-3-hydroxy-7-*O*-[(*E*)-3,7-dimethyl-7-hydroxyocta-2,5-dienyl] rotenoid. As far as we are aware, griffonianone A is the first example of a rotenoid bearing an *O*-geranyl side chain from a natural source.

Griffonianone B (**2**), was obtained in a yellow amorphous form. The molecular formula $C_{22}H_{18}O_7$ was determined by HREIMS (*m*/*z* 394.1080 $M^{+\cdot}$). It reacted positively with $FeCl_3$, and methylation of its phenolic hydroxyl group gave the monomethyl ether **3** indicating the partial structure $C_{22}H_{17}O_6$(OH). The ^{1}H NMR spectrum of **2** (Table 1) showed a typical isoflavone singlet for H-2 at δ 7.80 ppm. The ^{1}H NMR spectrum

further revealed a three proton singlet at δ 4.01 ppm due to one methoxy group and an AB-system ($J = 10$ Hz) at δ 5.74 and 6.81 ppm which, together with the six proton singlet at δ 1.58 ppm, suggested the presence of a 2,2-dimethylpyranyl ring. In the aromatic region, the ABX spin coupled system appearing at δ 6.88 ppm, (1H, *d*, 8.0 Hz), 6.97 ppm (1H, *dd*, 1.7 and 8.0 Hz) and 7.10 ppm (1H, *d*, 1.7 Hz) together with the two proton singlet at δ 6.00 ppm and the significant ion fragment observed at *m*/*z* 146, obtained from the molecular ion as a result of a RDA fission of ring C, clearly established the piperonyl nature of ring B. In its ^{13}C NMR spectrum (Table 1), the presence of signals at δ 114.9 and 129.4 ppm, indicated that the gem-dimethylpyranyl ring was fused angularly on ring A (Agrawal and Bansal, 1989). The lack of a chelated 5-hydroxyl signal between δ 11–13 ppm in its ^{1}H NMR spectrum led to the location of the methoxy group at the C-5 position on ring A. Thus, griffonianone B was identified as 6-hydroxy-5-methoxy-3′,4′-methylenedioxy-2″, 2″-dimethylpyrano [5″,6″:7,8] isoflavone (**2**). Further supporting evidence for this assignment was obtained from the chemical conversion of **2** to the known 5-methoxydurmillone (**3**) by methylation (Dagne et al., 1989; Fuendjiep et al., 1998a,b).

Griffonianone C (**4**), obtained as white crystals, (mp 138–139°C) (MeOH), was assigned the molecular formula $C_{27}H_{28}O_6$ by HREIMS (*m*/*z* 448.1894 $M^{+\cdot}$). The signals at δ 7.90 ppm (1H, *s*, H-2) and at δ 152.1 ppm (C-2) in its NMR spectra (Table 2) were suggestive of an isoflavone type skeleton. In addition to the isoflavone α-enone group (ν_{max} 1645 cm^{-1} and δ 174.0 ppm), the IR and ^{13}C NMR spectra showed a six membered ring carbonyl group (ν_{max} 1678 cm^{-1} and δ 195.4 ppm, respectively). The ^{1}H NMR spectrum further revealed the presence of a methoxy group at δ 3.79 ppm and an isolated proton at δ 6.89 ppm. As for compound **2**, the piperonyl nature of ring B was evident from its EIMS (*m*/*z* 146, RDA fission of ring C), its ^{1}H NMR spectrum which showed one methylenedioxy proton signal at δ 6.0 ppm and an ABX spin coupled system at δ 6.8–7.0 ppm and its ^{13}C NMR spectrum which indicated a methylenedioxy carbon signal at 102.1 ppm and two *ortho*

Table 1
^{1}H and ^{13}C NMR data for griffonianone-A (**1**) and B (**2**)

Position	Griffonianone-A (**1**)		Griffonianone-B (**2**)	
	^{13}C	^{1}H (mult, *J* (Hz))	^{13}C	^{1}H (mult, *J* (Hz))
2	76.2	4.38 (*m*)	150.6	7.80 (*s*)
3	67.5		125.2[b]	
4	191.3		175.1	
5	129.2	7.81 (*d*, 8.8)	150.6	
6	111.6	6.56 (*dd*, 2.2 and 8.8)	136.5	
7	166.4		145.4	
8	101.3	6.34 (*d*, 2.2)	106.4	
9	162.5		150.6	
10	111.0		114.9	
11	63.8	4.28 (*dd*, 2.1 and 15.7)[a]		
		4.40 (*dd*, 2.5 and 14.5)[a]		
1′	109.1		125.6[b]	
2′	148.3		110.0	7.10 (*d*, 1.7
3′	101.1	6.46 (*s*)	147.6	
4′	151.1		147.6	
5′	144.0		108.3	6.88 (*d*, 8.0)
6′	106.6	6.52 (*s*)	122.6	6.97 (*dd*, 1.7 and 8.0)
1″	65.3	4.41 (*d*, 6.1)		
2″	119.1	5.45 (*t*, 6.1)	78.7	
3″	141.1		114.9	5.74 (*d*, 10)
4″	42.1	2.72 (*d*, 5.6)[a]	129.4	6.81 (*d*, 10)
5″	123.8	5.27 (*dt*, 6.5 and 14.1)[a]	28.2	1.58 (*s*)
6″	140.4	5.50 (*d*, 15.6)[a]	28.2	1.58 (*s*)
7″	70.7			
8″	29.8	1.28 (*s*)		
9″	16.7	1.65 (*s*)		
10″	29.8	1.28 (*s*)		
OMe	55.8	3.65 (*s*)	62.5	4.01 (*s*)
	56.3	3.75 (*s*)		
OCH_2O			101.1	6.00 (*s*)

[a] Either the coupling constants should be identical pairwise or the spectrum is not first order and then the values are not coupling constants (rather observed splitting).

[b] Interchangeable assignments within the same column.

Table 2
^{1}H and ^{13}C NMR data for griffonianone C (**4**)[a] and compound (**8**)[b]

Position	Compound (**4**)		Compound (**8**)
	^{13}C	^{1}H (mult. *J* (Hz))	^{1}H (mult. *J* (Hz))
2	152.1	7.90 (*s*)	7.94 (*s*)
3	128.6		
4	174.0		
5	107.0	6.89 (*s*)	7.73 (*s*)
6	151.8		
7	195.4		
8	60.0		7.21 (*s*)
9	165.0		
10	121.0		
1′	125.0		
2′	110.1	7.08 (*d*, 1.7)	7.08 (*d*, 1.6)
3′	148.5		
4′	148.7		
5′	109.1	6.84 (*d*, 8.1)	6,41 (*d*, 8.0)
6′	123.7	6.95 (*dd*, 1.7 and 8.1)	6.96 (*dd*, 1.7 and 8.0)
1″ and 1‴	39.4	2.70 (*m*)[c]	
		2.81 (*m*)[c]	
2″ and 2‴	117.8	4.70 (*m*)[c]	
3″ and 3‴	137.0		
Me	18.8	1.50 (*s*)	
	26.8		
OMe	57.0	3.79 (*s*)	3.96 (*s*)
OCH_2O	102.1	6.00 (*s*)	5.98 (*s*)
$COCH_3$			2.35 (*s*)

[a] ^{1}H and ^{13}CNMR spectra measured in $CDCl_3$ at 500 and 125 MHz, respectively.

[b] ^{1}H NMR spectrum measured in $CDCl_3$ at 90 MHz.

[c] Coupling constants should be identical pairwise; apparently the spectrum is not first order and the observed values are rather the observed splittings of multiplets.

oxygenated aromatic carbon signals at δ 148.5 and 148.7 ppm (Table 2). Two gem-diisoprenyl groups attached to a saturated alicyclic quaternary carbon resonating at δ 60.00 ppm were detectable in its NMR spectra and easily assignable by comparison to the reported chemical shift values for similar substitution patterns in the literature (Ramiandrasoa et al., 1983). The ready removal of one of the isoprenyl group from the molecular ion to give the ion peak at m/z 380, appearing as base peak, suggested that the two isoprenyl groups were also adjacent to the carbonyl group at δ 195.4 ppm (Karanjgoakar et al., 1973). The substitution pattern of ring A was unambiguously established by long range heteronuclear coupling using the HMBC technique (Fig. 1). Long-range correlations were observed between H-5 (δ 6.89 ppm) and carbon signals at δ 165.0 ppm (C-9) and 174.0 ppm (C-4) on the one hand, and between H-1″ (δ 2.70 and 2.81 ppm) and the carbon signal at δ 165.0 ppm (C-9) on the other hand. In addition, the methoxy proton signal at δ 3.79 ppm was correlated with the carbon signal at δ 151.8 ppm (C-6). Final proof for the substitution pattern of ring A was obtained by the facile acid-catalyzed loss of one of the two gem-diisoprenyl groups at C-8 followed by cyclization or by addition of water to give a mixture of compounds **10** and **11**, respectively in the ratio (62:38) (estimated using ^{1}H NMR signal integration curve of their methoxy groups at 3.93 and 3.97 ppm respectively). Structures **10** and **11** were assigned to the reaction products on the basis of their NMR spectral data (Table 3) and were established by their HMBC spectra which showed the expected correlations (Figs. 2 and 3). From the above spectroscopic and chemical observations, griffonianone C was assigned structure **4**. This is the first report of the presence of such a modified ring A isoflavone in a member of the Leguminosae. The isolation of griffonianone C (**4**) along with a chalcone, a 3-aryl-4-hydroxycoumarin, a rotenoid and isoflavones (Yankep et al., 1997, 1998) raises interesting biogenetic questions, which must await further study.

Table 3
^{1}H and ^{13}C NMR[a] data for compounds (**10**) and (**11**)

	Compound (**10**)		Compound (**11**)	
Position	^{13}C	^{1}H (mult, J (Hz))	^{13}C	^{1}H (mult, J (Hz))
2	151.72	7.93 (*s*)	152.13	7.96 (*s*)
3	124.27		123.95	
4	175.65		175.86	
5	102.52	7.49 (*s*)	102.30	7.53 (*s*)
6	147.60		145.18	
7	148.85		148.73	
8	109.75		116.48	
9	150.18		150.75	
10	116.63		117.43	
1′	126.06		125.96	
2′	109.80	7.09 (*d*, 2.5)	109.76	7.09 (*d*, 2.5)
3′	147.50		147.60	
4′	147.60		147.60	
5′	108.31	6.84 (*d*, 8.9)	108.33	6.84 (*d*, 8.9)
6′	122.32	6.96 (*dd*, 2.5 and 8.9)	122.32	6.9 (*dd*, 2.5 and 8.9)
1″	26.53	1.43 (*s*)	29.16	1.31 (*s*)
2″	76.18		71.06	
3″	31.42	1.88 (*t*, 6.4)	42.30	1.76 (*m*)
4″	16.85	2.89 (*t*, 6.4)	17.97	2.96 (*m*)
OCH_3	56.21	3.93 (*s*)	56.35	3.97 (*s*)
OCH_2O	101.09	5.96 (*s*)	101.09	5.96 (*s*)

[a] ^{1}H and ^{13}C NMR spectra measured in $CDCl_3$ at 500 and 125 MHz, respectively.

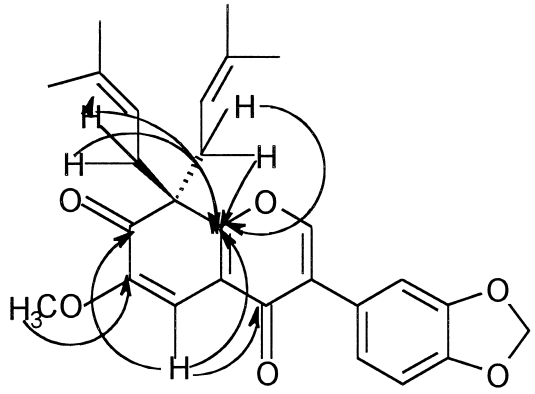


Fig. 1. Selected HMBC correlations for griffonianone C (**4**).

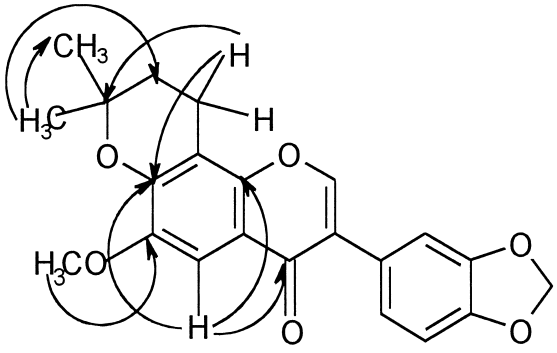


Fig. 2. Selected HMBC correlations for compound **10**.

3. Experimental

3.1. General

Melting points were determined on a Kofler hot-stage microscope apparatus and are not corrected. UV spectra were taken in MeOH. IR spectra were taken in KBr disks. EIMS were obtained with a H-SQ30 (Finigan MAT) instrument at 70 eV ionization energy. NMR spectra were taken on a Bruker AXR 300 spectrometer (^{1}H 300 MHz and ^{13}C 75 MHz) in $CDCl_3$ solution using TMS as internal standard, unless otherwise stated. Si gel 60 F_{254} (230–400 mesh, Merck) and 60 F_{254} (Merck) were used for column chromatography and for TLC,

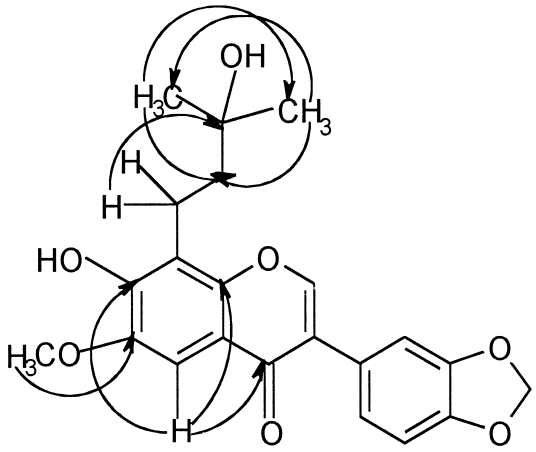


Fig. 3. Selected HMBC correlations for compound **11**.

respectively, with petrol containing increasing amounts of EtOAc as eluent. 20×20 cm self-made glass plates (0.5 mm layer of Si gel 60 $F_{254–360}$ (Merck)) were used for preparative TCL. Spots were viewed by UV illumination (254–366 mm).

3.2. Plant material

The root bark of *M. griffoniana* was collected in July, 1994 at Onguesse, in the Central province of Cameroon. A voucher specimen documenting the collection was identified at the National Herbarium, Yaounde, Cameroon, and is on deposit there.

3.3. Extraction and isolation

The powdered dried root bark of *M. griffoniana* (20 kg) was successively extracted with *n*-hexane (24 h, 3×20 l) and $CHCl_3$ (24 h, 3×20 l) at room temperature yielding residues (of 710 g *n*-hexane, and of 550 g $CHCl_3$).

The concentrated $CHCl_3$ extract (50 g) was subjected to flash column chromatography over Si gel (200 g). A total of 17 fractions of ca 500 ml each were collected and combined on the basis of their TLC analysis leading to two main series A and B. Fractions 3 and 4 eluted with petrol–EtOAc (70:30) gave series A (3 g), which was chromatographed over Si gel (80 g). A total of 46 fractions of ca 200 ml each were collected. Fractions 27–46 eluted with petrol–EtOAc (60:40) upon evaporation of the solvent afforded an oily residue which was further purified by preparative TLC (C_6H_6/petrol/ETOAc (3:6:1)) to give **4** (16 mg). Series B, resulting from the combination of fractions 8–10 eluted with petrol–EtOAc (60:40) (7.7 g) was further chromatographed over Si gel (225 g). A total of 98 fractions of ca 200 ml each were collected. From fractions 47–68 eluted with petrol–EtOAc (70:30) a white solid (50 mg) was obtained which was not soluble in common organic solvents. It was filtered and washed thoroughly with $CHCl_3$. Acetylation of this solid (20 mg) using Ac_2O-pyridine followed by separation on preparative TLC [petrol–EtOAc (70:30)], gave **7** (3 mg) and **8** (2 mg).

The EtOAc soluble part of the defatted $CHCl_3$ extract (15 g) was subjected to flash column chromatography over Si gel (200 g). A total of 25 fractions of ca 100 ml each were collected and combined on the basis of their TLC analysis. Repeated column chromatography over Sephadex LH20 with MeOH–$CHCl_3$ (50:50) of the combined fractions 24–26 (7.8 g), eluted with petrol–EtOAc (60:40) gave 2.6 g of defatted residue. This residue was further subjected to flash column chromatography over Si gel (75 g). Fractions of ca 100 ml each were collected. Fractions 4–9 eluted with petrol–EtOAc (85:15) afforded a residue which was further purified by preparative TLC [petrol–EtOAc (60:40)] to give **2** (16.4 mg). The residue (121 mg) obtained from fraction 18 eluted with petrol–EtOAc (80:20) was purified on preparative TLC [petrol–EtOAc (40:60)] yielding **1** (77 mg).

3.3.1. Griffonianone A (*1*)

Yellowish amorphous solid. IR: ν_{max} cm^{-1}: 3508 (OH), 1674 (C=O), 1620, 1655, 1510, 1420, 1375, 1290, 1180. 1H and ^{13}C NMR spectra (Table 1). EIMS: *m*/*z* (rel. int.): 496 $M^{+\cdot}$ (50), 463 [$M-H_2O-CH_3$] (**2**) 208 (100), 207 (26), 193 (**3**), 165 (**5**), 137 (11), 151 (**1**). HREIMS *m*/*z*: 496.2110 $M^{+\cdot}$ (calcd. for $C_{28}H_{32}O_8$, 496.2097).

3.3.2. Griffonianone B (*2*)

Yellow amorphous solid. IR: ν_{max} cm^{-1}: 3150, 2904, 1629, 1469, 1242, 1032, 930, 910, 832, 792, 910. ^1H and ^{13}C NMR spectra (Table 1). EIMS: *m*/*z* (rel. int.): 394 $M^{+\cdot}$ (100), 380 (22), 379 (25), 312 (24), 311 (14), 310 (15), 298 (15), 271 (16), 270 (78), 233 (3), 185 (14), 146 (13). HREIMS *m*/*z*: 394.1080 $M^{+\cdot}$ (calcd. for $C_{22}H_{18}O_7$ 394.1053).

3.3.3. Griffonianone B methylether (*3*) (=5-methoxy-durmillone)

Compound **2** (20 mg) was methylated with Me_2SO_4–K_2CO_3 in Me_2CO to give the methylether **3**, mp 130–141°, identical in all respects to a natural sample (Dagne et al., 1989; Fuendjiep et al., 1998).

3.3.4. Griffonianone C (*4*)

White crystals from MeOH. mp 138–139°. IR: ν_{max} cm^{-1}: 2919, 1678, 1649, 1621, 1496, 1421, 1236, 1191, 1103, 1035, 859, 737. ^1H and ^{13}C NMR spectra (Table 2). EIMS: *m*/*z* (rel. int.): 448 $M^{+\cdot}$ (3), 380 (100), 378 (8), 325 (15), 163 (10), 137 (14). HREIMS *m*/*z*: 448.1894 $M^{+\cdot}$ (calcd. for $C_{17}H_{28}O_6$, 448.1886).

3.3.5. Maximaisoflavone G acetate (*7*)

Amorphous solid. $M^{+\cdot}$ 354. UV, IR and ^1H NMR spectra were superimposable with the reported data (Rao et al., 1984).

3.3.6. 7-Acetoxy-6-methoxy-3′,4′-methylenedioxy isoflavone (**8**)

Amorphous solid. UV: ν_{max} nm: 268, 308. IR: ν_{max} cm^{-1}: 3050, 2850, 1740, 1635, 1460, 1660, 900, 790. ^1H NMR: (Table 2). EIMS: *m*/*z* (rel. int.): 354 $M^{+\cdot}$ (65), 312 (100), 166 (5), 146 (6).

3.3.7. Compounds **10** and **11**

Griffonianone C (**4**) (48 mg) was C-deisoprenylated (Dagne and Bekele, 1990) with 98% formic acid (8 ml) and concentrated sulfuric acid (2 drops) at room temperature to give a mixture (15 mg) of chromane **10**, and compound **11** in an amorphous form. IR: ν_{max} cm^{-1}: 3450, 2975, 1636, 1491, 1469, 1371, 1245, 1039. ^1H and ^{13}C NMR spectra (Table 3). EIMS: *m*/*z* (rel. int.): 398 (10), 380 (100), 325 (74), 334 (10), 322 (10), 235 (8), 179 (12), 146 (19).

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Lignan glucosides from *Acanthus ilicifolius*

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Abstract

From the aerial part of *Acanthus ilicifolius*, two lignan glucosides, (+)-lyoniresinol 3a-[2-(3,5-dimethoxy-4-hydroxy)-benzoyl]-*O*-β-glucopyranoside, and dihydroxymethyl-bis(3,5-dimethoxy-4-hydroxyphenyl) tetrahydrofuran-9(or 9′)-*O*-β-glucopyranoside have been isolated, together with eight known compounds. The structural elucidations were based on the analyses of physical and spectroscopic data. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Acanthus ilicifolius*; Acanthaceae; Aerial parts; Lignan glucosides; Acanfolioside

1. Introduction

Acanthus ilicifolius L. (Acanthaceae) is a spiny herb found in the mangroves of southern Thailand. In Thai traditional medicine, the plant is used as a purgative, and an anti-inflammatory, and the leaves dispensed with pepper (*Piper nigrum* L.) as tonic pills for longevity. In preliminary investigations of this plant, acanthicifoline (Cordell, 1999), a triterpenoidal saponin (Minocha and Tiwari, 1981) and 2-benzoxazolinone (Kapil et al., 1994) have been isolated. The present study deals with the isolation and structure elucidation of seven lignan glucosides (**1–7**), of which two are new (**3**,**4**), together with known phenylpropanoid glycosides (**8**,**9**) and megastigmane glucoside (**10**) from the aerial parts of the plant.

2. Results and discussion

The methanolic extract of the aerial part of *A. ilicifolius* was suspended in H_2O and defatted with Et_2O. The aqueous layer was subjected to column chromatography using a highly porous copolymer of styrene and divinylbenzene, and eluted with H_2O, 30% MeOH, 60% MeOH, MeOH and Me_2CO, successively. The fraction eluted with 60% MeOH was repeatedly subjected to column chromatography using silica gel, and octadecylsilyl silica gel, then by HPLC–ODS to afford ten compounds (**1–10**). Eight were known compounds; (+)-lyoniresinol 3a-*O*-β-glucopyranoside (**1**), (-)-lyoniresinol 3a-*O*-β-glucopyranoside (**2**) (Achenbach et al., 1992), alangilignoside C (**5**) (Yuasa et al., 1997), (8*R*, 7′*S*,8′*R*)-5,5′-dimethoxylariciresinol 4-*O*-β-glucopyranoside (**6**) (Ida et al., 1994), (+)-syringaresinol-*O*-β-glucopyranoside (**7**) (Kobayashi et al., 1985), verbascoside (**8**) (Miyase et al., 1982), β-hydroxyacteoside (**9**) (Kitakawa et al., 1984) and plucheoside B (**10**) (Uchiyama et al., 1989) by physical data and spectroscopic evidence.

The molecular formula of compound **3** was determined as $C_{37}H_{46}O_{17}$ by HR–FAB mass spectrometry. The ^{13}C NMR spectrum was similar to that of **1** (Table 1). In addition, the ^{13}C NMR signals of 1,3,4,5-tetrasubstituted symmetrical aromatic ring carbons, two equivalent methoxy carbons (δ 56.1) and one ester carbonyl carbon (δ 166.4) were observed. Comparison of the ^{13}C NMR spectrum of **3** with that of **1** revealed the downfield shift of C-2 (+0.6 ppm) and upfield shifts of C-1 and C-3 (−3.2 and −2.1 ppm, respectively) of the glucosyl moiety indicating that the additional unit is an

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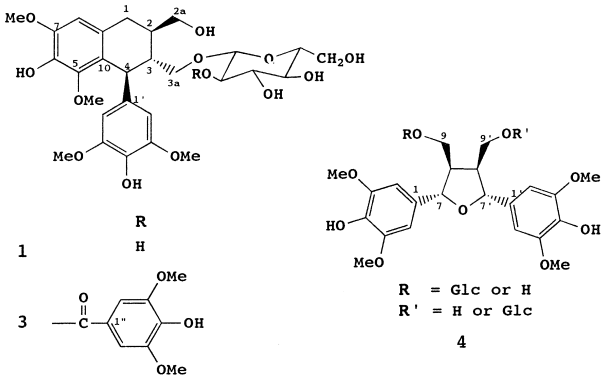
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PII: S0031-9422(00)00362-9

ester located at C-2 of the glucose moiety. Furthermore, the HMBC spectrum revealed a correlation between H-2 (δ 5.93) of the glucosyl moiety and the carbonyl carbon (δ 166.4) as shown in Fig. 1. Consequently, the structure of compound **3** was assigned as (+)-lyoniresinol 3a-[2-(3,5-dimethoxy-4-hydroxy)-benzoyl]-*O*-β-glucopyranoside, named acanfolioside.

Table 1
^{13}C NMR spectral data of compounds **1**, **3** and **4** (100 MHz, C_5D_5N)

Carbon no.	**1**	**3**	Carbon no.	**4**[a]
1	33.7	33.2	1	133.7
2	40.6	40.4	2	104.8
2a	65.4	65.6	3	149.2
3	46.1	45.3	4	136.9
3a	71.1	71.1	5	149.2
4	42.3	41.8	6	104.8
5	148.0	147.8	7	83.3
6	139.4	139.3	8	51.8
7	148.2	148.1	9	70.2
8	107.4	107.3		
9	129.4	129.2	1′	133.9
10	126.5	126.0	2′	104.8
			3′	149.2
1′	138.6	138.0	4′	136.9
2′	107.2	107.1	5′	149.2
3′	148.9	148.7	6′	104.8
4′	[b]	135.6	7′	83.4
5′	148.9	148.7	8′	54.9
6′	107.2	107.1	9′	61.2
G-1	105.4	102.2	G-1	105.0
2	75.2	75.8	2	75.1
3	78.7	76.3	3	78.6
4	71.6	71.8	4	71.4
5	78.4	78.6	5	78.5
6	62.8	62.6	6	62.6
1″		120.6	MeO-3,5	56.3
2″		108.4	MeO-3′,5′	56.3
3″		148.5		
4″		142.6		
5″		148.5		
6″		108.4		
MeO-5	59.6	59.5		
MeO-7	56.0	55.9		
MeO-3′,5′	56.4	56.4		
MeO-3″,5″		56.1		
-COO-		166.4		

[a] Assignments for chemical shift of 1–9 maybe exchanged with 1′–9′.
[b] Signal hidden under C_5D_5N.



The molecular formula of compound **4** was determined as $C_{28}H_{38}O_{14}$ by HR-FAB mass spectrometry. The ^{1}H and ^{13}C NMR spectra revealed the presence of one β-glucopyranosyl unit (δ 4.95, *d*, $J = 7.8$ Hz from ^{1}H NMR) together with two 1,3,4,5-tetrasubstituted symmetrical aromatic rings, two methylenes (δ 61.2 and δ 70.2) which have oxygen functions, four methines (δ 51.8, δ 54.9, δ 83.3 and δ 83.4), two of which have oxygen substituents, and two sets of equivalent methoxy carbons (δ 56.3). From these functionalities, the aglycone suggested a 2,5-diaryl tetrahydrofuranoid-type lignan. The relative configurations between C-7 and C-8, and C-7′ and C-8′ were established as *trans*-configurations and *cis*-configuration between H-8 (or 8′) (δ 3.12)

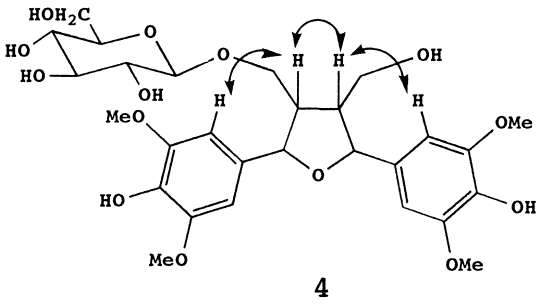


Fig. 2. The NOE correlations of **4**.

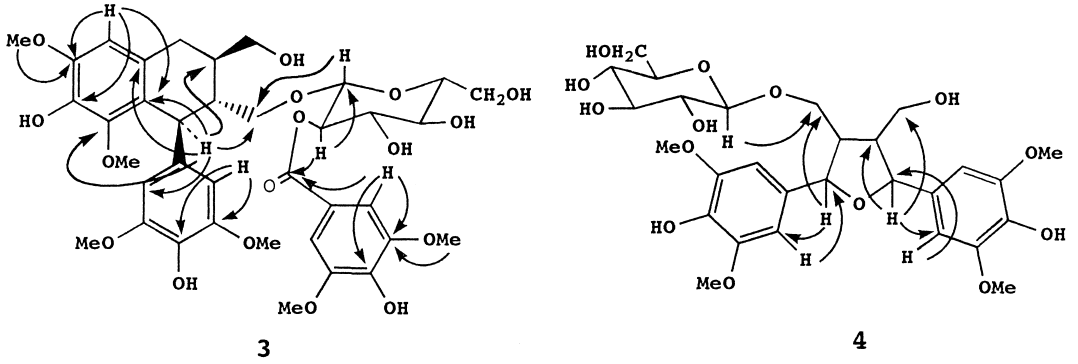


Fig. 1. The HMBC correlations of **3** and **4**.

and H-8′ (or 8) (δ 2.92) by an NOE experiment (Fig. 2). The glucosylation shift at C-9 (or 9′) (δ 70.2) and C-8 (or 8′) (δ 51.8) established the attachment of the glucosyl unit to C-9 (or 9′) of the aglycone, when compared with C-9′ (or 9) (δ 61.2) and C-8′ (or 8) (δ 54.9). Moreover, HMBC spectrum provided further confirmation from the three bonds correlation between anomeric proton (δ 4.95) and C-9 (or 9′) (δ 70.2) of the aglycone as shown in Fig. 1. The attachment of the glucosyl unit could not be determined to the position C-9 or C-9′ because of the small amount of sample. Thus, the structure of compound **4** was concluded to be dihydroxymethyl-bis (3,5-dimethoxy-4-hydroxyphenyl) tetrahydrofuran-9 (or 9′)-*O*-β-glucopyranoside.

3. Experimental

3.1. General

NMR spectra were recorded in C_5D_5N using a JEOL JNM A-400 spectrometer (400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR) and JNM-ECP 500 (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS) as internal standard. MS were recorded on a JEOL JMS-SX 102 spectrometer. Optical rotations were measured with a Union PM-1 digital polarimeter. Preparative HPLC was carried out on columns of ODS (150×20 mm i.d., YMC) with a Tosoh refraction index (RI-8) detector. MPLC was carried out on a column of Polyamide C-200. For CC, silica gel G 60 (Merck), YMC-gel ODS (50 μm, YMC) and highly porous copolymer of styrene and divinylbenzene (Mitsubishi Chem. Ind. Co. Ltd) were used. The solvent systems were: (I) EtOAc–MeOH–H_2O (4:1:0.1), (II) EtOAc–MeOH–H_2O (7:3:0.3), (III) EtOAc–MeOH–H_2O (6:4:1), (IV) 15% MeCN, (V) 20% MeCN, (VI) 30% MeCN, (VII) 95% MeCN, (VIII) 95–85% MeCN, (IX) 90–80% MeCN and (X) 25% MeOH. The spray reagent used for TLC was 10% H_2SO_4 in 50% EtOH.

3.2. Plant material

Acanthus ilicifolius L. was collected in April, 1997 from Satun Province, southern Thailand. The identification of the plant was confirmed by Prof. Vichiara Jirawongse, Dept. of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher sample is kept in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

3.3. Extraction and isolation

The dried aerial part (2.5 kg) of *A. ilicifolius* was extracted with hot MeOH. After removal of the solvent by evaporation, the residue (380.0 g) was defatted with Et_2O. The aqueous layer was subjected to a column of a highly porous copolymer of styrene and divinylbenzene (Mitsubishi Chem. Ind.) and this was eluted with H_2O, 30% MeOH, 60% MeOH, MeOH and Me_2CO, successively. The fraction eluted with 60% MeOH (16.0 g) was subjected to a column of silica gel (systems I, II and III, respectively) affording five fractions. Fraction 2 (2.88 g) was subjected to prep. HPLC-ODS chromatography (system IV) to provide compound **8** (557 mg). Fraction 3 (3.97 g) was further separated on a YMC-ODS column (system VI) to give 10 fractions. Fractions 3–2 (765 mg), 3–5 (960 mg) and 3–10 (455 mg) were subjected to MPLC-polyamide (system VII,VIII and IX, successively), then followed by prep. HPLC-ODS (systems X, IV and V) to afford compounds **1** (236 mg), **2** (44 mg), **4** (10 mg), **7** (28 mg), **9** (18 mg) and **10** (10 mg). Fractions 3-8 (246 mg) and 3–9 (257 mg) were purified by HPLC-ODS (system V) to give compounds **3** (27 mg), **5** (95 mg) and **6** (11 mg).

3.4. Acanfolioside (*3*)

Amorphous powder, $[\alpha]_D^{22}+28.3^\circ$ (MeOH, *c* 2.7); ^1H NMR (C_5D_5N): δ 7.76 (2H, *s*, H-2″,6″), δ 6.82 (2H, *s*, H-2′,6′), δ 6.65 (1H, *s*, H-8), δ 5.93 (1H, *t*, *J*=8.4 Hz, H-2 Glc), δ 5.16 (1H, *d*, *J*=8.0 Hz, H-1 Glc), δ 4.32–4.58 (6H, *m*, H-3,4,5,6 Glc and H-3a), δ 4.03 (1H, *dd*, *J*=10.5, 4.3 Hz, H-2a), δ 3.98 (1H, *d*, *J*=10.5 Hz, H-2a), δ 3.84 (1H, *dd*, *J*=9.6, 4.3 Hz, H-3a), δ 3.73 (3H, *s*, OMe-7), δ 3.72 (6H, *s*, OMe-3″,5″), δ 3.69 (3H, *s*, OMe-5), δ 3.68 (6H, *s*, OMe-3′,5′), δ 3.06 (1H, *dd*, *J*=15.0, 11.5 Hz, H-1), δ 2.96 (1H, *dd*, *J*=15.0, 4.6 Hz, H-1), δ 2.70 (1H, *m*, H-3), δ 2.16 (1H, m, H-2) ; ^{13}C NMR (C_5D_5N): Table 1; Negative HR-FAB-MS, *m*/*z*: 761.2612 $[M-H]^-$ ($C_{37}H_{45}O_{17}$ requires 761.2657).

3.5. Compound *4*

Amorphous powder, $[\alpha]_D^{22}$ −25.5° (MeOH, *c* 1.0); ^1H NMR (C_5D_5N): δ 7.24 (2H, *s*, H-2′,6′ or 2,6), δ 7.20 (2H, *s*, H-2,6 or 2′,6′), δ 5.64 (1H, *d*, *J*=8.1 Hz, H-7′ or 7), δ 5.60 (1H, *d*, *J*=7.7 Hz, H-7 or 7′), δ 4.94 (1H, *d*, *J*=7.8 Hz, H-1 Glc), δ 4.50 (1H, *dd*, *J*=11.2, 2.2 Hz, H-6 Glc), δ 4.36 (1H, *dd*, *J*=11.2, 5.3 Hz, H-6 Glc), δ 4.18–4.25 (2H, overlapped, H-4, 5 Glc), δ 4.48 (2H, *m*, H-9 or 9′), δ 4.26 (1H, *m*, H-9′ or 9), δ 4.12 (1H, *m*, H-9′ or 9), δ 4.06 (1H, *t*, *J*=7.8 Hz, H-2 Glc), δ 3.89 (1H, *t*, *J*=7.6 Hz, H-3 Glc), δ 3.78 (6H, *s*, OMe x 2), δ 3.76 (6H, *s*, OMe x 2), δ 3.12 (1H, *m*, H-8 or 8′), δ 2.92 (1H, *m*, H-8′ or 8); ^{13}C NMR (C_5D_5N): Table 1; Negative HR-FAB-MS, *m*/*z*: 597.2203 $[M-H]^-$ ($C_{28}H_{37}O_{14}$ requires 597.2183).

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PHYTOCHEMISTRY

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A malonylated anthocyanin and flavonols in blue *Meconopsis* flowers

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Abstract

The structures of the major anthocyanin and two flavonols from the blue flowers of *Meconopsis* were identified by NMR spectroscopy as being cyanidin 3-*O*-[(6-*O*-malonyl-2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside, kaempferol 3-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside and kaempferol *3*-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside respectively. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Meconopsis grandis*; Papaveraceae; Blue Himalayan poppy; Malonylated anthocyanin; Flavonols

1. Introduction

The blue colours of flowers are usually produced by deiphinidin-based anthocyanins, as in *Commelina communis* (Hayashi et al., 1958), *Hydrangea macrophylla* (Takeda et al., 1985), *Salvia patens* (Takeda et al., 1994), *Lupinus* (Bayer, 1959) and *Iris* (Asen et al., 1970), and only rarely by cyanidin derivatives, as in *Centaurea cyanus* (Bayer, 1958). The flower colour of *Meconopsis* is sky blue and is known to be produced by a cyanidin type of anthocyanin. However, little is known of the bluing of this flower colour. We reported previously that the blue colour of *Meconopsis* flowers was based on cyanidin 3-malonylsambubioside (**1**) (Takeda et al., 1996), although the site of malonyl substitution was not determined. In the present study the full structure determination of anthocyanin (**1**) is presented, together with the structures of two potential flavonol copigments, which were identified as kaempferol glycosides (**2** and **3**).

2. Results and discussion

2.1. Identification of anthocyanin

The structure of **1** was elucidated by NMR spectroscopic techniques including by ^{1}H–^{1}H COSY, ROESY, HOHAHA, HMQC and HMBC spectroscopy. In the ^{1}H NMR (500 MHz) spectrum of **1**, six characteristic signals at δ 8.88 (H-4), 8.41 (H-6′), 8.10 (H-2′), 7.06 (H-5′), 7.28 (H-8) and 6.89 (H-6) in the downfield region indicated that the aglycone was cyanidin (Table 1). The three 1H doublet signals at δ 5.24 ($J=7.2$ Hz), 5.56 ($J=7.6$ Hz) and 4.83 ($J=7.7$ Hz) were attributed to the anomeric protons of three sugar residues in the β-configuration. Analysis of the proton connectivity network of the sugar moieties using ^{1}H NMR, COSY and HOHAHA experiments allowed the anomeric protons (δ 5.24 and 5.56) to be correlated to the nonequivalent methylene protons of C-6 at δ 3.78 and 4.02, and δ 4.35 and 4.57, respectively: both methylene groups exhibited large coupling constants (Table 1), indicating the existence of two glucopyranoside moieties. The remaining sugar moiety was identified as xylose from its characteristic chemical shift and coupling constant parameters (Table 1). The attachment positions of the sugar residues to the aglycone were determined by HMBC and ROESY measurements. A correlation between the

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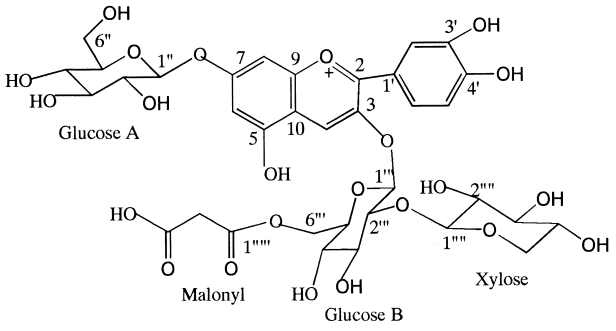
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anomeric proton of glucose A (δ 5.24) and C-7 of the aglycone (δ 167.3) was observed in the HMBC spectrum, indicating that glucose A was attached to position 7. Similarly, glucose B was shown to be glycosylated at the 3-OH of the aglycone, due to the correlation observed between the anomeric proton of glucose B (δ 5.56) and C-3 of the aglycone (δ 146.5) in the HMBC spectrum.

Dipolar interactions in the ROESY spectrum between the anomeric proton of glucose A with H-6 and H-8, and the anomeric proton of glucose B with H-4 also supported the localization of these glucose residues at C-7 and C-3 of the aglycone. The low field shift of C-2‴ (δ 81.3) in the ^{13}C NMR spectrum indicated that the 2‴-OH of glucose B was likely to be substituted (the α-effect) (Agrawal, 1992). This was confirmed by analysis of ROESY data which showed a dipolar interaction between H-2‴ of the glucose B and the anomeric proton of xylose, and by analysis of HMBC data (Table 1). These results demonstrated that **1** was a cyanidin 7-*O*-glucoside bearing a 2-β-xylosylglucose (sambubiose) at the 3-OH group.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for anthocyanin **1**

Position	δ_C	δ_H	J (Hz)	HMBC ^{13}C with ^{1}H
Aglycone				
2	166.1			4, 2′, 6′
3	146.5			1‴, 4
4	134.4	8.88 *s*		
5	158.3			4, 6
6	103.5	6.89 *d*	1.9	8
7	167.3			6, 8, 1″
8	95.5	7.28 *d*	1.3	6
9	156.5			4, 8
10	113.6			4, 6, 8
1′	121.1			2′, 5′
2′	119.0	8.10 *d*	1.5	6′
3′	147.8			2′, 5′
4′	157.2			2′, 5′, 6′
5′	117.6	7.06 *d*	8.8	
6′	130.1	8.41 *dd*	2.2, 8.7	2′
Glucose A				
1″	101.9	5.24 *d*	7.2	2″, 3″, 5″
2″	74.6	3.61 *t*	9.0	3″
3″	77.9	3.59 *t*	9.0	
4″	71.0	3.46 *m*		
5″	78.7	3.67 *ddd*	2.1, 5.8, 9.3	6″
6″-a	62.6	3.78 *dd*	5.8, 12.2	4″, 5″
6″-b	62.6	4.02 *dd*	1.6, 12.3	
Glucose B				
1‴	101.2	5.56 *d*	7.6	2‴
2‴	81.3	4.05 *t*	8.8	3‴, 1⁗
3‴	77.9	3.85 *t*	9.0	
4‴	71.0	3.53 *t*	9.5	
5‴	75.9	3.93 *ddd*	1.6, 7.7, 9.3	3‴, 6‴
6‴a	65.4	4.57 *dd*	1.6, 11.7	4‴, 5‴
6‴b	65.4	4.35 *dd*	7.4, 12.0	
Xylose				
1⁗	105.7	4.83 *d*	7.7	2⁗, 3⁗, 5⁗
2⁗	75.7	3.21 *dd*	7.9, 8.9	1⁗, 3⁗, 4⁗
3⁗	77.9	3.30 *t*	9.1	
4⁗	71.2	3.46 *m*		
5⁗a	67.2	3.75 *dd*	5.4, 11.9	1⁗, 4⁗
5⁗b	67.2	3.12 *t*	10.7	
Malonyl				
1⁗′	168.2			2⁗′, 6‴
2⁗′a	Not detected	3.43 *br s*		
2⁗′b	Not detected	3.43 *br s*		
3⁗′	168.8			2⁗′



Anthocyanin **1**

Observation of methylene protons at δ 3.43 (br s), the intensity of which reduced gradually in solution (CD_3OD acidified with TFA-d_4), and two carbonyl carbon at δ 168.2 and 168.8 in the ^{13}C NMR spectrum, confirmed the presence of a malonyl group. The downfield shift of the methylene protons (H-6‴) of glucose B (δ 4.35 and 4.57) indicated acylation at this position. Observation of a long-range correlation between C-1⁗′ of the malonyl moiety and H-6‴ of glucose B also confirmed the presence of a malonyl group at the 6‴-OH.

We previously reported the structure of *Meconopsis* anthocyanin to be cyanidin 3-malonylsambubioside 7-glucoside (Takeda et al., 1996). However, the position of malonic acid attachment as well as a configuration of sugar attachment was not determined. In order to understand the nature of blue colour developed with a cyanidin type of anthocyanin in *Meconopsis* flower, it will be important to determine the full structure of anthocyanin as well as copigments involved in the flower colour. As described above, the structure of **1** was determined as cyanidin 3-*O*-[(6-*O*-malonyl-2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside, that is, cyanidin 3-*O*-(6-*O*-malonyl-β-sambubioside)-7-*O*-β-D-glucopyranoside. This was further confirmed by HR FAB MS spectrometry: m/z 829.2087 $[M]^+$, $C_{35}H_{41}O_{23}$ requires 829.2038.

2.2. Flavonols

HPLC with a C_{22} column separated the main flavonol fraction, described previously as kaempferol 3-gentiobioside (Takeda et al., 1996), into two fractions,

compounds **2** and **3**, in the ratio of ca. 1:3. Shirai et al. (1999) reported the improvement of separation of triacylglycerol molecule species by HPLC with a C_{22} column. The application of the C_{22} column was effective for the separation of the above two compounds.

The ^{1}H NMR spectrum of **2** indicated the presence of a kaempferol moiety, δ 6.92 (H-3′, 5′), 8.16 (H-2′, 6′), 6.25 (H-6) and 6.44 (H-8), and two sugar moieties with the β-anomeric configuration [δ 4.18 (J = 7.8 Hz), 5.15 (J = 7.8 Hz)]. Using the anomeric proton at δ 4.18 as a starting point, analysis of ^{1}H NMR and COSY experiments allowed the identification, in sequence, of four oxymethine groups and one oxymethylene group. This sugar moiety was identified as β-glucose on the basis of the large coupling constants observed for all of the oxymethine protons, consistent with their mutually diaxial relationships. The second anomeric proton at δ 5.15 was shown to be derived from β-galactose, as demonstrated by the axial-equatorial coupling between H-3″ and H-4″ of J = 3.5 Hz, and between H-4″ and H-5″ of J = 2.9 Hz, and the axial–axial coupling between H-2″ and H-3″ of J = 9.8 Hz (Anet and Anet, 1971).

The linkage between the sugars and the aglycone was determined by a NOESY experiment. The correlation observed between the anomeric proton of galactose (δ 5.15) and H-2′, 6′ (δ 8.16) indicated that the kaempferol aglycone was substituted at the 3-OH position. The terminal glucose was shown to be linked in the β-configuration to the 6-OH of galactose due to the correlation between the anomeric proton of the glucose H-1″ (δ 4.18) and the methylene protons of the galactose H-6″ (δ 3.67 and 3.90). On the basis of these results, **2** was identified as kaempferol 3-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside.

The structure of **3** was confirmed by 1- and 2-D NMR experiments to be kaempferol 3-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside, kaempferol 3-gentiobioside as reported previously. In this study, however, we did not succeed in isolating a minor flavonol in the petals, kaempferol 3-xylosylgentiobioside, in sufficient quantity for analysis by NMR spectroscopy (Takeda et al., 1996).

3. Experimental

3.1. Plant materials

After collection of the blue petals of *M. grandis,* the materials (85.2 g) were immediately frozen and kept at −20°C.

3.2. Isolation of anthocyanin and flavonols

Pigments were extracted with HCO_2H–MeOH–H_2O (FMW, 1:10:9). After evapn, anthocyanin and flavonols were separated by HPLC on an ODS (Develosil, Nomura Chemical Co. Ltd., Aichi, Japan) column (1.6×30 cm) using a stepwise elution with FMW (10 min with 5:22:73 — 10 min with 5:37:58 — 15 min with 5:50:45), principally as reported previously (Ishikawa et al., 1999). The anthocyanin and flavonols obtained were purified further by HPLC on a C_{22} column (1.0×25 cm, Docosil, Senshu scientific Co. Ltd., Tokyo, Japan) using an isocratic elution with the following solvent systems: anthocyanin, HCO_2H–CH_3CN–H_2O 5:10:85; flavonols, HCO_2H–CH_3CN–H_2O 5:20:75, to yield compounds **1** (13 mg), **2** (8 mg) and **3** (32 mg).

3.3. NMR and MS spectroscopies

^{1}H and ^{13}C NMR spectra were obtained at 25°C, at 500 and 125 MHz, respectively, on a Bruker DMX-500 NMR spectrometer locked to the deuterium resonance of the solvents, CD_3OD containing 8% of CF_3CO_2D for anthocyanin and CD_3OD for flavonols. Chemical shifts are reported in ppm relative to the residual solvent signals (δ_H = 3.35, δ_C = 49.0). Determination of one-bond heteronuclear ^{1}H–^{13}C connectivities, and distinction of methyl, methylene and methine carbon signals were carried out with HMQC experiments, optimized for $^1J_{C-H}$ = 145 Hz. Two- and three-bond ^{1}H–^{13}C connectivities were determined using HMBC experiments optimized for $^nJ_{C-H}$ = 7.3 Hz. ^{1}H connectivities were determined by using COSY and HOHAHA experiments. The 2-D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 decoupling sequence (mixing time 100 ms) (Bax and Davis, 1985). Nuclear Overhauser effect measurements were performed by 2-D NOESY and ROESY experiments. HR FAB MS spectra were obtained on a Kratos concept-2H instrument. The mass spectrometer was operated at an accelerating voltage of 8 kV, using nitrobenzyl alcohol for the compound **1** and a mixture of dithiothreitol and thioglycerol for the compounds **2** and **3** as a matrix, respectively.

3.4. Kaempferol 3-O-6-O-β-D-glucopyranosyl)-β-D-galactopyranoside (2)

^{1}H NMR spectral data (CD_3OD): kaempferol: δ 6.25 (1H, *d*, J = 2.1 Hz, H-6), 6.44 (1H, *d*, J = 2.1 Hz, H-8), 8.16 (2H, *m,* H-2′, H-6′), 6.92 (2H, *m*, H-3′, H-5′); galactose: 5.15 (1H, *d*, J = 7.8 Hz, H-1″), 3.84 (1H, *dd*, J = 7.9, 9.9 Hz, H-2″), 3.58 (1H, *dd*, J = 3.5, 9.7 Hz, H-3″), 3.91 (1H, *dd*, J = 2.9, 3.3 Hz, H-4″), 3.68 (1H, *m*, H-5″), 3.67 (1H, *m*, H-6″a), 3.90 (1H, *m*, H-6″b); glucose: 4.18 (1H, *d*, J = 7.8 Hz, H-1‴), 3.08 (1H, *dd,* J = 8.0, 8.8 Hz, H-2‴), 3.20 (1H, *t*, J = 8.9 Hz, H-3‴), 3.24 (1H, *t,* J = 9.0, H-4‴), 3.11 (1H, *ddd*, J = 2.4, 5.8, 9.3 Hz, H-5‴),

3.63 (1H, *dd*, $J=6.0$, 12.0 Hz, H-6‴a), 3.83 (1H, *dd*, $J=2.3$, 12.0 Hz, H-6‴b). HR FAB MS: *m/z* 633.1436 $[M+Na]^+$, $C_{27}H_{30}O_{16}Na$ requires 633.1431.

3.5. Kaempferol 3-O-(6-O-β-D-glucopyranosyl)-β-D-glucopyranoside (**3**)

^1H NMR spectral data (CD_3OD): kaempferol: δ 6.24 (1H, *d*, $J=2.1$ Hz, H-6), 6.45 (1H, *d*, $J=2.1$ Hz, H-8), 8.15 (2H, *m*, H-2′, H-6′), 6.93 (2H, *m*, H-3′, H-5′); glucose A: 5.29 (1H, *d*, $J=7.4$ Hz, H-1″), 3.50 (1H, *dd*, $J=7.9$, 8.9 Hz, H-2″), 3.47 (1H, *t*, $J=9.1$ Hz, H-3″), 3.43 (1H, *t*, $J=9.4$ Hz, H-4″), 3.45 (1H, *ddd*, $J=1.6$, 5.2, 9.4 Hz, H-5″), 3.68 (1H, *dd*, $J=5.2$, 11.8 Hz, H-6″a), 4.02 (1H, *dd*, $J=1.6$, 11.8 Hz, H-6″b); glucose B: 4.18 (1H, *d*, $J=7.7$ Hz, H-1‴), 3.11 (1H, dd, $J=7.9$, 8.8 Hz, H-2‴), 3.21 (1H, *t*, $J=8.9$ Hz, H-3‴), 3.26 (1H, *dd*, $J=9.0$, 9.3 Hz, H-4‴), 3.05 (1H, *ddd*, $J=2.4$, 5.7, 9.2 Hz, H-5‴), 3.63 (1H, *dd*, $J=5.7$, 12.0 Hz, H-6‴a), 3.79 (1H, *dd*, $J=2.3$, 12.0 Hz, H-6‴b). HR FAB MS: *m/z* 633.1469 $[M+Na]^+$, $C_{27}H_{30}O_{16}Na$ requires 633.1431.

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PHYTOCHEMISTRY

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Phenylbutanoids and stilbene derivatives of *Rheum maximowiczii*

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Abstract

The methanol extract of the dried roots of *Rheum maximowiczii* afforded four phenylbutanoid and two stilbene derivatives. Their structures were established on the basis of chemical and spectroscopic studies. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Rheum maximowiczii*; Polygonaceae; Phenylbutanoid; Stilbene dimer

1. Introduction

Rheum maximowiczii Loinsk (Polygonaceae) grows in Central Asia, where it is a folk medicine used by the natives of Uzbekistan as a purgative, and for the treatment of stomach disorders. The genus *Rheum* is one of the world's important crude drugs, containing anthraquinones, glycosides, dianthrone glycosides and polyphenols, and the chemical constituents of this genus have been studied by many groups (Okabe, et al., 1973; Oshio, et al., 1974). However, the chemical constituents of *R. maximowiczii* have not been reported. As a part of our continuing study on the chemical constituents of medicinal plants of Uzbekistan, we have succeeded in the isolation and characterization of fifteen (**1**–**15**) compounds, including four new phenylbutanoids (**3–6**) and two dimeric stilbenes (**9** and **10**) from this plant. In this paper, we report the isolation and identification of these compounds.

2. Results and discussion

Repeated column chromatography of the ethyl acetate and *n*-BuOH soluble fractions from the methanol extract of the roots of *R. maximowiczii* yielded four new phenyl butanoids (**3–6**) and two new stilbene dimers (**9** and **10**), along with nine other known compounds (+)-rhododendrol (**1**), epirhododendrin (**2**) (Pan and Lundgren, 1994), lindleyin, (*S*)-4-(4-hydroxyphenyl)-2-butanone 4′-*O*-β-D-glucopyranoside (Kashiwada et al., 1986), torachrysone (Tsuboi et al., 1977), resveratrol, resveratrol 4′-*O*-β-D-glucopyranoside (Nonaka et al., 1977), emodin 8-*O*-β-D-glucopyranoside and physcion (Satake et al., 1990). The structures of the known compounds were identified by comparison with literature data.

Compound **3** showed the presence of hydroxyl (3271 cm^{-1}) and aromatic ring (1656, 1600 cm^{-1}) absorption bands in the IR spectrum. The ^{1}H and ^{13}C NMR spectral data of **3** were very close to those of (+)-rhododendrol (**1**), the evident difference between them being the chemical shift of C-2 (Table 1). 2D NMR spectral data also suggested that compounds **1** and **3** have the same skeleton. The negative ion FAB mass spectrum indicated that the molecular weight of **3** was 246 which was 80 mass units higher than that calculated for **1**. This increase in mass agreed with an esterification of one of the hydroxyl groups by $-PO(OH)_2$ (MW 81) or $-SO_2OH$ (MW 81). The acid moiety was presumed to be sulfate, because no couplings (J_{POC} and J_{POCC}) were observed in the ^{13}C NMR spectrum. From HR-FABMS analysis, the molecular formula of **3** was determined to be $C_{10}H_{13}O_5S$ (m/z:245.0473 $[M\text{-}H]^-$). The presence of a sulfur atom was confirmed further by elemental analysis (see Section 3). Furthermore, the acid hydrolysis of **3** gave **1** (identified by $[\alpha]_D$, ^{1}H and ^{13}C

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NMR data). The absolute configuration of C-2 was determined to be *S* by comparison of its optical rotation $\{[\alpha]_D^{25} + 30.8^\circ$ (*c* 0.7 MeOH)$\}$ with that reported in the literature (Das and Padma Rao, 1993). From these facts, the structure of **3** was determined to be (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-sulfate. The presence of a sulfate functionably is very rare in natural products.

The ^{1}H and ^{13}C NMR (Table 1) spectral data for compounds **4** and **5** were very similar to those of **2** except for the appearance of signals due to galloyl and acetyl moieties, respectively, suggesting that **4** and **5** have the same phenylbutanoid glucoside moiety as for **2**. The positions of the galloyl and acetyl functions were established by COLOC correlations between the carbonyl carbon and H_2-6 of glucose, and acylation shifts (Table 1). Acid hydrolysis of **4** and **5** gave glucose and **1**. Thus, the structures of **4** and **5** were determined as (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside and (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-acetyl)-β-D-glucopyranoside, respectively.

Compound **6** showed the molecular formula $C_{31}H_{42}O_{15}$ as determined by HR-FABMS. Its IR spectrum showed absorption bands due to hydroxyl (3402 cm^{-1}) and carbonyl (1709 cm^{-1}) groups. The ^{1}H and ^{13}C NMR (Table 1) spectral data of **6** were similar to those of **2** except for presence of the signals assignable to the galloyl and rhamnosyl moieties. In the COLOC spectrum of **6** (Fig. 1), the proton signals at δ 4.40 and 4.68 (H_2-6 of glucose) were correlated with the carbonyl carbon signal at δ_c 168.4, and the proton signal at δ 5.38 (H-1 of rhamnose) with the carbon signal at δ_c 139.7 (C-4 of galloyl). From these results, the connecting positions of the methoxyl, galloyl and rhamnose moieties were determined. Acid hydrolysis of **6** gave **1** which showed the optical rotation of $+20.0^\circ$ (*c* 0.08, MeOH). Hence, the structure of **6** was determined to be (*S*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-[6-*O*-(3,5-dimethoxy-4-*O*-α-L-rhamnopyranosylgalloyl)-β-D-glucopyranoside].

Maximol A (**9**) indicated the molecular formula $C_{28}H_{22}O_6$ by HR-FABMS. The IR spectrum indicated the existence of hydroxyl (3377 cm^{-1}) and aromatic groups (1601 cm^{-1}). The ^{1}H NMR spectrum of **9** (Table 2) exhibited 13 protons belonging to the aromatic rings, two *trans* olefinic protons [δ 6.78 and 6.97 (each 1H, *d*, $J = 16.2$ Hz)], and two methine protons [δ 4.40 and 5.39 (each 1H, *d*, $J = 8.3$ Hz)]. From the coupling pattern and integration in the ^{1}H NMR spectrum (Table 2), four aromatic rings (A, B, C and D) were recognized. On the basis of correlations in the COLOC spectrum, the aromatic rings of A and B were deduced to be connected with C-7 and C-8, respectively (Fig. 2). Long range correlations from the carbon signals at δ_c 94.9 (C-7), 107.7 (C-10, -14) to the proton signals at δ 7.17 (H-2, -6), 4.40 (H-8) were detected. Also, the carbon signals at δ_c 129.4 (C-7′) and 127.4 (C-8′) were correlated with the proton signals at δ 7.17 (H-2′) and 7.36 (H-6′) and 6.44 (H-10′, -14′) (Fig. 2). From these results, the connections between C-7′ and ring C, and between C-8′ and

Table 1
^{13}C NMR spectral data for compounds **1–6** (CD_3OD)[a]

Agylcone	1	2	3	4	5	6
C-1	24.2	21.8	20.5	22.0	22.1	22.3
C-2	66.3	76.7	77.2	78.8(a)	77.5	77.6
C-3	42.2	39.2	40.8	40.0	40.1	40.2
C-4	31.8	31.2	32.1	31.5	31.6	31.6
C-5	133.3	134.1	134.7	134.5	134.4	134.4
C-6,10	129.7	129.9	130.7	130.0	130.3	130.3
C-7,9	115.9	115.7	116.5	116.0	116.0	116.1
C-8	156.6	155.3	156.7	155.9	156.2	156.2
β-D-Glucose						
C-1		103.2		104.1	104.2	104.3
C-2		74.5		75.0	75.2	75.1
C-3		77.2		78.8(a)	77.9	78.0
C-4		70.8		71.7	71.7	72.1
C-5		76.9		75.3	75.5	75.3
C-6		62.2		64.9	64.8	65.6
Galloyl moiety						
C-1				121.4		127.1
C-2,6				110.2		107.8
C-3,5				146.3		154.5
C-4				139.7		140.0
−COO−				168.4		167.3
α-L-Rhamnose						
C-1						103.4
C-2						72.2
C-3						72.0
C-4						73.6
C-5						71.3
C-6						17.0
−COO−					172.8	
$-CH_3$					20.7	
$-OCH_3$						56.6

[a] Spectra were measured in CD_3OD at 100 MHz (δ values). Assignment of carbon signals was achieved by analysis of ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra. (a) Overlapping signals.

Table 2
^{1}H NMR spectral data for compounds **9** and **10** (CD_3OD)[a]

	9	10
2(6)	7.17, 2H, *d*, $J = 8.3$ Hz (a)	7.17, 2H, *d*, $J = 10.7$ Hz (a)
3(5)	6.78, 2H, *d*, $J = 8.3$ Hz (a)	6.78, 2H, *d*, $J = 10.7$ Hz
7	5.39, 1H, *d*, $J = 8.3$ Hz	5.31, 1H, *d*, $J = 8.8$ Hz
8	4.40, 1H, *d*, $J = 8.3$ Hz	4.32, 1H, *d*, $J = 8.8$ Hz
10(14)	6.13, 2H, *d*, $J = 2.4$ Hz	6.04, 2H, *t*, $J = 2.4$ Hz
12	6.21, 1H, *t*, $J = 2.4$ Hz	6.15, 1H, *t*, $J = 2.4$ Hz
2′	7.17, 1H, *s* (a)	6.88, 1H, *brs*
5′	6.84, 1H, *d*, $J = 8.3$ Hz	6.70, 1H, *d*, $J = 8.3$ Hz
6′	7.36, 1H, *d*, $J = 8.3$ Hz	7.17, 1H, *d*, $J = 8.3$ Hz (a)
7′	6.97, 1H, *d*, $J = 16.2$ Hz	6.44, 1H, *d*, $J = 12.7$ Hz
8′	6.78, 1H, *d*, $J = 16.2$ Hz (a)	6.33, 1H, *d*, $J = 12.7$ Hz
10′(14′)	6.44, 2H, *d*, $J = 2.4$ Hz	6.22, 2H, *d*, $J = 2.4$ Hz
12′	6.16, 1H, *t*, $J = 2.4$ Hz	6.11, 1H, *t*, $J = 2.4$ Hz

[a] Spectra were measured in CD_3OD at 400 MHz (δ values). Assignment of proton signals was achieved by analysis of ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra. (a) Overlapping signals.

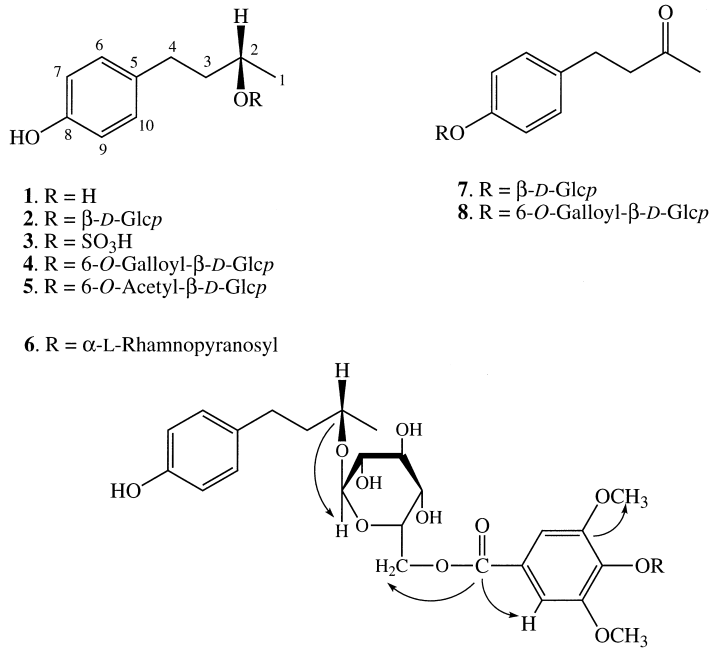


Fig. 1. ^{13}C-^{1}H long-range correlations.

ring D were determined. The degree of unsaturation in **9** was 18; **9** was established to have dihydrobenzofuran moieties of a stilbene dimer biosynthesized from resveratrol. The relative structure of **9** was revealed by an NOE experiment, wherein the proton signal at δ 5.39 (H-7) was correlated with the proton signals at δ 7.17 (H-2, -6) and 6.13 (H-10, -14), and the proton signal at δ 4.40 (H-8) was correlated with proton signals at δ 7.17 (H-2, -6) and 6.13 (H-10, -14). These facts clearly showed that the configuration of rings A and B was *trans*. Thus, the structure of maximol A (**9**) was assigned as shown. According to a previous paper (Langcake and Pryce, 1977), **9** was synthesized from resveratrol by treatment with horseradish peroxidase and H_2O_2. This is the first time that **9** has been isolated from a natural source.

Maximol B (**10**) ($C_{28}H_{22}O_6$) showed very similar ^{1}H and ^{13}C NMR spectral data to those of **9** except for double bond signals [**10**:δ 6.78 and 6.97 (each 1H, *d*, $J=16.2$ Hz), **9**:δ 6.33 and 6.44 (each 1H, *d*, $J=12.7$ Hz)] and benzofuran proton signals [**10**:δ 4.40 and 5.39 (each 1H, *d*, $J=8.3$ Hz), **9**:δ 4.32 and 5.31 (each 1H, *d*, $J=8.8$ Hz)]. From the coupling constant of the double bond, its geometry of **10** was determined to be *cis*. That evidence was supported by the NOESY spectrum (Fig. 2). *Cis* orientation of the benzofuran ring was deduced from an NOE correlation between δ 5.31 (H-7) and 4.32 (H-8) (Fig. 2), and the coupling constant values at H-7/H-8 ($J=8.8$ Hz), (Kurihara et al., 1990). From these results, the structure of maximol B (**10**) was determined as shown.

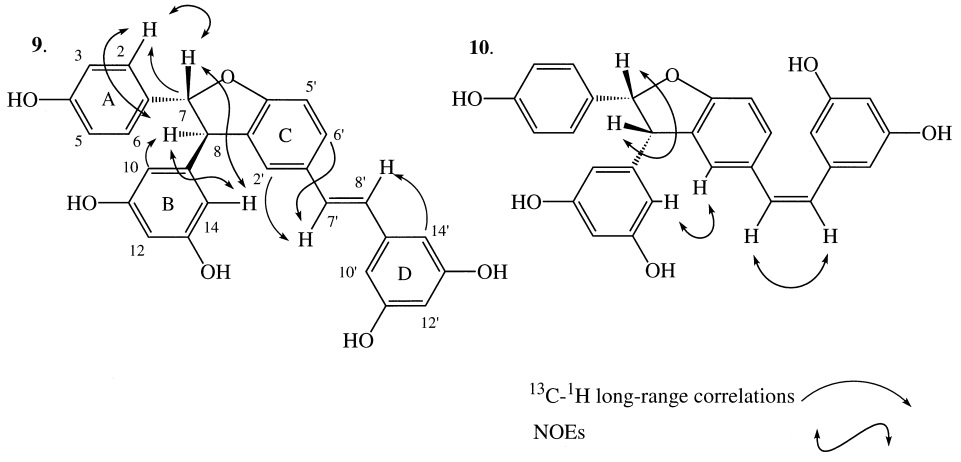


Fig. 2. ^{13}C-^{1}H long-range correlations of **9** and NOE correlations of **9** and **10**.

Epirhododendrin (**2**) was obtained in high yield (23% of the dry weight of *n*-BuOH soluble layer). A previous paper (Fujita et al., 1990) reported that epirhododendrin is more stable than (+)-rhododendrol (1.3% of the dry weight of AcOEt soluble layer), and is useful for pharmaceuticals and skin-lightening cosmetics, and as a melanin inhibitor (Ikemoto and Nakatsugawa, 1998). Therefore, *R. maximowiczii* is an important source of phenylbutanoids.

3. Experimental

3.1. General

^{1}H NMR: 400 MHz with TMS as int. stand: ^{13}C NMR: 100 MHz: CC: silica gel 60 (Merck), Sephadex LH-20 (Pharmacia) and Toyo pearl HW-40 (TOSOH); HPLC: GPC H-2002 (SHODEX), ODS: D-ODS-5 (YMC).

3.2. Plant material

The roots of *R. maximowiczii* were collected in June 1998 from Uzbekistan. Herbarium specimens were deposited in the herbarium of Institute of Botany, Academy of Sciences, Uzbekistan. This plant was identified by Dr Olimjon K. Kodzhimatov.

3.3. Extraction and fractionation

The MeOH extracts (261 g) of the dried roots of *R. maximowiczii* (1.9 kg) were partitioned between EtOAc and H_2O. The EtOAc layer was concentrated to give a residue (46 g), which was subjected to silica gel column chromatography. The column was eluted with solvents of increasing polarity ($CHCl_3$–MeOH) to give 15 frs. (frs. 1–15). Fr. 11 (714 mg) was applied to a Toyo pearl column, eluted with $CHCl_3$–MeOH (2:1) to give 6 frs. (11.1–11.6). Fr. 11.2 (66 mg) was sepd. by HPLC (GPC) with MeOH to give **5** (18 mg). Fr. 12 (1.6 g) was subjected to Toyo pearl column chromatography eluted with $CHCl_3$–MeOH (2:1) to give 7 frs. (12.1–12.7). Fr. 12.6 (242 mg) was purified by HPLC (ODS) with CH_3CN–H_2O (7:3) to give **9** (30 mg) and **10** (5 mg). Fr. 15 (5 g) was applied to a Toyo pearl column eluted with $CHCl_3$–MeOH (2:1) to give 7 frs. (15.1–15.7). Fr. 15.2 (165 mg) was chromatographed on a silica gel column using $CHCl_3$–MeOH (8:2 to 1:1) to give **6** (21 mg). The water layer was partitioned between *n*-BuOH and H_2O to give the *n*-BuOH soluble part (128 g). Half (64 g) of the *n*-BuOH layer was separated by using silica gel chromatography to yield 12 frs. (1.1–1.12) and **2** (15 g). Fr. 1.8 (673 mg) was chromatographed on Sephadex LH-20 with MeOH to give 4 frs. (1.8.1–1.8.4). Fr. 1.8.2 (82 mg) was purified by HPLC (ODS) with MeOH–H_2O (7:3) to give **3** (33 mg). Fr. 1.9 (1.2 g) was chromatographed on Sephadex LH-20 with MeOH to give 9 frs. (1.9.1–1.9.9). Fr. 1.9.2 (220 mg) was purified by HPLC (ODS) with MeOH–H_2O (7:3) to give **4** (27 mg).

3.4.1. (*S*)-4-(4-Hydroxyphenyl)-2-butanol 2-*O*-sulfate (**3**)

Hygroscopic white amorphous powder, $[\alpha]_D^{25}$ +20.9° (*c* 1.3, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3271, 2951, 1656, 1600, 1518, 1451, 1381, 1220, 1042, 951; UVλ_{max}^{MeOH} nm (logε): 278 (3.2). 223 (3.8); HR-FABMS: *m*/*z* 245.0473 [M-H]$^-$, calcd. for $C_{10}H_{13}O_5S$, 245.0484; ^{1}H NMR spectral data (CD_3OD) δ: 7.02 (2H, *d, J* = 8.3 Hz, H-6, 10), 6.67 (2H, *d, J* = 8.3 Hz, H-7, 9), 4.47 (1H, *m*, H-2), 2.58 (2H, *m*, H-4), 1.89 (1H, *m*, H-3), 1.76 (1H, *m*, H-3), 1.33 (3H, *d, J* = 6.3 Hz, H-1); ^{13}C NMR spectral data: Table 1; Found: S, 7.62. $C_{10}H_{14}O_5S{\cdot}10\ H_2O$ requires: S, 7.52%.

3.4.2. (*S*)-4-(4-Hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-galloyl)-β-D-glucopyranoside (**4**)

Pale orange oil, $[\alpha]_D^{25}$−41.6° (*c* 1.0, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3393, 1702, 1614, 1515, 1448, 1348, 1040; UV λ_{max}^{MeOH} nm (log ε): 277 (4.2), 220 (4.7); HR-FABMS *m*/*z*:503.1455 $[M+Na]^+$, calcd. for $C_{23}H_{28}O_{11}Na$, 503.1529; ^{1}H NMR spectral data (CD_3OD) δ:7.31 (2H, *s*, H-2, 6 of gal), 7.06 (2H, *d, J* = 8.3 Hz, H-6, 10), 6.82 (2H, *d, J* = 8.3 Hz, H-7, 9), 4.67 (1H, *d, J* = 10.6 Hz, H-6 of glc), 4.54 (1H, *dd, J* = 5.8, 10.6 Hz, H-6 of glc), 4.48 (1H, *d, J* = 7.7 Hz, H-1 of glc), 3.70 (1H, *m*, H-2), 3.67–3.45 (4H, *m*, H-2–5 of glc), 2.69 (2H, *m*, H-4), 1.85 (1H, *m*, H-3), 1.67 (1H, *m*, H-3), 1.23 (3H, *d, J* = 6.2 Hz, H-1); ^{13}C NMR spectral data: Table 1.

3.4.3. (*S*)-4-(4-Hydroxyphenyl)-2-butanol 2-*O*-(6-*O*-acetyl)-β-D-glucopyranoside (**5**)

Colorless powder, $[\alpha]_D^{25}$−40.2° (*c* 0.8, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3400, 1722, 1620, 1516, 1376, 1036; UV λ_{max}^{MeOH} nm (log ε): 278 (3.0), 223 (3.6); HR-FABMS *m*/*z*: 369.1557 [M-H]$^-$, calcd. for $C_{18}H_{25}O_8$, 369.1549; ^{1}H NMR spectral data (CD_3OD) δ:7.02 (2H, *d, J* = 8.3 Hz, H-6, 10), 6.67 (2H, *d, J* = 8.3 Hz, H-7, 9), 4.45 (1H, *d, J* = 10.0 Hz, H-6 of glc), 4.30 (1H, *d, J* = 8.2 Hz, H-1 of glc), 4.18 (1H, *dd, J* = 5.8, 10.0 Hz, H-6 of glc), 3.70 (1H, *m*, H-2), 3.50–3.20 (4H, *m*, H-2–5 of glc), 2.65 (2H, *m*, H-4), 2.08 (3H, *s*, −$COOCH_3$), 1.84 (1H, *m*, H-3), 1.72 (1H, *m*, H-3), 1.28 (3H, *d, J* = 6.2 Hz, H-1); ^{13}C NMR spectral data: Table 1.

3.4.4. (*S*)-4-(4-Hydroxyphenyl)-2-butanol 2-*O*-[6-*O*-(3,5-dimethoxy-4-*O*-α-L-rhamnopyranosylgalloyl)-β-D-glucopyranoside] (**6**)

Pale yellow oil, $[\alpha]_D^{25}$−13.8° (*c* 1.3, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3402, 1709, 1596, 1516, 1338, 1132, 1073; UV λ_{max}^{MeOH} nm (log ε): 270 (4.2); HR-FABMS *m*/*z*:653.2427 [M-H]$^-$, calcd. for $C_{31}H_{41}O_{15}$, 653.2445; ^{1}H NMR spectral data (CD_3OD) δ:7.02 (2H, *d, J* = 8.3 Hz, H-6,

10), 6.68 (2H, *d*, $J=8.3$ Hz, H-7, 9), 5.38 (1H, *br s*, H-1 of rham), 4.68 (1H, *d*, $J=10.6$ Hz, H-6 of glc), 4.40 (1H, *dd*, $J=5.8$, 10.6 Hz, H-6 of glc), 4.38 (1H, *d*, $J=7.7$ Hz, H-1 of glc), 4.25 (1H, *m*, H-5 of rham), 4.12 (1H, *br s*, H-2 of rham), 3.95 (1H, *m*, H-3 of rham), 3.89 (6H, *s*, $-OCH_3$), 3.72 (1H, *m*, H-2), 3.56 (1H, *m*, H-4 of rham), 3.46–3.17 (4H, *m*, H-2–5 of glc), 2.65 (2H, *m*, H-4), 1.84 (1H, *m*, H-3), 1.72 (1H, *m*, H-3), 1.19 (6H, *d*, $J=6.2$ Hz, H-1, H-1 of rham); ^{13}C NMR spectral data: Table 1.

3.4.5. Maximol A (**9**)

Brown amorphous powder, $[\alpha]_D^{25}-16.3^\circ$ (*c* 0.75, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3377, 1601, 1488, 1237, 1152, 997; UV λ_{max}^{MeOH} nm (logε): 310 (4.6), 211 (4.8); HR-FABMS *m*/*z*:455.1420 $[M+H]^+$, calcd. for $C_{28}H_{23}O_6$, 455.1485; ^{13}C NMR spectral data (CD_3OD) δ:132.3 (C-1), 128.7 (C-2, 6), 116.3 (C-3, 5), 158.7 (C-4), 94.9 (C-7), 58.7 (C-8), 145.3 (C-9), 107.7 (C-10, 14), 159.9 (C-11, 13), 102.5 (C-12), 132.8 (C-1′), 124.1 (C-2′), 132.4 (C-3′), 161.0 (C-4′), 110.3 (C-5′), 128.7 (C-6′), 129.4 (C-7′), 127.4 (C-8′), 141.1 (C-9′), 105.8 (C-10′, 14′), 159.6 (C-11′, 13′), 102.7 (C-14′); ^1H NMR spectral data: Table 2.

3.4.6. Maximol B (**10**)

Brown amorphous powder, $[\alpha]_D^{25}+99.4^\circ$ (*c* 0.34, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3371, 1631, 1469, 1286, 1144, 1047; UV λ_{max}^{MeOH} nm (logε): 298 (4.5), 205 (4.9); HR-FABMS *m*/*z*:453.1309 $[M-H]^-$, calcd. for $C_{28}H_{21}O_6$, 453.1338; ^{13}C NMR spectral data (CD_3OD) δ:132.8 (C-1), 128.6 (C-2, 6), 116.3 (C-3, 5), 158.7 (C-4), 94.7 (C-7), 58.7 (C-8), 145.1 (C-9), 107.7 (C-10, 14), 159.8 (C-11, 13), 102.5 (C-12), 131.7 (C-1′), 127.3 (C-2′), 131.4 (C-3′), 160.3 (C-4′), 109.8 (C-5′), 130.7 (C-6′), 131.0 (C-7′), 129.7 (C-8′), 140.9 (C-9′), 108.4 (C-10′, 14′), 159.2 (C-11′, 13′), 102.4 (C-14′); ^1H NMR spectral data: Table 2.

3.5. Hydrolysis of compounds **2–6**

A solution of **2** (10 mg) in MeOH (10 ml) was treated with 10% HCl (10 ml), and the mixture was refluxed for 6 h. After cooling, the reaction mixture was diluted with water (30 ml) and extracted with EtOAc (30 ml). The organic layer were combined and concentrated in vacuo. The residue was purified by GPC (MeOH) to give compound **2a** (4 mg), identified by ^1H NMR, ^{13}C NMR, TLC and $[\alpha]_D$ comparison to (+)-rhododendrol (Das et al., 1993). Compounds **3–6** were identified by the same method.

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Canthin-6-one and β-carboline alkaloids from *Eurycoma harmandiana*

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Dedicated to Professor Vichiara Jirawongse on the occasion of his 82nd birthday

Abstract

Two alkaloids, canthin-6-one 9-*O*-β-glucopyranoside and 7-hydroxy-β-carboline 1-propionic acid, were isolated from the roots of *Eurycoma harmandiana*, together with the five known canthin-6-one alkaloids, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, 9,10-dimethoxycanthin-6-one, canthin-6-one and canthin-6-one *N*-oxide, and the two known β-carboline alkaloids, β-carboline 1-propionic acid and 7-methoxy-β-carboline 1-propionic acid. Their structures were based on analyses of spectroscopic data. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Eurycoma harmandiana*; Simaroubaceae; Alkaloid; Canthin-6-one 9-*O*-β-glucopyranoside; 7-hydroxy-β-carboline 1-propionic acid; Canthin-6-one; β-Carboline

1. Introduction

Eurycoma harmandiana Pierre (Simaroubaceae, Thai name: Ian-don) is a small shrub distributed on the border regions between Thailand and Laos. In North-eastern Thai traditional medicine, the root is used as a bitter tonic, as well as an aphrodisiac, anticancer and antimalarial agent. No phytochemical investigation has been carried out on this species. The present study deals with the isolation and structural elucidation of nine alkaloids (**1–9**), consisting of the two new alkaloids (**1**, **8**) together with the known canthin-6-one alkaloids (**2–6**) and the known β-carboline alkaloids (**7**,**9**) from the roots of this plant.

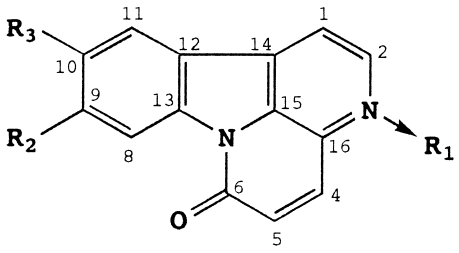
2. Results and discussion

The ethanolic extract of the roots of *E. harmandiana* was suspended in H_2O and extracted with CH_2Cl_2, EtOAc and n-BuOH, successively. The n-BuOH extract was subjected to a column of a highly porous copolymer of styrene and divinylbenzene, and eluted successively with H_2O, MeOH and Me_2CO. The fraction eluted with MeOH, and the CH_2Cl_2 extract were then repeatedly subjected to silica gel column chromatography, and reversed phase siliga gel chromatography to afford nine compounds (**1–9**). Five were identified as the known canthin-6-one alkaloids; 9-hydroxycanthin-6-one (**2**), 9-methoxycanthin-6-one (**3**) (Kardono et al., 1991), 9,10-dimethoxycanthin-6-one (**4**) (Mitsunaga et al., 1994), canthin-6-one (**5**) (Koike and Ohmoto, 1985) and canthin-6-one *N*-oxide (**6**) (Ohmoto et al., 1981) together with the two known β-carboline alkaloids; β-carboline 1-propionic acid (**7**) and 7-methoxy-β-carboline 1-propionic acid (**9**) (Kardono et al., 1991) by analysis of both physical and spectroscopic data.

Compound **1** was obtained as a yellow amorphous powder and determined as $C_{20}H_{18}O_7N_2$ by HR-FAB mass spectrometry. The ^{13}C NMR spectrum revealed the presence of one β-glucopyranosyl unit in addition to 14 carbon signals for the aglycone. Enzymatic hydrolysis of **1** with crude hesperidinase (Kohda and Tanaka, 1975) gave 9-hydroxycanthin-6-one (**2**). The ^1H NMR

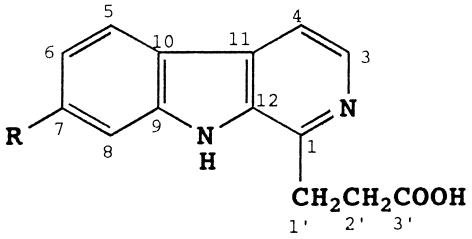
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	R_1	R_2	R_3
1	-	OGlc	H
2	-	OH	H
3	-	OMe	H
4	-	OMe	OMe
5	-	H	H
6	O	H	H

Glc : β-glucopyranosyl



	R
7	H
8	OH
9	OMe

spectrum of **1** showed two pairs of doublets ascribed to *ortho*-coupled signals at δ 8.15 (1H, *d*, $J=5.0$ Hz), δ 8.75 (1H, *d*, $J=5.0$ Hz) and at δ 8.08 (1H, *d*, $J=9.8$ Hz), δ 6.93 (1H, *d*, $J=9.8$ Hz), and the AMX type signals at δ 8.12 (1H, *d*, $J=2.0$ Hz), δ 7.27 (1H, *dd*, $J=8.6$, 2.0 Hz) and δ 8.23 (1H, *d*, $J=8.6$ Hz). These spectral data were similar to those of **2**, except that the ^{1}H NMR signals for H-8 and H-10 were downfield shifted by about 0.16 and 0.30 ppm, respectively, which established the attachment of the glucosyl unit to C-9. Furthermore, the ^{13}C NMR spectrum also revealed the attachment of a glucosyl unit to C-9 due to the downfield shift of C-9 (0.9 ppm) together with upfield shifts of C-8 (1.6 ppm) and C-10 (2.5 ppm). Thus, compound **1** was concluded to be canthin-6-one 9-*O*-β-glucopyranoside.

Compound **8** was obtained as an amorphous powder and determined as $C_{14}H_{12}O_3N_2$ by HR-FAB mass spectrometry. The ^{1}H NMR spectrum revealed a pair of vicinal protons at δ 8.13 (1H, *d*, $J=5.3$ Hz) and δ 7.74 (1H, *d*, $J=5.3$ Hz), and the AMX type signals at δ 7.93 (1H, *d*, $J=8.6$ Hz), δ 6.69 (1H, *dd*, $J=8.6$, 2.0 Hz) and δ 6.90 (1H, *d*, $J=2.0$ Hz) together with two sets of methylene protons [δ 2.82 (2H, *t*, $J=7.3$ Hz) and δ 3.27 (2H, *t*, $J=7.3$ Hz)]. The ^{1}H and ^{13}C NMR spectra were very similar to those of 7-methoxyl-β-carboline 1-propionic acid (**9**) but no methoxy signal was observed, indicating the presence of a hydroxyl group. The hydroxyl group was assigned to C-7 by the irradiation of N–H at δ 11.28 which induced a 5% NOE at H-8 [δ 6.90 (1H, *d*, $J=2.0$ Hz)]. Consequently, 7-hydroxy-β-carboline 1-propionic acid was proposed for compound **2**.

The biological activities of the isolated compounds have not been investigated. However, there are own biological activities for the known compounds **2**, **3**, **5**, and **6** which are cytotoxic (Anderson et al., 1983; Kardono et al., 1991), and compound **7** (Kardono et al., 1991) has antimalarial activity. These activities are in agreement with their traditional uses in North-east Thailand.

3. Experimental

3.1. General

NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ using a JEOL JNM A-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) and JNM-ECP 500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS) as internal standard. MS were recorded on a JEOL JMS-SX 102 spectrometer. For CC, silica gel G 60 (Merck), RP-18 (50 μm, YMC) and highly porous copolymer of styrene and divinylbenzene (Mitsubishi Chem. Ind. Co. Ltd) were used. The solvent systems were: (I) EtOAc–MeOH (9:1), (II) EtOAc–MeOH–H_2O (4:1:0.1), (III) EtOAc–MeOH–H_2O (7:3:0.3), (IV) EtOAc–MeOH–H_2O (6:4:1), (V) 30–50% MeOH, (VI) CH_2Cl_2, (VII) CH_2Cl_2–MeOH (19:1), (VIII) CH_2Cl_2–MeOH (9:1), (IX) CH_2Cl_2–MeOH (4:1) and (X) 40–100% MeOH.

3.2. Plant material

The roots of *E. harmandiana* were collected in May, 1998 from the Nong Khai Province, North-east Thailand. The identity of the plant was confirmed by Professor Vichiara Jirawongse, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher sample is kept in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Table 1
^{1}H NMR spectral data of compounds **1** and **8** (400 MHz, DMSO-d_6)

H	**1**	H	**8**
1	8.15 (1H, *d*, *J* = 5.0 Hz)	3	8.13 (1H, *d*, *J* = 5.3 Hz)
2	8.75 (1H, *d*, *J* = 5.0 Hz)	4	7.74 (1H, *d*, *J* = 5.3 Hz)
4	8.08 (1H, *d*, *J* = 9.8 Hz)	5	7.93 (1H, *d*, *J* = 8.6 Hz)
5	6.93 (1H, *d*, *J* = 9.8 Hz)	6	6.69 (1H, *dd*, *J* = 8.6, 2.0 Hz)
8	8.12 (1H, *d*, *J* = 2.0 Hz)	8	6.90 (1H, *d*, *J* = 2.0 Hz)
10	7.27 (1H, *dd*, *J* = 8.6, 2.0 Hz)	1′	2.82 (2H, *t*, *J* = 7.3 Hz)
11	8.23 (1H, *d*, *J* = 8.6 Hz)	2′	3.27 (2H, *t*, *J* = 7.3 Hz)
Glc-1	5.02 (1H, *d*, *J* = 8.0 Hz)	N–H	11.28 (1H, *bs*)

Table 2
^{13}C NMR spectral data of compounds **1** and **8** (100 MHz, DMSO-d_6)

C	**1**	C	**8**
1	114.3	1	142.9
2	145.9	3	137.4
4	139.9	4	111.8
5	128.2	5	122.5
6	158.9	6	109.7
8	104.5	7	158.3
9	159.7	8	96.6
10	116.5	9	142.3
11	124.4	10	113.8
12	118.2	11	127.8
13	139.9	12	133.9
14	129.3	1′	31.6
15	131.8	2′	27.9
16	135.3	3′	174.2
G-1	101.0		
G-2	73.2		
G-3	77.2		
G-4	69.5		
G-5	76.5		
G-6	60.5		

3.3. Extraction and isolation

The dried root (7.0 kg) of *E. harmandiana* was extracted with 95% EtOH. After removal of the solvent by evaporation, the residue (200 g) was extracted with CH_2Cl_2, EtOAc and n-BuOH, successively. The n-BuOH extract (45.0 g) was subjected to a column of highly porous copolymer of styrene and divinylbenzene, and eluted with H_2O, MeOH and Me_2CO, successively. The fraction eluted with MeOH was chromatographed on a column of silica gel (systems I, II, III and IV, successively) affording nine fractions. Fraction 5 and 6 were further purified by RP-18 using system V to provide compounds **1** (75.0 mg), **7** (35.0 mg), **8** (30.7 mg) and **9** (20.7 mg).

The CH_2Cl_2 extract (90.0 g) was subjected to a column of silica gel (systems VI, VII, VIII and IX) affording six fractions. Fraction 2 was subjected to repeated chromatography on silica gel column using a gradient system (CH_2Cl_2 to 5% MeOH in CH_2Cl_2) followed by RP-18 reversed phase column chromatography to afford compounds **2** (367.0 mg), **3** (900.0 mg), **4** (8.0 mg), **5** (250.4 mg) and **6** (27.5 mg).

3.4. Compound *1*

Yellow amorphous powder. UV $\lambda_{max}^{MeOH+HCl}$ (log ε) nm: 285 (3.16), 321 (3.14), 367 (3.22); ^{1}H NMR: Table 1; ^{13}C NMR: Table 2; Positive HR-FAB-MS, *m*/*z*: 399.1205 $[M+H]^+$ ($C_{20}H_{19}O_7N_2$ requires 399.1192).

3.5. Enzymatic hydrolysis of compound *1*

Compound **1** (25.0 mg) was dissolved in 0.5 ml of DMSO. A solution of crude hesperidinase (Kohda and Tanaka, 1975) (100 mg in 20 ml of H_2O) was added. After stirring at 37°C for 12 h, the mixture was extracted with CH_2Cl_2. The CH_2Cl_2 extract was evaporated to provide 9-hydroxycanthin-6-one (**2**) (14.0 mg) whose structure was identified by ^{1}H and ^{13}C NMR spectra analysis.

3.6. Compound *2*

Amorphous powder. UV $\lambda_{max}^{MeOH+HCl}$ nm (log ε): 300 (2.74), 318 (2.76), 346 (2.80); ^{1}H NMR: Table 1; ^{13}C NMR: Table 2; Positive HR-FAB-MS, *m*/*z*: 257.0891 $[M+H]^+$ ($C_{14}H_{13}O_3N_2$ requires 257.0926).

Acknowledgements

We would like to thank the Research Center for Molecular Medicine, Hiroshima University for the use of its NMR facilities.

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The absolute configuration of (+)-isoshinanolone and in situ LC–CD analysis of its stereoisomers from crude extracts☆

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Dedicated to Professor Johnson Ayafor, Cameroon, who unexpectedly passed away on 10 November 2000

Abstract

The absolute configuration of (+)-isoshinanolone, a wide-spread acetogenic metabolite from higher plants, has been determined by X-ray structure analysis of its new dibromide; accordingly, this natural tetralone is 3*R*,4*R*-configured, in agreement with previous degradative results. In addition, a first chiroptical on-line stereoanalysis for isoshinanolones is presented, i.a. by HPLC on a chiral phase coupled to CD spectroscopy, giving pure CD spectra of all of the four stereoisomers of isoshinanolone directly from stereoisomeric mixtures. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Dioncophyllum thollonii*; Dioncophyllaceae; Structural elucidation; X-ray crystallography; Stereochemistry; Circular dichroism; HPLC–CD coupling; Tetralones; Isoshinanolone

1. Introduction

cis-Isoshinanolone (**1a**) (Tezuka et al., 1973) is a wide-spread tetralone isolated from a series of different plant families, like Iridaceae (Kumar et al., 1985), Plumbaginaceae (Gunaherath et al., 1983; Bhattacharyya and de Carvalho, 1986), Nepenthaceae (Likhitwitayawuid et al., 1998), Dioncophyllaceae (Lavault and Bruneton, 1980; Hanson et al., 1981), Ancistrocladaceae (Bringmann et al., 1991; Bringmann and Pokorny, 1995; Anh et al., 1997), and Ebenaceae (Tezuka et al., 1973; Bin Zakaria et al., 1984; Zhong et al., 1984; Richomme et al., 1991). In cell cultures of *Ancistrocladus heyneanus* (Ancistrocladaceae) (Bringmann et al., 1998a, 1999a) and *Triphyophyllum peltatum* (Dioncophyllaceae) (Bringmann et al., 2000a), we have recently found enhanced formation of both, *cis*- and *trans*-isoshinanolones and similar metabolites, upon stimulation by chemical, physical, or biotic stress. Compound **1a** and related naphthoquinones originate from polyketide precursors (Bringmann et al., 1998a) identical to those from which both molecular halves of the co-occuring naphthylisoquinoline alkaloids (Bringmann and Pokorny 1995; Bringmann et al., 1998b) are formed (Bringmann et al., 2000b).

For a long time, the elucidation of the absolute configuration of **1a** had to rely on a sometimes dubious comparison of its frequently varying optical rotation[1] with that of a sample previously investigated as its dibenzoate, using the Exciton Chirality method (Tezuka et al., 1973). We have recently described a procedure for the oxidative degradation of *cis*- and *trans*-isoshinanolones (**1a** and **1b**) to give 2-methylsuccinic acid, microanalytically analyzed as its (stereochemically known) diester, and have likewise presented methods for the direct distinction between the four stereoisomeric forms of isoshinanolone, by chromatography

☆ Part 146 in the series 'Acetogenic isoquinoline alkaloids'. For part 145, see Bringmann et al. (2001).

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[1] This variability is in part due to the occasional co-occurrence of the difficult-to-separate 4-epimeric *trans*-diastereomer **1b** (occasionally named '*epi*-isoshinanolone') (Zhong et al., 1984). For a comprehensive treatment of reports on the additional occurrence of yet two further isoshinanolone stereoisomers, *ent*-**1a** ('*neo*-isoshinanolone') (Kumar et al., 1985) and *ent*-**1b** (again '*epi*-isoshinanolone') (Bhattacharyya and de Carvalho, 1986), see Bringmann et al. (1999b).

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on a chiral HPLC phase (Bringmann et al., 1999b). Given the wide-spread occurrence of **1** in the plant kingdom, its importance as a stress induced biosynthetic product upon impairment of naphthylisoquinoline formation, and the numerous apparent mistakes made in the attribution of the absolute configuration of this compound, we here report on the ultimate proof of the absolute configuration of (+)-*cis*-isoshinanolone (**1a**) as 3*R*,4*R*, by an X-ray structure analysis of its dibromide **2** using the heavy atom method. Furthermore, we present an on-line CD analysis in combination with a separation on a chiral chromatographic phase that permits us to determine the absolute stereostructures of all four stereoisomeric forms in one run.

By this LC–CD method (Bringmann et al., 1999c), the assignment of the correspondingly predominating enantiomer both for the *cis*- and the *trans*-diastereomers of isoshinanolone (**1a** or **1b** — or their enantiomers) is possible right from crude plant extracts, even by the use of an *achiral* HPLC phase (Fig. 1).

2. Results and discussion

Enantiomerically pure (+)-*cis*-isoshinanolone (**1a**) was obtained by isolation from *Dioncophyllum thollonii* (Dioncophyllaceae) as described earlier (Bringmann et al., 1999b). As a pure compound, it shows no tendency to crystallize — in contrast to the racemate of its *trans*-isomer (**1b**/*ent*-**1b**), which is easily available through 'biomimetic' (but non-enantioselective) reduction of plumbagin (Tezuka et al., 1973). But even that racemic synthetic material did not give crystals suited for an X-ray structure analysis (Bringmann and Messer, unpublished results). In order to improve the crystallization tendency of *cis*-isoshinanolone (**1a**) and, simultaneously, introduce heavy atoms allowing for an absolute configurational assignment, we have now brominated **1a**. Thus, treatment with 2.2 eq. of bromine gave mainly the dibromide **2**, with the two halogen substituents at C-5 and C-7, as evidenced by the fact that the previous three aromatic protons of **1a** with their typical appearance (essentially a '*d-dd-d*' pattern) had become a single one-proton singlet for the remaining H-6 ($\delta = 7.94$), and further proven by heteronuclear 2D techniques, in particular by HMBC interactions of H-6 both to C-8 ($\delta = 159.2$) and C-4a ($\delta = 142.6$).

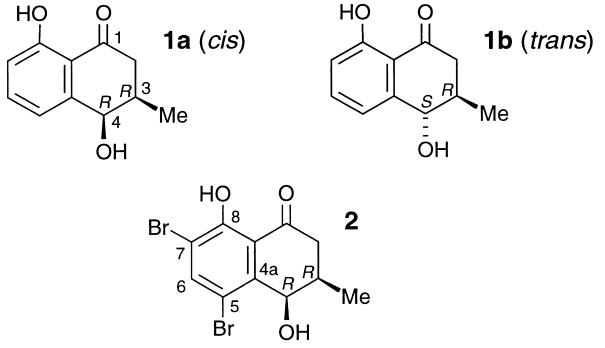


Fig. 1. Absolute stereostructures of natural (+)-*cis*- and (+)-*trans*-isoshinanolone (**1a** and **1b**, respectively) and the novel 5,7-dibromide **2** of **1a**.

Different from **1a**, its dibromide **2** did indeed give crystals, luckily with sufficient quality for an X-ray structure analysis, even for a determination of the absolute configuration. Fig. 2 shows the structure of **2** in the crystal, with the absolute stereostructure as determined by the X-ray diffraction. Of the two possible half-chair conformations of the partially saturated cyclohexene moiety, only the one with the 3-methyl group in an equatorial and the 4-hydroxy function in an axial position is found. The other imaginable conformation, with an axial 3-methyl group and the 4-OH function equatorial, appears to be energetically largely disfavored due to a 1,3-allylic strain between that OH group and the 5-bromo substituent, which thus rigidifies the previously more flexible tetralone framework. This may be one of the reasons for the better crystallization properties of **2** as compared to those of **1**. Besides likewise confirming the bromination sites (C-5 and C-7) and the relative configuration (3,4-*cis*), this first X-ray structure analysis of an isoshinanolone derivative unambiguously establishes the stereostructure of **2** and thus confirms the full absolute configuration of natural (+)-*cis*-isoshinanolone (**1a**) itself, as 3*R*,4*R*. The result is in agreement with previous EC–CD investigations on a dibenzoate derivative (Tezuka et al., 1973) and with our degradative results (Bringmann et al., 1999b).

With the full absolute stereostructure of **1** now being entirely sure, the availability of a stereoanalytic tool for the rapid and unambiguous attribution of newly isolated isoshinanolones or even stereoisomeric mixtures thereof, seemed rewarding in view of the wide-spread occurrence of such tetralones in plants (see above). For this purpose, we have now coupled the chromatographic analysis on a chiral HPLC phase for the separation of the four stereoisomers of **1** elaborated earlier (Bringmann et al., 1999b), with the in situ analysis of the CD spectra of the resulting peaks, by the

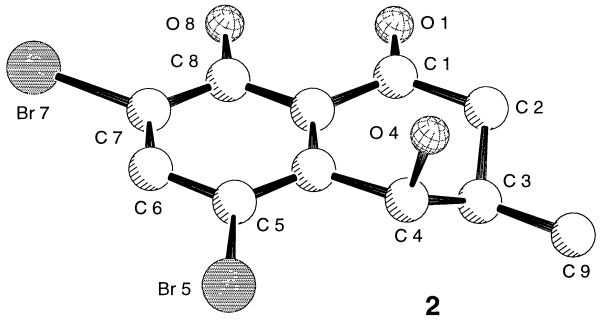


Fig. 2. Structure of 5,7-dibromoisoshinanolone (**2**) in the crystal; hydrogen atoms have been omitted for reasons of clarity.

HPLC–CD technique recently introduced into phytochemical analysis (Bringmann et al., 1999c).

As can be clearly seen (Fig. 3), the CD behavior of **1** is strongly dominated by the stereocenter in the benzylic 4-position next to the aromatic chromophore. Independent of a relative *cis*- or *trans*-configuration and thus regardless of the configuration at C-3, the CD spectra show a positive first Cotton effect at 255 nm and a stronger second negative one at 214 nm for both of the isomers with 4*R*-configuration (**1a** and *ent*-**1b**; Fig. 3, center and right, dotted lines), while the CD curves obtained for the 'enantiomeric' 4*S*-forms (*ent*-**1a** and **1b**) are — as expected — opposite (full lines). The CD influence of the stereocenter at C-3, by contrast, is only very small, manifested by a very weak positive Cotton effect around 333 nm for the 3*R*-configuration, negative for 3*S*. The effect is, however, not strong enough to establish an attribution of the configuration at C-3.

Since, however, an in situ determination of the relative configuration is easily possible, e.g. by analysis of the corresponding NMR coupling constants (*cis*: 2.5 Hz, *trans*: 8.0 Hz), as part of our LC–NMR methodology (Bringmann et al., 1999d), the absolute configuration at both, C-3 and C-4, can now be assigned by simple comparison of the on-line CD and NMR spectra with those of the peaks of the pure isomers. This complements the already known (Bringmann et al., 1999b) attribution by the chromatographic behavior on a chiral HPLC phase.

And since the two *cis*- and *trans*-diastereomers of isoshinanolone (**1**) can likewise be separated on a usual RP_{18} HPLC phase, and with the CD spectra of all of the four pure isomers known, it has now become possible to determine the major enantiomers out of mixtures of *cis*- and *trans*-isoshinanolone by HPLC even on such an *achiral* phase, using the LC–CD coupling technique presented here, also in crude plant extracts.

As exemplified for an extract of *D. thollonii* in Fig. 4, the minor *trans*-diastereomer of isoshinanolone eluting at 11.4 min shows a positive CD effect at 214 nm. This clearly indicates that the *trans*-isoshinanolone of this plant is — at least mainly — 4*S*-configured and thus has structure **1b**. Its major *cis*-diastereomer ($R_t = 11.6$ min) consists of 3*R*,4*R*-configured *cis*-isoshinanolone (i.e. **1a**) predominantly, as obvious from the negative CD effect at 214 nm.

The presented HPLC–CD coupling technique, combined with the HPLC–NMR coupling method described earlier (Bringmann et al., 1999d), permits a fast and easy detection and full stereochemical assignment of isoshinanolones (**1**), directly in crude plant extracts, even without the necessity of a resolution on a chiral HPLC phase.

3. Experimental

3.1. General

Mps: uncorr. NMR spectra were recorded in $CDCl_3$ with the solvent as int. standard ($CDCl_3$, δ 7.26 and δ 77.01, resp.). EIMS: 70 eV. CC: silica gel (60–200 mesh, Merck). Isoshinanolone (**1**) was available from previous isolation work (Bringmann et al., 1999b).

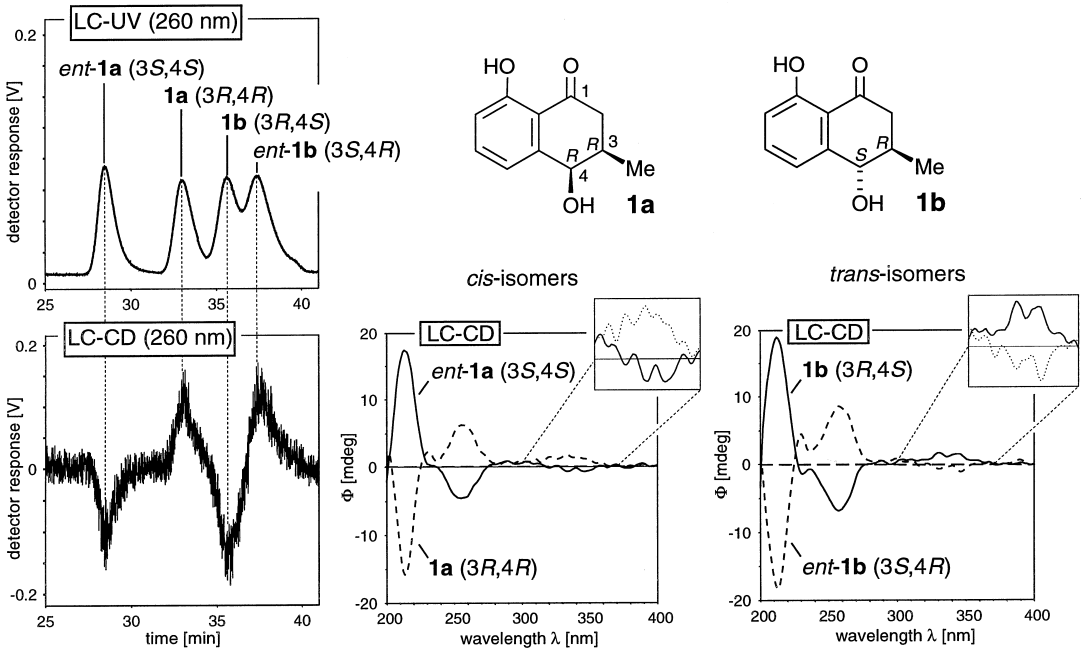


Fig. 3. LC–UV and LC–CD chromatograms of the four stereoisomers of **1** at 260 nm on a chiral phase (left). Stop-flow LC–CD spectra of the pure isomers (center: *cis*-isomers **1a** and *ent*-**1a**; right: *trans*-isomers **1b** and *ent*-**1b**).

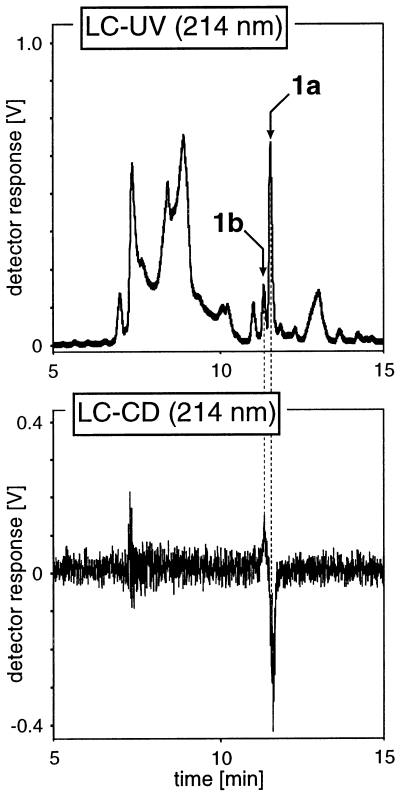


Fig. 4. LC–UV and LC–CD chromatograms of a plant extract of *Dioncophyllum thollonii* at 214 nm on an achiral phase: *trans*-isoshinanolone (R_t = 11.4 min), *cis*-isoshinanolone (R_t = 11.6 min).

3.2. Plant material

Plant material of *D. thollonii* was present from a previous collection by Dr A.M. Louis in Rabi Kounga (Gabon) in October 1996. A voucher specimen (No. 1) has been deposited at Herb. Bringmann, University of Würzburg.

3.3. Partial synthesis of 5,7-dibromo-cis-isoshinanolone (2)

To a stirred soln. of 15.0 mg (78.0 μmol) of *cis*-isoshinanolone (**1a**) in 5 ml of CH_2Cl_2 at 0°C under argon a solution of 9.30 μl (172 μmol) of bromine in 2 ml of CH_2Cl_2 was added dropwise over a 2 min period. After 1 h, the soln. was poured into 10 ml of H_2O, and the aq. layer was washed two times with CH_2Cl_2 (10 ml). The combined org. layers were washed consecutively with 5% Na_2CO_3 (10 ml), and brine (10 ml), then dried ($MgSO_4$) and the solvent was evpd. in vacuo. The residue was passed through a silica gel column using CH_2Cl_2–MeOH (50:1) yielding 5,7-dibromo-*cis*-isoshinanolone (**2**) (38.8 mg, 111 μmol, 78%) as yellow needles (from CH_2Cl_2). Mp 144–145°C. $[\alpha]_D^{25}$ + 13.2° ($CHCl_3$, *c* 0.2). CD: $\Delta\varepsilon_{227}$ −2.7, $\Delta\varepsilon_{263}$ + 0.6 (EtOH; *c* 0.011). UV λ_{max}^{EtOH} nm: 224, 353. IR ν_{max}^{KBr} cm^{-1}: 3546 (O−H), 2962 (C=C), 2363, 1628 (C=O), 1418, 1324, 1213, (C−O), 1184, 1161, 1015, 980, 887, 840, 796, 690. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.19 (3H, *d*, *J* = 6.7 Hz, CH_3), 2.34 (1H, m_c, H-3), 2.51 (1H, *dd*, *J* = 17.4, 3.6 Hz, H_{eq}-2), 2.97 (1H, *dd*, *J* = 17.4, 13.4 Hz, H_{ax}-2), 4.96 (1H, *d*, *J* = 1.8 Hz, H-4), 7.94 (1H, *s*, H-6), 8.40 (1H, *s*, OH-8). ^{13}C NMR (50 MHz, $CDCl_3$): δ 17.3 (CH_3), 33.6 (C-3), 39.4 (C-2), 70.0 (C-4), 112.4 (C-8a), 112.7 (C-5), 116.8 (C-7), 142.5 (C-6), 142.6 (C-4a), 159.2 (C-8), 205.0 (C-1). The ^{13}C assignments were achieved by HMQC and HMBC experiments. EIMS *m*/*z* (rel. int.): 352/350/348 $[M]^+$ (45/100/49), 334/332/330 $[M-H_2O]^+$ (12/22/11), 310/308/306 $[M-C_2H_2O]^+$ (26/49/28), 281/279/277 $[M-C_2H_2O-CHO]^+$ (35/74/39), 253/251 $[M-Br]^+$ (41/40). HRMS: $C_{11}H_{10}Br_2O_3$. Found 347.899, calcd. 347.899.

3.4. X-Ray structure analysis of compound 2

Crystals suited for an X-ray structure analysis were obtained from CH_2Cl_2. The X-ray data were obtained on a Bruker AXS P4 diffractometer using graphite-monochromated Mo K_α radiation (λ = 0.71073 Å) in ω-scan mode in the range of 1.75° < θ < 27.5°. Crystal dimensions, 0.25×0.55×0.02 mm, $C_{11}H_{10}Br_2O_3$, M_r = 350.01, orthorhombic system, space group $P2_12_12_1$, with *a* = 8.253(1), *b* = 16.227(1), *c* = 17.319(1) Å, *V* = 2319.5(2) $Å^3$, *Z* = 8, D_c = 2.005 g cm^{-3}, μ(MoK_α) = 69.8 cm^{-1}, and *F*(000) = 1360. Unit cell parameters were determined by least-squares refinement using 60 centered reflections within 7.3° < θ < 17.4°. A total of 7047 reflections were collected in ω-scan mode to $2\theta_{max}$ = 55° (*h*: −10→1, *k*: −1→21, *l*: −22→22), of which 5294 were unique. The structure was solved by direct phase determination and refined by full-matrix anisotropic least-squares with the aid of the program Shelxtl-Plus (Siemens). In refinements, weights were used according to the scheme $w = 1/[\sigma^2(F_o)]$. The final refinement converged to *R* = 0.071 and R_w = 0.061, for 290 parameters and 3879 observed reflections with $F > 3\sigma(F)$, ratio data-to-parameter were being 13.38. The electron density of the largest difference peak was found to be 1.896 $eÅ^{-3}$, while that of the largest difference hole was 1.892 $eÅ^{-3}$. All non-hydrogen atoms were refined anisotropically. The hydrogen positions were calculated and refined using a riding model and were considered with isotropic thermal parameters.

Atomic coordinates, bond lengths and angles, and thermal parameters may be obtained from the Cambridge Crystallographic Data Centre on quoting the depository number CCDC 141840.

3.5. HPLC separation and detection of the four stereoisomeric forms of 1

The chiral analytical separation was carried out on a Chiralcel OD-H column (250×4.6 mm, 5 μm, Daicel

Chemical Ltd.) at room temp., elution with *n*-hexane–*iso*-PrOH (98:2), 1 ml min^{-1}. Achiral analytical separation was performed on a Waters RP_{18} Symmetry column (250×4.6 mm, 4 μm) at room temp., 0.8 ml min^{-1}, gradient mode (MeCN–H_2O, 20:80, 7 min 50:50, 15 min 80:20). Simultaneous UV and CD detection was performed on a J-715 Spectropolarimeter (Jasco GmbH Deutschland) with standard flow cell at 214 or 260 nm. CD spectra were measured in stop-flow mode.

Acknowledgements

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Saponins from the roots of *Zygophyllum gaetulum* and their effects on electrically-stimulated guinea-pig ileum

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Abstract

A new ursanolide, 3β-*O*-α-L-rhamnopyranosyl-(1- > 2)-*O*-α-L-arabinopyranosyl-(1- > 2)-β-D-glucopyranosyl)oxy]-ursan-20β,28-olide, zygophyloside N (**1**), and three known quinovic acid glycosides were isolated from the methanolic extract of the roots of *Zygophyllum gaetulum*. The crude extract, some fractions and 3-*O*-β-D-glucopyranosyl quinovic acid 28-β-D-glucopyranosyl ester, significantly and dose-dependently, are able to reduce the electrically-induced contractions of isolated guinea-pig ileum. The structure of compound (**1**) was determined, using 1D and 2D NMR techniques. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Zygophyllum gaetulum*; Zygophyllaceae; Roots; Triterpene glycosides; Zygophyloside N; Electrically-stimulated guinea-pig ileum

1. Introduction

The aerial parts of *Zygophyllum gaetulum* Emb. & Maire (Zygophyllaceae) have many traditional uses in the Moroccan indigenous system of medicine, including antispasmodic, antieczema, and the treatments of diabetes and stomach and liver pain (Bellakhadar et al., 1991). Three new bisdesmosidic triterpene saponins, zygophylosides I, L and M, possessing 3β-hydroxy-urs-20(21)-en-28-oic acid or 3β-hydroxy-27-nor-oleanolic-acid as aglycons and three known quinovic acid glycosides, two of them having an $-SO_3H$ residue on the sugar moieties, were isolated from the aerial parts (Safir et al., 1998). Ursane glycosides with an $-SO_3H$ group on the sugar moiety seem to be characteristic metabolites of the genus *Zygophyllum* (Ahmad et al., 1990, 1993; Hassanean et al., 1993; Pollmann et al., 1997). Extracts of the aerial parts, some partially purified fractions as well as zygophyloside M and 3-*O*-β-D-glucopyranosyl quinovic acid 28-β-D-glucopyranosyl ester were reported to reduce both electrically-stimulated contractions and morphine withdrawal in isolated guine-pig ileum (Capasso et al., 1998).

Also the roots are used as an antispasmodic remedy as an alternative to the aerial parts in Moroccan folk medicine. In this paper we report both on the saponin content of the roots of *Z. gaetulum* and on the antispasmodic activity of the extracts of *Z. gaetulum* tested by using electrically-induced contractions of guinea-pig ileum (E.C.I.). Subsequent bioassay-direct fractionation of the active fractions has led to the isolation of the new saponin zygophyloside N (**1**) together with three known quinovic acid glycosides, also present in the aerial parts.

2. Results and discussion

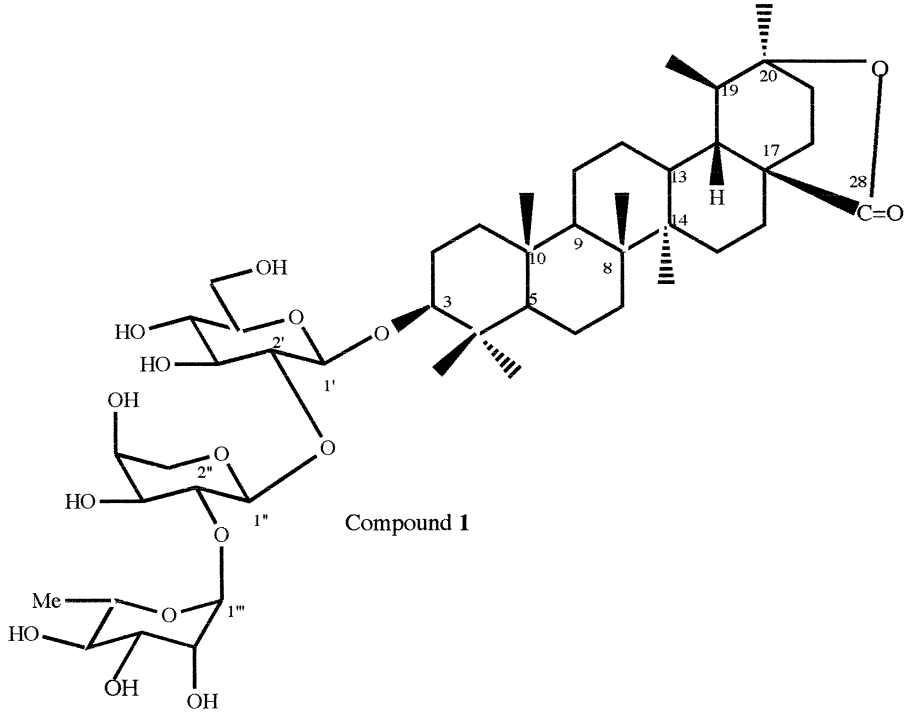
The roots of *Z. gaetulum* were successively extracted with petroleum ether, chloroform and methanol and the resulting extracts tested for antispasmodic activity using E.C.I. Fig. 2 shows that the MeOH extract (MR) of *Z. gaetulum*, at the used concentrations, reduced dose-dependently the E.C.I. while the antispasmodic effects of the petroleum and chloroform extract were not significant (data not shown). The IC_{50} value for MR extract was 150 μg (C.L. = 120–152) almost superimposable to that of the MeOH extract of the aerial parts [IC_{50} 145 μg (C.L. 112-179)] (Capasso et al., 1998). The MR extract was subjected to preliminary purification

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by gel-filtration on Sephadex LH-20 column to yield five main fractions, which were tested under the same experimental conditions. Only fractions C and D were able to reduce significantly the E.C.I. [IC_{50} was 75 μg (C.L. = 69-82) for C, 67 μg (C.L. = 60-77) for D, Fig. 2] and showed the same relative order of potency. In comparison to the whole extract, these fractions were very potent, suggesting that they can contain a higher concentration of the active principles responsible for a marked inhibitory activity on E.C.I. The inhibition appears 2–4 min after the extract administration, it is enhanced with time and lasts for the whole recording period (15 min). In order to identify the molecules responsible for the activity, fractions C and D were chromatographed by reversed-phase HPLC to give compounds **1**–**4** (Fig. 1). Compound **1** was a new saponin, the remaining saponins were identified as 3-*O*-(2-*O*-sulfo-β-D-glucopyranosyl) quinovic acid 28-β-D-glucopyranosyl ester (Zygophyloside G) (**2**), 3-*O*-(2-*O*-sulfo-β-D-quinovopyranosyl) quinovic acid 28-β-D-glucopyranosyl ester (Zygophyloside E) (3) and 3-*O*-β-D-glucopyranosyl quinovic acid 28-β-D-glucopyranosyl ester (4), previously isolated from *Z. propinquum* and from the aerial parts of *Z. gaetulum* (Ahmad et al., 1993; Pollmann et al., 1997). Fig. 2 shows that only compound **4** exerts appreciable inhibiting activity on E.C.I. In fact, whereas saponins **1**, **2** and **3** (data not shown) are inactive at all the tested concentractions (1×10^{-4}–5×10^{-5}–1×10^{-5} M), glycoside **4** shows a good activity. Also in this case the inhibition appeared 2–4 min after the administration, it enhanced with time and lasted for all the recording period (15 min). Although decoctions both of the aerial parts and of the roots of *Z. gaetulum* are used in Moroccan folk medicine as an antispasmodic remedy (Bellakhadar et al., 1991), there are no data in the literature on the pharmacological effects of the roots. The results of the present study indicate that both the MeOH extract and the partially purified fractions C and D are able to reduce dose-dependently the electrically-stimulated contractions of guinea-pig ileum. Glycoside **4** seems to be one of the active sources present in the extract. These data can validate the traditional uses of the roots of *Z. gaetulum* as an antispasmodic remedy as an alternative to the aerial parts and confirm that the antispasmodic activity could be ascribed to the triterpene saponin content. The active compound (**4**), isolated both from the aerial parts and from the roots of *Z. gaetulum* is a triterpenic glycoside of the ursane series. In a previous paper (Capasso et al., 1998) we suggested that, between the bisdesmosidic triterpene glycosides isolated from the aerial parts of *Z. gaetulum*, the active compounds inhibiting E.C.I. were those possessing only a sugar unit (like compound **4**) at C-3 of the triterpenic aglycon. Also the absence of an $-SO_3H$ group on the sugar unit seems to be essential for the antispasmodic activity. Compound **1**, possessing three sugar residue at C-3, was inactive in our experiments confirming the above hypothesis.

The structural elucidation of compound **1** proceeded as follows. Zygophyloside N (**1**) ($C_{47}H_{76}O_{16}$ by MS, DEPT and ^{13}C NMR analyses) gave a quasi-molecular ion peak at m/z 895 $[M\text{-}H]^-$ and prominent peaks due



Compound **1**

Fig. 1. Compound **1** isolated from *Zygophyllum gaetulum*.

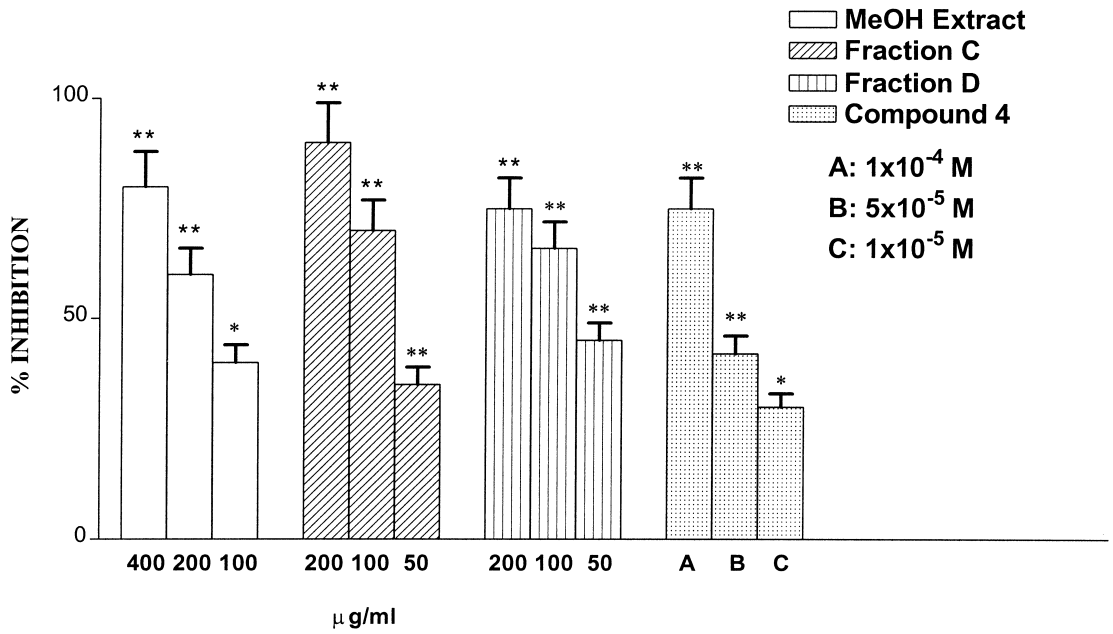


Fig. 2. The effects of MeOH extract (400, 200 and 100 μg/ml), partially purified fractions C and D (200, 100 and 50 μ/g/ml), and pure compound **4** (10^{-4}, 5×10^{-5}, and 1×10^{-5} M) from the roots of *Z.gaetulum* on electrically stimulated guinea-pig ileum. Results are expressed as mean ± S.E.M. $^{*}P<0.05$, ($n=6$).

to the loss of a deoxyhexose and a pentose in its negative FABMS. The IR spectrum showed absorption bands at 3600 (hydroxyl) and 1740 (δ-lactone) cm^{-1}. The NMR spectral data of saponin **1** (Tables 1 and 2) revealed the feature of a saturated triterpenic acid containing an-OR (δ_C 85.9) and a secondary hydroxyl group at C-3β (δ_C 90.4, δ_H 3.18, *dd*, $J=11.1$ and 4.5 Hz, H-3ax) linked to the sugar moiety.

The ^1H NMR spectrum of **1** displayed signals for five tertiary Me (δ 0.84, 0.87, 0.95, 0.97, 1.09), a downfield tertiary Me (δ 1.32) and one secondary Me (δ 1.05, d, $J=6.5$ Hz) groups in the aglycon moiety indicating a pentacyclic triterpene skeleton of the ursane or of the taraxastane series (Yahara et al., 1997; Khan et al., 1998). Full assignments of the proton and carbon signals of the aglycon part of **1** were obtained by ^1H–^1H DQF–COSY and HSQC spectra. A combination of COSY and HSQC experiments showed the following connectivities C-3–C-2–C-1 for ring A, C-5–C-6–C-7 for ring B, C-16–C-15 for ring D, and C-13–C-18–C-19–Me-29 for rings D/E, starting from the well resolved signals at δ 3.18 (H-3), 0.75 (H-5), 2.02 (H-16 eq) and 1.05 (Me-29). These data indicated that **1** had a 3-β-hydroxy-ursane skeleton carrying a −COOR (δ 176.6) group at C-28, an −OR group linked at C-19 or C-20 and a glicosyl moiety at C-3 (Khan et al., 1998; Yahara et al., 1997; Begum et al., 1997). The location of the-OR on ring E at C-20 was deduced by: the low-field shift of Me signal at δ_H 1.32 (*s*, Me-30) indicating that it was due to a Me on a carbon bearing an oxygen; the resonance of Me-29 at δ 1.05 (*d*, $J=6.5$ Hz) shifted to downfield (ca. +0.09 ppm), with respect to Me-29 signals in ursolic acid derivatives (Safir et al., 1998), by the presence of the −OR at C-20; the multiplicity of H-18 signal resonating as a broad multiplet at δ 1.16; all of the ^{13}C NMR resonances of ring E. The above mentioned properties indicated that −OR was at C-20 position and in the lactone form with the −COOR (δ 176.6) at C-28.

This interpretation was unambiguously confirmed by the HMBC (8 Hz) spectrum of **1** which showed significant cross-peaks, due to $^2J_{C-H}$ and $^3J_{C-H}$ correlations, between Me-29 (δ 1.05) and C-20, C-19 and C-18, between Me-30 (δ 1.32) and C-19, C-20 and C-21. Other diagnostic correlations between H-3 (δ 3.18) and Me-23, Me-24 and C-1′ of a sugar unit led us to confirm the proposed structure.

The stereochemistry at C-17,18 (*cis*-linkage of D/E rings) was derived by the ROESY spectrum which showed key correlations peaks between H-19α (δ 1.55) and Me-27α (δ 0.97) and Me-30α (δ 1.32) signals, between Me-29β (δ 1.05) and H-18β (δ 1.16) and H-16 eq (δ 2.02) signals. Other diagnostic ROEs were recorded between H-5α and H-3α and between H-1′ of the first sugar unit and both H-2′ of the glucose and H-3 of the aglycon. Therefore the structure of the aglycon of saponin **1** is 3β-hydroxyursan-20β,28-olide. This is the first report of a naturally occurring saturated pentacyclic triterpene of the ursane series with a C-20,28 lactone. However similar compounds of the taraxastane series have been previously described (Das and Mahato, 1982).

The sugar residue was determined to be 3-*O*-α-L-rhamnopyranosyl-(1->2)-α-L-arabinopyranosyl-(1->2)-

Table 1
^{13}C and ^{1}H NMR data and ^{13}C–^{1}H long-range correlations of aglycon of compound **1** (600 MHz)[a]

Position	δ_C	Dept	δ_H	HMBC connectivities
1	40.0	CH_2	0.98 *m*, 1.69 *m*	
2	26.0	CH_2	1.01 *m*, 1.71 *m*	
3	90.4	CH	3.18 *dd* (11.1, 4.5)	C-23, C-24, Glc-1′
4	40.2	C	–	
5	57.1	CH	0.75 *m*	C-23, C-24, C-25
6	18.7	CH_2	1.35 *m*, 1.53 *m*	
7	34.9	CH_2	1.41 *m*	
8	42.0	C		
9	51.8	CH	1.38 *m*	
10	37.8	C	–	
11	23.9	CH_2	1.91 *m*	
12	30.5	CH_2	0.93 *m*, 1.31 *m*	
13	44.4	CH	1.18 dt (12.0, 3.4)	
14	41.6	C	–	
15	28.3	CH_2	1.03 *m*, 1.14 *m*	
16	32.0	CH_2	1.85 *ddd* (13.0, 13.0, 4.5) 2.02 *ddd* (13.0, 4.5, 3.1)	
17	49.9	C	–	
18	49.2	CH	1.16 *br m*	
19	43.3	CH	1.55 *dd* (11.5, 6.5)	
20	85.9	C	–	
21	32.9	CH	1.57 *m*, 1.65 *m*	
22	39.8	CH_2	1.67 *m*	
23	16.6	CH_3	0.84 *s*	C-3, C-4, C-5, C-24
24	28.2	CH_3	1.09 *s*	C-3, C-4, C-5, C-23
25	16.5	CH_3	0.95 *s*	C-1, C-5, C-9, C-10
26	16.6	CH_3	0.87 *s*	C-7, C-9, C-14
27	15.8	CH_3	0.97 *s*	C-13, C-14, C-15
28	176.6	C	–	
29	18.6	CH_3	1.05 *d* (6.5)	C-18, C-19, C-20
30	24.0	CH_3	1.32 *s*	C-19, C-20, C-21

[a] Chemical shifts values (in CD_3OD) are in ppm and *J* in Hz are presented in parentheses. All signals were assigned by ^{1}H–^{1}H DQF-COSY, ^{1}H–^{13}C HSQC and HMBC.

Table 2
^{13}C and ^{1}HNMR data[a] of sugar moiety of compound **1** in CD_3OD

Position[c]	δ_C	δ_H (J_{HH} in Hz)[b]
Glc-1′	105.5	4.43 *d* (7.8)
Glc-2′	79.5	3.40 *dd* (9.0, 7.8)
Glc-3′	76.4	3.55 *t* (9.0)
Glc-4′	73.4	3.47 *t* (9.0)
Glc-5′	77.6	3.56 *ddd* (9.0, 4.5, 3.0)
Glc-6′	63.0	3.72 *dd* (12.0, 4.5) 3.84 *dd* (12.0, 3.0)
Ara-1″	101.3	5.01 *d* (5.2)
Ara-2″	75.7	3.88 *dd* (5.2, 8.5)
Ara-3″	72.3	3.80 *dd* (8.5, 3.0)
Ara-4″	67.4	3.78 *m*
Ara-5″	63.2	3.43 *dd* (12.0, 3.0) 3.93 *dd* (12.0, 2.0)
Rha-1‴	102.2	5.03 *d* (1.5)
Rha-2‴	72.1	3.84 *dd* (1.5, 2.0)
Rha-3‴	72.0	3.67 *dd* (2.0, 8.5)
Rha-4‴	73.6	3.37 *t* (8.5)
Rha-5‴	69.9	3.84 *dd* (8.5, 6.6)
Rha-6‴	17.8	1.29 *d* (6.6)

[a] Assignments confirmed by 1D TOCSY and 2D COSY, HSQC, HMBC experiments.
[b] ^{1}H–^{1}H coupling constants in the sugar spin–spin were measured from TOCSY and COSY spectra in Hz.
[c] Glc = β-D-glucopyranosyl, Ara = α-L-arabinopyranosyl, Rha = α-L-rhamnopyranosyl.

β-D-glucopyranoside linked to C-3 of the aglycone by use of monodimensional TOCSY (Davis and Bax, 1985), 2D DQF-COSY and HSQC experiments. Three anomeric signals at δ_H 4.43, 5.01, and 5.03 were easily identified in the ^{1}H spectrum of **1**. They correlated to carbons at δ_C 105.5, 101.3, 102.2, respectively in the HSQC spectrum. Starting from these well-resolved anomeric proton signals, the other proton sugar signals could be assigned by 1D TOCSY and 2D DQF-COSY spectra. In the 1D TOCSY spectrum of **1**, H-1 signal at δ 4.43 showed connectivities to four methines and a methylene; the COSY spectrum established the proton sequence within this monosaccharide as H-1 (δ 4.43), H-2 (δ 3.40), H-3 (δ 3.55), H-4 (δ 3.47), H-5 (δ 3.56), H_2-6 (δ 3.72 and 3.84). Similar observations on the other sugar residue and analysis of the ^{13}C data led to the identification of a rhamnopyranose and an arabinopyranose units (Safir et al., 1998). The absence of any glycosylation shift for the carbon resonances of the rhamnopyranose suggested this sugar to be the terminal unit, while glycosylation shifts were observed for C-2 (ca. 6 ppm) of the arabinopyranose and glucopyranose units. The ^{1}H NMR as well as ^{13}C NMR data of key carbons (C-2, C-3 and C-5), indicated a β-configuration at the anomeric positions of the D-glucopyranosyl unit ($J_{H1-H2} = 7.5$ Hz) and an α-configuration for the L-rhamnopyranose ($J_{H1-H2} = 1.5$ Hz). L-Arabinose in the pyranose form was evident from ^{13}C NMR data. However, the value of its J_{H1-H2} coupling constant (5.2 Hz), midway between that reported for methyl-α-arabinopyranoside (4 Hz) and methyl-β-arabinopyranoside (8 Hz), was not diagnostic owing the high conformational mobility of arabinopyranosides ($^{4}C_1 <-> {}^{1}C_4$). As we also reported in our previous work (Safir et al., 1998), evidence of α-L-arabinopyranoside was obtained from ROESY spectrum which showed nuclear Overhauser effects from C-1″ to C-2″, C-3″, and C-5″ indicative of α-L-arabinopyranoside in rapid conformational exchange from $^{4}C_1 <-> {}^{1}C_4$ conformation.

Key correlation peaks in the HMBC spectrum between H-1 of the glucose unit at δ 4.43 and C-3 (δ_C 90.4) of the aglycone and between H-2″ of arabinose (δ_H 3.88) and C-1‴ of the terminal rhamnose unit (δ_C 102.2) allowed the sequence of the trisaccharide chain at C-3 to be determined as shown in Fig. 1. Thus **1** is 3β-[(*O*-α-L-rhamnopyranosyl-(1->2)-*O*-α–arabinopyranosyl-(1->2)-β-D-glucopyranosyl)oxy]-ursan-20β,28-olide.

The structural correlation between zygophyloside N (**1**), the major compound present in the roots of *Z. gaetulum* and zygophyloside I 3β-[*O*-α-L-rhamnopyranosyl-(1->2)-

O-α-L-arabinopyranosyl-(1- > 2)-β-D-glucopyranosyl)-oxy]-urs-20(21)-en-28-oic acid 28-(2-*O*-sulfo-β-D-glucopyranosyl) ester, the major compound present in the aerial part of the plant (Safir et al., 1998), suggests their possible interrelationship. It may be reasonably be presumed that Zygophyloside I undergoes spontaneous lactonization by hydrolysis of sugar residue at C-28 followed either by direct attack of the −COOH group (C-28) on the double bond at C-20 or by the initial hydration of the double bond forming a tertiary alcohol.

3. Experimental

3.1. General experimental procedures

A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^{1}H and 150.86 for ^{13}C using the UXNMR software package was used for NMR measurements in CD_3OD solutions. 2D experiments: ^{1}H–^{1}H DQF-COSY, inverse detected ^{1}H–^{13}C HSQC and HMBC, and ROESY were obtained by employing the conventional pulse sequences as described previously (Safir et al., 1998). The 1D TOCSY (selective excitation spectra) were acquired using waveform generator-based GAUSS shaped pulses, with mixing time ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm in 1% w/v solutions in MeOH. FABMS were recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (XE atoms of energy of 2–6 kV). HPLC separation were performed with a Waters model 6000A pump equipped with a U6K injector and a Model 401 refractive index detector.

3.2. Plant material

Zygophyllum gaetulum Emb. Maire was collected in September 1992 near Zreouila at 27 km from Goulmine, South of Morocco. The plant was identified by Dr. A. Khaoudji of the Department de Biologie, Faculté des Sciences, Rabat, Maroc. A voucher specimen (ZGR1) of the plant is deposited at the herbarium of Laboratoire de Chimie des Plantes et de Synthèse Organique et Bioorganique, Faculté des Sciences, Rabat, Maroc.

3.3. Extraction and isolation

Air dried roots of *Z. gaetulum* (500 g) were sequentially extracted at room temp. for 24 h with petroleum ether, $CHCl_3$, and MeOH to afford 3.95, 4.06 and 10.03 g of dried residue, respectively. Part of the MeOH residue (4 g) was chromatographed on a Sephadex LH-20 column (100 x 5 cm) eluting with MeOH. Fractions of 8 ml were collected and combined by TLC similarity [silica gel plates, *n*-BuOH–AcOH–H_2O (60:15:25) and $CHCl_3$–MeOH– H_2O (80:18:2)] into five main fractions A–E. Fractions C and D containing saponin mixtures (211 mg) were further chromatographed by RP-HPLC (μ-Bondapak C_{18} column, 30 cm × 7.8 mm, flow rate 1.5 ml/min) with MeOH–H_2O (1:1) as solvent system. Fraction C, yielded compounds (**1**) ($t_R = 5$ min, 15.8 mg) and 4 ($t_R = 25$ min, 7 mg); fr. C provided zygophyloside G (**2**) ($t_R = 18$ min, 10 mg), zygophyloside E (**3**) ($t_R = 21$ min, 8 mg) and compound **4**, 3-*O*-β-D-glucopyranosyl quinovic acid 28-β-D-glucopyranosyl ester ($t_R = 25$ min, 9 mg). The known saponins were identified by comparison of their NMR spectra with literature data (Ahmad et al., 1993; Pollmann et al. 1997; Safir et al. 1998).

3.3.1. Compound *1*

$[\alpha]_D^{25} + 148.5°$ (MeOH; *c* 0.5); IR (MeOH) ν_{max} 3600 (OH), 1740 (δ-lactone C=O) cm^{-1}; negative FABMS *m/z*: 895 $[M\text{-}H]^-$, 749 $[(M\text{-}H)\text{-}146]^-$, 617 $[(M\text{-}H)\text{-}(146+132)]^-$; NMR data for the aglycone moiety are reported in Table 1, for the sugar moiety in Table 2.

3.4. Pharmacology screening

3.4.1. Animals

Male Charles River guinea-pigs (180–200 g) were used for all the experiments. The animals were housed in a colony cage (4 guinea-pigs each) under conditions of standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (22±1°C) and room humidity (60±10%) conditions for at least 1 week before the experimental sessions. Food and water were available ad libitum.

3.4.2. Transmurally stimulated guinea-pig ileum test

The animals were sacrified by CO_2 and bled. Guinea-pig ileum was prepared as described previously (Capasso et al., 1998). Pieces of ileum, 2–3 cm long were set up in 10 ml organ bath containing Tyrode solution with 5% CO_2 in 95% oxygen: the solution was maintained at 37°C. Whole ileum preparation was placed between platinum electrodes and connected to a 85/2/50 M.A.R.B. Stimulator (DITTA M.A.R.B. Chiesina Uzzanese, Pistoia, Italy). An isotonic transducer and unirecord model poligraph was used for measurements of isotonic contractions (Ugo Basile, Italy). A resting tension of 0.5 g was applied. After a 30 min equilibration period, the preparation was stimulated for 5 ms pulse delivered transmurally at a frequency of 10 s at supramaximal voltage (25 V). In these conditions, the preparation showed a contraction mean of 60±57 cm. The inhibition of ileal contractions by drugs was expressed as percentage of basal value (mean±S.E.M.).

3.4.3. Experimental procedure

The MeOH extract was dissolved in dimethylsulfoxide (DMSO, Merck) whereas both the partially purified

fractions (A–E) and pure compounds (**1–4**) were dissolved in distilled water. In preliminary experiments, the administration of DMSO up to 100 μl was added to the organ bath to determine the baseline contraction. After these experiments, the effects of the extracts, partially purified fractions, and pure compounds on the electrically-induced contractions of guinea-pig ileum (E.C.I.) were investigated according to the following experimental schedule:

(a) MeOH extract at concentrations of 400–200–100 μg/ml organ bath: 15 min contact period;

(b) Partially purified fractions A-E at concentrations of 200–100–50 μg/ml organ bath: 15 min contact period;

(c) Pure compounds (**1–4**) at concentrations of 1×10^{-4}- 5×10^{-5}- 1×10^{-5} M: 15 min contact period.

3.5. Statistical analysis

Regression methods were used for statistical analysis and critical significance set at $P < 0.05$ for transmurally stimulated guinea-pig ileum test.

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Five naphthalene glycosides from the roots of *Rumex patientia*

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Abstract

Three novel and two known naphthalene glycosides were isolated from the roots of *Rumex patientia* L. (Polygonaceae). The structures of the new compounds were established, respectively as 2-acetyl-3-methyl-6-carboxy-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranoside, 4,4′-binaphthalene-8,8′-*O*,*O*-di-β-D-glucopyranoside and 2-acetyl-3-methyl-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranosyl (1→3) β-D-glucopyranoside on the basis of spectral analysis. The other napthalene glycosides were determined as nepodin-8-*O*-β-D-glucopyranoside and torachrysone-8-*O*-β-D-glucopyranoside by comparison of their spectral data with those previously reported. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Rumex patientia*; Polygonaceae; Naphthalene glycosides; Rumexoside; Labadoside; Orientaloside

1. Introduction

Rumex species are wide spread plants in the flora of Turkey (Davis, 1967, 1988). The roots of *Rumex patientia* are used in some parts of Turkey for its purgative, constipate, depurative and tonic properties (Baytop, 1984). Chemical studies on *R. patientia* have been carried out by some investigators and the presence of the anthraquinone, naphthalene, tannin and napthoquinone derivatives has been reported (Sharma et al., 1977; Suri et al., 1978; Demirezer and Kuruüzüm, 1997). Here, we report the isolation from this plant of five naphthalene glycosides, including the structure elucidation of three new compounds (**1**–**3**).

2. Results and discussion

Five naphthalene glycosides (**1**–**5**) were isolated from the MeOH extract of the roots of *R. patientia*. Compounds **1**–**3** were described as 2-acetyl-3-methyl-6-carboxy-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranoside (**1**), 4,4′-binaphthalene-8,8′-*O*,*O*-di-β-D-glucopyranoside (**2**) and 2-acetyl-3-methyl-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranosyl (1→3) β-D-glucopyranoside (**3**) on the basis of spectral analysis. Naphthalenes **4** and **5** were identified as nepodin-8-*O*-β-D-glucopyranoside and torachrysone-8-*O*-β-D-glucopyranoside by comparison of their spectral data with those previously reported (Tsuboi et al., 1977; Nishina et al., 1991).

Compounds **1**–**3** were obtained as amorphous materials. The UV spectra of **1**–**3** suggested that their hydroxy naphthalene were observed.

The IR spectrum of compound **1** showed the presence of a carboxy group (1732 cm^{-1}). Its mass spectrum exhibited the $[M-H]^+$ peak at m/z 421 and $[M-COOH]^+$ peak at m/z 377. In the ^{13}C NMR spectrum, a carbon signal at δ 127.80 proved the presence of an carboxy group at C-6 in compound **1**. Generally 1D (Table 1) and 2D NMR spectral data of **1** were similar to those of nepodin-8-glucoside (**4**) except for the presence of signals due to the carboxy moiety. Thus compound **1** was assigned as 2-acetyl-3-methyl-6-carboxy-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranoside.

In the ESI mass spectrum of compound **2**, the peak of highest mass number was observed at m/z 777 $[M+Na]^+$ confirming the molecular weight of **2** corresponding to the molecular formula $C_{38}H_{42}O_{16}$. Its UV

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Table 1
^{13}C and ^{1}H NMR spectral data for compounds **1** and **3**[a]

	Compound **1**				Compound **3**			
Position	C atom	δ_C (ppm)	δ_H (ppm), *J* (Hz)	HMBC	C atom	δ_C (ppm)	δ_H (ppm), *J* (Hz)	HMBC
1	C	153.02	–		C	152.65	–	
2	C	125.18	–		C	126.57	–	
3	C	133.55	–		C	138.94	–	
4	CH	118.17	7.14 *s*	C-2, CH_3	CH	118.56	7.14 s	C-2, C-5, CH_3
5	CH	127.34	7.23 *d* (4)	C-7	CH	123.99	7.40 *m*	C-4, C-8
6	C	127.80	–		CH	128.62	7.42 *m*	C-8, C-10
7	CH	122.59	7.41 *d* (4)	C-8, C-9	CH	112.92	7.36 *m*	C-5
8	C	154.59	–		C	157.14	–	
9	C	111.09	–		C	114.30	–	
10	C	136.30	–		C	135.14	–	
3-CH_3	CH_3	19.63	2.22 *s*	C-2, C-3, C-4	CH_3	20.47	2.29 *s*	C-2, C-3, C-4
2-CO$\underline{CH_3}$	CH_3	31.65	2.50 *s*	$\underline{CO}CH_3$	CH_3	31.13	2.65 *s*	
2-$\underline{C}OCH_3$	C	205.16	–		C	208.26	–	
6-COOH	C	170.50	–		–	–	–	
Glucose								
1′	CH	103.02	5.03 *d* (7.3)	C-8	CH	105.30	5.14 *d* (7.8)	C-8
2′	CH	73.45	3.24–3.68[b]		CH	73.65[c]	3.61 *m*[d]	
3′	CH	74.43	3.24–3.68[b]		CH	75.04	3.57 *m*	
4′	CH	69.97	3.24–3.68[b]		CH	71.69	3.31 *m*	
5′	CH	76.03	3.24–3.68[b]		CH	78.09	3.49 *m*	
6′	CH_2	63.26	4.12 *dd* (12/6.8)		CH_2	62.55	3.62 *m*	
			4.31 *dd*			3.74 *m*		
Glucose								
1″	CH	–	–		CH	99.99	4.90 *d* (7.5)	C-3′
2″	CH	–	–		CH	73.71[c]	3.63 *m*[c,d]	
3″	CH	–	–		CH	75.24	3.71 *m*	
4″	CH	–	–		CH	71.42	3.48 *m*	
5″	CH	–	–		CH	77.25	3.76 *m*	
6″	CH_2	–	–		CH_2	67.51	3.84 *m*	
							3.96 *m*	

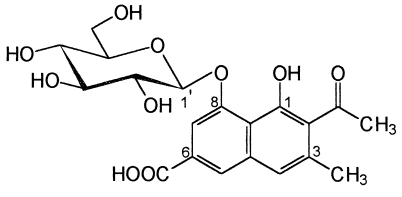
[a] Assigments are based on APT, COSY, HMQC and HMBC.
[b] Signal pattern unclear due to overlapping.
[c] Values for C-2′ and C-2″ can be interchanged.
[d] Values for H-2′ and H-2″ can be interchanged.

and IR spectra indicated the presence of a hydroxy naphthalene skeleton. The ^{1}H NMR spectrum (Table 2) of **2** was similar to that of **4** whereas its ^{1}H NMR spectrum contains resonances for two anomeric protons of two sugar units at δ 5.10 (*d*, H-1′, $J = 7.0$ Hz) and δ 5.11 (*d*, H-1″, $J = 7.0$ Hz). The anomeric proton and other proton signals of sugars are characteristic for glucose. The anomeric configuration of glucose was proposed to be β on the basis of the coupling constant ($J = 7$ Hz) and the chemical shifts. The ^{1}H NMR spectrum of **2** was significant in that it indicated duplication of resonances attributable to the glucosidic naphthalene function. ^{13}C NMR assigments were supported by the APT experiment are shown in Table 2. ^{1}H, ^{1}H-Homonuclear COSY spectrum of **2** showed the symetrically same spin system for double naphthalene ring and double sugar units. The assigments for all protonated carbons and protons of **2** were determined with short range and long range heteronuclear correlations spectra (HMQC and HMBC). Thus the dimeric naphthalene glucosides were clearly defined in the COSY, HMQC and HMBC spectra of compound **2**. However, the long range ^{1}H, ^{13}C correlations of H-1′ with C-8 and H-1‴ with C-8″ supports the position of the attachment of the sugars. The lack of the typical H-4/H-4″ signals in the ^{1}H NMR spectrum of **2** suggested that C-4 and C-4″ are the positions of dimerization. In addition, the downfield shift ($\Delta\delta = 5.16$ ppm) in the ^{13}C NMR spectra of C-4/C-4″ in comparison to that of nepodin-8-glucoside (**4**) is supporting this point. Evidence for the dimer glucosidic naphthalene structure was confirmed by a combination of the mass, 1D NMR (^{1}H, ^{13}C) and 2D NMR (COSY, HMQC, HMBC) spectrometry. Therefore, compound **2** was determined as 4,4″-binaphthalene-8,8″-*O*,*O*-di-β-D-glucopyranoside.

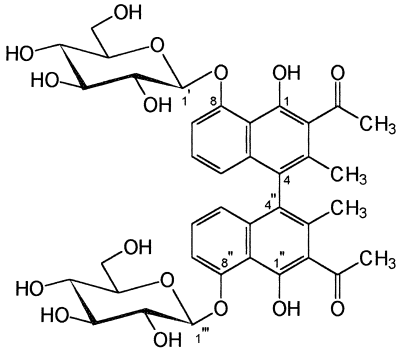
The UV and IR spectra of compound **3** were similar to those of **1**. Its ^{1}H and ^{13}C NMR spectra (Table 1) displayed many similarities with those of **4**, especially

for the resonances assigned to the naphthalene moiety and the glucose unit. However, the set of additional protons, apart from the β-anomeric proton at δ 4.90 (*d*, $J = 7.5$ Hz) and the corresponding carbon signals, were in agreement with the presence of another hexose unit, which was identified as β-glucose by COSY and HMQC experiments. The heteronuclear multiple bond correlation (HMBC) experiment observed between H-1′ of inner glucose and C-8 of the naphthalene aglycone and H-1″ of terminal glucose and C-3′ of the inner glucose. Consequently, compound **3** was established as 2-acetyl-3-methyl-1,8-dihydroxynaphthalene-8-*O*-β-D-glucopyranosyl (1→3) β-D-glucopyranoside.

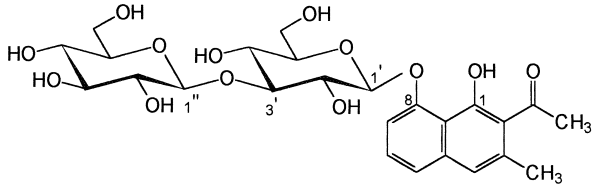
Compound **1**–**3** were isolated from nature for the first time, for which **rumexoside**, **labadoside**, and **orientaloside** are proposed as the trivial names, respectively. To our knowledge this is the first report of the occurrence of the dimeric type naphthalene in the genus *Rumex* until now.



1



2



3

3. Experimental

UV spectra were determined in spectroscopic grade MeOH on a Shimadzu UV-160 A spectrophotometer. IR spectra were measured on a Perkin-Elmer FTIR 1720 X spectrometer as pressed KBr disks. NMR spectra were recorded on Bruker AMX 300 and AMX 500 NMR, operating at 300 and 500 MHz for proton and 75.5 MHz for carbon. EIMS (70 eV) and DCIMS were recorded on Finnigan MAT 311 A and Varian MAT 731, respectively. TLC was carried out on precoated silica gel 60 F 254 aluminium sheets (Merck). For column chromatography, normal phase silica gel 60 (0.063–0.200 mm, Merck), reversed phase silica gel (Li Chroprep RP-18, Merck), and polyamide (Polyamid-MN-Polyamid SC 6, Macherey-Nagel, Düren) were used. Compounds were detected by UV flourescence and/or spraying with vanillin–H_2SO_4 reagent followed by heating at 100°C for 5–10 min.

3.1. Plant material

The roots of *R. patientia* L. were collected from Bor-Niğde (1050 m), Turkey in September 1996. A voucher specimen has been deposited at the herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 96003).

Table 2
^{13}C and ^{1}H NMR spectral data for compound **2**[a]

Position	C atom	δ_C (ppm)	δ_H (ppm), *J* (Hz)	HMBC
1, 1″	C	158.47	–	
2, 2″	C	126.32	–	
3, 3″	C	132.10	–	
4, 4″	C	126.11	–	
5	CH	120.49	6.57 *d* (8)	C-4, C-7, C-9
5″	CH	120.49	6.53 *d* (8)	
6	CH	128.01	7.22 *dd* (8/8)	C-8, C-10
6″	CH	128.01	7.20 *dd* (8/8)	
7	CH	111.04	7.33 *d* (8)	C-5, C-8, C-9
7″	CH	111.04	7.32 *d* (8)	
8, 8″	C	154.95	–	
9, 9″	C	113.83	–	
10, 10″	C	135.18	–	
3-CH_3	CH_3	16.70	1.75 *s*	C-2, C-3, C-4
3″-CH_3	CH_3	16.64	1.74 *s*	
2, 2″-$COCH_3$	CH_3	31.36	2.59 *s*	$COCH_3$
2, 2″-$COCH_3$	C	205.88	–	
Glucose				
1′	CH	102.83	5.11 *d* (7)	C-8
1‴	CH	102.83	5.10 *d* (7)	C-8″
2′, 2‴	CH	73.55	3.17–3.80[b]	
3′, 3‴	CH	76.32	3.17–3.80[b]	
4′, 4‴	CH	69.92	3.17–3.80[b]	
5′, 5‴	CH	77.85	3.17–3.80[b]	
6′, 6‴	CH_2	60.81	3.17–3.80[b]	

[a] Assignments are based on APT, COSY, HMQC and HMBC.
[b] Signal pattern unclear due to overlapping.

3.2. Extraction and isolation

Powdered dried material (500 g) was extracted with MeOH (3×2.5 l) at 40°C. The MeOH extracts were combined and evaporated to dryness in vacuo (100 g). The crude extract (40 g) was fractionated by column chromatography on polyamide by gradient elution with H_2O–MeOH mixtures.

The fractions eluted with 50% MeOH in water were purified by repeated silica gel column chromatographies eluted with $CHCl_3$–MeOH (95:5, 90:10) to give compound **1** (8 mg).

The fractions eluted with 60% MeOH were subjected to a polyamide column, eluting with H_2O–MeOH (60:40→20:80) to give 13 fractions. The fractions 5–7 were rechromatographed over silica gel columns using EtOAc–MeOH–H_2O (100:5:1→100:17:13) solvent system and a sephadex LH-20 column using MeOH to yield compound **2** (8.6 mg).

The fractions eluted with 80% MeOH were rechromatographed an a polyamide column using MeOH gradient as eluent and a silica gel column using EtOAc–MeOH–H_2O (100:5:2→100:17:13) solvent system to give compound **3** (16 mg).

The fractions eluted with 70% MeOH were applied to a polyamide column using increasing amounts of MeOH in H_2O (40–100% MeOH). The fractions eluted with 40–60% MeOH were subjected to MPLC using RP-18 material as stationary phase and H_2O–MeOH gradient as solvent system (30–100%). Fractions 33–36 were further purified by silica gel column using EtOAc–MeOH–H_2O (100:17:13) to yield compounds **4** (12 mg) and **5** (14 mg).

3.3. Rumexoside (*1*)

Amorphous; ESIMS m/z: 868 $[2M+Na]^+$, 445 $[M+Na]^+$, 421 $[M-H]^-$, 377 $[M-COOH]^-$, 215 $[(M-C_6H_{10}O_5)-COOH]^-$; UV λ_{max} (MeOH) nm: 224, 260, 301, 334; IR ν_{max} (KBr) cm^{-1}: 3399 (OH), 1732 (COOH), 1690 (C=O), 1605 (C=C, arom.), 1079 (C−O); ^1H (300 MHz; DMSO–D_2O) and ^{13}C NMR (75.5 MHz; DMSO-d_6): Table 1.

3.4. Labadoside (*2*)

Amorphous; ESIMS m/z: 777 $[M+Na]^+$, 615 $[(M-C_6H_{10}O_5)+Na]^+$; UV λ_{max} (MeOH) nm: 230, 314, 337; IR ν_{max} (KBr) cm^{-1}: 3399 (OH), 1693 (C=O), 1621 (C−C), 1078 (C−O); ^1H (300 MHz; DMSO–D_2O) and ^{13}C NMR (75.5 MHz; DMSO–D_2O): Table 2.

3.5. Orientaloside (*3*)

Amorphous; DCIMS m/z: 234 $[aglycon+NH_4]^+$, 217 $[aglycon+H]^+$, 197 $[C_6H_{10}O_5+NH_4+NH_3]^+$, 180 $[C_6H_{10}O_5+NH_4]^+$; UV λ_{max} (MeOH) nm: 225, 260, 301, 333, 345; IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 1632 (C−C), 1028 (C−O); ^1H (300 MHz; CD_3OD) and ^{13}C NMR (75.5 MHz; CD_3OD): Table 1.

Acknowledgements

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PHYTOCHEMISTRY

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Editorial comment

Who benefits from biopiracy?☆

In the last decade, much attention has focused on tropical plants and micro-organisms as sources of novel bioactive molecules. The expectations of indigenous peoples and the governments of developing countries for large financial returns for their traditional knowledge of plant uses have been raised. Many lawyers and non-governmental organisations (NGOs) have become engaged in protecting indigenous people's rights in bioprospecting and from biopiracy. The claims of biopiracy targeted at multi-national companies and other large organisations in developed counties are many. The result is that many companies or organisations of the developed countries no longer work on natural products or have greatly reduced their efforts in this specific area for fear of bad press and the inability to get legal agreements, since NGOs vigorously contest them using media such as the internet. At the same time combatorial chemistry allows the companies to buy synthetic chemicals for their screening programmes in vast numbers at much lower cost than natural products and with shorter lead development times due to the synthetic route already being available. Who are the winners and losers in these developments? Are tropical plants really that more valuable than others and will claims of biopiracy damage income to less well developed countries?

Clear winners in the biopiracy war are the "biopiracy" lawyers who often have large salaries and a vested interest in making the process of reaching legal agreement for bioprospecting long and complex. Irresponsible NGOs are easily able to whip up support for their actions claiming to be protecting indigenous people's rights from patent wielding "multi-nationals". Some NGOs appear to seek notoriety by wild claims on internet sites prior to investigating the facts and even slight inaccuracies, deliberate or not, can lead to claims of apparent exploitation. Many of these NGOs are against patents in any form and so their campaigns will be critical of almost any commercial bioprospecting venture since most companies cannot invest the large sums of money required into such programmes without securing a legal position over the potential products. Clear losers are the indigenous peoples who often have no direct say in the campaigns on their behalf and who might benefit more from patents than "protection" by action groups of well-heeled activists in cities. Often the NGOs quote "spokespeople" from the indigenous people who can have a vested interest due to commercial operations of their own. The other losers are the world's sick who may not benefit from new treatments for their diseases as companies run scared of natural products debate and direct action groups.

There can be little doubt that there are unscrupulous individuals in companies or other powerful bodies who are purely motivated by profit margins. However, in today's world of mass communication no business or academic body can afford to attract substantiated bad press accusing exploitation of the poor. Indeed, they probably stand to gain more from a perception of ethical business practise. On the issue of sustainable production of the plants they are working on, companies would be very foolish, commercially, not to protect their source plants (ideally in the country of origin where they are likely to grow best) from a very early stage of product development in case synthesis of any new chemical they discover is difficult or expensive. Attempts to patent whole genomes or plants themselves (such as neem) or traditional preparations with little innovation are not worth the paper they are written on and deserve ridicule and legal contest. However, good specific patents on products derived from indigenous people's plants or micro-organisms, with a fair distribution of revenue to the indigenous people themselves to use as they think fit, should be applauded and not hindered by prolonged discussion or inaccurate reporting. Such prolonged discussions are precisely what has driven many companies out of bioprospecting completely. Well developed tropical countries such as Brazil are perfectly able to exploit their own resources without external help, but other tropical countries are not yet in that position and could lose out if their natural products are not studied commercially. Since the distribution of species often does not respect political borders, countries may witness the development of products from their

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flora by their richer neighbours. However, the instigation of training programmes as a component of joint commercial ventures to establish the capability of natural products discovery within developing countries would be a good way to progress if NGOs and lawyers give the leeway for these to be discussed.

Perhaps the saddest thing about the recent biopiracy "investigations", primarily in tropical countries, is that expectations of large financial returns are unrealistic and are leading to fewer companies who are prepared to go through the minefield set by activists and bioprospecting lawyers. Yet one has only to look at some of the drugs developed recently, such as taxanes, artemisinin and etoposide, to realise that temperate species (of developed countries) are also very productive in bio-active and valuable natural products. Advances in analytical and purification methods and the ability to produce screen-ready purified natural products in libraries allows the rapid discovery of new chemicals in common native species of the UK, Europe and the USA (Fig. 1). For example, DMDP and DAB, bioactive pyrrolidine alkaloids initially found in tropical legume species, are major components of bluebells and other members of the Hyacinthaceae native to Europe. Indeed the Hyacinthaceae appear to produce a much wider range of related structures than do the tropical legumes. INBIO have been developing the production of DMDP as a nematocide from native leguminous trees in Costa Rica and yet potential sources are clearly common worldwide.

Tropical ecologists have often proposed that tropical plants should produce a wider range of defensive chemicals because herbivores and pathogens can be active throughout the year and consequently the selective pressures are higher. This has often been accepted as fact in justifying travel and collection in tropical regions by natural product chemists in the USA, Germany and the UK in particular. However, the reverse argument is that the tropics often offer good prolonged growing conditions where lost leaf or seed production can be replaced without the time constraint of a cold winter ahead. The extremes of seasonality and the ferocity of herbivore attack in temperate regions could also be argued to increase the requirement for good chemical defences. Such factors are combined with the necessity to survive frosts, frozen soil and drying winds, which in themselves select natural products with potentially interesting properties such as betaines, sugar alcohols and hydroxyproline. Although there is greater biological diversity in the tropics, in terms of natural products it is not clear whether the greater number discovered in tropical plants does not just reflect the greater recent effort. The presence of endophytic fungi in many plants as well as uptake of chemicals produced by soil micro-organisms may well lead to the future discovery of many more apparent "tropical plant" products in temperate species. For example, *Taxomyces* in Yew produces taxanes and this could explain the recent discovery of taxanes in Hazel and the macrocyclic trichothecines reported from



Fig. 1. Temperate plants are a proven source of valuable natural products but they have been largely ignored by drug and agrochemical discovery programmes in recent years. Companies such as MolecularNature Limited are using chemical fingerprinting approaches to identify potentially valuable compounds in common European and North American species.

Baccharis spp. are most likely to be products of soil micro-organisms taken up by the plant

The "theft" of genes for natural products production is sometimes also mentioned but is not a credible concern since production of most are controlled by several genes and even if a valuable product could be found in a tropical plant and produced using genetic manipulation, the research cost and timescale is most likely to be prohibitive. Justifiable concerns over possible transfer of genes out of GM plants are also going to restrict future development of new GM crops for chemical production. (NB the production of cyanogenic glycosides by both *Lotus* and its specialised herbivore *Zygaena* and the alkaloid swainsonine by *Rhizoctonia leguminicola* and its host legumes strongly suggests transfer of genes between organisms already occurs naturally and genes moved by GM technology may well be even more readily transferrable.)

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Cytosolic aldolase is a ripening related enzyme in strawberry fruits (*Fragaria* × *ananassa*)

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Abstract

Two aldolase isoenzymes have been isolated from ripe strawberry fruits (*Fragaria* × *ananassa* cv. Camarosa and Elsanta) and partially purified by DEAE anion exchange and Sephacryl size exclusion chromatography. The isoenzymes were identified as class I cytosol and plastid aldolase on the basis of their chromatographic behavior on DEAE–cellulose columns, native molecular weight, pH optimum pattern, K_m value for D-fructose-1,6-bisphosphate, tendency to be inactivated by lower pH values and SDS–PAGE subunit determination of 40 and 38 kDa, respectively. Total aldolase activity and distribution of both aldolase isoenzymes was also investigated at different stages of strawberry fruit ripening. Strawberries in the green and white ripening stage showed the same ratio of the two isoenzymes as green leaves with 15 and 8% cytosol aldolase activity, respectively. During strawberry fruit development the overall total aldolase activity decreased until the pink ripening stage and then increased due to a rise of cytosol aldolase yielding up to 75% in red strawberries. A cDNA putatively encoding the cytosolic form of aldolase in strawberry was cloned during the course of this study. Both microarray and RNA gel blot analyses showed that the cytosolic aldolase gene expression is induced during ripening as detected for the cytosolic aldolase enzyme. We suggest that induction of the cytosolic aldolase both at the levels of transcription and translation might be part of a ripening related stress response in the receptacle tissue. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Strawberry fruits; *Fragaria* × *ananassa*; Rosaceae; D-Fructose-1,6-bisphosphate; Aldolase; Microarrays

1. Introduction

Although strawberries (*Fragaria* × *ananassa*) represent an economically important fruit in Europe and America biochemical studies on strawberry ripening physiology have until now, been restricted to investigations on ascorbic acid metabolism (Loewus, 1963; Kim and Chung, 1998), partial purification of polygalacturonase (Nogata et al., 1993), UDP-glucose:-flavonoid *O*-glucosyl transferase (Cheng et al., 1994), peroxidase (Civello et al., 1995; López-Serrano and Barceló, 1997), lipoxygenase (Pérez et al., 1999a), alcohol acyltransferase (Pérez et al., 1993, 1996), hydroperoxide-lyase (Pérez et al., 1999a), invertase (Poovaiah and Veluthambi, 1985) and phenylalanine ammonia lyase (PAL) (Given et al., 1988). Reasons for the limited number of studies are the high content of polyphenolics and pectins in unripe and ripe strawberry fruits hampering enzyme isolation, the short harvest period and storage shelf life as well as the low protein content.

Thus several groups have generated cDNA libraries from wild and cultivated strawberries to investigate regulation of ripening, improve shelf life or flavor formation (Manning, 1998; Medina-Escobar et al., 1998; Moyano et al., 1998; Medina-Escobar et al., 1997). In addition, Aharoni et al. (2000) reported on the use of microarray technology for large scale gene expression studies in strawberry (for review on microarray technology see Lemieux et al., 1998; Van Hal et al., 2000). Microarrays containing 1701 cDNAs were used for monitoring gene expression in four consecutive stages of

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strawberry fruit development. More than 400 cDNAs showed significant change in expression during development. A strawberry acyltransferase gene was identified, putatively encoding the enzyme performing the last committed step in volatile ester biosynthesis.

Recently, we started studies on the flavor formation in strawberry fruits and found that D-fructose-1,6-bisphosphate represents the major precursor of the strawberry key aroma compound 2,5-dimethyl-4-hydroxy-3(2H)-furanone (Roscher et al., 1998). In work with bananas the increase in carbon dioxide output during the 'climacteric' was shown to be accompanied by a large increase in the content of D-fructose-1,6-bisphosphate (Barker and Solomos, 1962) during ripening. However, in apples another climacteric fruit this earlier finding was not confirmed (Barker, 1968).

Our aim was to investigate D-fructose-1,6-bisphosphate degrading enzymes in strawberry fruits in order to find strategies to improve strawberry flavor. We already showed that increased D-fructose and D-fructose-6-phosphate levels caused higher concentrations of aroma-active furanones as well as their non-volatile derivatives (Schwab, 1998; Pérez et al., 1999b).

Therefore, this paper deals for the first time with the isolation and characterization of two aldolase isoenzymes and a gene putatively encoding one of them from ripening strawberry fruits as well as their time-course of activity during strawberry development.

2. Results

2.1. Detection of aldolase activity in ripening strawberry fruits

A crude enzyme extract obtained from red strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta) was analyzed for its ability to degrade ^{14}C-labeled D-fructose-1,6-bisphosphate. Polyvinylpyrrolidone was used during the preparation of the extract to avoid inactivation of enzymes by polyphenols. After an incubation period of 2 h anion exchange radio-HPLC as well as radio-TLC analysis showed mono-phosphorylated and non-phosphorylated products. The incubation was repeated with unlabeled D-fructose-1,6-bisphosphate and the mono-phosphorylated compounds were collected after separation by anion exchange HPLC. After treatment with alkaline phosphatase, reduction with $NaBH_4$ or $NaBD_4$ and acetylation unlabeled or labeled glycerol triacetate was identified by GC–MS, respectively. Thus, the presence of aldolase in the crude strawberry enzyme extract was expected.

Aldolase activity in the crude enzyme extract was determined with an optical test based on the coupled enzymatic reaction with triose phosphate isomerase and glycerol-3-phosphate dehydrogenase. The reaction was assayed at 25°C by monitoring NADH utilization at 334 nm.

2.2. Preliminary purification of aldolase from ripening strawberry fruits

A crude extract was prepared from ripe strawberry fruits (*Fragaria* × *ananassa* cv. Camarosa) and pectin was precipitated by adding ammonium sulfate up to 20% saturation. Removal of pectin was necessary to avoid clogging of the anion-exchange column used for the following purification step. After centrifugation the supernatant was dialyzed against 0.02 M Tris (pH 7.7) until the conductivity was less than 1.5 mS. This protein extract contained half of the enzymatic activity as extracts obtained from cultivar Elsanta. The extract was applied to a DEAE–cellulose ion-exchange resin and eluted by a linear gradient of 0–0.4 M KCl in column buffer. Two aldolase isoenzymes were obtained (Fig. 1). The elution pattern of the two aldolases was similar to that published for the resolution of aldolase from the cytosol and chloroplast (Nishimura and Beevers, 1981; Lebherz et al., 1984; Krüger and Schnarrenberger, 1985; Botha and O'Kennedy, 1989; Moorhead et al., 1994). After lyophilization of the combined fractions containing the aldolase isoenzymes the individual isoenzymes were further purified by size exclusion chromatography on Sephacryl S300 using 0.2 M Tris as eluting buffer. Both aldolase isoenzymes showed a molecular mass of approx. 160 kDa confirming the classification as class I aldolases (Krüger and Schnarrenberger, 1983). Table 1 displays the preliminary purification protocol for the strawberry fruit aldolases. Strawberry fruits contain a high percentage of water and only small amounts of protein. The specific catalytic activities of 0.036 and 0.034 U mg^{-1} for the two isoenzymes are relatively low

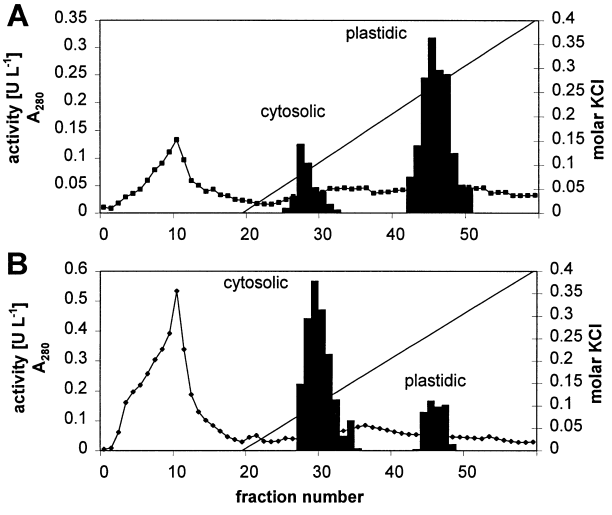


Fig. 1. Separation of aldolase isoenzymes obtained from green (A) and red strawberry fruits (B) by chromatography on DEAE–cellulose.

Table 1
Preliminary purification protocol for the strawberry aldolase isoenzymes

Sample	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification
Crude extract	100	443.2	0.85	0.0019	100	1
Dialysate	110	97.5	0.62	0.0063	73	3
DEAE–cellulose	Cytosolic: 28	8.0	0.10	0.0125	12	7
	Plastidic: 32	4.5	0.03	0.0066	4	4
Sephacryl S300	Cytosolic: 23	0.54	0.0192	0.036	2.2	19
	Plastidic: 20	0.21	0.0072	0.034	0.8	18

compared with purified aldolase (Hodgson and Plaxton, 1998). This implies that the preparations still contain additional proteins but due to the low protein contents further purification steps were not performed.

2.3. Characterization of aldolase isoenzymes isolated from ripening strawberry fruits

After denaturation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the partially purified enzymes was performed to characterize the isoenzymes as the cytosolic and plastidic aldolase. SDS–PAGE revealed several protein-staining bands indicating that the two strawberry fruit aldolase preparations were not yet homogeneous (data not shown). However, isoenzyme 1 and 2 could be differentiated by the occurrence of a 40 and a 38 kDa subunit as expected for cytosolic and plastidic aldolase, respectively (Lebherz et al., 1984). These subunits were easily resolved by co-electrophoresis of a mixture of the two enzymes. Due to their chromatographic behavior on DEAE–cellulose, their native molecular weight and their subunits molecular weights aldolase isoenzyme 1 and 2 were identified as cytosol and plastid aldolase, respectively.

The cytosolic aldolase shows a fairly broad pH maximum from pH 7.5 to 8.5 whereas the second isoenzyme exhibits a distinct maximum at pH 8.0. Aldolase activities were also measured in the absence and presence of 1 mM $MgCl_2$ or 1 mM EDTA. No difference in activity was observed with or without $MgCl_2$ or EDTA as expected for class I aldolases (Gross et al., 1994; Pelzer-Reith et al., 1994).

When the K_m (D-fructose-1,6-bisphosphate) was determined, typical Michaelis–Menten kinetics were obtained with K_m of 35 μM for the cytosolic aldolase and 40 μM for the plastid aldolase. These data are in agreement with properties of class I aldolases (Pelzer-Reith et al., 1994).

2.4. Time-course of aldolase activity during strawberry ripening

In order to estimate the percentage of cytosol and plastid aldolase in strawberry fruits during ripening, we separated the respective isoenzymes by ion-exchange chromatography on DEAE–cellulose at different stages of fruit development. Two replicates for each ripening stage were performed. In Fig. 1 representative analyses are shown for red and green strawberry fruits. The activity peaks at low KCl concentration represent the cytosolic aldolase and the peaks at high KCL concentration the plastid aldolase (Krüger and Schnarrenberger, 1983, 1985). From the results of the time-course experiments summarized in Fig. 2 it is evident that the cytosol aldolase represents only approx. 8 and 15% of the total aldolase activity in green and white fruits whereas in white and red strawberries the portion of cytosolic aldolase increases to 50 and 75%, respectively. Total aldolase activity per strawberry fresh weight increased gradually during strawberry ripening. However, extracts obtained from strawberries in the green ripening stage contained only half of the proteins that were extractable from white, pink and red fruits. Thus, the total aldolase activity calculated per mg extracted protein decreased until the pink ripening stage and then increased.

2.5. Heat-stability and recovery of activity after acid treatment

The heat-stability and recovery of activity after acid treatment was determined with the crude enzyme preparation obtained from green (85% plastid aldolase) and red strawberries (75% cytosol aldolase). The cytosolic isoenzyme was quite stable to prolonged heating at 50°C. After 30 min 74% of the initial enzymatic activity was recovered. In contrast, the plastid aldolase was heat-sensitive as incubation at 50°C for 30 min resulted in a 40% loss in enzymatic activity. Another striking difference between the cytosolic and plastidic form of spinach leaf isoenzymes is their ability to regain catalytic activity following treatment at pH 2.3. We found that after 10 min incubation at pH 2.3 and then readjustment to pH 8.0 cytosol and plastid aldolase regained 82 and 62% of their initial enzymatic activity, respectively.

2.6. Cloning and sequence analyses of the strawberry cytosolic aldolase gene

We recently reported on partial sequencing of approximately 1100 cDNA clones randomly picked

from a strawberry ripe fruit cDNA library (Aharoni et al., 2000). Sequence homology search (using the BLAST program) identified two similar cDNAs putatively encoding the *S*trawberry *C*ytosolic *A*ldolase (*SCA1*). Sequencing one of the cDNAs (1409 bp long) revealed a predicted open reading frame encoding 358 residues protein with estimated molecular weight of 38.5 kDa. Aldolase is a highly glycosylated enzyme. This fact might explain the slight difference between the molecular weight of the native enzyme and the translated protein of the cDNA clone. Protein sequence alignments show that *SCA1* is 85% identical to its first three, cytosolic aldolases (class I) homologs from common ice plant (accession T12416), rice (accession S65072) and chickpea (accession O65735). The homology with plant plastidic aldolases (pea, accession Q01516; rice, accession BAA02730; spinach, accession CAA47293) was much lower compared to the cytosolic proteins (between 48 and to 56% identity). It is apparent that the *SCA1* protein is the cytosolic form of aldolase in strawberry since it does not contain in its *N*-termini the characteristic transit peptide of plant plastidic aldolases (Pelzer-Reith et al., 1993).

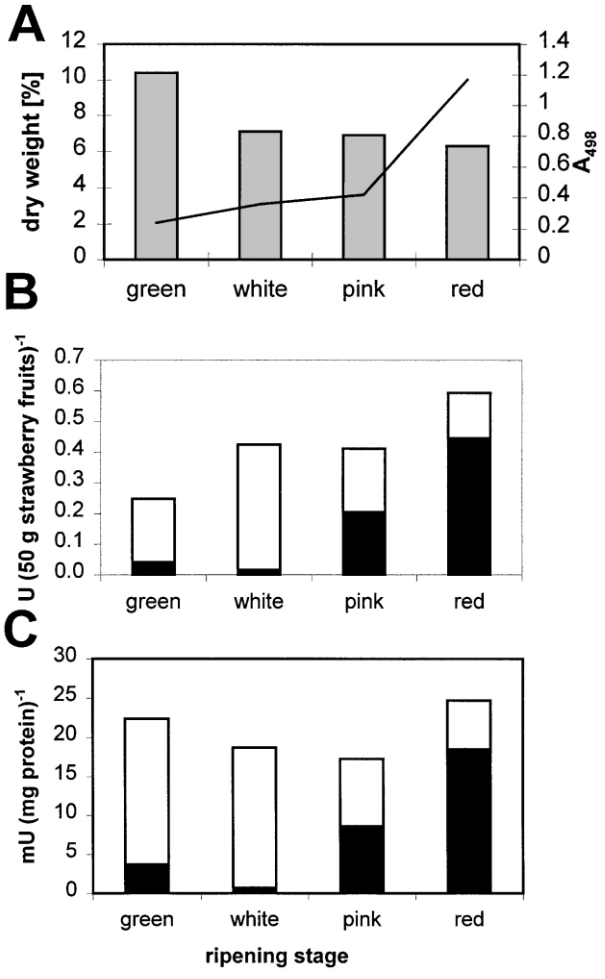


Fig. 2. Dry weight (bars) and development of anthocyanines (A_{498}) (line) during strawberry fruit ripening (A). Distribution of cytosolic (black bars) and plastidic aldolase (white bars) during strawberry fruit development calculated per strawberry fresh weight (B) and protein (C).

2.7. Microarray and RNA gel blot analyses show that SCA1 expression is induced in the ripening receptacle

Previous experiments using gene expression arrays, containing 1701 strawberry cDNAs (Aharoni et al., 2000) provided us with quantitative data on *SCA1* expression in strawberry. Gene expression analyses in four consecutive stages of strawberry fruit development (green, white, pink and red) revealed that *SCA1* shows a 3-fold increase in expression between the green and the red stage. A different microarray experiment comparing expression between strawberry receptacle and achene tissues (A. Aharoni et al., unpublished data, 2000) showed that *SCA1* is highly expressed in the receptacle (4.5-fold higher expression in receptacle compared to achenes). Up regulation of *SCA1* expression during development and ripening was also detected by RNA gel blot analysis (Fig. 3). *SCA1* expression was not restricted to reproductive tissues as high level of expression was also detected in strawberry leaf tissue.

3. Discussion

Important in flavor, carbohydrates represent one of the main soluble components in the non-climacteric strawberry fruits and provide energy and building blocks for metabolic changes. D-Glucose and D-fructose are present in equivalent concentrations in ripe strawberries (2.3 and 2.2 g per 100 g fr. wt, respectively), and account for about 83% of the total carbohydrates (Wrolstad and Shallenberger, 1981). It is believed that in ripening fruits carbohydrates are primarily degraded by glycolysis rather than the oxidative pentose phosphate pathway (Tucker, 1993).

One of the essential enzymes in glycolysis, gluconeogenesis, and the Calvin cycle is D-fructose-1,6-bisphosphate

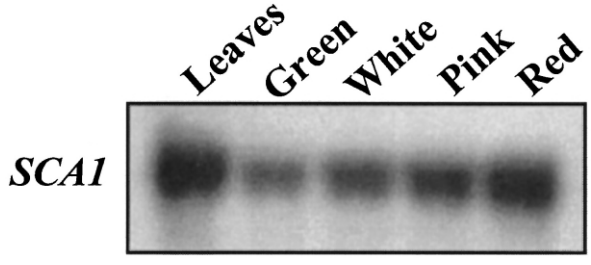


Fig. 3. *SCA1* gene expression in strawberry. RNA gel blot of total RNA isolated from leaves and from green, white, pink and red strawberry fruit. Blots were hybridized with the entire *SCA1* cDNA.

aldolase. Two aldolase isoenzymes have been isolated from green pea shoots, spinach leaves and corn leaves (Anderson and Pacold, 1972; Gasperini and Pupillo, 1983; Lebherz et al., 1984). One is restricted to the cytosol and the other to the chloroplasts. Genetic analyses proved that the two aldolases are coded for by different gene loci and immunochemical comparisons revealed great structural differences in the amino-acid sequences of the two proteins (Weeden and Gottlieb, 1980). Quantitation of the two aldolases in green leaf tissue showed that the cytosol aldolase represents only 5–15% of the total activity and the plastid enzyme represents the remaining 85–95% (Krüger and Schnarrenberger, 1985). A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the level of sugars and starch, and inhibits growth of potato plants (Haake et al., 1998).

Relatively little is known about the aldolases from non-photosynthetic plant tissues. Characterization of homogeneous cytosolic aldolase preparations from non-green plant tissue have thus far only been reported for the germinating *Phaseolus vulgaris* seeds (Botha and O'Kennedy, 1989), castor oil seeds (Hodgson and Plaxton, 1998), spinach seeds (Krüger and Schnarrenberger, 1985) and carrot storage root enzymes (Moorhead and Plaxton, 1990). The contribution of cytosolic aldolase to the total aldolase activity of non-green tissues appears to be much greater than in green leaves and may account for more than 50% of the total aldolase activity present (Krüger and Schnarrenberger, 1985). This indicates quantitative changes in the proportion of the two aldolases during the progress of greening. To our knowledge there is no study on the aldolase isoenzymes from fruits.

We have separated a strawberry protein extract on DEAE–cellulose and showed that ripening strawberry fruits contain two aldolase isoenzymes. The isoenzymes were characterized by their elution order on an anion exchange column, molecular weight, subunit molecular weight, pH optimum, K_m value, Mg^{2+} dependency, heat-stability and recovery of activity after acid treatment. On the basis of their properties the isoenzymes were assigned as cytosol and plastid aldolase, respectively.

We showed for the first time that although the total enzymatic aldolase activity first decreased and later increased during strawberry development the ratio of the two isoenzymes changed significantly (Fig. 2). In strawberries in the green and white ripening stage the plastid aldolase represents 15 and 8% of the total aldolase activity, respectively. These values correspond very well to the reported distribution in green leaves of spinach, corn, wheat and barley (Lebherz et al., 1984; Krüger and Schnarrenberger, 1985; Boldt et al., 1994). In pink and red strawberries the proportion of the cytosolic aldolase increases dramatically accounting for 50 and 75% of the total aldolase activity, respectively. This implies a function of cytosolic aldolase during strawberry fruit ripening. Apparently there is continuous new cytosolic aldolase expression as long as the fruits ripen. In contrast the chloroplast aldolase is gradually degraded during the ripening process.

It has been reported that during strawberry ripening cell enlargement is accompanied by major changes in the cell wall and subcellular structure. At petal fall, starch grains are present in the plastids and Golgi apparatus and ribosomes are abundant (Knee et al., 1977). During development the plastids lose their starch grains, eventually degenerating in ripe fruit. This observation explains the decreasing enzymatic activity of plastidic aldolase during the late stages of strawberry fruit development as shown in this paper. Although changes in the plastids might be considered a senescence characteristic, the mitochondria in ripe fruit remain normal (Manning, 1998). This finding together with the observed increased enzymatic activity of cytosolic aldolase during strawberry fruit ripening provides evidence that enhanced carbohydrate degradation proceeds via glycolysis rather than the oxidative pentose phosphate pathway.

During the course of this study we have cloned a cDNA putatively encoding the cytosolic form of aldolase from strawberry fruit (*SCA1*). Expression analyses both with microarrays and RNA gel blots showed that *SCA1* mRNA level increases during strawberry ripening. This observation is in agreement with the ripening enhanced activity of the cytosolic aldolase enzyme detected with the partially purified protein. *SCA1* expression suggests that it is involved in an important process occurring in the ripening receptacle.

Dramatic increase in aldolase mRNA and protein levels is often associated with various stress situations. They include anaerobic conditions (Andrews et al., 1994), desiccation and salt stress (Minhas and Grover, 1999). In these studies the induction of aldolase was normally accompanied by the induction of additional glycolytic (for example enolase and glyceraldehyde-3-phosphate dehydrogenase) and other enzymes involved in alcohol fermentation (for example, alcohol dehydrogenase and pyruvate decarboxylase). Minhas and Grover (1999) suggested that glycolytic and fermentation enzymes have a sufficient degree of flexibility to adjust to increased energy demand and supply of intermediates during stress conditions. We suggest that during strawberry fruit ripening, the receptacle passes through elevated stress conditions which may result in the induction of glycolytic and fermentation enzymes such as aldolase and pyruvate decarboxylase. The expression of the latter showed similar induction to aldolase during strawberry fruit ripening (Aharoni et al., 2000).

4. Experimental

4.1. General experimental procedures

Tris base, SDS and all other reagents were of analytical grade and were supplied by Sigma. Protein standards for SDS–PAGE and size exclusion chromatography were from Amersham Pharmacia Biotech. All solutions were prepared with Milli-Q-processed water. D-[U-^{14}C]-fructose-1,6-bisphosphate (153.4 mCi mmol^{-1}) was purchased from Sigma. Solvents and chemicals were purchased from Roth, Fluka, Aldrich and Merck. Protein concentrations were routinely determined by the dye-binding method of Bradford (1976) with bovine serum albumin as the protein standard. The protein concentration was also assessed by using the Pierce Chemical Co.-prepared BCA reagent. The native M_r of aldolase was estimated by gel filtration at room temperature on a calibrated Sephacryl S300 HR column 1.6×70 cm) equilibrated with 0.2 M Tris–HCl (pH 7.7). Calibration was performed with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa). Fractions (2 ml) were collected with a flow rate of 0.2 ml min^{-1} and assayed for A_{280} and aldolase activity. The native molecular mass of aldolase was determined from a plot of K_D (partition coefficient) versus log (molecular mass) for the protein standards. SDS–PAGE was performed with a Hoefer mini-gel apparatus (Pharmacia Biotech) in a discontinuous system. Polypeptides were resolved on 12% (w/v) polyacrylamide 1.0 mm-thick slab gels containing 0.1% sodium dodecyl sulfate (separating gel) (ratio of acrylamide to *N*,*N*′-methylenebisacrylamide 37.5:1) according to Laemmli (1970) with 3% (w/v) polyacrylamide for the stacking gel. All samples were preincubated in the presence of SDS sample buffer (250 mM Tris pH 6.8, 10% SDS, 40% (w/v) glycerol, 20% (w/v) mercaptoethanol, 0.005% (w/v) Bromophenol Blue) for 3 min at 100°C before being loaded on to the gel. Gels were run at a constant current of 40 mA until they reached the constant voltage of 150 V. For the determination of subunit M_r by SDS–PAGE, a plot of relative mobility versus log (molecular mass) was constructed with the following standard proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Gels were stained for protein with Coomassie blue or with silver (Heukeshoven and Dernick 1985). The metabolism of D-[U-^{14}C]-fructose-1,6-bisphosphate was monitored using high-performance liquid chromatography (HPLC). The HPLC equipment consisted of two Waters 64 pumps equipped with a Canberra Packard FlowOne radioactivity detector. The HPLC was fitted with a Macherey–Nagel Nucleosil 100-10 SB anion exchange column (25 cm×4 mm i.d., particle size 10 μm). The profiling HPLC gradient was conducted at a flow rate of 1 ml min^{-1}, utilizing water and 1 M ammonium formate. The gradient proceeded from 0 to 100% 1 M ammonium formate over 20 min. A Berthold TLC linear analyzer was used with argone:-methane (90:10 v/v) as counting gas at 1380 V. GC–MS consisted of a Finnigan MAT 44 quadrupol mass spectrometer coupled to a Varian 3300 GC equipped with Finnigan MAT PCDS software. A J&W DB5 WCOT fused silica capillary column (25 m×0.25 mm i.d. film thickness 0.25 μm), which was programmed from 60 to 300°C at 4°C min^{-1} was used with 3 ml min^{-1} of helium gas. Significant MS operating parameters were: ionization voltage 70 eV (electron impact ionization); ion source and interface temperature, 250°C; scan range 50 to 500 U, scan duration 1 s. Isolated phosphorylated and non-phosphorylated compounds were hydrolysed by 1 U alkaline phosphatase (Boehringer Mannheim) at room temperature overnight and concentrated to dryness. The residue was dissolved in 2 ml acetonitile:water (85:15, v/v) and the dissolved metabolites reduced with 1 ml of 1% $NaBH_4$ or $NaBD_4$ for 1 h. The excess borohydride or borodeuteride was destroyed with 15% acetic acid and the solution was concentrated to dryness. The boric acid in the residue was removed as methylborate by repeated evaporation with methanol in vacuo. Acetylation was performed using pyridine–acetanhydride as previously described (Beuerle et al., 1999).

4.2. Plant material

Strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta) were purchased from a local market, whereas strawberry fruits (*Fragaria* × *ananassa* cv. Camorosa) were obtained from Hortofruticola de Bonares (Bonafru), Bonares, Spain. Fruits were frozen in liquid N_2 and stored at −20°C until used. For time-course studies of developing strawberries, fruits of different ripening stages were harvested at the same sampling date.

4.3. Enzyme assay and kinetic studies

The aldolase reaction was routinely coupled with the triose phosphate isomerase and glycerol-3-phosphate dehydrogenase reactions and assayed for 60 min at 25°C by monitoring NADH utilization at 10 min intervals at 334 nm with a Beckman DU 600 spectrophotometer. Standard assay conditions for aldolase were 50 mM Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 1 mM D-fructose-1,6-bisphosphate, 0.1 U ml^{-1} glycerol-3-phosphate dehydrogenase, 1 U ml^{-1} triose phosphate isomerase, 0.15 mM NADH in a final volume of 1 ml. All assays were initiated by the addition of D-fructose-1,6-bisphosphate and were corrected for NADH oxidase activity, when necessary, by omitting D-fructose-1,6-bisphosphate from the reaction mixture. One unit of activity is defined as the amount of

enzyme resulting in cleavage of 1 μmol D-fructose-1,6-bisphosphate (equivalent to 2 μmol NADH oxidized) per min at 25°C and specific activity as units per mg of protein. In all cases the rate of reaction was proportional to concentration of enzyme assayed and remained linear with respect to time. Apparent K_m values were determined from the Michaelis–Menten equation by a Lineweaver–Burk plot.

4.4. Preparation of the clarified extract

Frozen strawberry fruits (50 g fr. wt) of different ripening stages and 7.5 g PVPP were homogenized with 100 ml of water in an OMNI mixer (Sorvall) for 2×30 s. The pH was immediately adjusted to 7.7 with 5–8 ml 1 N NaOH depending on the ripening stage. All other standard methods used led to gel formation in the tubes. The suspension was centrifuged for 25 min at 34 000 ***g***. The crude extract was either dialyzed against 20 mM Tris–HCl (pH 7.7) and lyophilized or directly used for the further purification steps.

4.5. Separation of pectic material

Saturated ammonium sulfate solution was added within 30 min to bring the solution to 20% saturation. The slightly turbid solution was stirred for 30 min in the cold and then centrifuged for 25 min at 34 000 ***g***. The residue consisting of jelly-like pectic material was discarded as it showed no aldolase activity. The supernatant was dialyzed against 20 mM Tris (pH 7.7) until the conductivity was less than 1.5 mS.

4.6. Chromatography on diethylaminoethyl (DEAE)–cellulose

Aldolase isoenzymes were separated by chromatography on DEAE–cellulose (Sigma). A column (1.5×9.5 cm) was prepared and equilibrated with 20 mM Tris–HCl (pH 7.7). The dialyzed crude extract was applied to the ion-exchange resin and eluted by a linear gradient of 200 ml of 0–0.4 M KCl in 20 mM Tris–HCl (pH 7.7) at a flow rate of 0.4 ml min^{-1}. Fractions (4 ml) were collected. The elution pattern of the two aldolase isoenzymes was similar to published data (Krüger and Schnarrenberger, 1983; Botha and O'Kennedy, 1989). The ratios of the activities for cytosolic and plastidic aldolase in the extracts were calculated from the activities of the individual fractions in the respective peaks. Fractions containing cytosolic and plastidic aldolase were pooled separately and lyophilized.

4.7. Size exclusion chromatography on Sephacryl S300

Lyophilized aldolase isoenzymes were dissolved in 2–4 ml of 20 mM Tris–HCl (pH 7.7) and applied to a Sephacryl S300 size exclusion column (2.8×100 cm). Proteins were eluted with 0.2 M Tris–HCl (pH 7.7) at a flow rate of 0.2 ml min^{-1}. Fractions (4 ml) were collected and assayed for A_{280} and aldolase activity. Fractions containing aldolase were combined and lyophilized.

4.8. Dry weight calculation and anthocyanin determination

For the calculation of the dry weight, 5 g of strawberries of different ripening stages were weighed, homogenized and dried at 100°C until constant weight of the residue. Extracts obtained by homogenization of 1 g of strawberries of different ripening stages in 1 ml of water were recorded at 498 nm for the determination of anthocyanins.

4.9. Sequence and expression analyses of SCA1

DNA sequencing was performed using the Applied Biosystems (Foster City, CA) dye terminator cycle sequencing Ready Reaction kit and the Applied Biosystems 310 DNA sequencer. Sequence homology search was conducted with the advanced basic local alignment search tool, BLAST X server (Altschul et al., 1990) and the National Center for Biotechnological Information (www.ncbi.nlm.nih.gov, BankIt359018, AF308587) non-redundant protein database. Software used for DNA and protein sequence analyses was the DNASTAR program (DNASTAR Inc., Madison, WI). Total RNA from greenhouse-grown strawberry plants (cultivar Elsanta) was prepared as described by Schultz et al. (1994). For RNA gel blot analysis, 10 μg of glyoxal (1.5 M)-denaturated total RNA was electrophorized and blotted onto Hybond N^+ membrane (Amersham). After fixation (2 h 80°C) blots were hybridized as described by Angenent et al. (1992). The hybridization probe was made by random labelling oligonucleotide priming (Feinberg and Vogelstein, 1984) of the entire *SCA1* cDNA. RNA gel blot was washed two times for 30 min each in 0.1 X SSC and 0.1% SDS at 65°C. Detailed discription of the strawberry microarray experiments including array fabrication, hybridization and data analyses were described previously (Aharoni et al., 2000).

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Biotransformation of cedrol by *Curvularia lunata* ATCC 12017

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Dedicated to Dr Earle V. Roberts in celebration of his service of over 30 years to the Chemistry Department, University of the West Indies, Mona Campus.

Abstract

Bioconversion of cedrol (**1**) with the *Curvularia lunata* was investigated in two different growth media. Five products were obtained in potato dextrose broth, whereas nine compounds were produced in a medium containing beef extract. Only three of the metabolites: 3β-hydroxycedrol (**2**), 3α-hydroxycedrol (**3**) and 12-hydroxycedrol (**4**) were common to both media. They were also obtained as the major products in each case. Three new derivatives have also been identified. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Curvularia lunata* ATCC 12017; Cedrol; Biotransformation; Hydroxylation; Cedrane; Sesquiterpene

1. Introduction

Curvularia lunata is a deuteromycete best known in chemistry for the 11β-hydroxylation of steroids (Holland, 1992). Strain ATCC 12017 (synonyms: IMI 61535, CBS 215.54, NRRL 2380) has been used previously to effect the biotransformation of eudesmane (Garcia-Granados et al., 1991) and aromadendrane (de Lima et al., 1999) sesquiterpenes as well as the diterpene sclareol (Kouzi and McChesney, 1990; Aranda et al., 1991).

The sesquiterpene cedrol (**1**) is isolated from the volatile oil of *Juniperus* sp. Odiferous analogues of this compound have potential as high value products in the perfume industry. This terpene has been biotransformed by several microorganisms previously including *Aspergillus niger* (Wang et al., 1972), *Beauveria sulfurescens* (Lamare et al., 1987), *Cephalosporium aphidicola* (Hanson and Nasir, 1993), *Glomerella cingulata* (Miyazawa et al., 1995) and *Mucor plumbeus* (Fraga et al., 1996). Extensive transformations of **1** have also been carried out by Abraham (Abraham et al., 1987) using *Rhizopus stolonifer, Streptomyces bikiniensis, Verticillium tenerum, Streptoverticillium reticuli* and *Corynespora cassicola*. Maatooq (Matooq et al., 1993), using *Streptomyces griseus* and *Bacillus cereus*, also produced several hydroxycedrane derivatives. No report of the biotransformation of **1** using *C. lunata*, however, has been encountered.

In an ongoing programme to examine the metabolism of terpenes by fungi (Hanson et al., 1994; Buchanan and Reese, 2000), **1** was fed to *C. lunata*. The microorganism was cultivated in two different liquid media previously used in biotransformation studies with this fungus, viz, potato dextrose broth (PDB; Chen and Wey, 1990), and a beef extract medium (BEM; Garcia-Granados et al., 1991). Five known products were obtained from growth in PDB. Metabolism of **1** was, however, more extensive in BEM, resulting in the isolation of nine analogues, three in common with those from PDB. In addition three new derivatives: 7-hydroxycedrol (**10**), 2,12-dihydroxycedrol (**11**) and 7,12-dihydroxycedrol (**12**) were obtained.

2. Results and discussion

Incubation of cedrol (**1**) with *C. lunata* for 14 days in PDB resulted in the production of 3β-hydroxycedrol (**2**), 3α-hydroxycedrol (**3**), 12-hydroxycedrol (**4**), 3β-hydroxycedrene (**6**) and 3-oxocedrol (**7**). Transformation of **1** with *C. lunata* in BEM gave 2-hydroxycedrol (**5**), 4β-hydroxycedrol (**8**), 10β-hydroxycedrol (**9**), 7-hydroxycedrol (**10**), 2,12-dihydroxycedrol (**11**) and 7,12-dihydroxycedrol (**12**) in addition to the major metabolites **2**, **3** and **4**. The known compounds **2**–**9** were identified by

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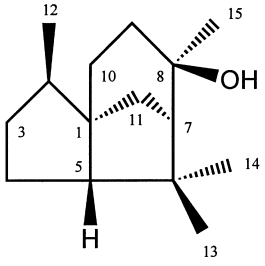
comparison of their spectral data with that in the literature (Hanson and Nasir, 1993 [**2**, **4**, **6**, **7**]; Fraga et al., 1996 [**2**, **3**, **4**, **5**]; Abraham et al., 1987 [**2**, **3**, **4**, **5**, **8**, **9**]; Miyazawa et al., 1995 [**2**, **3**, **4**]; Matooq et al., 1993 [**2**, **3**, **4**, **5**, **8**, **9**]; Lamare et al., 1987 [**3**]) (Fig. 1).

Data from the HREIMS of **10** suggested a molecular formula of $C_{15}H_{26}O_2$ ($[M]^+ = 238.1930$) which indicated the presence of an additional hydroxyl group in the molecule. The ^{13}C NMR spectrum revealed that insertion of the new hydroxyl group (δ_C 85.1) occurred simultaneously with the disappearance of a methine. Finally, downfield shifts in resonances of the adjacent carbons (C-6, C-8 and C-11) justified the positioning of the new hydroxyl group at C-7.

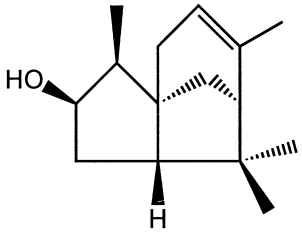
The structure of 2,12-dihydroxycedrol (**11**) followed from the ^1H NMR spectrum that contained two doublets (δ_H 3.52, $J = 11.0$; 3.64, $J = 11.0$ Hz) that were characteristic of a primary alcohol. The methyl doublet of the starting material was also absent. The ^{13}C NMR spectrum contained a $CH_2{-}O$ signal at δ 66.7 that was therefore assigned to C-12. A third hydroxyl group was identified in this isomer, notably borne by a non-protonated carbon atom (δ_C 83.7). The carbon shifts seen within the molecule bore a striking resemblance to those of **5** with hydroxylation having occurred at C-2. Compound **11** was thus assigned as the 2,12-dihydroxy derivative.

The methyl doublet of the substrate was also missing from the ^1H NMR spectrum of **12** pointing to hydroxylation having occurred at C-12. This C-12 methyl group was replaced by an oxygen bearing methylene (δ_C 63.6) in the ^{13}C NMR spectrum. The presence of a third hydroxyl function was confirmed by HREIMS that gave a molecular formula of $C_{15}H_{26}O_3$ ($[M]^+ = 254.1881$). This hydroxyl group was borne on a non-protonated carbon (C-7). A comparison of the carbon resonance values of this metabolite with both **4** and **10** verified congener **12** as 7,12-dihydroxycedrol.

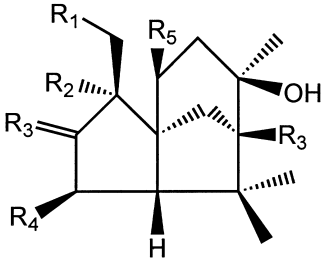
Bioconversion reactions of cedrol in PDB were more regioselective. The oxidation reactions occurred at only two centres, C-3 and -12. The hydroxylases developed by the fungus in BEM, however, appeared to be less selective with hydroxylation occurring at C-2, -3, -4, -7, -10 and -12. Preliminary molecular modeling studies on **11** and **12** indicate that both metabolites may have resulted from a single enzyme system. Both compounds could



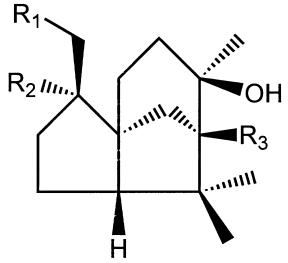
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6



2: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = \beta OH, \alpha H$
3: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = \alpha OH, \beta H$
4: $R_1 = OH$, $R_2 = R_4 = R_5 = H$, $R_3 = H, H$
5: $R_1 = R_4 = R_5 = H$, $R_2 = OH$, $R_3 = H, H$
7: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = O$
8: $R_1 = R_2 = R_5 = H$, $R_4 = OH$, $R_3 = H, H$
9: $R_1 = R_2 = R_4 = H$, $R_5 = OH$, $R_3 = H, H$



10: $R_1 = R_2 = H$, $R_3 = OH$
11: $R_1 = R_2 = OH$, $R_3 = H$
12: $R_1 = R_3 = OH$, $R_2 = H$

Fig. 1. Cedrol and biotransformation products.

Table 1
C-13 NMR chemical shifts for cedranes **1**–**12**

	Compounds											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
C-1	52.0	50.7	52.7	53.2	57.5	52.0	52.6	52.9	60.0	49.3	58.4	48.5
C-2	41.4	50.1	46.0	50.3	79.6	45.8	48.4	38.0	38.1	42.3	83.7	51.1
C-3	37.0	81.4	79.9	32.1	36.5	79.0	220.0	46.7	34.5	36.4	36.5	30.6
C-4	25.3	32.1	32.7	25.8	21.5	33.2	35.9	73.5	23.8	26.5	22.0	26.9
C-5	61.0	52.6	61.4	61.1	59.1	56.8	58.2	61.4	53.4	57.2	59.2	57.0
C-6	43.4	42.6	42.9	43.0	44.9	47.4	44.1	45.1	44.1	45.6	44.2	45.0
C-7	56.5	60.9	54.2	57.4	53.5	55.0	52.3	63.0	59.7	85.1	54.2	85.3
C-8	75.1	74.8	74.9	75.0	74.9	140.2	74.2	74.9	74.7	77.2	74.9	77.7
C-9	35.3	35.3	35.0	34.9	35.6	119.0	35.2	34.7	43.5	34.8	35.7	34.5
C-10	31.6	34.2	33.9	30.8	30.0	39.9	34.4	31.6	71.5	31.3	29.3	31.3
C-11	41.9	43.1	42.4	42.5	41.2	40.9	37.7	42.7	35.0	45.5	37.8	46.1
C-12	15.5	12.4	9.6	63.7	24.3	9.4	8.9	15.3	15.4	15.2	66.7	63.6
C-13	27.6	29.5	27.4	27.4	28.4	26.0	27.8	30.1	28.6	25.4	28.4	25.2
C-14	28.9	27.3	29.1	29.0	27.5	27.4	30.0	28.5	29.3	24.7	27.5	24.8
C-15	30.2	30.2	29.7	30.1	30.3	24.7	30.1	30.6	32.4	26.1	30.2	25.2

have been obtained because the rigid bridged system of the substrate may have two possible orientations in the active site of the enzyme. This idea of a triangular relationship between binding and hydroxylation sites within enzymes is not new (Holland, 1983; Lamare and Furstoss, 1990) and in fact, cedrol (**1**) has been used by Fraga (Fraga et al., 1996) to map the three dimensional topology of enzyme systems in *Mucor plumbeus*.

In summary cedrol was biotransformed by *C. lunata*, growing in PDB, to five known compounds. When the microorganism was grown on a BEM the sesquiterpene was converted to nine analogues, three of which were isolated from the other fermentation. Three of the metabolites, however, have not been reported previously.

3. Experimental

Cedrol (**1**) was obtained commercially from the Maybridge Chemical Company, UK. Column chromatography was performed with Kieselgel silica (40–63 μm). Compounds on thin layer chromatography (TLC) plates were visualised by the use of the ammonium molybdate/sulphuric acid spray reagent followed by heating at 120°C. IR data was acquired on a Perkin Elmer FTIR Paragon 1000 instrument using KBr disks. Optical rotations were acquired on a Perkin Elmer 241 polarimeter. High resolution electron impact mass spectrometry (HREIMS) was done on a Kratos MS50 instrument at an ionising voltage of 70 eV. ^{1}H and ^{13}C NMR spectra were generated in deuterated chloroform at 200 and 50 MHz respectively using a Bruker AC200 instrument. Tetramethylsilane (TMS) was used as the internal standard. ^{13}C NMR assignments are listed in Table 1. *C. lunata* ATCC 12017 was obtained from the American Type Culture Collection, Rockville, MD, USA.

The fungus was maintained on PDA slants. PDB was comprised of potato dextrose broth (24 g l^{-1}; Chen and Wey, 1990). BEM was composed of peptone (1 g l^{-1}), yeast extract (1 g l^{-1}), beef extract (1 g l^{-1}) and glucose (5 g l^{-1}) in water (Garcia-Granados et al., 1991). One 14-day-old slant was used to inoculate four 500 ml conical flasks each containing 125 ml of liquid medium. The flasks were incubated at 200 rpm at 27°C. An EtOAc solution (2 ml) containing 10% of the total mass of the substrate was fed 24 h after inoculation. The remaining 20, 30 and 40% of the substrate was fed at 36, 48 and 60 h after inoculation respectively. The fermentation was allowed to proceed for 10 days after the last feed. The pH was measured and the mycelium was filtered from the broth. Broth extraction utilised EtOAc (3×500 ml). The mycelium was homogenised in EtOAc. The extracts were dried with sodium sulphate, concentrated in vacuo, and analysed by TLC.

Cedrol (**1**) (1 g) was fed to *C. lunata* in 20 flasks of each medium as outlined above. After incubation the fungus was harvested to give broth extracts (0.818 and 0.883 g) and mycelial extracts (0.442 and 0.540 g) from PDB and BEM respectively. The pH at the end of each fermentation was 6.5. Analysis of both extracts by TLC indicated the presence of biotransformed compounds. The broth and mycelial extracts of each fermentation were combined and subjected to column chromatography using increasing concentrations of EtOAc in petrol. Untransformed cedrol was recovered (PDB, 555 mg; BEM, 337 mg). Three new metabolites, **10**, **11** and **12**, were obtained

from the extracts of the fermentation in BEM. They were eluted in solutions of 3, 5 and 10% EtOAc in petrol respectively.

3.1. 3β-Hydroxycedrol (**2**) (PDB, 78 mg; BEM, 84 mg)

Cubes from MeOH; m.p. 147–148°C, $[\alpha]_D^{25}$: +3.41° ($c=3.8$, $CHCl_3$) [lit. m.p. 149.5–152.4°C, $[\alpha]_D^{20}$: +3.21° ($c=1.0$, $CHCl_3$) (Miyazawa et al., 1995)]; IR ν_{max} cm^{-1}: 3280 (OH); ^1H NMR: δ 0.91 (3H, *d*, $J=7.3$ Hz, H-12), 1.01 (3H, *s*, H-14), 1.26 (3H, *s*, H-13), 1.34 (3H, *s*, H-15), 1.64 (1H, *m*, $w/2=3.7$ Hz, H-4), 1.90 (1H, *m*, $w/2=7.0$ Hz, H-11), 2.17 (1H, *m*, $w/2=3.7$ Hz, H-5), 4.29 (1H, *q*, $J=4.1$ Hz, H-3).

3.2. 3α-Hydroxycedrol (**3**) (PDB, 24 mg; BEM, 56 mg)

Needles from MeOH; m.p. 161–162°C, $[\alpha]_D^{25}$: +13.62° ($c=7.8$, $CHCl_3$) [lit. m.p. 159.5–162.7°C, $[\alpha]_D^{20}$: +12.87° ($c=1.0$, $CHCl_3$) (Miyazawa et al., 1995)]; IR ν_{max} cm^{-1}: 3331 (OH); ^1H NMR: δ 0.96 (3H, *d*, $J=7.0$ Hz, H-12), 1.03 (3H, *s*, H-14), 1.26 (3H, *s*, H-13), 1.34 (3H, *s*, H-15), 1.80 (1H, *m*, $w/2=11.0$ Hz, H-4), 3.61 (1H, *ddd*, $J=5.1$, 9.8, 10.1 Hz, H-3).

3.3. 12-Hydroxycedrol (**4**) (PBD, 156 mg; BEM, 368 mg)

Needles from EtOH; m.p. 121–122°C, $[\alpha]_D^{25}$: +8.00° ($c=13.7$, $CHCl_3$) [lit. m.p. 124.9–127.2°C, $[\alpha]_D^{20}$: +9.78° ($c=1.0$, $CHCl_3$) (Miyazawa et al., 1995)]; IR ν_{max} cm^{-1}: 3317 (OH); ^1H NMR: δ 1.01 (3H, *s*, H-14), 1.26 (3H, *s*, H-15), 1.33 (3H, *s*, H-13), 1.50 (1H, *m*, $w/2=3.5$ Hz, H-4), 3.49 (1H, *m*, $w/2=12.9$ Hz, H-12), 3.68 (1H, *m*, $w/2=12.9$ Hz, H-12).

3.4. 2-Hydroxycedrol (**5**) (BEM, 24 mg)

Oil; $[\alpha]_D^{25}$: +10.52° ($c=12.9$, $CHCl_3$) [lit. m.p.137°C, $[\alpha]_D$: +10.6° ($c=1.0$, $CHCl_3$) (Abraham et al., 1987)]; IR ν_{max} cm^{-1}: 3472 (OH); ^1H NMR: δ 1.05 (3H, *s*, H-15), 1.18 (3H, *s*, H-14), 1.28 (3H, *s*, H-13), 1.30 (3H, *s*, H-12), 1.52 (1H, *m*, $w/2=5.9$ Hz, H-9), 1.61 (1H, *m*, $w/2=5.9$ Hz, H-10), 1.77 (1H, *m*, $w/2=7.5$ Hz, H-3), 1.80 (3H, *m*, $w/2=7.5$ Hz, H-4), 1.86 (1H, *m*, $w/2=4.0$ Hz, H-11).

3.5. 3β-Hydroxycedrene (**6**) (PDB, 39 mg)

Oil; $[\alpha]_D^{25}$: −35.22° ($c=13.3$, $CHCl_3$) [lit. oil. (Hanson and Nasir, 1993)]; IR ν_{max} cm^{-1}: 3400 (OH), 1466; ^1H NMR: δ 0.89 (3H, *d*, $J=7.3$ Hz, H-12), 0.96 (3H, *s*, H-14), 1.04 (3H, *s*, H-13), 1.41 (1H, *m*, $w/2=3.0$ Hz, H-2), 1.62 (1H, *m*, $w/2=3.0$ Hz, H-3), 1.67 (3H, *dd*, $J=1.5$, 3.8 Hz, H-15), 1.76 (1H, *m*, $w/2=4.5$ Hz, H-7), 1.83 (1H, *d*, $J=7.3$ Hz, H-2), 1.90 (1H, *d*, $J=2.5$ Hz, H-5), 4.34 (1H, *q*, $J=5.0$ Hz, H-3), 5.21 (1H, *bs*, H-9).

3.6. 3-Oxocedrol (**7**) (PDB, 21 mg)

Cubes from EtOH; m.p. 94–96°C, $[\alpha]_D^{25}$: −28.10° ($c=3.5$, $CHCl_3$) [lit. m.p. 113–114°C (Hanson and Nasir, 1993)]; IR $-_{max}$ cm^{-1}: 3460 (OH), 1731 (CO); ^1H NMR: δ 0.96 (3H, *d*, $J=1.6$ Hz, H-14), 1.00 (3H, *d*, $J=7.6$ Hz, H-12), 1.30 (3H, *s*, H-13), 1.31 (1H, *m*, $w/2=1.9$ Hz, H-11), 1.35 (1H, *m*, $w/2=1.9$ Hz, H-11), 1.40 (1H, *s*, H-10), 1.41 (3H, *s*, H-15), 1.57 (1H, *m*, $w/2=7.0$ Hz, H-7), 1.78 (1H, *m*, $w/2=3.8$ Hz, H-10), 2.10 (1H, *d*, $J=7.3$ Hz, H-2), 2.18 (1H, *d*, $J=1.0$ Hz, H-5), 2.28 (1H, *d*, $J=5.7$ Hz, H-4), 2.37 (1H, *m*, $w/2=8.9$ Hz, H-4).

3.7. 4β-Hydroxycedrol (**8**) (BEM, 17 mg)

Oil; $[\alpha]_D^{25}$: +5.19° ($c=1.9$, $CHCl_3$) [lit. m.p. 118°C, $[\alpha]_D$: +5.9° ($c=1.0$, $CHCl_3$) (Abraham et al., 1987)]; IR ν_{max} cm^{-1}: 3340 (OH); ^1H NMR: δ 0.92 (3H, *d*, $J=7.0$ Hz, H-12), 1.26 (3H, *s*, H-15), 1.35 (3H, *s*, H-14), 1.41 (3H, *s*, H-13), 4.31 (1H, *ddd*, $J=2.9$, 6.3, 6.6 Hz, H-4).

3.8. 10β-Hydroxycedrol (**9**) (BEM, 60 mg)

Cubes from EtOH; m.p. 93–94°C, $[\alpha]_D^{25}$: −9.52° ($c=26.5$, $CHCl_3$) [lit. m.p. 88°C, $[\alpha]_D$: −9.8° ($c=1.0$, $CHCl_3$) (Abraham et al., 1987)]; IR ν_{max} cm^{-1}: 3416 (OH); ^1H NMR: δ 0.91 (3H, *d*, $J=6.6$ Hz, H-12), 1.02 (3H, *s*, H-14), 1.27 (3H, *s*, H-13), 1.36 (1H, *d*, $J=10.1$ Hz, H-11), 1.45 (3H, *s*, H-15), 1.59 (1H, *t*, $J=10.1$ Hz, H-7), 1.72 (1H, *m*, $w/2=6.2$ Hz, H-5), 1.80 (1H, *m*, $w/2=4.1$ Hz, H-9), 1.89 (1H, *m*, $w/2=4.1$ Hz, H-3), 1.95 (1H, *m*, $w/2=4.1$ Hz, H-2), 3.95 (1H, *d*, $J=4.8$ Hz, H-10).

3.9. 7-Hydroxycedrol (**10**) (BEM, 3 mg)

Oil; $[\alpha]_D^{25}$: +14.53° ($c=1.1$, $CHCl_3$); EIMS *m/z* 238.1930 ($[M]^+$, $C_{15}H_{26}O_2$, 238.1932), 193.1590 (64), 165.1277 (30), 154.0991 (22); IR ν_{max} cm^{-1}: 3566 (OH); ^1H NMR: δ 0.88 (3H, *d*, $J=7.0$ Hz, H-12), 0.97 (3H, *s*, H-14), 1.21 (3H, *s*, H-13), 1.35 (3H, *s*, H-15), 1.40 (1H, *m*, $w/2=5.3$ Hz, H-11), 1.62 (1H, *m*, $w/2=4.0$ Hz, H-2), 1.80 (1H, *m*, $w/2=5.3$ Hz, H-11), 2.35 (1H, *m*, $w/2=7.6$ Hz, H-5).

3.10. 2,12-Dihydroxycedrol (**11**) (BEM, 13 mg)

Oil; $[\alpha]_D^{25}$: +10.42° ($c=3.5$, $CHCl_3$); EIMS *m/z* 254.1884 ($[M]^+$, $C_{15}H_{26}O_3$, 254.1882), 223.1699 (100), 165.1281 (30), 147.1178 (49); IR ν_{max} cm^{-1}: 3453 (OH); ^1H NMR: δ 1.01 (3H, *s*, H-15), 1.27 (3H, *s*, H-14), 1.31 (3H, *s*, H-13), 1.59 (1H, *m*, $w/2=6.7$ Hz, H-9), 1.73 (1H, *m*, $w/2=4.2$ Hz, H-4), 3.52 (1H, *d*, $J=11.0$ Hz, H-12), 3.64 (1H, *d*, $J=11.0$ Hz, H-12).

3.11. 7,12-Dihydroxycedrol (**12**) (BEM, 10 mg)

Oil; $[\alpha]_D^{25}$: +8.90° ($c=2.7$, $CHCl_3$); EIMS m/z 254.1881 ($[M]^+$, $C_{15}H_{26}O_3$, 254.365), 236.1775 (94), 165.1277 (20), 147.1179 (21); IR ν_{max} cm^{-1}: 3416 (OH); ^1H NMR: δ 0.98 (3H, *s*, H-14), 1.22 (3H, *s*, H-13), 1.35 (3H, *s*, H-15), 1.47 (1H, *m*, $w/2=3.2$ Hz, H-3), 1.62 (1H, *m*, $w/2=3.2$ Hz, H-2), 1.71 (1H, *d*, $J=2.2$ Hz, H-11), 1.75 (1H, *t*, $J=1.6$ Hz, H-5), 3.54 (1H, *dd*, $J=7.3$, 11.4 Hz, H-12), 3.67 (1H, *dd*, $J=6.6$, 11.1 Hz, H-12).

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Ripening-related changes in raspberry cell wall composition and structure

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Abstract

Cell walls were prepared from the fruit of two cultivars of raspberry at three stages of ripening; green, white and red (ripe). The cultivars, Glen Clova and Glen Prosen, are subjectively classified, at harvest by growers, as soft and firm fruit, respectively. The cell walls were analysed for neutral sugar composition, uronic acid content, degree of methyl esterification, lignin and ferulic acid-derived dehydrodimers. Solid-state ^{13}C NMR and diffuse reflectance infrared (DRIFT) spectra were acquired for the cell wall residues. For both cultivars the progression from green to white produced minimal changes, save for a reduction in pectin. NMR analyses indicated that the solubilized pectin was acetylated. Progression to the red (ripe) stage, in both cultivars, was accompanied by a reduction in the ordered cellulose and a dramatic reduction in pectin content and the degree of methyl-esterification. Significantly, the softer fruit (Glen Clova) exhibited greater reductions in both parameters, implicating increased pectin hydrolysis, as one of the main factors contributing to the difference in firmness between the cultivars. A relative increase in cell wall-associated protein was seen at the red stage. The nature and function of the protein(s) are, as yet unknown. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Rubus*; Ripening; Cell-wall; Firmness; Structure

1. Introduction

The mechanisms and processes behind the chemical and structural changes in the cell walls of fruit during ripening have been the focus of much research (Brady, 1987; Waldron et al., 1997; Brownleader et al., 1999). However, the ripening of soft fruit, such as strawberry and, in particular, raspberry, has been poorly documented. For raspberry at least, there may be several reasons for this; a limited market size, a short post-harvest shelf-life and, to a lesser extent, the difficulty in separating the cell wall from the seed.

Recent studies have shown that the inclusion of fresh fruit in the diet improves general health. These improvements arise from an increased consumption of cell wall-related dietary fibre (Eastwood, 1992; MacDougall et al., 1995) and ingestion of antioxidant plant phenolics (Sarkar et al., 1996), such as the anti-carcinogen, ellagic acid (Maas et al., 1991; Rommel and Wrolstad, 1993). The promotion of raspberries, or for that matter any other fruit, as being beneficial for health will mean that more data will be required regarding the composition and structure of the fruit (both walls and contents) during ripening, at harvest and during storage. To this end we studied raspberry and have devised a simple, but effective, method to fractionate the fruit cell walls and seeds.

Within the raspberry family there are, like most fruit, varietal differences with respect to specific parameters, especially sweetness and firmness. The latter is particularly important since excessive handling leads to bruising and spoilage resulting in unpalatable fruit with a reduced shelf-life. The (bio)chemical basis behind these differences, in particular firmness, have yet to be established.

Here we report the changes in cell wall composition and structure, at three stages of ripening, of two cultivars of raspberry, Glen Clova and Prosen, subjectively classified by growers as soft and firm, respectively.

2. Materials and methods

Two raspberry genotypes, Glen Clova and Prosen, were selected from the breeding program of SCRI. The fruits were sampled from field-grown plants at three

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stages of ripening; green, white and red (ripe). The fruits were homogenized in 20 mM sodium acetate (pH 4.5) containing 0.05% Triton X-100 then poured onto a 0.5 mm sieve. The homogenate was then gently pressed through the sieve using the flat bottom of a beaker. The material passing through was collected and re-sieved as before until no seeds remained. The resultant cell wall suspension was then washed twice with distilled water, centrifuged (2000 ***g***) and the pellet was boiled in 80% ethanol for 30 min, washed (×2) with neat ethanol, filtered and dried at 40°C for 16 h to give a crude cell wall (CCW) preparation. The CCWs were subject to Soxhlet extraction for 16 h with acetone as the solvent. (This removed virtually all residual colour although the red stage CCW did retain a pink tinge.) Following Soxhlet extraction the CCWs were kept in a dessicator until required. Karl–Fischer analysis showed that the CCWs had water contents of 7±1%.

These residues were used for diffuse reflectance Fourier-transform infra-red (DRIFT) and solid-state NMR spectroscopic analyses as described by Stewart et al. (1997). Briefly, Fourier-transform infrared spectroscopy was performed on a Bruker IFS 66 FT-IR spectrometer using a Diffuse Reflectance FT-IR (DRIFT) cell (Graseby-Specac, Worthington, UK). Two hundred scans were acquired at a resolution of 4 cm^{-1}. Cross Polarisation/Magic Angle Spinning Nuclear Magnetic Resonance (CP/MAS NMR) spectra were acquired on a Chemagnetics 300 MHz NMR spectrometer operating at 75.46 MHz using an air-bearing probe. The spinning rate was 4 kHz, contact time 1 ms, acquisition time 41 ms, sweep width 400 ppm and a delay between pulses of 2 s. Ten thousand transients were accumulated for each sample.

Neutral monosaccharides were determined using the phenol–sulphuric method as described by Stewart et al. (1995). The uronic acids were determined using the modified sulfamate/*m*-hydroxydiphenyl method of Filisetti-Cozzi and Carpita (1991) whilst the degree of methyl esterification was determined by measuring the methanol released following hydrolysis of the ester using the method of Knee (1978). Ester-bound cinnamic acid-derived dehydrodimers were analysed for using reverse phase HPLC following alkaline (1 M NaOH) hydrolysis of the CCWs as described by Grabber et al. (1995). Lignin content was determined using the acetyl bromide/perchloric acid method as described by Iiyama and Wallis (1988) but the levels were low ($\leqslant 1$ [±0.5]%) and are not reported here. All analyses are the mean of triplicates with the exception of the uronic acid and methyl ester data which are the mean of five analyses.

Fruit firmness was determined by recording the force necessary to penetrate the skin of a raspberry druplet. A penetrometer (Pioden Controls Ltd, UK) with a load-cell (UF1), range 0–100 g was used in conjunction with a motorized platform. The penetrator was a needle tapered to a 0.5 mm diameter flat face and advanced at 0.3 mm s^{-1} with a full penetration depth of 5 mm. The force, recorded as milli-Newtons (mN), was recorded after each motorized advance. The reading prior to druplet burst was taken to be the firmness.

3. Results and discussion

The compositional data of the CCWs are shown in Table 1 and the corresponding DRIFT and solid-state NMR spectra in Figs. 1 and 2, respectively. The progression from green to white produced minimal changes in the composition of the CCWs and these were largely restricted to a reduction in the level of pectin (as uronic acid) and the degree of pectin methyl-esterification. The corresponding DRIFT spectra show little, if any, change. This change is restricted to a broadening of the pectic CO_2H/CO_2^- and methyl ester $-CH_3$ absorbances centered at 1600–1660 and 1420 cm^{-1}, respectively, in the white stages of both cultivars. This is probably due to cell wall hydrolysis (Iannetta et al., 1999) producing a range of different chemical environments thereby broadening the range of absorbance frequencies. There is evidence of reduced level of uronic acid and degree of methyl esterification in the solid-state NMR spectra. There are small but significant reductions in intensity of the pectic acid/ester carbonyl resonance at 176–171 ppm and the associated ester OCH_3 at 53 ppm. There are no observable reductions in the pectic carbohydrate signals as these are masked by the overwhelming presence of the other cellulose and non-cellulosic-derived signals. However there is a reduction in the acetyl CH_3 resonance at 22 ppm. Where the plant cell wall is concerned the acetyl group is invariably associated with non-cellulosic polysaccharides (NCPs) (Hulleman et al., 1994; Stewart et al., 1995) and since the principal change in NCP has been a reduction in pectin it is likely that the acetyl groups are present on pectin. Acetylated pectins have previously been reported in other fruit (MacDougall et al., 1996).

Although the cell wall neutral sugar compositional data showed little change over the progression from green to white the pectin contents and measured firmness of both of the cultivars reduced by similar levels of 19 and 12%, and, 31 and 33%, respectively, for Glen Clova and Glen Prosen, respectively (Table 2).

It was with the progression from the white to red (ripe) stage that the most significant changes in both composition and cell wall structure occurred and where difference between cultivars became evident. Both cultivars experienced a net reduction in NCPs (almost entirely accounted for by pectin) of 27 and 24.8% for Glen Clova and Glen Prosen, respectively. (The corresponding reductions on the progression from green to white were 10.0 and 9.7%, respectively.) This suggests

Table 1
The neutral sugar, uronic acid and methyl esterification content of CCWs prepared from Glen Prosen and Glen Clova raspberries at the green, white and red (ripe) stages[a]

	Rha	Ara	Xyl	Man	Gal	Glc	UA	%Me
Glen Clova								
Green	0.4 (0.1)	2.2 (0.1)	23.2 (1.0)	0.5 (0.1)	1.7 (0.3)	0.6 (0.2)	26.8 (1.4)	39 (5)
White	0.3 (0.1)	2.2 (0.1)	23.5 (0.8)	0.5 (0.1)	1.2 (0.2)	0.7 (0.2)	21.6 (1.5)	31 (6)
Red	0.3 (0.1)	2.1 (0.1)	26.6 (0.4)	0.4 (0.1)	1.1 (0.1)	0.5 (0.1)	5.5 (1.1)	7 (4)
Glen Prosen								
Green	0.4 (0.1)	2.9 (0.1)	24.1 (0.3)	0.7 (0.1)	2.7 (0.1)	1.3 (0.1)	28.0 (1.3)	41 (6)
White	0.4 (0.1)	3.0 (0.1)	24.0 (0.2)	0.6 (0.1)	2.7 (0.1)	1.4 (0.2)	22.0 (1.1)	35 (4)
Red	0.2 (0.1)	2.1 (0.2)	24.6 (0.3)	0.6 (0.1)	2.1 (0.2)	1.4 (0.1)	9.7 (0.8)	14 (3)

[a] Figures in parentheses are the standard errors. Non-cellulosic neutral monosaccharide and uronic acid contents are expressed as mg/100 mg cell wall. The non-cellulosic neutral monosaccharide contents are the mean of triplicates whilst. The uronic acid contents and % methyl esterification are the mean of 5 replicates.

that pectin hydrolysis had advanced, at least during the latter stages of ripening, such that the lower molecular weight fragments were lost during preparation of the CCWs.

Varietal differences were evident within the individual neutral monosaccharide composition. Glen Prosen experienced a relative reduction in arabinose and galactose and to a lesser extent, rhamnose. The concurrent reduction of these monosaccharides is often indicative of rhamnogalacturonan degradation and has been reported in other fruit (Gross and Sams, 1984; Huysamer et al., 1997). Glen Clova underwent small reductions in all the neutral monosaccharides with the exception of xylose, the level of which rose significantly. However, by far the most significant change, in both genotypes, was the large reduction in the levels of pectin and degree of methyl ester. These were reduced by 74.5 and 77.4% and 55.9 and 60.0%, respectively for Glen Clova and Glen Prosen. This varietal difference in the pectin levels may give some clues to the origin of the measured differences in firmness. At the red (ripe) stage Glen Prosen was significantly firmer (37%) than Glen Clova (Table 2). The firmness measurements were done on individual druplets, a complex tissue comprised of hundreds of cells (B. Williamson, personal communication, 2000). It is logical, therefore, to assume that this difference in firmness may be contributed to, at least in part, by cell-to-cell adhesion. It is now accepted that there are several factors contributing to cell-to-cell adhesion. Two of these are pectin (and its relative degree of methylation) (Jarvis, 1984; Brady, 1987) while another is the presence of intercellular cinnamic acid-derived bridges in the form of ferulic acid dehydrodimers (Parker and Waldron, 1995; Parr et al., 1996; Waldron et al., 1996). Analyses showed that none of the CCWs analyzed here showed any evidence of having these dimers (data not shown). This suggests that pectin may be one of the key factors determining varietal firmness with reduced levels of pectin backbone and methyl ester hydrolysis during ripening in the firmer variety, Glen Prosen, leading to a comparatively greater molecular weight pectic polymer, more able to maintain cell wall cohesion. Indeed it has been previously shown that, at the red (ripe) stage, Glen Clova (softer fruit) had a relatively greater pectin methylesterase (PME) activity than Glen Prosen, thereby allowing polygalacturonase (PG) more substrate to work with leading to a reduced residual uronic acid content (Iannetta et al., 1999).

At the red stage there was also evidence of extensive changes in the structure of the cell walls. Their DRIFT spectra are significantly different from those obtained from the green and white fruit (Fig. 1). The intensity of the ester C=O peak, centred at 1740 cm^{-1}, is almost double that seen in for the other stages, despite a dramatic reduction in the level of pectin methyl ester (O-$\underline{CH_3}$, 1420 cm^{-1}, Table 1). This is may due to digestion by cell wall hydrolases (Iannetta et al., 1999) during ripening with the non-acetylated regions of the cell wall polymers being digested first resulting in a consequential increase in acetylation.

The DRIFT spectra also show a significant reduction in crystalline or "ordered" cellulose-related absorbances. This was reflected in the relative reduction in absorbance at 1100 cm^{-1} and the increase in intensity and line broadening of the amorphous cellulose-related absorbance at ~900 cm^{-1} (Michell, 1990). (Although xyloglucan can also absorb at this frequency it is reported to accounts for less than 6% of the total cell wall in raspberry; Iannetta et al., 1998.)

The progression from green to white had minimal effect on the degree of ordered cellulose save an apparent reduction in the C_4 resonance, attributed to crystalline cellulose (Stewart et al., 1997), at 89 ppm with a concomitant increase in the corresponding amorphous cellulose peak at 85 ppm (Fig. 2). Also the $C_{2,3,5}$ resonances were slightly less sharp, or defined, at the resonance maxima (73 ppm). However, the progression to the red (ripe) stage saw an large change in the ordered cellulose resonances, with reductions in the intensity of the $C_{1,4,\&6}$ resonances (65, 89 and 105 ppm) and increases in

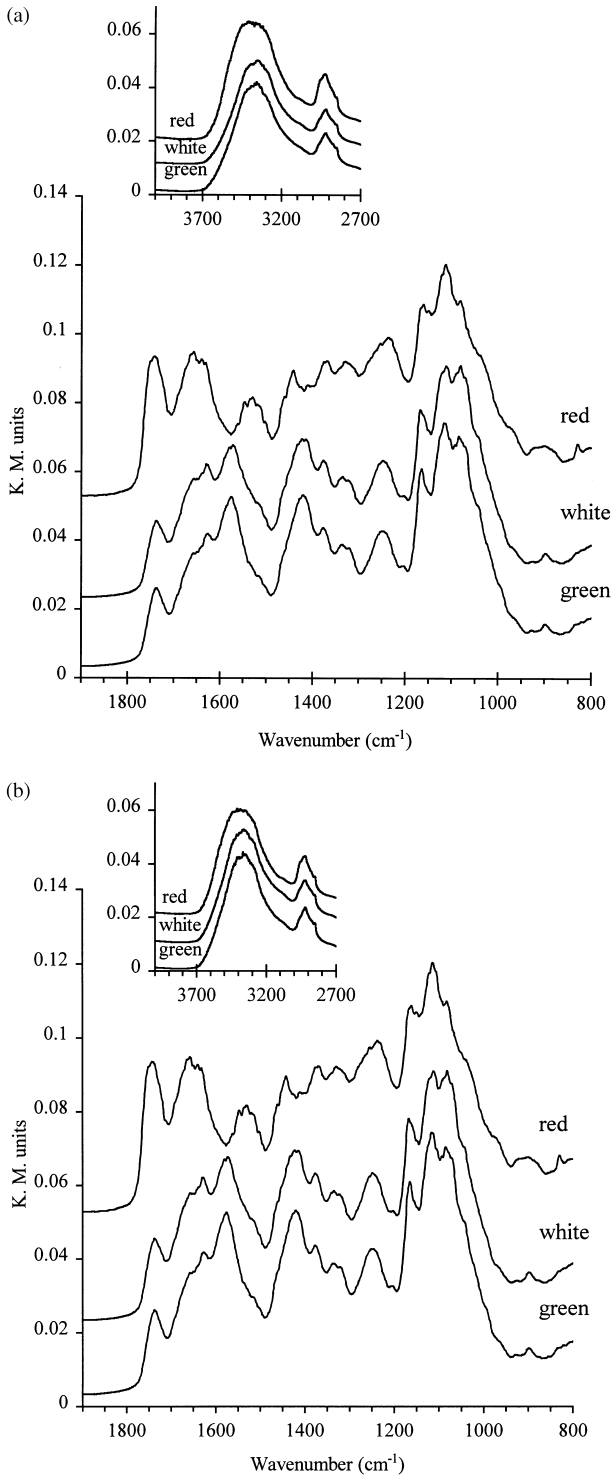


Fig. 1. The DRIFT spectra of crude cell wall preparations from green, white and red (ripe) fruit from (a) Glen Clova and (b) Glen Prosen, respectively.

the corresponding amorphous resonances (63, 85 and 101 ppm) (Earl and Vanderhart, 1981; Attala and VanderHart, 1984). Also, the main $C_{2,3,5}$ resonances collapsed to give one main peak with an upfield shoulder. This is in agreement with the reported increase in cellulase

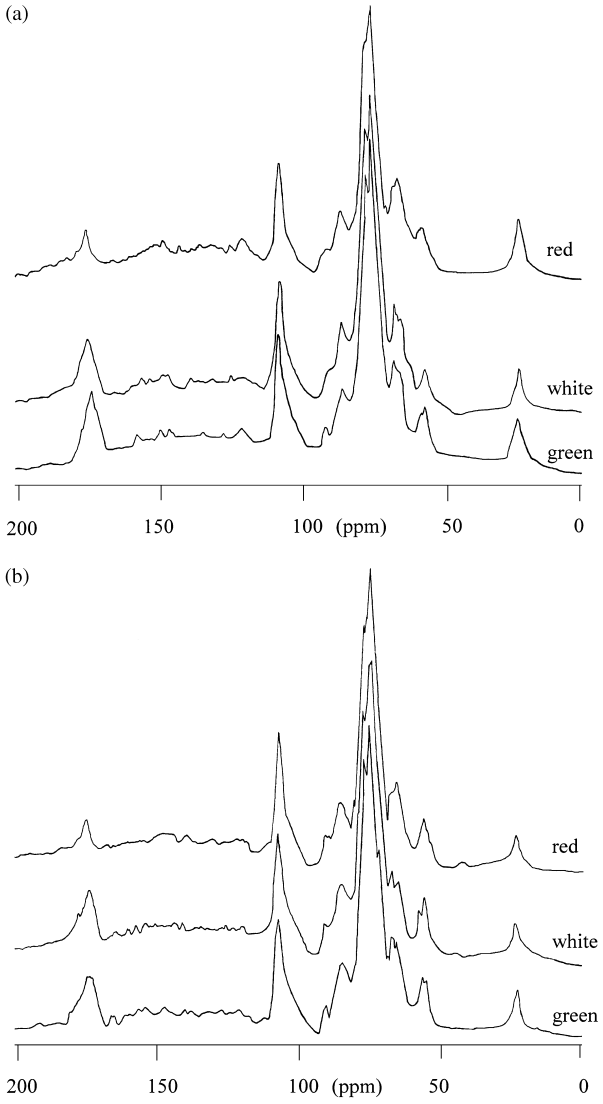


Fig. 2. The solid-state NMR spectra of crude cell wall preparations from green, white and red (ripe) fruit from (a) Glen Clova and (b) Glen Prosen, respectively.

activity on progression from the white to red stage in raspberry fruit (Iannetta et al., 1999).

The pectic methyl ester resonance at 53 ppm also reduced in intensity, at the red ripe stage, but the corresponding acid/ester carbonyl resonance centred at ~174 ppm was still significant despite a large reduction in uronic acid content (Table 1). This may be due to the digestion of the cell wall by glycosylases resulting in a relative increase in cell wall associated protein. The carbonyl resonance of the (protein) amide bond is also, centred at 174 ppm (Kemp, 1991), and the concurrent relative reduction and increase of pectic and protein carbonyl resonance intensities, respectively, results in a small but significant resonance at 174 ppm. This relative increase in protein was corroborated by the corresponding DRIFT spectra (Fig. 1). At both the red stages, the spectra have significant absorbances at 1650

Table 2
The measured firmnesses (mN) of druplets from Glen Clova and Glen Prosen at three stages of ripening

Genotype	Glen Clova	Glen Prosen
Subjective firmness (at harvest)	Soft	Firm
Ripening stage		
Green	605	748
White	415	497
Red	133	210

and 1550 cm^{-1}, regions classically associated with amide (protein) absorbance (Kemp, 1991). Slight additional evidence for this is seen at the higher wavenumber end of the DRIFT spectra (Fig. 1 inset). The water hydration absorbance at 3100–3700 cm^{-1} is similar in both intensity and line shape for the green and white stages within variety. However, at the red (ripe) stage the absorbance has broadened and the maxima has shifted from 3360 to 3410 cm^{-1}, the region of absorbance for the main N−H stretch of secondary amides (including proteins) (Kemp, 1991). Further supportive evidence for the presence of protein in the red (ripe) stage cell walls is seen in the increase in the shoulder of the absorbance centered at 2900 cm^{-1}, a region in which there is an additional, albeit weak, N−H stretch reported to be representative of N−H hydrogen bonding (Kemp, 1991). The origins of these protein absorbances are, as yet, unclear although one possible explanation may be that during the preparation of the CCWs the cytoplasmic proteins have become associated/complexed with the cell wall phenolics and intractably-bound anthocyanins. The complexation of proteins and phenolics has been well reported (McManus et al., 1985).

4. Conclusion

Raspberry appears to follow a classic fruit ripening scenario involving pectin demethylation, hydrolysis and solubilization particularly at the red (ripe) stage. At this latter stage extensive cellulose degradation was evident, as was an apparent relative increase in cell wall bound protein. Hydrolytic reduction of pectin molecular weight and demethylation appears to play a key role in contributing to the difference in firmness between the two genotypes and is likely to contribute to the short shelf-life of raspberry.

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Formation and occurrence of dopamine-derived betacyanins

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Abstract

In light of the fact that the main betaxanthin (miraxanthin V) and the major betacyanin (2-descarboxy-betanidin) in hairy root cultures of yellow beet (*Beta vulgaris* L.) are both dopamine-derived, the occurrence of similar structures for the minor betacyanins was also suggested. By HPLC comparison with the betacyanins obtained by dopamine administration to beet seedlings, enzymatic hydrolysis, LCMS and ^{1}H NMR analyses, the minor betacyanins from hairy roots were identified as 2-descarboxy-betanin and its 6′-*O*-malonyl derivative. A short-term dopamine administration experiment with fodder beet seedlings revealed that the condensation step between 2-descarboxy-*cyclo*-Dopa and betalamic acid is the decisive reaction, followed by glucosylation and acylation. From these data a pathway for the biosynthesis of dopamine-derived betalains is proposed. Furthermore, the occurrence of these compounds in various cell and hairy root cultures as well as beet plants (Fodder and Garden Beet Group) is shown. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Beta vulgaris*; Chenopodiaceae; Hairy roots; Fodder beets; Betacyanins; 2-Descarboxy-betanidin; 2-Descarboxy-betanin; 6′-*O*-Malonyl-2-descarboxy-betanin

1. Introduction

Betalains, the yellow betaxanthins and red–violet betacyanins, are a group of water-soluble pigments characteristic of certain members of the plant order Caryophyllales and some higher fungi (Steglich and Strack, 1990), e.g. red beet (*Beta vulgaris*) and fly agaric (*Amanita muscaria*). The betaxanthins are conjugates of betalamic acid with amino acids or the corresponding amines (including dopamine), while almost all betacyanins are derivatives of betanidin, the conjugate of betalamic acid with *cyclo*-Dopa. The only two enzymes known to be involved in the biosynthesis of the basic skeleton of betalains are tyrosinase (Mueller et al., 1996; Steiner et al., 1996, 1999) and Dopa dioxygenase (Girod and Zryd, 1991a; Terradas and Wyler, 1991; Hinz et al., 1997; Mueller et al., 1997a,b). Whereas tyrosinase is responsible for the formation of Dopa and its oxidation to *cyclo*-Dopa, the Dopa dioxygenase, hitherto only known from fly agaric, catalyzes the Dopa extradiol cleavage, leading to the formation of the chromophore betalamic acid. The decisive step, the linkage of betalamic acid with *cyclo*-Dopa (or *cyclo*-Dopa 5-*O*-glucoside) or amino acids/amines, was suggested to be a non-enzymatic reaction (Trezzini and Zryd, 1990; Terradas and Wyler, 1991) as this condensation process showed neither an amino acid specificity nor a stereoselectivity (Hempel and Böhm, 1997; Schliemann et al., 1999). For the attachment of glucose in betacyanin formation, it is unclear whether the transferase reaction occurs at the *cyclo*-Dopa and/or betanidin level (Vogt et al., 1997, 1999). Moreover, the biosynthesis of dopamine-derived betaxanthin (miraxanthin V) and 2-descarboxy-betanidin has not been investigated thus far.

Until now, the only exception from the *cyclo*-Dopa-derived betacyanins is the dopamine-derived 2-descarboxybetanidin, a minor betacyanin pigment in flowers of *Carpobrotus acinaciformis* (L.) L. Bol. (Aizoaceae) (Piattelli and Impellizzeri, 1970), a xerophilous plant native to South Africa. Recently, 2-descarboxy-betanidin has also been identified (Schliemann et al., 1999) as a betacyanin constituent of hairy root cultures from yellow beet [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Golden Beet'] (nomenclature of cultivated plants according to

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Lange et al., 1999). The other co-occurring minor betacyanins had not been identified at that time due to their low abundance. While administration of the precursors of betalains (tyrosine and derived compounds) to this hairy root culture led to melanin formation due to high tyrosinase activity (Steiner et al., 1999), the administration of dopamine to fodder beet seedlings led to the formation of betacyanins with retention and UV–VIS-spectral properties identical with those of the minor betacyanins in hairy roots. A short-term dopamine administration experiment with fodder beets thus revealed: (i) an alternative pathway in betalain biosynthesis; and (ii) the condensation reaction with betalamic acid takes place prior to glucosylation. In addition, the structures and the distribution of these dopamine-derived betacyanins in various plants are reported here.

2. Results and discussion

The presence of nine betalain pigments (**1**–**9**) has been detected by their characteristic spectral properties in hairy root cultures of yellow beets (Fig. 1A). Betalamic acid (**1**) (Fig. 2) and betanin (**2**) (Fig. 3) as well as its 15*R*-isoform (isobetanin, **2′**) has been readily identified as described recently (Schliemann et al., 1999). Because of the relatively high concentration of dopamine in these hairy root cultures, and in light of the fact that the main betaxanthin (miraxanthin V, **3**) and the major betacyanin, 2-descarboxy-betanidin (**7**), are both dopamine-derived (Schliemann et al., 1999), the occurrence of similar structures for its minor betacyanins was also suggested. Dopamine administration to de-rooted fodder beet plants and HPLC analysis (Fig. 1B) of the hypocotyl extract showed the presence of three major betacyanins (compounds **5**, **7**, **8**) whose retention times and UV–VIS data corresponded to some pigments of the hairy root extract (Fig. 1A). The former structure elucidation of 2-descarboxy-betanidin (**7**) from *C. acinaciformis* (Piattelli and Impellizzerri, 1970) and hairy roots of *B. vulgaris* (Schliemann et al., 1999) was based on comparison with authentic synthetic standard (Minale and Piattelli, 1965; Schliemann et al., 1999). The possibility that this compound was an artefact due to decarboxylation of betanidin was excluded by proving that during heat treatment of betanidin the carboxy group was not split off from C-2, but from C-15 with an additional migration of the C-17/C-18 double bond to the C-14/C-15-position (Minale and Piattelli, 1965; Dunkelblum et al., 1972) leading to 15-descarboxy-betanidin.

The identity of compound **7** (R_t 30.6 min; λ_{max} 533 nm) as 2-descarboxy-betanidin was further confirmed by LCMS analysis which showed the correct protonated molecular ion $[M+H]^+$ at m/z 345 (base peak). Large scale dopamine administration to seedlings of *B. vulgaris* yielded sufficient amounts for isolation and purification by

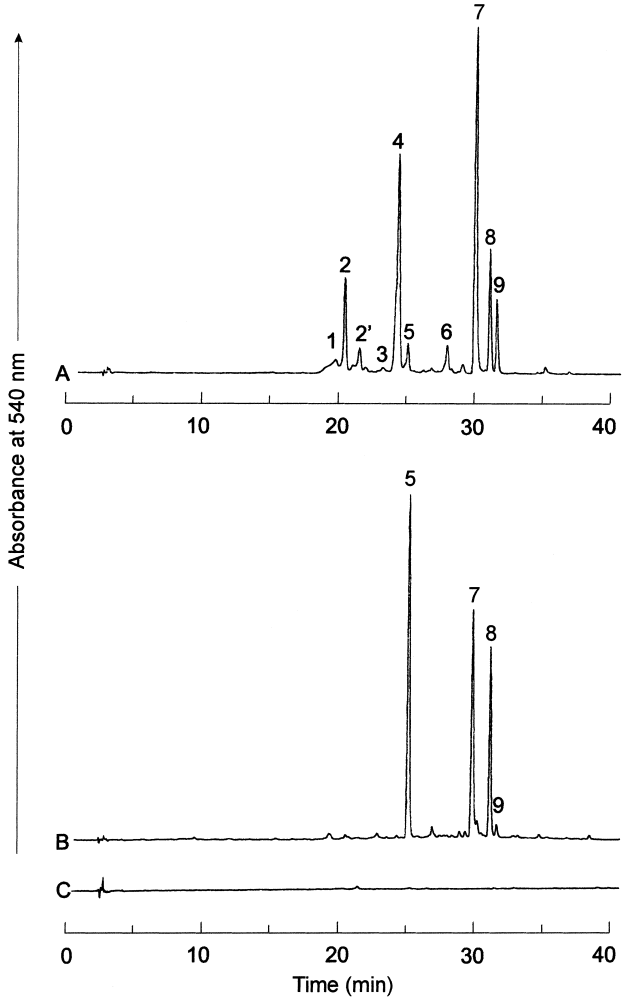


Fig. 1. HPLC patterns of betacyanins in *Beta vulgaris*: (A) hairy root culture (BVL 5A); (B) hypocotyls of fodder beet plants ('Altamo') (28-day-old) after administration of dopamine for 48 h; (C) control (for B); Full scale of A_{540}, (A) 0.2 AU; (B) and (C) 0.1 AU. (Note: Peaks **1** and **3** correspond to the main betalains in hairy root cultures, but are scarcely detectable at 540 nm). For peak identification see Table 1; compound numbers refer to Figs. 2 and 3.

preparative HPLC. The purified compounds were characterized by both analytical HPLC (2-descarboxy-betanin, **5**: R_t 25.5 min, λ_{max} 532 nm; 6′-*O*-malonyl-2-descarboxy-betanin, **8**: R_t 31.8 min, λ_{max} 535 nm; unknown dopamine-derived betacyanin, peak **9**: R_t 32.3 min, λ_{max} 533 nm) and by LCMS analyses (see Table 1). The $[M+H]^+$ ion of **5** was observed at m/z 507. From the mass difference (162) between **5** (m/z 507) and **7** (m/z 345) the presence of an additional hexose was suggested in agreement with the shorter R_t of **5** in HPLC. This assumption was confirmed by treating **5** with β-glucosidase which resulted in almost complete hydrolysis to the aglycone **7** within 10 min. Thus, the presence of glucose in **5** connected in a β-glucosidic linkage with **7** was established. On the basis of its retention characteristics, [betanidin 5-*O*-glucoside (betanin), **2** (Fig. 3), was found to be more polar

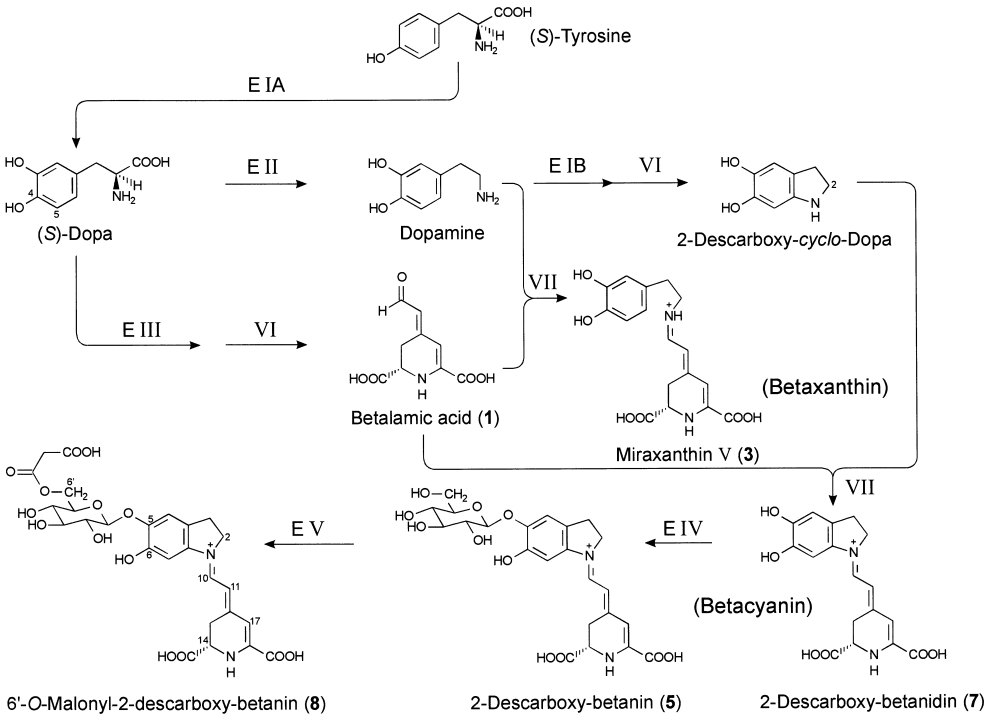


Fig. 2. Proposed biosynthetic pathway to dopamine-derived betalains in *Beta vulgaris* (hairy root culture). Enzymically catalyzed steps: **E IA**, hydroxylating activity of tyrosinase; **E IB**, oxidizing activity of tyrosinase; **E II**, Dopa decarboxylase; **E III**, Dopa 4,5-dioxygenase; **E IV**, glucosyltransferase; **E V**, malonyltransferase. Spontaneous reactions: **VI**, cyclization reaction; **VII**, condensation reaction (aldimine formation). Compound numbers of this pathway refer to Table 1.

Table 1
Retention time, HPLC-PDA and MS data of betalains from hairy root cultures of *Beta vulgaris* L. subspec. *vulgaris* (Garden Beet Group) 'Golden Beet', line 5A[a]

Compound	R_t (min)	HPLC-PDA λ_{max} (nm)	M_r	LCMS $[M+H]^+$	Betalain
1	19.5	405	220	–[b]	Betalamic acid
2	21.8	537	550	551	Betanin
2′	22.3	536	550	–[b]	Isobetanin
3	23.6	457	346	347	Miraxanthin V
4	24.8	539	636	637	Phyllocactin
5	25.5	532	506	507	2-Descarboxy-betanin
6	28.5	536	522	523	Unknown betacyanin
7	30.6	533	344	345	2-Descarboxy-betanidin
8	31.8	535	592	593	6′-*O*-Malonyl-2-descarboxy-betanin
9	32.3	533	650	651	Dopamine-derived betacyanin

[a] Peak numbers correspond to Fig. 1, compound numbers correspond to the formula numbers in Figs. 2 and 3.
[b] Not determined.

than betanidin 6-*O*-glucoside (gomphrenin I) (Heuer et al., 1992)] and the fact that in *Beta* species, hitherto only betanidin derivatives glucosylated at the 5-*O*-position have been found, the new dopamine-derived betacyanin **5** was thought to be a 5-*O*-linked betacyanin. The characteristic signals of **5** in 1D and 2D ^{1}H (COSY) NMR spectroscopic analyses confirmed the proposed structure. In keeping with previous data (Heuer et al., 1994), the small chemical shift difference between H-4 and H-7 of 0.06 ppm is characteristic of substitution at the hydroxyl group at C-5 of the aglycone, as opposed to that at C-6 where differences of ca. 0.8 ppm are to be expected. The double doublet of H-2 at 5.46 ppm of betanidin was not detected, but was instead replaced by a two proton triplet at 4.40 ppm, which indicated the absence of the carboxyl group at the C-2 position as a consequence of the incorporation of dopamine into **5**. The LCMS, enzymatic hydrolysis and NMR spectral data establish that compound **5** is 2-descarboxy-betanidin 5-*O*-*β*-D-glucoside (2-descarboxy-betanin).

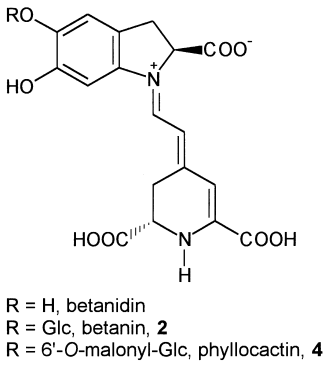


Fig. 3. Structure scheme of *cyclo*-Dopa-derived betacyanins.

Compound **8** (Fig. 1) was less polar than **5**, indicating the possible presence of an acyl moiety (Heuer et al., 1992). LCMS analysis of **8** showed a $[M+H]^+$ ion at *m*/*z* 593, which, in a daughter-ion scan, resulted in an intense peak at *m*/*z* 345, indicating that **8** belongs to the dopamine-derived betacyanins. From the mass difference (86) between **8** (*m*/*z* 593) and **5** (*m*/*z* 507) the presence of a malonyl residue can be deduced. In fact, the presence of a dicarboxylic acid as acylating residue was confirmed by detection of three consecutive losses of CO_2 (two from the betalamic acid part and one from the acyl group) in its ESMS (negative ion mode using API-CID). This is consistent with the similar fragmentation pattern (loss of four CO_2) found for phyllocactin [(betanidin 5-*O*-(6′-*O*-malonyl-*β*-D-glucoside), **4**] isolated from flower petals of Christmas cactus (*Schlumbergera* × *buckleyi*) and analyzed by LCMS under the same conditions. The linkage of the malonyl residue at the 6-*O*-position of glucose in phyllocactin was recently verified by ^{1}H NMR spectroscopic analysis (Kobayashi et al., 2000). Therefore, the site of malonyl attachment in **8** is most likely the same as in **4** (Fig. 3). This was confirmed from the ^{1}H NMR spectral data for **8** where the low field chemical shifts of H-6′A/H-6′B (4.62 and 4.32 ppm), respectively, provide definitive evidence that the acyl system is bound to C-6′ of the glucose moiety. Hence, the combination of LCMS and NMR data identified **8** as 6′-*O*-malonyl-2-descarboxy-betanin [2-descarboxy-betanidin 5-*O*-(6′-*O*-malonyl-*β*-D-glucoside)]. This result is further supported by the detection of phyllocactin (**4**) (Fig. 3), co-occurring in hairy root cultures of yellow beet (as evidenced by HPLC co-chromatography and LCMS analysis). Compound **9** (Fig. 1B), occurring in very low concentration in the extract from dopamine administration, was also found in hairy root cultures (Fig. 1A). As **9** is a result of dopamine administration and showed a characteristic daughter ion at *m*/*z* 345, it belongs unequivocally to the group of dopamine-derived betacyanins, but the $[M+H]^+$ ion at 651 did not match with any plausible structural proposal. Compound **6** (Fig. 1A) gave a $[M+H]^+$ ion at *m*/*z* 523 with a mass difference (16) between peak **6** (*m*/*z* 523) and compound **5** (*m*/*z* 507). Interestingly, this molecular ion yielded in the daughter ion scan mode neither a daughter ion at *m*/*z* 345 ($[2\text{-descarboxy-betanidin}+H]^+$, **1**) nor at *m*/*z* 389 ($[\text{betanidin}+H]^+$), but at *m*/*z* 361, a hitherto unknown betacyanin aglycone. In addition to the recently found betalains from hairy roots cultures (betalamic acid, **1**, betanin/isobetanin, **2**, **2′**, miraxanthin V, **3**, and 2-descarboxy-betanidin, **7**) (Schliemann et al., 1999) in the course of the present study compounds **5** and **8** were identified as 2-descarboxy-betanin and its 6′-*O*-malonyl conjugate, respectively, and are new dopamine-derived betacyanins. Furthermore, phyllocactin (**4**), often the main pigment from flowers and fruits of Cactaceae (Piattelli and Imperato, 1969), was detected in hairy root material. Its occurrence was formerly also observed in leaves of *Kochia scoparia*, stems of *Salsoda soda* and petioles of *Spinacia oleracea* (all Chenopodiaceae as *B. vulgaris*) (Steglich and Strack, 1990). The retention time, HPLC-PDA and MS data of the betalains from hairy root cultures are summarized in Table 1.

It is still under debate whether the condensation reaction (aldimine formation) with betalamic acid takes place before or after glucosylation of the *cyclo*-Dopa moiety in betacyanin biosynthesis. The betanidin 5-*O*-glucosyltransferase (Vogt et al., 1997) and the corresponding recombinant enzyme, expressed in *Escherichia coli* (Vogt et al., 1999), exhibited no activity towards *cyclo*-Dopa, thus favouring the first alternative in *Dorotheanthus bellidiformis* (Aizoaceae). The detection and isolation of *cyclo*-Dopa 5-*O*-glucoside from red beets (Wyler et al., 1984) in appreciable amounts points to the second pathway in some other plants. To prove which alternative is used for the formation of dopamine-derived betacyanins in fodder beets, a short-term dopamine administration experiment was performed (Fig. 4). 2-Descarboxy-betanidin (**7**)

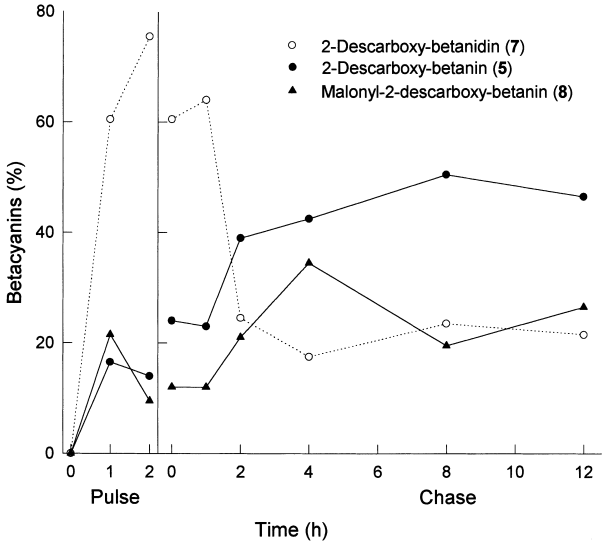


Fig. 4. Short-term dopamine administration experiment with hypocotyls of fodder beet plants ('Altamo') (28-day-old). Compound numbers correspond to the biosynthetic pathway in Fig. 2.

accounts for nearly 80% of the total betacyanins at the end of the pulse phase, thus clearly demonstrating that the condensation reaction between betalamic acid (**1**) and 2-descarboxy-*cyclo*-Dopa takes place prior to the glucosylation step. The chase phase is characterized by a direct precursor–product relationship, thus confirming the former assumption. The reaction sequence (Fig. 2) observed in fodder beets obviously holds true also for the hairy root system, as **7** is there the main betacyanin component (Fig. 1A).

Using the purified dopamine-derived betacyanins as standards, the occurrence of these compounds was examined in different plants, hairy root and cell cultures by analytical HPLC and co-injection experiments (Table 2). Only in hairy root cultures derived from yellow beets is **7** the prevailing betacyanin, followed by **8** and **5**. The occurrence of these dopamine-derived betacyanins is accompanied by a high abundance of the dopamine-derived betaxanthin **3**. In red beet hairy root cultures, in hypocotyls of beet plants (Garden and Fodder Beet Group) and its cell suspension cultures, the concentrations of these betacyanins are either very low or not detectable. Likewise, callus cultures of *Portulaca grandiflora* did not contain any dopamine-derived betacyanin, but did contain a small amount of **3**, showing the involvement of dopamine in betaxanthin biosynthesis. From these data, and in light of a former study in which tyrosinase activity was correlated with the betalain content (Steiner et al., 1999), it can be concluded that the formation of these betacyanins only takes place when both the dopamine level and the tyrosinase activity are high. Under these conditions 2-descarboxy-*cyclo*-Dopa does compete with dopamine in the condensation reaction with betalamic acid, which leads to a simultaneous formation of **3** and **7**. While **3** is a biosynthetic end product, **7** can be further conjugated with glucose, giving **5**, and can finally be acylated to form **8**. The results of the structure elucidation and the dopamine administration experiments are summarized in a pathway of the biosynthesis of dopamine-derived betalains in hairy root cultures (Fig. 2). By combination of different enzymatic and spontaneous reactions, the characteristic pattern of the predominant dopamine-derived betalains in hairy root cultures is realized, in addition to the low amount of co-occurring **2**, which is otherwise the prevailing betacyanin pigment in *Beta* species.

From the present work and a recent tyrosinase study (Steiner et al., 1999) the question arose how the plant channels Dopa into *cyclo*-Dopa, betalamic acid and dopamine in the betalain biosynthetic pathways. Concerning dopamine, it could be speculated that most of this precursor in hairy root cultures of yellow beets has

Table 2
Occurrence of dopamine-derived betacyanins in hypocotyls, hairy root and cell cultures of *Beta vulgaris* and callus culture of *Portulaca grandiflora*[a]

Plant material	Total betacyanin (BC) content [nmol (g fw)$^{-1}$][b]	2-Descarboxy-betanin (% of total BC)	2-Descarboxy-betanidin (% of total BC)	6′-0-Malonyl-2-descarboxy-betanin (% of total BC)	Miraxanthin V [nmol (g fw)$^{-1}$]
Beta vulgaris L. subsp. vulgaris (Garden Beet Group) 'Golden Beet'					
Hairy roots (7-day-old)					
BVL5A	97	3	37	10	546
BVL5D	133	5	56	6	1219
BVL7	96	3	31	15	925
BV red[c]	308	3	1	6	51
Hypocotyls (28-day-old)					
(Garden Beet Group)					
'Golden Beet Geel'	45	n.d.[d]	0.5	1.0	321
'Renova'	857	0.3	0.3	0.2	32
'BV 61243'	1426	n.d.	0.9	n.d.	51
(Fodder Beet Group)					
'Altamo'	3.4	n.d.	0.5	n.d.	41
'Fumona'	1.9	n.d.	n.d.	n.d.	19
'Brigadier'	6.1	n.d.	0.2	n.d.	81
Cell suspension culture (14-day-old)					
BV[e]	198	1.5	n.d.	1.1	1.0
Portulaca grandiflora					
Callus culture (14-day-old)					
PG K64	34	n.d.	n.d.	n.d.	3.2

[a] Each value is the average of duplicate samples.
[b] In betanin equivalents, fw, fresh weight.
[c] *B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Egyptian Flat Round'.
[d] Not detectable.
[e] *B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Rote Kugel'.

to be transported directly into the vacuole for the formation of the main betaxanthin, miraxanthin V (**3**), whereas a minor part is oxidized by tyrosinase to 2-descarboxy-*cyclo*-Dopa, leading to the dopamine-derived betacyanins.

3. Experimental

3.1. Plant material

Different fodder beets [*B. vulgaris* L. subsp. *vulgaris* (Fodder Beet Group) 'Altamo', 'Fumona' and 'Brigadier'] and red and yellow beet plants [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Renova', 'BV61243' and 'Golden Beet Geel'] were grown from seeds in a greenhouse and cultivated in soil under natural day-light conditions. For betalain analysis hypocotyls of seedlings (4-weeks old) were harvested. Hairy root and suspension cultures of *B. vulgaris* L. and callus culture of *P. grandiflora* Hook., line K64, were maintained as described (Steiner et al., 1999). A Christmas cactus [*Schlumbergera* × *buckleyi* (T. Moore) Tjaden] was cultivated in a greenhouse and the flower petals were harvested after full flower opening.

3.2. HPLC

Analytical and semi-preparative HPLC was performed with a system from Waters (Milford, MA, USA) including the separation module 2690. The liquid chromatograph was equipped with a 5-μm Nucleosil C_{18} column (250×4 mm i.d.; Macherey-Nagel, Düren, Germany) and the following solvent and gradient systems were used. Solvent system 1: A, 1.5% aq. H_3PO_4; B, MeCN; linear gradient from 100% A to 76% A in (A + B) within 40 min. The flow rate was 1 ml min^{-1}. Solvent system 2: A, 1% aq. HOAc; B: MeCN; gradient as in solvent system 1. Compounds were detected at 540, 475 and 405 nm or by maxplot detection between 400 and 650 nm (photodiode array detection). Injection volume was 20 or 50 μl for analytical (solvent system 1) and 100 μl for semi-prep. HPLC (solvent system 2).

For preparative HPLC the liquid chromatograph (System Gold; Beckman Instruments, München, Germany) was equipped with a 10 μm-Nucleosil 100-10 C_{18} column (VarioPrep; 250×40 mm i.d.; Macherey-Nagel, Düren, Germany). The separation of the dopamine administration extract was performed with a linear gradient within 90 min from 100% solvent A (aq HCO_2H, pH 3) to 10% solvent A in solvent B (MeOH) (at a flow rate of 10 ml min^{-1} (injection volume: 2 ml; detection at 540 and 475 nm). **3**: R_t 59–60 min; **5**: R_t 61–63 min; **7**/**8**/**9**: R_t 66–70 min. For subsequent purification the betacyanin fractions were concentrated and separated by semi-prep. HPLC (fraction collection: 30 s).

3.3. LCMS and NMR spectroscopy

Positive and negative electrospray mass spectra were obtained using a Finnigan MAT TSQ 7000 instrument [electrospray voltage 4.5 kV (positive ions), 3.5 kV (negative ions); capillary 220°C; sheath gas: N_2] coupled with a Micro-Tech Ultra-Plus Micro LC system equipped with a 4-μm C_{18} column (100×1 mm i.d., ULTRASEP). For LC, a gradient system starting from 10% B (0.2% aq AcOH in MeCN) in 90% A (0.2% aq AcOH) to 50% B in (A + B) within 10 min was used, followed 10 min further isocratic elution at a flow rate of 70 μl min^{-1} (injection volume: 2 μl). The negative ion ESMS spectra were recorded using an APICID offset voltage of 10 V. The collision-induced dissociation (CID) mass spectra were obtained during LC analysis under the following conditions: collision energy (collision cell) −40 eV (positive ions); collision gas: argon; collision pressure: 1.8 mTorr. All mass spectra are averaged, with the background subtracted.

1 D and 2 D ^{1}H (COSY) NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the major deuterium resonance of the solvent, CD_3OD, containing a trace of DC1. All chemical shifts are given in ppm relative to TMS and coupling constants in Hz.

Compound **2** [betanidin 5-*O*-β-D-glucoside (betanin)] LCMS (positive ion mode) (*m*/*z*, rel. int.): 551 $[M+H]^+$ (100).

Compound **3** (miraxanthin V) LCMS (positive ion mode) (*m*/*z*, rel. int.): 347 $[M+H]^+$ (100).

Compound **4** [phyllocactin, betanidin 5-*O*-(6′-*O*-malonyl-β-D-glucoside) LCMS (positive ion mode) (*m*/*z*, rel. int.): 637 $[M+H]^+$ (100); negative ion mode: 635 $[M-H]^-$ (95), 591 $[M-H-CO_2]^-$ (100), 547 $[M-H-2CO_2]^-$ (88), 503 $[M-H-3CO_2]^-$ (67), 459 $[M-H-4CO_2]^-$ (20).

Compound **5** [2-descarboxy-betanin, 2-descarboxy-betanidin 5-*O*-β-D-glucoside] LCMS (positive ion mode) (*m*/*z*, rel. int.): 507 $[M+H]^+$ (100); daughter ion scan mode of *m*/*z* 507: 345 [2-descarboxy-betanidin + H]$^+$ (100). ^{1}H NMR (600 MHz, CD_3OD/DCl): δ 8.67 (1H, *d*, J_{10-11} = 11.9 Hz, H-10), 7.36 (1H, *s*, H-7), 7.30 (1H, *s*, H-4), 6.49 (1H, *s*, H-17), 6.39 (1H, *d*, J_{11-10} = 12.0 Hz, H-11), 4.87 (H-1′), 4.59 (1H, *dd*, J_{14-13A} = 7.0, J_{14-13B} = 5.6, H-14), 4.40 (2H, *t*, $J_{2A/B-3A/B}$ ~7 Hz, H-2A/B), 3.96 (1H, *dd*, $J_{6'A-5'}$ = 2.0 Hz, $J_{6'A-6'B}$ = 12.1 Hz, H-6′A), 3.75 (1H, *dd*, $J_{6'B-5'}$ = 5.3 Hz, $J_{6'B-6'A}$ = 12.0 Hz, H-6′B), 3.66* (H-13A), 3.56 (1H, *dd*, $J_{2'-1'}$ ~8 Hz, $J_{2'-3'}$ = ~9 Hz, H-2′), 3.50* (H-5′), 3.33* (H-3A/B), 3.29* (H-13B), 3.6–3.3 (m, H-3′, H-4′). The chemical shifts of the signals marked * were determined from the cross peaks in the 2D COSY spectrum.

Compound **6**, LCMS (positive ion mode) (*m*/*z*, rel. int.): 523 $[M+H]^+$ (100); daughter ion scan mode of *m*/*z* 523: 361 (100).

Compound **7** (2-descarboxy-betanidin), LCMS (positive ion mode) (*m*/*z*, rel. int.): 345 $[M+H]^+$ (100).

Compound **8** [2-descarboxy-betanidin 5-*O*-(6′-*O*-malonyl)-*β*-D-glucoside], LCMS (positive ion mode) (*m*/*z*, rel. int.): 593 $[M+H]^+$ (100); daughter ion scan mode of *m*/*z* 593: 345 [2-descarboxy-betanidin + H]$^+$ (100); negative ion mode: 591 $[M-H]^-$ (73), 547 $[M-H-CO_2]^-$ (73), 503 $[M-H-2CO_2]^-$ (100), 459 $[M-H-3CO_2]^-$ (79). ^{1}H NMR (600 MHz, CD_3OD/DCl): δ 8.67 (1H, *d*, J_{10-11} = 12.1 Hz, H-10), 7.36 (1H, *s*, H-7), 7.24 (1H, *s*, H-4), 6.49 (1H, *s*, H-17), 6.39 (1H, *d*, J_{11-12} = 12.3 Hz, H-11), 4.88* (H-1′), 4.62 (1H, *dd*, $J_{6'A-5'A}$ = 1.9 Hz, $J_{6'A-5'B}$ = 12.0 Hz, H-6′A), 4.57 (1H, *dd*, J_{14-13A} = 5.7 Hz, J_{14-13B} = 6.8 Hz, H-14), 4.40 (2H, *t*, $J_{2A/B-3A/B}$ = 7.5 Hz, H-2A/B), 4.32 (1H, *dd*, $J_{6'B-5A'}$ = 6.7 Hz, $J_{6'B-5'B}$ = 11.9 Hz, H-6′B), 3.73* (H-5′), 3.67* (H-13A), 3.57* (H-2′), 3.36* (H-3A/B), 3.27* (H-13B), 3.7–3.3 (m, H-3′, H-4′). The chemical shifts of the signals marked * were determined from the cross-peaks in the 2D COSY spectrum. The methylene protons of the malonyl moiety were not unambiguously identified because of signal overlap and the probability that they have exchanged with the solvent. A second betanidin spin system (~18%) was evident from signals at 8.70 and 6.05 ppm that presumably belong to the 11*Z*-isomer.

Compound **9**, LCMS (positive ion mode) (*m*/*z*, rel. int.): 651 $[M+H]^+$ (100), 345 [2-descarboxy-betanidin + H]$^+$ (14); daughter ion scan mode of *m*/*z* 651: 345 [2-descarboxy-betanidin + H]$^+$ (100).

3.4. Administration of dopamine to fodder beet hypocotyls

An aq solution of dopamine×HCl (5 ml, 10 mM) was administered to five de-rooted 28-day-old fodder beet plants ('Altamo') via the hypocotyls for 48 h, which were then extracted for HPLC analysis. Controls were treated with distilled water. In a large scale administration experiment, 470 'Altamo' plants were treated under the same conditions.

3.5. Isolation and purification of dopamine-derived betacyanins

After 48 h dopamine administration and metabolism, the hypocotyls (25.8 g) were harvested, frozen in liquid N_2, homogenized in a mortar and extracted with 100 ml 80% aq MeOH containing 50 mM ascorbate for 30 min. After centrifugation at 14,000 *g* for 10 min at 4°C, the supernatant was removed and its betacyanin content was photometrically quantified. For the calculation the molar extinction coefficient for betanin (62×10^6 cm^2 mol^{-1}; Wyler et al., 1959) was used with the results expressed as betanin equivalents (yield: 2.8 μmol betanin equivalents). The extract was concentrated in vacuo at 30°C to 10 ml. By analytical HPLC four betacyanins (peak **5**: R_t 25.5 min, λ_{max} 532 nm; peak **7**: R_t 30.6 min, λ_{max} 533 nm; peak **8**: R_t 31.8 min, λ_{max} 535 nm; peak **9**: R_t 32.3 min, λ_{max} 533 nm) were detected, each of which was purified in a two-step procedure. First, by prep. HPLC of half of the concentrated extract, compound **5** (0.27 μmol betanin equivalents) and a mixture of **7**/**8**/**9** (0.52 μmol betanin equivalents) were obtained. Subsequently, final purification of **5** and separation of **7**, **8** and **9** was achieved by semi-prep. HPLC (solvent system 2).

3.6. Preparation of 2-descarboxy-betanidin, 7

7 was synthesized as described recently (Schliemann et al., 1999) and used as an authentic standard (R_t 30.6 min, λ_{max} 533 nm).

3.7. Enzymatic hydrolysis of 2-descarboxy-betanin, 5

Twenty nmol **5** (in 200 μl 0.1 M KPi-buffer, pH 5.0) was added to 200 μl *β*-glucosidase (almond) (2 mg ml^{-1} in 0.1 M KPi-buffer, pH 5.0) at 37°C. After 0, 10, 20, 30 and 60 min, two 25 μl aliquots of the hydrolysis mixture were removed and added to 50 μl MeOH for protein precipitation. After centrifugation (5 min, 14,000 *g*), the supernatant was analyzed by HPLC (solvent system 1). A control was stopped by the addition of 50 μl MeOH immediately after the addition of *β*-glucosidase.

3.8. Isolation of phyllocactin, 4, from flower petals of Christmas cactus

For comparison by HPLC and LCMS, **4** was isolated from flower petals of Christmas cactus [*Schlumbergera* × *buckleyi* (T. Moore) Tjaden], purified by prep. HPLC and characterized by LCMS and ^{1}H NMR (Kobayashi et al., 2000).

3.9. Extraction and quantification of betalains

The plants and cell cultures were extracted and the betalains were quantified as recently described (Schliemann et al., 1999). The betalains were quantified photometrically using the mean molar extinction coefficients for betaxanthins (48×10^6 cm^2 mol^{-1}; Girod and Zryd, 1991b) and for betanin (62×10^6 cm^2 mol^{-1}; Wyler et al., 1959). After HPLC analysis (solvent system 1) of the extracts, the peak areas of individual compounds were compared with those of standard compounds. For the assignment of the dopamine-derived betacyanins, the R_t and λ_{max} of the betacyanin peaks were compared with those of metabolically obtained standard compounds and additionally confirmed co-chromatographically in HPLC. Quantitative data were derived and expressed as percentage of total betacyanin content (in betanin equivalents). All experiments were performed in duplicate and mean values were obtained.

3.10. Short-term dopamine administration experiment

For the pulse experiment, an aq solution of dopamine× HCl (5 ml, 10 mM) was administered to five de-rooted 28-day-old fodder beet plants ('Altamo'). After 0, 1 and 2 h, the hypocotyls (0.5 g) were extracted as described above and analyzed by HPLC (solvent system 1). For the chase experiment, after 2 h of dopamine administration (pulse phase), the dopamine solution was replaced by distilled water. After 0, 1, 2, 4, 8, 12, 24 and 48 h, the hypocotyls (0.5 g) were extracted, the extracts analyzed and then the betacyanins were quantified as described above. The areas of compounds **5**, **7**, and **8** were added and the concentration of the individual compounds were expressed as percentage of the sum. The experiments were performed in duplicate.

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Seasonal variation in hypericin content of *Hypericum perforatum* L. (St. John's Wort)[☆]

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Abstract

Hypericin and pseudohypericin, bioactive constituents in St. John's Wort (*Hypericum perforatum*), have been determined in the soft tops of the plant that are most likely to be browsed by foraging livestock. In two consecutive seasons, the hypericin/pseudohypericin concentration in a broad leaf biotype varied from a winter minimum of less than 100 ppm to a summer maximum approaching 3000 ppm. In contrast the narrow leaf biotype increased from similar winter values to summer maxima approaching 5000 ppm. The latter biotype was slower in returning to low levels of hypericin/pseudohypericin. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Hypericum perforatum*; Clusiaceae; St. John's Wort; Hypericin; Pseudohypericin; Seasonal variation; Soxhlet extraction; UV-Vis spectrometry; Toxicity; Pasture

1. Introduction

Hypericum perforatum L. (Family: Clusiaceae). St. John's Wort, native to Europe, Asia and N. Africa, has reached weed status in Australia, New Zealand, S. Africa and other temperate regions around the world (Campbell and Delfosse, 1984) where it is frequently listed as a poisonous plant (Watt and Breger-Brandwijk, 1962). This perennial, 0.3–1.2 m herb is gaining increasing popularity as a medicinal plant (Cellarova et al., 1994; Fornasiero et al., 1998) due to anti-viral (Hudson et al., 1991; Lopez-Bazzocchi et al., 1991) and anti-retroviral activity (Meruelo et al., 1988; Lavie et al., 1989), and use in the treatment of mild to moderately severe depression (Mauri and Pietta, 2000; Laudahn, 1992). Consequently, many investigations have been directed at understanding and enhancing hypericin production by studying leaf structures (Fornasiero et al., 1998), field cultivation (Buter et al., 1998) and cell culture establishment (Cellarova et al., 1994; Kartnig et al., 1996).

H. perforatum bioactive constituents are not completely understood. Possible candidates include hypericin and related bisanthraquinones (Cellarova et al., 1994; Giese, 1980), flavonoids such as amentoflavone (Kartnig et al., 1996) and phloroglucinols such as hyperforin (Mauri and Pietta, 2000). Of these, pseudohypericin is most significant in contributing two to four times as much as hypericin itself in *H. perforatum* (Mauri and Pietta, 2000; Buter et al., 1998; Kartnig et al., 1996; Giese, 1980; Piperopoulos et al., 1997; Brolis et al., 1998). These derivatives also exist in precursor forms as protohypericin and protopseudohypericin (Cellarova et al., 1994; Giese, 1980; Southwell and Campbell, 1991). Light sensitive precursors of protohypericin have been proposed as the 10–10′ coupled emodin bianthrone for plants such as *H. perforatum* (Giese, 1980; Banks et al., 1976; Brockman, 1957; Gill and Gimenez, 1991) or confirmed as the 5–5′ coupled austrovenetin for the toadstool *Dermocybe austroveneta* (Gill and Gimenez, 1991). These precursor components are only available after extraction in the dark. In addition, the acid catalysed cyclisation product, cyclopseudohypericin is present in dark-adapted extracts of *H. perforatum* (Giese, 1980).

Methods available for the determination of hypericin usually involve solvent extraction followed by either spectrophotometric assay for total hypericins (Cellarova

[☆] The authors use the name 'hypericin' as a generic label for hypericin type pigments as suggested by Watt and Breger-Brandwijk (1962).

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et al., 1994; Southwell and Campbell, 1991) or HPLC determination of individual constituents (Buter et al., 1998; Kartnig et al., 1996) including HPLC-MS systems (Mauri and Pietta, 2000; Piperopoulos et al., 1997; Brolis et al., 1998) using electrospray and diode array detection.

Earlier investigation of *H. perforatum* in Australia indicated a higher hypericin concentration in the narrow leaf variety compared to the broad leaf and the flowers compared to the leaves and the stems (Southwell and Campbell, 1991). Hypericin concentrations ranged from 40 ppm in broad-leaf variety stem material to 5190 ppm in narrow-leaf variety flowers and were consistent with values reported from other parts of the world (Watt and Breger-Brandwijk, 1962; Mathis and Ourisson, 1963).

The toxic principle is thought to be hypericin and pseudohypericin which, along with their precursor 'proto' analogues act as photosensitising agents and cause disease in foraging livestock such as sheep, cattle and horses (Giese, 1980). *H. perforatum* toxins only display significant pharmacological activity when ingestion of the plant by an animal is followed by a period of exposure of the animal to bright sunlight (Bourke, 2000). In vitro and in vivo studies that do not include bright light activation may fail to demonstrate significant biological activity for hypericin compounds.

Although known to be more toxic in flower when hypericin concentrations are highest (Southwell and Campbell, 1991), no seasonal studies of hypericin concentration or toxicity in Australia have been reported. This investigation reports the seasonal variation in the concentration of hypericin and its derivatives in the tops of the two most common biotypes of *H. perforatum* plants that are likely to be browsed by livestock when no alternative forage is available. This data, coupled with concurrent toxicity investigations, can now be applied to the grazing management system currently under investigation in Australia for suppressing wort growth (Bourke and Southwell, 1999).

2. Results and discussion

A modified Southwell and Campbell (1991) method was used to determine the 'total hypericin' concentrations of the air dried soft growth material of St. John's Wort plants. Sheep selectively graze these soft growth portions which are known to contain higher concentrations of hypericins than the more lignified supporting stalks (Southwell and Campbell, 1991; Bourke and Southwell, 1999). Soxhlet extraction with *t*-butyl methyl ether removed almost all of the chlorophyll from the plant material which, after in vacuo residual solvent removal, was further extracted using absolute ethanol to remove hypericins and other polar constituents.

The λ 592 nm absorptions of the ethanolic solutions were measured in order to obtain estimates of the concentrations of hypericins. Each estimate included hypericin and the similarly absorbing pseudohypericin, protohypericin and protopseudohypericin (Mauri and Pietta, 2000; Banks et al., 1976). The absorption of the co-occurring phloroglucinols and flavonoids (Mauri and Pietta, 2000; Mabry et al., 1970) was sufficiently distant from λ 592 nm not to interfere.

The concentrations of total-hypericins (minima, maxima, mean values) over a 22 month period for both the broad leaf and the narrow leaf variety are shown in Table 1. The growth stages of the plants sampled developed as follows: early January to mid March; seed capsules formed on the flower stems, late March to mid June; existing upright stems under went senesence and new green prostrate growing stems developed at the base of each plant, late June to early September; basal stems remained green but their growth became static, mid September to early November; new upright growing flower stems appeared and developed rapidly, mid November to late December; flowers appeared in ever increasing numbers. Initially, moderate autumn levels reduced to near zero for the winter period from late July to the end of August. With spring and new season growth including flower spikes, the concentration of hypericins increased rapidly to peak in early summer (December) when flowering had almost finished and fruiting capsules were forming. Total hypericins then decreased as flowers were lost and capsules were maturing over summer. Autumn saw a steady decline to the winter minimum levels and the onset of a second similar annual cycle.

The second year cycle recorded higher summer hypericins for both biotypes. This year experienced higher rainfall (1312 mm), but lower average daily sunlight hours (7.0) and a lower average daily maximum temperature (17.9°C), compared to below average rainfall (655 mm), higher sunlight hours (8.1) and a higher maximum temperature (18.9°C) for the first year. The average annual rainfall for this region is 903 mm, daily sunlight hours 7.5 and daily maximum temperature 17.6°C. The greater concentrations in the wetter year suggest that, contrary to some other secondary metabolites [e.g. proline analogues (Naidu et al., 1987) and some essential oils (Lawrence 1986)], hypericin is not enhanced by water stress.

The narrow leaf biotype was confirmed as containing approx. 75% more hypericin than the broadleaf biotype at their summer flowering peak times. In addition, the narrow leaf biotype took much longer throughout the autumn to return to low winter levels of hypericin. This difference may have been associated with a much earlier onset of frosts at the broad leaf field site, which was located in a low lying, flat, basin with poor air drainage, compared to the narrow leaf field site, which was located on a high, steep, slope with excellent air drainage. A comparison plot of the seasonal variation for the broad

Table 1
The total-hypericins content variation (ppm) in the broadleaf and narrowleaf biotypes of *H. perforatum* over two seasons

Broadleaf biotype					Narrowleaf biotype				
Date	Min.	Max.	*n*	Mean	Date	Min.	Max.	*n*	Mean
1/04/1997	395	467	2	431	3/04/1997	941	1047	2	994
23/04/1997	305	333	2	319	24/04/1997	1029	1042	2	1036
13/05/1997	403	427	2	415	15/05/1997	863	892	2	878
3/06/1997	207	242	2	225	5/06/1997	263	481	3	359
25/06/1997	210	225	2	218	26/06/1997	162	320	3	229
14/07/1997	115	154	2	134	17/07/1997	75	82	2	79
7/08/1997	37	81	2	59	6/08/1997	72	142	7	97
28/08/1997	44	65	2	55	28/08/1997	0	36	2	18
17/09/1997	34	42	2	38	17/09/1997	106	124	2	115
9/10/1997	109	109	2	109	9/10/1997	165	265	2	215
29/10/1997	402	523	2	462	29/10/1997	614	810	3	704
19/11/1997	815	1302	3	1049	19/11/1997	2062	2303	2	2183
10/12/1997	1841	2848	8	2147	11/12/1997	3388	4438	8	3775
7/01/1998	651	898	3	756	7/01/1998	2673	3128	2	2900
5/02/1998	532	631	2	581	5/02/1998	2439	2860	2	2650
26/02/1998	449	532	2	490	26/02/1998	1042	1370	3	1199
12/03/1998	397	403	2	400	12/03/1998	890	1637	8	1205
2/04/1998	292	447	2	370	2/04/1998	1267	1319	2	1293
24/04/1998	319	329	2	324	24/04/1998	1054	1193	2	1124
13/05/1998	214	221	2	218	13/05/1998	491	625	2	558
4/06/1998	109	229	2	169	26/06/1998	270	362	2	316
26/06/1998	80	133	2	107	24/07/1998	274	286	2	280
23/07/1998	92	173	7	147	18/08/1998	210	247	2	229
7/08/1998	80	89	2	85	8/09/1998	206	309	2	257
8/09/1998	74	142	2	108	30/09/1998	520	527	2	523
30/09/1998	147	223	2	185	21/10/1998	466	469	2	468
21/10/1998	155	260	2	208	11/11/1998	944	1018	2	981
11/11/1998	286	303	2	294	2/12/1998	4743	4864	2	4803
2/12/1998	622	674	2	648	18/12/1998	4376	4442	2	4409
18/12/1998	2468	3106	3	2728	8/01/1999	3598	4634	8	4010
8/01/1999	1108	1310	2	1209	29/01/1999	2675	2743	2	2709
29/01/1999	472	541	2	507	12/02/1999	2896	3408	3	3115
12/02/1999	448	507	2	477					

leaf variety and the narrow leaf variety is shown in Fig. 1.

Investigations on the toxicity of St. John's Wort pastures suggest that 160–200 ppm hypericin pastures should be safe for grazing Merino sheep that are neither pregnant nor lactating and have at least a four month cover of wool (Bourke and Southwell, 1999, 2000). This extrapolation from Fig. 1 indicates that July to September would be suitable months for grazing the broad leaf biotype with extension by a few weeks given favourable climatic conditions. However, more caution is required for the more toxic narrow leaf biotype where Fig. 1 indicated July to September as suitable for the first season but a lesser period as suitable during a wet year when hypericin concentrations are elevated. Although the above is applicable to these two specific sites, comparable concentrations from additional populations (Southwell and Campbell, 1991) when related to the growth stage and plant part analysed, suggest that these conclusions will be valid for all St. John's wort infested pastures.

3. Experimental

3.1. Plant material

H. perforatum plant samples were collected at two field sites on the Central Tablelands of New South Wales, Australia every 3 weeks for a 2 year period. Both sites consisted of natural wort infestations of long standing on government owned lands. Neither site had been sprayed with herbicide, burnt, mechanically slashed, mown, or grazed by livestock, for several years prior to this experiment nor at any stage during it. The broad leafed biotype site, was located in a low lying, flat, basin, on a deep but well drained basalt derived soil, at Orange. The narrow leafed biotype site was

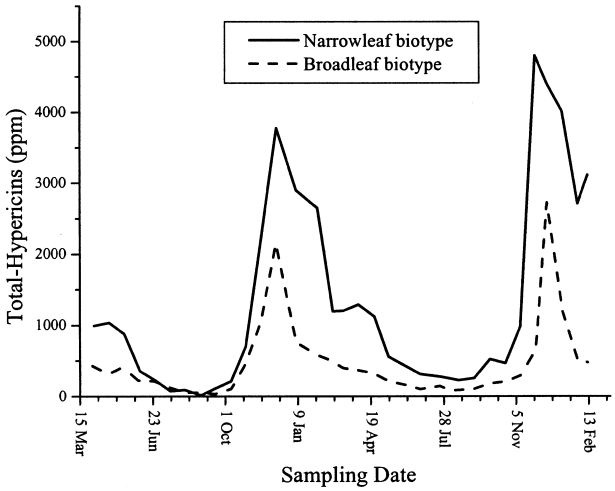


Fig. 1. Comparison of the mean total-hypericin concentrations in the narrow and broad leaf biotypes of *H. perforatum* forage over two consecutive seasons.

located on a high, steep, slope, on a shallow but well drained granite derived soil, at Tuena. The sample for hypericin determination was taken from a bulked collection of all soft growth material present on 50 plants selected at random on each sampling occasion. Soft growth consisted of leaves, small fine stems and, when present, flowers and immature fruit capsules. Previous observation had shown that these were the plant parts which sheep selectively graze in preference to the coarser stalks. Plant material was air dried at room temperature indoors, away from sunlight. Results reported are from April 1997 to February 1999. The growth stages of wort plants were observed and recorded at each 3 weekly sample collection. The field sites sampled were not grazed, mown or defoliated by other mechanical or chemical means during the collection period.

3.2. Leaf analysis

Hypericin concentrations were determined using a modified Southwell and Campbell (1991) Soxhlet extraction method in which *t*-butyl methyl ether replaced diethyl ether as solvent for the extraction of chlorophyll. Dried, ground plant material (approx. 2.0 g) was accurately weighed into a Soxhlet thimble and extracted with *t*-butyl methyl ether (200 ml) by Soxhlet until chlorophyll ceased extracting (4 h). The thimble was dried in vacuo (16 h) and re-extracted with absolute ethanol (200 ml) until the red hypericin pigments ceased extracting (8 h). The ethanolic solution was made up to 500 ml with EtOH and the visible spectrum measured from λ 500–700 nm. Hypericin concentrations were determined using the ε_{592} 41 600 value for absorption maximum in EtOH (Knox and Dodge, 1985) equivalent to that obtained by us from an authentic sample. Where chlorophyll was incompletely removed, an adjustment equivalent to 15% of the λ 660 nm chlorophyll absorption was made to allow for the contribution by the secondary λ 608 nm chlorophyll band. The linearity of hypericin absorption at λ 592 was established for absorption values from 0 to 1.8 (i.e. 0 to 5500 ppm).

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The ecological and taxonomic importance of flower volatiles of *Clusia* species (Guttiferae)

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Abstract

The chemical composition of floral volatiles of sixteen species of *Clusia* (Guttiferae) belonging to four different taxonomic sections of the genus was investigated. The volatiles were extracted from fresh petals by microhydrodistillation and analysed by GC/MS. The composition of the volatiles was in part, but not always, related to the taxonomic position of the species, and to a minor extent to the type of pollinator observed on the flowers as revealed by clustering analysis. The composition of the volatile components of female and male flowers belonging to the same species (*C. grandiflora, C. lanceolata, C. paralicola, C. parviflora* and *C. spiritu-sanctensis*) was found to be almost identical. Field bioassays showed the petal extracts to be attractive to pollinating bees.
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Keywords: *Clusia* spp.; Guttiferae; Floral scents; Pollinators; Taxonomy

1. Introduction

The neotropical genus *Clusia* L. (Guttiferae) comprises about 250–300 species grouped in a number of more or less natural taxonomic sections (Planchon and Triana, 1860a,b; Engler, 1925; Gustafsson and Bittrich, 1999). Life forms include hemiepiphytes (sometimes stranglers), lianas, shrubs and trees. Recent studies show a high variability in CO_2 uptake patterns in the genus, possibly in part responsible for the success of *Clusia* in various habitats (Franco et al., 1990). Like other Guttiferae (Clusiaceae), most tissues of the plants contain latex channels and various colours of latex have been reported by plant collectors. While the vast majority of *Clusia* species are dioecious, a few are known to be hermaphroditic or to include races with hermaphrodite flowers. Apomixis is known from *C. rosea* Jacq. and races of *C. minor* L. (Maguire, 1976; Hammel, 1986). The genus *Clusia* is notable for its enormous variety in floral morphology, especially in the androecium (Planchon and Triana, 1860a,b; Engler, 1888), and this morphological variability is paralleled by a great diversity in the reproductive biology (Armbruster, 1984; Bittrich and Amaral, 1996, 1997). Rewards for pollinators include pollen, nectar and often floral resin; in some species, however, the female flowers are rewardless and are thus pollinated by "mistake-pollination" (Correia et al., 1993; Bittrich and Amaral, 1996). Bees of various sizes belonging to several taxonomic groups, beetles, hummingbirds, sphingids, and flies have been observed as flower visitors (for a review see Lopes and Machado, 1998). Floral resin is an extremely rare reward in angiosperms, and besides in *Clusia* spp. it is known only from *Dalechampia* L. (Euphorbiaceae) (Armbruster, 1984) and *Clusiella* Planch. & Triana (Guttiferae). The chemistry of the floral resin of some *Clusia* species was recently investigated (de Oliveira et al., 1996, 1999), and various isoprenylated benzophenones were found to be the major components.

Floral scents are found in the vast majority of animal-pollinated plants with the exception of ornithophilous flowers (Proctor et al., 1996). The scents mostly serve to attract or orient pollinators over long or short distances (Dobson, 1994). Rarely are fragrant essential oils also the reward for pollinating male euglossine bees (Williams, 1983). In the Guttiferae, the latter phenomenon

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was observed in species of the genus *Tovomita* Aubl., and the chemistry of their fragrant chemical components has been recently investigated by Nogueira et al.(1998). Floral scents are generally a mixture of several volatile organic substances and are most often emitted by the petals, but other floral parts may also play a role in scent production and emission (Dobson, 1994). The type of scent and the time of its emission may be typical for flowers attracting a certain group of pollinators, thus forming part of the so-called floral syndromes (Faegri and Van der Pijl, 1979; Dobson, 1994). On the other hand, like all inheritable characters, the chemical compounds in the scents may also reflect taxonomic affinities of a species. Like most plant features, scents and their chemical composition reflect both evolutionary history and actual ecological conditions.

The present study investigates the volatile oils in the petals of several species of *Clusia* belonging to four taxonomic sections of the genus. The objective is to determine if the floral scent chemistry is related to the type of pollinator observed or rather to the taxonomic position of the species in the genus.

2. Results and discussion

The flower volatiles in 16 *Clusia* species were analysed, and identified as shown in Table 1, where the species are grouped by taxonomic section. Replicate analyses of some species (*C. grandiflora, C. spiritu-sanctensis, C. fluminensis, C. lanceolata, C. hilariana*) revealed no significant changes in chemical composition, only a little variation in relative abundance.

Floral fragrance data were first organised by grouping the components into 4 different classes (Knudsen et al., 1993), which allowed a better visualisation of the molecular variation ensemble (Table 1). It thus became apparent that in the section *Chlamydoclusia* (*C. grandiflora*, *C. nemorosa* (male), *C. nemorosa* (hermaphrodite) and *C. rosea*) there is a predominance of fatty acid derivatives and benzenoid compounds. The section *Cordylandra* can a priori be subdivided into two subgroups, one characterised by the predominance of volatile components possessing isoprenoid skeletas (subgroup 1 : *C. pernambucensis*, *C. fluminensis*, *C. weddelliana*) and the other with a predominance of fatty acid derivatives (subgroup 2 : *C. panapanari*, *C. spiritu-sanctensis*, *C. burchellii*, *C. renggerioides*); *C. paralicola* stands apart in having flowers with a very particular scent ascribed to the strong presence of indole (80.1%), but when indole is omitted the relative abundance of the remaining components provides a pattern (fatty acid derivatives, 49.5%; isoprenoids, 31.0%; benzenoids, 13.7%) that is consistent with that of subgroup 2 of section *Cordylandra*. In section *Criuva*, the presence of a diterpene (kaurene) and the predominance of benzenoid compounds characterise the species *C. criuva* and *C. parviflora* (probably better treated as a subspecies of *C. criuva*), while *C. flava* differs in having a rather strong banana-like odour attributed to the presence of 16.4% 3-methyl-1-butanyl acetate (isoamyl acetate) and 9.0% 3-methyl-4-heptanone. In section *Phloianthera*, the floral volatiles of both species studied (*C. hilariana* and *C. lanceolata*) have a composition very similar to those in section *Cordylandra* subgroup 1, with a predominance of isoprenoids.

A dendrogram (Fig. 1) reflecting the overall similarity of the essential oils of the investigated *Clusia* species was produced using the data (components were grouped into 4 different classes) depicted in Table 1, and a second one (Fig. 2) was produced from the data matrix grouping the essential oil components into 10 compound classes (Table 2). Both dendrograms are similar, but the second one showed a better separation between the species belonging to sections *Chlamydoclusia* and *Cordylandra*. This phenogram was therefore chosen as the basis for further discussion. Based on the chord distances, dendrogram 2 shows five different groups with *C. paralicola* forming an isolated group by itself. The second group contains the species of section *Cordylandra* (*C. fluminensis, C. pernambucensis, C. weddelliana*) together with species of section *Phloianthera* (*C. hilariana, C. lanceolata*). The third group contains species belonging to section *Criuva* (*C. parviflora* and *C. criuva*). *Clusia renggerioides, C. burchellii* and *C. spiritu-sanctensis* belonging to section *Cordylandra* form the fourth group, and finally, the fifth group is formed by species belonging to section *Chlamydoclusia* (*C. grandiflora, C. nemorosa* (male), *C. nemorosa* (herm.) and *C. rosea*), except for *C. panapanari* and *C. flava*, which belongs to section *Cordylandra* and *Criuva*, respectively. Dendrogram 2 thus shows some correlation with the taxonomic subdivisions based on morphological characters, but does not completely agree with them. For example, it mainly agrees with the taxonomic group of section *Chlamydoclusia*, where the group is disturbed only by the presence of *C. panapanari* (section *Cordylandra*) and *C. flava* (section *Criuva* s. lat.). In other cases, however, even closely related species like *C. burchellii* and *C. weddelliana* or *C. fluminensis* and *C. spiritu-sanctensis* (all sect. *Cordylandra*), had floral scents with a considerably different chemical composition, as reflected by their position in dendrogram 2. When pollinator type is superimposed on the dendrogram, there is also some degree of correlation, though it is less complete. A canonical correspondence analysis (CCA) (ter Braak, 1986) showed that roughly 38% of the total inertia could be explained by the first two axes using taxonomic groups as the "environmental" variables, while a similar analysis with pollinator types as the "environmental" variables accounted for only 20% of the total inertia in the first two axes. This suggests

Table 1
Floral essential oil composition of sixteen *Clusia* species belonging to four taxonomic sections

	Taxonomic sections																		
	Chlamydoclusia					*Cordylandra*								*Criuva*			*Phloianthera*	
			nemorosa															
Compounds	RI	*grandiflora*	male	herm.	*rosea*	*paralicola*	*spiritu-sanctensis*	*panapanari*	*renggerioides*	*burchellii*	*fluminensis*	*pernambucensis*	*weddelliana*	*parviflora*	*criuva*	*flava*	*hilariana*	*lanceolata*
Fatty acid derivatives																		
Methyl pentanoate	–	–	–	5.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isoamyl acetate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	16.4	–	–
3-Methyl-4-heptanone	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	9.0	–	–
3-Methyl valeric acid	941	–	–	–	–	–	–	–	–	1.5	tr	–	–	–	–	–	–	–
Pentyl propanoate	952	–	–	–	–	–	–	–	–	5.5	–	–	–	–	–	–	–	–
2-Octanol	962	–	–	–	–	–	1.1	1.1	tr	–	–	–	–	–	–	–	–	–
Octanal	968	–	–	–	–	–	–	–	0.5	–	–	–	–	–	–	–	–	–
Hexanoic acid	981	–	–	0.2	–	–	–	tr	1.9	–	–	–	–	–	–	–	–	–
Octen-3-ol?	992	–	–	–	–	–	–	–	–	1.0	–	–	–	–	–	–	–	–
Ethyl hexanoate	997	–	–	–	–	–	–	–	0.5	–	–	–	–	–	–	–	–	–
Decane	1000	–	–	–	–	–	–	–	–	0.8	–	–	–	–	–	0.3	–	–
Octanol	1067	–	–	–	–	–	–	–	1.7	0.8	–	–	–	–	–	–	–	–
Ethyl heptanoate	1093	–	–	–	–	–	–	–	5.4	–	–	–	–	–	–	–	–	–
Undecane	1100	–	–	0.5	–	–	–	–	–	0.6	tr	tr	–	–	–	54.6	–	–
Nonanal	1102	–	–	0.4	–	–	–	0.9	1.4	3.4	–	–	–	–	–	–	–	–
Isopentyl valerate	1106	–	–	–	–	–	–	–	–	2.2	–	–	–	–	–	–	–	–
2-Ethyl hexanoic acid	1123	–	–	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–
Nonanol	1169	–	–	–	–	–	–	–	2.4	–	–	–	–	–	–	–	–	–
Octanoic acid	1183	–	–	–	–	–	–	–	2.5	–	–	–	–	–	–	–	–	–
Dodecane	1200	–	–	0.2	–	–	–	1.0	–	–	–	–	–	–	tr	–	–	–
Nonanoic acid	1283	0.6	–	–	–	–	–	–	21.6	–	–	–	–	–	–	–	–	tr
Tridecane	1300	–	1.0	0.5	–	–	–	tr	–	tr	–	–	–	0.2	–	3.3	–	tr
γ-Nonalactone	1359	–	–	–	–	–	–	–	1.4	–	–	–	–	–	–	–	–	–
Decanoic acid	1374	–	–	–	–	–	–	–	1.5	–	–	–	–	–	–	–	–	–
Tetradecane	1400	0.8	18.6	10.0	13.4	2.5	–	tr	–	5.3	–	–	3.1	–	2.5	–	0.2	1.9
Dodecanal	1407	–	–	0.4	–	–	–	–	–	tr	–	–	–	–	–	–	–	–
γ-Decalactone	1463	–	–	–	–	–	–	–	0.4	–	–	–	–	–	–	–	–	–
Dodecanol	1473	–	–	3.6	10.9	5.0	3.5	15.9	tr	1.6	–	–	1.9	0.2	2.1	–	–	2.2
Pentadecane	1500	1.8	6.3	2.9	5.1	1.9	0.7	2.7	0.2	1.6	–	–	1.4	0.6	2.0	tr	–	–
Dodecanoic acid	1562	–	–	–	–	–	0.6	1.1	–	–	–	–	–	–	–	–	–	–
1-Hexadecene	1592	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hexadecane	1600	0.6	1.7	1.0	2.2	–	0.6	3.3	–	tr	–	–	–	–	–	–	–	–
Tetradecanal	1611	–	–	–	–	–	tr	–	0.4	–	–	–	–	–	–	–	–	–
(Z)-Methyl jasmonate	1647	–	–	–	–	–	–	–	–	–	–	–	–	14.6	–	–	–	–
(Z)-Methyl epi-jasmonate	1677	–	–	–	–	–	–	–	tr	–	–	–	–	7.4	1.5	–	–	–
Tetradecanol	1677	–	1.7	1.3	4.3	–	2.2	6.4	–	–	–	–	–	–	–	–	–	0.8
Heptadecane	1700	0.4	1.0	0.4	–	–	0.6	1.7	tr	–	–	–	–	–	–	–	0.6	tr
Tetradecanoic acid	1762	–	–	1.5	–	–	3.1	–	–	tr	1.1	–	–	–	–	–	–	–
Pentadecanol	1778	–	–	–	–	–	–	2.7	–	–	–	–	–	–	–	–	–	–
1-Octadecene	1793	1.0	–	–	–	–	–	–	0.3	–	0.1	–	–	–	–	–	0.5	–
Octadecane	1800	0.6	0.9	0.5	–	–	tr	2.3	0.2	–	–	–	–	–	–	–	0.3	0.2
Hexadecanol	1881	1.2	1.2	3.2	2.5	–	8.6	–	tr	4.0	0.2	–	–	0.7	–	–	–	0.4
Nonadecane	1900	–	–	0.6	–	–	tr	0.8	tr	–	–	–	–	–	–	–	–	tr
Heptadecenol	1945	–	–	–	–	–	–	–	–	2.8	–	–	–	–	–	–	–	–
15-Hexadecanolide	1959	–	–	–	–	–	24.3	–	–	–	–	–	–	–	–	–	–	–
Hexadecanoic acid	1963	12.5	4.3	4.9	7.2	–	25.5	–	–	10.2	0.9	–	–	–	–	–	–	1.7
1-Eicosene	1992	3.3	–	–	–	–	–	–	tr	–	–	–	–	–	–	–	1.0	–
Eicosane	2000	tr	0.9	0.4	4.7	–	tr	–	0.2	–	–	–	–	–	–	–	0.6	0.3
Hexadecyl acetate	2009	–	–	0.7	–	–	–	–	–	–	–	–	–	0.8	1.4	–	–	–
Octadecanol	2084	1.2	–	2.0	6.5	–	2.2	1.1	–	11.6	–	–	–	tr	–	–	–	0.4
Heneicosane	2100	4.8	2.0	0.9	1.6	–	tr	–	–	–	–	–	–	–	–	–	0.2	0.2
(Z)-9-Octadecenoic acid	2147	–	–	–	–	–	13.4	–	–	4.0	–	–	–	–	–	–	–	–
1-Docosene	2195	tr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.9	tr

(*Table continued on next page*)

Table 1 (*continued*)

	Taxonomic sections																	
	Chlamydoclusia					*Cordylandra*								*Criuva*			*Phloianthera*	
			nemorosa															
Compounds	RI	*grandiflora*	male	herm.	*rosea*	*paralicola*	*spiritu-sanctensis*	*panapanari*	*renggerioides*	*burchellii*	*fluminensis*	*pernambucensis*	*weddelliana*	*parviflora*	*criuva*	*flava*	*hilariana*	*lanceolata*
Docosane	2200	4.9	1.7	0.4	–	–	tr	–	0.3	–	–	–	–	–	–	–	0.6	0.3
Octadecanol acetate	2208	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	tr
Eicosanol	2273	–	1.6	0.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tricosane	2300	12.5	5.0	0.9	2.5	–	2.2	–	–	–	–	–	–	–	2.4	–	0.2	0.6
Tetracosane	2400	4.0	1.7	0.6	–	–	1.0	–	–	–	–	–	–	–	tr	–	0.5	0.4
subtotal		50.7	49.6	44.6	60.9	9.4	89.6	41.0	42.6	56.9	2.3	–	6.4	24.5	11.9	83.6	5.6	9.4
Isoprenoids																		
Limonene	1031	–	–	–	–	–	1.0	–	0.8	0.7	0.5	0.2	–	–	–	–	–	–
iso-3-Thujanol	1135	–	–	0.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Camphor	1142	–	–	–	–	–	–	–	–	–	tr	tr	–	–	–	–	–	–
3-*neo*-Thujanol	1151	0.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Menthone	1152	–	–	–	–	–	–	–	0.9	–	tr	–	–	–	–	–	–	–
α-Felandren-8-ol	1167	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	–	–	–
α-Terpineol	1190	–	–	–	–	–	–	3.9	–	–	tr	tr	–	–	1.2	–	–	–
cis-Sabinene hydrate acetate	1213	–	–	–	–	–	–	–	6.1	–	–	–	–	–	–	–	–	–
Carvone	1242	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	–	–
Geraniol	1252	–	–	–	–	–	–	–	0.4	–	–	–	–	–	–	–	–	–
Thymol	1291	–	–	–	–	–	–	1.1	–	–	–	–	–	–	–	–	–	–
trans-Carvyl acetate	1333	–	–	–	–	–	–	–	–	–	0.1	–	–	–	–	–	–	–
δ-Elemene	1338	–	–	–	–	–	–	–	–	–	–	0.7	–	–	–	–	–	–
δ-Cebebene	1349	–	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	0.5
Isoledene	1372	–	–	–	–	–	–	–	–	–	tr	1.2	–	–	–	–	–	–
Linalool isobutyrate	1372	–	–	–	–	–	–	tr	–	–	–	–	–	–	–	–	–	0.4
α-Copaene	1377	–	–	–	–	0.5	–	–	–	0.8	1.0	tr	6.3	tr	–	6.0	0.6	25.0
β-Cubebene	1391	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.5	–
β-Elemene	1394	–	–	–	–	–	–	–	–	–	1.8	7.4	–	–	–	–	–	–
α-Cedrene	1408	–	–	–	–	–	–	–	–	–	0.3	–	–	–	–	–	–	–
α-Gurjunene	1410	–	–	–	–	–	–	–	–	–	–	0.6	–	–	–	–	–	tr
α-*cis*-Bergamotene	1415	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.2	–
β-Cedrene	1419	–	–	–	–	–	–	–	–	–	3.2	–	–	–	–	–	–	–
β-Caryophyllene	1419	–	1.5	–	–	–	–	–	0.5	0.8	–	7.5	26.8	3.0	–	0.3	3.9	6.4
Aristolene	1429	–	–	–	–	–	–	–	–	–	12.5	–	–	–	–	–	–	–
β-Gurjunene	1431	–	–	–	–	–	–	–	0.2	–	9.6	0.6	–	–	–	–	–	0.7
trans-β-Bergamotene	1436	–	–	–	2.0	–	–	–	–	–	–	–	–	1.6	–	–	–	–
Aromadendrene	1442	–	–	–	–	–	–	–	–	–	–	3.4	–	–	–	–	0.4	0.9
Geranyl acetone	1452	1.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α-Humulene	1454	–	–	–	–	–	–	–	0.9	tr	1.2	2.1	5.4	0.6	–	0.6	tr	2.5
trans-β-Farnesene	1456	–	–	3.8	–	–	–	–	–	tr	tr	–	4.5	–	–	–	3.6	3.5
Alloaromadendrene	1465	–	–	–	–	–	–	–	–	–	0.5	0.7	–	0.2	–	tr	13.9	1.5
Drima-7,9(11)-diene	1471	–	–	–	–	–	–	–	–	–	–	0.5	–	–	–	–	–	–
β-Acoradiene	1473	–	–	–	–	–	–	–	–	–	0.6	–	–	–	–	–	–	–
γ-Muurolene	1479	–	6.2	–	–	–	–	–	–	–	6.8	5.4	1.8	–	–	tr	1.4	1.3
Germacrene D	1482	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	–	1.2
Ar-Curcumene	1483	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.9	–
Neryl isobutyrate?	1485	–	–	–	–	–	–	–	–	–	–	–	–	0.2	–	–	–	–
γ-Curcumene	1488	–	–	–	–	–	–	–	–	–	2.8	–	–	–	–	–	–	–
cis-β-Guaiene?	1487	–	–	–	–	–	–	–	–	–	–	4.3	–	–	–	–	0.8	0.3
β-Selinene	1494	–	–	–	–	–	–	–	–	–	5.9	–	–	–	–	–	tr	0.8
Ledene	1495	–	–	–	–	–	–	–	–	–	8.9	8.4	–	–	–	–	–	3.6
Valencene	1496	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.8	–
Bicyclogermacrene	1497	–	–	–	–	–	–	–	–	–	–	–	9.3	–	–	tr	–	–
α-Muurolene	1499	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	tr	2.3
α-Bulnesene	1505	–	–	–	–	–	–	–	–	–	–	10.8	–	–	–	–	–	–
β-Bisabolene	1508	–	–	–	–	–	tr	–	–	–	tr	–	–	–	–	–	1.8	–

(*Table continued on next page*)

Table 1 (*continued*)

	Taxonomic sections																		
	Chlamydoclusia					*Cordylandra*								*Criuva*			*Phloianthera*		
			nemorosa																
Compounds	RI	*grandiflora*	male	herm.	*rosea*	*paralicola*	*spiritu-sanctensis*	*panapanari*	*renggerioides*	*burchellii*	*fluminensis*	*pernambucensis*	*weddelliana*	*parviflora*	*criuva*	*flava*	*hilariana*	*lanceolata*	
(E,E)-α-Farnesene	1509	2.8	3.3	–	–	–	–	–	–	tr	–	–	1.0	–	–	1.6	tr	0.3	
trans-β-Guaiene	1513	–	–	–	–	–	–	–	–	–	1.4	–	–	–	–	–	–	–	
Dehydro-Ar-α-himachalene	1514	–	–	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–	
γ-Cadinene	1516	–	1.6	–	–	–	–	–	–	–	2.0	–	–	–	–	–	2.2	0.7	
β-Curcumene	1517	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	–	0.2	
α-Irone	1522	–	–	–	–	–	–	–	–	–	–	–	–	–	0.6	–	–	–	
7-*epi*-α-Selinene	1523	–	–	–	–	–	–	–	–	–	tr	1.7	–	–	–	–	–	–	
δ-Cadinene	1525	–	2.6	–	–	3.1	–	–	0.5	–	4.2	1.5	4.1	tr	–	6.5	9.0	11.0	
Cadina-1,4-diene	1536	–	–	–	–	–	–	–	–	–	0.5	–	–	–	–	–	1.5	0.9	
α-Cadinene	1542	–	–	–	–	–	–	–	–	–	0.7	1.0	–	–	–	–	tr	0.2	
α-Calacorene	1543	–	–	–	–	–	–	–	0.4	–	0.2	0.5	–	–	–	–	0.9	tr	
Elemol	1551	–	–	–	–	–	–	–	–	–	–	1.6	–	–	–	–	–	–	
Germacrene B	1559	–	–	–	–	–	–	–	tr	–	tr	0.9	–	–	–	–	–	tr	
epi-Longipinanol	1560	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.7	0.2	
β-Calacorene	1563	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	tr	tr	
(E)-Nerolidol	1563	–	–	–	–	–	–	–	tr	2.1	tr	–	0.7	–	4.0	–	–	–	
Ledol	1569	–	–	–	–	–	–	–	–	–	1.1	1.0	tr	–	–	–	–	1.2	
Caryophyllene alcohol	1570	–	–	–	–	–	–	–	1.4	–	–	–	2.9	–	–	–	1.5	tr	
Spathulenol	1580	–	–	–	–	–	–	–	tr	–	0.5	0.9	–	–	–	–	0.8	–	
Globulol	1586	–	–	–	–	–	–	–	–	–	1.4	2.6	3.7	–	–	–	3.4	2.2	
Caryophyllene oxide	1586	–	–	–	–	–	–	–	2.2	–	–	–	–	–	–	–	–	–	
Viridiflorol	1592	–	–	–	–	–	–	–	0.5	–	1.3	2.4	2.3	–	–	–	tr	0.8	
Guaiol?	1594	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.3	1.2	
cis-dihydro-Occidentalol?	1602	–	–	–	–	–	–	–	–	tr	–	1.3	–	–	–	–	–	–	
α-Cedrol	1604	–	–	–	–	–	–	–	–	–	4.3	–	1.9	–	–	–	–	–	
cis-Isolongifolanone	1610	–	–	–	–	–	–	–	–	–	–	0.6	–	–	–	–	–	–	
epi-Cedrol	1613	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.4	0.3	
10-*epi*-γ-Eudesmol	1620	–	–	–	–	–	–	–	–	–	1.7	1.2	–	–	–	–	2.3	–	
1-*epi*-Cubenol	1629	0.3	–	–	–	0.8	0.3	–	2.1	–	tr	–	–	–	–	–	6.8	tr	
γ-Eudesmol	1632	–	–	–	–	–	–	–	–	–	–	4.5	–	–	–	–	–	–	
α-Acorenol	1634	–	–	–	–	–	–	–	–	–	0.3	–	–	–	–	–	–	1.9	
epi-α-Cadinol	1643	0.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
epi-α-Muurolol	1643	–	–	–	–	1.5	tr	–	2.0	–	5.1	3.0	1.0	–	–	–	–	3.8	
Cubenol	1644	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8.4	–	
α-Muurolol	1646	–	–	–	–	–	–	–	tr	–	tr	–	tr	–	–	–	–	0.9	
Vulgarone B	1649	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.5	
α-Cadinol	1656	0.6	–	–	–	–	0.5	–	1.8	–	9.2	9.2	2.9	–	–	–	2.7	1.4	
Bulnesol	1666	–	–	–	–	–	–	–	–	–	–	1.5	–	–	–	–	–	–	
14-OH-9-*epi*-β-Caryophyllene	1672	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	–	0.3	
β-Bisabolol	1674	–	–	–	–	–	–	–	–	–	2.6	–	–	–	–	–	tr	0.5	
Khusinol	1674	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.0	–	
5-*neo*-Cedranol?	1678	–	–	–	–	–	–	–	–	1.0	0.7	–	–	–	–	–	–	–	
Acorenone	1685	–	–	–	–	–	–	–	0.6	–	0.6	0.2	–	–	–	–	–	–	
epi-α-Bisabolol	1685	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	–	
α-Bisabolol	1687	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	–	–	
(Z)-α-*trans*-Bergamotol	1694	–	–	–	–		–	–	–	–	–	–	–	–	1.1	–	–	–	
Selin-7(11)-en-4-ol	1696	–	–	–	–	–	–	–	0.3	–	–	–	–	–	–	–	–	–	
(2Z, 6E)-Farnesol	1697	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.5	tr	
14-OH-α-Humulene	1711	–	–	–	–	–	–	–	–	–	0.1	–	–	–	–	–	–	–	
Oplopenone?	1717	–	–	–	–	–	–	–	–	–	–	1.2	–	–	–	–	–	–	
(E,E)-Farnesol	1722	tr	–	–	–	–	1.1	–	–	–	–	–	–	–	1.3	–	–	–	
Zerumbone?	1731	–	–	–	–	–	–	–	0.8	–	–	–	–	–	–	–	–	–	
Bisabolone	1746	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.5	–	
Aristolone	1762	–	–	–	–	–	–	–	–	–	0.4	–	–	–	–	–	–	–	
(Z)-*trans*-Bergamotol?	1800	–	–	–	–	–	–	–	–	tr	tr	–	–	–	–	–	–	–	

(*Table continued on next page*)

Table 1 (*continued*)

	Taxonomic sections																	
	Chlamydoclusia					*Cordylandra*								*Criuva*			*Phloianthera*	
			nemorosa															
Compounds	RI	*grandiflora*	male	herm.	*rosea*	*paralicola*	*spiritu-sanctensis*	*panapanari*	*renggerioides*	*burchellii*	*fluminensis*	*pernambucensis*	*weddelliana*	*parviflora*	*criuva*	*flava*	*hilariana*	*lanceolata*
14-OH-δ-Cadinene	1805	–	–	–	–	–	–	–	–	–	0.1	–	–	–	–	–	–	–
(E,E)-Farnesyl acetate	1837	–	–	–	–	–	–	–	–	–	–	–	–	0.3	–	–	0.2	–
Pimaradiene	1941	–	–	–	–	–	–	–	0.2	–	–	–	1.4	–	–	–	–	–
Kaur-16-ene	2043	–	–	–	–	–	–	–	–	–	–	–	–	11.5	1.8	–	–	–
subtotal		6.6	15.2	4.6	2.0	5.9	2.9	5.0	22.8	5.4	94.1	90.6	76.0	17.4	10.8	15.8	76.7	79.4
Benzenoids																		
Benzaldehyde	980	–	–	–	–	–	–	–	–	–	–	–	–	0.4	–	–	–	–
Benzyl alcohol	1034	–	–	0.3	–	–	–	–	–	–	–	–	–	0.5	–	–	–	–
Benzeneacetaldehyde	1043	–	–	–	–	–	–	–	–	–	–	–	–	13.4	–	–	–	–
Phenyl ethyl alcohol	1109	–	–	–	–	–	–	–	0.7	–	–	tr	–	12.1	–	–	–	–
Veratrole	1142	–	–	–	–	–	–	–	0.9	–	–	–	–	–	–	–	–	–
1,2-Dimethoxybenzene	1148	–	–	–	–	–	–	–	–	–	–	–	–	0.4	2.0	–	–	–
p-Anisaldehyde	1249	–	3.3	–	–	–	–	–	–	–	–	–	–	–	58.7	–	–	–
p-Anisyl alcohol	1278	–	2.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl 2-methoxybenzoate	1335	–	–	–	–	–	–	–	–	–	–	–	–	0.2	–	–	–	–
Eugenol	1354	6.6	1.0	13.3	1.1	–	–	–	tr	–	–	–	–	–	–	–	–	–
4-*tert*-Amyl phenol	1393	–	–	–	–	–	0.4	–	–	–	–	–	–	–	–	–	–	–
Vanillin	1394	0.5	–	–	–	–	–	1.4	1.1	–	–	–	–	–	–	–	–	–
Methyl eugenol	1402	–	–	–	–	–	–	–	2.0	–	–	–	–	3.5	–	–	–	–
1,3,5-Trimethoxybenzene	1405	–	–	–	–	–	–	–	tr	–	–	–	–	0.5	6.5	–	–	–
Isoeugenol	1448	tr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2,4-di-*tert*-Butyl phenol	1512	–	–	–	–	–	tr	1.2	–	–	–	–	–	–	–	–	–	–
Ethyl 4-ethoxy-benzoate	1522	–	–	–	–	–	0.5	2.4	–	1.4	–	–	–	–	–	–	–	–
Rheosmin	1550	5.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3-Methoxy-4-methyl acetophenone	1601	–	–	–	–	–	–	–	–	–	–	–	3.7	–	–	–	–	–
subtotal		12.7	6.9	13.6	1.1	–	0.9	5.0	4.7	1.4	–	–	3.7	31.0	67.2	–	–	–
Nitrogen-containing compounds																		
Benzeneacetonitrile	1135	–	–	–	–	–	–	–	1.0	–	–	–	–	6.2	–	–	–	–
Indole	1288	–	–	–	–	80.1	–	–	–	–	–	–	–	0.3	–	–	–	–
Subtotal		–	–	–	–	80.1	–	–	1.0	–	–	–	–	6.5	–	–	–	–
Others																		
M$^+$ *154*	987	–	–	–	–	–	–	–	–	1.4	–	–	–	–	–	–	–	–
M$^+$ *129*	1231	–	–	–	–	1.1	–	–	–	–	–	–	–	–	1.0	–	–	–
M$^+$ *196*	1233	–	4.5	9.0	–	–	–	2.0	–	10.5	–	–	–	–	–	–	–	–
M$^+$ 204	1436	–	–	–	–	–	–	–	–	–	–	1.1	–	–	–	–	–	–
M$^+$ 199	1544	–	–	–	–	–	–	3.9	–	–	–	–	–	–	–	–	–	–
M$^+$ 204	1602	–	–	–	–	–	–	–	1.0	–	–	–	–	–	–	–	–	–
M$^+$ 222	1604	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4.6	1.9
M$^+$ 222	1613	–	–	–	–	–	–	–	–	–	1.1	–	–	–	–	–	–	–
M$^+$ 204	1619	–	–	–	–	–	–	–	0.7	–	–	–	–	–	–	–	–	–
M$^+$ 222	1621	–	–	–	–	–	–	–	–	–	0.9	–	–	–	–	–	–	–
M$^+$ 232	1645	–	–	–	–	–	0.7	1.2	–	–	–	–	–	–	–	–	–	–
M$^+$ *224*	1673	–	–	–	–	–	–	–	1.0	–	–	–	–	–	–	–	–	–
M$^+$ *252*	1702	–	–	–	–	–	–	–	–	–	–	–	–	1.1	–	–	–	–
M$^+$ *199*	1764	–	–	–	–	–	–	–	3.4	–	–	–	–	–	–	–	–	–
M$^+$ 208	1809	–	–	–	–	–	–	–	–	–	–	–	–	8.5	1.7	–	–	–
M$^+$ 236	1821	–	–	–	–	–	–	–	–	–	–	1.0	–	–	–	–	–	–
Unidentified		26.3	19.8	24.7	7.4	–	2.0	27.0	6.9	21.9	–	–	–	3.3	–	–	0.5	1.3
Total		96.3	98.5	96.8	71.4	96.5	96.1	85.1	91.4	97.5	98.4	92.7	86.1	92.3	92.6	99.4	87.4	92.0

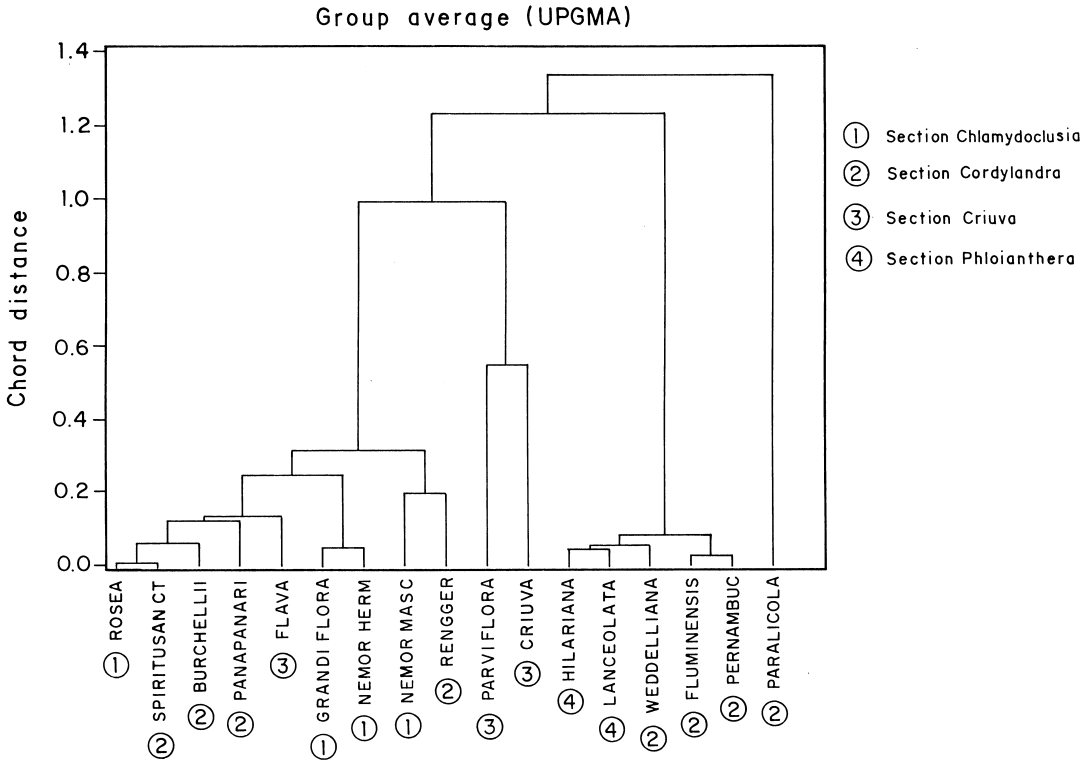


Fig. 1. Dendrogram showing clustering of *Clusia* species using UPGMA with chord distance based on floral fragrance data matrix (fatty acid derivatives, isoprenoids, benzenoids, nitrogen-containing compounds) depicted in Table 1.

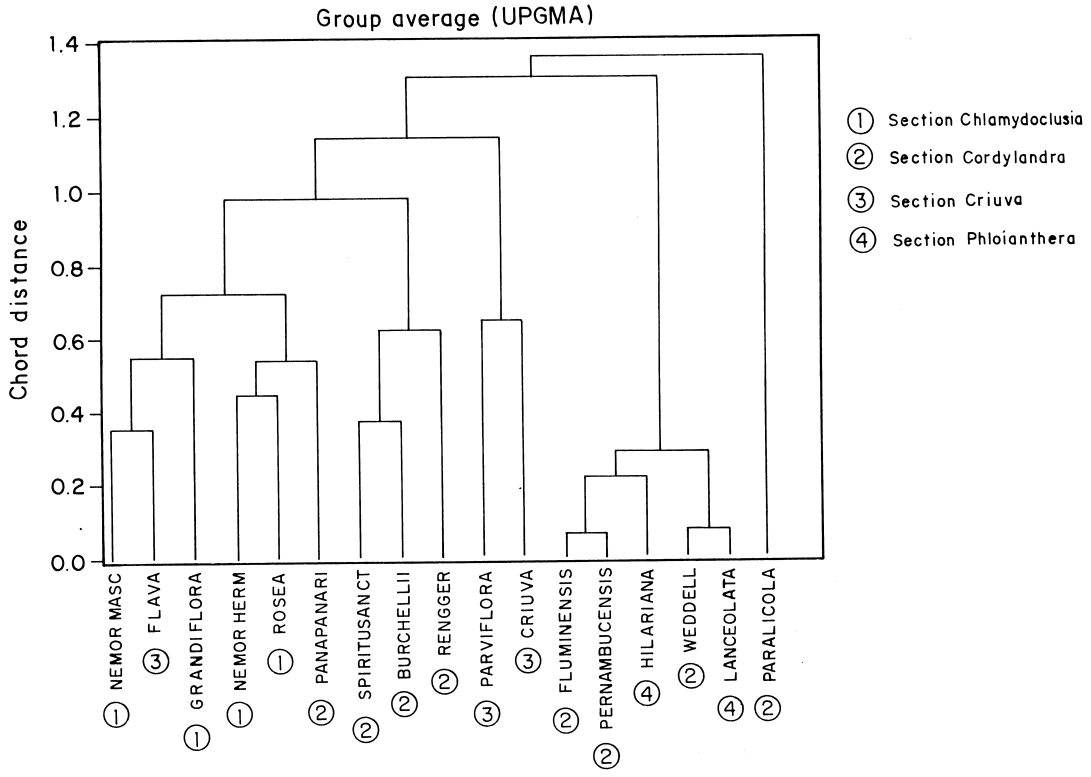


Fig. 2. Dendrogram showing clustering of *Clusia* species using UPGMA with chord distance based on floral fragrance data matrix depicted in Table 2.

Table 2
Flower volatile components of sixteen *Clusia* species grouped into 10 different classes

	Taxonomic sections																
	Chlamydoclusia				*Cordylandra*								*Criuva*			*Phloianthera*	
		nemorosa															
Compounds	*grandiflora*	male	herm.	*rosea*	*paralicola*	*spiritu-sanctensis*	*panapanari*	*renggerioides*	*burchellii*	*fluminensis*	*pernambucensis*	*weddelliana*	*parviflora*	*criuva*	*flava*	*hilariana*	*lanceolata*
Benzenoids	12.7	6.9	13.6	1.1	–	0.9	5.0	4.7	1.4	–	–	3.7	31.0	67.2	–	–	–
Monoterpenes	–	–	–	–	–	1.0	–	0.8	0.7	0.5	0.2	–	–	–	–	–	–
Oxygen–containing monoterpenes	2.0	–	0.8	–	–	–	5.0	7.4	–	0.1	–	–	0.2	2.6	–	–	0.4
Long chain hydrocarbons	35.2	40.8	19.8	29.5	4.4	5.1	11.8	1.2	8.3	0.1	–	4.5	0.8	6.9	58.2	5.6	3.9
Oxygen–containing long chain hydrocarbons	2.4	4.5	18.2	24.2	5.0	41.9	28.1	13.7	32.9	0.2	–	1.9	23.7	5.0	25.4	–	3.8
Sesquiterpenes	–	15.2	3.8	2.0	3.6	–	–	2.7	1.6	64.1	59.2	59.2	5.4	–	15.8	43.4	63.8
Oxygen–containing sesquiterpenes	4.6	–	–	–	2.3	1.9	–	11.7	3.1	29.4	31.2	15.4	0.3	6.4	–	33.3	15.2
Carboxylic acids	13.1	4.3	6.6	7.2	–	42.6	1.1	27.7	15.7	2.0	–	–	–	–	–	–	1.7
Diterpenes	–	–	–	–	–	–	–	0.2	–	–	–	1.4	11.5	1.8	–	–	–
Nitrogen–containing compounds	–	–	–	–	80.1	–	–	1.0	–	–	–	–	6.5	–	–	–	–

that the taxonomic subgroups explain more of the total variation in the volatile data set than does the pollinator type. An ordination of the data using principal components analysis (PCA) showed that the apparently isolated position of *C. paralicola* in the dendrogram is somewhat misleading, and that this species occupies an intermediate position among all of the other species.

As expected, there are also patterns at the ecological level concerning the pollinators, taxonomic grouping and the chemical composition of the floral volatiles. Thus, flowers of species in section *Chlamydoclusia* are mainly pollinated by bees of the tribe *Euglossini*, and flowers in section *Cordylandra* by *Trigonini* bees, while those of the section *Phloianthera* attract *Euglossini* (large flowers) or *Trigonini* (small flowers). Specimens of the section *Criuva* s. lat. seem to mostly attract beetles.

The combination of chemical analyses of floral odours and behavioural experiments of the flower-visiting insects is of special importance (Dobson, 1994). Field bioassays were performed to test if the floral volatiles were responsible for insect attraction. Two tests were conducted; in one, fresh dichloromethane flower extract was put onto filter paper and, after solvent evaporation, was suspended in branches of *C. lanceolata* (section *Phloianthera*) close to filter papers treated only with dichloromethane. In the second test, petals of fresh flowers were squeezed on pieces of black cloth and suspended in branches close to pieces of black cloth without squeezed petals. Filter papers with flower scent and pieces of cloth impregnated with petal fluid both attracted *Trigona spinipes* bees, which visit the flowers for resin collection. In another experiment we combined flowers of different species. When flowers of *C. grandiflora* (section *Chlamydoclusia*) were placed in the *C. lanceolata* tree they were not visited, although they are resiniferous and visited by *Trigona spinipes* bees regularly on their mother plant ca. 100 m away. When we placed two petals of *C. lanceolata* inside the much larger flower of *C. grandiflora*, however, resin-collecting Trigonas almost immediately visited this flower and started to collect its resin.

3. Conclusions

The chemical composition of the floral scents in *Clusia* reflects only in part the taxonomic group to which each species belongs. Discordance between groups in the dendrogram and the taxonomic sections may have different causes. First, the traditional sections of *Clusia* based on morphological characters may be unnatural (not monophyletic). This seems unlikely, however, since the sections included in this analysis are rather well supported by several morphological characters. Analysis of macromolecular characters largely supports this conclusion (Gustafsson and Bittrich, 1999). Second, adaptation to different pollinators may have resulted in a relatively rapid development of new floral scents. The banana-like flavour of the flowers of *C. flava* is almost certainly an adaptation to pollination by weevils (Hammel, 1986) and is known to have evolved independently in other flowering plants, e.g. certain Annonaceae. Third, alteration of floral scents may have played an important role during speciation. The fact that species of section *Cordylandra*, members of a most probably natural (monophyletic) section and all mainly or exclu-

sively pollinated by small bees of tribe Trigonini, often show very different composition in their floral scents, cannot be explained by causes 1 or 2. One important element in the reproduction of flowering plants is the avoidance of unwanted cross-pollination. Plants depending on cross-pollination (as in all dioecious species) have to ensure that their pollen is transported to the stigmas of conspecific plants and conversely that their stigma receives pollen only from a conspecific plant. Failing to achieve this may lead to pollen/ovule loss, hybridization resulting in maladapted offspring, and eventually extinction of the species. As our field experiments have shown, the floral scents in *Clusia* may be of considerable importance in attracting *Trigona* bees to flowers of a specific species. Several species of *Clusia* occur sympatrically or even syntopically (e.g. *C. renggerioides* and *C. panapanari* of section *Cordylandra*, and *C. grandiflora* and *C. nemorosa* of section *Chlamydoclusia*). While crossing experiments (Bittrich and Amaral, unpublished results) have shown that many species of *Clusia*, even from different sections, can be artificially crossed rather easily, natural hybrids are rare as documented in herbarium records of *Clusia* specimens from Brazil (V. Bittrich, unpublished results). This suggests that the importance of different floral scents is probably high both for speciation and for maintaining the integrity of the species already evolved. Another problem for the construction of the phenogram based on the composition of the floral scents is how best to form groups of its chemical components. We have presented two different possibilities, with 4 and 10 principal groups respectively, but obviously there are several others. We doubt, however, that different groupings would affect the basic conclusions of our study.

4. Experimental

4.1. Methods and plant material

The essential oils of petals were obtained from species cultivated in the Fazenda Santa Elisa of the Instituto Agronômico (IAC), Campinas, São Paulo State, Brazil by microhydrodistillation of ca. 5 g of fresh petals. The oils were extracted from the distillation water (ca. 40 ml) with dichloromethane (3 × 10 ml), dried over anhydrous sodium sulphate and reduced to ca. 0.5 ml at room temperature under reduced pressure on a rotatory evaporator. Two or three replicate analyses were used for some species (*C. grandiflora, C. spiritu-sanctensis, C. fluminensis, C. lanceolata, C. hilariana*). Voucher specimens of the species are deposited in the Herbarium UEC (coll. nrs. M.C.E. Amaral & V. Bittrich). *C. burchellii* Engl. (#97/3 masc.)., *C. criuva* Cambess. (#97/247 masc.), *C. flava* Jacq. (#97/6 masc.), *C. fluminensis* Planch. & Triana (#97/249 masc.), *C. grandiflora* Splitg. *(#95/152* masc., *95/153* fem.), *C. hilariana* Schltdl. (#97/248 masc.), *C. lanceolata* Cambess. (#96/27 masc., #96/27a, fem.), *C. nemorosa* G. Mey. (#95/151 masc., 95/150 hermafr.), *C. panapanari* (Aubl.) Choisy (#95/156 fem.), *C. paralicola* G. Mariz. (#97/5 masc., #97/5a fem.), *C. parviflora* Engl. nom. illeg. (#97/7 fem., #97/7a masc.), *C. pernambucensis* G. Mariz (#95/186 masc.), *C. rosea* Jacq. (#95/154 fem.), *C. renggerioides* Planch. & Triana (#97/1 masc.), C. *spiritu-sanctensis* G. Mariz & B. Weinberg (#95/185 masc., #95/185a fem.), *C. weddelliana* Planch. & Triana (#97/4 masc.).

4.2. GC and GC/MS

Analyses were carried out using a HP-5990/5970 system equipped with J&W Scientific DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm); column temperatures were programmed from 60 to 240°C at 3°C min^{-1} for integrating purposes.

4.3. GC

Injector and detector temperatures were 220 and 285°C respectively. Hydrogen was used as carrier gas at a flow rate 1.16 ml min^{-1} in the split mode, with an injection volume, 1.0 μl solution of about 10 mg of essential oil in dichloromethane. The retention indices were obtained by co-injecting the oil sample with a C_{11}–C_{24} linear hydrocarbon mixture (retention index from 900 to 1099 range was obtained by extrapolation).

4.4. GC/MS

The carrier gas was helium and the temperature program was the same as that for the GC experiments. The mass spectra were taken at 70 eV with a scanning speed of 0.84 scan s^{-1} from m/z 40 to 550.

The oils were analysed by GC and GC/MS, and identification was made on the basis of standard compound co-injection (both commercial and synthetic, characterised by IR, ^{1}H, ^{13}C NMR spectra) and comparison of retention indices (Van den Dool and Kratz, 1963) as well as by computerised matching of the acquired mass spectra with those stored in the Wiley/NBS mass spectral library of the GC/MS data system and other published mass spectra (Adams, 1995).

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Flavonoid characters contributing to the taxonomic revision of the *Hebe parviflora* complex

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Abstract

Comparative flavonoid chemistry is a key element of a multidisciplinary study aimed at a revision of the genus *Hebe*, New Zealand's largest genus of flowering plants. One aspect of this study has been an investigation of the *Hebe parviflora* complex. A recent botanical paper on this topic marshalls generalised flavonoid data and morphological characters to support the recognition of two species in this complex, *Hebe stenophylla* and *Hebe parviflora*, which are clearly distinguishable from each other and from the related *Hebe traversii* and *Hebe strictissima*. A detailed study of the flavonoid chemistry and the distributional data used to support these conclusions are presented here. Six new compounds have been isolated in this study, including 6-hydroxyapigenin-7-*O*-β-[2-*O*-β-xyloxyloside] and-7-*O*-β-[2-*O*-β-xyloglucoside], 6-hydroxyluteolin-7-*O*-β-[2-*O*-β-xyloxyloside] and, luteolin-, 6-hydroxyluteolin- and 4′-*O*-methylluteolin-7-*O*-β-[6-*O*-β-xyloglucoside]. Other flavonoids include apigenin and luteolin 7- and 4′- mono-, di- and possibly tri-*O*-glycosides, 8-hydroxyluteolin 7- and 8-*O*-glucosides, and kaempferol and quercetin 3-*O*-mono- and di-glycosides. New structure assignments are supported with detailed ^{1}H and ^{13}C NMR data, including HMQC and HMBC measurements.
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Keywords: *Hebe parviflora*; *Hebe stenophylla*; Scrophulariaceae; Chemotaxonomy; Flavonoids; 6-Hydroxyapigenin-7-*O*-glycosides; 6- and 8-Hydroxyluteolin-7-*O*-glycosides; 8-Hydroxyluteolin-8-*O*-glucoside

1. Introduction

Hebe is New Zealand's largest genus of flowering plants. According to the latest monograph (Allan, 1961), it contains about 100 species. However this monograph is now seriously out of date and a major new multidisciplinary study of *Hebe* taxonomy has recently been initiated. Comparative flavonoid chemistry comprises a key element of this study, and the present communication provides details of the flavonoid chemistry and flavonoid distribution that contributed to the recent paper on the taxonomic revision of the *Hebe parviflora* complex (Bayly et al., 2000). In this revision, the two entities long informally recognised in the *Hebe parviflora* complex, are assigned species status and named *H. parviflora* and *H. stenophylla* (with three varieties recognised within *H. stenophylla* on morphological grounds). Additionally, these two species were shown to be distinguishable from the related species, *Hebe strictissima* and *Hebe traversii*. The bulk of the flavonoid chemistry and the detailed distributional data used to support the revised taxonomy have yet to be reported. To date, the only flavonoid paper relating to this study, describes the structure elucidation of one known and one novel flavone glycoside, 6-hydroxyluteolin-7-*O*-[2-*O*-xyloglucoside] and -7-*O*-[2-*O*-xyloxyloside] respectively, from *H. stenophylla* (Mitchell et al., 1999). The balance of the flavonoid studies is reported in the current paper.

2. Results

Leaf samples from 20 *H. stenophylla* (including all three varieties), 15 *H. parviflora*, six *H. traversii* and four *H. strictissima* plants were supplied for flavonoid analyses. Samples of *H. stenophylla*, *H. parviflora* and *H. strictissima* cover the full geographic range of these

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species, while samples of *H. traversii* are from a more limited geographic area. The oven dried specimens were extracted with $MeOH/H_2O$, and 2D-PCs run on each crude extract. Flavonoid high performance liquid chromatography (HPLC) profiles of these extracts were found to be very complex due to the presence of numerous hydroxycinnamic acid derivatives (which ran well clear of most flavonoid glycosides in the 2D-PCs). Therefore, prior to HPLC, the extracts were routinely cleared of these derivatives using a short cellulose column eluted with 2% HOAc. The 2D-PC profiles were used to assign numbers to each visible flavonoid (spot), and these profiles were correlated to the HPLC profiles by extraction of individual 2D-PC spots followed by co-HPLC with the crude extract. Additional compounds revealed by HPLC were assigned the spot number followed by a, b or c. A composite 2D-PC profile representing all flavonoid glycosides observed in the studied species is presented in Fig. 1, and flavonoid structure and HPLC retention time data appear in Table 1. Because this work forms part of a much larger study, the spot (= compound) numbering system is discontinuous. This is because compounds found in other studied *Hebe* species are not included.

Presence/absence data for all flavonoids in every plant sample are detailed in Table 2. Samples are labelled here with collector's or herbarium numbers [see Bayly et al. (2000) for further explanation of these codes]. The data presented are the raw data used in summarized form for the submitted taxonomic revision paper on the *H. parviflora* complex (Bayly et al., 2000), and represent a combination of information obtained from both 2D-PC and HPLC. HPLC was of particular value in the identification of components which overlap or cochromatograph on 2D-PC. It was also helpful in the detection of "trace" levels of components difficult to recognise by 2D-PC.

The flavonoid glycosides found in the *H. parviflora* complex and related species, comprise a limited range of structural types which are recognisable both from the colour of their 2D-PC spots in NH_3/UV and from their on-line recorded absorption spectra. On these criteria the flavonoids fall roughly into ten groups as follows.

2.1. Group 1 (apigenin-7-O-glycosides)

Group 1 compounds (**10**, **12**, **17** and **22**) possess absorption spectra with maxima at about 267 and 337 nm and turn a fluorescent green in NH_3/UV on a 2D-PC. They thus resemble apigenin-7-*O*-glycosides (Mabry et al., 1970). The lowest level glycoside in the group, **10**, was shown to be identical to apigenin-7-*O*-glucoside by its 2D-PC properties, absorption spectrum and by HPLC cochromatography with an authentic sample.

The absorption spectrum and 2D-PC properties of **12** suggest that it is an apigenin-7-*O*-diglycoside and this was confirmed by its ^1H NMR spectrum, which contains two sugar anomeric proton signals (δ 5.03 and δ 4.17, $J = 7$ Hz) in addition to the aromatic proton signals of an

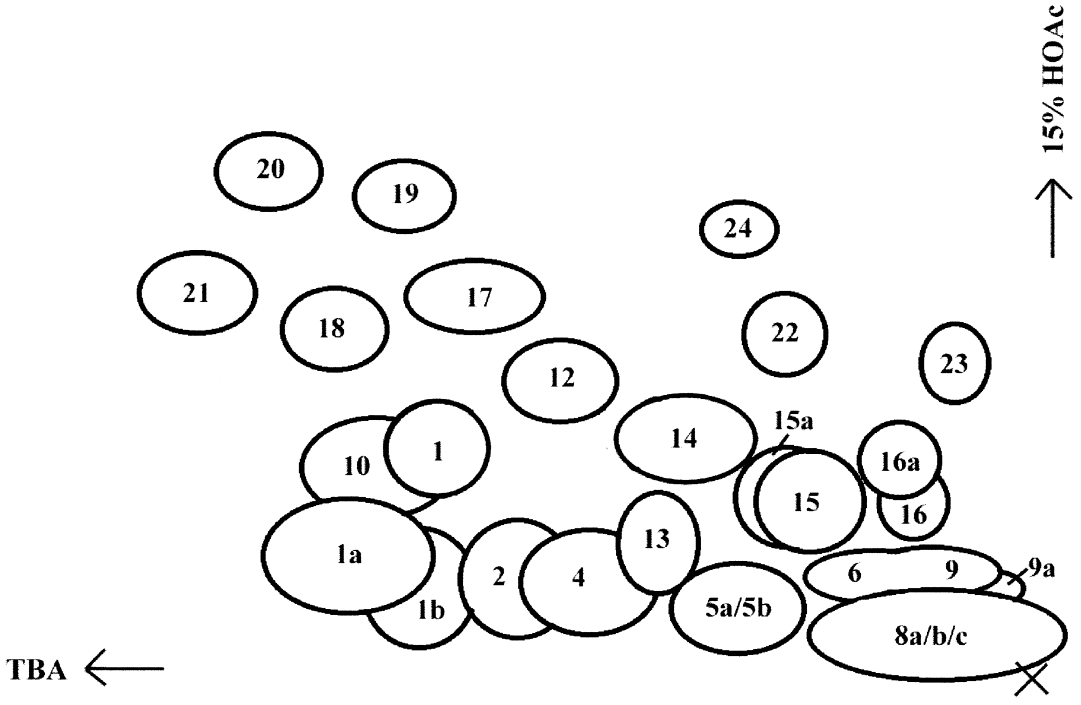


Fig. 1. Composite 2D-PC representation of flavonoids encountered in the *Hebe parviflora* complex. The structures of flavonoids represented by these spots are detailed in Table 1.

Table 1
Structures and HPLC retention times (RT) for the flavonoids found in species of the *Hebe parviflora* complex

Compound no. (= Spot no.)	Structure[a]	RT[b]
1	Luteolin-4′-*O*-β-[6-*O*-α-rhamnosylglucoside]?	35.3
1a	Luteolin-4′-*O*-β-glucoside	36.2
1b	Luteolin-3′-*O*-β-glucoside	37.5
2	8-Hydroxyluteolin-8-*O*-β-glucoside	37.5
4	Luteolin-7-*O*-β-glucoside	33.7
5a	8-Hydroxyluteolin-7-*O*-β-glucoside	32.8
5b	6-Hydroxyapigenin-7-*O*-β-[2-*O*-β-xyloxyloside]	37.5
6	6-Hydroxyapigenin-7-*O*-β-[2-*O*-β-xyloglucoside]	34.4
8a	6-Hydroxyluteolin-7-*O*-β-[2-*O*-β-xyloxyloside]	35.1
8b	6-Hydroxyluteolin-7-*O*-β-[2-*O*-β-xyloglucoside]	32.4
8c	6-Hydroxyluteolin-7-*O*-β-glucoside	33.7
9	6-Hydroxyluteolin-7-*O*-β-[2-*O*-β-glucoglucoside]	29.8
9a	6-Hydroxyluteolin-7-*O*-β-[6-*O*-β-xyloglucoside]	30.8
10	Apigenin-7-*O*-β-glucoside	36.3
12	Apigenin-7-*O*-β-[6-*O*-β-xyloglucoside]	35.1
13	Luteolin-7-*O*-β-D-glucuronide	33.8
14	Luteolin-7-*O*-β-[6-*O*-α-rhamnosylglucoside]	33.1
15	Luteolin-7-*O*-β-[6-*O*-β-xyloglucoside]	32.8
15a	4′-*O*-Methylluteolin-7-*O*-β-[6-*O*-β-xyloglucoside]	33.7
16	Luteolin-3′, 7-di-*O*-β-glucoside	31.6
16a	Luteolin-7-*O*-triglycoside?	30.9
17	Apigenin-7-*O*-β-[6-*O*-α-rhamnoglucoside]	35.1
18	Quercetin-3-*O*-β-glucoside	33.6
19	Quercetin-3-*O*-β-[6-*O*-α-rhamnoglucoside]	32.8
20	Kaempferol-3-*O*-β-[6-*O*-α-rhamnoglucoside]	34.8
21	Kaempferol-3-*O*-β-glucoside	35.6
22	Apigenin-7-*O*-triglycoside?	32.2
23	Luteolin-7-*O*-triglycoside?	29.4
24	Apigenin-6,8-di-C-glucoside	28.0

[a] Sugars all pyranosides; ? = structure tentative.
[b] RT values accurate to ±0.1.

apigenin nucleus. ^{13}C NMR defined the sugars as xylose and glucose (Markham, 1982). The terminal sugar (H-1 at δ4.17) was identified as xylose by TOCSY studies which correlated this anomeric proton, and its H–H coupled H-2, to the five sugar carbons representing xylose. That this xylose is linked 1–6 to the glucose is evidenced by the downfield shift of the glucose C-6 to 68.8 ppm. Compound **12** is thus identified as apigenin-7-*O*-β-[6-*O*-β-xylopyranosylglucopyranoside], a compound which has been reported only once previously (Markham, et al., 1987). In a like manner, compound **17** was shown to be the known apigenin-7-*O*-β-[6-*O*-α-rhamnosylglucopyranoside].

The fourth compound in this group, **22**, was rarely encountered in this survey and appears to be of no taxonomic significance. It is thought to be an apigenin-7-*O*-triglycoside on the basis of its absorption spectrum and 2D-PC characteristics.

2.2. Group 2 (luteolin-3′ and 4′-O-glycosides)

Compounds in this group all have similar absorption spectra, **1** and **1a** (λ_{max} 247, 267, 288 sh, 337 nm) and **1b** (λ_{max} 240, 267, 288 sh, 341 nm), and apart from **1b** which turns olive green, are unaffected by NH_3 on a 2D-PC. In these features they resemble luteolin-3′ and 4′-*O*-glycosides (Markham and Porter, 1974).

Compound **1a** possesses an ^{1}H NMR spectrum typical of a luteolin monoglycoside. The ^{13}C NMR spectrum confirms this and defines the sugar as β-glucopyranose. Shifts in the signals of C-4′ (−1.5 ppm) and C-1′ (+2.7 ppm) relative to luteolin (Markham and Chari, 1982), together with the lack of an effect of NH_3 on its 2D-PC spot, define this compound as luteolin-4′-*O*-β-glucopyranoside.

Compound **1** bears the same 2D-PC relationship to **1a** as apigenin-7-rutinoside (**17**) does to apigenin-7-glucoside (**10**), and with the same absorption spectrum as **1a**, is considered to be a luteolin-4′-*O*-diglycoside and probably the rhamnoglucoside. This compound (and the related **14a**, a possible 4′-*O*-xyloglucoside of luteolin which is found in other *Hebe* species) is rarely encountered at greater than trace levels in this complex (Table 1) and is therefore not investigated further. Compound **1b** was identified as luteolin-3′-*O*-β-glucopyranoside by direct HPLC comparison with an authentic sample.

2.3. Group 3 (luteolin-7-O-glycosides)

Compounds **4**, **13**, **14**, **15**, **15a**, **16a** and **23** all possess absorption spectra with maxima about 254, 271 and 348 nm and (apart from **15a**) turn yellow fluorescent on paper in NH_3/UV, features that are characteristic of luteolin-7-*O*-glycosides (Mabry et al., 1970). The base compounds in this group are **4** and **13**. Compound **4** gave ^{1}H and ^{13}C NMR spectra identical with those of luteolin-7-*O*-β-glucopyranoside (Markham and Geiger, 1994), and **13**, a compound which unlike **4** is mobile on paper in water, gave a similar ^{1}H NMR spectrum but with a distinctive H-5″ doublet at ca δ 3.9 reminiscent of the 7-*O*-glucuronide (Markham and Geiger, 1994). The ^{13}C NMR spectrum confirmed the glucuronide formulation and thereby defined **13** as luteolin-7-*O*-β-glucuronopyranoside.

None of the derivative compounds in this group, **14**, **15**, **16a** or **23** showed significant mobility in H_2O on paper, which indicates that they do not contain glucuronic acid (Markham, 1982) and therefore are not derivatives of luteolin-7-glucuronide (**13**). Compounds **14** and **15** were isolated for nmr studies as they are important indicator compounds for distinguishing *H. parviflora* from *H. stenophylla*. ^{1}H and ^{13}C NMR (Table 3) studies defined **14** and **15** as luteolin-7-*O*-β-[6-*O*-α-rhamnopyranosylglucopyranoside] and luteolin-7-*O*-β-[6-*O*-β-xylopyranosylglucopyranoside] respectively. The sugar identities were evident from the ^{13}C NMR spectra and the interglycosidic linkage from the C-6″ signal downfield shifts of 5.4 and 7.6 ppm respectively,

Table 2
The distribution of flavonoid glycosides in *Hebe* specimens representing the *H. parviflora* complex and related species[ab]

Sample	A/L-mono-glycs						6/8OH-A/L-mono/di-glycs								A/L-di/tri-glycs										K/Q-glycs			
	10	**4**	**1b**	**16**	**1a**	**13**	**2**	**5a**	**5b**	**6**	**8c**	**8a**	**8b**	**9**	**9a**	**12**	**1**	**14**	**15**	**15a**	**24**	**22**	**16a**	**17**	**23**	**18**	**21**	**19**	**20**
	A-7-*O*-glucoside	L-7-*O*-glucoside	L-3′-*O*-glucoside	L-7, 3′-di-*O*-glucoside	L-4′-*O*-glucoside	L-7-*O*-glucuronide	8OHL -8-*O*-glucoside	8OHL -7-*O*-glucoside	6OHA -7-*O*-[2-*O*-xylo xyloside]	6OHA -7-*O*-[2-*O*-xylo glucoside]	6OHL -7-*O*-glucoside	6OHL -7-*O*-[2-*O*-xylo xyloside]	6OHL -7-*O*-[2-*O*-xylo glucoside]	6OHL -7-*O*-[2-*O*-gluco glucoside]	6OHL -7-*O*-[6-*O*-xylo glucoside]	A-7-*O*-[6-*O*-xylo glucoside]	L-4′-*O*-[6-*O*-rhamno glucoside]?	L-7-*O*-[6-*O*-rhamno glucoside]	L-7-*O*-[6-*O*-xylo glucoside]	4′OMeL -7-*O*-[6-*O*-xylo glucoside]	A-6,8 -di-C-glucoside	A-7-*O*-triglycoside?	L-7-*O*-triglycoside?	A-7-*O*-[6-*O*-rhamno glucoside]	L-7-*O*-triglycoside?	Q-3-*O*-glucoside	K-3-*O*-glucoside	Q-3-*O*-[6-*O*-rhamno glucoside]	K-3-*O*-[6-*O*-rhamno glucoside]
H. stenophylla																													
MJB983(s)		+			tr		+	+	tr	+	+	+	+	+															
MJB645(s)		+			tr		+	+	tr	+	+	+	+	+															
MJB651(s)		+			tr		+	+	tr	+	+	+	+	+															
MJB863(s)		+			tr		+	+	+	+	+	+	+	+															
MJB864(s)		+					+	+	tr	+	+	+	+	+															
MJB687(s)		+			tr		tr	+	tr	+	+	+	+	+															
MJB688(s)		+			tr		tr	+	tr	+	+	+	+	+															
MJB689(s)		+					tr	+	tr	+	+	+	+	+													tr		tr	
PJB008(s)		+					tr	+	+	+	+	+	+	+															
PJB009(s)		+					tr	+	+	+	+	+	+	+															
MJB647(s)		+			tr		+	+	+	+	+	+	+	+															
MJB1040(s)		+			+		+	+	+	+	+	+	+	+	?								tr				?			
MJB1041(s)		+						+	+	+	+	+	+	+	+					?			tr						
MJB1033(s)		+			?		+	+	+	+	+	+	+	+	+														
MJB1034(s)		+					+	+	+	+	+	+	+	+	+														
WELT81504(o)		+			?		+	+	+	+	+	+	+	+	+					?			tr				+			
WELT81505(o) ?		+			?		+	+	+	+	+	+	+	+	+					?			tr							
PGJ2350(h)								+	tr	+	tr	+	+	+																
PGJ2352(h)		+						+	tr	tr	+	+	+	+	tr								tr				tr			
PGJ2344(h)		+						+	+	+	tr	+	+	+	tr								tr							
H. parviflora																													
MJB529	+	+		+	+	+	tr	+					+			+	+	+	+					+		tr			
MJB530	+	+		tr	+	+	+	+					+				+	+	+							+			
MJB653		+	+	+	+	+	+	+					+			+	+?	+	+					+					
MJB652		+	+	+	+	+	+	+								+	tr?	+	+					tr		tr		tr	
MJB665		+	+		+	+	+	+					tr				tr	+	+										
MJB883		+	+	+	+		+	+					tr		+	tr	tr	+	+				+	+					
MJB655		+	+	+	+	+	+	+					tr				tr	+	+				?						
PGJ2258		+	+	?	+	+	+	+					tr			tr	tr?	+	+				+	+					
MJB654		+	+	+	+	+	+	+					tr			tr	tr	+	+				?	tr		tr			
MJB426		+		+	+	+										tr		+	+				+	tr?					
MJB423		tr			+	+	tr											+	+				+			+		+	
MJB986		+			+	+	tr	+					+?		tr	tr	+?	+	+				+	+		tr		+	
MJB987	+	+			+	+	+								tr		+	+	+			+	+					+	
MJB1016	+	+			+	+		+							+	tr		+	+	+			+	+				+	
MJB1017	+	+			+	+	+	+					tr					+	+				+					+	
H traversii																													
MJB1012					tr		tr	+	+	+		+	+	+		tr			tr			+?	+			+	+	+	+
MJB996		tr			+		+	+	tr	+		+	+	+									+			+		+	+
MJB995					+		+	+	tr	+		+	+	+		tr							+			tr	tr	+	+
MJB1010		tr			+		+	+	tr	+		+	+	+		+				+			+					+	+
MJB837		+		+			+	+					+	+												+		+	+
MJB838		+		+	tr	tr		tr?	tr	tr		+	+	+															
H strictissima																													
MJB998		+			+	+							+?		+	tr		+	+		+	+	+					+	
MJB999	+	+			+	+		+					tr		+	+		+	+				+					+	
MJB1001	+	+			+	+							tr		+	+		+	+		tr	tr	+		+			+	
MJB1008		+			+	+							+		+	tr		tr	+		+	?	+			tr		+	

[a] glc, glucoside; glyc, glycoside; xyl, xylosyl; rha, rhamnosyl; glcU, glucuronide; A, apigenin; L, luteolin; K, kaempferol; Q, quercetin.

[b] +, significant presence; tr, present in trace quantity; ?, doubtful presence (or identity unproven); (s), variety *stenophylla;* (o), variety *oliveri;* (h), variety *hesperia.*

Table 3
^{13}C NMR spectral data for new and unusual flavonoids found in the *Hebe parviflora* complex[a,b]

Carbon no.		6-Hydroxy-apigenin derivatives		6-Hydroxy-luteolin derivatives				Luteolin derivatives	
		5b	**6**	**8a**	**8b**	**9**	**9a**	**15**	**15a**
Aglycone	2	164.3	164.1	164.3	164.4	164.3	164.4	164.5	164.2
	3	102.7	102.5	102.4	102.6	102.5	102.5	103.1	103.9
	4	182.6	182.4	182.3	182.4	182.3	182.2	181.8	182.0
	5	146.0	146.2	146.4	146.3	146.5	146.6	161.1	161.2
	6	130.9	130.7	130.7	130.8	130.6	130.0	99.5	99.7
	7	150.7	150.8	150.6	150.9	151.0	151.2	162.9	163.0
	8	94.3	94.6	94.2	94.5	93.9	93.9	94.7	94.9
	9	149.1	148.9	148.8	149.0	149.0	149.0	156.9	157.0
	10	106.1	106.2	106.2	106.2	105.9	105.8	105.3	105.5
	1′	121.1	121.2	121.5	121.6	121.6	121.6	121.4	123.0
	2′	128.5	128.4	113.4	113.5	113.4	113.5	113.6	113.2
	3′	116.0	115.9	145.8	145.8	145.7	145.6	145.7	146.8
	4′	161.5	161.2	149.8	149.8	149.7	149.7	149.9	151.4
	5′	116.0	115.9	116.0	116.0	115.9	116.0	116.0	112.3
	6′	128.5	128.4	119.0	119.0	118.9	119.0	119.2	119.0
Xyl/Glc	1″	100.2	99.8	100.2	99.8	99.4	100.9	99.9	100.0
	2″	82.1	82.6	82.1	82.6	81.6	73.0	73.0	73.1
	3″	74.8	75.5	75.1	75.5	75.6	75.6	76.2	76.3
	4″	68.8	69.2	68.8	69.2	69.2c	69.5a	69.4a	69.4
	5″	65.7	77.2	65.8	77.2	76.2a	75.5	75.5	75.7
	6″	–	60.5	–	60.6	60.3b	68.1	68.2	68.5
Xyl/Glc	1‴	105.1	105.2	105.1	105.2	103.2	103.9	104.0	104.2
	2‴	74.2	74.2	74.1	74.2	74.4	73.2	73.3	73.4
	3‴	76.0	75.9	76.0	76.0	77.0	76.4	76.4	76.5
	4‴	69.3	69.2	69.3	69.4	69.6c	69.2a	69.3a	69.5
	5‴	65.7	65.7	65.8	65.8	76.7a	65.6	65.6	65.6
	6‴	–	–	–	–	60.5b	–	–	–
Methoxyl									55.9

[a] Spectra measured in DMSO-d_6 at ambient temperature (in ppm and referenced to the DMSO multiplet centred at 39.5 ppm).
[b] Assignments bearing the same letter in the same spectrum are interchangeable.

relative to luteolin-7-*O*-glucoside (**4**). The structures of **16a** and **23**, possibly luteolin-7-*O*-triglycosides, were not determined.

Compound **15a** is unique in this group of compounds in that although it possesses a luteolin-7-glycoside type absorption spectrum, its 2D-PC spot is unaffected by NH_3. The ^{1}H NMR spectrum closely resembles that of luteolin-7-*O*-β-[6-*O*-β-xylopyranosylglucopyranoside] (**15**) except for the additional presence of a three-proton methoxyl signal at δ 3.88. This *O*-methyl group was also evident in the ^{13}C NMR spectrum (Table 3) at 55.9 ppm. An HMBC spectrum revealed a strong long-range correlation between the methoxyl proton signal and a carbon signal at 151.4 ppm. This signal represents C-4′ as it cross-correlates in the same spectrum with the H-6′ (δ 7.59) and H-2′ (δ 7.48) signals. Thus the methoxyl group is placed at the 4′-position in **15a**. The ^{13}C NMR spectrum confirmed that the sugar moiety is identical to that of **15** and accordingly that **15a** is 4′-*O*-methylluteolin (diosmetin)-7-*O*-β-[6-*O*-β-xylopyranosylglucopyranoside], a new natural product.

2.4. Group 4 (luteolin-7,3′-di-O-glycosides)

Compound **16** with absorption maxima at 240/246, 266 and 341 nm, resembled the group 2 compounds, but turned bright yellow-green fluorescent on the 2D-PC in NH_3/UV. Its 2D-PC characteristics resemble those of luteolin-7,3′-diglycosides (Markham and Porter, 1974), and indeed it was found to be spectrophotometrically and chromatographically identical with authentic luteolin-7,3′-di-*O*-β-glucopyranoside (ex *Reseda luteola* L.).

2.5. Group 5 (8-hydroxyluteolin-8-O-glycosides)

The sole compound in this group, **2**, has an absorption spectrum with maxima at 257 sh, 270, 292 sh, and 350 nm and its 2D-PC spot is unaffected by NH_3. Compound **2** has a luteolin-like absorption spectrum and an ^{1}H NMR spectrum showing only one A-ring proton (δ 6.18). That this signal represents H-6 is evident from the excellent match with the spectrum of 8-hydroxy luteolin-8-*O*-glucuronide (Markham and Geiger, 1994).

A one proton anomeric doublet at δ 4.62 ($J=7.5$ Hz) indicates the presence of a single sugar which was identified as glucose from the ^{13}C NMR spectrum. Linkage of this sugar to the 8-hydroxyl was evident from the close similarity (i) of the ^{1}H NMR spectra of **2** and 8-hydroxyluteolin-8-*O*-glucuronide, and (ii) of the absorption spectra of **2** and 8-hydroxyluteolin-8-*O*-glycosides (Markham and Porter, 1975). The absorption spectra, unlike those of the 7-*O*-glycosides, resemble that of luteolin (Markham and Given, 1988). Compound **2** is therefore 8-hydroxyluteolin (hypolaetin)-8-*O*-β-glucopyranoside.

2.6. Group 6 (6-hydroxyapigenin-7-O-glycosides)

Compounds **5b** and **6** both give spectra with maxima at about 282 and 334 nm and were unaffected by NH_3 on a 2D-PC. Both compounds have similar ^{1}H and ^{13}C NMR (Table 3) spectra, with the major differences being in the sugar carbon region of the ^{13}C NMR spectra. The ^{1}H NMR spectra defined the oxygenation pattern of both as being either 5,6,7,4′ or 5,7,8,4′, with the single A-ring proton resonating at ca. δ7.0 ($J=7.5$ Hz). This differs markedly from the chemical shift of H-6 in **2** and approximates to that of the H-8 in 6-oxygenated flavones such as baicalein (Markham and Geiger, 1994). 6-Oxygenated substitution patterns are also indicated by the ^{13}C shifts (ca. 95 ppm) of the protonated A-ring carbons (Markham and Chari, 1982). Both compounds contain two sugars as evidenced by the two sugar H-1 doublets at ca. δ 5.15 and δ 4.5 ($J=7.5$ Hz). The ^{13}C NMR resonances in the sugar carbon regions, however, differed in that **6** clearly contains a pentose/hexose combination and **5b**, a pentose/pentose combination. When a set of signals assignable to a terminal xylose (Markham, 1982) is removed from the ^{13}C NMR spectrum of **6**, the remaining signals match with those of a 2-*O*-glycosylated glucoside (Markham and Chari, 1982). The diglycoside is thus defined as a xylo (1-2) glucoside, (sambubioside). HMBC studies linked the glucose H-1 with C-7 at 150.7 ppm and the xylose H-1 to the glucose C-2 at 82.1 ppm, thereby defining **6** as 6-hydroxyapigenin-7-*O*-β-[2-*O*-β-xylopyranosylglucopyranoside]. The dipentose ^{13}C NMR signal pattern in **5b** was identical with that of the recently reported 6-hydroxyluteolin-7-*O*-β-[2-*O*-β-xylopyranosylxylopyranoside], **8a** from the same plant (Mitchell et al., 1999), and accordingly **5b** is assigned the 6-hydroxyapigenin-7-*O*-β-[2-*O*-β-xylopyranosylxylopyranoside] structure. Both **5b** and **6** are new natural products.

2.7. Group 7 (8-hydroxyluteolin-7-O-glycosides)

The sole representative of this group, **5a**, gave an absorption spectrum with maxima at 253 sh, 275, 296 sh and 340 nm and was unaffected by NH_3 on the 2D-PC. The absorption spectrum and an ^{1}H NMR spectrum are both supportive of a luteolin-type oxygenation pattern with additional oxygenation at C-6 or C-8. The single A-ring proton signal at δ 6.60 is within the acceptable range for an H-6 in an 8-oxygenated flavone or flavonol (Markham and Geiger, 1994), and the HMQC-correlated A-ring carbon signal at 100.4 ppm is in the region expected for a C-6 (Markham and Chari, 1982). The ^{13}C NMR spectrum also reveals the presence of a single glucosyl moiety. This glucose is considered to be attached to the 7-hydroxyl group because the H-6 singlet at δ6.60 is shifted downfield by about 0.3 ppm relative to its position in the spectrum of the 8-*O*-glucoside (**2**). Such an effect on the ring proton *ortho* to the glycosylation site is of the order expected (Markham and Geiger, 1994) and indeed, the H-6 resonance for the 7-*O*-glucoside of 8-hydroxyquercetin (gossypetin) is at δ 6.63 (Markham and Geiger, 1994). Further support for the 7-*O*-glycosylation in **5a** derives (i) from the HMBC correlation of the glucose H-1 signal with a carbon signal at ca 149 ppm and not with the C-8 signal at 126.9 ppm, and (ii) from the absorption spectrum which matches that of a 7-*O*-glycosylated 8-hydroxyluteolin but not that of an 8-*O*-glycosylated derivative (Markham and Given, 1988). Compound **5a** is therefore assigned the structure 8-hydroxyluteolin-7-*O*-β-glucopyranoside.

2.8. Group 8 (6-hydroxyluteolin-7-O-glycosides)

Compounds **8a**, **8b**, **8c**, **9**, and **9a** all gave spectra with maxima at 230 sh, 255, 280 and 343 nm and were largely unaffected by NH_3 on a 2D-PC. Two compounds in this group, **8a** (6-hydroxyluteolin-7-*O*-β-[2-*O*-β-xylopyranosylglucopyranoside]) and **8b** (6-hydroxyluteolin-7-*O*-β-[2-*O*-β-xylopyranosylglucopyranoside]), have formed the basis of a recent paper (Mitchell et al., 1999), the former being the first recorded example of a flavone or flavonol xyloxyloside. The other compounds in this group also appear to be 7-*O*-glycosides of 6-hydroxyluteolin. Compound **8c** is produced by mild acid treatment of **8b** and with a longer HPLC retention time was identified as 6-hydroxyluteolin-7-*O*-β-glucopyranoside.

The ^{1}H NMR spectrum of **9** contains two, one proton anomeric sugar proton signals. An HMBC experiment showed that the more upfield of the two, δ 4.61, correlated strongly with the most downfield sugar carbon (81.6 ppm), thus confirming the presence of a disaccharide moiety. The H-1 signal of the flavonoid attached sugar (δ 5.20) correlated with C-7 at 151.0 ppm. A dihexoside formulation for the disaccharide was indicated by the ^{13}C NMR spectrum (Table 3), and the pattern of sugar carbon signals was identical with that of published sophorosides (Markham and Chari, 1982). Compound **9** is therefore identified as 6-hydroxyluteolin-7-

O-β-[2-*O*-β-glucopyranosylglucopyranoside], a compound which has been found only once previously (Williams and Harborne, 1994).

The aglycone moiety and the glycosylation site in **9a** are defined by the absorption spectrum and the ^{1}H and ^{13}C NMR (Table 3) spectra as being the same as in the other members of this group such as **8a**, **8b** and **9**. Attached to this aglycone is a disaccharide (H-1″/1‴ doublets at δ 5.00 and 4.19), the ^{13}C NMR signal pattern of which matches closely with those of **15** and **15a** (Table 3), and is thus defined as 6^{G}-xyloglucosyl. Cross correlation in the HMBC spectrum of the H-1″ signal with that of C-7 at 151.2 ppm, confirms the 7-hydroxyl as the site of glycosylation. Compound **9a** is accordingly assigned the structure 6-hydroxyluteolin-7-*O*-β-[6-*O*-β-xyloglucoside], a new natural product.

2.9. Group 9 (flavonol-3-O-glycosides)

Compounds, **18** and **19**, gave spectra with absorption maxima at about 255/270 and 354 nm and **20** and **21** had maxima at about 264 and 345 nm. They all turned a dull yellow-green on paper in NH_3. The absorption spectra and the 2D-PC mobilities suggested that these compounds are flavonol-3-*O*-glycosides and indeed, they co-chromatographed on HPLC with authentic samples of quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-[6-*O*-rhamnoglucoside] and kaempferol-3-*O*-[6-*O*-rhamnoglucoside], respectively.

2.10. Group 10 (flavone-C-glycosides)

The only representative, compound **24**, gave an absorption spectrum with maxima at 271 and 334 nm. On paper its spot turned olive in NH_3. Compound **24** was chromatographically (HPLC, 2D-PC) and spectrophotometrically identical with authentic vicenin-2 (apigenin-6, 8-di-C-glucoside) from *Passiflora incarnata*.

3. Discussion

The flavonoids identified in this study comprise the major flavonoids found in the *H. parviflora* complex and related species. Six of the flavonoids are new natural products: 6-hydroxyapigenin-7-*O*-[2-*O*-xyloxyloside] (**5b**) and-7-*O*-sambubioside (**6**), and 6-hydroxyluteolin-7-*O*-[2-*O*-xyloxyloside] (**8a**), and 7-*O*-[6-*O*-xyloglucoside] (**9a**), and luteolin-(**15**) and diosmetin-7-*O*-[6-*O*-xyloglucoside](**15a**). Several others such as **22**, and **23** for which confirmatory data are still required may also be novel. It is anticipated that these often minor and sporadically occurring components will be more readily accessible from the many *Hebe* species not included in the *H. parviflora* complex.

The relationship between the wide variety of flavonoids accumulated in species of the *H. parviflora* complex can to a large extent be rationalised in terms of the biosynthetic enzyme systems active in this group of plants. Thus, the apigenin-7- and luteolin-7- and-4′-*O*-monoglucosides are all further elaborated by the separate addition of xylose and rhamnose to produce the 6-*O*-xyloglucosides (**12**, **15**) and 6-*O*-rhamnoglucosides (**17**, **14**, **1**) and additionally by 4′-*O*-methylation (**15a**). Luteolin 3′-*O*-glucosylation is seen in some *H. parviflora* and where present, this commonly also results in the production of the luteolin-7, 3′-di-*O*-glucoside (**16**) from the ubiquitous luteolin-7-*O*-glucoside (**4**) substrate. The 6- and 8-hydroxylated flavones are the other major aglycone types accumulated by this complex and they too are distinctively glycosylated. 8-Hydroxyluteolin is found glycosylated at either the 7-(**5a**) or 8-hydroxyl (**2**). In contrast, the 6-hydroxylated apigenin and luteolin are found glycosylated only at the 7-hydroxyl. The 7-*O*-[2-*O*-xyloxyloside] derivatives (**5b**, **8a**) and 7-*O*-[2-*O*-xyloglucoside] derivatives (**6** and **8b**) of both are found, and for the more abundant 6-hydroxyluteolin, the 7-*O*-[2-*O*-glucosylglucoside] (**9**) and 7-*O*-[6-*O*-xyloglucoside] (**9a**) are often produced. Flavonol specific enzymes appear to be restricted to a 3-*O*-glucosyl-transferase and a rhamnosyl-transferase capable of using the 3-glucosides as substrates to produce the 6^{G}-*O*-rhamnoglucosides.

The biosynthetic relationships between compounds accumulated in each entity have been of value in elucidating the taxonomy of this complex (Bayly et al., 2000), which is proposed to comprise two species, *H. stenophylla* and *H. parviflora*, both of which are shown to be distinct from the related *H. traversii* and *H. strictissima*. As can be seen from the data in Table 2, *H. parviflora* is primarily distinguished from *H. stenophylla* by the presence of glycosylating enzymes capable of using the apigenin- and luteolin-monoglucosides as substrates (e.g. as in **14** and **15**), and by the relative absence of 6-oxygenated flavones (**5b**, **6**, **8a**, **8b**, **8c**, **9**, **9a**). Although the flavonoid profiles of the related species, *H. traversü* and *H. strictissima*, resemble those of *H. stenophylla* and *H. parviflora* respectively, they are distinguished from these and from one another by various presence/absence combinations of compounds **2**, **4**, **9a**, **13**–**15**, and **18**–**21** (Bayly et al., 2000). For example the flavonoid profile of *H. traversii*, although similar to that of *H. stenophylla*, differs in that it is rich in flavonol glycosides. The absence of luteolin-7-*O*-glucuronide (**13**) and the luteolin-7-*O*-diglycosides (**14**, **15**) distinguishes it from *H. parviflora*. *H. strictissima* is distinguished from *H. parviflora* both by the absence of 8-hydroxyluteolin-8-glucoside (**2**) from, and the presence of **9a** in, its flavonoid profile. In fact, *H. strictissima* is the only species in this complex that both lacks **2** and ubiquitously accumulates **9a**.

4. Experimental

4.1. Plant material

Leaf samples of 20 *H. stenophylla*, 15 *H. parviflora*, six *H. traversii* and four *H. strictissima* specimens were collected from wild populations. Details of the locations of samples are given in Bayly et al., 2000. Voucher specimens were prepared and stored [see Bayly et al. (2000) for details]. Freshly collected samples were oven dried at 100°C and stored for later analysis.

4.2. Extraction and flavonoid analyses

Dried plant material was ground and extracted at ambient temperature with MeOH:H_2O (4:1). For 2D-PC profiling of individual samples, the extract from either 12.5 or 25 mg d.w. of plant material was chromatographed on sheets of Whatman 3MM paper in TBA and 15% HOAc as previously described (Bayly et al., 2000). A composite 2D-PC which includes all readily detected spots from all samples was prepared manually by superimposing the patterns of spots evident under UV (366 nm). Exposure of each 2D-PC to NH_3 vapour under UV gave colour responses in the observed spots. For HPLC analyses, the clarified extract was applied to a small microcrystalline cellulose (Merck) column to remove the high level of phenolic acid derivatives. The column was first eluted with 2% aq. HOAc and the flavonoids then removed with EtOH:H_2O (4:1). The resultant flavonoid glycoside fraction was analysed on a Merck Superspher 100 end capped RP-18 column (12.5×0.4 cm I.D.; 4 μm) using a Waters (Milford, MA, USA) 600E solvent controller, a Waters 996 photodiode array detector, a Jasco (Tokyo, Japan) 851-AS intelligent sampler and Millennium^{32} (Milford, MA, USA) Version 3.05.01 software. Injection volume was 20 μl and elution was performed using a flow rate of 0.8 ml min^{-1} at 24°C. The solvents, water adjusted to pH 2.5 with orthophosphoric acid (A) and acetonitrile (B), were mixed using a linear gradient starting with 100% A, decreasing to 91% over 12 min, to 87% over the next 8 min, and to 67% over the next 10 minutes. After holding at this composition for 2 min, A was decreased to 57% over 10 min and then held at this level until the end of the 60-min analysis. Spectral data for all peaks were accumulated in the range 220–400 nm, and standard chromatograms were plotted at 352 nm. An added internal standard (penta-*O*-methylquercetin) provided a reference peak for retention time comparisons between samples.

Presence/absence data for individual samples (see Table 2) were determined as described in the Results section.

4.3. Flavonoid isolation

Individual compounds of interest were isolated from the most suitable available plant material by a series of standard procedures. Extract obtained (as above) was first passed through a short microcrystalline cellulose column (as above) and the aqueous ethanol fraction reduced in volume and applied to a polyamide (CC-6 Macherey-Nagel) column. Chromatography was carried out by gradually changing the solvent from 0.5% aq. HOAc to 0.5% methanolic HOAc. Fractions eluted were monitored by HPLC (see above) for the compound(s) of interest and the appropriate fractions reduced in volume and rechromatographed by 1D-PC, or on LH-20. Purified compounds from 1D-PCs were freed of carbohydrate contaminants by rechromatography on LH-20 (solvent: MeOH:H_2O:HCO_2H, 65:35:0.1) prior to NMR spectroscopy studies. These procedures were used only when the identity of the compound was not evident from 2D-PC, HPLC and absorption spectra data. The structures of many of the more common flavonoids were confirmed by HPLC cochromatography with authentic standards from the personal collection of K.R.M. The structures and tentative structures of flavonoids so isolated are detailed in Table 1, and ^{13}C NMR spectra in Table 3.

4.4. Data on new flavonoids

HPLC retention times, approximate R_f values, and NMR data are presented in Tables 1 and 3, and in Fig. 1, and relevant 2D NMR details are discussed under Results. Absorption spectra (ex HPLC) are also detailed (for groups of compounds) under Results. ^{1}H NMR data (300 MHz, δ in DMSO-d_6) for new flavonoids are as follows.

4.4.1. Compounds **5b/6**

7.96–7.93 (2H, *d*, *J* = 8.7 Hz, H-2′6′); 6.98–7.05 (1H, s, H-8); 6.93–6.94 (2H, *d*, *J* = 9 Hz, H-3′5′; 6.81–6.82 (1H, *s*, H-3); 5.16–5.14 (1H, *J* = 7.2 Hz, H-1 xyl); 4.55–4.57 (1H, *d*, *J* = 7.5 Hz, H-1 xyl/H-1 glc).

4.4.2. Compounds **8a** and **8b**

See Mitchell et al, 1999.

4.4.3. Compound **9a**

7.43 (2H, *m*, H-2′6′); 6.95 (1H, *s*, H-8); 6.93 (1H, *d*, *J* = 8.3 Hz, H-5′); 6.67 (1H, *s*, H-3); 5.00 (1H, *d*, *J* = 7.3 Hz, H-1 glc); 4.19 (1H, *d*, *J* = 7.6 Hz, H-1 xyl).

4.4.4. Compound **9**

7.41 (1H, *br. s*, H-2′); 7.42 (1H, br. *d*, *J* = ca 10 Hz, H-6′); 6.97 (1H, *s*, H-8); 6.90 (H, *d*, *J* = 8.1 Hz, H-5′); 6.70 (1H, *s*, H-3); 5.20 (1H, *d*, *J* = 7.3 Hz, H-1 glc); 4.61 (1H, *d*, *J* = 7.8 Hz, H-1 glc terminal).

4.4.5. Compound **15**

7.46 (2H, m, H-2′6′); 6.93 (1H, *d*, *J* = ca 9 Hz, H-5′); 6.78 (1H, *d*, *J* = ca. 2 Hz, H-8); 6.73 (1H, *s*, H-3); 6.47 (1

H, *d*, *J* = ca. 2 Hz, H-6); 5.06 (1H, *d*, *J* = ca. 7 Hz, H-1 glc); 4.17 (1H, *d*, *J* = ca. 7 Hz, H-1 xyl).

4.4.6. Compound **15a**

7.59 (1H, *dd*, *J* = 8.5, 2 Hz, H-6′); 7.48 (1H, *d*, *J* = 2 Hz, H-2′); 7.15 (1H, *d*, *J* = 8.5 Hz, H-5′); 6,81 (1H, *s*, H-3); 6.81 (1H, *d*, *J* obscure*d*, H-8); 6.48 (1H, *d*, *J* = 2 Hz, H-6); 5.05 (1H, *d*, *J* = 7.5 Hz, H-1 glc); 4.17 (1H, *d*, *J* = 7.5 Hz, H-1 xyl).

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Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaetia* sp.

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Abstract

Ambuic acid, a highly functionalized cyclohexenone, was isolated and characterized from *Pestalotiopsis* spp. and *Monochaetia* sp. these being biologically related endophytic fungi associated with many tropical plant species. This compound was found in representative isolates of these fungal species obtained from rainforest plants located on several continents. The relevance of ambuic acid to the biology of the association of these fungi to their host plants is also discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Endophytic fungi; Rainforest; Antimycotic; Ambuic acid; Cyclohexenone

1. Introduction

Pestalotiopsis spp. and *Monochaetia* sp. (a related fungal genus) are generally found as endophytic fungi of rainforest plants. However, the former fungus appears more frequently than the latter. One of the most common *Pestalotiopsis* sp. that does occur as an endophyte is *P. microspora*. Organisms virtually identical to the taxonomic description of *P. microspora* are numerous. These fungi have usually been isolated as leaf and stem pathogens of economically important tropical plants such as the palms, pines, loquats, guavas, mangoes and a large number of ornamental plants (Raj, 1993). Generally, this fungus is viewed as a relatively weak plant pathogen, but at times it may act in a more severe manner and cause major plant loss. Most often, however, no signs or symptoms of these fungi are to be found by an external examination of the plant. Culturing of the inner tissues of surface sterilized stems, petioles or leaves of plants commonly will yield pure cultures of *P. microspora* (or related fungi).

Furthermore, besides being widespread, another feature of *Pestalotiopsis* spp. is its wide genetic and biological diversity (Li et al., 1996). We have other indications that enormous variability must exist in this organism arising by mutation, genetic crossing, or by as yet undiscovered mechanisms such as genetic exchange with its hosts. It also appears that *P. microspora* is a microbial factory of bioactive secondary metabolites and these compounds may contribute to the biological interactions occurring between the fungus and its plant host.

Thus, this report describes a novel organic acid, ambuic acid, that is present in isolates of *Pestalotiopsis* spp. and a *Monochaetia* sp. obtained as endophytes from several of the major representative rainforests of the world. It structure has been determined by NMR and mass spectroscopic methods and some of its biological activities examined.

2. Results and discussion

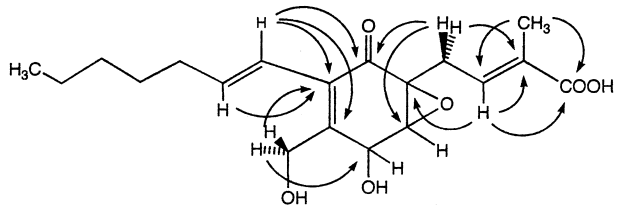
Pestalotiopsis spp. and *Monochaetia* sp., the fungal endophytes, were isolated from the inner tissues of surface-sterilized plant materials including leaves, stems and petioles. The plant materials were collected in rainforests of

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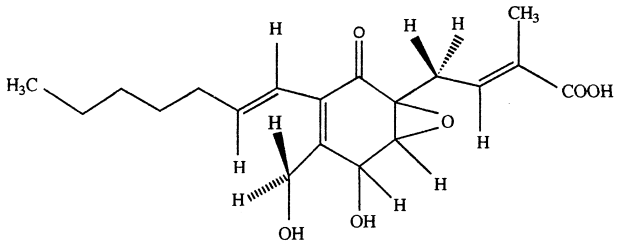
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several countries. The fungi were obtained as pure colonies by picking and culturing hyphal tips as they grew from the plant materials (Strobel et al., 1996). The fungi were identified on the basis of the structure of the asexual fruiting bodies (acervuli) and conidia that they produced on gamma-irradiated carnation leaves (Strobel et al., 1996). Ambuic acid (1) was isolated from the methylene chloride extract of the fermentation medium of each of the fungi that are the subjects of this report. It was purified by silica gel column chromatography using a bioassay guided fractionation procedure with *Pythium ultimum* as the test organism. This organism was selected because it is a representative of the oomycetes (water molds) which are some of the most potent plant pathogens in environments in which excessive water is present such as in the rainforests. After removal of the elution solvent the whitish powder was redissolved in ethyl acetate, hexane 1:1 v/v and recrystallized. Since the crystals were unsuitable for X-ray analysis, a series of NMR spectroscopic techniques were used to acquire structural data. Electrospray mass spectroscopy yielded a prominent peak at (M + Na) + = 373.4 and thus the M_r of ambuic acid is 350. Elemental analysis could account for C = 64.8% (theoretical = 65.1%), and H = 7.58% (theoretical = 7.4%) with no detectable nitrogen, thus the empirical formula for ambuic acid is $C_{19}H_{26}O_6$.

The one dimensional ^1H and ^{13}C NMR spectra were consistent with the mass spectral and elemental analytical data. However, the structure of ambuic acid (**1**) was established using 2-D NMR spectroscopic methods. All carbon–carbon connections (excluding C1–C2) were directly determined using 2D INADEQUATE analyses. Bonding at C1 was determined from HMBC interactions of the C18 and C3 protons (δ_H 1.86 and 6.67, respectively) with C1 (δ_C. Proton chemical shifts were established from HMQC data (Table. 1). Geometry about the C2–C3 double bond was established from an NOE interaction between the protons on C18 (δ_H1.86) and those on C4 (δ_H2.78)(**1**). Likewise, the *trans* configuration of the C11–C12 double bond was verified from the magnitude of the proton–proton scalar coupling ($^3J_{HH}$ 15.9) between the protons on C11 and C12 (**1**). All proposed assignments are consistent with DEPT data used to assign carbon types. NMR data alone did not allow an unambiguous determination of whether the epoxide is attached to the C5–C6 or C6–C7 positions. However, the anisotropic ^1H shifts at C19 shows that this methylene is conformationally restrained rather than freely rotating. This is consistent with a hydroxyl group at C7 acting as a hydrogen bond acceptor for the OH at C19 thus restricting rotation. We, therefore, propose the C7 hydroxyl C5–C6 epoxide structure shown (**1**). This arrangement provides for the formation of the highly favored six member intramolecular hydrogen bonded ring (Etter, 1990). Relative stereochemistry of ambuic acid was not completely established in this study due to the lack of suitable H-H interactions. Specifically, the similarity of the H-C6–C7-H dihedral angles (as determined via computer generated models) in both the *cis* and *trans* stereochemistries ($\theta = 61^\circ$ for *cis* and -68° for *trans*) prevented use of the Karplus relationship to differentiate these stereoisomers. Likewise, NOE measurements are ambiguous as the C6-H ... H-C7 distances (2.50 Å for *cis* vs. 2.56 Å for *trans*) are remarkably similar in both forms. Overhauser interactions of the protons at C19 with the H at C7 are also unsatisfactory as the distance of the closest methylene H to the C7 proton differs by less than 1 Å in the two stereoisomers. The remainder of the cyclohexenone ring is devoid of hydrogen atoms, thereby preventing utilization of other protons to resolve stereochemical issues. Therefore, structure (**2**) of ambuic acid is currently proposed with no relative stereochemical certainty at positions C-5, 6 and 7.



(**1**)



(**2**)

Ambuic acid contains a cyclohexenone moiety similar to that found in another antibiotic- tetracycline (Evans, 1968). Similar hexa-substituted cyclohexenone groups are also found in gedunin and anthothecol from wood extracts and in andirobin that was isolated from *Carapa guyanensis* seeds (Akisanya et al., 1961; Bevan et al.; 1963; Ollis and Ward, 1964). No prior observations appear to have been made of bioactive, but structurally similar analogs, related to ambuic acid.

It is to be noted that one of the chief structural difference between *P. microspora* and *P. guepinii* that make them taxonomically distinct is the number of appendages on the conidiospores (Strobel et al., 1996, 1999). In the case of *P. guepinii* the conidiospore has two apical appendages, while with *P. microspora* there are generally three (Sutton, 1980). In the case of *Monochaetia* sp. there is only one appendage, but recently we have shown that a few UV generated mutants of *P. microspora* yield a fungus that resembles *Monochaetia* sp.

Table 1
A complete list of assignments and important HMBC ^{1}H–^{13}C correlations in ambuic acid

Carbon no.	$\delta^{13}C$	$\delta^{1}H$[a]	HMBC
1	171.2	–	–
2	131.9	–	–
3	136.6	6.67 (*t*, *J* = 7.5)	C1, C2, C4, C5, C18
4	28.8	2.78 (*q* of *d*, *J* = 7.8, 15.8)	C2, C3, C5, C6, C10
5	61.3	–	–
6	61.1	3.74 (*d*, *J* = 2.8)	C4, C5, C7, C8
7	66.0	4.81(*s*)	C8 and C9
8	150.7	–	–
9	132.0	–	–
10	196.0	–	–
11	122.8	6.13 (*d*, *J* = 15.9)	C8, C9, C10, C13
12	140.3	5.83 (*m*)	C9, C13, C14
13	34.6	2.15 (*q*, *J* = 7.0)	C11, C12, C14, C15
14	30.0	1.43 (*m*)	C12, C13, C15
15	32.6	1.32 (*m*)	C16
16	23.6	1.32 (*m*)	C17
17	14.5	0.90 (*t*, *J* = 6.9)	C15, C16
18	13.0	1.86 (*s*)	C1, C2, C3, C5
19	60.3	4.39 (*d*, J = 12.9)	C7, C8, C9
		4.51 (*d*, *J* = 12.9)	

[a] Proton assignments made exclusively from HMQC data.

(Sutton, 1980; Ford and Strobel, unpublished). Furthermore, there is 98% or more homology among the 18s rDNAs of *Pestalotiopsis* sp. and *Monochaetia* sp. (Worapong, Long and Strobel, unpublished) which more firmly establishes their genetic relationship. Thus, it appears that the fungi discussed in this report are very closely biologically related. Perhaps, therefore, it is not surprising that ambuic acid was successfully isolated from these two fungal genera- *Pestalotiopsis* spp. and *Monochaetia* sp. (Table 2). However, each fungal isolate varied in the quantities of ambuic acid in the culture fluid (Table 2).

Ambuic acid (**2**) is only one of a number of novel secondary products that have relatively recently been isolated and described from *P. microspora*. Torreyanic acid, a selectively cytotoxic quinone dimer, was isolated from a *P. microspora* originally obtained as an endophyte associated with the endangered tree- *Torreya taxifolia* (Florida torreya) (Lee et al., 1996). Other products of this same fungus included pestaloside, an aromatic β glucoside, and two pyrones–pestalopyrone and hydroxypestalopyrone (Lee et al., 1995). These products also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes-pestalotiopsins A and B (Pulici et al., 1996a). Other novel sesquiterpenes produced by this fungus are 2-α-hydroxydimeninol and a highly functionalized humulane (Pulici et al., 1996b and c). Variation in the amount and kinds of products found in this fungus is dependent upon both the cultural conditions of the organism as well as the original plant source from which it was isolated. Now, however, for the first time, we note that at least one secondary product, ambuic acid (**2**) is produced by a number of isolates of *P. microspora* that were originally cultured from a wide range of different plants from several continents supporting rainforests.

Each of the fungi in this report were obtained from either tropical or temperate rainforest plants. In the case of *Monochaetia* sp. it was isolated from *Taxus wallichiana* in three separate Himalyan foothill regions of Nepal ranging from the central district (Kakari) to the Dadeldhura district in the far west. *Pestalotiopsis guepinii*, on the other hand, was obtained from the exceedingly rare Wollemi pine located in a secreted area of the Wollemi National Park northwest of Sydney, Australia. However, of the various isolates of *P. microspora* examined, the one of greatest interest was the one from a highland area of Papua New Guinea (Table 2). It was in Papua New Guinea that the perfect stage (sexual form — *Pestalosphareia hansensii*) of *P. microspora* was first discovered (Shoemaker and Simpson, 1981). This was a significant finding since its discovery completed the description of the life cycle of this important organism. We have decided to give acknowledgement to this area of the world by virtue of the name suggested for this novel organic acid — ambuic acid. It turns out that certain highland areas of Papua New Guinea are the source of a yellow clay that is commonly used by local tribes people for facial decoration during the special time of singsing celebrations. Ambua, in the Huli language means yellow. Coincidently, the methylene chloride extract of each of the fungi in this study is also yellow.

Table 2
Isolates of *Pestalotiopsis* spp. and *Monochaetia* sp. producing ambuic acid and the source plant and country from which they were obtained

Fungal isolate and MSU collection no.	Source country	Source plant	Ambuic acid mg/l
P. microspora MSU 21	Germany	*Taxus baccata* (European yew)	203
P. microspora MSU 24	Florida, USA	*Torreya taxifolia* (Florida torreya)	288
P. microspora MSU 191	S. Carolina, USA	*Taxodium disticum* (Bald cypress)	87
P. guepinii MSU 214	Wollemi National Park Australia	*Wollemia nobelis* (Wollemi pine)	200
P. microspora MSU	Highlands — Papua New Guinea	*Dendrobium speciosum* (orchid)	125
Monochaetia sp. MSU-NC 202	Himalayan foothills, Nepal	*Taxus wallichiana* (Nepalese yew)	220

The biological activity of ambuic acid was determined on a number of phytopathogenic fungi using a simple agar plate test. While no activity was exhibited against *Pyricularia oryzae*, *Rhizoctonia solani*, or *Botrytis cinerea*, ambuic acid was active against several fusarium species, *Diplodia natelensis,* and *Cephalosporium gramineum* (Table 3). In plate-well dilution assays, ambuic acid was active against *Pythium ultimum* (the bioassay test organism used in purification) with a minimum inhibitory concentration of 7.5 μg per ml. Ambuic acid was inactive against the major fungal pathogens of humans below the maximum concentration tested of 80 μg per ml. Since it is active against some plant pathogenic fungi, it may be that the role played by this compound in the fungus-plant relationship is that of providing protection to the plant by virtue of its antimycotic activity. This observation suggests that the relationship of *Pestalotiopsis* spp. to its host plant can, at times, be more symbiotic than pathogenic.

3. Experimental

3.1. General spectroscopic precedures

A 2D INADEQUATE analysis was performed on a 21 mg sample dissolved in 30 μl of d4-methanol. Acquisition was performed using a Varian INOVA 500 MHz spectrometer operating at 125.894 MHz equipped with a Varian nanoprobe (Barbara, 1994). A sample spinning speed of 2000 Hz was used for analysis. Subsequent collection of additional ambuic acid provided for a second 2D INADEQUATE analysis on 131 mg in 0.7 ml d_6-DMSO to establish three ^{13}C-^{13}C bonds missing (C5–C10, C9–C10, and C8–C19) from the initial analysis. For consistency with HMQC and HMBC results, only ^{13}C shifts in methanol are reported here. INADEQUATE data interpretation was performed using previously described software (Dunkel et al., 1990, 1992a,b). ^{1}H, ^{13}C, HMQC, and HMBC data were collected on a Varian INOVA spectrometer operating at 500.62 for ^{1}H, HMQC, and HMBC and 125.89 MHz for ^{13}C. NOE difference data were collected using a Bruker DRX spectrometer operating at 250.13 MHz. All reported data were obtained in d_4-methanol.

An electrospray mass spectrum of ambuic acid was obtained on a home built instrument with the sample dissolved in methanol/water/acetic acid 50:50:1 by vol. The sample was injected into the instrument with a flow rate of 2 μl per min with a spray voltage of 2.2 kv via the loop injection method. The ultraviolet absorption spectrum of ambuic acid was obtained in a Beckman DU 50 spectrometer with the compound dissolved in methanol.

Table 3
Antimycotic activity of ambuic acid against a variety of plant pathogenic fungi

Plant pathogenic fungus	Zone of inhibition on a potato dextrose agar plate after 5 days in the presence of 25 μg of ambuic acid
Pyricularia oryzae	No zone of inhibition
Rhizoctonia solani	No zone of inhibition
Botrytis cinerea	No zone of inhibition
Fusarium solani	0.5 cm
Fusarium cubense	0.7 cm
Helminthosporium sativum	1.2 cm
Diplodia natelensis	0.7 cm
Cephalosporium gramineum	1.4 cm[a]
Pythium ultimum	2.0 cm

[a] Zone of inhibition did not appear until after 10 days due to the relatively slow growth of this fungus.

3.2. Molecular modelling

Geometry of potential stereoisomers of ambuic acid were obtained via an ab initio energy minimization procedure using the B3LYP hybrid method with the D95* basis (Lee et al., 1988; Becke, 1993).

3.3. Fungal isolation and microscopy

Plant samples (leaves, twigs, petioles) were surface sterilized with 70% ethanol. After evaporation of the solvent from the plant surface in a decontaminated laminar flow hood, the outer layers of plant tissue were removed aseptically. Small pieces of underlying tissues were removed and placed on water agar plates. After a period of initial growth, hyphal tipping was done to recovered pure cultures of endophytic fungi. These were transferred to potato dextrose agar and allowed to develop over a course of 10 days. The cultures were then transferred to sterile carnation leaves on water agar which generally facilitates the development of fungal fruiting structures (Strobel et al., 1996). Fungal cultures were examined by both light and scanning electron microscopy using techniques previously described (Strobel et al., 1996). Fungi resembling *Pestalotiopsis* sp. or *Monochaetia* sp. were selected for further culturing and examination for ambuic acid production.

3.4. Culture conditions

Each of the fungi used in this study were grown in still conditions in 2-l Erlenmeyer flasks containing 1-l of M-1-D medium supplemented with 1 g of soytone per l at 23–24°C for 3 weeks (Strobel et al., 1996). All cultures were stored in distilled water blanks at 4°C as well as at −70°C.

3.5. Ambuic acid isolation

Culture fluids were filtered free of mycelia and subsequently extracted twice with equal volumes of methylene chloride and the organic phase taken to dryness by flash

evaporation. Approximately 200–300 mg of residue remained after evaporation. The residue (200 mg) was dissolved in methylene chloride and placed on a 3 × 30 cm column of silica gel. The column was eluted with a mixture of chloroform, methanol, and acetic acid 7:1:0.1 (v/v). The first 150 ml of column eluate was discarded and the next 80–100 ml of eluate contained the active compound. The solvent was removed by flash evaporation and a whitish powder was collected. Crystallization in E to Ac, and hexane 1:1 (v/v) yielded small needle like crystals. Purity of the preparations was checked by HPLC column chromatography on a 4.6 × 25 cm Dupont Zorbox RX column with methanol as the mobile phase and a flow rate of 1.0 ml per min. The retention of ambuic acid was 7.2 min. and only a single peak appeared. Furthermore, thin layer chromatography, run on Merck silica gel (0.25 mm) plates, showed that ambuic acid had R_Fs of 0.58, 0.24 and 0.55 in the following systems, respectively: chloroform, methanol, water 7:1:0.1 v/v; chloroform, acetonitrile, water 6:2:0.1 v/v and ethyl acetate; hexane 3:1 v/v. On TLC the compound absorbed short wave UV light (254 nm).

3.6. Bioassay procedures

During the purification procedure, samples (20–30 μl) of solution were placed in the center on the surface of a Petri plate containing potato dextrose agar. The solution was dried by exposure to the air under a laminar flow hood. Then four agar plugs containing *P. ultimum* were placed on the plate, one in each quadrant located at 1.5 cm of the center of the plate. After incubation for 2 days, a zone of inhibition in the center of the plate was taken as a positive indication of bioactivity. Other plant pathogenic fungi were tested in the same manner by placing 10 μl of a methanolic solution containing 25 μg of ambuic acid at the center of the plate. The plates were incubated at 23° C for 5 days, unless otherwise indicated, and the diameter of the zone of inhibition that developed was measured. Tests conducted to measure the activity of ambuic acid against human pathogenic fungi were as previously described (Strobel et al., 1999). Minimum inhibitory concentrations of ambuic acid affecting *P. ultimum* were also determined as previously described (Strobel et al., 1999).

3.7. Ambuic acid (**3**). Small needle-like crystals, m.p. = 126–128° C

UV λ_{max} (methanol) (log ε): 272 (2.93).

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Lactone diterpenes from the aquatic plant *Potamogeton natans*

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Abstract

Four lactone diterpenes and two related glucosides with a labdane skeleton have been isolated from the aquatic plant *Potamogeton natans*. The structures of three new compounds were determined as 19-acetoxy-20-oxo-8(17),13-*ent*-labdadien-15→16 lactone, 8(17), 13-*ent*-labdadien-15→16,19→20 dilactone and 6′-acetyl-19-glucopyranosyloxy-8(17),13-*ent*-labdadien-15→16 lactone, respectively, by means of spectral analysis. Antialgal assays showed inhibitory activity for some compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Potamogeton natans*; Potamogetonaceae; *Raphidocelis subcapitata*; *ent*-Labdane; Lactone diterpenes; Antialgal activity; Spectroscopic analysis

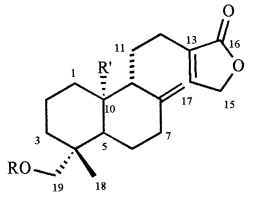
1. Introduction

The first observation that aquatic plants could interfere with the phytoplankton is due to Hasler and Jones (1949), who referred a reduction of the algal population in ponds where some Potamogetonaceae were present. In vitro studies have evidenced that many aquatic plants produce metabolites which inhibit the algal growth so that in many cases allelopathy may justify this interference (DellaGreca et al., 1998 and ref cit.).

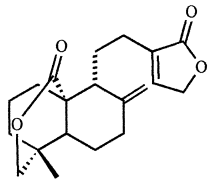
Recently (DellaGreca et al., 2000) we have reported that the Potamogetonacea *Ruppia maritima* produces labdane diterpenes with strong antialgal activity, which encouraged us to study another relative, *Potamogeton natans*, a fresh water Potamogetonacea known as water tongue. In this paper, we describe the isolation, structure determination, and antialgal activity of three novel labdane diterpene lactones.

2. Results and discussion

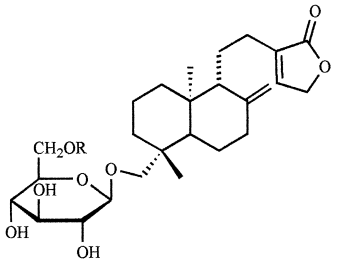
P. natans was air-dried, powdered and progressively extracted with light petrol, ethyl acetate and MeOH. Chromatographic separation of the extracts gave three new compounds **3**, **5** and **6**, together with three known compounds, andrograpanin (**1**), andrograpanin acetate (**2**) (Fujita et al., 1984), and neoandrographolide (**4**) (Chan et al., 1971).



1 R = H R′ = CH_3
2 R = Ac R′ = CH_3
3 R = Ac R′ = CHO



6



4 R = H
5 R = Ac

Compound **3**, $[\alpha]_D$ −2.6°, isolated from petrol extract, was identified as 19-acetoxy-20-oxo-8(17),13-*ent*-labdadien-15→16 lactone. The molecular peak at *m*/*z* 374 in

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the EI mass spectrum and the elemental analysis indicated a molecular formula $C_{22}H_{30}O_5$. The IR spectrum revealed adsorptions at 1750, 1735 and 1716 cm^{-1} indicating the presence of three carbonyl groups in the molecule.

The ^1H (Table 1) and ^{13}C (Table 2) resonances of **3** were assigned by a combination of COSY, NOESY, DEPT, HMQC and HMBC experiments. The ^1H NMR showed the presence of an aldehydic proton at δ 9.81, two mutually coupled multiplet signals at δ 7.09 (1 H) and at δ 4.76 (2 H) of the α,β unsaturated γ-lactone moiety, two broad singlets of an exocyclic methylene protons at δ 4.96 and 4.63, an AB system downshifted at δ 3.89 and 3.75 of a methylene group, and two tertiary methyls at δ 2.01 and 0.99.

The ^{13}C NMR showed the peaks of the aldehydic carbon at δ 206.6 and two further carbonyl signals at δ 174.2 and 170.9. The aldheydic function was assigned to C-20 on the basis of the HMBC spectrum. The aldehydic proton was correlated to C-1 at δ 31.9, C-5 at δ 53.5, C-9 at δ 54.3, and C-10 at δ 53.3. In the same experiment interactions were evidenced between the H-18 methyl at δ 0.99 with the carbons C-4 at δ 37.3 and C-5. These carbons were also correlated to the H-19 methvlene protons at δ 3.89 and 3.75, which gave long range correlations with the methyl at δ 20.9 and the acetyl carbonyl at δ 170.9. Finally, a NOESY experiment evidenced NOE effects between the aldehydic proton and the acetyl methyl indicating an α orientation for both groups.

Compound **5**, $[\alpha]_D -34.4^\circ$, isolated from the MeOH extract, was identified as 19-[6-(*O*)-acetylglucopyranosyl]-8(17),13-*ent*-labdadien-15→16 lactone. FAB mass spectrum and the elemental analysis suggested a molecular formula $C_{28}H_{42}O_9$.

The ^1H NMR (Table 1) showed the coupled signals at δ 5.98 for H-14 proton and 4.61 for the H-15 protons, according with the endocyclic α,β unsaturated γ-lactone moiety. In the spectrum were also present the H-17 protons of the exomethylene group as two broad singlets at δ 4.85 and 4.64, the anomeric doublet at δ 4.30 and the H-18 and H-20 methyls as singlets at δ 0.98 and 0.70. Another methyl signal at δ 2.01 suggested the presence, in the molecule, of an acetyl group which was positioned at C-6′ of the glucosidic moiety on the basis of the downfield shift of the H-6′ protons at δ 4.28 and 3.84. The HMBC experiment showed long range correlations between the glucose carbon C-6′ at δ 63.6 and the acetyl protons at δ 2.01, and between the carbonyl carbon of the acetyl group at δ 174.1 with the methylene protons H-6′. Alkaline hydrolysis of **5** gave the neoandrographolide **4**.

The structure of dilactone was attributed to the compound **6**. The EI–MS spectrum showed the molecular peak at *m/z* 330. suggesting a molecular formula $C_{20}H_{26}O_4$, in accordance with the ^{13}C NMR and the elemental analysis. The IR spectrum, beside the signals of the α,β unsaturated γ-lactone at 1726 cm^{-1}, indicated the presence of a further peak at 1745 cm^{-1}.

The ^1H NMR spectrum showed the signals assignable to the α,β unsaturated γ-lactone, as multiplets at δ 7.18 (H-14) and 4.78 (H-16), the broad singlets H-17 at δ 4.96 and 4.75, a tertiary methyl group as a singlet at δ 0.92 (H-18), and two double doublets centred at δ 4.19 and 4.03. The ^{13}C NMR spectrum (Table 2) showed, beside the signals of the γ-lactone moiety, the carbonyl signal at δ 173.0 and the methylene carbon at δ 76.4. The HMBC spectrum showed interactions between the H-19 protons with the methyl carbon at δ 23.8 and, vice versa, between the methyl protons and the carbon at δ 76.4. Moreover, the carbon at δ 173.0 showed correlation with both the H-19 methylene protons and the methyne H-9 at δ 1.75. These data were in accordance with a carboxylic carbon for C-20 forming a δ-lactone with the hydroxyl group at C-19, which has been supported by the resistance to alkaline hydrolysis also observed for γ-lactone and some diterpenoid δ-lactones (White and Manchand, 1973). The relative stereochemistry

Table 1
Selected ^1H NMR spectral data of compounds **1** –**6** (in $CDCl_3$)

H	**1**[a]	**2**[a]	**3**	**4**[a,b]	**5**[b]	**6**
14	7.10 *m*	7.10 *m*	7.09 *m*	6.10 *m*	5.98 *m*	7.18 *m*
15	4.75 *m*	4.77 *m*	4.76 *m*	4.72 *m*	4.61 *m*	4.78 *m*
17	4.55 *s*	4.59 *s*	4.63 *s*	4.63 *s*	4.64 *s*	4.75 *s*
	4.83 *s*	4.87 *s*	4.96 *s*	4.83 *s*	4.85 *s*	4.96 *s*
18	0.95 *s*	0.97 *s*	0.99 *s*	0.94 *s*	0.98 *s*	0.92 *s*
19	3.36 *d*	3.85 *d*	3.75 *d*	3.63 *d*	3.84 *d*	4.03 *dd*
	(11.0 Hz)	(11.0 Hz)	(10.8 Hz)	(10.4 Hz)	(10.0 Hz)	(11.9, 0.4 Hz)
	3.72 *d*	4.22 *d*	3.89 *d*	3.86 *d*	4.28 *d*	4.19 *dd*
	(11.0 Hz)	(11.0 Hz)	(10.8 Hz)	(10.4 Hz)	(10.0 Hz)	11.9, 2.1 Hz)
20	0.63 *s*	0.70 *s*	9.81 *s*	0.66 *s*	0.70 *s*	–
1′	–		–	4.30 *d*	4.30 *d*	–
				(8.0 Hz)	(7.8 Hz)	
OAc	–	2.04 *s*	2.01 *s*		2.01 *s*	–

[a] Data assigned according with Fujita et al. (1984).
[b] Recorded in CD_3OD.

Table 2
^{13}C NMR spectral data of compounds **1**–**6** (in $CDCl_3$)

Carbon	1[a]	2	3	4[a,b]	5[b]	6
1	39.0	38.9	31.9	40.1	40.8	36.9
2	18.9	18.9	19.1	20.3	20.8	20.8
3	35.3	36.2	38.0	34.4	35.1	41.1
4	39.5	39.5	37.3	40.8	41.4	33.4
5	56.2	56.2	53.5	57.9	58.4	49.5
6	24.5	24.5	24.0	24.7	25.2	28.0
7	38.5	38.4	36.5	36.8	38.1	36.1
8	147.5	147.2	145.1	149.6	149.9	145.0
9	56.4	56.4	54.3	57.9	58.3	52.6
10	38.8	37.3	53.3	40.3	39.3	51.2
11	21.7	21.7	22.2	20.3	21.6	23.7
12	24.4	24.4	23.9	25.8	26.5	24.9
13	134.8	134.9	134.2	135.2	138.6	134.7
14	143.9	143.8	144.0	140.8	148.1	143.9
15	70.1	70.1	70.2	69.4	69.9	70.2
16	174.4	174.3	174.2	171.0	174.1	174.3
17	107.0	107.0	108.1	107.6	108.5	108.4
18	27.1	27.5	26.6	28.1	28.9	23.8
19	65.0	66.7	66.4	65.1	68.7	76.4
20	15.2	15.2	206.6	16.2	16.6	173.0
glc-1′	–	–	–	104.2	104.7	–
glc-2′	–	–	–	75.3	75.9	–
glc-3′	–	–	–	78.2	79.0	–
glc-4′	–	–	–	71.8	72.4	–
glc-5′	–	–	–	78.3	78.8	–
glc-6′	–	–	–	62.9	63.6	–
ac-1	–	171.3	170.9	–	174.1	–
ac-2	–	20.9	20.9	–	20.8	–

[a] Data assigned according with Fujita et al. (1984).
[b] Recorded in CD_3OD.

was determined on the basis of the NOESY experiment which showed NOE effect between the H-5 and H-9 protons.

The toxicity of compounds **1**–**6** was tested on the unicellular green alga *Raphidocelis subcapitata* CCAP 278/4 currently used for aquatic toxicity assays (OECD, 1994).

These compounds were not particularly toxic to the alga. In fact, as described in Fig. 1, compounds **1** and **2** show similar trends of toxicity. They are slightly toxic even at high concentrations and the data do not allow calculation of their IC_{50} values in the range of concentrations tested. Compound **3** showed no dose-dependency. This could be compatible with an autoxidative process of tertiary aldehydes (Caputo et al., 1974). Compound **4**, contrary to **1**–**3** stimulated the algal growth until the toxic concentration of 28.30 μmol/l (log $C = 1.45$). Compound **5**, like **4**, showed a growth stimulation at lower concentrations even if with a different slope starting to be toxic at 6 μmol/l with $IC_{50} = 53.70$ μmol/l (log $C = 1.73$). These results are in agreement with the well-known detoxification process of compounds in plants occurring by glucosylation (Harborne, 1977). Finally compound **6** showed a stimulation of growth at lower concentration, but was toxic with $IC_{50} =$ 44.16 μmol/l (log $C = 1.65$) in higher concentration.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer in 0.05 M soln of $CDCl_3$ or CD_3OD at 37°C. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^1J_{HC} = 145$ Hz) and HMBC (optimised for $^1J_{HC} = 7$ Hz). Optical rotations were measured on a Perkin–Elmer 343 polarimeter. IR spectra were determined in $CHC1_3$ solns on a FT–IR Perkin–Elmer 1740 spectrometer. EI mass spectra were obtained with a Kratos MS 80 apparatus. FAB mass were obtained with a Kratos VG ZAB 2SE. UV spectra were obtained on a Perkin–Elmer Lambda 7 spectrophotometer in EtOH solns. HPLC apparatus consisted of a pump (Shimadzu LC-10AD), and a RI detector (Shimadzu RID-10A) equipped with a SiO_2 column (Maxsil 10 μm, 250×10 mm i.d., Phenomenex) or RPl8 SiO_2 column (Kromasil 10 μm, 250×10 mm i.d., Phenomenex). Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm film thickness. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (230–400 mesh) under a medium pressure or on Sephadex LH-20® (Pharmacia).

3.2. Plant material

P. natans was collected from the Volturno River (South Italy) and identified by Professor Antonino Pollio. A voucher specimen is deposited at the Dipartimento di Biologia Vegetale of University Federico II of Naples.

3.3. Extraction and isolation

The air dried and powered plant material (13 kg) was successively extracted with light petrol, EtOAc and MeOH exhaustively. The light petrol extract (66 g) was chromatographed on silica gel eluting with hexane and EtOAc solns. Most of the extract consisted of a complex mixture of lipids which was eluted with hexane. The fraction eluted with hexane–EtOAc (17:3) was separated on Sephadex LH-20 using hexane–$CHCl_3$–MeOH (3:1:1) as eluent, and was purified by HPLC using a preparative silica gel column and a mixture of hexane–$i Pr_2O$ (3:1) as eluent to give pure **2** (15 mg) and **3** (11 mg).

The EtOAc extract (79 g) was chromatographed on silica gel eluting with hexane and EtOAc mixtures. Hexane eluted waxes and lipids. Fraction eluted with hexane–EtOAc (3:1) was further chromatographed on preparative TLC [C_6H_6–EtOAc (3:2)] to give compounds **1** (51 mg) and **6** (13 mg).

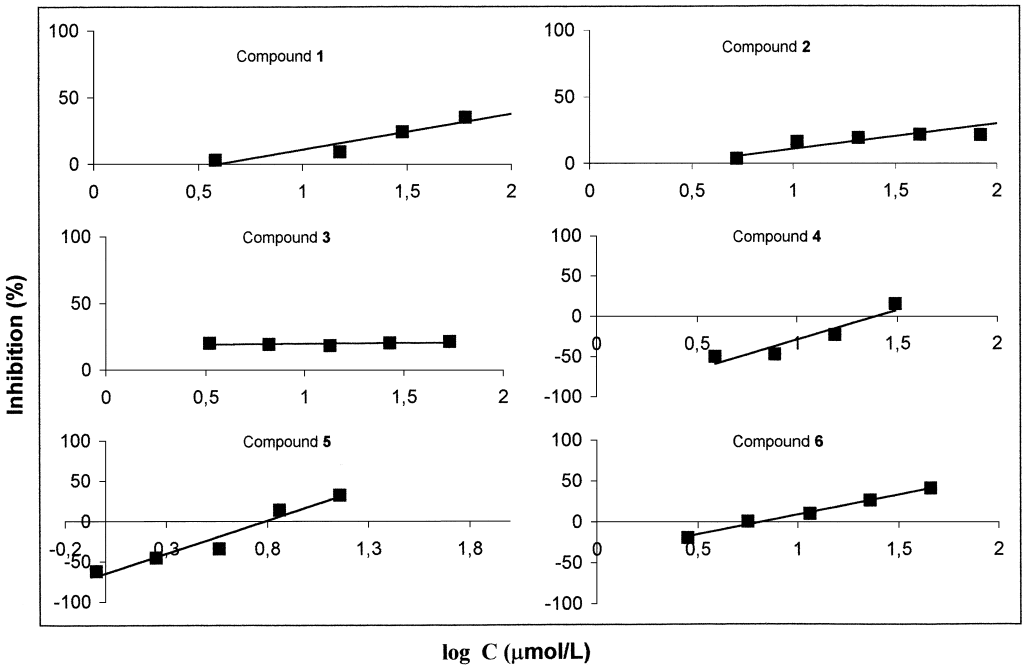


Fig. 1. Inhibition (%) of algal growth of the compounds **1**–**6**.

The methanolic extract (120 g) was treated with H_2O and EtOAc. The aq. layer, concentrated to 500 ml, was chromatographed on Amberlite XAD-4 and the MeOH fraction (2 g) was chromatographed on Sephadex LH-20 [MeOH–H_20 (1:1)] to give a mixture of glycosides which were separated on prep. RP18 eluting with MeOH–H_2O (4:1) to give pure **4** (21 mg) and **5** (8 mg).

3.4. Compound characterisations

3.4.1. 19-Hydroxy-8(17),13-ent-labdadien-15→16-lactone (*1*)

Colourless oil; $[\alpha]_D$−36.4° ($CHCl_3$, *c* 0.75); UV λ_{max} nm (log ε): 215 (3.65); IR ν_{max} cm^{-1}: 3587, 1758, 1645, 886. ^1H NMR and ^{13}C NMR: see Table 1 and 2. EI–MS (probe 70 eV) *m/z*: 318 $[M]^+$, 300 $[M–H_2O]^+$, 287 $[M–CH_2OH]^+$. (Found: C, 75.1; H, 9.3. $C_{20}H_{30}0_3$ requires C, 75.4, H, 9.5%).

3.4.2. 19-Acetoxy-8(17),13-ent-labdadien-15→16 lactone (*2*)

Colourless oil; $[\alpha]_D$−69.6° ($CHCl_3$, *c* 0.54); UV λ_{max} nm (log ε): 214 (3.51); IR ν_{max} cm^{-1}: 1740, 1715, 1645, 906; ^1H NMR and ^{13}C NMR: see Table 1 and 2; EIMS (probe 70 eV) *m/z*: 360 $[M]^+$, 300 $[M–CH_3COOH]^+$. (Found: C, 73.0; H, 9.0. $C_{22}H_{32}0_4$ requires: C, 73.3, H, 8.9%).

3.4.3. 19-Acetoxy-20-oxo-8(17),13-ent-labdadien-15→16 lactone (*3*)

Colourless oil; $[\alpha]_D = -2.6°$ ($CHC1_3$ *c* 0.79); UV λ_{max} nm (log ε): 210 (3.70); IR ν_{max} cm^{-1}: 1750, 1735, 1716, 1645, 906. ^1H NMR and ^{13}C NMR: see Table 1 and 2; EIMS *m/z* 374 $[M]^+$ 314, $[M–CH_3COOH]^+$. (Found: C, 71.0; H, 8.3. $C_{22}H_{30}O_5$ requires: C, 70.6, H, 8.1%).

3.4.4. 19-β-D-Glucopyranosyloxy-8(17),13-ent labdadien-15→16-lactone (*4*)

$[\alpha]_D = -34.4°$ (MeOH, *c* 0.57); UV λ_{max} nm (log ε): 211(3.87); ^1H NMR and ^{13}C NMR: see Table 1 and 2; FABMS *m/z* 481 $[M+1]^+$ (Found: C, 65.5; H, 8.3. $C_{26}H_{40}0_5$ requires: C, 65.0, H, 8.4%).

3.4.5. (6′-Acetyl) 19-β-D-glucopyranosyloxy-8(17),13-ent-labdadien-15→16-lactone (*5*)

$[\alpha]_D = -39.9°$ (MeOH, *c* 0.47); UV λ_{max} nm (log ε): 211 (3.58); ^1H NMR and ^{13}C NMR: see Table 1 and 2; FABMS *m/z* 523 $[M+1]^+$. (Found: C, 64.1; H, 8.1. $C_{28}H_{42}O_9$ requires: C, 64.4, H, 8.2%).

3.4.6. 8(17),13-ent-Labdadien-15→16,19→20 dilactone (*6*)

Colourless oil $[\alpha]_D = -15.6°$ ($CHCl_3$, *c* 0.85); UV λ_{max} nm (log ε): 211 (3.58); IR ν_{max} cm^{-1}: 1745, 1726, 748; ^1H NMR and ^{13}C NMR: see Table 1 and 2. EI–MS: *m/z* 330 $[M]^+$, 315 $[M–CH_3]^+$. (Found: C, 73.3; H, 7.8. $C_{20}H_{26}O_4$ requires: C, 73.3; H, 7.9%).

3.5. Algal growth inhibition test

Tests were performed following the Toxkit™ technology in microbiotest (Persoone, 1998). The Algaltoxkit F™ (Creasel, Belgium) is a 0–72 h algal growth inhibition assay with *Raphidocelis subcapitata*. This test uses microalgae immobilised in a matrix of alginate from which

they can be set free and transferred into the algal culturing medium to resume their growth immediately prior to performing the toxicity test (Algaltoxkit F™, 1996).

A preliminary screening in 10-fold dilution series (range finding test) was performed to determine the 0–100% tolerance range of organisms to toxicants before definitive tests were carried out to determine 50% effect threshold.

Single chemicals of high purity were initially dissolved in DMSO and then diluted further in double-deionised water to make the final stock solns. The highest DMSO concentration in the test samples did not exceed 0.01% (v/v). Two control series were carried out at the same time as the test, one without solvent and the other with its maximum concentration.

The algal test was performed by de-immobilising algae from beads of alginate with EGTA. *R. subcapitata* was inoculated (1×10^4 cells/ml) in polystyrene cuvettes with 10 cm pathlength used as test vials, already containing 25 ml of test solns that were prepared in five toxicant concentrations for each compound using OECD medium (OECD, 1994; ISO, 1987). Three replications were used for each concentration and control. Cuvettes were placed in a growth chamber at 25°C under continuous illumination (8000 lux). Optical densities of the algal suspension in the wells were measured daily for 3 days at 670 nm using a Jenway 6300 spectrophotometer (Jenway, UK). Mean values of the optical density observed for each concentration and control were calculated and from these data the algal growth inhibition was calculated by integrating the mean values from t_0 to t_{72} h (area under the curve) according to the procedures outlined in OECD Guideline 201, estimating the median inhibition (IC50) and then operating the log-transformation.

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Germacranolides from *Mikania guaco*☆

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Dedicated to Professor Dr. H. Rimpler, Institut für Pharmazeutische Biologie, Universität Freiburg on the occasion of his 65th birthday.

Abstract

Fourteen novel sesquiterpene lactones of the germacranolide type have been isolated from the aerial parts of *Mikania guaco*: six costunolide, two melampolide and six germacra-4-*trans*,10(14),11(13)-trien-12,6α-olide derivatives. Except for one compound all the others possess a carbonyl function at C-9. Eight were obtained in the form of four isomer pairs which were difficult to separate. Structure elucidation was based on mass and 1D and 2D NMR measurements. Low energy conformations were obtained by quantum mechanical calculations. Pyrrolizidine alkaloids could not be detected. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Mikania guaco*; Asteraceae; Structure elucidation; Sesquiterpene lactones; Germacranolides

1. Introduction

Mikania guaco Humb. et Bonpl. (Asteraceae, tribe Eupatorieae) is a herbaceous creeper and climbs over shrubs and trees. The plant has a longstanding reputation as an anti-inflammatory remedy in the traditional medicine of Central America (Morton, 1981). Some time ago, four sesquiterpene lactones (Sls) of the eudesmanolide type were isolated, but pharmacological studies have not been undertaken (Castro et al., 1986). The search for Sls with potential anti-inflammatory activity has prompted us to reinvestigate the aerial parts of *M. guaco*. The present communication reports the isolation and structure elucidation of 14 new sesquiterpene lactones of the germacranolide type.

2. Results and discussion

The lipophilic extract of the aerial parts of *M. guaco* was separated using common CC in addition to high and low pressure as well as thin-layer chromatography (see Experimental). Bioguided fractionation using the agar plate diffusion assay for antibacterial activity as well as the electrophoretic mobility shift assay with the transcription factor NF-κB as molecular target for anti-inflammatory activity (Lyß et al., 1997) led to detection of 14 Sls (**1**–**14**) (Fig. 1), eight of which were obtained in the form of four isomer pairs differing only in the acyl moiety.

Compound **1** was obtained in a mixture with **2** as a white amorphous powder. TLC analysis using two different solvent mixtures revealed one spot which gave a brownish colour after spraying with anisaldehyde-sulfuric acid reagent. The mixture could not be resolved completely by HPLC analysis, but both compounds could eventually be separated with heavy losses. This was shown with compound **2** which was obtained in an amount of 1.5 mg. Therefore structure elucidation was carried out on the mixture.

The molecular mass of 420 was deduced from the mass spectrum (CI, isobutane, ESI). Moreover, fragment ions indicated the occurrence of an *O*-acetyl and an *O*-C_5-acyl moiety (see Experimental). The molecular formula $C_{22}H_{28}NaO_8$ was calculated by high resolution molecular mass measurement on the basis of the ion at *m*/*z* 443.

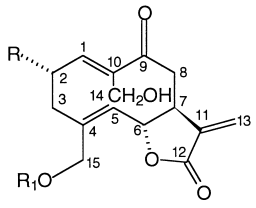
The ^{13}C NMR spectrum displayed 28 carbon signals with different intensities, 22 of higher and six signals of lower intensity. The 22 signals were assigned by DEPT-135 to the resonances of seven quaternary, six CH, six

☆ Part V in the series Phytochemical and biological studies of Costa Rican Asteraceae.

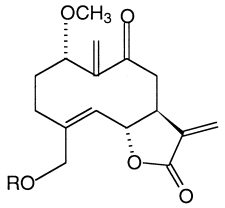
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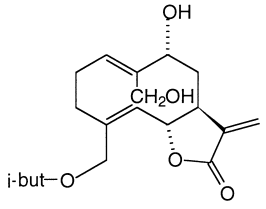
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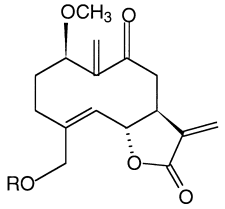
1 R: OAc; R_1: i-val
2 R: OAc; R_1: 2-mebut
3 R: H; R_1: i-but
4 R: H; R_1: i-val
5 R: H; R_1: 2-mebut



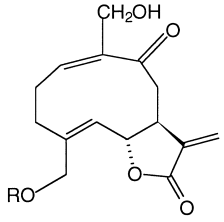
9 R: i-but
13 R: i-val
14 R: 2-mebut



6



10: R: i-but
11: R: i-val
12: R: 2-mebut



7 R: i-val
8 R: 2-mebut

Fig. 1. Structures of the isolated sesquiterpene lactones.

CH_2, and three CH_3 carbon atoms. Five signals could be readily assigned to an isovaleryl and two to an acetoxyl moiety (see Table 1) (Budesinsky and Saman, 1995). The remaining 15 carbons indicated the occurrence of a sesquiterpene skeleton. The chemical shifts of five signals with lower intensity agreed with those of 2-methylbutyric acid (Budesinsky and Saman, 1995). Except for one carbon signal at δ 61.9 no further one with lower intensity could be detected. It follows that the main compound (**1**) differed from the minor (**2**) only in the C_5-acyl moiety and that both compounds possessed the same skeleton.

The presence of an α-methylene-γ-lactone moiety was evident from ^{13}C NMR signals at δ 120.8, 137.1 and 168.5. Consequently, in the ^1H NMR spectrum two characteristic one-proton doublets appeared downfield at δ 6.31 (H-13a) and 5.60 (H-13b), both being coupled to H-7 (δ 2.80). The coupling constants between H-6 and H-7 ($J_{6,7}=9.5$ Hz) together with the allylic coupling between H-7 and H-13 ($J_{7,13}=3.0$ Hz) indicated *trans* attachment of the α-methylene-γ-lactone ring (Samek, 1970), which was bound to a germacradienolide system. Two double bonds at Δ^4 and $\Delta^{1,10}$ could be deduced from two carbon signals (δ_C 134.6 and 133.1) typical for methine groups and from 2D COSY studies (Marco et al., 1997). The cyclodecadiene ring exhibits an oxygen function at C-2. Its presence was corroborated by the carbon signal at δ 70.1 and its location at C-2 followed from ^1H–^1H COSY, GHSQCR and GHMQCR analysis. The large coupling $J_{1,2}=10.4$ Hz agreed with an α-configuration (Bohlmann et al., 1982a; Vasquez et al., 1990). This value was similar to that reported for tamaulipin A angelate, for which the molecular structure was established by X-ray diffraction analysis (Vasquez et al., 1990). An α H-2 would have shown a smaller coupling constant ($J_{1,2}=6$ Hz) (Marco et al., 1997). The hydroxy group was esterified by acetic acid as proved by correlations between the ester carbonyl of the acetoxy group and H-2 in the GHMQCR spectrum (Table 3).

The 2D COSY spectrum only showed geminal couplings of the protons at C-8 and vicinal couplings of H-8 to H-7. Consequently, C-9 must be quarternary and resonated at δ 203.0. This was unambiguously confirmed by a GHMQCR experiment which exhibited correlations between the signal at δ_C 203 (C-9) and H-8α and β, H-7 and H-1 (see Table 3). The neighbouring oxygen function induced a downfield shift for C-8 to δ 40.5 (Vasquez et al., 1990) compared with an unsubstituted germacranolide, e.g. tamaulipin A angelate (δ 27.9). Although, the signal of C-1 should also be significantly shifted downfield, because of the conjugation between the carbonyl group and the double bond at $\Delta^{1,10}$. However, the resonance for C-1 at δ 134.6 showed only a slight downfield shift of about 8 ppm in comparison to a 1,10 double bond without any oxygen substitution (Vasquez et al., 1990). This could be explained by a keto group which is not coplanar with the 1,10-double bond, but most probably lies below the plane as already assumed earlier in the case of a costunolide derivative with a C-9 keto group where the same effects were observed (Bohlmann et al., 1981), in agreement with earlier studies which showed that the influence of a carbonyl group on neighbouring atoms strongly depends on its torsion angle (Montecalvo and St. Jacques, 1975). In **1** and **2,** the typical upfield signals of C-14 and C-15 methyl groups were replaced by two characteristic signals of oxygen bearing methylene groups in the ^1H and ^{13}C NMR spectrum. Assignment was achieved by GHSQCR and GHMQCR spectra. The magnitude of the ^{13}C NMR shifts suggested that they were bound to trans double bonds, respectively (Herz and Kumar, 1981; Lange and Lee, 1986; Pearce et al., 1986). The resonance at δ_C 59.1 was assigned to C-14, because of long range couplings of the respective doublet at δ_H 4.43 to C-1, 9 and 10 in the GHMQCR spectrum (Table 3) and the allylic coupling observed between H-1 and H-14a in the 2D COSY. Furthermore, C-14 contains a hydroxyl since in the 2D COSY spectrum both H-14 protons were coupled with the broad signal of the OH group. The remaining two one proton

Table 1
^{13}C NMR-spetral data of compounds **1**–**8** (75 MHz, $CDCl_3$)

C	1[bd]	2[bd]	3[b]	4	5	6[c]	7[e]	8[e]
1	134.6 CH	134.6 CH	141.0	141.0	141.0	127.6	136.7 CH	136.7 CH
2	70.1 CH	70.1 CH	26.8	26.8	26.8	26.0	23.8 CH_2	23.8 CH_2
3	41.1 CH_2	41.1 CH_2	35.3	35.4	35.4	35.2	34.0 CH_2	34.0 CH_2
4	139.4 C	139.4 C	140.0	139.9	139.9	139.7	138.4 C	138.5 C
5	133.1 CH	133.0 CH	130.8	130.9	130.9	131.4	128.7 CH	128.6 CH
6	78.6 CH	78.6 CH	78.9	78.9	78.9	79.8	78.4 CH	78.4 CH
7	48.3 CH	48.3 CH	48.8	48.8	48.8	41.4	47.8 CH	47.8 CH
8	40.5 CH_2	40.5 CH_2	40.1	40.2	40.2	35.7	42.5 CH_2	42.5 CH_2
9	203.0 C	203.0 C	203.6	203.6	203.6	69.3	202.6 C	202.6 C
10	146.5 C	146.5 C	–[f]	146.0	146.0	141.4	143.5 C	143.5 C
11	137.1 C	137.1 C	137.6	137.7	137.7	137.9	137.7 C	137.7 C
12	168.5 C	168.5 C	168.8	168.8	168.8	170.0	169.1 C	169.1 C
13	120.8 CH_2	120.8 CH_2	120.4	120.4	120.4	119.8	119.9 CH_2	119.9 CH_2
14	59.1 CH_2	59.1 CH_2	58.4	58.5	58.5	59.2	66.1 CH_2	66.1 CH_2
15	61.8 CH_2	61.9 CH_2	62.0	61.8	61.3	61.7	60.5 CH_2	60.5 CH_2
1′	170.4 C	170.4 C						
2′	20.9 CH_3	20.9 CH_3						
1″	172.5 C	176.2 C	176.8	172.7	176.2	176.9	172.6 C	176.4 C
2″	43.1 CH_2	41.0 CH	34.0	43.3	41.0	34.0	43.3 CH_2	41.0 CH
3″	25.6 CH	26.7 CH_2	19.0a	25.7	26.7	18.9	25.7 CH	26.7 CH_2
4″	22.4[a] CH_3	11.6 CH_3	18.9a	22.4	11.7	18.9	22.4 CH_3	11.6 CH_3
5″	22.3[a] CH_3	16.5 CH_3		22.4	16.6		22.4 CH_3	16.5 CH_3

[a] Assignment interchangeable.
[b] Assignments determined by GHSQCR and GHMQCR correlations.
[c] Assignments determined by GHSQCR correlations.
[d] Multiplicities from DEPT spectrum.
[e] Multiplicities from APT spectrum.
[f] not detectable.

Table 2
^{13}C NMR spectral data of compounds **9**–**14** (75 MHz, $CDCl_3$)[a]

C	9	10	11	12	13	14
1	77.9 CH	76.7 CH	76.6 CH	76.6 CH	77.9 CH	77.9 CH
2	36.0 CH_2	33.9 CH_2	33.9 CH_2	33.9 CH_2	36.1 CH_2	29.8 CH_2
3	29.7 CH_2	32.5 CH_2	32.4 CH_2	32.4 CH_2	29.8 CH_2	29.8 CH_2
4	143.2 C	144.8 C	144.6 C	144.6 C	142.7 C	142.7 C
5	127.3 CH	126.2 CH	126.4 CH	126.4 CH	127.5 CH	127.5 CH
6	77.7 CH	78.7 CH	78.6 CH	78.6 CH	77.7 CH	77.7 CH
7	46.9 CH	50.2 CH	50.2 CH	50.2 CH	46.9 CH	46.9 CH
8	39.8 CH_2	35.6 CH_2	35.6 CH_2	35.6 CH_2	39.8 CH_2	39.8 CH_2
9	199.7 C	201.9 C	201.9 C	201.9 C	199.7 C	199.7 C
10	151.1 C	152.4 C	152.4 C	152.4 C	151.1 C	151.1 C
11	138.1 C	138.0 C	138.0 C	138.0 C	138.1 C	138.1 C
12	168.9 C	169.1 C	169.1 C	169.1 C	168.9 C	168.9 C
13	119.6 CH_2	118.6 CH_2	118.6 CH_2	118.6 CH_2	119.6 CH_2	119.6 CH_2
14	123.4 CH_2	123.4 CH_2	123.5 CH_2	123.5 CH_2	123.5 CH_2	123.5 CH_2
15	63.4 CH_2	61.7 CH_2	61.4 CH_2	61.4 CH_2	63.3 CH_2	63.3 CH_2
OCH_3	56.0 CH_3	55.5 CH_3	55.7 CH_3	55.7 CH_3	56.0 CH_3	56.0 CH_3
1′	176.6 C	176.4 C	172.6 C	176.0 C	172.5 C	176.2 C
2′	33.9 CH	33.9 CH	43.2 CH_2	41.0 CH	43.1 CH_2	40.9 CH
3′	18.9[b] CH_3	18.9[b] CH_3	25.6 CH	26.6 CH_2	25.6 CH	26.6 CH_2
4′	18.8[b] CH_3	18.8[b] CH_3	22.4 CH_3	11.6 CH_3	24.6 CH_3	11.6 CH_3
5′			22.4 CH_3	16.4 CH_3	22.4 CH_3	16.4 CH_3

[a] Assignments determined by GHSQCR and GHMQCR correlations, multiplicities by DEPT analysis.
[b] Assignment interchangeable.

Table 3
^{1}H NMR data as well as GHMQCR and NOESY correlations of compounds **1** and **2** (300 MHz, $CDCl_3$)

H	**1**	**2**					**1**	**2**	**A**[e]	**B**[e]
	δ (ppm)		mult	GHMQCR, corr. with C[d]	NOESY, corr. with H[d]	H	J (Hz)			
1	5.54	5.54	*d*	2, 9, 10, 14	3α, 5, 7	1/2β	10.4	10.4	10.7	10.2
2β	5.40	5.40	*m*	1, 1′, 3, 10	14a, 15a	2β/3α	10.4	10.4	10.4	6.2
3α	2.29[a]	2.29[a]	*m*[b]	2, 4, 5, 15	1, 5	2β/3β	5.3	5.3	5.8	9.3
3β	2.84	2.84	*m*[b]	2, 4, 5, 15	15a	5/6	9.5	9.5	9.8	9.4
5	5.08	5.08	*d*	3, 6, 7, 15	1, 3α, 7	6/7	9.5	9.5	8.6	9.6
6	4.80	4.80	*t*[c]	3, 4, 5	8β, 14b	7/8α			1.0	10.3
7	2.80	2.80	*m*[b]	6, 9, 10, 11	3α, 5	7/8β	11.4	11.4	10.7	2.8
8α	2.77[a]	2.77[a]	*m*[b]	6, 7, 9, 10, 11		7/13a	3.0	3.0		
8β	2.98	2.98	*dd*	6, 7, 9	6, 14b	7/13b	3.0	3.0		
13a	6.31	6.31	*d*	7, 12		8α/8β	12.5	12.5		
13b	5.60	5.60	*d*	7, 12		14a/14b	13.9	13.9		
14a	4.43	4.43	*d (br)*	1, 9, 10	2, 3″ (**2**)	14b/OH	5.6	5.6		
14b	4.10	4.10	*dd*		2, 6, 8β	15a/15b	13.7	13.7		
15a	4.74	4.74	*d*	1″, 3, 4, 5	3β					
15b	4.65	4.65	*d*	1″, 3, 4, 5	3β	3″/4″	6.6			
OH at C-14	2.50	2.50	*m*			3″/5″	6.6			
2′	2.08	2.08	*s*	1′, 2		2″/5″		7.0		
2″	2.28[a]		*d*	1″, 3″, 4″, 5″	4″, 5″	3″/4″		7.4		
3″	2.14		*m*	2″, 4″, 5″						
4″	0.98		*d*	2″, 3″	2″					
5″	0.98		*d*	2″, 3″	2″					
2″		2.46	*m*	1″, 3″						
3″		1.72	*m*	1″, 4″						
3″		1.52	*m*	1″, 4″						
4″		0.93	*t*	2″, 3″						
5″		1.20	*d*	1″, 2″, 3″						

[a] Assignment determined by GHSQCR correlations.
[b] Multiplicity not determined (signal overlap).
[c] Pseudo triplet.
[d] Valid for **1** and **2.**
[e] Calculated coupling constants for conformation A and B of **1** and **2**.

doublets at δ_H 4.65 and 4.74, exhibiting a geminal coupling, were assigned to H-15a and H-15b and the carbon signal at δ_C 61.82 to C-15. The latter assignment as well as the esterification of the C-15 hydroxy group by isovaleric and 2-methylbutyric acid was confirmed by the GHMQCR spectrum (correlations of H-15a and b with C-3, 4 and 5 and the ester carbonyl of both C_5-acyloxy groups). The two substances were neither heliangolides (1,10-*trans*, 4-*cis*; $J_{6,7}$ and $J_{7,13} = 2$ Hz) nor melampolides (1,10-*cis*, 4-*trans*) but *trans*-1(10),*trans*-4-germacradienolides, as indicated by the magnitude of the coupling constants $J_{7,13}$ (> 3 Hz), $J_{6,7}$ (9.5 Hz) and by the chemical shifts for C-14 and C-15 (about δ 60) as well as by the NOESY spectrum. Assuming that H-7 is normally α-orientated, as shown by X-ray analysis (Witt and Watkins, 1978; Picher et al., 1984), the interactions between H-7, H-5 and H-1 in a NOESY experiment confirmed α-orientation, respectively. Hence, the ten-membered ring is in the $^{15}D_5$, $_1D^{14}$-conformation (= UU) for which crossed, trans double bonds and a double chair conformation was proved by X-ray diffraction (Witt and Watkins, 1978; Picher et al., 1984; Watson and Kashyap, 1986; Vasquez et al., 1990). Moreover, the relative stereochemistry at C-2 and C-6 was confirmed by the NOESY spectrum. The NOE between H-2 and H-14 as well as H-14 and H-6 showed the α-configuration of the C-2 acetoxy group and the *trans*-fused lactone ring.

The conformation of Sl **1** and **2** based on extensive NMR studies was in accordance with the mostly common one found for *trans*,*trans*-germacradiene-6,12-olides in solution (Watson and Kashyap, 1986). To find out whether further conformations may exist we calculated possible low-energy conformations of compound **1**. Two conformations, showing an up–up arrangement (UU-conformation) of the two hydroxymethyl groups, but differing in the orientation of the C-9 carbonyl group, were obtained (see Fig. 2, **1**/**2**: A and B). The theoretical values of the coupling constants were deduced from these molecular models (see Table 3). As expected, only the conformation, in which the carbonyl group showed antiparallel orientation to the 1,10 double bond, was in good agreement with the NMR data. Thus, in this case only one conformation was present, as it was previously shown for 14-acetoxy-8α-hydroxycostunolide

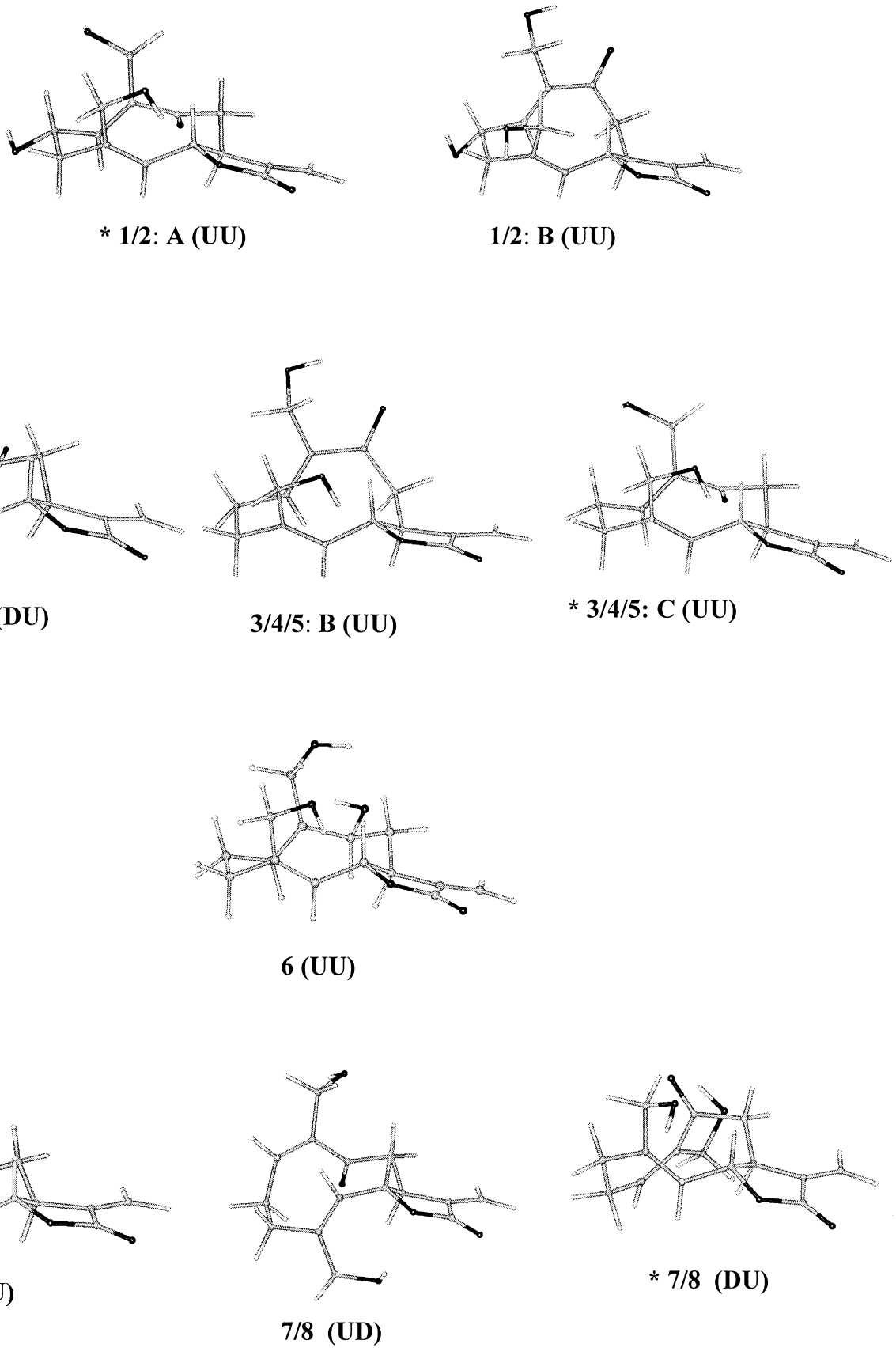


Fig. 2. Low energy conformations of the isolated sesquiterpene lactones **1**–**10** without acyl side chains as found by quantum mechanical calculations (*mainly occurring conformations considering the NMR data) (*continued on next page*).

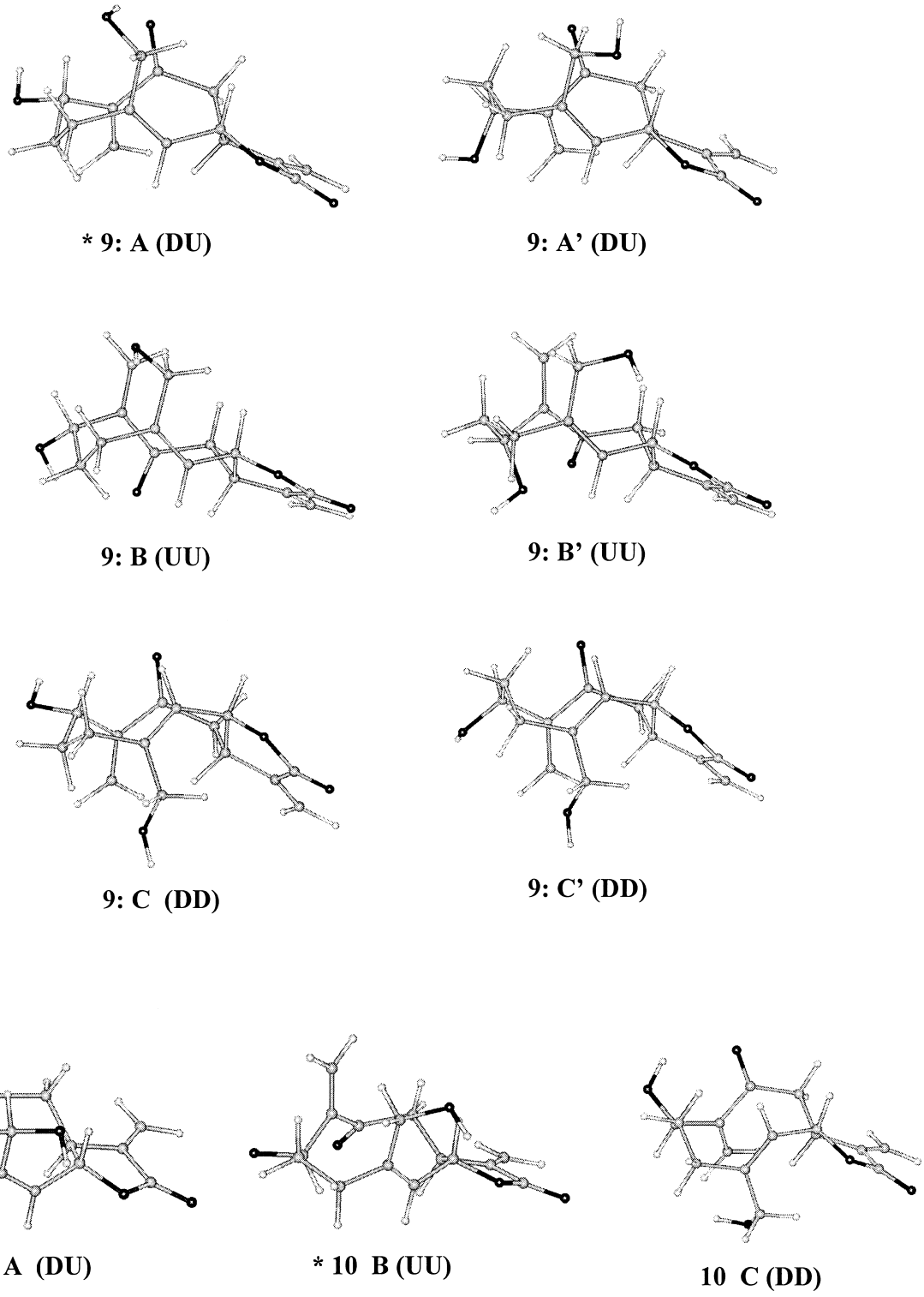


Fig. 2 (*continued*).

(Bohlmann et al., 1985). To the best of our knowledge, compounds **1** and **2** are here described for the first time. These costunolide derivatives can be described as 2α-acetoxy-14-hydroxy-15-isovaleryloxy-9-oxo-costunolide (**1**) and 2α-acetoxy-14-hydroxy-15-(2-methylbutyryloxy)-9-oxo-costunolide (**2**), respectively. The skeleton without the acyl moieties could be named miguanin, so Sl **1** is 2α-acetoxy-15-isovaleryl-miguanin and **2** 2α-acetoxy-15-(2-methylbutyryl)-miguanin.

Compound **3** gave a grey-brownish colour with anisaldehyde-sulfuric acid reagent and possessed a molecular mass of 348 (ESI–MS). Its ^{13}C NMR (see Table 1)

revealed the presence of 18 carbon atoms, four of which could be readily identified as belonging to an isobutyryloxy substituent (Budesinsky and Saman, 1995). The remaining carbon signals resembled those obtained from the skeleton of Sl **1** and **2**, except for C-1, C-2 and C-3 and to a lesser extent for C-5 and C-14. The signal of the quarternary C-10 could not be detected. The respective protons bound to these carbon atoms differed in their chemical shifts from those found for compounds **1** and **2** in the ^{1}H NMR spectrum, too. The other signals as well as the experimental coupling constants were identical. Thus, the occurrence of a germacrolide, the same as in **1** and **2**, was confirmed (see Table 4). Here, the C-15 hydroxy group was esterified by isobutyric acid indicated by the correlation of the ester carbonyl and both protons at C-15 in the GHMQCR spectrum. The differences in the chemical shifts could be explained when an unsubstituted C-2 was assumed. The lack of an oxygen function induced a pronounced upfield shift of C-2 and, to a lesser degree, of C-3 and a downfield shift of C-1. The signal for H-1 appeared as doublets of doublets and was shifted ca. 0.2 ppm downfield compared to **1**. Assignment of the stereochemistry followed from the NOESY spectrum by which the occurrence of a [$^{15}D_5$, $_1D^{14}$] was confirmed. Molecular models were created and theoretical values of the coupling constants calculated. From the three possibilities (A: DU, B: UU and C: UU) (see Fig. 2) only conformation C was in full agreement with the NOESY experiment and the coupling constants (see Table 4). Notations refer to the orientation of the methyl groups attached to C-10 and C-4, respectively. Thus, compound **3** is 14-hydroxy-15-isobutyryloxy-9-oxo-costunolide or 15-isobutyryl-miguanin, which has been found for the first time.

Compound **4** was isolated in a mixture with **5** as a colourless resin and showed the same behaviour in TLC analysis as the mixture of **1** and **2**. The occurrence of a pair of isomers was confirmed by the ESI and GC/MS mass spectra with a molecular mass of 362. ^{1}H and ^{13}C NMR data revealed the same skeleton as in compound **3** (see Tables 1 and 4), but differences in the acyl moiety. Thus, Sl **4** was 15-isovaleryl-miguanin and **5** 15-(2-

Table 4
^{1}H NMR data of compounds **3**–**5** as well as GHMQCR and NOESY correlations of **3** (300 MHz, $CDCl_3$)

H	**3**				**4**	**5**			**3**	**4**	**5**	**A**[e]	**B**[e]	**C**[e]
	δ (ppm)	mult	GHMQCR, corr. with C	NOESY, corr. with H	δ (ppm)		mult	H	J (Hz)					
1	5.74	*dd*	9	5, 8β	5.73	5.73	*dd br*	1/2α[d]	5.9	6.0	6.0	11.2	4.8	4.4
2a	2.54[a]	*m*[b]	3, 4		2.54	2.54	*m*[b]	1/2β[d]	11.0	11.0	11.0	4.3	10.6	11.2
2b	2.22[a]	*m*[b]	1, 3		2.16–2.22	2.16–2.22	*m*	2α/3α				11.7	9.4	5.4
3a	2.70[a]	*m*[b]	2, 4, 5, 15		2.70	2.70	*m*[b]	2α/3β				2.2	0.4	1.4
3b	2.20[a]	*m*[b]	2, 4, 5, 15		2.16–2.22	2.16–2.22	*m*	2β/3α				4.4	8.9	12.5
5	4.92	*d*	3, 7, 15	1, 7	4.92	4.92	*d*	2β/3β				11.6	9.6	5.4
6	4.83	*t*[c]	4, 5, 8	8β, 15b	4.81	4.81	*t*[c]	5/6	10.0	10.0	10.0	11.4	9.6	10.2
7	2.84	*m*[b]		5	2.84	2.84	*m*	6/7	10.0	10.0	10.0	9.1	9.5	8.8
8α	2.75	*dd*	6, 7, 9, 11		2.75	2.75	*dd*	7/8α	1.7	1.7	1.7	2.6	10.3	1.0
8β	3.07	*dd*	6, 7, 9	1, 6, 14b	3.06	3.06	*dd*	7/8β	10.3	10.3	10.3	6.1	3.0	10.1
13a	6.30	*d*	7, 11, 12		6.30	6.30	*d*	7/13a	3.4	3.3	3.3			
13b	5.61	*d*	7, 12		5.61	5.61	*d*	7/13b	3.2	3.3	3.3			
14a	4.28	*d*	1, 9		4.28	4.28	*d*	8α/8β	12.5	12.5	12.5			
14b	4.16	*d*	1, 9	8β	4.15	4.15	*d*	14a/14b	13.1	13.6	13.6			
15a	4.73	*d*	1′, 3, 4, 5	6	4.71	4.71	*d*	15a/15b	13.7	13.6	13.6			
15b	4.64	*dd*	1′, 3, 4, 5		4.65	4.65	*d*							
OH at	1.84	*s br*			1.80	1.80	*m*	2′/3′	7.1					
C-14								2′/4′	7.1					
2′	2.61	*sept*	1′, 3′, 4′		2.24		*m*[b]							
3′	1.21	*d*	1′, 2′		2.13		*m*[b]	3′/4′		6.6				
4′	1.21	*d*	1′, 2′		0.98		*d*	3′/5′		6.6				
5′					0.98		*d*							
								2′/5′			7.1			
2′						2.44	*m*	3′/4′			7.3			
3′a						1.70	*m*							
3′b						1.50	*m*							
4′						0.92	*t*							
5′						1.18	*d*							

[a] Assignment determined by GHSQCR correlations.
[b] Multiplicity not determined (signal overlap).
[c] Pseudo triplet.
[d] 1/2a and 1/2b for **3**, **4** and **5.**
[e] Calculated coupling constants for different conformations (A, B and C) of **3**–**5**.

methyl-butyryl)-miguanin. For these Sls the UU-conformation, as for compound **3**, can be assumed, too.

Compound **6** was obtained as a colourless resin which gave a violet colour after spraying with anisaldehyde-sulfuric acid. A molecular mass of 350 was deduced from the ESI and CI (isobutane) mass spectra. ^{13}C NMR data were close to those of Sls **3**–**5** except for the signals of C-7, C-8, C-9 and C-10. For compound **6** the signal of the keto carbonyl at C-9 (δ_C 203) was replaced by a signal at δ_C 69.26, which corresponds to an oxygenated methine group. In agreement with this the signals of C-1, C-7, C-8 and C-10 were shifted upfield. The ^1H NMR and 2D COSY spectra were in full agreement with these observations, displaying among others the double doublet at δ 4.78 of H-9 which coupled with the two protons at C-8. The stereochemistry of the protons at C-3 and C-8 was deduced by the NOESY spectrum and that of H-9 from the coupling constants. In fact, coupling constants of $J_{8\alpha,9} = 5.6$ Hz and $J_{8\beta,9} = < 1$ Hz agreed with an α-orientated C-9 hydroxy group (Rustaiyan et al., 1986; Bruno et al., 1991), whereas a β-orientation would give values of $J_{8\alpha,9} =$ about 2 Hz and $J_{8\beta,9} =$ about 10 Hz (Bohlmann et al., 1982b; Rustaiyan et al., 1986). The $^{15}D_5$, $_1D^{14}$-conformation was confirmed by the NOESY spectrum. NOEs involving H-7 and H-5 as well as H-5 and H-1 indicated that these substituents were below the plane, the NOE between H-6β and H-15a showed an above position. Quantum mechanical calculations proposed only one low energy form (see Fig. 2). The theoretical values of the coupling constants calculated from this molecular model were in good agreement with the experimental ones (see Table 5). Slight differences were only obtained for $J_{1,2\alpha}$ and $J_{1,2\beta}$. Again Sl **6**, which is 9α,14-dihydroxy-15-isobutyryloxy-costunolide, has not been found in nature up to now.

Compounds **7** and **8**, which were present as a mixture, had the same molecular mass ($M_r = 362$, ESI–MS) and similar spectroscopic data as Sls **4**–**5**. Again, **7** and **8**

Table 5
^1H NMR data and NOESY correlations of compounds **6**–**8** (300 MHz, $CDCl_3$)

H	**6** δ (ppm)	mult	NOESY, corr. with H	**7** δ (ppm)	**8** δ (ppm)	mult	NOESY, corr. with H[d]	H	**6**	**6**[f]	**7** *J* (Hz)	**8**	**7**/**8**[g]
1	5.45	*t(br)*	5	5.83	5.83	*t(br)*	14b	1/2α[e]	6.8	4.3	8.8	8.8	3.2
2α	2.33[ab]	*m*[c]		2.24	2.24	*m*[c]		1/2β[e]	9.3	11.2	8.8	8.8	4.6
2β	2.33[ab]	*m*[c]		2.74	2.74	*m*	15b	2α/3α		5.8			2.8
3α	2.18[a]	*m*[c]	5, 8α	1.75	1.75	*m*[c]	5	2α/3β		1.2			13.2
3β	2.55[a]	*m*[c]		2.57	2.57	*ddd*		2β/3α		12.2			4.2
5	4.99	*d*	1, 3α, 7	5.09	5.09	*d*	3α, 7	2β/3β		5.8			2.5
6	4.62	*dd*	8β, 15b	4.73	4.73	*dd*	8β	5/6	10.3	10.4	10.4	10.4	11.3
7	3.15	*m*	5	3.25	3.25	*m*	5, 14a	6/7	8.8	8.5	9.8	9.8	9.8
8α	2.42	*ddd*	3α	3.36	3.36	*dd*		7/8α	1.5	1.0	3.7	3.7	1.6
8β	2.07	*ddd*	6	2.29	2.29	*dd*	6	7/8β	9.3	9.5	12.2	12.2	11.8
9	4.78	*dd*						7/13a	3.7		3.2	3.2	
13a	6.27	*d*		6.26	6.26	*d*		7/13b	3.2		3.2	3.2	
13b	5.54	*d*		5.49	5.49	*d*		8α/8β	14.2		14.2	14.2	
14a	4.09	*d*		4.50	4.50	*dd*	7	8α/9	5.6	5.8			
14b	3.89	*d*		4.24	4.24	*d*	1	8β/9	< 1	1.2			
15a	4.59	*d*		4.65	4.65	*d*		14a/14b	12.0		12.5	12.5	
15b	4.54	*d*	6	4.36	4.36	*d*	2β	14a/OH			6.8	6.8	
OH at C-14				1.63	1.63	*m*		15a/15b	12.7		13.2	13.2	
2′	2.59	*sept*		2.20		*m*		2′/3′	7.1				
3′	1.20	*d*		2.08		*m*		2′/4′	7.1				
4′	1.20	*d*		0.95		*d*		3′/4′			6.6		
5′				0.95		*d*		3′/5′			6.6		
2′					2.38	*m*		2′/5′				7.1	
3′a					1.68	*m*		3′/4′				7.4	
3′b					1.48	*m*							
4′					0.90	*t*							
5′					1.14	*d*							

[a] Assignment determined by GHSQCR correlations.
[b] 2a and 2b.
[c] Multiplicity not determined (signal overlap).
[d] Valid for **4** and **5**.
[e] 1/2a and 1/2b for **6**.
[f] Calculated coupling constants from the model structure of **6**.
[g] Calculated coupling constants for the DU conformation of compound **7** and **8**.

should be isomers because only one pseudomolecular ion at m/z 363 appeared besides other signals from Na and K adducts. The ^{13}C and ^{1}H NMR data revealed the presence of a mixture of two components in different proportion, one with an isovaleryloxy (**7**) and the other with a 2-methylbutyryloxy residue (**8**) (see Tables 1 and 5). Differences in the chemical shifts of C-1, C-2, C-10 and C-14 suggested a germacranolide, but of a subtype different from Sls **1**–**6**. This was confirmed by the chemical shift of C-14 which was shifted downfield from ca 58 to 66.1 ppm and was characteristic for a vinylic hydroxymethyl group located on a 1,10-*cis* double bond (Asada et al., 1984; Lange and Lee, 1986). In the ^{1}H NMR spectrum, the H-1 signal appears as a broad triplet (Asada et al., 1984; Ming et al., 1989) while in germacrolides the corresponding signal exists as a double doublet. Additionally, the chemical shift suggests again a *cis* relationship with the hydroxymethyl group. That **7** and **8** were 1,10-*cis*,4-trans-germacran-12,6-olides with a *trans*-fused γ-lactone ring (melampolides) was also confirmed by the coupling constants $J_{1,2\alpha}$ and $J_{1,2\beta} = 8.8$ Hz, $J_{5,6} = 10.4$ Hz as well as $J_{7,13a}$ and $J_{7,13b} = 3.2$ Hz (Samek, 1970; Asada et al., 1984; Ming et al., 1989). The assignment of the relative stereochemistry of the protons at C-2, C-3 and C-8 was achieved by a NOESY spectrum. Quantum mechanical calculations afforded three low energy conformers (see Fig. 2) which differed mainly in the orientation of the substituents at the double bonds: UU, UD and DU. NOEs including H-1/H-14a, H-5/H-14a and H-7/H-14a showed a *cis*-configuration of the 1,10-double bond as well as orientation of both substituents below the plane. No NOE could be observed between H-5 and H-15, but between H-5 and H-7. Therefore a 4,5-*trans* double bond with an up arrangement of C-15 was present. Due to these observations only the DU-conformation which is in agreement with the X-ray structure of melampolide is possible (Ming et al., 1989) In contrast, Bohlmann et al. (1984) postulated for 8α-(2′,3′-epoxy-2′-methylbutyryloxy)-9-oxo-germacra-4*E*,1(10)-*Z*-dien-6β,12-olide a UD conformation. Theoretical values of the coupling constants calculated from the DU conformer agreed only partly with the experimental ones. These differences in the coupling constants $J_{1,2\alpha}$ and $J_{1,2\beta}$ may be explained by a different behaviour of this part of the molecule in solution than in the vacuum used for calculation. Altogether, compound **7** could be identified as 14-hydroxy-15-isovaleryloxy-9-oxo-melampolide and **8** as 14-hydroxy-15-(2-methylbutyryloxy)-9-oxo-melampolide. Both melampolides are described for the first time.

Compound **9** was separated from **10** by preparative TLC. Silica gel plates were developed several times in diethylether and afforded a colourless resin which gave a grey-brownish colour after spraying with anisaldehyde-sulfuric acid. The molecular mass of 362 was deduced from the mass spectra (CI, isobutane and ESI) and agreed with the molecular formula $C_{20}H_{26}O_6$. Fragment ions at m/z 275 [M + H–$C_4H_8O_2^+$] and 257 [M + H–H_2O–$C_4H_8O_2$] in the CI mass spectrum indicated the occurrence of a C_4-acyl acid residue which was identified as isobutyric acid according to the signals in the ^{1}H and ^{13}C NMR spectra (see Tables 2 and 6). The remaining chemical shifts in the ^{1}H and ^{13}C NMR spectra (^{1}H-^{1}H COSY, GHMQCR, GHSQCR and DEPT) revealed that compound **9** partly possessed the same structure elements found in Sl **3**, e.g. a 6,7-*trans*-α-methylene-γ-lactone ring, a carbonyl group at C-9, a Δ^4 double bond and a hydroxymethyl group at C-15 esterified with isobutyric acid (see Tables 2 and 6). However, the corresponding signals for C-1 and C-14 at about δ_C 140 and δ_C 60, respectively were replaced by signals at 77.9 and 123.4. The first signal corresponds to an oxygen substituted methine carbon, which correlated in the GHSQCR spectrum with a broad doublet that was assigned to H-1 in the COSY experiment. Additionally, a signal appearing at δ_C 56.0 corresponds to a methoxy group, which must be located at C-1 since only one –CH–O– group was observed. The signal at δ_C 123.4 correlated with two downfield one-proton singulets in the GHSQCR spectrum at δ_H 6.08 and 5.92 and well agreed with an exocylic methylene group attached to C-10. This fact was confirmed by long range couplings between H-14a and H-14b and H-1 and correlations of the exocyclic protons to C-1, C-9 and C-10 in the GHMQCR spectrum. Due to the presence of the carbonyl function at C-9 the C-1 signal was shifted downfield about 10 ppm compared with values reported in the literature for unsubstituted compounds (Segal et al., 1983; Sanz et al., 1989; Marco et al., 1991). Since the coupling constants $J_{1,2\alpha}$ and $J_{1,2\beta}$ show values of similar magnitude (Geissman, 1970; Segal et al., 1983; Sanz et al., 1989; Marco et al., 1991), the relative stereochemistry at C-1 was determined from the NOESY spectrum. NOEs between H-7α and H-14 as well as H-14 and the methoxy group showed that H-1 was β. Moreover, *trans*-configuration of the Δ^4 double bond as well as the position of C-15 above the plane were confirmed by an NOE between H-5 and H-7α. Calculation of low energy conformations afforded the three conformers DU, UU and DD, which existed in two forms, respectively (see Fig. 2). These subtypes differed in the position of C-2, which is either above or below the plane. Similar conformations have already been reported from the structural related germacranolide lucentolide (Marco et al., 1997). Here, the DU conformer predominated, as it was also reported for 8β-acetoxy-1β-peroxycostunolide (Schmeda-Hirschmann et al., 1985). NOESY experiments as well as the comparison of the experimental with the calculated coupling constants from the force field molecular models provided evidence for the occurrence of the DU conformer A with C-2 facing down (see Fig. 2 and Table 6). Thus, compound **9** is 1α-methoxy-15-isobutyryloxy-9-oxo-germacra-4-*trans*-10 (14),11(13)-trien-12,6α-olide.

Table 6
^{1}H NMR data as well as GHMQCR and NOESY correlations of compounds **9** and **10** (300 MHz, * 400 MHz)

H	**9** δ * (ppm)	mult	GHMQCR, corr. with C	NOESY, corr. with H	**10** δ * (ppm)	mult	GHMQCR, corr. with C	NOESY, corr. with H	H	**9**	A[d]	A′[d] J (Hz)	**10**	B[e]
1	4.03	*d(br)*			4.23	*dd*		3α	1β/2α	8	9	5.4		
2α	2.03[a]	*m*[b]		5	2.15[a]	*m*[b]		5	1β/2β		1.2	1.5		
2β	2.14[a]	*m*[b]			1.78	*m*		14	1α/2α				2	1.2
3α	2.51[a]	*m*[b]		5	2.12[a]	*m*[b]		1	1α/2β				10	10.5
3β	2.10[a]	*m*[b]			2.50	*m*[b]			2α/3α		3.6	0.4		3.6
5	5.31	*d*	3, 7, 15	2α, 3α, 7	5.19	*d*	3, 7, 15	2α, 7	2α/3β		13.5	7.9		3.0
6	4.69	*m*			4.44	*t*[c]		15a	2β/3α		2.9	12.2		13.5
7	3.30	*m*		5, 14b	3.15	*m*		5	2β/3β		3.5	0.5		3.6
8α	3.50	*dd*	6, 7, 9	14b	2.67	*dd*	6, 7, 9, 11		5/6	10	11.6	11.3	10	11.5
8β	2.38	*dd*	6, 7, 9, 10, 11		2.90	*t(br)*	6, 7, 9	14, 15b	6/7		8.9	9.6	10	9.4
13a	6.26	*d*	7, 11, 12		6.22	*d*	7, 11, 12		7/8α	4	2.8	1.8	1.5	1.0
13b	5.50	*d*	7, 11, 12		5.50	*d*	7, 11, 12		7/8β	12	12.4	12	12	9.6
14a	6.08	*s*	1, 9, 10	OCH_3	5.99	*d*	1, 9, 10	2β, 8β, OCH_3	7/13a	3			3	
14b	5.92	*s*	1, 9, 10	7, 8α	5.99	*d*	1, 9, 10	2β, 8β, OCH_3	7/13b	3			3	
15a	4.54	*d(br)*	1′, 4		4.60	*dd*	1′, 3, 4, 5	6	8α/8β	14			12	
15b	4.38	*d(br)*	1′, 4		4.46	*d*	1′, 3, 4, 5	8β	14a/14b				1.5	
OCH_3	3.17			14a	3.18	*s*	1	14	15a/15b	13			13	
2′	2.56	*sept*	1′, 3′, 4′		2.55	*sept*	1′, 3′, 4′		2′/3′	7			7	
3′	1.17	*d*	1′, 2′		1.16	*d*	1′, 2′		2′/4′	7			7	
4′	1.17	*d*	1′, 2′		1.16	*d*	1′, 2′							

[a] Assignment determined by GHSQCR correlations.
[b] Multiplicity not determined (signal overlap).
[c] Pseudo triplet.
[d] Calculated coupling constants from model structures of **9.**
[e] Calculated coupling constants from model structure of **10**.

Compound **10** had MS and NMR features similar to those of **9** suggesting the occurrence of isomers. Both Sls differed in the stereochemistry of the methoxy group at C-1 and in the conformation of the skeleton. The most conspicuous difference was observed in the multiplicity of the signal for two H-14, which resonated at δ_H 5.99 and appeared as a doublet. Furthermore, in the ^{13}C NMR spectrum chemical shifts for C-2, C-8 and C-15 were shifted upfield, that of C-3 and C-7 downfield of 2–3 ppm. The NOESY experiment confirmed β-orientation of the methoxy group and of the exocyclic methylene group at C-10 as well as the orientation of C-14 and C-15 above the plane by interactions between H-2β, H-8β, H-14, H-15 and the methoxy group. Therefore, only the UU conformation (B) which was obtained by quantum mechanical calculation agreed with the NMR data, but not the low energy forms of a DU (A) and DD (C) conformation (see Fig. 2). Theoretical values of the coupling constants were also in good agreement with the experimental ones (see Table 6). Thus, compound **10** possessed the same conformation as gallicin, which lacks the carbonyl function at C-9 and is substituted by a hydroxy instead of a methoxy group (González et al., 1979). Compound **10** is 1β-methoxy-15-isobutyryloxy-9-oxogermacra-4-*trans*,10(14),11(13)-trien-12,6α-olide, a new epimer of Sl **9**.

Both epimers can be easily distinguished by the different behaviour of the methylene protons at C-14. The following explanation may be applicable: In the DU conformation A of compound **9** the distance between the oxygen at C-9 and one proton at C-14 is 3.88 Å, and 2.64 Å between the oxygen at C-9 and the other proton at C-14; on the other hand in compound **10** with the β-methoxy group, these values were 3.71 and 3.3 Å, respectively. The distances were calculated using HyperChem™. The influence of the two electronegative oxygen atoms on both protons is much more regular for compound **10** than for **9** and hence both protons at C-14 might be chemically equivalent and resonated as one signal.

Compounds **11** and **12** were obtained and identified as a mixture for the reasons mentioned above. The mass spectra (CI, isobutane, negative mode and ESI) showed one molecular ion peak at *m*/*z* 376 and one pseudomolecular ion peak at *m*/*z* 377. ^{1}H and ^{13}C NMR data agreed well with those of compound **10** (see Tables 2 and 7), with the isobutyryloxy moiety replaced by isovaleryloxy in **11** and 2-methylbutyryloxy in **12**. Comparison of the experimental and theoretical coupling constants confirmed the same conformation (UU, B) as found for Sl **10**. **11** and **12** have been isolated for the first time.

An additional mixture of Sls contained compounds **11** and **12** and a further pair of isomers. The mass spectra [EI, CI (NH_3)] from which a molecular weight of 376 was determined did not give any hint about the complexity of the mixture. However, ^{1}H and ^{13}C NMR spectra revealed the occurrence of two isomeric pairs. Besides the signals

Table 7
^{1}H NMR data of compounds **11**–**14** ($CDCl_3$, 300 MHz)

H	11 δ (ppm)	12 δ (ppm)		13 δ (ppm)	14 δ (ppm)	mult	H	11 J (Hz)	12 J (Hz)	13 J (Hz)	14 J (Hz)
1	4.24	4.24	*dd*	3.99	3.99	*d(br)*	1β/2α			8	8
2α	2.15[a,b]	2.15[a,b]	*m*[c]	2.03[a,d]	2.03[a,d]	*m*[c]	1β/2β				
2β	1.80[a,b]	1.80[a,b]	*m*[c]	2.15[a,d]	2.15[a,d]	*m*[c]	1α/2α	2.5	2.5		
3α	2.12[a,b]	2.12[a,b]	*m*[c]	2.48[a,d]	2.48[a,d]	*m*[c]	1α/2β	10	10		
3β	2.50[a,b]	2.50[a,b]	*m*[c]	2.10[a,d]	2.10[a,d]	*m*[c]	5/6	10.5	10.5	10	10
5	5.20	5.20	*d*	5.28	5.28	*d*	6/7	10.5	10.5		
6	4.46	4.46	*t*[e]	4.68[a]	4.68[a]	*m*	7/8α	2	2	4	4
7	3.15	3.15	*m*	3.28[a]	3.28[a]	*m*	7/8β	12	12		
8α	2.67[b]	2.67[b]	*dd*	3.47[b]	3.47[d]	*dd*	7/13a	3	3	3	3
8β	2.90[b]	2.90[b]	*t(br)*	2.36[a,d]	2.36[a,d]	*m*[c]	7/13b	3	3	3	3
13a	6.23	6.23	*d*	6.21	6.21	*d*	8α/8β	12	12	14	14
13b	5.51	5.51	*d*	5.47	5.47	*d*	15a/15b	13	13		
14a	6.00	6.00	*d*	6.04	6.04	*s*	3′/4′	6.6	7.4	6.6	7.4
14b	6.00	6.00	*d*	5.90	5.90	*s*	3′/5′	6.6		6.6	
15a	4.62	4.62	*dd*	4.54[a]	4.54[a]	*d*	2′/5′		7.1		7
15b	4.45	4.45	*d*	4.34[a]	4.34[a]	*d*					
OCH_3 at C-9	3.19	3.19	*s*	3.15	3.15	*s*					
2′	2.20		*m*[c]	2.18[a]		*m*[c]					
3′	2.10		*m*[c]	2.06[a]		*m*[c]					
4′	0.93		*d*	0.93[a]		*d*					
5′	0.93		*d*	0.93[a]		*d*					
2′		2.40	*m*[c]		2.38[a]	*m*[c]					
3a′		1.65	*m*[c]		1.64[a]	*m*[c]					
3b′		1.45	*m*[c]		1.43[a]	*m*[c]					
4′		0.89	*t*		0.87[a]	*m*[c]					
5′		1.15	*d*		1.10	*d*					

[a] Assignment determined by GHSQCR correlations.
[b] Assignment according to **10**.
[c] Multiplicity not determined (signal overlap).
[d] Assignment according to **9**.
[e] Pseudo triplet.

for **11** and **12**, those for two further Sls differing in the acyl moiety, but possessing both the skeleton of compound **9**, were detected (see Tables 2 and 7). Compound **13** was the isovaleryloxy and **14** the 2-methylbutyryloxy derivative of **9**. The same conformation (DU), as determined for **9** can be assumed.

The known 6-methoxyflavone eupafolin was isolated and identified by MS and comparative TLC analysis with the authentic compound (Liu and Mabry, 1981). β-Amyrin, β-amyrin acetate as well as stigmasterol and β-sitosterol were identified by comparative GC/MS analysis, too.

All Sls presented here possess a germacranolide skeleton. The eudesmanolides which were previously found in the aerial parts of *M. guaco* from a different location in Costa Rica could not be detected. This difference may be explained by the occurrence of geographical variability which has also been found in the case of *M. micrantha* (Cuenca et al., 1988). Germacranolides of the germacrolide and melampolide type are characteristic Sls of the genus *Mikania*. However, whereas more often Sls with a 7,8 attached lactone ring have been found in other *Mikania* species, only germacradiene-6,12-olides were detected in *M. guaco* investigated here. These Sls were isolated by bio-guided fractionation using the transcription factor NF-κB as molecular target. This protein is a pivotal mediator in the immune system and regulates the transcription of various inflammatory cytokines (Lyß et al., 1997). All Sls were studied in more detail for their antiinflammatory activity which will be reported in a subsequent paper.

The genus *Mikania* belongs to the tribe Eupatorieae from which most species with pyrrolizidine alkaloids are known, like in the tribe Senecioneae (Asteraceae) (Mattocks, 1968,1986). Unsaturated representatives of these secondary metabolites are toxic. They possess especially hepatotoxic properties. Therefore, it is essential to clarify if plants used in traditional medicine are free of this type of alkaloids. Saturated and unsaturated pyrrolizidine alkaloids can be distinguished by the reagent of Dann and Mattocks (detectable limit 0.5–1.0 μg) after TLC analysis (Mattocks, 1968; Röder and Neuberger, 1988). Here we proved that no type of pyrrolidizidine alkaloids could be detected in the methanolic extract of

the aerial parts from *M. guaco* under these conditions. The Dragendorff reagent, an unspecific reagent for alkaloids, also gave a negative result. From this point of view preparations from *M. guaco* are quite safe. However, it has to be kept in mind that due to the presence of Sls, external use of *M. guaco* preparations can induce contact dermatitis (Hausen and Vieluf, 1997).

3. Experimental

3.1. General experimental procedures

Optical rotation was measured with Perkin-Elmer 241 polarimeter at 27°C. Mass spectra were recorded in the direct inlet mode using chemical ionization with NH_3 or isobutane as reactant gas (CI-MS) on a Finnigan MAT 8200 (130 eV) or Finnigan MAT 44S, EI–MS on a Finnigan MAT 44 (70 eV), ESI–MS on a Finnigan TSQ 7000; GC/MS on a Finnigan GC 9610/MS 4500, 70 eV using an OV-1-DF capillary column (25 m×0.25 mm I.D., 0.25 μM film-thickness), carrier He at 24 ml/min (total flow), split 1:24, column flow 1.0 ml/min, gradient: 120–270°C, rate: 10°C/min, GC/MS-CI with isobutane as reactant gas on a Varian 3700/MAT 44S, 170 eV, DB-1 CB (5 m×0.32 mm×0.25 μm) carrier He, split 1:20, gradient: 50–300°C, rate 20°C/min; GC analyses were carried out with a Varian 3700 equipped with a split injector and FID, using an OV-1-DF capillary column (25 m×0.25 mm I.D., 0.25 μM film-thickness), carrier nitrogen at 25 ml/min, split 1:42.7, column flow 0.59 ml/min, temperatures: injector and detector 290°C, gradient as above, retention time (RT) of the standard santonin: 10.92 min; NMR spectra were recorded with a Varian UNITY 300 (300/75 MHz), compounds **9** and **10** partly with a Bruker Arx 400 (400 MHz). All NMR spectra were taken at room temperature with TMS as internal standard.

TLC for the detection of Sls was performed using silica 60 plates (Merck) with CH_2Cl_2–EtOAc–MeOH (8:1:1) (system I) and toluene–EtOAc (1:2) (system II) as solvents and anisaldehyde–H_2SO_4 as visualizing agent, heating at 120°C. TLC for the detection of pyrrolizidine alkaloids was carried out using silica 60 plates with CH_2Cl_2–MeOH–NH_3 (25%) (85:14:1) as solvent and the reagent of Dann and Mattocks (Röder and Neuberger, 1988). Here plates were subsequently sprayed with 30% H_2O_2, acetanhydride-benzene (80–100°C)-toluene (1:4:5) and finally with *p*-dimethylaminobenzaldehyde (2.00 g), 37% HCl (54.0 ml), EtOH (90%) (ad 100.0 ml). The plates were heated at 120°C for 30 min after each spraying. Monocrotaline was used for comparison. Low pressure chromatography was carried out with a pump from Latek (model S1990) combined with a UV detector and with columns from Latek LC-1/2-23 and Kronlab Classic CL 15/450 and 20/450.

3.2. Calculation of the conformations for Sls **1**, **3**, **6**, **7**, **9**, **10** and the coupling constants

We generated low-energy conformations of the Sls using the conformational search option of ChemPlus® (v. 2.0), which is operated under the Molecular Modelling package Hyperchem® (v. 5.1 Professional). Energy minimizations were performed with Hyperchem's semi-empirical quantum mechanical method AM1 using the Polak–Ribiere minimization algorithm.

Starting structures were created with Hyperchem and initially minimized to an RMS gradient < 0.01 kcal mol^{-1} Å^{-1}. All rotatable cyclic bonds were included as variable torsions and allowed to be changed simultaneously. The search was performed applying a usage directed search method and standard settings for duplication tests. A search run was terminated after energy minimization of 2500 unique starting geometries. Acyl side chains of the respective Sls were not included in the conformational search. The resulting structures were energy minimized to an RMS gradient as above. Coupling constants were calculated with PCMODEL™ using the MOPAC form of the conformations created with HyperChem™.

3.3. Plant material

Aerial parts of *M. guaco* were collected near Rio Corinto, Costa Rica, in March 1995 and identified by L. Poveda, Professor of Botany, Universidad National, Costa Rica. Voucher specimens (no. FM 1022) are deposited at the Herbarium of Inventory Program, INBio, Costa Rica.

3.4. Extraction and isolation

Dried, powdered aerial parts of *M. guaco* (1147 g) were exhaustively extracted with *n*-hexane–Et_2O–MeOH (1:1:1) at room temp. The crude extract (48.5 g) was treated with MeOH at −20°C for 48 h. After filtering and evaporation of the solvent a viscous residue (29.8 g) was obtained. The MeOH soluble part (in four portions) was separated by CC on Sephadex LH-20 with MeOH as solvent. Nine fractions were obtained. They were studied for their antibacterial activity in the agar plate diffusion test (Burkhardt, 1992) using *Bacillus subtilis* and *Staphylococcus aureus* as well as for their antiinflammatory activity in the NF-κB EMSA (Lyß et al., 1997). Fractions 4–6 showed inhibitory activity in both assays. Further isolation of the Sls followed from fractions 5 and 6. During the isolation procedure studies on the antibacterial activity were repeated from time to time. CC of fraction 5 on Sephadex LH-20 with cyclohexane–CH_2Cl_2-MeOH (7:4:1) gave 10 subfractions (5.1–5.10).

Subfr. 5.2 (3.14 g) was submitted to CC on silica gel with CH_2Cl_2–EtOAc mixtures of increasing polarities (9:1–4:6) and afforded 15 fractions (5.2.1.–5.2.15). CC

of 5.2.14 (440 mg) on silica gel 60 using CH_2Cl_2–EtOAc–MeOH (8:1:1) yielded eight fractions. Further separation of subfr. 5.2.14.4 (166 mg) on silica gel 60 with *n*-hexane–EtOAc mixtures (100–50%, rate 5%) afforded 10 fractions. Subfr. 5.2.14.4.6. gave 21.8 mg of a mixture of **1** and **2**, subfr. 5.2.14.4.10 a mixture of **4** and **5** (6.8 mg). A further subfr. 5.2.14.4.7 (12.3 mg), containing compounds **1** and **2**, was separated by HPLC on a RP-18 column (Hypersil ODS, 5 μm, 12.5×4.6 cm) with a water-MeOH gradient (flow: 3.2 ml/min, MeOH–H_2O, 45–60%, within 45 min) and 1.5 mg of compound **2**, nearly pure, was obtained.

Subfr. 5.7 (88 mg) was chromatographed on RP-18 (Eurosil Bioselect, 20–45 μm) by low pressure chromatography with a H_2O–MeOH mixture (58:42) and subsequently on a RP-18 column (Hypersil ODS, see above) by HPLC with H_2O–MeOH (65:35). Thus, compound **3** (6 mg) and **6** (5 mg) were isolated.

Subfr. 5.2.14.5 (152 mg) was submitted to low pressure chromatography on RP-18 (Eurosil Bioselect, 20–45 μm) with a H_2O–MeOH gradient (40–65%) which afforded 5.5 mg of a mixture of **7** and **8**.

CC of subfr. 5.2.6 (543 mg) on Sephadex LH-20 with cyclohexane–CH_2Cl_2–MeOH (7:4:1) yielded five fractions. Subfr. 5.2.6.4 (150 mg) was separated by low pressure chromatography on RP-18 (Eurosil Bioselect, 20–45 μm) and 60% aqueous MeOH as eluent. Prep. TLC, by which silica gel G plates were developed four times with Et_2O, was carried out with subfr. 5.2.6.4.3 (50.6 mg) yielding 8.3 mg of **9** and 6.0 mg of **10**. Both compounds were slightly impure. Subfr. 5.2.6.4.10 afforded 31 mg of a mixture of **11**, **12**, **13** and **14**, which gave a broad peak in HPLC. Further separation attempts were unsuccessful.

Subfr. 5.2.6.3 (122 mg) was rechromatographed on Sephadex LH-20 with cyclohexane–CH_2Cl_2–MeOH (7:4:1). One of the subfr. (80 mg) was submitted to CC on silica gel 60 using mixtures of *n*-hexane–EtOAc (100–50%, rate 5%). One fraction (2.2 mg) yielded **11** and **12** in a mixture.

Fraction 6 (4.65 g) was subjected to CC on Sephadex LH-20 with cyclohexane–CH_2Cl_2–MeOH (7:4:1) and gave 18 subfractions (6.1–6.18). Low pressure chromatography of subfr. 6.11 (417 mg) on RP-18 (Eurosil Bioselect, 20–45 μm) using 50% aqueous MeOH afforded an additional amount of **3** (8.2 mg).

The flavone eupafolin was obtained from fraction 8 (410 mg). Identification of the triterpenes occurred in subfraction 6.4, while that of the sterols in subfr. 5.2.5.

3.5. 2α-Acetoxy-15-isovaleryl-miguanin (*1*) and 2α-acetoxy-15-(2-methylbutyryl)-miguanin (*2*)

RT: 15.67 min (**1**), 15.80 min (**2**); R_f: 0.64 (I) and 0.46 (II) (**1** and **2**); optical rotation: dextrorotatory (**2**), no value given, because of the small amount; UV (online): $\lambda_{max} = 209.9$ nm (**1** and **2**); CI–MS (isobutane) *m/z* 421 $[m+H]^+$ (11), 415 (9), 391 $[M+H–CH_2O]^+$ (6), 361 $[M+H–CH_3COOH]^+$ (5), 331 $[M+H–CH_2O–CH_3COOH]^+$ (100), 319 $[M+H–C_5\text{-acid}]^+$ (8), 289 $[M+H–CH_2O–C_5\text{-acid}]^+$ (4), 258 (8), 229 $[M+H–CH_2O–CH_3COOH\text{-}C_5\text{-acid}]^+$ (23), 185 (6), 169 (10), 103 (6); ESI–MS *m/z* 475 $[M+Na+CH_3OH]^+$ (44), 443 $[M+Na]^+$ (100), 415 $[M+Na+CH_3OH–CH_3COOH]^+$, 413 $[M+Na–CH_2O]^+$ (32), 385 $[M+Na+CH_3OH–CH_2O–CH_3COOH]^+$ (2), 383 $[M+Na–CH_3COOH]^+$ (3), 353 $[M+Na–CH_2O–CH_3COOH]^+$ (28).

3.6. 15-Isobutyryl-miguanin (*3*)

Amorphous, optical rotation: dextrorotatory, value not given, because of the small amount; UV (online): $\lambda_{max} = 219.3$ nm; RT: 12.16 min; R_f: 0.57 (I) and 0.34 (II); ESI–MS *m/z* 387 $[M+K]^+$ (100), 371 $[M+Na]^+$ (37), 366 $[M+H_2O]^+$ (22), 349 $[M+H]^+$ (3).

3.7. 15-Isovaleryl-miguanin (*4*) and 15-(2-methylbutyryl)-miguanin (*5*)

RT: 12.59 min (**4**) and 12.59 (**5**); R_f: 0.57 (I) and 0.36 (II) (**4** and **5**); GC/CI–MS (isobutane): *m/z* 363 $[M+H]^+$ (56), 349 (9), 333 $[M+H–CH_2O]^+$ (10), 261 $[M+H–C_5\text{-acid}]^+$ (100), 243 (10), 231 $[M+H–CH_2O–C_5\text{-acid}]^+$ (18), 103 (16), 85 (30); ESI–MS *m/z* 401 $[M+K]^+$ (100), 385 $[M+Na]^+$ (23), 380 $[M+H_2O]^+$ (18), 363 $[M+H]^+$ (2).

3.8. 9α,14-Dihydroxy-15-isobutyryloxy-costunolide (*6*)

RT: 12.17 min; R_f: 0.39 (I) and 0.12 (II); optical rotation: dextrorotatory, values omitted, because of the small amount; UV (online): $\lambda_{max} = 205.2$ nm; CI–MS (isobutane) *m/z* 351 $[M+H]^+$ (3), 349 (16), 333 $[M+H–H_2O]^+$ (59), 263 $[M+H–C_4H_8O_2]^+$ (38), 245 $[M+H–H_2O–C_4H_8O_2]^+$ (50), 233 (22), 89 (100), 71 (19); ESI–MS *m/z* 389 $[M+K]^+$ (100), 373 $[M+Na]^+$ (23), 368 $[M+H_2O]^+$ (4).

3.9. 14-Hydroxy-15-isovaleryloxy-9-oxo-melampolide (*7*) and 14-hydroxy-15-(2-methylbutyryloxy)-9-oxo-melampolide (*8*)

RT: 15.82 (**7** and **8**); R_f: 0.52 (I) and 0.26 (II); UV (online) $\lambda_{max} = 200.6$ nm (**7** and **8**); ESI–MS *m/z* 401 $[M+K]^+$ (100), 385 $[M+Na]^+$ (45), 363 $[M+H]^+$ (2).

3.10. 1α-Methoxy-15-isobutyryloxy-9-oxo-germacra-4-trans,10(14),11(13)-trien-12,6α-olide (*9*)

RT: 15.42 min, R_f: 0.73 (I) and 0.47 (II); α_D 0 ($CHCl_3$); CI–MS (isobutane) 363 $[M+H]^+$ (41), 345 $[M+H–H_2O]^+$ (3), 277 (26), 275 $[M+H\text{-}i\text{-butyric acid}]^+$ (32), 257 $[M+H\text{-}H_2O\text{-}i\text{-butyric acid}]^+$ (9), 245 (69), 231 (15), 215 (14), 199 (19), 89 (100), 71 (27); ESI–MS 401

$[M+K]^+$ (11), 385 $[M+Na]^+$ (100), 363 $[M+H]^+$ (2), 243 (4).

3.11. 1β-Methoxy-15-isobutyryloxy-9-oxo-germacra-4-trans,10(14),11(13)-trien-12,6α-olide (**10**)

RT: 15.59 min; R_f: 0.67 (I) and 0.49 (II); α_D 0 ($CHCl_3$); CI–MS (isobutane) 363 $[M+H]^+$ (14), 277 (65), 275 (16), 245 (59), 231(15), 201 (20), 103 (19), 89 (100), 71 (28); ESI–MS 401 $[M+K]^+$ (26), 385 $[M+Na]^+$ (100), 363 $[M+H]^+$ (9), 243 (32).

3.12. 1β-Methoxy-15-isovaleryloxy-9-oxo-germacra-4-trans,10(14),11(13)-trien-12,6α-olide (**11**) and 1β-methoxy-15-(2-methylbutyryloxy)-9-oxo-germacra-4-trans,10-(14),11(13)-trien-12,6α-olide (**12**)

RT: 16.86 min; R_f: 0.70 (I) and 0.51 (II) (**11** and **12**); CI-MS (isobutane, negative ions) 376 $[M]^-$ (14), 274 (10), 252 (100), 101 (72); ESI–MS 415 $[M+K]^+$ (47), 399 $[M+Na]^+$ (100), 377 $[M+H]^+$ (6), 243 (14).

3.13. 1α-Methoxy-15-isovaleryloxy-9-oxo-germacra-4-trans,10(14),11(13)-trien-12,6α-olide (**13**) and 1α-methoxy-15-(2-methylbutyryloxy)-9-oxo-germacra-4-trans,10-(14),11(13)-trien-12,6α-olide (**14**)

RT: 16.71; R_f: 0.77 (I) and 0.49 (II) (**13** and **14**); EI–MS 376 $[M]^+$ (2), 345 (22), 317 (26), 274 (2), 261 (2), 242 (2), 231 (1), 215 (4), 185 (2), 169 (3), 161 (4), 137 (6), 133 (4), 105 (10), 91 (9), 85 (51), 81 (13), 57 (100), 53 (29), 41 (35); CI–MS (NH_3) 394 $[M+NH_4]^+$ (100), 377 $[M+H]^+$ (5).

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Five saponins from the root bark of *Aralia elata*

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Abstract

Five saponins, 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-oleanolic acid 28-*O*-β-D-glucopyranosyl ester (aralia-saponin V), 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-echinocystic acid 28-*O*-β-D-glucopyranosyl ester (aralia-saponin VI), 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-hederagenin 28-*O*-β-D-glucopyranosyl ester (aralia-saponin VII), 3-*O*-{β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→ 3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranosyl}-caulophyllogenin 28-*O*-β-D-glucopyranosyl ester (aralia-saponin VIII), 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl(1→3)]-α-L-arabinopyranosyl}-hederagenin 28-*O*-β-D-glucopyranosyl ester (aralia-saponin IX), were isolated from the root bark of *Aralia elata* (Miq.) Seem., together with four known compounds. Their structures were determined on the basis of chemical and spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Aralia elata*; Araliaceae; Saponin; Oleanolic acid; Echinocystic acid; Hederagenin; Caulophyllogenin

1. Introduction

The root bark of *Aralia elata* (Miq.) Seem. (Araliaceae) is used in Japan for treatment of diabetes, and has been used as a tonic, anti-arthritic and anti-diabetic agent in Chinese traditional medicine. As regards the biological active constituents of *A. elata*, many triterpenoidal saponins were reported to have hypoglycemic activity and cytoprotective effects on carbon tetrachloride-induced hepatic injury (Yoshikawa et al., 1996; Saito et al., 1993). In the course of our studies, twenty-three saponins have been isolated from the root bark of *A. elata* (Song et al., 2000). The present paper deals with structure elucidation of five additional saponins, named aralia-saponins V-IX (**1**–**5**), together with four known compounds.

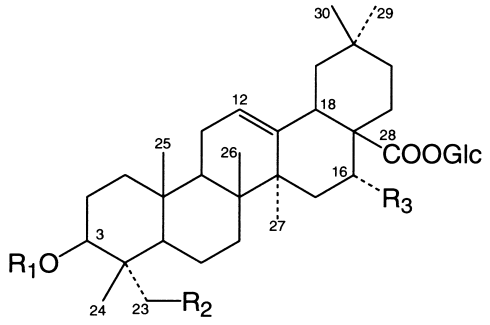
2. Results and discussion

The air-dried root bark of *A. elata* was extracted with methanol. The methanol extract was suspended in water, washed with petroleum ether and chloroform, and then extracted with butanol saturated with water. The butanol soluble part was repeatedly subjected to normal and reversed-phase silica gel chromatography to give eleven saponins. The known compounds were identified as congmuyenoside A (**6**) (Kuang et al., 1996), stipuleanoside R_1 (**7**) (Yoshikawa et al., 1996), stipuleanoside R_2 (**8**) (Yoshikawa et al., 1996), and araloside A (**9**) (Jiang et al., 1992) by comparing their spectral data with those reported previously.

Compound **1** (aralia-saponin V) showed a quasi-molecular ion peak at m/z 1127 $[M+Na]^+$ in the positive ion electrospray ionization (ESI)-MS, and at m/z 1103 $[M-H]^-$ in the negative ion ESI-MS. The HR-FABMS revealed the molecular formula of **1** to be $C_{54}H_{88}O_{23}$. The IR spectrum showed the presence of an ester carbonyl group at 1740 cm^{-1} and an olefinic group at 1660 cm^{-1}. The ^1H NMR spectrum revealed signals due to seven tertiary methyl groups (δ 0.78, 0.86, 0.88, 1.06, 1.06, 1.23, 1.23), one trisubstituted olefinic proton (δ 5.39) and four anomeric protons (δ 4.81, 5.36, 5.70, and 6.31). The ^{13}C NMR spectrum showed signals of a pair of olefinic carbons at δ 122.1 and 144.1, four anomeric carbons at δ 95.7, 103.8, 104.6, and 104.9, and a carbonyl carbon at δ 176.4. On acid hydrolysis, oleanolic

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	R_1	R_2	R_3
1	-Glc^{2}-Glc $\|^{3}$ Glc	H	H
2	-Glc^{2}-Glc $\|^{3}$ Glc	H	OH
3	-Glc^{2}-Glc $\|^{3}$ Glc	OH	H
4	-Glc^{2}-Glc $\|^{3}$ Glc^{3}-Glc	OH	OH
5	-Ara^{2}-Glc $\|^{3}$ Glc	OH	H

acid was identified as an aglycone and the monosaccharide unit obtained was identified as only glucose by co-chromatography on TLC with authentic samples; its absolute configuration was determined as a D-form by the method developed by Hara et al. (1987). The spin systems for sugars were assigned on the basis of spectroscopic evidence obtained by ^{1}H–^{1}H COSY, HMQC, and HOHAHA experiments. The sugar protons and carbons were assigned as shown in Tables 1 and 2. The sugar linkages were determined on the basis of the HMBC spectrum. Namely, long-rang couplings ($^{3}J_{HCOH}$) were observed between a proton signal at δ 6.31 (glc-H-1) and a carbonyl carbon signal at δ 176.4 (C-28). A proton signal at δ 4.81 (glc-H-1′) correlated with a carbon signal at δ 89.3 (C-3), whereas a proton signal at δ 5.70 (glc-H-1″) and 5.36 (glc-H-1‴) correlated with carbon signals at δ 79.2 (glc-C-2′) and 88.6 (glc-C-3′), respectively. This indicated glycosylation at C-3 with a [glucopyranosyl (1→2)]-[glucopyranosyl (1→3)]-glucopyranosyl moiety and at C-28 with a glucopyranosyl moiety. By comparison of the ^{13}C NMR spectral data of **1** with those of oleanolic acid (Jiang et al., 1992), the signal due to C-3 shifted to downfield by approximately 10 ppm, the signals due to C-2 and C-4 shifted to higher field by 1.39 ppm and 0.7 ppm, respectively, and the signal due to C-28 also shifted to a higher field by 3.6 ppm. This indicated that the two sugar moieties of **1** were linked at both C-3 and C-28 of oleanolic acid. Furthermore, the characteristic ion peaks at m/z 1103 $[M-H]^-$, 941 $[M\text{-glc-H}]^-$, 779 $[M\text{-2glc-H}]^-$, 617 $[M\text{-3glc-H}]^-$, and 455 $[M\text{-4glc-H}]^-$ in the negative ion ESI-MS confirmed the sugar linkages. The anomeric configurations of the sugar moieties were determined to be a β-form on the basis of the $J_{H\text{-}H}$ values (8.0 Hz). From the above evidence, the structure of **1** was concluded to be 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-oleanolic acid 28-*O*-β-D-glucopyranosyl ester.

The molecular formula of **2** (aralia-saponin VI), $C_{54}H_{88}O_{24}$, was assigned by the presence of quasi-molecular ion peaks at m/z 1143 $[M+Na]^+$ in the positive ion ESI-MS and at m/z 1119 $[M-H]^-$ in the negative ion ESI-MS, and by HR-FABMS. The IR spectrum showed the presence of an ester carbonyl group at 1738 cm^{-1} and an olefinic group at 1628 cm^{-1}. Compound **2** displayed four anomeric proton signals at δ 4.81, 5.34, 5.70, and 6.30 in the ^{1}H NMR spectrum, and four anomeric carbon signals at δ 95.3, 103.8, 104.6, and 104.9 in the ^{13}C NMR spectrum. In comparison of the ^{13}C NMR spectral data with those reported for various echinocystic acid glycosides (Yoshikawa et al., 1995; Nagao et al., 1993), **2** was suggested to be an echinocystic acid derivative with four glucose moieties at both C-3 and C-28. The NMR spectral signals due to the sugar moiety were superimposable between **2** and **1**, indicating that **2** possesses the same sugar as **1**. On acid hydrolysis, **2** gave D-glucose only. By detailed analysis of the 2D NMR (^{1}H–^{1}H COSY, HMQC, and HOHAHA), the spin systems for sugars were assigned as shown in Tables 1 and 2. On the other hand, in the HMBC spectrum of **2**, a proton signal at δ 6.30 (glc-H-1) showed a correlation with a carbonyl carbon signal at δ 175.9 (C-28). A proton signal at δ 4.81 (glc-H-1′) correlated with a carbon signal at δ 89.3 (C-3), and proton signals at δ 5.70 (glc-H-1″) and 5.34 (glc-H-1‴) correlated with carbon signals at δ 79.3 (glc-C-2′) and 88.6 (glc-C-3′), respectively. This indicated glycosylation at C-3 with a [glucopyranosyl (1→2)]-[glucopyranosyl (1→3)]-glucopyranosyl moiety and at C-28 with a glucopyranosyl moiety. Each glucose was determined to be in the β-configuration from the respective coupling constants (J = 7.5–8.0 Hz). Moreover, significant fragment ions observed at m/z 1119 $[M-H]^-$, 957 $[M\text{-glc-H}]^-$, 795 $[M\text{-2glc-H}]^-$, and 633 $[M\text{-3glc-H}]^-$ in the negative ion ESI-MS, confirmed the linkages of sugars. The structure of **2** was consequently concluded to be 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-echinocystic acid 28-*O*-β-D-glucopyranosyl ester.

Compound **3** (aralia-saponin VII) showed quasi-molecular ion peaks $[M+Na]^+$ at m/z 1143 in the positive ion ESI-MS, and at m/z 1119 $[M-H]^-$ in the negative ion

Table 1
^{1}H NMR spectral data for compounds **1**–**5** in C_5D_5N (500 MHz)

	1	2	3	4	5
Aglycon					
3	3.24(*dd, J* = 4.0, 11.5 Hz)	3.26(*dd, J* = 4.0, 11.5 Hz)	4.15(*dd, J* = 4.0, 11.5 Hz)	4.12(*dd, J* = 4.0, 11.5 Hz)	4.11(*dd, J* = 4.0, 11.5 Hz)
12	5.39(br s)	5.58(br s)	5.39(br s)	5.56(br s)	5.39(br s)
16		5.26(br s)		5.05(br s)	
18	3.56(*dd, J* = 4.5, 14.0 Hz)	3.49(*dd, J* = 4.0, 14.0 Hz)	3.13(*dd, J* = 4.0, 14.0 Hz)	3.48(*dd, J* = 4.0, 14.0 Hz)	3.14(*dd, J* = 4.0, 12.0 Hz)
23	1.23(s)	1.21(s)	3.67(*d, J* = 11.0 Hz)	3.64(*d, J* = 11.0 Hz)	3.64(*d, J* = 11.0 Hz)
			4.27(*d, J* = 11.0 Hz)	4.26(*d, J* = 11.0 Hz)	4.24(*d, J* = 11.0 Hz)
24	1.06(s)	1.06(s)	1.04(s)	1.03(s)	1.03(s)
25	0.78(s)	0.82(s)	0.84(s)	0.89(s)	0.88(s)
26	1.06(s)	1.09(s)	1.08(s)	1.11(s)	1.07(s)
27	1.23(s)	1.81(s)	1.16(s)	1.74(s)	1.14(s)
29	0.88(s)	0.98(s)	0.85(s)	0.94(s)	0.85(s)
30	0.86(s)	1.02(s)	0.86(s)	0.97(s)	0.84(s)
3-*O*-Sugar	Glc	Glc	Glc	Glc	Ara
1′	4.81(*d, J* = 8.0 Hz)	4.81(*d, J* = 7.5 Hz)	5.00(*d, J* = 7.8 Hz)	4.98(*d, J* = 7.5 Hz)	4.99(*d, J* = 7.5 Hz)
2′	4.34	4.35	4.30	4.27	4.67
3′	4.24	4.22	4.03	4.02	4.13
4′	4.01	3.98	4.05	4.28	3.39
5′	3.80	3.82	3.62	3.63	3.51(*d, J* = 11.6 Hz)
					4.08(*d, J* = 11.6 Hz)
6′	4.45	4.43	4.43	4.48[a]	
Glc					
1″	5.70(*d, J* = 8.0 Hz)	5.70(*d, J* = 7.5 Hz)	5.71(*d, J* = 7.8 Hz)	5.64(*d, J* = 8.0 Hz)	5.49(*d, J* = 8.0 Hz)
2″	4.06	4.06	4.09	4.07	4.03
3″	4.25	4.25	4.18	4.14	3.65
4″	4.14	4.12	4.28	4.13	4.11
5″	3.87	3.84	3.82	4.22	4.15
6″	4.44	4.44	4.50	4.34[a]	4.38[a]
Glc					
1‴	5.36(*d, J* = 8.0 Hz)	5.34(*d, J* = 7.5 Hz)	5.29(*d, J* = 7.8 Hz)	5.29(*d, J* = 8.0 Hz)	5.22(*d, J* = 8.0 Hz)
2‴	4.02	4.00	4.01	3.99	3.95
3‴	4.19	4.18	4.18	4.22	4.18
4‴	4.12	4.13	4.20	3.98	4.15
5‴	4.17	4.18	4.00	3.95	3.90
6‴	4.23	4.34	4.21	4.40[a]	4.32[a]
Glc					
1⁗				5.13(*d, J* = 8.0 Hz)	
2⁗				4.01	
3⁗				3.90	
4⁗				4.02	
5⁗				4.12	
6⁗				4.44[a]	
C-28-Glc					
1	6.31(*d, J* = 8.0 Hz)	6.30(*d, J* = 8.0 Hz)	6.30(*d, J* = 8.0 Hz)	6.32(*d, J* = 8.0 Hz)	6.29(*d, J* = 8.0 Hz)
2	4.18	4.13	4.17	4.15	4.15
3	4.26	4.23	4.25	4.25	4.24
4	4.31	4.30	4.35	4.30	4.31
5	4.01	3.98	4.00	4.02	3.98
6	4.52[a]	4.52[a]	4.42	4.32[a]	4.49[a]

[a] Chemical shifts may be interchangeable in each column.

ESI-MS. HR-FABMS assigned the molecular formula $C_{54}H_{88}O_{24}$. Compound **3** displayed four anomeric proton signals at δ 5.00, 5.29, 5.71, and 6.30 in the ^{1}H NMR spectrum, and four anomeric carbon signals at δ 95.7, 103.7, 103.9, and 104.5 in the ^{13}C NMR spectrum. The ^{1}H NMR and ^{13}C NMR spectra of **3** were similar to those of **1**, especially in the sugar region. However, methyl protons at C-23 in **1** were replaced by oxygenated methyl protons (δ_H 3.67, 4.27, δ_C 64.8), suggesting that the aglycone of **3** is hederagenin. On acid hydrolysis, **3** yielded hederagenin and only D-glucose as a sugar component. On the basis of the 2D NMR (^{1}H-^{1}H COSY, HMQC, and HOHAHA) experiments, the spin systems for sugars were assigned as shown in Tables 1 and 2. In the

Table 2
^{13}C NMR spectral data for compounds **1**–**5**

No.	1	2	3	4	5
1	38.7	38.8	38.7	38.7	38.7
2	26.5	26.5	26.0	25.9	25.9
3	89.3	89.3	83.0	82.9	82.4
4	39.8	39.5	42.1	43.4	43.5
5	55.7	55.9	48.1	47.2	47.8
6	18.5	18.5	18.2	18.2	18.1
7	33.9	33.4	33.1	33.1	32.7
8	39.5	40.1	39.9	40.0	39.8
9	47.9	47.1	47.9	47.9	48.1
10	36.9	36.9	36.9	36.8	36.8
11	23.7	23.8	23.8	23.8	23.8
12	122.1	122.7	122.8	122.6	122.8
13	144.1	144.2	144.1	144.4	144.0
14	42.1	42.0	41.7	41.9	42.1
15	28.2	36.1	28.2	36.1	28.2
16	23.4	74.1	23.6	74.1	23.3
17	46.9	49.1	46.1	48.9	46.9
18	41.7	41.3	43.5	41.2	41.6
19	46.2	47.1	46.9	47.2	46.1
20	30.7	30.8	30.7	30.7	30.7
21	33.9	35.9	33.9	35.9	33.9
22	32.5	32.2	32.8	32.2	32.5
23	27.9	27.9	64.8	64.7	64.6
24	16.7	16.7	13.3	13.3	13.3
25	15.5	15.6	16.0	16.1	16.1
26	17.4	17.5	17.5	17.5	17.5
27	26.0	27.2	25.9	27.1	25.9
28	176.4	175.9	176.4	175.9	176.4
29	33.1	33.2	32.5	33.1	33.0
30	23.6	24.6	23.4	24.5	23.6
3-*O*-Sugar	Glc	Glc	Glc	Glc	Ara
1′	104.9	104.9	103.9	103.7	104.3
2′	79.2	79.3	79.3	79.1	77.3
3′	88.6	88.6	88.9	88.6	83.4
4′	69.9	69.9	69.8	70.9	68.6
5′	77.8	77.6	77.6	77.5	65.8
6′	63.3	63.3	62.3	62.3	
Glc					
1″	103.8	103.8	103.7	103.5	104.3
2″	76.4	76.4	76.3	76.2	75.9
3″	78.7	78.6	78.6	77.9	77.5
4″	71.5	71.0	71.5	71.4	71.9
5″	77.7	77.9	77.7	78.5	78.6
6″	62.1	62.1	62.3	62.3	62.1
Glc					
1‴	104.6	104.6	104.5	103.9	104.8
2‴	75.4	75.4	75.3	74.1	75.9
3‴	78.6	78.8	78.6	87.6	78.2
4‴	72.5	72.5	72.2	69.8	71.4
5‴	78.6	78.5	78.6	77.6	78.4
6‴	62.2	62.2	62.4	62.1	62.9
Glc					
1⁗				105.1	
2⁗				75.1	
3⁗				78.5	
4⁗				69.8	
5⁗				77.9	
6⁗				61.9	
C-28-Glc					
1	95.7	95.3	95.7	95.8	97.8
2	74.1	74.3	74.1	74.1	74.1
3	78.8	79.1	78.8	78.8	78.8
4	71.1	71.0	71.1	72.0	71.0
5	79.3	79.3	79.2	79.3	79.3
6	62.5	62.6	63.1	63.0	62.3

HMBC spectrum, signals of anomeric protons at δ 5.00 (H-1′), 5.71 (H-1″) and 5.29 (H-1‴) showed correlations with carbon signals of at δ 83.0 (C-3 of aglycone), 79.3 (C-2′) and 88.9 (C-3′), respectively. Furthermore, long-range couplings ($^3J_{HCOH}$) were also observed between a proton signal at δ 6.30 (H-1) and a carbonyl carbon signal at δ 176.4 (C-28). The glucose moiety were determined to be in the β-configuration from the coupling constants ($J = 7.8$–8.0 Hz). Moreover, the significant fragment ions observed at m/z 1119 $[M\text{-}H]^-$, 957 $[M\text{-glc-}H]^-$, 795 $[M\text{-}2glc\text{-}H]^-$, 633 $[M\text{-}3glc\text{-}H]^-$, 471 $[M\text{-}4glc\text{-}H]^-$ in the negative ion ESI-MS confirmed the linkages of sugars. Based on the above evidence, the structure of **3** was concluded to be 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl}-hederagenin 28-*O*-β-D-glucopyranosyl ester.

Compound **4** (aralia-saponin VIII) showed quasi-molecular ion peaks $[M + Na]^+$ at m/z 1321 in the positive ion ESI-MS, and at m/z 1297 $[M\text{-}H]^-$ in the negative ion ESI-MS. The molecular formula $C_{60}H_{98}O_{30}$ was assigned by HR-FABMS. Compound **4** displayed five anomeric proton signals at δ 4.98, 5.13, 5.29, 5.64, and 6.32 in the ^1H NMR spectrum, and five anomeric carbon signals at δ 95.8, 103.5, 103.7, 103.9, and 105.1 in the ^{13}C NMR spectrum. On acid hydrolysis, **4** yielded only D-glucose as a sugar component. The ^1H NMR and ^{13}C NMR spectra were similar to those of **3**. However, in the aglycone region, **4** showed a signal at δ 64.7 in ^{13}C NMR spectrum and signals at δ 5.05 (br *s*, H-16) and 1.74 (*s*, H_3-27) in ^1H NMR spectrum, characteristic signals for caulophyllogenin (Yoshikawa et al., 1995). In the sugar region, **4** showed a set of additional signals corresponding to an inner β-D-glucopyranosyl unit in the ^{13}C NMR spectrum. In the HMBC spectrum, an anomeric proton signal at δ 4.98 (H-1′) showed a correlation with a signal of a carbon due to C-3 of the aglycone moiety at δ 82.9, and an anomeric proton signal at δ 5.13 (H-1⁗) showed a correlation with a carbon signal due to C-3‴ of the middle glucosyl at δ 87.6. In addition, anomeric proton signals at δ 5.64 (H-1″) and 5.29 (H-1‴) showed correlations with carbon signals due to C-2′ and C-3′ of the inner glucosyl at δ 79.1 and 88.6, respectively. Furthermore, long-range couplings ($^3J_{HCOH}$) were also observed between the proton signal at δ 6.32 (H-1) and the carbonyl carbon signal at δ 175.9. These data indicated the presence of a [glucopyranosyl (1→2)]-[glucopyranosyl (1→3) glucopyranosyl (1→3)]-glucopyranosyl moiety at C-3 and a glucopyranosyl moiety at C-28 in the structure of **4**. The configuration of each glucose was determined to be in the β-form from examination of the respective coupling constant values ($J = 7.5$–8.0 Hz). Based on the above data, the structure of **4** was concluded to be 3-*O*-{β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranosyl}-caulophyllogenin 28-*O*-β-D-glucopyranosyl ester.

Compound **5** (aralia-saponin IX) was assigned the molecular formula $C_{53}H_{86}O_{23}$ by HR-FABMS. Compound **5** displayed four anomeric proton signals at δ 4.99, 5.22, 5.49, and 6.29 in the ^{1}H NMR spectrum, and four anomeric carbon signals at δ 97.8, 104.3, 104.3, and 104.8 in the ^{13}C NMR spectrum. By comparison of the spectral data between **5** and **3**, the signals for the aglycone part were virtually identical to each other, indicating that **5** was a hederagenin derivative possessing four sugar moieties at C-3 and C-28. On acid hydrolysis, **5** yielded hederagenin, and D-glucose and L-arabinose as sugar components. By detailed analysis of the 2D NMR (^{1}H–^{1}H COSY, HMQC, and HOHAHA), the spin systems for the sugars were assigned as shown in Tables 1 and 2. In the HMBC spectrum, the signals of anomeric protons at δ 4.99 (H-1′), 5.49 (H-1″) and 5.22 (H-1‴) showed a correlation with carbon signals at δ 82.4 (C-3 of aglycone), 77.3 (C-2′) and 83.4 (C-3′), respectively. Furthermore, long-rang couplings ($^3J_{HCOH}$) were also observed between a proton signal at δ 6.29 (H-1) and a carbonyl carbon signal at δ 176.4 (C-28). The configurations of glucose and arabinose were determined to be β and α, respectively, according to their coupling constants (J = 8.0 and 7.5 Hz, respectively). Moreover, the significant fragment ions observed at m/z 1089 $[M\text{-}H]^-$, 927 $[M\text{-glc-}H]^-$, 765 $[M\text{-}2glc\text{-}H]^-$, 603 $[M\text{-}3glc\text{-}H]^-$, 471 $[M\text{-}3glc\text{-}ara\text{-}H]^-$ in the negative ion ESI-MS confirmed the linkages of sugars. The structure of **5** was consequently concluded to be 3-*O*-{β-D-glucopyranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-α-L-arabinopyranosyl}-hederagenin 28-*O*-β-D-glucopyranosyl ester.

3. Experimental

3.1. General

Optical rotations were measured with a Jasco DIP-360 automatic polarimeter. IR spectra were measured with a Jasco FT/IR-230 infrared spectrometer. ^{1}H and ^{13}C NMR spectra were measured with a JNM-LA400WB Lambda (Jeol) NMR (^{1}H, 400 MHz; ^{13}C, 100 MHz) or a Varian UNITY 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer. ESI-MS were measured with a Perkin–Elmer SCIEX API III biomolecular mass analyzer. HR-FABMS were measured with a Jeol JMS-700 mass spectrometer with a resolution of 5000 and glycerol containing a faint amount of NaCl. GC were performed on a Shimadzu GC-17A instrument.

Column chromatography was carried out on silica gel 60 (Merck, 70–230 mesh). Medium pressure liquid chromatography (MPLC) was carried out on a LiChroprep RP-18 (Merck, size A). Preparative HPLC was carried out on a Gilson instrument with a 231XL injector, a 119 UV/VIS detector and a TSK-gel ODS-80TM column (21.5×300 mm, Tosoh). Spots were detected after spraying with ethanol–5% H_2SO_4.

3.2. Plant material

The root bark of *A. elata* was collected at Xinbin (Liaoning, China), and its botanical source was confirmed by Professor Qishi Shun, Shenyang Pharmaceutical University (SPU), Shenyang, China. The specimen is deposited in the Department of Natural Products of SPU.

3.3. Extraction and isolation

Dried bark (3.5 kg) of *A. elata* was extracted three times with methanol (3×7 l) for 2 h under conditions of reflux, and the combined extracts were concentrated in vacuo. The resulting extract (175 g), was next suspended in water, and successively extracted with petroleum ether, chloroform and butanol saturated with water to give the respective extracts after solvent removal. The butanol-soluble portion (100 g) was subjected to column chromatography on silica gel 60 (10×100 cm) with $CHCl_3$–MeOH–H_2O (7:3:0.5) to give six fractions (fr. I–VI). Fraction VI (28.9 g) was further applied to a silica gel 60 column with elution $CHCl_3$–MeOH–H_2O (6:4:1) to give three fractions (VIa–VIc). Subraction VIa (5.8 g) was separated with RP-18 MPLC [MeOH– H_2O (6:4)] to give **7** (12 mg) and **9** (10 mg). Fraction VIb (14.2 g) was applied to preparative HPLC [MeOH–0.1% TFA/H_2O (7:3→10:0), 5 ml/min, monitored at 205 nm] to afford **1** (6 mg), **2** (5 mg), **3** (28 mg), **6** (7 mg), and **8** (24 mg). Fraction VIc (2.6 g) was applied to preparative HPLC [MeOH–0.1% TFA H_2O (7.5:2.5→10:0), 5 ml/min, monitored at 205 nm] to afford **4** (7 mg) and **5** (12 mg).

3.4. Aralia-saponin V (*1*)

Colorless amorphous powder, $[\alpha]_D$ −11.3° (c = 0.2, pyridine). IR (KBr) cm^{-1}: 3420, 2920, 2850, 1740, 1660, 1550, 1460, 1380, 1080, 1030. ^{1}H NMR: Table 1. ^{13}C NMR: Table 2. Positive ion ESI-MS m/z: 1127 $[M+Na]^+$, 965 $[M+Na\text{-glc}]^+$, 803 $[M+Na-2glc]^+$, 641 $[M+Na-3glc]^+$. Negative ion ESI-MS m/z: 1103 $[M\text{-}H]^-$, 941 $[M\text{-glc-}H]^-$, 779 $[M\text{-}2glc\text{-}H]^-$, 617 $[M\text{-}3glc\text{-}H]^-$, 455 $[M\text{-}4glc\text{-}H]^-$. HR-FABMS m/z: 1127.5634 $[M+Na]^+$ (calcd. for $C_{54}H_{88}O_{23}Na$: 1127.5615).

3.5. Aralia-saponin VI (*2*)

Colorless amorphous powder, $[\alpha]_D$ −18.8° (c = 0.2, pyridine). IR (KBr) cm^{-1}: 3400, 2860, 1738, 1628, 1450, 1394, 1310, 1210, 1040, 890. ^{1}H NMR: Table 1 . ^{13}C NMR: Table 2. Positive ion ESI-MS m/z: 1143 $[M+Na]^+$, 981 $[M+Na\text{-glc}]^+$, 819 $[M+Na\text{-}2glc]^+$, 657 $[M+Na\text{-}3glc]^+$. Negative ion ESI-MS m/z: 1119 $[M\text{-}H]^-$, 957 $[M\text{-glc-}H]^-$, 795 $[M\text{-}2glc\text{-}H]^-$, 633 $[M\text{-}3glc\text{-}H]^-$. HR-FABMS m/z: 1143.5571 $[M+Na]^+$ (calcd. for $C_{54}H_{88}O_{24}Na$: 1143.5564).

3.6. Aralia-saponin VII (3)

Colorless amorphous powder, $[\alpha]_D + 23.1°$ ($c = 0.3$, pyridine). IR (KBr) cm^{-1}: 3400, 2920, 2850, 1740, 1660, 1550, 1460, 1380, 1080, 1030. ^1H NMR: Table 1. ^{13}C NMR: Table 2. Positive ion ESI-MS m/z: 1143 $[M + Na]^+$, 981 $[M + Na\text{-glc}]^+$. Negative ion ESI-MS m/z: 1119 $[M\text{-}H]^-$, 957 $[M\text{-glc-}H]^-$, 795 $[M\text{-}2glc\text{-}H]^-$, 633 $[M\text{-}3glc\text{-}H]^-$, 471 $[M\text{-}4glc\text{-}H]^-$. HR-FABMS m/z: 1143.5583 $[M + Na]^+$ (calcd. for $C_{54}H_{88}O_{24}Na$: 1143.5564).

3.7. Aralia-saponin VIII (4)

Colorless amorphous powder, $[\alpha]_D + 23.1°$ ($c = 0.3$, pyridine). IR (KBr) cm^{-1}: 3400, 2920, 1740, 1640, 1550, 1460, 1394, 1300, 1260, 1080, 1035, 995. ^1H NMR: Table 1. ^{13}C NMR: Table 2. Positive ion ESI-MS m/z: 1321 $[M + Na]^+$, 1159 $[M + Na\text{-glc}]^+$, 997 $[M + Na - 2glc]^+$. Negative ion ESI-MS m/z: 1297 $[M\text{-}H]^-$, 1135 $[M\text{-glc-}H]^-$, 973 $[M\text{-}2glc\text{-}H]^-$, 811 $[M\text{-}3glc\text{-}H]^-$. HR-FABMS m/z: 1321.6042 $[M + Na]^+$ (calcd. for $C_{60}H_{98}O_{30}Na$: 1321.6041).

3.8. Aralia-saponin IX (5)

Colorless amorphous powder, $[\alpha]_D + 13.3°$ ($c = 0.61$, pyridine). IR (KBr) cm^{-1}: 3400, 2920, 2850, 1740, 1660, 1550, 1460, 1380, 1080, 1030. ^1H NMR: Table 1. ^{13}C NMR: Table 2. Positive ion ESI-MS m/z: 1113 $[M + Na]^+$, 951 $[M + Na\text{-glc}]^+$. Negative ion ESI-MS m/z: 1089 $[M\text{-}H]^-$, 927 $[M\text{-glc-}H]^-$, 765 $[M\text{-}2glc\text{-}H]^-$, 603 $[M\text{-}3glc\text{-}H]^-$, 471 $[M\text{-}3glc\text{-}ara\text{-}H]^-$. HR-FABMS m/z: 1113.5437 $[M + Na]^+$ (calcd. for $C_{53}H_{86}O_{23}Na$: 1113.5459).

3.9. Acid hydrolysis of saponins

Compound **1** (2 mg) was heated with 7% H_2SO_4 at 80°C for 12 h. The reaction mixture was neutralized with 1 N NaOH and filtered. The filtrate was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was concentrated to dryness to afford oleanolic acid as an aglycone, which was identified by direct comparisons of the R_f value on TLC and ^1H and ^{13}C NMR spectral data with those of an authentic sample. The water layer was concentrated and glucose ($R_f = 0.38$) was identified by cellulose TLC with a solvent system of pyridine–ethyl acetate–acetic acid–water (36:36:7:21). The absolute configuration of the sugar was determined by the method of Hara et al. (1987); after being desalted with Amberlite MB-3 and dried, the residue was dissolved in pyridine (0.1 ml), then 0.1 M L-cysteine methyl ester hydrochloride in pyridine (0.2 ml) was added to the sugar solution. The mixture was kept at 60°C for 1.5 h, dried in vacuo, and trimethylsilylated with hexamethyl disilazane–trimethyl chlorosilane (HMDS–TMCS) (0.1 ml) at 60°C for 1 h. After partitioning between hexane (0.3 ml) and water (0.3 ml), the hexane layer was subjected to GC [column, DB-1, J.&W. Scientific, 0.25 mm i.d.×30 m; column temperature, 50–230°C, 15°C/min then 230°C, 18 min; carrier gas, He]. The sugar derivative thus obtained had a retention time of 21.4 min, identical with that of an authentic D-glucose derivative. Under the same conditions, a derivative of L-glucose showed a retention time of 22.0 min.

On acid hydrolysis, the aglycones of **3** and **5** were both identified as hederagenin by direct comparisons of ^1H and ^{13}C NMR spectral data with those of an authentic sample. The aglycones of **2** and **4** were identified as echinocystic acid (Yoshikawa et al., 1995; Nagao et al., 1993) and caulophyllogenin (Yoshikawa et al., 1995), respectively, by comparing their spectral data with those reported. Of the sugar moieties, **2**, **3**, and **4** yielded D-glucose, but **5** gave L-arabinose and D-glucose, which showed R_f values of 0.61 and 0.38, respectively [cellulose TLC with solvent system of pyridine–ethyl acetate–acetic acid–water (36:36:7:21), and GC retention times of 16.4 and 21.4 min, respectively, after derivatization]. Under the same conditions, a D-arabinose derivative had a retention time of 17.0 min.

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Lepidimoic acid increases fructose 2,6-bisphosphate in *Amaranthus* seedlings

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Abstract

This study examines the influence of the growth promoter, lepidimoic acid, on the level of an important cytosolic signal metabolite, fructose 2,6-bisphosphate (Fru-2,6-P_2), which can activate pyrophosphatedepedent:phosphofructokinase (PFP, EC 2.7.1.90), and on glycolytic metabolism in *Amaranthus caudatus* seedlings. Fru-2,6-P_2 concentrations were respectively increased by approximately 2-, 3- and 4-fold when the seedlings were treated with 0.3, 3 and 30 mM lepidimoic acid. Exogenous lepidimoic acid also affected levels of glycolytic intermediates in the seedlings. The increase in fructose 1,6-bisphosphate and decreases in fructose 6-phosphate and glucose 6-phosphate were found in response to the elevated concentration of lepidimoic acid. These results suggest that lepidimoic acid may affect glycolytic metabolism in the *Amaranthus* seedlings by increasing the activity of PFP due to increasing level of Fru-2,6-P_2. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Amaranthus caudatus*; Amaranthaceae; Growth-promoting substance; Lepidimoic acid; Fructose 2,6-bisphosphate; Glycolysis; Phosphofructokinase

1. Introduction

Fructose 2,6-bisphosphate (Fru-2,6-P_2) is a low-molecular weight cytosolic regulator that is found many eukaryotes (Stitt, 1990; Plaxton, 1996). In plants, Fru-2,6-P_2 activates PPi-dependent:phosphofructokinase (PFP) and inhibits cytosolic fructose 1,6-bisphosphatase (FBPase, EC 3.3.3.11) (Black et al., 1987; Hatzfeld et al., 1989). The level of Fru-2,6-P_2 in plants fluctuates depending on physiological and environmental conditions such as aging, wounding, nutrition and photosynthetic light (Van Schaftingen and Hers, 1983; Paz et al., 1985; Murley et al., 1998; Whittaker and Botha, 1999). Exogenous application of ethylene also increased the level of Fru-2,6-P_2 (Stitt et al., 1986).

Since the reaction catalyzed by PFP is readily reversible, PFP can catalyze both the glycolytic formation of fructose 1,6-bisphosphate (Fru-1,6-P_2) and Pi, and the gluconeogenetic formation of fructose 6-phosphate (Fru-6-P) and PPi. Thus, interaction of PFP and Fru-2,6-P_2 was considered an important mechanism for switching plant sugar metabolism between glycolysis and gluconeogenesis (Smyth and Black, 1984; Wu et al., 1984; Mertens et al., 1987; Stitt, 1990; Plaxton, 1996).

A new plant growth-promoting substance, lepidimoide was recently isolated from the mucilage of germinating cress (*Lepidium sativum* L.) as the sodium salt of 2-*O*-rhamnopyranosyl-4-deoxy-α-L-*threo*-hex-4-enopyranosiduronate (Hasegawa et al., 1992) and its free carboxylic acid, lepidmoic acid, was isolated from the exudate of germinating *Arabidopsis thaliana* (Fig. 1; Yokota-Tomita et al., 1998). These substances were found in exudates from all plant species studied (Yamada et al., 1995) and promoted the growth of shoots in several germinating seeds (Goto et al., 1995; Yamada et al., 1997). At a dose of 3 mM, the substances caused 150–200% increases in elongation of *Amaranthus caudatus* shoots (Yamada et al., 1996). These seedlings may accelerate respiratory metabolism to produce metabolic energy and biosynthetic precursors for the enhanced growth.

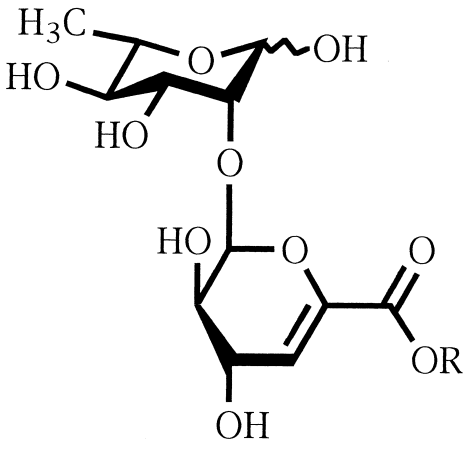
The object of this research was to investigate the effect of lepidimoic acid on glycolytic metabolism. Thus, levels of Fru-2,6-P_2 and glycolytic intermediates, and activity of PFP were determined in *Amaranthus* seedlings treated with lepidimoic acid.

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2. Results and discussion

Fig. 2 shows the effect of lepidimoic acid on the level of Fru-2,6-P_2 in *Amaranthus* seedlings. Lepidimoic acid increased Fru-2,6-P_2 level in the seedlings at concentrations greater than 0.3 mM and the level was increased with increasing doses up to 30 mM. At 0.3, 3 and 30 mM doses of lepidimoic acid, the Fru-2,6-P_2 levels in the seedlings were 1.9-, 3.2- and 4.1-fold, respectively, greater than that in non-treated seedlings.



R; Na Lepidimoide
R; H Lepidimoic acid

Fig. 1. Chemical structures of lepidimoide and lepidimoic acid.

Fig. 3 shows the effect of Fru-2,6-P_2 on PFP activity in crude extract obtain from *Amaranthus* seedlings. PFP activity was measured in the glycolytic direction from Fru-6-P to Fru-1,6-P_2 with various amounts of exogenously supplied Fru-2,6-P_2. Fru-2,6-P_2 activated markedly PFP and the dose-response curve was linear at least up to 50 pmol ml^{-1} Fru-2,6-P_2, supporting the data previously described (Van Schaftingen et al., 1982; Kesy and Kowalczyk, 1987). PFP was activated 2-, 4- and 6-fold by 10, 30 and 50 pmol ml^{-1} Fru-2,6-P_2, respectively.

The interconversion of Fru-6-P and Fru-1,6-P_2 in glycolysis and gluconeogenesis has been recognized as an important regulatory site for the flow of carbon between carbohydrate storage and carbohydrate use (Black et al., 1987; Plaxton, 1996). The reactions are catalyzed essentially by two irreversible enzymes ATP-dependent:phosphofructokinase; (PFK, EC 2.7.1.11, conversion of Fru-6-P to Fur-1,6-P_2) and FBPase (conversion of Fur-1,6-P_2 to Fru-6-P). The third enzyme, PFP readily reversible in vivo, catalyzes both glycolytic and gluconeogenic direction depending on the amount

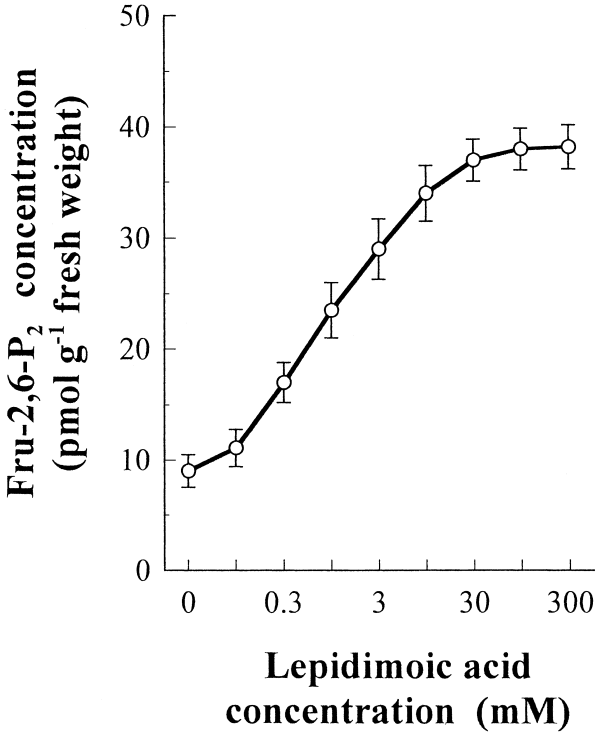


Fig. 2. Effect of lepidimoic acid on Fru-2,6-P_2 level in *Amaranthus* seedlings. Five-day-old seedlings were treated with lepidimoic acid for 24 h and extracted for Fru-2,6-P_2 determination. Means ±S.E. from three replicate experiments with at least three assays for each determination are shown.

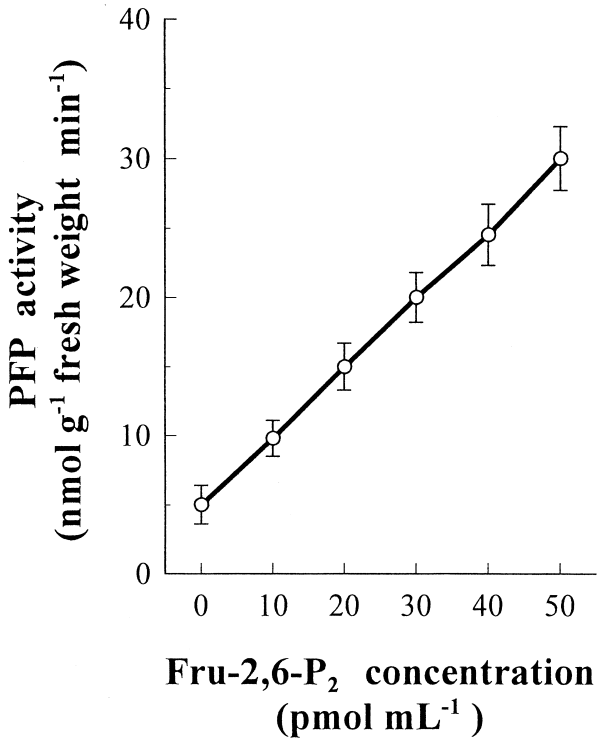


Fig. 3. Effect of Fru-2,6-P_2 on PFP activity in extract of *Amaranthus* seedlings. Five-day-old *Amaranthus* seedlings were extracted and PFP was assayed with exogenous Fru-2,6-P_2. Means ±S.E. from three replicate experiments with at least three assays for each determination are shown.

of substrates (Nielsen, 1995; Whittaker and Botha, 1999). Although an absolute function is still unclear (Nielsen, 1994, 1995), PFP is expected to play an integral function in plant carbohydrate metabolism because it is potently activated by Fru-2,6-P_2 (Stitt, 1990; Plaxton, 1996). Much research on PFP suggests that it is an adaptive enzyme exhibiting physical and kinetic properties that are responsive to a variety of environmental, developmental and tissue-specific signals (Black et al., 1987; Tobias et al., 1992; Plaxton, 1996). In fact, germinating seedlings of maize and castor bean showed marked changes in PFP activity substantially with tissue age, whereas PFK activity remained relatively constant throughout their developmental stage (Kruger and Beevers, 1985; Black et al., 1987; Tobias et al., 1992).

Table 1 shows the levels of the glycolytic intermediates in the *Amaranthus* seedlings after 24 h of incubation with lepidimoic acid. Lepidimoic acid affected the levels of the intermediates. Significant changes were an increase in Fru-1,6-P_2 level and decreases in glucose 6-phosphate (Gru-6-P) and Fru-6-P levels in response to the elevated concentration of lepidimoic acid. The level of Fru-1,6-P_2 in the seedlings at a dose of 30 mM lepidimoic acid was 160% of that of non-treated seedlings (0 mM), and the levels of Fru-6-P and Gru-6-P in the seedlings at the same dose of lepidimoic acid were 53 and 67% of those in the non-treated seedlings, respectively. The increase in Fru-1,6-P_2 and decreases in Fru-6-P and Gru-6-P in seedlings may be caused by activation of PFP toward the glycolytic direction (Black et al., 1987; Stitt and Vasella, 1988).

In summary, lepidimoic acid increased the level of Fru-2,6-P_2 in *Amaranthus* seedlings at concentrations greater than 0.3 mM (Fig. 2). The increase in Fru-2,6-P_2 may activate PFP (Fig. 3). The accumulation of Fru-1,6-P_2 and decreases of Fru-6-P and Gru-6-P were found in *Amaranthus* in response to lepidimoic acid (Table 1). These results suggest that lepidimoic acid may affect glycolytic metabolism in the seedlings by increasing the activity of PFP due to increasing the levels of Fru-2,6-P_2.

Table 1
Effect of lepidimoic acid on the concentration of glycolytic intermediates in *Amaranthus* seedlings[a]

	Intermediate concentration (nmol g^{-1} fresh weight)			
	Lepidimoic acid concentration (mM)			
Intermediate	0	0.3	3	30
Glucose 6-phosphate	197±17	178±15	151±14	132±14
Fructose 6-phosphate	51±5	45±4	39±4	27±3
Fructose 1,6-bisphosphate	27±3	31±3	37±3	44±4
Dihydroxyacetone phosphate	37±4	42±4	44±4	46±4
3-Phosphoglycerate	35±3	37±3	40±3	42±4
Phosphoenolpyruvate	31±2	34±3	36±4	38±3
Pyruvate	29±3	31±2	33±3	35±4

[a] Five-day-old *Amaranthus* seedlings were grown in the presence of lepidimoic acid for 24 h, and the intermediates were determined by enzymatic analysis of $HClO_4$ extracts. Means±S.E. from three replicate experiments with at least three assays for each determination are shown.

3. Experimental

3.1. Lepidimoic acid

Lepidimoic acid was isolated from exudate of germinating *Arabidopsis thaliana* as described previously (Yokota-Tomita et al., 1998).

3.2. Plant materials and treatment

Seeds of *Amaranthus caudatus* were sterilized in a 2% (w/v) solution of NaOCl for 15 min, rinsed in distilled H_2O for 20 min (4×), and germinated on two sheets of moist filter paper (No 1, Toyo, Tokyo) in the dark at 25°C. After 5 days, uniform seedlings were transferred to 9-cm Petri dishes, each containing two sheets of filter paper moistened with several concentrations of 10 ml aq. solution of lepidimoic acid, and grown for one further day in the dark at 25°C.

For quantification of glycolytic intermediates and Fru-2,6-P_2 levels, and determination of PFP activity in the *Amaranthus* seedlings, three Petri dishes for each treatment were removed, and the seedlings were harvested, frozen immediately in liquid N_2 and stored at −80°C until extraction.

3.3. Measurement of Fru-2,6-P_2

The frozen seedlings (1 g fr. wt. eq.) were ground to a fine powder in a mortar containing liquid N_2 using a pestle and homogenized with 5 ml of ice-cold 50 mM NaOH. The homogenate was incubated for 30 min on ice with occasional shaking and centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatant was heated for 5 min at 80°C, cooled on ice, and centrifuged again at 10,000 ***g*** for 5 min (Mertens et al., 1990). The resulting supernatant was used to quantify Fru-2,6-P_2.

Quantification was carried out spectrophotometrically by monitoring the oxidation of NADH at 340 nm for 15 min at 30°C in a 1-ml reaction mixture according to Van Schaftingen and Hers (1983): 50 mM Tris–acetate (pH 8.0), 2 mM magnesium acetate, 5 mM Fru-6-P, 0.15 mM NADH, 1.2 units of aldolase (EC 4.1.2.13), 1.8 units of glycerol-3-phosphate dehydrogenase (GDH, EC 1.1.1.8), 7.5 units of triosephosphate isomerase (TIM, EC 5.3.1.1.), 0.5 units of potato PFP (Sigma) and up to 0.1 ml of the extract. The assay was initiated by adding 0.5 mM PPi after 5 min preincubation. The amount of Fru-2,6-P_2 in each sample was calculated by

comparing the extent of activation with that induced by known amounts of standard Fru-2,6-P_2. The recovery of Fru-2,6-P_2 added to the extraction medium containing the tissue powder before homogenation was 87±6% as calculated from 5 replications of the test run.

3.4. Extraction and assay of PFP

Tissue powder (1 g fr. wt. eq.), which was obtained from *Amaranthus* seedlings grown without lepidimoic acid, was prepared as described above and homogenized with 3 ml ice-cold solution containing 100 mM Tris–HCl (pH 7.8), 2 mM EDTA, 1 mM magnesium acetate, 5 mM DTT and 5% (w/v) insoluble PVP at 4°C for 30 min with occasional shaking. The homogenate was centrifuged at 30,000 ***g*** for 30 min and the supernatant was used immediately for enzyme assay.

PFP was assayed spectrophotometrically by monitoring the oxidation of NADH in the absence or presence of Fru-2,6-P_2 in the 1-ml reaction mixture according to Smyth et al. (1984): 100 mM HEPES-NaOH (pH 7.8), 4 mM magnesium acetate, 2 mM Fru-6-P, 0.15 mM NADH, 1.2 units of aldolase, 14 units of TIM, 1.8 units of GDH, 0.05 ml of the extract, and Fru-2,6-P_2 (0, 10, 20, 30, 40 and 50 pmol ml^{-1}; Sigma). The assay was started by the addition of 1 mM PPi to the reaction mixture.

3.5. Quantification of glycolytic intermediates

Tissue powder (1 g fr. wt. eq.) was extracted with 3 ml of ice-cold 0.45 M $HClO_4$, and the homogenate was incubated for 30 min on ice with occasional shaking and centrifuged at 30,000 *g* for 30 min at 4°C. The supernatant was neutralized to pH 7.0–7.5 with 2.5 M K_2CO_3 and incubated on ice for 10 min. The precipitated $KClO_4$ was removed by centrifugation (3000 ***g***, 5 min) and the resulting supernatant was used for analysis of intermediates.

Quantification was carried out spectrophotometrically by monitoring the oxidation/reduction of NADH/NAD^+ at 340 nm for 15 min at 30°C in 1-ml of TEA buffer (pH 7.6) according to the procedure described by Bergmeyer (1983). The recovery of glycolytic intermediates through the quantification process was 92±6, 87±5, 89±8, 90±7, 88±6, 86±6 and 89±7% for Glu-6-P, Fru-6-P, F-1,6-P_2, dihydroxyacetone phosphate, 3 phosphoglycerate, phosphoenolpyruvate and pyruvate, respectively, as calculated from 5 replications of the test run with pure glycolytic intermediates in the extracts.

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Monooxygenases involved in GA_{12} and GA_{14} synthesis in *Gibberella fujikuroi*

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Abstract

A microsomal preparation from mycelia of the gibberellin (GA)-producing fungus *Gibberella fujikuroi* catalyzed the first two steps in the conversion of the biosynthetic intermediate GA_{12}-aldehyde to gibberellic acid (GA_3). [^{14}C]GA_{12}-Aldehyde was converted to radiolabelled GA_{14}, the major product, together with smaller amounts of non-hydroxylated GA_{12}. The microsomal activities required reduced pyridine nucleotides and molecular oxygen. However, GA_{12} and GA_{14} synthesis differed markedly in the preferred electron source. Formation of GA_{12} required NADH or NADPH, while GA_{14} synthesis from GA_{12}-aldehyde occurred only with NADPH. Marked differences were also found in the activating effect of FAD. When NADPH was the reductant, the rate of GA_{14} synthesis was enhanced 3.5 times by 5 μM FAD while this flavin nucleotide did not alter the synthesis of GA_{12}. In contrast, GA_{12} synthesis was activated 3.8 times by 50 μM FAD in the presence of NADH. Both activities were inhibited by carbon monoxide and cytochrome c. These properties suggest that the 3β-hydroxylation of GA_{12}-aldehyde and further oxidation of carbon 7 are catalyzed by cytochrome P-450 monooxygenases in *Gibberella fujikuroi*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Gibberella fujikuroi*; Gibberellin; Oxidases; Monooxygenases

1. Introduction

The main steps of gibberellic acid (GA_3) biosynthesis in *Gibberella fujikuroi* have been elucidated from experiments with labelled precursors administered into intact mycelia (Cross et al., 1968; Bearder et al., 1975; Hedden et al., 1978; MacMillan, 1997). Results from these studies demonstrated that *ent*-kaurene and GA_{12}-aldehyde are key intermediates (Bearder et al., 1975; Evans and Hanson, 1975) which, by further sequential oxidations, give the 3-hydroxylated lactone GA_4 that is finally transformed into GA_3 (Pitel et al., 1971) (Fig. 1). Since metabolic studies have not identified several steps of the sequence after GA_{12}-aldehyde (Bearder et al., 1975; MacMillan, 1997), there is an interest in developing cell-free systems for studying these transformations in vitro. Furthermore, the nature of the fungal oxidases involved in GA_3 synthesis could differ considerably from those employed in plants. This adds an additional interest in determining evolutionary differences between both systems. In fact, relatively few reactions of the pathway have been studied and characterized in fungal cell-free extracts (MacMillan, 1997) and thus the properties of many of the enzymes involved in GA_3 synthesis in the fungus are unknown. Enzymes studied in vitro include *ent*-kaurene synthase (Fall and West, 1971), *ent*-kaurene oxidase (Ashman et al., 1990) and *ent*-kaurenoic acid 7β-hydroxylase (Jennings et al., 1993). The latter two are microsomal monooxygenases related to cytochrome P-450. No enzymatic activities after GA_{12}-aldehyde have been characterized in *G. fujikuroi* cell-free systems, although [^{14}C]GA_{14} was detected as a minor product from *ent*-[^{14}C]kaurenoic acid in incubations with mycelial extracts (Jennings et al., 1993).

Recently, a cluster containing the GA-biosynthetic genes of *G. fujikuroi* was described (Tudzynski and Hölter, 1998). This cluster contains several cytochrome P-450 monooxygenase genes of as yet unknown function (Tudzynski and Hölter, 1998); some would be expected to encode *ent*-kaurene oxidase and *ent*-kaurenoic acid hydroxylase, which have properties expected for cytochrome P-450 s (Ashman et al., 1990; Jennings et al., 1993). Here we report the enzymatic activities, after

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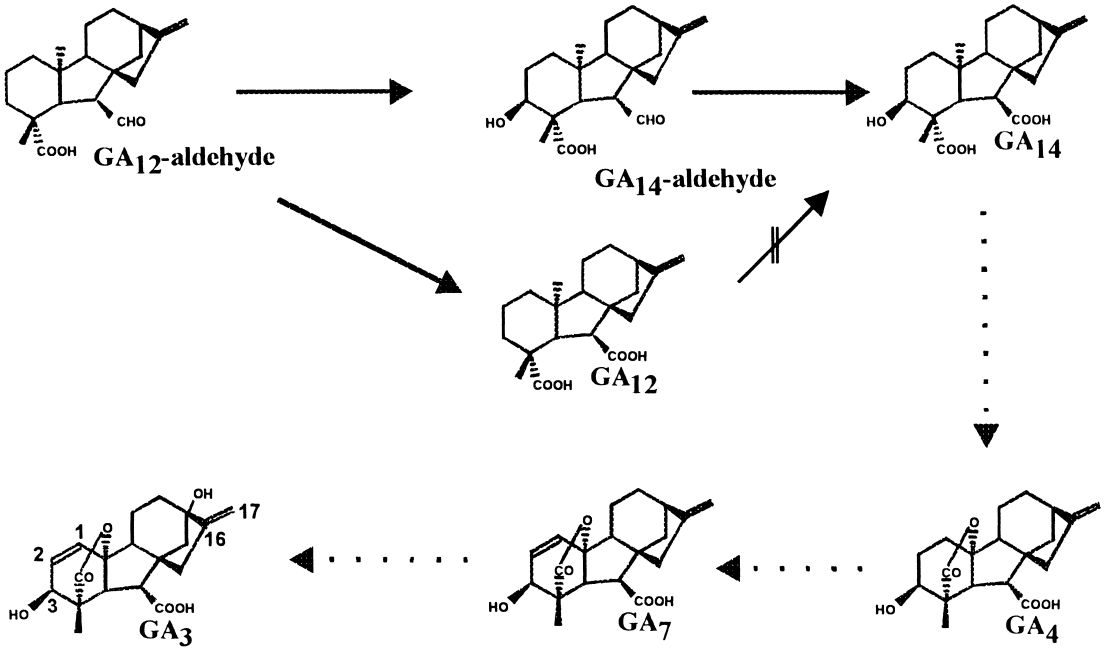


Fig. 1. Metabolic steps in the conversion of GA_{12}-aldehyde to GA_{12} and GA_{14} (full arrows), and in the hypothetical further conversion of GA_{14} to GA_3 (dotted arrows).

GA_{12}-aldehyde, that are present in a partially purified preparation obtained from *G. fujikuroi* mycelia. We found that only the enzymes that catalyze the first two steps of the sequence were active in our extracts. These were characterized and compared with other oxidases of GA biosynthesis already described for *G. fujikuroi*.

2. Results and discussion

2.1. Products obtained from [^{14}C]GA_{12}-aldehyde

A cell-free preparation was obtained from the mycelia of *G. fujikuroi* by ammonium sulfate precipitation of the 10 000 ***g*** supernatant. In the presence of NADPH, this fraction efficiently transformed [^{14}C]GA_{12}-aldehyde into two main products, as determined by HPLC (Fig. 2A). The major product with a retention time of 17–18 min was identified by GC/MS as [^{14}C]GA_{14} while that eluting after 29 min was identified as [^{14}C]GA_{12} (Table 1). The predominance of [^{14}C]GA_{14} was expected since it is an intermediate on the main 3β-hydroxylation pathway in *G. fujikuroi* (Hedden et al., 1974; MacMillan, 1997). Some unmetabolized [^{14}C]GA_{12}-aldehyde remained (R_t 31 min) and polar material eluting at 14–15 min contained [^{14}C]GA_{14} 17-nor-16-one, probably an artifact formed by non-specific oxidation of the 16,17-double-bond.

The formation of GA_{14} and GA_{12} indicates the presence in the extract of 3β-hydroxylase and 7-oxidase activities: both activities are involved in GA_{14} synthesis while only 7-oxidase participates in the synthesis of GA_{12} (Fig. 1). Neither GA_{12} nor GA_{14} was metabolized

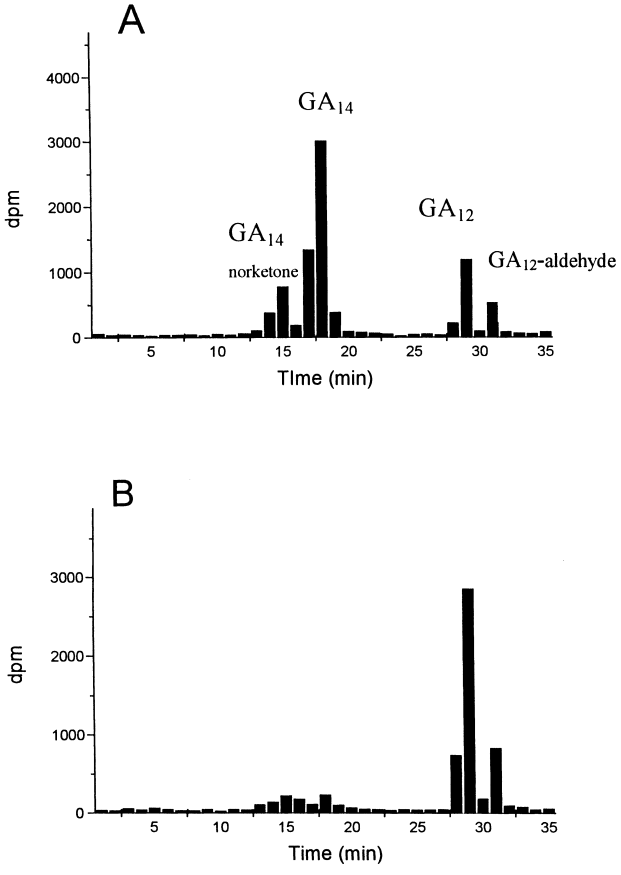


Fig. 2. HPLC profiles of products from incubation of [^{14}C]GA_{12}-aldehyde with fungal enzymes and (A) 1 mM NADPH, 5 μM FAD or (B) 1 mM NADH, 50 M FAD. Chromatographic conditions as described in Section 3. Retention times for GA_{12}-aldehyde, GA_{12} and GA_{14} are 31, 29 and 18 min, respectively.

Table 1
GC/MS analysis of the products obtained from [^{14}C]GA_{12}-aldehyde and the fungal enzymes

Products and authentic samples	KRI	*m/z* (% relative intensity)
[^{14}C]GA_{14} norketone (fractions 14–15)	2647	M^+ 458(2), 443(5), 400(22), 338(19), 311(25), 295(28), 265(20), 237(25), 162(9), 131(200), 129(30)
GA_{14} norketone[a]	2661	M^+ 450 (trace), 435(12), 394(22), 334(10), 307(40), 289(40), 261(44), 233(41), 168(12), 129(100)
[^{14}C]GA_{14} (fractions 17–18)	2481	M^+ 456(10), 441(12), 424(81), 396(43), 334(38), 306(98), 293(98), 291(60), 263(63), 235(100), 203(39), 173(43), 131(94)
GA_{14}[a]	2492	M^+ 450(2), 433(15), 416(58), 388(39), 326(26), 298(95), 287(95), 259(63), 231(96), 199(32), 171(39), 129(100)
[^{14}C]GA_{12} (fractions 28–29)	2316	M^+ 338(12), 320(5), 306(10), 293(19), 278(49), 261(13), 247(100), 245(54), 233(18)
GA_{12}[a]	2332	M^+ 330(12), 312(8), 300(11), 287(18), 270(61), 255(19), 241(100), 239(29), 227(25)

[a] Data taken from library spectra (Gaskin and MacMillan, 1992).

when incubated with the enzyme preparation in the presence of different cofactors (not shown), in particular, [^{14}C]GA_{12} was not transformed into [^{14}C]GA_{14}, thus confirming that 3β-hydroxylation occurs before oxidation of carbon 7. Furthermore, the 3β-hydroxylase is specific for the aldehyde substrate, in agreement with previous results from incubations with intact mycelia (Hedden et al., 1974, MacMillan, 1997). Since no [^{14}C]GA_{14}-aldehyde was detected in the incubations, 3β-hydroxylation would appear to limit the formation of GA_{14} from GA_{12}-aldehyde, although it is possible that GA_{14}-aldehyde is not released from the enzyme active site before oxidation at C-7 occurs.

When [^{14}C]GA_{12}-aldehyde was incubated in the presence of NADH and the fungal enzymes, only [^{14}C]GA_{12} was produced (Fig. 2B), indicating different specificities of the 3β-hydroxylase and 7-oxidase activities for electron source. The 3β-hydroxylase is specific for NADPH and cannot accept electrons from NADH, in contrast to the 7-oxidase that is active in the presence of both pyridine nucleotides. This result provides a way of studying the 7-oxidase activity independently of 3β-hydroxylase. *ent*-Kaurenoic acid 7β-hydroxylase, which oxidizes C-7, but in the kaurenoid rather than gibberellane skeleton, can also utilize NADPH or NADH (Jennings et al., 1993) and thus may be related to GA_{12}-aldehyde 7-oxidase, which catalyzes a further oxidation step at a similar position.

The enzymatic activities for GA_{12} or GA_{14} synthesis were very stable retaining full activity for several months when stored at −20 or −70°C.

2.2. Localization of oxidases involved in GA_{12} and GA_{14} synthesis

The activities for conversion of [^{14}C]GA_{12}-aldehyde to [^{14}C]GA_{12} and [^{14}C]GA_{14} were associated with the 100 000 *g* pellet (P100) obtained from the mycelial extracts (Table 2) and thus they are membrane-bound. No 7-oxidase or 3β-hydroxylase activities were found in incubations with the soluble fraction (100 000 *g* supernatant). The stability of these enzymes in the microsomal fraction was lower than in the partially purified fraction obtained by ammonium sulfate precipitation of the 10 000 *g* supernatant. The resuspended microsomal pellet (P100) was totally inactivated in 4 h at 4°C and lost most of its activity when stored for 2 days at −70°C. For this reason most of the results reported here were obtained using the ammonium sulfate precipitate. Two of the enzymes involved in early GA metabolism in *G. fujikuroi, ent*-kaurene oxidase and *ent*-kaurenoic acid 7-hydroxylase, are microsomal monooxygenases (Ashman et al., 1990; Jennings et al., 1993), although both activities are partially solubilized in the presence of high ionic strength buffers (Jennings et al., 1993). Thus, a main part of GA synthesis occurs bound to membranes in *G. fujikuroi*.

2.3. Cofactor requirement

The enzymes involved in GA_{12} and GA_{14} synthesis were active only in the presence of reduced pyridine nucleotides, as expected for monooxygenases (Table 3). Strong activation by 5 μM FAD was obtained for [^{14}C]GA_{14} synthesis, while the conversion of [^{14}C]GA_{12}-aldehyde to [^{14}C]GA_{12} was stimulated by this flavin nucleotide only when NADH was the reductant. [^{14}C]GA_{12} was synthesized at a higher rate with NADPH than with NADH but similar rates were obtained in the presence of 50 μM FAD. These results indicate that the 3-hydroxylase and 7-oxidase differ in the source of electrons for oxidation as well as in the activating effect of flavin nucleotides.

Both oxidases require molecular oxygen as demonstrated by the strong inhibition of GA_{12} and GA_{14} synthesis in N_2 or He (Table 4). In the presence of NADPH the rate of GA_{12} synthesis was reduced to 48 and 19% in N_2 and He, respectively, while under the same conditions GA_{14} synthesis decreased to 26 and 14% of the respective control value. A similar result was found for GA_{12} synthesis in the presence of NADH, which was reduced to 21% in the presence of He.

Table 2
Distribution of oxidases involved in [^{14}C]GA_{12} and [^{14}C]GA_{14} synthesis

Fraction[a]	Volume (ml)	[^{14}C]GA_{12} synthesis (nkat×10^8)	[^{14}C]GA_{14} synthesis (nkat×10^8)
S10	11.2	196[b]	92[b]
P100	1.0	253[c]	166[c]
S100	11.0	3[b]	10[b]

[a] S10: 10 000 *g* supernatant; P100: 100 000 *g* pellet; S100: 100 000 *g* supernatant.
[b] Assayed with 80 μl of enzyme.
[c] Assayed with 10 μl of enzyme.

Table 3
Cofactor requirements for [^{14}C]GA_{12} and [^{14}C]GA_{14} synthesis from [^{14}C]GA_{12} aldehyde

Cofactor	[^{14}C]GA_{12} synthesis (nkat×10^8)[a]	[^{14}C]GA_{14} synthesis (nkat×10^8)[a]
None	4±0.5	3±0.4
1 mM NADPH	122±8	90±5
1 mM NADPH, 5 mM FAD	130±7	319±14
1 mM NADPH, 1 mM NADH	121±7	89±4
1 mM NADPH, 1 mM NADH, 5 μM FAD	131±5	324±13
1 mM NADH	56±2	3±0.7
1 mM NADH, 50 μM FAD	148±5	4±1
0.5 mM Fe^{2+}, 4 mM 2-oxoglutarate, 4 mM ascorbate	4±0.7	3±0.6
1 mM $NADP^+$	4±0.7	4±0.5
1 mM NAD^+	3±0.5	3±0.7

[a] Assays with 20 μl of enzyme preparation (10 000 *g* supernatant precipitated with 70% ammonium sulfate and desalted through Sephadex G-25). Values reported are the means of three independent determinations.

Table 4
Oxygen requirement for 7-oxidase and 3β-hydroxylase[a]

Cofactor	Atmosphere[b]	[^{14}C]GA_{12} synthesis (nkat×10^8)	% Activity	[^{14}C]GA_{14} synthesis (nkat×10^8)	% Activity
NADPH	air	65±2	100	272±5	100
	N_2	31±7	48	72±7	26
NADPH	air	36±1	100	162±5	100
	He	7±1	19	23±5	14
NADH	air	189±11	100	4±0.4	0
	He	40±7	21	2±0.3	0

[a] Assays in the presence of NADPH (1 mM) were carried out with 10 μl of enzyme solution and 5 μM FAD. Assays in the presence of NADH (1 mM) were carried out with 80 μl of enzyme solution and 50 μM FAD.
[b] A sealed tube containing the enzyme was flushed with He or N_2 for 5 min. After that, substrates were added and the mixture was incubated for activity determination. Values reported are the means of three independent determinations.

2.4. Activation by FAD

The rate of [^{14}C]GA_{12}-aldehyde metabolism in the presence of pyridine nucleotides was strongly influenced by the addition of FAD to the reaction medium. Moreover, this effect showed marked differences when [^{14}C]GA_{12} or [^{14}C]GA_{14} synthesis was monitored (Fig. 3). The rate of [^{14}C]GA_{14} synthesis increased with increasing FAD concentration in the range of 0.1–4 μM (Fig. 3A). Half saturation was obtained at 0.5 μM FAD giving a maximum rate of 146×10^{-8} nkat, 3.5 times higher than the rate obtained in the absence of this flavin nucleotide (42×10^{-8} nkat). In contrast, the rate of [^{14}C]GA_{12} synthesis in the presence of NADPH was not altered by FAD (Fig. 3A). Electrons required by microsomal monooxygenases are transferred from the reduced pyridine nucleotides by carrier proteins that may be flavoproteins (Donaldson and Luster, 1991; Guengerich, 1991). During protein isolation and extraction, flavin cofactors can

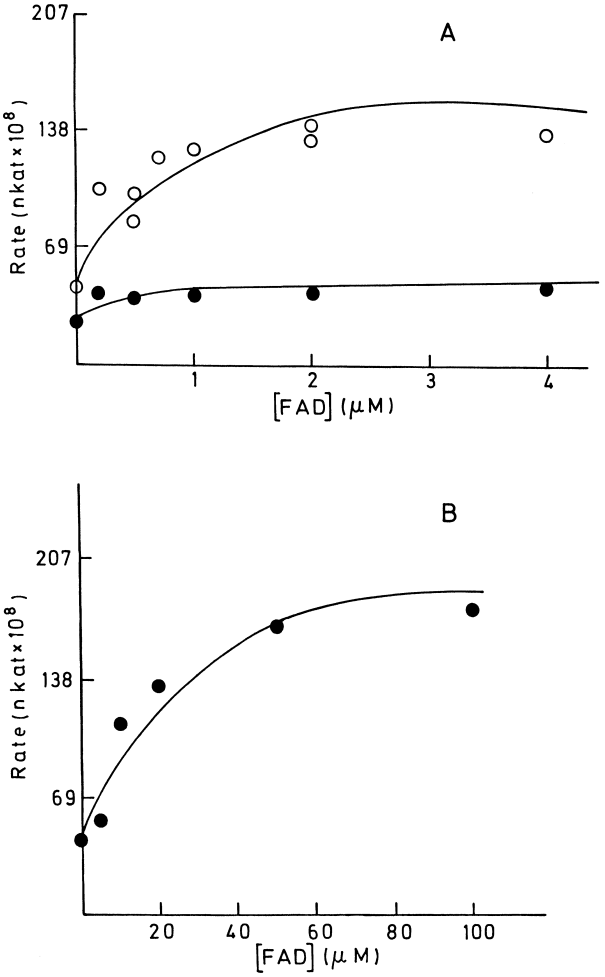


Fig. 3. Effect of FAD (A) on the rate of synthesis of [^{14}C]GA_{12} (●) and [^{14}C]GA_{14} (○) from [^{14}C]GA_{12}-aldehyde and 1 mM NADPH or (**B**) on the rate of synthesis of [^{14}C]GA_{12} (●) from [^{14}C]GA_{12}-aldehyde and 1 mM NADH. Assays contained 10 μl of enzyme solution (**A**) or 20 μl of enzyme (**B**).

partially dissociate from their apoproteins resulting in loss of activity that can be restored by added FAD. The results shown in Fig. 3 indicate the participation of a flavoprotein in electron transfer from NADPH into the gibberellin 3-hydroxylase. This is not the case for the 7-oxidase since [^{14}C]GA_{12} synthesis was not altered by FAD (Fig. 3A). In contrast to the above results, [^{14}C]GA_{12} synthesis in the presence of NADH was activated by adding FAD in the range of 10–100 μM (Fig. 3B). Maximum activation gave a rate of 188×10^{-8} nkat, 3.8 times higher than the rate in the absence of FAD. Half saturating effect was obtained at a concentration of 20 μM FAD, 40 times higher than that found for 3-hydroxylase activity. The results shown in Fig. 3 suggest that distinct flavoproteins differing in their affinities for FAD, participate in the reactions catalyzed by the 3-hydroxylase and 7-oxidase. Furthermore, electrons from NADPH or NADH may be delivered to the 7-oxidase through different pathways since participation of a flavoprotein could be demonstrated only in the presence of NADH.

NADPH and FAD are typically required by cytochrome P-450-linked enzymes (Donaldson and Luster, 1991; Guengerich, 1991; Porter and Coon, 1991) and have been reported to be essential for *G. fujikuroi ent*-kaurene oxidase and *ent*-kaurenoic acid 7-hydroxylase activities (Ashman et al., 1990; Jennings et al., 1993). P-450 monooxygenases are associated with cytochrome P-450 reductase, a flavoprotein that transports electrons from NADPH, which would account for the activating effect of flavin nucleotides found in these reactions.

2.5. CO and cytochrome c inhibition

The requirement for pyridine nucleotides and activation by FAD of the enzymes that catalyze the synthesis of GA_{12} and GA_{14} are characteristic of cytochrome P-450 s. To investigate this point further we studied the effect of carbon monoxide and of cytochrome c, inhibitors of P-450 enzymes, on these reactions. The reduced, ferrous form of cytochrome P-450 has a high affinity for carbon monoxide (Donaldson and Luster, 1991), which inhibits enzyme activity by binding strongly to the heme ferrous iron and preventing oxygen from binding (Sono et al., 1996). We found that treatment of the fungal enzyme preparation with CO in the presence of a reductant, strongly inhibited GA_{12} and GA_{14} synthesis (Table 5). This was obtained in the presence of NADPH, dithionite or ascorbate as reductants. In all experiments inhibition of the 3-hydroxylase activity was greater than inhibition of the 7-oxidase, suggesting that the enzymes have different affinities for carbon monoxide. Utilization of [^{14}C]GA_{12}-aldehyde was also assayed in the presence of 200 μM cytochrome c (Table 5). Under these conditions [^{14}C]GA_{12} synthesis was inhibited by 50% while [^{14}C]GA_{14} synthesis decreased up to 32%. Cytochrome c has been reported as an effective artificial electron acceptor for the cytochrome P-450 reductase (Mihaliak et al., 1993) and may thus prevent reduction of cytochrome P-450 s.

Table 5
Inhibition by carbon monoxide and cytochrome c

Inhibitor	Reductant[a]	[^{14}C]GA_{12} synthesis (nkat$\times10^8$)	% Activity	[^{14}C]GA_{14} synthesis (nkat$\times10^8$)	% Activity
None	None	85±4	100	297±13	100
0.2 mM Cytochrome c	None	42±2	50	95±6	32
CO	NADPH	49±2	57	61±5	20
CO	Dithionite[b]	18±1	21	15±2	5
CO	Ascorbate[b]	45±4	53	50±4	17

[a] Reductant added to the enzyme solution to obtain the ferrous form of cytochrome P-450. This solution was flushed with carbon monoxide for 5 min in sealed tubes. Afterwards ^{14}C-GA_{12} aldehyde (10 000 dpm), NADPH (1 mM) and FAD (5 μM) were added and the mixture was incubated in air for assay. Values reported are means of three independent determinations.

[b] 3 mM sodium dithionite; 1 mM sodium ascorbate.

The properties described here for the oxidases involved in the two-step conversion of GA_{12}-aldehyde to GA_{14} as part of gibberellic acid biosynthesis in *G. fujikuroi*, correspond to those of cytochrome P-450 monooxygenases. The two oxidases differ in their preferred electron source and in the flavoproteins involved in electron transfer. The enzymes involved in *ent*-kaurenoic acid formation and its subsequent oxidation have also been shown to correspond to cytochrome P-450 monooxygenases, and thus this type of enzyme plays a major role in gibberellic acid synthesis in the fungus. This is reinforced by the recent finding that several P-450 genes are present in the GA-biosynthesis gene cluster of *G. fujikuroi* (Tudzynski and Hölter, 1998) and suggest that a main part of the pathway could occur on membranes and be mediated by monooxygenases.

3. Experimental

3.1. Culture conditions

The wild-type strain ACC 917 of *Gibberella fujikuroi* was utilized for these studies. Cultures were maintained in potato dextrose agar and used for inoculating into liquid 40% ICI medium (Geissman et al., 1966). After 4 days, the culture was transferred into 0% ICI medium and grown for an additional 7 days for enzyme extraction. Liquid cultures were grown at 28°C under light with orbital shaking at 150 rpm.

3.2. Substrate synthesis

[^{14}C]GA_{12}-aldehyde was synthesized from *R*-[2-^{14}C]mevalonic acid (Amersham) by incubation with pumpkin endosperm enzymes and appropriate cofactors

(Graebe et al., 1974; Lange and Graebe, 1993). [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} were separated from the reaction products by reversed phase HPLC on a C_{18} column with on-line radiomonitoring (Lange et al., 1993) and their identity was confirmed by combined gas chromatography–mass spectrometry (GC/MS) after derivatization to methyl esters trimethylsilyl ethers (Lange et al., 1993). [^{14}C]GA_{14} was produced from [^{14}C]GA_{12}-aldehyde by incubation with the enzyme preparation from *G. fujikuroi* in the presence of 1 mM NADPH (see below).

3.3. Preparation of cell-free extracts

Mycelia from cultures grown for seven days in 0% ICI liquid medium (Geissman et al., 1966) were harvested by vaccuum filtration. At this stage the culture was slightly pigmented. The mycelia were washed with ultrapure water (500 ml) followed by 50 mM Tris–HCl pH 7.5 (500 ml). About 700 mg of washed mycelia were suspended in 10 ml of the extraction solution (50 mM Tris–HCl; 5 mM EDTA; 4 mM DTT; 2.5 M sucrose and 10 mg/ml bovine serum albumin, BSA) and stored frozen at −20°C until preparation of the extract. Disruption was accomplished with the frozen mycelia (−30°C) under 28 000 psi in a chilled X-Press (AB BIOX, Sweden, Edebo, 1983). After thawing, additional 10 ml of the extraction solution were added to the crushed mycelia and the homogenate was centrifuged at 10 000 *g* for 20 min. The supernatant was precipitated with 70% ammonium sulfate and the pellet resuspended in 50 mM Tris–HCl containing 4 mM DTT and desalted on a PD-10 column (Pharmacia) equilibrated in the same solution. Glycerol (5%) was added to the eluted high molecular weight fraction which was stored frozen at −20°C or −70°C. For preparation of microsomes, the 10 000 *g* supernatant was centrifuged at 100 000 *g* for 1 h. The pellet was supended in 50 mM Tris–HCL, 4 mM DTT and 5% glycerol (0.5 ml).

3.4. Enzyme assays

Incubations with [^{14}C]GA_{12}-aldehyde, [^{14}C]GA_{12} or [^{14}C]GA_{14} were carried out in a final volume of 100 μl containing the enzyme preparation (10–80 μl), labelled substrate (10 000 dpm, 0.25 μM), oxidase cofactors and 50 mM Tris–HCl pH 7.5. The mixture was incubated with shaking for 1 h at 30°C and the reaction stopped by adding 10 μl of acetic acid and 1 ml of pH 3.0 water into the reaction media. The samples were further purified on C_{18} SepPak cartridges and then analysed by HPLC.

3.5. HPLC conditions

Products were separated on a C_{18} column (Symmetry, Waters) in a Waters 600 HPLC instrument. Elution conditions were: isocratic 15 min run with 75:25, MeOH:H_2O pH 3.0 followed by a linear 10 min gradient to 100% MeOH which was maintained for 10 min. The flow rate was 1 ml/min, 1 ml fractions were collected and the radioactivity in the eluate was measured by liquid scintillation counting.

3.6. Product identification

Radioactive fractions from HPLC were methylated with ethereal diazomethane and converted to trimethylsilyl ethers by heating in *N*-trimethylsilyl-*N*-fluoroacetamide at 90°C for 30 min (Gaskin and MacMillan, 1992). These were analyzed by combined gas chromatography–mass spectrometry by comparison with authentic gibberellin standards (Gaskin and MacMillan, 1992).

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Ferulic acid is esterified to glucuronoarabinoxylans in pineapple cell walls

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Abstract

The ester-linkage of ferulic acid (mainly *E*) to polysaccharides in primary cell walls of pineapple fruit (*Ananas comosus*) (Bromeliaceae) was investigated by treating a cell-wall preparation with 'Driselase' which contains a mixture of endo- and exo-glycanases, but no hydroxycinnamoyl esterase activity. The most abundant feruloyl oligosaccharide released was *O*-[5-*O*-(*E*-feruloyl)-α-L-arabinofuranosyl](1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX). This indicated that the ferulic acid is ester-linked to glucuronoarabinoxylans in the same way as in the primary walls of grasses and cereals (Poaceae). Glucuronoarabinoxylans are the major non-cellulosic polysaccharides in the pineapple cell walls. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Ananas comosus*; Pineapple; Bromeliaceae; Commelinoid monocotyledons; Glucuronoarabinoxylans; Feruloyl esters; Oligosaccharides; 'Driselase'; Plant cell walls; *O*-[5-*O*-(*E*-feruloyl)-α-L-arabinofuranosyl](1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose

1. Introduction

The hydroxycinnamic acid (HCA) ferulic acid (FA) occurs ester-linked to the primary cell walls (PCWs) of all families of the commelinoid group of monocotyledons. This group contains approximately half of all the monocotyledon families and comprises the following orders recognized by the Angiosperm Phylogeny Group (1998): Arecales, Commelinales, Poales, Zingiberales and some unplaced taxa (Harris and Hartley, 1980; Rudall and Caddick, 1994; Harris et al., 1997; Smith and Harris, 1999; Harris, 2000). However, most of the research done on FA ester-linked to monocotyledon cell walls (CWs) has been done on economically important species of the family Poaceae (Poales) which comprise the grasses and cereals. In contrast to the monocotyledons, FA occurs ester-linked to the PCWs of dicotyledons only in the order Caryophyllales as defined by Cronquist (1988) (Hartley and Harris, 1981). This order contains the family Chenopodiaceae that has a number of economically important species, including spinach (*Spinacia oleracea*) and sugar beet (*Beta vulgaris*).

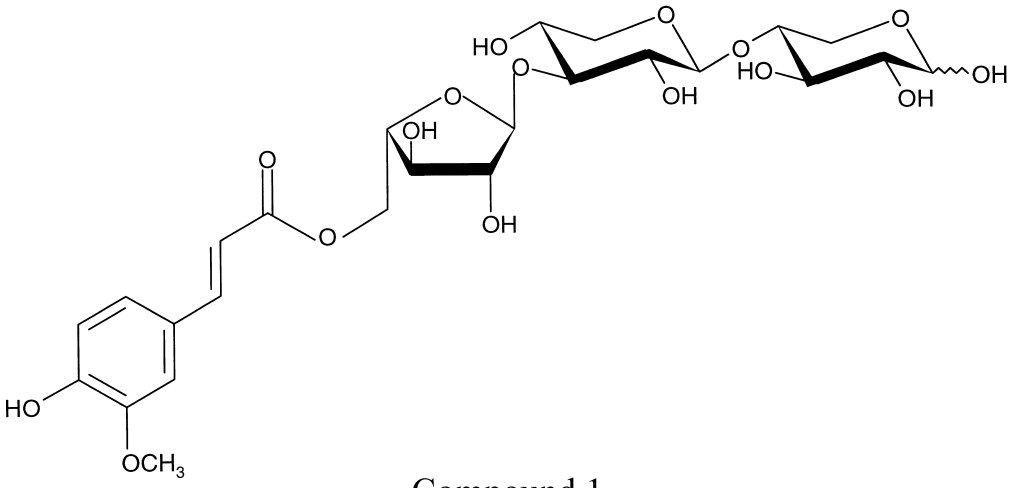
FA is ester-linked to polysaccharides in PCWs, but it is linked to different polysaccharides in the PCWs of the Poaceae and Chenopodiaceae. The polysaccharides to which the FA is ester-linked and the exact location of the link were determined by treating isolated PCWs with commercial fungal 'cellulases' such as 'Driselase' which is from the fungus *Irpex lacteus* and contains a mixture of endo- and exo-glycanases, but lacks hydroxycinnamoyl esterase activity. Feruloyl oligosaccharides (FOs) were isolated from such enzymic digests and their structures determined. In enzymic digests of Poaceae PCWs, the most abundant FO was usually *O*-[5-*O*-(*E*-feruloyl)-α-L-Ara*f*](1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p* (FAXX) (Fig. 1), indicating that the FA is ester-linked to glucuronoarabinoxylans (GAXs), typically the most abundant non-cellulosic polysaccharides in these PCWs (Kato and Nevins, 1985; Ishii and Hiroi, 1990; Ishii, 1997; Wende and Fry, 1997a). GAXs consist of a backbone of Xyl residues substituted with mostly single Ara and GlcA (or 4-*O*-MeGlcA) residues linked at C(O)3 and C(O)2 respectively to the Xyl residues. The FA is esterified via its carboxyl group to the C(O)5 hydroxyl of single α-L-Ara*f* residues located on the C(O)3 of Xyl*p* residues in the polysaccharide backbone. In contrast,

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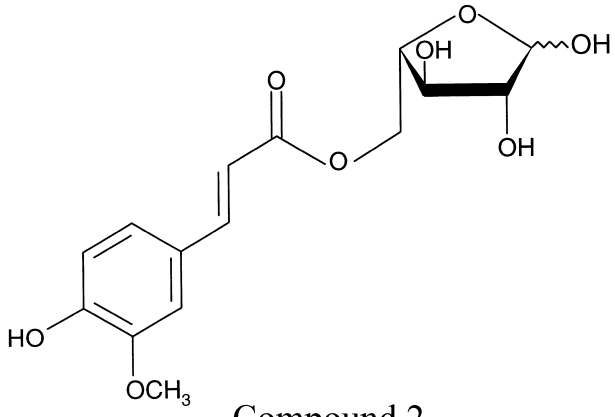
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Compound 1



Compound 2

Fig. 1. Structure of Compound **1** *O*-[5-*O*-(*E*-feruloyl)-α-L-arabinofuranosyl](1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) and Compound **2** 5-*O*-feruloyl-L-arabinose.

the two most abundant FOs in enzymic digests of PCWs of the Chenopodiaceae were usually *O*-[2-*O*-*E*-feruloyl-α-L-Ara*f*]-(1→5)-L-Ara*f* and *O*-[6-*O*-*E*-feruloyl-α-D-Gal*p*]-(1→4)-D-Gal*p* from the PCWs of spinach and sugar beet (Fry 1982, 1983; Ishii and Tobita, 1993; Colquhoun et al., 1994; Ishii, 1994; Ralet et al., 1994). The structures of these indicated that the FA is ester-linked to pectic polysaccharides, the most abundant non-cellulosic polysaccharides in these PCWs (Renard and Thibault, 1993). The FA is ester-linked via its carboxyl group, to C(O)2 hydroxyls of Ara*f* residues in the backbone of α-(1→5)-linked pectic arabinan chains; and to C(O)6 hydroxyls of Gal*p* residues in the backbone of β-(1→4)-linked pectic galactan chains.

Analyses of the monosaccharide compositions of PCWs of commelinoid monocotyledon families in addition to the Poaceae indicated that GAXs were major components (Harris et al., 1997). The only exception was the palm family, the Arecaceae (Carnachan and Harris, 2000). More detailed studies of the polysaccharides in the PCWs of species in families of the order Poales (Smith and Harris, 1999) and of the polysaccharides in the PCWs of pineapple (*Ananas comosus*, Bromeliaceae) fruit (Smith and Harris, 1995) confirmed the presence of GAXs, similar in structure to those in the PCWs of the Poaceae.

In the present study, we have investigated whether the FA in pineapple fruit PCWs is linked to GAXs and, if so, if it is linked to these polysaccharides in the same way as in the Poaceae. We have done this by isolating PCWs, treating them with 'Driselase', and structurally characterizing the most abundant FO released.

2. Results and discussion

2.1. Ester-linked hydroxycinnamic acids

E-FA was the most abundant HCA detected after TLC (Solvents 1 and 2) of alkaline hydrolysates of PCWs from pineapple fruit. The next most abundant HCA was *Z*-FA, followed by small amounts of *E*-*p*-coumaric acid

(PCA), and trace amounts of *Z*-PCA. In addition, a component was detected that had the same R_f (0.09, Solvent 2) as 5-5′-dehydrodiferulic acid ('diferulic acid'). After treatment with diazotized *p*-nitroaniline, it gave a purple colour that faded on spraying with Na_2CO_3, as does 5-5′-dehydrodiferulic acid (Hartley and Jones, 1976). However, unlike 5-5′-dehydrodiferulic acid, which fluoresces purple in UV radiation before and after treatment with NH_3, this component did not fluoresce. Small amounts of a further two unknown phenolic components which also did not fluoresce were detected. One of these (R_f 0.55, Solvent 1) stained orange with *p*-nitroaniline changing to blue on treatment with Na_2CO_3, and the other (R_f 0.7, Solvent 1) stained red with *p*-nitroaniline changing to blue/green on treatment with Na_2CO_3. Our results are consistent with a quantative analysis of the HCAs released from pineapple fruit CWs using the same conditions as the present study (Hartley, 1987). In this analysis, the following amounts of HCAs (mg/g of cell walls) were found: *E*-FA 17.7; *Z*-FA 4.2; *E*-PCA 2.1; and *Z*-PCA 0.5.

2.2. Separation of FOs from PCWs

PCWs were incubated with 'Driselase' and the material solubilized passed through a C_{18} cartridge to bind FOs and *p*-coumaroyl oligosaccharides (PCOs). These oligosaccharides were eluted to give a 'cell-wall extract' (CWE) that was subjected to PC (Solvent 3). This separated seven compounds that in UV radiation fluoresced blue changing to green on exposure to NH_3, indicating they were FOs (Harris and Hartley, 1976). The R_f values of these compounds are shown in Table 1. The most intensely fluorescing compound (Compound **1**) had the same mobility (R_f 0.52) as reference FAXX. Identical results were obtained in an experiment in which the PCWs were incubated with 'Driselase' in aqueous NaN_3 (0.02%) instead of H_2O. Identical results were also obtained when the CWE was subjected to TLC in Solvent 3. After PC and TLC (Solvent 3) no spots corresponding to *O*-[5-*O*-(*E*-*p*-coumaroyl)-α-L-Ara*f*]-(1→3)-*O*-β-D-Xyl*p*-(1→4)-DXyl*p* PAXX were found in the pineapple PCWs indicating the absence of PCOs. Furthermore, no free FA or PCA was detected.

PC (Solvent 3) of equivalent extracts of PCWs of Italian ryegrass and maize, both Poaceae species, also gave a series of spots that showed similar fluorescence behaviour; the most intensely fluorescent spot also had the same R_f as reference FAXX (Table 1). If Compound **1** is indeed FAXX, then the other compounds are probably FOs derived from GAXs, similar to those isolated and identified by other workers from Poaceae CWs (Ishii, 1997). This is supported by the fact that the fluorescent compounds from the maize CWE had identical R_f values to those from the pineapple CWE and showed similar fluorescence intensities. However, the R_f values of the other fluorescent spots from Italian ryegrass CWE differed slightly from those from the pineapple CWE: the spot at the origin and the R_f 0.19 spot were absent, but there was a spot at R_f 0.28.

In contrast, the CWE of spinach (Chenopodiaceae) gave two spots (R_f 0.48 and 0.71) that fluoresced intensely blue, changing to green on exposure to NH_3, and a third faint spot that showed the same fluorescence behaviour, but none of these had the same R_f as reference FAXX. From the R_f values of the two major FOs, the less mobile compound is probably feruloyl galactobiose [*O*-(6-*O*-*E*-feruloyl-α-D-Gal*p*)-(1→4)-D-Gal*p*] and the more mobile compound is probably feruloyl arabinobiose [*O*-(2-*O*-*E*-feruloyl-α-L-Ara*f*)-(1→5)-L-Ara*f*] (Fry, 1982, 1983, 1988; Ishii and Tobita, 1993; Ishii, 1994).

2.3. Characterization of Compound **1**

Compound **1** was eluted from the paper and again subjected to PC (Solvent 3). Only one fluorescent spot resulted with the same R_f (0.52) as reference FAXX. Saponification of Compound **1** followed by TLC (Solvents 1 and 2) gave *E*-FA as the only HCA. FA was also the only HCA in acid hydrolysates of Compound **1** (2.0 and 2.5 M TFA) and in *Aspergillus niger* 'cellulase' hydrolysates of Compound **1** that were subjected to PC (Solvent 3 and 4). 'Cellulase' preparations from *Aspergillus niger* are known to contain hydroxycinnamoyl esterase activity (Hartley and Jones, 1976).

Table 1
Separation of feruloyl oligosaccharides in cell-wall extracts by PC using Solvent 3[a]

R_f	Cell-wall extracts				
	Pineapple	Italian ryegrass	Maize	Spinach	Reference mixture of FAXX and PAXX
Origin	+ +		+ +		
0.19	+		+		
0.28		+			
0.31	+/−		+/−		
0.39	+ +	+ +	+ +		
0.45	+	+	+		
0.48				+ + +	
0.52	+ + +	+ + +	+ + +		+ + +
0.55					+ + +
					B→P
0.61	+ +	+/−	+ +		
0.63				+/−	
0.71				+ + +	

[a] The cell-wall extracts were obtained by incubating cell walls with 'Driselase', passing the solubilized material through a C18 cartridge, and eluting the bound material with MeOH. Intensity of fluorescence: +/− = faint; + = weak; + + = moderate; + + + = strong. Fluorescence colour of the spots in UV radiation was blue, changing to green after exposure to NH_3, unless otherwise indicated. B→P = blue, changing to purple after exposure to NH_3.

PC (Solvents 3 and 4) of the acid and *Aspergillus niger* 'cellulase' hydrolysates also showed they contained Xyl and Ara as the only monosaccharides. This was confirmed by converting the neutral monosaccharides in the acid (2.5 M TFA) hydrolysate to alditol acetates and separating them by GC; the molar ratio of Xyl and Ara was 2.1:1. A trace of Glc was also detected (by GC) in the acid hydrolysates, but this monosaccharide was also found in hydrolysates of 'paper' controls in which chromatography paper with no Compound **1** was eluted and processed. Linkage analysis showed that terminal-Ara*f*, and 3- and 4-linked Xyl*p* residues were present in the molar ratios 1.0:1.1:0.9, respectively. Small amounts of 4-Glc*p* residues were also detected, but were also detected in 'paper' controls (as above). These results are consistent with Compound **1** being FAXX (Fig. 1).

Compound **1** gave a ^{1}H NMR spectrum that was identical to that obtained by Mueller-Harvey et al. (1986) for FAXX isolated from barley straw. The signals of the monosaccharide protons were assigned as shown in Table 2. The coupling constant $J_{1,2}$ for the Ara residue was <2 Hz which is consistent with the Ara being α- rather than β-linked to the non-reducing Xyl (Xyl′) (Joseleau et al., 1977; Mueller-Harvey et al., 1986). The low-field resonances of H-5 in Ara (4.30 and 4.47 ppm) indicate that the arabinose was esterified at O-5; H-5 in Ara would be expected to resonate at ~3.8 ppm (Mueller-Harvey et al., 1986).

FAB-MS of Compound **1** showed ions at *m*/*e* 591 and 613 corresponding to $590+H^+$ (1) and $590+Na$ (23), indicating a molecular weight of 590 which is consistent with Compound **1** being FAXX.

Further evidence that Compound **1** was FAXX was obtained by subjecting it to mild acid hydrolysis (0.1 M TFA, 1 h, 100°C) and analysing the products. These hydrolysis conditions hydrolyse Ara*f* linkages more readily than glycopyranosyl linkages and feruloyl ester linkages. PC (Solvent 3) of the hydrolysate gave a spot, Compound **2** (R_f 0.76), which fluoresced blue in UV radiation and green after exposure to NH_3, indicating it was a feruloyl ester. A trace of FA was also detected.

Table 2
Assignments of signals from monosaccharides in the ^{1}H NMR spectrum of Compound **1**

	H-1	H-2	H-3	H-4	H-5ax	H-5eq
α-L-arabinose	5.32	4.17	4.02	4.37	4.30	4.47
	(5.32)[a]	(4.19)	(4.02)	(4.37)	(4.30)	(4.46)
β-D-xylose′	4.43	3.37	3.55	3.66	3.30	3.97
(non-reducing)	(4.43)	(3.39)	(3.55)	(3.66)	(3.30)	(3.97)
α-D-xylose	5.13	3.52	3.72	3.80	3.66	3.83
	(5.14)	(3.51)	(3.72)	(3.78)	(3.68)	(3.86)
β-D-xylose	4.54	3.20	3.52	3.72	3.32	4.02
	(4.54)	(3.21)	(3.51)	(3.74)	(3.32)	(4.01)

[a] Values in parentheses are assignments of signals in the ^{1}H NMR spectrum of FAXX by Mueller-Harvey et al. (1986).

PC (Solvent 3) of the hydrolysate also showed the presence of Xyl and xylobiose. Treatment of reference xylobiose with 0.1 M TFA under the same conditions showed that it was partly hydrolysed to Xyl. Thus, Compound **1** consisted of Compound **2** linked to xylobiose.

2.4. Characterisation of Compound 2

Complete acid hydrolysis (2.5 M TFA) of Compound **2** followed by PC (Solvent 3) showed FA as the only HCA. PC (Solvent 4) followed by staining with $AgNO_3$–NaOH showed the presence of Ara, and trace amounts of Glc and Xyl. Thus, Compound **2** is FA ester-linked to Ara and is consistent with this being 5-*O*-feruloyl-L-Ara (Fig. 1).

2.5. Conclusions

The evidence presented showed that the most abundant FO released when PCWs from pineapple fruit were treated with 'Driselase' was FAXX. Thus, in the PCWs of this species, FA is ester-linked to GAXs in the same way as in the Poaceae. As 'Driselase' contains many glycanase enzymes, it is possible that other monosaccharide residues are linked to the Ara to which the FA is esterified. Indeed, by using mild acid hydrolysis, it has been shown that the GAXs of many species of Poaceae have FO side-chains instead of the simple side-chain 5-*O*-feruloyl-L-Ara (Wende and Fry, 1997a–c).

3. Experimental

3.1. Chemicals

'Driselase' (Sigma) was purified using the method of Borneman et al. (1990) to give a solution containing 11.9 mg/ml protein. 'Cellulase' from *Aspergillus niger* was obtained from Sigma. Mixtures of *Z*- and *E*-isomers of FA, PCA and sinapic acid were obtained by exposing solutions of the *E*-isomers (5 mg/ml in MeOH) to UV radiation (maximum λ 365) for 8 h (Hartley and Jones, 1975) and *p*-hydroxybenzoic acid were used as standards. A mixture of *O*-[5-*O*-(*E*-feruloyl)-α-L-Ara*f*](1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p* (FAXX) and *O*-[5-*O*-(*E*-*p*-coumaroyl)-α-L-Ara*f*]-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p* (PAXX) was donated by Dr. R.D. Hartley, formerly of the USDA, Athens, GA, USA.

3.2. Plant material

Pineapple [*Ananas comosus* (L.) Merr. cv. Smooth Cayenne] fruit was obtained from a store. Seedlings of Italian ryegrass (*Lolium multiflorum* Lam. cv. Grasslands Paroa) and spinach (*Spinacia oleracea* L. cv. English Improved) were grown in potting mix; maize

(*Zea mays* L. cv. Treasure F1 hybrid) seedlings were grown in sterilized vermiculite.

3.3. Isolation of PCWs

Unlignified PCWs were isolated by the procedure of Smith and Harris (1995) from: the flesh of pineapple fruit, spinach petioles, 4-day old maize coleoptiles, and fully expanded leaf blades of Italian ryegrass seedlings at the stage of growth when the third leaf had just emerged. The cell walls were ground to a fine powder and then further dried over SiO_2. All procedures, including the isolation of the PCWs, were carried out using a halogen reflector lamp with a UV filter (Sylvania) to prevent *E*–*Z* isomerisation (Hartley and Jones, 1975).

3.4. 'Driselase' digestion of PCWs

This was done by a modification of the method of Borneman et al. (1990). PCWs (10 mg) were suspended in H_2O (1 ml), 'Driselase' (20 μl) was added and the mixture was shaken (20 h at 37°C). In another experiment, NaN_3 (0.02%, 1 ml) (Hartley, 1973) was used instead of H_2O. Controls in which PCWs or 'Driselase' were omitted were also carried through the procedure. The tubes were centrifuged (23 000 ***g***, 10 min) and the supernatants retained. The pellets were washed in H_2O (0.5 ml, 2×) and centrifuged as before; the washings and supernatants were combined and heated (100°C, 10 min) to inactivate the enzymes, and when cool, filtered (cellulose acetate, 0.2 μm). The filtrate was passed through a pre-conditioned C-18 solid phase extraction cartridge (100 mg, 1 ml); FOs and PCOs bound to the solid phase. Unbound material was removed by elution with H_2O (5 ml), and FCOs and PCOs were removed by eluting with MeOH (5 ml). This MeOH fraction was evaporated to dryness in a stream of air to leave the 'cell-wall extract' (CWE).

3.5. Saponification of hydroxycinnamoyl esters

NaOH (1 M, 1 ml) was added to the esters (PCWs, CWE and Compound **1**) under Ar at 20°C for 20 h with gentle shaking. The solution was adjusted to pH 2.5 with 6 M HCl and passed through a pre-conditioned C-18 solid phase extraction cartridge (as above), washed with H_2O (5 ml) and subjected to TLC (Solvents 1 and 2) to separate the HCAs. Reference standards, chromatographed under the same conditions, were *E*- and *Z*-PCAs, *E*- and *Z*-FAs, *E*- and *Z*-sinapic acids and *p*-hydroxybenzoic acid.

3.6. Acid hydrolysis of feruloyl esters

Complete hydrolysis was done under Ar using 2 M TFA (0.5 ml, 121°C, 1 h) (Albersheim et al., 1967) and 2.5 M TFA (0.5 ml, 100°C, 1 h) (Mueller-Harvey et al., 1986). Partial acid hydrolysis was done using 0.1 M TFA (0.5 ml, 100°C, 1 h) (Fry, 1988). Hydrolysates were evaporated to dryness in a stream of air at 20°C.

3.7. TLC

This was done using cellulose plates (Schleicher and Schüll, Avicell, F1440). HCAs were separated with Solvent 1, HCOOH–H_2O (1:24 v/v) or Solvent 2, PhMe-HCOOH–H_2O (8:9:3 v/v, upper phase) (Hartley and Jones, 1976). Solvent 1 separated *E*- and *Z*-isomers, but Solvent 2 did not. FOs were separated with Solvent 3, *n*-BuOH–HOAc–H_2O (12:3:5 v/v) (BAW) (Fry, 1988). HCAs and FOs were located by UV radiation (max λ 365 nm) (before and after exposure to NH_3). Phenolic compounds, including HCAs, were also located by spraying with diazotized *p*-nitroaniline, followed by spraying with Na_2CO_3 and recording the colours after each spray (Swain, 1953).

3.8. PC

Descending PC was done on Whatman No. 1 chromatography paper. FOs were separated with Solvent 3 and detected as described above. Monosaccharides and disaccharides were separated using Solvents 3 and 4 [EtOAc–pyridine–H_2O (8:2:1 v/v)] and were detected by staining with aniline hydrogen phthalate (Fry, 1988) or with $AgNO_3$–NaOH (Trevelyan et al., 1950).

3.9. Neutral-monosaccharide composition

The monosaccharides in the 2.5 M TFA hydrolysates were reduced to alditols using $NaBH_4$ (20 mg/ml, 0.5 ml) in 1 M NH_4OH, for 60 min at 20°C. The reaction was stopped with 18 M AcOH (100 μl). MeOH and 18 M HOAc (9:1 v/v, 1 ml) was added and evaporated to dryness in a stream of air. This step was repeated 3×; MeOH (1 ml) was added and evaporated. The alditols were acetylated (Blakeney et al., 1983) and the resulting alditol acetates separated by GC (Smith and Harris, 1995).

3.10. Linkage analysis

This was done by the method of Harris et al. (1984) as described by Smith and Harris (1995) except that evaporations were done at 40 and not 90°C. Partially methylated alditol acetates were identified by their mass spectra and GC retention times relative to *myo*-inositol hexaacetate as described by Smith and Harris (1995), and were quantified as described by Rae et al. (1985).

3.11. FAB-MS

Compound **1** in MeOH was placed on the probe and allowed to dry. Glycerol (1 ml) was used as the matrix

material. The atom gun (8 keV, 1 m amp) was fired using xenon. Spectra were obtained by scanning from 70 to 1500 m/e at 5 s decade^{-1}.

3.12. NMR

^{1}H NMR spectra were obtained using a Bruker DRX 400 MHz spectrometer. A 2.5 mm inverse detection microprobe and the standard Bruker presaturation programme "zgpr" were used. The sample was dissolved in 250 μl of 100% D_2O (Aldrich) and was externally referenced to 3-(trimethylsilyl)-1-propane sulfonic acid sodium salt in D_2O. The probe temperature was 27°C.

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Book review

Mycorrhizal Biology
Edited by K.G. Mukerji, B.P. Chamola and J. Singh, Kluwer Academic/Plenum Publishers, New York, 2000, 336 pp., ISBN 0-30604-629-4, $145

The book *Mycorrhizal Biology* is a compilation of chapters by different authors, the majority being researchers in institutions in India. The editors of the book indicate that it was their goal to provide a review of mycorrhizae with respect to microbial biotechnology, microbial ecology and applied mycology. Thus, the emphases are on the fungal side of the symbiosis. The choice of topics was influenced strongly by the problems facing crop production in India that include salinity, contamination by metals, poor soil structure and limited access to fertilizers. As a consequence, several chapters focus on areas that have not been previously targeted in other current reviews of mycorrhizae. The two chapters on mycorrhizae in micropropagation of plants and the chapter on the impact of agricultural pesticides on the mycorrhizal fungi are such examples. With this directive, the book has an applied rather than a basic science approach. Perhaps it would have been more informative for the prospective reader to use the term "Applied" in the title.

Most of the chapters are factual in nature. Some have a brief summary in which areas for future studies are outlined. Others discuss specific research findings almost in a paper-like format. Because one of the strengths of the book is that each chapter is referenced extensively the book makes a useful library volume. Unfortunately, in spite of the 2000 publication date, most of the references are from 1997 and earlier so that the information is not as current as other in print Mycorrhizal reviews. This time lag is especially detrimental in the area of molecular biology, molecular genetics and biochemistry. The lack of a combined reference index for the whole volume is disappointing. Also, the text is marred by numerous typographical and grammatical errors.

In general, the text is not strong in chemistry, biochemistry and molecular biology of the plant in the mycorrhizal associations. Thus, it is not the best acquisition for a reader requiring summaries of current information in these areas. The chapter by Mandelbaum and Piche ("The role of root exudates in arbuscular mycorrhizal initiation") introduces the linkage between biology and the chemical scene. For instance, whether plant secondary products may deter mycorrhizal growth in some of the non-mycorrhizal brassicas is discussed. Similarly, whether other phytochemicals initiate changes in the fungal gene expression essential for the symbiosis to become established is reviewed. The book exemplifies still how little is known at the molecular/biochemical level about mycorrhizal symbiosis, especially in comparison with the knowledge base for the other plant root symbionts, the nitrogen-fixing bacteria.

The whole-organism approach of the volume reiterates information presented in the classic book *Mycorrhizal symbiosis* by Harley and Smith published in 1983. Several of the chapters in *Mycorrhizal Biology* introduce the extensive diversities of the types of fungi and the morphologies of the symbiosis collectively termed mycorrhizae. Indeed the volume is timely in consideration of biological diversity and the potential for its rapid loss through current agricultural practices. For instance, in the chapter on mycorrhizae in orchids a novel mycorrhizal fungus (*Piriformospora indica*) is discussed. In this same chapter we are reminded of the fine line between the fungi that act as beneficials for the plant host in the mycorrhizal association and those that become detrimental as pathogens. Thus, those readers with a biochemical and molecular background may be stimulated towards formulating novel research directions by the content of the chapters.

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L-Tyrosine β-naphthylamide is a potent competitive inhibitor of tyramine *N*-(hydroxycinnamoyl)transferase in vitro

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Abstract

L-Tyrosine β-naphthylamide, a synthetic substrate designed to measure tyrosine aminopeptidase activity, is a potent inhibitor of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT) purified from elicited tobacco cell-suspension cultures. The inhibition is competitive, with the inhibitor binding reversibly to the tyramine binding site of the enzyme. Similar results were obtained with THT extracted from elicited potato cell-suspension cultures. K_i values were found to be 0.66 μM for the enzyme from tobacco and 0.3 μM for the enzyme from potato. L-Tyrosine 7-amido-4-methylcoumarin, a fluorogenic substrate for tyrosine aminopeptidases, the structure of which is close to that of L-tyrosine β-naphthylamide, was also a powerful inhibitor, but slightly less effective with K_i values of 0.72 and 0.42 μM for tobacco and potato THT, respectively. L-Tyrosine β-naphthylamide was rapidly hydrolysed when fed in vivo to tobacco or potato cell cultures or when incubated in crude enzymic extracts prepared from these cultures. This hydrolysis, which is presumably catalysed by aminopeptidases, precludes the use of L-tyrosine amides as inhibitors of THT in vivo. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Nicotiana tabacum*; *Solanum tuberosum*; Hydroxycinnamoyl transferase; Inhibition; L-Tyrosine β-naphthylamide; L-Tyrosine 7-amido-4-methylcoumarin; Tyrosine aminotransferase

1. Introduction

The enzyme hydroxycinnamoyl-CoA: tyramine *N*-hydroxycinnamoyl transferase, (THT, EC 1.2.2.110) catalyses the condensation of various hydroxycinnamoyl-CoA esters with tyramine. The amides formed by THT have repeatedly been found associated with cell wall fractions and their deposition together with other phenolics in the cell wall is believed to create a barrier against pathogens by reducing cell wall digestibility (Negrel et al., 1996; Facchini et al., 1999; McLusky et al., 1999). THT is a rational target to attempt to inhibit the synthesis of hydroxycinnamoyltyramines to evaluate the role played by these amides in the reinforcement of cell walls. THT has so far been purified from potato, tobacco, and opium poppy cell-suspension cultures (Hohlfeld et al., 1996; Negrel and Javelle, 1997; Yu and Facchini, 1999). Both potato and tobacco THT cDNA clones have been isolated, creating the opportunity to generate THT-downregulated plants using an antisense RNA strategy (Farmer et al., 1999; Schmidt et al., 1999). In a parallel approach, we have been screening for THT inhibitors which might be used in vivo. We previously reported that various L-tyrosine esters are competitive inhibitors of tobacco THT, the most active being L-tyrosine benzyl ester (Negrel and Javelle, 1997). In this paper we report that L-tyrosine β-naphthylamide (L-Tyr-NA, Fig. 1), a commercially available substrate used to measure tyrosine aminopeptidase activity, is a remarkably powerful inhibitor of both tobacco and potato THTs. However, when fed to tobacco or potato cell-suspension cultures, L-Tyr-NA was rapidly hydrolysed, most likely by aminopeptidases. This hydrolysis precludes the use of L-Tyr-NA as a THT inhibitor in vivo.

2. Results and discussion

2.1. Inhibition of THT in vitro

During the course of our research on the inhibition of tobacco THT by L-tyrosine derivatives, we found that L-Tyr-NA, a synthetic substrate for tyrosine aminopeptidase, was a very potent inhibitor of the transferase

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(Table 1). THT catalyses the transfer of ferulic acid from feruloyl-CoA to tyramine in an ordered bi bi mechanism that initially involves binding of feruloyl-CoA to the enzyme (Hohlfeld et al., 1995; Negrel and Javelle, 1997). The inhibition pattern observed in the presence of L-Tyr-NA was similar to that previously described with L-tyrosine benzyl ester (Negrel and Javelle, 1997). The inhibition was completely reversed in the presence of high concentrations of tyramine (Fig. 2A), whereas uncompetitive inhibition was observed when increasing concentrations of feruloyl-CoA were added in the presence of fixed concentrations of tyramine (Fig. 2B). These results show that L-Tyr-NA does not inhibit the initial binding of feruloyl-CoA to the transferase, but instead competes with tyramine for the same binding site. This was further confirmed by analysing the structure–activity relationships of several amino acid naphthylamides. L-Tyr-NA was much more active than phenylalanine β-naphthylamide, whereas the arginine derivative was totally inactive (Table 1). As expected, L-Tyr-NA was also a powerful inhibitor of the recombinant tobacco transferase, in displaying 92% inhibition at 50 μM in the presence of 25 μM feruloyl-CoA and 125 μM tyramine. Interestingly, L-tyrosine 7-amido 4-methyl coumarin (L-Tyr-AMC, Fig. 1), a sensitive fluorogenic substrate for tyrosine aminopeptidase (Zimmerman et al., 1977), was also a strong inhibitor of tobacco THT, although slightly less potent than L-Tyr-NA. Both compounds inhibited potato THT, and were about 10-fold

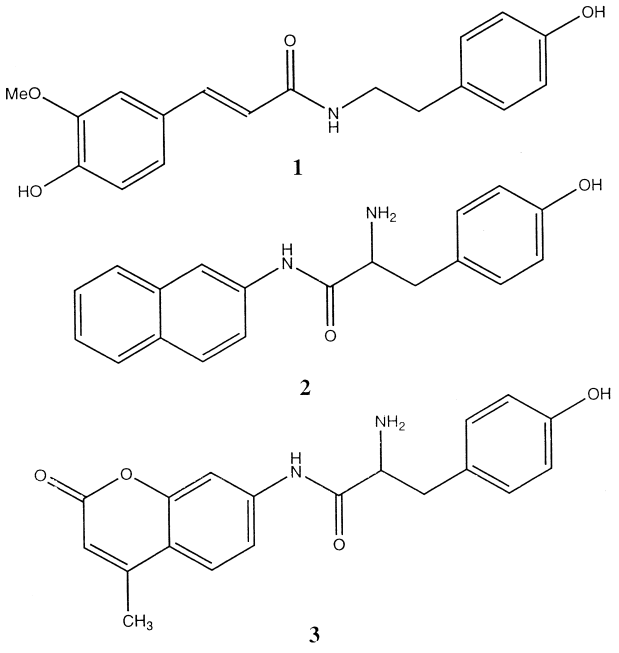


Fig. 1. Structures of feruloyltyramine (**1**), L-tyrosine β-naphthylamide (**2**) and L-tyrosine 7-amido-4-methylcoumarin (**3**).

Table 1
Inhibition of tobacco or potato THT by various amino acid amides or esters

Inhibitor	K_i (μM)[a]	
	Tobacco	Potato
L-Tyrosine β-naphthylamide	0.66	0.3
L-Tyrosine 7-amido 4-methylcoumarin	0.72	0.42
L-Tyrosine benzyl ester	3	3.4
L-Phenylalanine β-naphthylamide	112	91
L-Arginine β-naphthylamide	–[b]	–

[a] K_i values were calculated from Dixon plots, after measuring the inhibition of THT at different inhibitor concentrations, from 10 to 200 μM.

[b] No detectable inhibition.

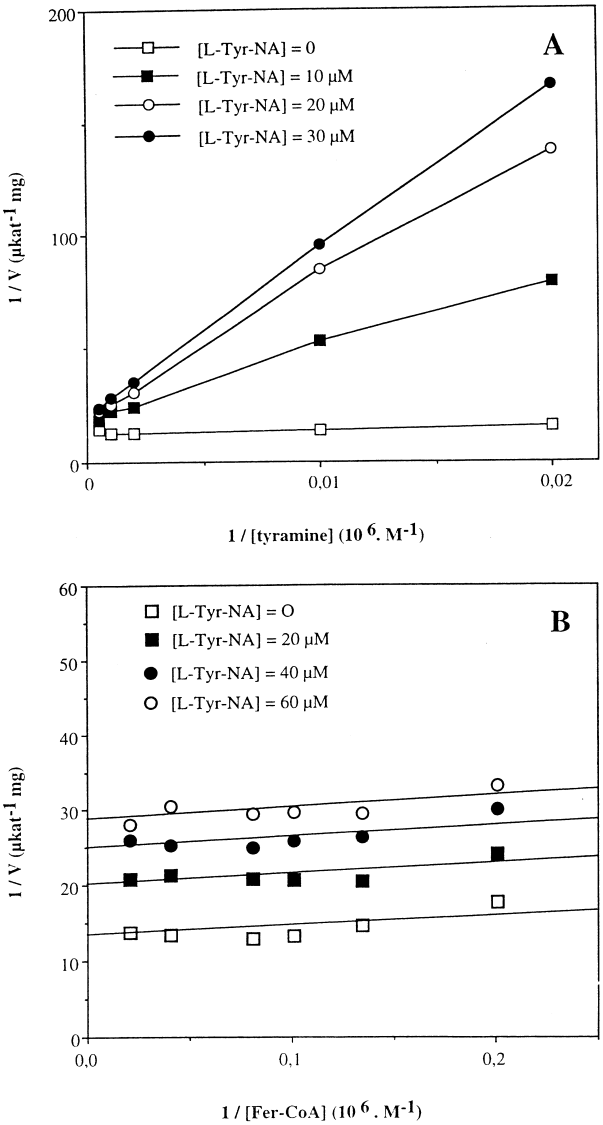


Fig. 2. Lineweaver–Burk plots of THT activity showing the variations of 1/V at constant and saturating concentration of one substrate (**A**: [feruloyl-CoA] = 25 μM; **B**: [tyramine] = 250 μM), with varying concentrations of the second substrate, in the absence or presence of various concentrations of L-Tyr-NA.

more potent than L-tyrosine benzyl ester (Table 1). The fact that the highest inhibition was obtained with the naphthylamine derivative is interesting because it provides a clue about the mode of action of this family of inhibitors. The structure of L-Tyr-NA is notable because it is intermediate between tyramine and a feruloyltyramine analogue (Fig. 1). Taking the mechanism of action of THT into account, it is likely that the naphthylamide moiety of the inhibitor, which is comparable to a rotationally constrained analogue of ferulic acid, can interact with the feruloyltyramine binding site after L-Tyr-NA has bound to the tyramine binding site of the enzyme-feruloyl-CoA complex. This likely explains the high affinity of THT for L-Tyr-NA and the fact that L-Tyr-AMC is less effective than L-Tyr-NA. Thus the mode of action of these inhibitors seems different from that of other specific inhibitors which have been designed to inhibit acyl-CoA amine acyltransferases, such as nonhydrolysable acyl-CoA analogues (Paige et al., 1990, Rudnick et al., 1991) or multisubstrate analogues (Cullis et al., 1982, Erwin et al., 1984).

2.2. Metabolism of L-Tyr-NA

We next considered the possibility of using L-Tyr-NA as an inhibitor of THT in vivo. Since amino acid naphthylamides are known substrates of aminopeptidases (Bodansky, 1971), we first tried to incubate L-Tyr-NA in crude protein extracts to detect a possible tyrosine aminopeptidase activity. Both L-Tyr-NA and L-Tyr-AMC were rapidly hydrolysed when incubated in enzyme extracts from tobacco or potato cell-suspension cultures. The hydrolysis of L-Tyr-NA was monitored photometrically whereas hydrolysis of L-Tyr-AMC was detected fluorometrically. Aliquots of the enzyme extracts were analysed by HPLC at regular intervals to check the nature of the products formed during the reaction. As expected, tyrosine and β-naphthylamine were the only products formed from L-Tyr-NA (Fig. 3) whereas tyrosine and 7-amino-4-methylcoumarin were rapidly formed from L-Tyr-AMC. The specific activities of tyrosine aminopeptidase in crude enzyme extracts prepared from potato or tobacco cell-suspensions one week after sub-culture were 6 and 1 nkat/mg protein, respectively. The rate of hydrolysis of L-Tyr-NA was similar in extracts from elicited and non-elicited cells. L-Tyr-NA was also hydrolysed when fed in vivo to tobacco or potato cell-suspension cultures. When added at a concentration of 10^{-4} M to the culture medium of tobacco cell suspensions, Tyr-NA was almost completely taken up after 3 h (Fig. 4). It was then rapidly metabolised inside the cells and became undetectable after 8 h. At that time β-naphthylamine could be detected in cell extracts but it was also quickly metabolised and became undetectable after 24 h (Fig. 4). Similar results were obtained with potato cell cultures (data not shown). Aminopeptidases are widespread enzymes in higher plants (Taylor, 1993; Chao et al., 1999). The rapid hydrolysis of L-Tyr-NA and L-Tyr-AMC therefore precludes their use in vivo as THT inhibitors. However, it may be possible to synthesize L-Tyr-NA analogues which would be resistant to aminopeptidases. Potent inhibitors of choline acetyltransferase resistant to cholinesterase have been obtained by synthesizing keto analogues of acetylcholine (Rama Sastry et al., 1988). Substitution of a ketone group for the amide bond of L-Tyr-NA may prevent the hydrolysis by aminopeptidases while keeping the inhibitory properties of the molecule. The preliminary results presented in this

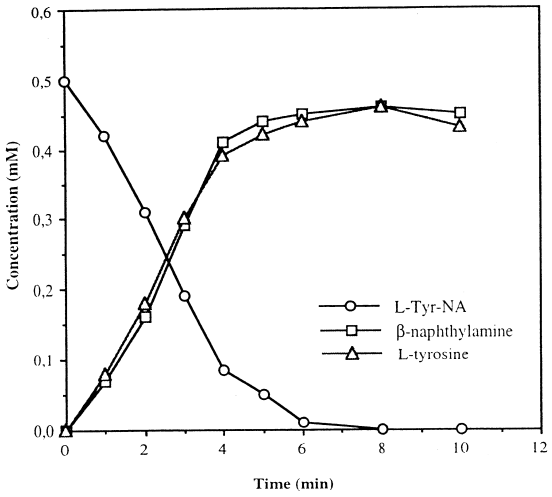


Fig. 3. Time course of hydrolysis of L-Tyr-NA in a crude enzyme extract from tobacco cells. The incubation mixture contained 1.14 mg protein and 0.5 μmol L-Tyr-NA in a total volume of 1 ml.

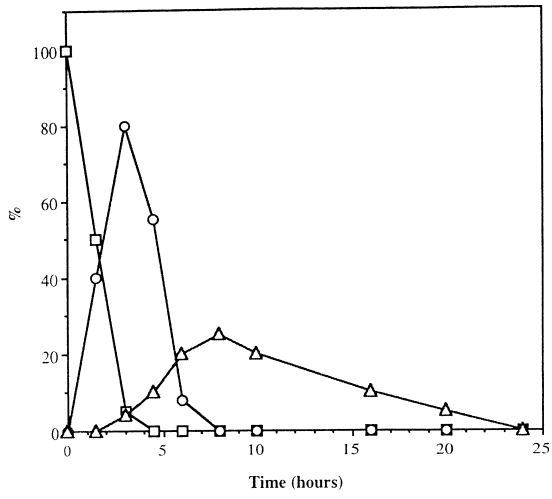


Fig. 4. Uptake and metabolism of L-Tyr-NA in tobacco cell-suspension cultures. Results are expressed as a percentage of the initial quantity of L-Tyr-NA (25 μmol) added to the culture medium: L-Tyr-NA in the culture medium (□), L-Tyr-NA (○) and β-naphthylamine (△) extracted from the cells.

paper show the usefulness of amino acid naphthylamides as tools to study the mechanism of action of amine *N*-hydroxycinnamoyltransferases and to identify the three-dimensional structure of the active site of these enzymes.

3. Experimental

3.1. Chemicals and substrates

L-Tyr-NA, L-Tyr-AMC, L-phenylalanine β-naphthylamide, L-arginine β-naphthylamide and L-tyrosine benzyl ester were all purchased from Sigma. Feruloyl-CoA was prepared by transesterification of feruloyl *N*-hydroxysuccinimide ester as previously described (Negrel and Javelle, 1997).

3.2. THT purification

The inhibition of tobacco THT was studied using an enzyme purified to apparent homogeneity from cell-suspension cultures of tobacco (*Nicotiana tabacum* L. cv Xanthi), as previously described (Negrel and Javelle, 1997). The potato enzyme was partially purified using the same protocol from pronase-elicited cell-suspension cultures of *Solanum tuberosum* L. (cv BF15), originally initiated from tuber callus tissue culture. The specific activity of the potato enzyme was 58 nkat/mg protein. It was completely free of tyrosine aminopeptidase activity. Inhibition of the recombinant tobacco enzyme was studied using a crude bacterial extract obtained after lysis of the XLOLR E. coli transformant (clone 10) in 0.1 M Tris–HCl buffer pH 7.5 containing 1 mg ml^{-1} lysosyme and 10 mM ME (Negrel and Javelle, 1997).

3.3. Enzyme assays

THT activity was measured photometrically as previously described (Negrel and Javelle, 1997). Addition of inhibitors did not interfere with the assay at 356 nm. None of the inhibitors tested was used as substrate by THT, using feruloyl-CoA as acyl donor. Tyrosine aminopeptidase activity was measured photometrically at 328 nm using L-Tyr-NA as substrate. The incubation mixture contained 200 μl protein extract, 800 μl 0.1 M Tris–HCl buffer pH 7.5 and 10 μl L-Tyr-NA 50 mM. Hydrolysis of L-Tyr-NA occured over a wide range of pH (5–10) with an optimum at pH 7.5. When L-Tyr-AMC was used as a substrate, the activity was measured in the same conditions but with a fluorometer, using activation and emission wavelengths of 380 and 460 nm, respectively.

3.4. HPLC

Products were separated on a Waters Novapack C18 column (3.9×300 mm, 4 μm) and detected using a two-channel UV detector at 280 and 328 nm. The following elution system was applied: linear gradient elution within 45 min from 10 to 85% MeOH in water acidified at pH 2 with 4% HCO_2H and 2 g/l ammonium formiate, using a flow rate of 0.8 ml/min. Retention times: L-tyrosine 5.45 min, β-naphthylamine 25.65 min, 7-amino-4-methylcoumarin 29.46 min, L-tyrosine benzylester 30.98 min, L-Tyr-AMC 32.50 min, L-Tyr-NA 36.43 min.

3.5. Metabolism of L-Tyr-NA

To monitor the degradation of L-Tyr-NA in vivo, 9.2 mg L-Tyr-NA dissolved in 2 ml MeOH 50% were added to 250 ml tobacco or potato cell-suspension cultures 1 week after sub-culture. Samples (25 ml) were withdrawn for analysis by pipetting from the treated cultures. Cells were then collected by centrifugation, frozen in liquid nitrogen and kept at −80°C until analysed. Aliquots of the supernatant were also frozen in order to monitor the uptake of L-Tyr-NA from the culture medium. Cells were extracted twice in MeOH acidified with 1% acetic acid (2 ml/ g fr. wt.). After centrifugation the MeOH extracts were pooled, evaporated to dryness, and taken up in 5 ml MeOH–H_2O–AcOH (3:1:1). Aliquots (20 μl) were then analysed by HPLC.

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Interaction of metal ions with lupin seed conglutin γ

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Abstract

Various metal ions were capable of aggregating and precipitating conglutin γ, an oligomeric glycoprotein purified from *Lupinus albus* seeds, at neutral pH values. The most effective metal ions, at 60-fold molar excess to the protein, were Zn^{2+}, Hg^{2+} and Cu^{2+}; a lower influence on the physical status of conglutin γ was observed with Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Sn^{2+} and Pb^{2+}, while Mg^{2+}, Ca^{2+} and Mn^{2+} had no effect at all. The insolubilisation of the protein with Zn^{2+}, which is fully reversible, strictly depended on both metal concentration and pH, with middle points of the sharp transitions at three-fold molar excess and pH 6.5, respectively. Conglutin γ is also fully retained on a metal affinity chromatography column at which Zn^{2+} and Ni^{2+} were complexed. A drop of pH below 6.0 and the use of chelating agents, such as EDTA and imidazole, fully desorbed the protein. A slightly lower binding to immobilised Cu^{2+} and Co^{2+} and no binding with Mg^{2+}, Cd^{2+} and Mn^{2+} were observed. The role of the numerous histidine residues of conglutin γ in the binding of Zn^{2+} is discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Lupinus albus*; Leguminosae; White lupin; Interactions; Metals; Zinc; Conglutin γ

1. Introduction

Lupin seed conglutin γ is an abundant oligomeric glycoprotein of still unknown function. However, several unusual features make this protein an interesting one to investigate both from the molecular and functional point of view. Conglutin γ accounts for about 5% of the total proteins of mature lupin seeds. It is a tetrameric protein, each monomer consisting of two disulphide-linked polypeptides of 29 and 17 kDa (Restani et al., 1981). The heavy chain is glycosylated (Duranti et al., 1981). Conglutin γ is deposited in the protein bodies along with the storage proteins, but it is not cleaved at germination (Duranti et al., 1994a) and is actively secreted upon heat-treatment of mature seeds (Duranti et al., 1994b). Moreover, this protein has been localised in the extracellular spaces of germinating cotyledons (Duranti et al., 1994a) and in the seedling radicles (Duranti et al., 1996). Amino acid sequence of *Lupinus albus* conglutin γ deduced from the nucleotide sequence of the corresponding gene (Scarafoni et al., 1998) has proved to be homologous to the 7S basic globulin (Bg7S) of soybean, which showed protein kinase activity (Komatsu et al., 1994) and receptor-like properties, being capable of binding insulin-related peptides (Komatsu and Hirano, 1991) and leginsulin, a hormone-like peptide purified from soybean seeds (Watanabe et al., 1994). Apart from this sequence homology, which is also partially shared with other scarcely characterised plant proteins (Satoh et al., 1992; Chung et al., 1997), conglutin γ did not show any similarity with known catalytic or biologically active proteins.

In the search of a function of this protein in the seeds and other tissues where it has been located, we have undertaken a study aimed at evidencing some molecular property which might help understanding its role. In this work we present the results obtained by treating purified conglutin γ with various divalent and trivalent metal ions and report its preferential interaction with zinc ions.

2. Results

The effect of a number of divalent and trivalent metal ions on the solubility of purified conglutin γ in buffered neutral solutions at fixed protein and metal concentrations has been tested as a preliminary approach to

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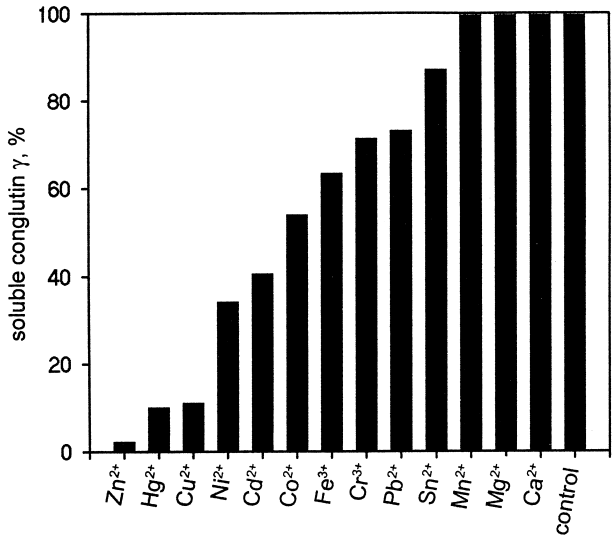


Fig. 1. Solubility of conglutin γ in the presence of metal ions. Conglutin γ (17 μM) was incubated with each metal at a final concentration of 1 mM, as detailed in the Methods. Soluble conglutin γ in the supernatants was determined spectrophotometrically. Each result, is expressed as percent of soluble conglutin γ in the control without metals and represent the average of two separate assays.

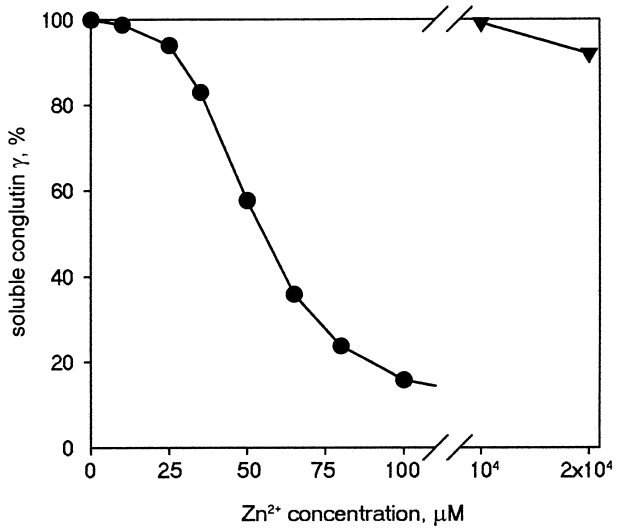


Fig. 2. Dependence of conglutin γ insolubilisation on Zn^{2+} concentration. Conglutin γ (20 μM) was treated with various Zn^{2+} concentrations at pH 7.5 (●-●) and 4.5 (▼-▼) and the amount of soluble fraction, spectrophotometrically assessed, was expressed as a percentage of the control without Zn ions. Results are the average of two separate assays.

evaluate the interaction of the protein with these ions (Fig. 1). Three metal ions, namely Mg^{2+}, Ca^{2+} and Mn^{2+}, showed no effect on the physical status of conglutin γ. Other metals, namely Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Sn^{2+} and Pb^{2+}, to various extent decreased conglutin γ solubility. However, the most striking effects were observed with Zn^{2+}, Hg^{2+} and Cu^{2+} which caused an almost complete insolubilisation of the protein. When conglutin γ precipitated by these metals was resuspended in a buffered solution containing 50 mM EDTA, the protein became soluble again with a yield $\geqslant$ 90%, suggesting an almost complete reversibility of the process. Thus, metal-induced insolubilisation did not implicate denaturation of conglutin γ, which would have prevented a complete resolubilisation of the protein.

Since the main aggregation effect was observed with Zn^{2+}, we decided to investigate in greater detail the modalities of interaction of conglutin γ with this ion. To this purpose, the dependence of conglutin γ insolubilisation on Zn^{2+} concentration at two pH values, which will be shown to be critical for the phenomenon to occur, has been studied (Fig. 2). While at acidic pH no significant difference from the control experiment was observed at any Zn^{2+} concentration, at pH 7.5 conglutin γ insolubilisation was sharply dependent on Zn^{2+} concentration. When the Zn^{2+} concentration was equimolar to the protein, solubility was scarcely affected, but already at five-fold metal excess, precipitation of conglutin γ was almost complete. The mid point of this transition occurred at about three-fold molar excess to the protein.

Loss of solubility of conglutin γ by Zn ions was also strongly pH dependent (Fig. 3), in that a relatively small pH change dramatically altered the physical status of conglutin γ in the presence of the metal, being its insolubilisation negligible at pH 4.5, but quantitative at pH 7.5. The mid point of this transition was around pH 6.5.

Since metal-induced protein insolubilisation is a relatively undetermined phenomenon which may not necessarily reflect the occurrence of a specific interaction, we decided to investigate the binding of conglutin γ to a Metal Affinity Chromatography (MAC) matrix by coupling various divalent metal ions. The results obtained with MAC were fairly consistent with the solubility trials, in that most, but not all, of the metals causing a significant loss of solubility of conglutin γ at a fixed metal concentration, also bound the protein to the stationary phase (Fig. 4). Again, we focussed on the interaction of conglutin γ with Zn^{2+} and studied the dependence of the protein binding to the Zn^{2+}-coupled matrix (ZnAC) under different experimental conditions. Conglutin γ binding was quantitative at neutral pH, while at acidic pH, complete desorption of the protein took place, as shown by the corresponding elution profile in Fig. 5. The transition was as sharp as in the insolubilisation trials, with a mid point very close to pH 6.0. In addition to the drop of pH below 6.0, two different metal chelating agents, namely EDTA and imidazole, did quantitatively elute the bound protein (Fig. 5). Non-precipitating Zn^{2+} concentrations in the elution buffer, that is around 5 μM in the conditions used for ZnAC, did not desorb the protein, seemingly because they were far too low to compete with the concentration of the

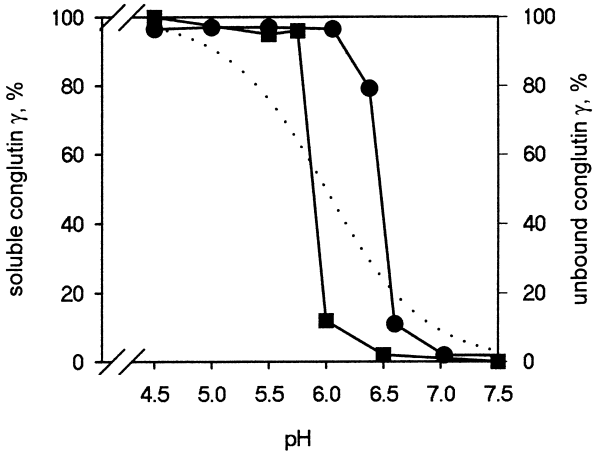


Fig. 3. Dependence of conglutin γ insolubilisation and binding to ZnAC on the pH. Conglutin γ (20 μM) was treated with 1 mM Zn^{2+} at the pH indicated. The amount of soluble fraction, spectrophotometrically assessed, was expressed as percent of the control without Zn ions (●-●). Results are the average of two separate assays. The dependence of conglutin γ binding to ZnAC on pH is expressed as percent of the unbound conglutin γ with respect to the total loaded protein (■-■). The titration of histidine imidazole group (...) is also reported as percent of positively charged amino group.

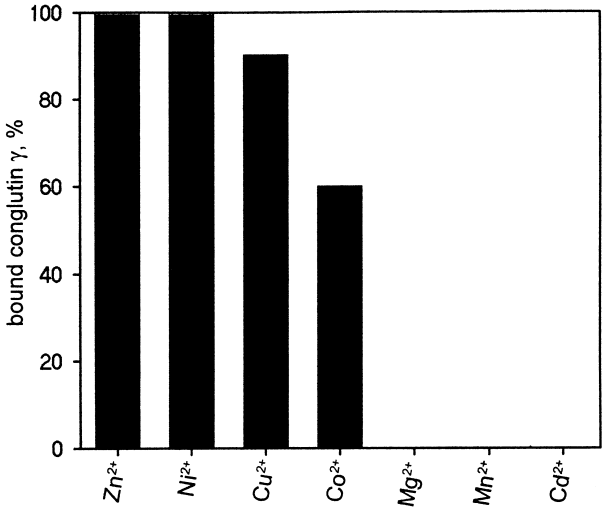


Fig. 4. Effect of various metal ions on the binding of conglutin γ to MAC. Conglutin γ (13 μM) was loaded on a column of iminodiacetic acid immobilised on cross-linked 4% beaded agarose complexed with the metal ions indicated. The amount of bound conglutin γ is expressed as percent of the total loaded protein.

metal bound to the matrix, claimed to be in the range of 30-40 mM by the manufacturers. However, when conglutin γ pre-treated with equimolar concentrations of Zn^{2+}, was loaded on the ZnAC column, the binding of the protein was reduced to 15% of the control (not shown).

3. Discussion

It has long since been known that the interaction of metal ions with proteins can lead to aggregation and consequently precipitation of the protein molecules (Hughes and Klotz, 1956). This phenomenon has been used to get selective precipitation of a protein from a mixture. This was the case of hemoglobin, which has been shown to precipitate from the serum with various metal ions, including Zn^{2+} and Cu^{2+} as the most effective agents (Frantzen et al., 1997). In some cases metal precipitation of the proteins is accompanied by denaturation, which usually significantly reduce their solubility (Adhikari et al., 1998). However, this does not seem to be the case of conglutin γ, which immediately becomes again soluble by simply complexing the precipitating metal with EDTA.

The finding that conglutin γ underwent aggregation and precipitation with various metals, in particular with those affecting also the solubility of hemoglobin, prompted us to investigate further the modalities of this interaction, especially with Zn ions, which had showed the greatest effects on the solubility of the protein. Indeed, pH dependence of both conglutin γ precipitation by

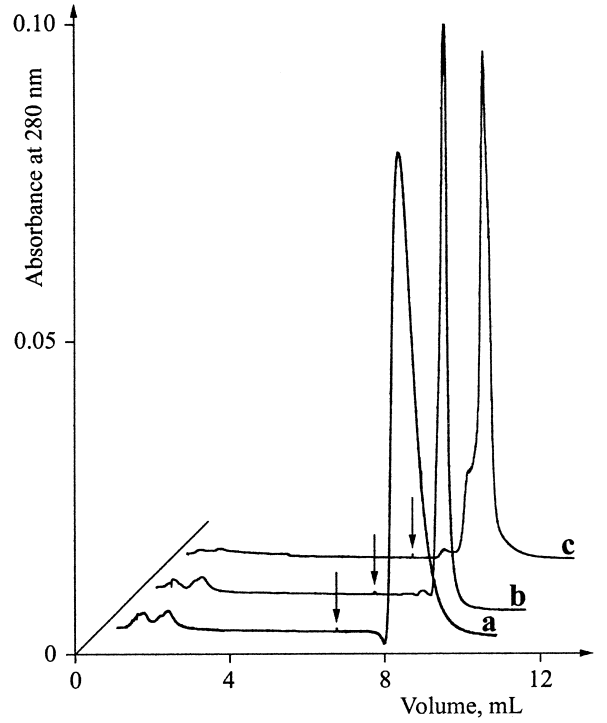


Fig. 5. ZnAC elution profiles of conglutin γ. The protein (400 μg) was loaded on the column as described in the section 4.4. After washing with 50 mM Tris-HCl, pH 7.5, containing 500 mM NaCl, the following elution buffers were applied (arrows): sodium acetate, pH 4.5 (a); washing buffer containing 250 mM imidazole (b); washing buffer containing 10 mM EDTA (c).

Zn^{2+} and binding to ZnAC matrix appeared to be very strict and both transitions closely corresponded to the pK_a of 6.0 of histidine side chain amino group (Fig. 3). Although this is an indirect evidence of the involvement

of histidine side chains in the interaction of conglutin γ with Zn ions, and possibly with the other metals too, these two independent results strongly suggest that this is the case. The involvement of these side chains in the binding of Zn ions has already been reported for various proteins, including bovine prolactin (Permyakov et al., 1997), carbonic anhydrase (Hunt et al., 1999), histatin-5 (Melino et al., 1999) and retinal-binding protein (Müller and Skerra, 1994). Indeed, *Lupinus albus* conglutin γ is relatively rich of histidine residues (19 out of 419 amino acid residues) (Scarafoni et al., 1998). On the other hand, the involvement of-SH groups in the interaction of conglutin γ with Zn^{2+} can be excluded since all the 12 cysteine residues of the protein are involved in disulphide bridges (Scarafoni et al. 1998; Duranti, M., unpublished results), and the alkaline pK_a of the thiol groups is far from the pH at which the observed transitions occur.

With the notable exception of Cd^{2+} which, despite aggregating about 60% of conglutin γ in our experimental conditions, did not bind the protein in MAC, the other metals tested behaved similarly both in MAC and as precipitating agents, with Zn^{2+}, Ni^{2+} and Cu^{2+} as the most effective ligands, Co^{2+} as less good ligand, Mn^{2+} and Mg^{2+} as non-ligands nor precipitating agents. This finding suggests that, although the two distinct phenomena not obviously relate to each other, they might have a common molecular basis.

The quantitative desorption of conglutin γ from the ZnAC column promoted by acidic pH and metal-chelating agents indicates a fully reversible binding of the protein to the matrix, as it was observed in the insolubilisation trials. The possibility that conglutin γ precipitates in the column, in analogy to the observed metal-induced insolubilisation of the free protein, cannot be excluded. However, if this is the case, the complete reversibility of the binding suggests that insolubilisation is not likely to involve conglutin γ unfolding, which, according to other studies (Duranti et al., 2000), would prevent prompt resolubilisation and desorption of the protein to take place.

Although the present results do not provide sufficient information to shed light on the physiological significance of the metal binding by conglutin γ, the possibility of a specific function of the metal could not be excluded. Further studies, aimed to assess the binding parameters, the stability of the apo- and holo-protein and the molecular determinants of the metal interaction with conglutin γ, are in progress.

Moreover, since some of the metals causing significant aggregation of conglutin γ belong to the class of toxic heavy metals, such as Cr^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+}, the possibility of exploiting the conglutin γ properties described in this paper for detoxification processes could be envisaged and is also currently being investigated.

4. Experimental

4.1. General procedures

The concentration of the conglutin γ solutions was determined by measuring the absorbance at 280 nm assuming an absorption coefficient of one for a 1 mg ml^{-1} solution (Duranti et al., 2000). Molar concentrations of conglutin γ were calculated considering the protein in its monomeric form, that is with M_r 46,000.

Chromatographic elutions were followed at 280 nm using a LKB Uvicord monitor.

All the metals used were in the form of chloride salts.

4.2. Purification of conglutin γ from mature lupin seeds

Conglutin γ was purified essentially as described by Duranti et al. (1994b). The purified conglutin γ was found to be homogeneous by SDS-PAGE (not shown). Purified conglutin γ was precipitated with ammonium sulphate (80% saturation) and then resuspended in 50 mM Tris-HCl, pH 7.5 containing 50 mM EDTA in order to remove any metal ion present in the preparations. Removal of salts and metal-EDTA complexes was carried out by gel filtration using a G25 Sephadex column.

4.3. Metal ion-induced insolubilisation of conglutin γ

Solutions of conglutin γ, from 17 to 20 μM in 50 mM Tris–HCl buffer, pH 7.5 have been used for the insolubilisation assays at room temperature with 1 mM concentrations of the various metal ions. The resulting mixtures were stirred and then centrifuged at 10,000 × ***g*** for 20 min. Protein concentration in the supernatants was spectrophotometrically determined against a blank containing the corresponding metal concentration in the buffer. Assays with Zn ions at different pH values were carried out by using the following buffers: 50 mM sodium acetate, pH 4.5; 50 mM 2-morpholinoethanesulfonic acid (MES), pH from 5.5 to 6.5; 50 mM Tris–HCl, pH 7.5.

4.4. Metal affinity chromatography (MAC)

A column (2 cm × 1 cm) of iminodiacetic acid immobilized on cross-linked 4% beaded agarose (SIGMA) was set up and equilibrated in 50 mM Tris–HCl buffer, pH 7.5 containing 500 mM NaCl (binding buffer) and 2 mg ml^{-1} of the desired metal ion. The metal excess was washed with 10 volumes of the binding buffer. One millilitre of 13 μM conglutin γ solution was applied to the column. Adsorbed conglutin γ was eluted with 50 mM sodium acetate buffer, pH 4.5 containing 500 mM NaCl, and with the binding buffer containing either 10 mM EDTA or 250 mM imidazole.

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Patterns of phenylpropanoids in non-inoculated and potato virus Y-inoculated leaves of transgenic tobacco plants expressing yeast-derived invertase

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Abstract

The patterns of secondary metabolites in leaves of yeast invertase-transgenic tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were analyzed. Plants expressing cytosolic yeast-derived invertase (cytInv) or apoplastic (cell wall associated) yeast invertase (cwInv) showed a characteristic phytochemical phenotype compared to untransformed controls (wild-type plants). The level of phenylpropanoids decreased in the cytInv plants but increased in the cwInv plants, which showed an induced de novo synthesis of a caffeic acid amide, i.e. *N*-caffeoylputrescine. In addition, the level of the coumarin glucoside scopolin was markedly enhanced. Increased accumulation of scopolin in the cwInv plants is possibly correlated with the induction of defense reactions and the appearance of necrotic lesions similar to the hypersensitive response caused by avirulent pathogens. This is consistent with results from potato virus Y-infected plants. Whereas there was no additional increase in the coumarins in leaves following infection in cwInv plants, wild-type plants showed a slight increase and cytInc a marked increase. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Nicotiana tabacum*; Biotechnology; Sugar sensing; Yeast invertase; Potato virus Y (PVY); Phenylpropanoids; Caffeic acid conjugates; Scopolin

1. Introduction

It has previously been shown that transgenic tobacco plants, expressing vacuolar or apoplastic yeast-derived invertase, develop necrotic lesions similar to hypersensitive responses caused by avirulent pathogens (Herbers et al., 1996). This phenomenon was accompanied by increased resistance to potato virus Y (PVY). Thus, it is tempting to assume that the accumulation of soluble sugars in these transgenic plants (Sonnewald et al., 1991) induces the production of secondary metabolites, possibly phenolics, shown to be involved in defense responses (Matern and Kneusel, 1988; Hahlbrock and Scheel, 1989; Dixon et al., 1994). Indeed, it was found that defense-related genes are inducible by soluble sugars, for example those encoding enzymes involved in the phenylpropanoid metabolism, i.e. chalcone synthase (Tsukaya et al., 1991), phenylalanine ammonia-lyase and peroxidase (Herbers et al., 1996). This holds also true for the sesquiterpenoid metabolism. Herbers et al. (1996) described sugar-inducible expression of the gene encoding 5-*epi*-aristolochene synthase, a pivotal enzyme in the formation of the tobacco-characteristic phytoalexin, capsidiol (Bailey et al., 1975).

These results suggested the formation of defensive secondary compounds as a result of genetic manipulation of the carbohydrate metabolism in tobacco plants and prompted us to perform the present phytochemical study. We also show here significant changes in the patterns of phenolic metabolites in plants expressing yeast-derived invertase.

2. Results and discussion

Greenhouse-grown wild-type and yeast-derived invertase-expressing transgenic tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were obtained as described

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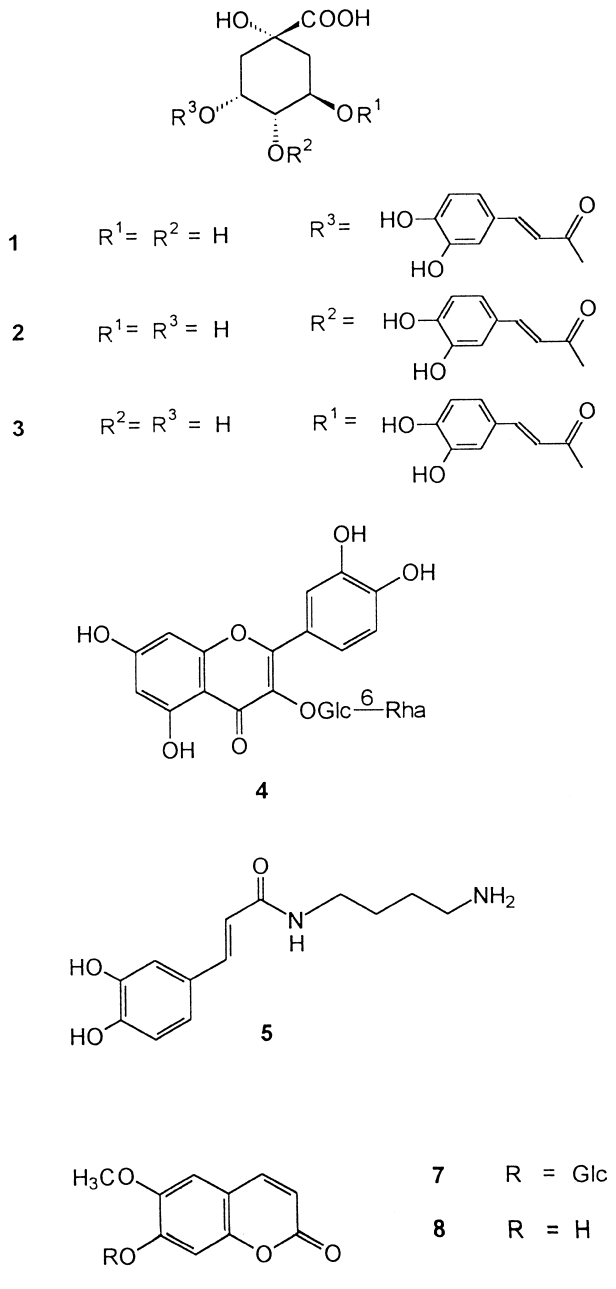
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previously (Herbers et al., 1996). Mature plants were inoculated with PVY or water for mock inoculation. Source leaves were harvested three days (3 dpi; early infection stage) and nine days (9 dpi; fully infected) after inoculation along with leaves of wild-type control plants. Leaves from these plants were analyzed by HPLC with regard to possible changes in the pattern of secondary metabolites (phenylpropanoids, isoprenoids).

HPLC analyses of methanolic leaf extracts showed patterns of several major and minor UV light-absorbing soluble phenolic compounds (Fig. 1A–C). The main ones from wild-type plants were identified by GC–MS according to Fuchs and Spiteller (1996) to be the 3-*O*-, 4-*O*- and 5-*O*-isomers of caffeoyl quinates [neochlorogenic (**1**), cryptochlorogenic (**2**) and chlorogenic acid (**3**); see (scheme 1)]. These caffeic acid esters are well known tobacco constituents (Jean and Reid, 1959; Koeppe et al., 1969). Quercetin 3-*O*-rutinoside (rutin, **4**), known to be a predominant flavonoid in tobacco species (Snook et al., 1992), was identified by comparison with a standard compound. Rutin showed high quantitative variations, however, there was in the cytInv plants an increase at 3 dpi from 0.95 to 1.4 nmol and a decrease at 9 dpi from 2.1 to 1.2 nmol g^{-1} dry wt. compared to the wild-type plants. In the cwInv plants rutin slightly decreased to ca. 0.5 nmol at 3 and 9 dpi. It should also be noted that tryptophan (peak 6 in Fig. 1) increased in leaves of some cwInv plants (see Fig. 1C). Its level was also enhanced in some cytInv plants and wild-type controls after infection with PVY (not shown).

Leaves of cytInv plants showed the same chromatographic profile of phenylpropanoids [photodiode array (PDA)-HPLC)] as wild-type controls (Fig. 1A B), but the amount of the caffeic acid esters was reduced (Fig. 2). In the cwInv plants, however, the accumulation of new compounds was observed (peak **5** and several minor unidentified peaks marked with arrows in Fig. 1C). The unidentified peaks showed UV spectra characteristic to phenolic compounds (not shown). Fig. 2 shows the levels of compound **5** in the cwInv plants, adding to the caffeic acid esters. This compound was isolated by preparative HPLC as described from elicited potato cell cultures (Keller et al., 1996) and identified by electrospray ionization mass spectrometry (ESI–MS/MS) as *N*-caffeoylputrescine. The positive ESI–MS of **5** showed the protonated molecular ion at *m*/*z* 251 $[M+H]^+$. The fragmentation patterns observed in the MS/MS of the $[M+H]^+$ ion showed a signal at *m*/*z* 234 which is formed by the loss of NH_3. The fragments at *m*/*z* 163 and *m*/*z* 89 are characteristic for the caffeoyl and putrescine ($[amine+H]^+$) moieties, respectively.

Compound **5** was not detected in leaves of wild-type or cytInv plants prior to or after infection with PVY. However, it has been shown that it is particularly abundant in tobacco flowers (Buta and Izac, 1972; Leubner-Metzger and Amrhein, 1993) as well as in cell cultures of *Nicotiana tabacum* (Misuzaki et al., 1970, 1971; Berlin, 1981). It has been found that compound 5 is induced in cell cultures treated with an elicitor preparation from *Phytophthora infestans*, which hints at a possible role in plant defense responses against fungi (Keller et al., 1996).



Scheme 1.

Analyses of methanolic extracts by HPLC with fluorescence detection revealed the accumulation of two major fluorescent compounds in leaves of all plants studied (Fig. 1D–F). They were identified as the coumarin glucoside scopolin and its aglycone, scopoletin, by LC–MS (not shown) and comparison with standard compounds (HPLC-PDA). It was most interesting to

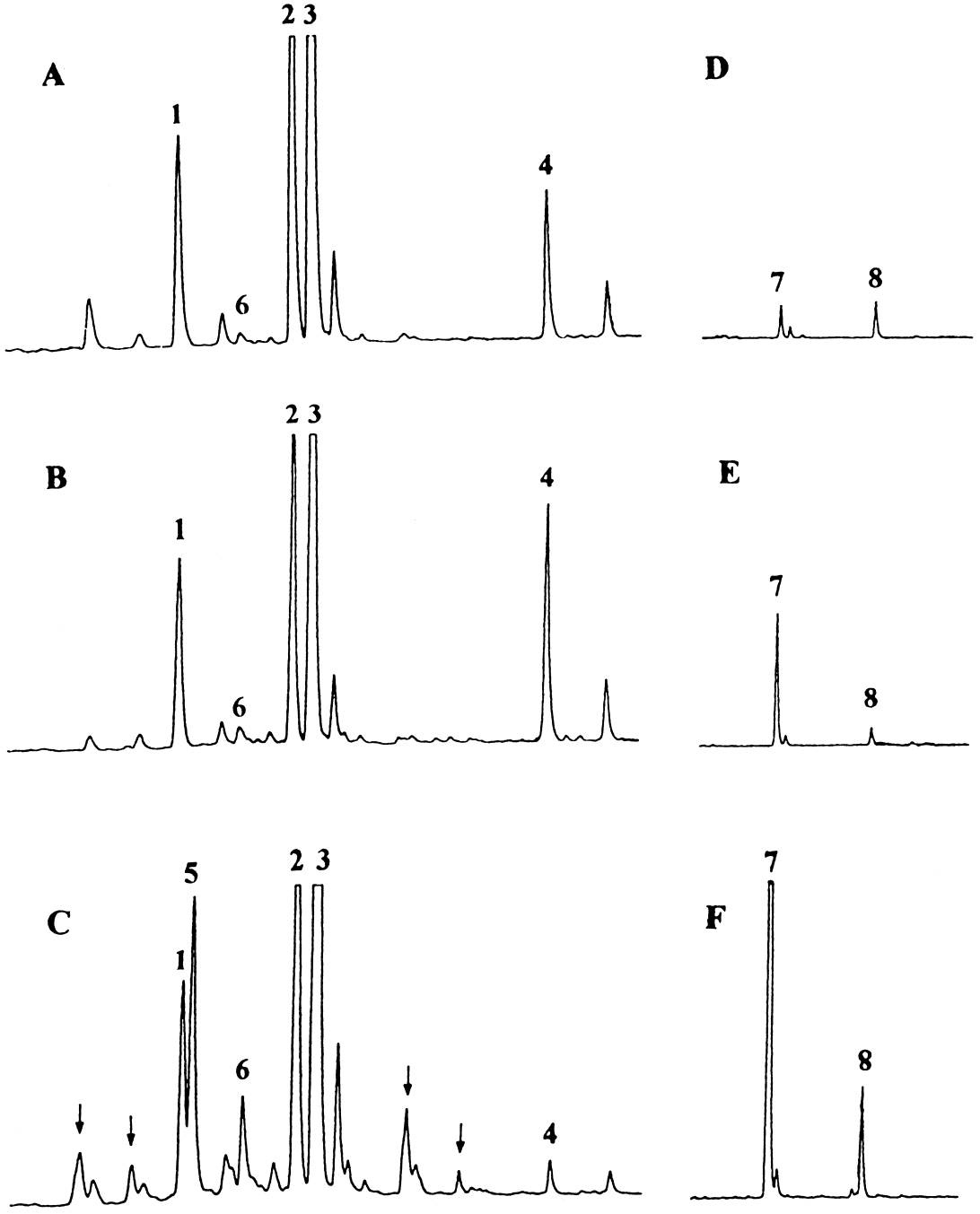


Fig. 1. Representative HPLC diagrams of methanolic extracts from leaves of wild-type tobacco plants (**A**, **D**), cytInv plants (**B**, **E**) and cwInv plants (**C**, **F**). **A**, **B**, **C**: 5- to 30-min traces of maxplot detection between 210 and 500 nm (0.2 full scale absorbance). **D**, **E**, **F**: 10- to 30-min traces of fluorescence detection; fluorescence excitation at 380 nm, emission at 430 nm (50 mV scale). Peak identification: **1**, neochlorogenic acid (λ_{max}, 325 nm); **2**, cryptochlorogenic acid (λ_{max}, 326 nm); 3, chlorogenic acid (λ_{max}, 326 nm); **4**, rutin (λ_{max}, 356 nm); **5**, caffeoylputrescine (λ_{max}, 319 nm); **6**, tryptophan (λ_{max}, 218 and 278 nm); arrows in trace C, unidentified UV light-absorbing compounds; **7**, scopolin; **8**, scopoletin.

note that the level of scopolin was more than 20-fold higher in leaves of cwInv plants compared to wild-type and cytInv plants (Fig. 2). Furthermore, when necrotic and non-necrotic leaf areas of the cwInv plants were separately analyzed, it was found that scopolin accumulation correlated with the formation of necrotic lesions (Table 1). These results are consistent with those obtained from PVY-infected plants. Whereas there was no additional increase in fully infected cwInv plants, wild-type plants showed a slight and cytInv plants a marked increase of the levels of scopolin after PVY infection.

It is known that coumarins are involved in plant defense reactions. Scopoletin has been found to express antimicrobial activity (El Modafar et al., 1995; Valle et al., 1997). Scopolin accumulation was observed after

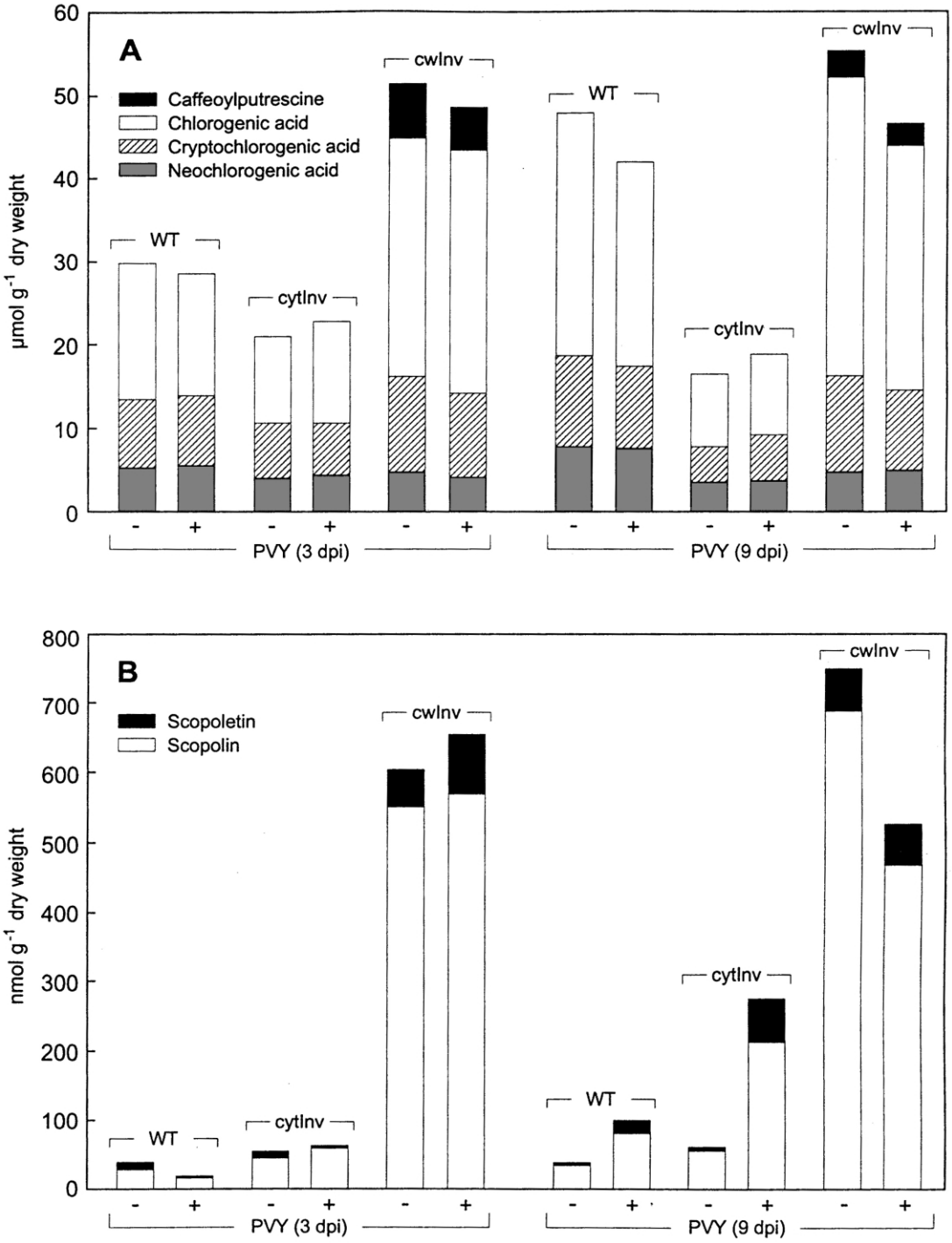


Fig. 2. Levels of the caffeic acid conjugates neochlorogenic acid (**1**), cryptochlorogenic acid (**2**), chlorogenic acid (**3**) and caffeoylputrescine (**5**) in panel A and the coumarins scopolin (**7**) and scopoletin (**8**) in panel B in wild-type, cytInv and cwInv tobacco plants without PVY infection (–PVY) and with PVY infection (+PVY) after 3 days (3 dpi; early stage) and 9 days (9 dpi; fully infected) of treatment. Data (µmol caffeic acid conjugates and nmol coumarins g^{-1} dry wt.; standard deviations up to 30%) represent the mean from three individual plants, three leaves from each plant pooled.

TMV (tobacco mosaic virus) inoculation of tobacco (Fritig and Hirth, 1971) and in potato tubers infected by *Phytophthora infestans* (Hughes and Swain, 1960). Tobacco plants sprayed with scopoletin prior to TMV infection showed reduced formation of necrotic lesions and it was suggested that high levels of scopolin and scopoletin in an amphidiploid tobacco hybrid contributes to its high disease resistance (Ahl-Goy et al., 1993). Sharan et al. (1998) described the effects of methyl jasmonate and *Fusarium solani* elicitor on stimulation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco cell

Table 1
Levels of scopolin in necrotic and non-necrotic leaf areas of cwInv plants. The data (nmol g^{-1} dry wt) represent the mean values±S.D. obtained from three independent experiments

Leaf number[a]	Scopolin (**7**)	
	Leaf area without lesions	Leaf area with lesions
6	37.5±7.5	n.d.[b]
7	72.5±5.5	2625±350
8	160.0±20.0	2475±300
9	n.d.	2775±375

[a] Leaves counted from the top to the base.
[b] n.d., complete separation of the different areas not possible.

cultures. Thus, increased accumulation of scopolin in the cwInv plants, reaching highest concentrations in the necrotic leaf areas, is consistent with the induction of defense reactions. The induction of a whole set of defense responses including scopolin accumulation was also observed in tobacco plants with de-regulated tetrapyrrole biosynthesis (Mock et al., 1999), exhibiting highest levels of scopolin in the necrotic leaf areas.

Nonpolar compounds (isoprenoids), extracted with methylene chloride from leaves of transgenic plants, did not show any qualitative changes in the chromatographic profiles in comparison to wild-type plants (although the HPLC system was optimized for detection of capsidiol with standard compound). All attempts failed to detect sesquiterpenoid phytoalexins such as capsidiol, characteristic of tobacco (Bailey et al., 1975), either in the transgenic plants or in potato virus Y(PVY)-inoculated ones. However, it was noted that the amounts of most nonpolar compounds in the cytInv and cwInv plants, including carotenoids, were markedly reduced. Two major carotenoids were recognized by their characteristic visible absorption spectrum (Zscheile et al., 1942) and were identified as lutein and violaxanthin by cochromatography (PDA-HPLC) with standard compounds. Their levels were reduced to more than 50% in both cytInv and cwInv plants compared to wild-type plants (data not shown). No corresponding differences were observed between PVY-infected and uninfected plants.

3. Conclusions

The most interesting results were obtained with the cwInv plants which showed marked changes of the patterns of phenylpropanoids, especially caffeic acid conjugates and the coumarin scopolin. *N*-Caffeoylputrescine was synthesized de novo and the level of scopolin dramatically increased. Based on these results, further studies should focus on changes of the enzyme activities and expression of the encoding genes involved in the biosyntheses of these compounds, i.e. the hydroxycinnamoyl–CoA: putrescine hydroxycinnamoyltransferase (Meurer-Grimes et al., 1989) in the formation of *N*-caffeoylputrescine as well as the pivotal enzyme of coumarin biosynthesis catalyzing the 2′-hydroxylation of hydroxycinnamic acids (Matern, 1991). This enzyme is essential for the lactone formation leading to the basic coumarin skeleton (Strack, 1997).

It should finally be stressed that the induced accumulation of phenylpropanoids could only be detected in the cwInv plants and not in the cytInv ones, where they significantly decreased. These differences may explain in part the resistance response of cell wall invertase-expressing tobacco plants towards PVY infection, as has been reported by Herbers et al. (1996). Since fungal pathogens may secrete invertases to hydrolyze extracellular sucrose in the apoplast of infected plants, plants may recognize apoplastic sugar perturbations and respond by induction of PR proteins and the production of defensive secondary compounds. The role of extracellular sugars and cell wall invertases as amplifiers of plant defense responses has recently been proposed (Herbers et al., 2000). Future experiments will have to clarify whether metabolic or signaling functions of sugars prevail in invertase-expressing tobacco plants.

4. Experimental

4.1. Material

Plants (*Nicotiana tabacum* cv. Samsun NN) were grown in soil with a 16-h-light and 8-h-dark regime. Sunlight was supplemented with artificial light between 200 and 250 μmol m^{-2} s^{-1}. The transgenic plants used were A-41-10 (cwInv) and U-In5 (cytInv) (von Schaewen et al., 1990; Sonnewald et al., 1991). Infection with potato virus Y (PVY) was performed as described (Herbers et al., 1996).

Leaf samples were harvested 5 h after beginning of the light period. Plants had developed 15–17 leaves at sampling time. Data were obtained from 3 individual plants of each genotype and/or treatment. Each sample was pooled of 3 different mature source leaves.

Standard tryptophan was purchased from Merck (Darmstadt, Germany), rutin from Roth (Karslruhe, Germany), chlorogenic acid from Serva (Heidelberg), and scopoletin from Sigma (Deisenhofen, Germany).

4.2. Extraction and HPLC of soluble phenolics

Lyophilized leaf material (20 mg) was powdered and extracted (3×1 ml each) with MeOH–H_2O (4:1) in 2 ml-safe-lock tubes in the presence of zirconia beads (1 mm diam.) using a bead beater (Bio Spec Products, Inc., Bartlesville, OK, USA). After centrifugation the combined supernatants were evaporated and the residue

redissolved in 1 ml of MeOH-H_2O (4:1). The extracts were centrifuged again and 20 μl samples were injected onto a Nucleosil C_{18} column (5 μm; 250×4 mm i.d.; Macherey-Nagel, Düren, Germany). Separation was achieved using a 25-min linear gradient at 1 ml min^{-1} from 4 to 25% MeCN in 1.5% aq. H_3PO_4 followed by a gradient from 25 to 90% MeCN within 3 min. Phenolic compounds were photometrically detected (maxplot between 210 and 500 nm) by a Waters 996 photodiode array (PDA) detector. Scopolin and scopoletin were analyzed from the same extracts by a Waters 474 scanning fluorescence detector (excitation wavelength 380 nm; emission wavelength 430 nm).

4.3. Extraction and HPLC of nonpolar compounds

Lyophilized leaf material (20 mg) was extracted with CH_2Cl_2 (4×1 ml each) using a bead beater and centrifuged. The combined extracts were dried and the residues redissolved in 1 ml 2-propanol. The extracts were centrifuged again and 20-μl samples injected onto a Nucleosil C_8 column (5 μm; 250×4 mm i.d.; Macherey-Nagel, Düren, Germany). Separation was achieved using a 25-min linear gradient at 1 ml min^{-1} from 30 to 80% MeCN in 1.5% aq. H_3PO_4 and another 25-min gradient from 80 to 100% MeCN. The compounds were photometrically detected (PDA; maxplot between 210 and 500 nm). Putative carotenoids were characterized by their specific online absorption spectrum in the visible region, i.e. 410–480 nm.

4.4. LC–MS of N-Caffeoylputrescine

ESI–CID–MS (*m*/*z*, rel. int.): 251 $[M+H]^+$ (53), 234 (5), 163 (100), 145 (7), 135 (10), 89 (10). The positive ion ESI mass spectra and the ESI–MS/MS measurements were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220°C; sheath gas nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1×100 mm, SEPSERV). *N*-Caffeoylputrescine was separated at 70 μl min^{-1} with a 15-min gradient from 20 to 90% MeOH in H_2O (containing 0.2% HOAc) followed by 10 min of isocratic elution at 90% MeOH in H_2O. The collision-induced dissociation (CID) mass spectrum of *m*/*z* 251 ($[M+H]^+$, *N*-caffeoylputrescine) was performed during the HPLC run under the following conditions: collision energy-25 eV; collision gas argon, collision pressure 1.8×10^{-3} Torr. All mass spectra were averaged and background subtracted.

4.5. Preparative HPLC and GC–MS

N-Caffeoylputrescine was isolated by preparative HPLC as described elsewhere (Keller et al., 1996). Neochlorogenic, cryptochlorogenic and chlorogenic acids were identified by GC–MS according to Fuchs and Spiteller (1996).

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Predicting quantitative phytochemical markers in single *Echinacea* plants or clones from their DNA fingerprints

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Abstract

Amplified restricted fragment length polymorphism (AFLP™) data analysis was found to be a statistically significant predictor of phytochemical markers in cultivated *Echinacea purpurea* germplasm and some related wild species. Over 50 accessions grown under greenhouse conditions were subjected to AFLP analysis and the same assessed for content of tetraene and cichoric acid by high pressure liquid chromatography. The first and second canonical correlation of DNA variables and the phytochemical variables were significant. Individual regressions of cichoric acid and dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutyl amide predicted by DNA polymorphism analysis against actual HPLC determined values were nearly linear. Mantel's test showed that there was a weak correlation but a strong association of values of the phytochemical variables and the DNA polymorphism data. Crown Copyright © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Echinacea*; AFLP; Cichoric acid; Dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutyl amide; DNA fingerprinting

1. Introduction

At least two attributes of quality control are required in the trade of herbal plants used for pharmaceutical purposes. One is authentication of species identity whereas the other is prediction of the concentration of active phytochemicals. Authentication is often intimately related to prediction. Identifying a particular strain or species correctly will ensure that the right plant material will be used for a specific treatment of an ailment. Authenticity may unfortunately not always predict the actual level of particular active principles in authenticated plant materials. In the "*Echinacea* germplasm enhancement project" (Baum, 1999; Baum et al., 1999) the objective is to achieve both authenticity at the level of species and prediction of the amounts of specific phytochemicals associated with selected lines using molecular tools, specifically fingerprinting by AFLP™ (amplified restricted fragment length polymorphism; Vos et al., 1995). Generation of DNA fingerprints by AFLP have been compared with other commonly used fingerprinting techniques [see, for instance, Powell et al. (1996) and McGregor et al. (2000)]. We are also investigating the possibility of phytochemical prediction for a broad range of wild and cultivated material. The basis for the prediction of phytochemical markers is the DNA fingerprint. The aim of this paper is to address the possibility of predicting the level of dodeca- 2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutyl amide (**1**, **2**) ("tetraene") and cichoric acid (**3**), two well documented bioactive phytochemicals in *Echinacea* (Bauer, 1998) in particular lines or clonal materials from their DNA fingerprints.

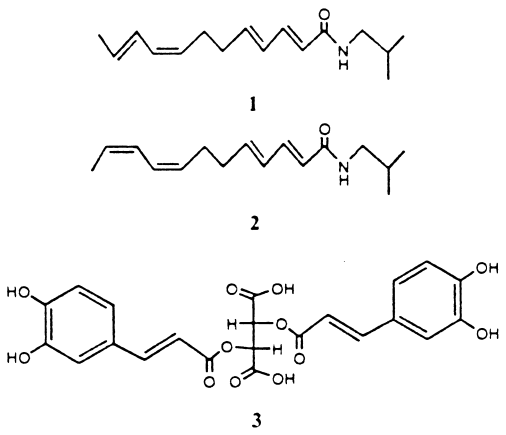
2. Results

2.1. DNA fingerprints

The AFLP data set consisted of 232 binary variables for 52 plants from the following combination of primers (see Experimental section of this paper). Eco-ACC/Mse-CCG yielded 28 unambiguous scorable DNA polymorphisms;

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Eco-ACC/Mse-CGC yielded 57 polymorphisms; Eco-AGC/Mse-CCG yielded 54 polymorphisms; Eco-AGG/Mse-CCG yielded 53 polymorphisms; Eco-AGG/Mse-CGC yielded 40 polymorphisms. The resulting data matrix contained 12,064 observations (not shown).

2.2. Regression analysis

The plot of the predicted values of cichoric acid **3** by the DNA data as a function of the actual HPLC determined values of cichoric acid (Fig. 1) indicated a near linear relationship between them. The best fit interpolated line (not shown) indicated a slight tendency toward some quadratic relationship at the extreme high values. The plot of the 95% confidence intervals for each prediction, shown in Fig. 1, fits the linear model. Similar results were obtained with the relationships between tetraene and DNA as a predictor. The plot of the predicted values of tetraenes by the DNA data over the actual HPLC determined values of tetraene **1** and **2** (Fig. 2) shows a nearly linear relationship between them. The interpolation line in both cases does not represent symmetry as it is an interpolation among the readings and need not be expected to replace the deviations about zero. Nonetheless, it may indicate, more than in the cichoric acid **3** case, a greater deviation from the linear relationship. The plot of the 95% confidence intervals for each prediction, shown in Fig. 2, fits the linear model as in the case of cichoric acid **3**.

Cook's D_i values (i.e. for each i reading) for both cichoric acid **3** and "tetraene" **1** and **2** are very near zero. Their range is 0.0–0.2, and only in one case does it reach 0.458 in cichoric acid **3** and 0.382 for tetraene **1** and **2**. For example, the plot of Cook's D_i for each observation (not shown) points to ASG-86 with a D value of 0.382, as one with the most influence on β but the relatively low value will only cause a very small shift.

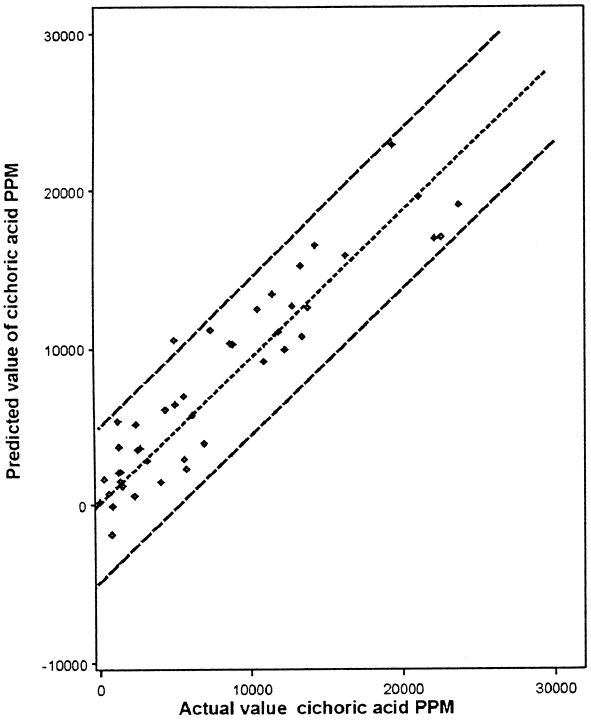


Fig. 1. Regression plot of the upper and lower bounds of a 95% confidence interval (long dashes) for each individual prediction of cichoric acid around the mean (short dashes). Data source: phytochemical (Table 1), and AFLP based on 232 binary variables.

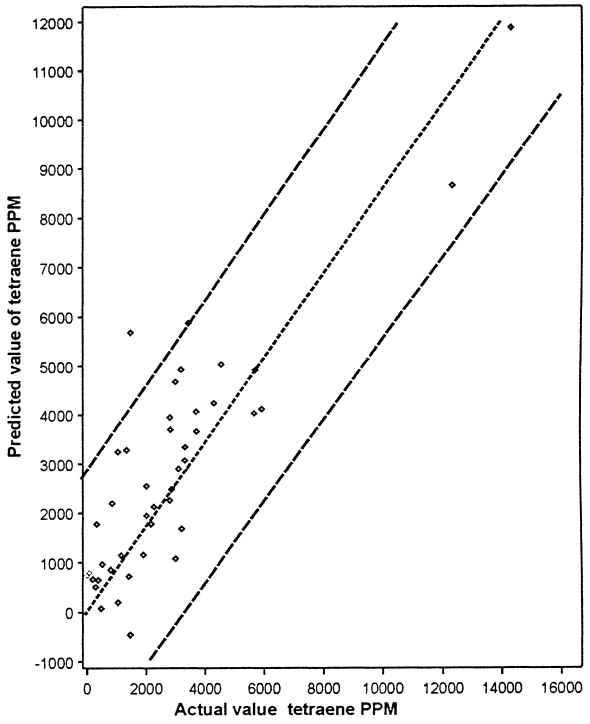


Fig. 2. Regression plot of the upper and lower bounds of a 95% confidence interval (long dashes) for each individual prediction of tetraene around the mean (short dashes). Data source: see Fig. 1.

2.3. Partial least squares analysis

The results indicate that there was no one set of components among the DNA variables that yielded good linear models for all the chemical variables.

2.4. Canonical correlation analysis

The first canonical correlation of DNA variables and the phytochemical variables has a coefficient of 0.93 which is very high. The probability level for the null hypothesis that all the canonical correlations are 0 is 0.0001 which is highly significant. The second canonical correlation has a coefficient of 0.85 which is also large and significant ($Pr > F$ is 0.019). The first standardized canonical variable has coefficients that suppress each other as in the two DNA bands with the highest weight −0.6876 and 0.5857 but the coefficients with the most weight act together in negative direction. This is in contrast to the second standardized canonical variable which has coefficients with contributions in the same direction, the highest being −0.4277 and −0.3682. The first standardized canonical variable has most weight for cichoric acid **3**, −1.0021, but has a negligibly positive (0.0251) impact for tetraene **1** and **2**. The second standardized canonical variable has negligible weight for cichoric acid **3** (−0.0735) and considerably more weight for tetraene **1** and **2** (1.0045).

The canonical redundancy analysis shows that the two canonical variables of the DNA data are good predictors of the chemical data as the total proportion of variance explained of 0.7925. The opposite is not true, since the canonical variables of the chemistry are not good predictors of DNA with a total variance explained of 0.947. The squared multiple correlations indicate that the first canonical variable of the DNA data has a high predictive power for cichoric acid **3** (0.8669) but practically none for tetraene **1** and **2**. The second canonical variable, however, has a high predictive power for both cichoric acid **3** (0.8673) and tetraene **1** and **2** (0.7177). The squared multiple correlations between the DNA data and the first canonical variables of the chemical data indicate the relative magnitude of the contribution of specific DNA bands to predict a high or low amount of cichoric acid **3** or tetraene **1** and **2** (Table 2). For instance, D12-440, i.e. Eco-ACC/Mse-CGC band size 440 bp, has the highest weight in contribution to the prediction of the amount of both cichoric acid **3** and tetraene **1** and **2**. The only other significant predictor of cichoric acid **3** is H12-182, i.e. Eco-AGG/Mse-CGC band size 182 bp. For tetraene **1** and **2**, H12-182, D10-270, D12-295 and D10-088 also contribute more highly than the others in Table 2.

A low correlation, $r = 0.19906$, between the DNA similarity matrix and the chemical similarity matrix, both based on Gower's coefficient of resemblance, was found. However, the Mantel t test, based on 1000 random permutations yielded a significant value $t = 3.3891$ ($P = 0.9996$), meaning that the null hypothesis, that there is no association between the two matrices, was rejected.

3. Discussion

This study has demonstrated for the first time that AFLP DNA fingerprints can be used to predict phytochemical concentration in whole aerial plants, at least for cichoric acid **3** and tetraene **1** and **2**. Previously, we have shown (Baum et al., 1999) with AFLP-based fingerprints that even single *Echinacea* plants can be differentiated from one another. Therefore, both the authentication of plant clone identity and the prediction of concentration of active phytochemicals is feasible using AFLP methods.

In the present study, the deviations around the linear regression (Figs. 1 and 2) may be due to a combination of random effects, variation due to sampling and measurement and most likely the influence of the environment on the product concentration synthesized during the life cycle of the plant. Another small source of variation could be related to the extraction of compounds from different plants. To minimize the latter source of variation we used a validated HPLC method in which extraction efficiencies were high (Bergeron et al., 2000). The Cook's D value can be interpreted as the Euclidean distance between a particular predicted observation Y and all the estimated predicted Y. It measures the shift in the estimated predicted Y caused by deleting the ith observation from the estimation of β, the entire vector of the estimated regression coefficient (Rawlings, 1988). Thus, the low Cook's D values obtained support the view that omitting any single data point hardly influences the results.

The disappointing results with partial least squares is perhaps due to the rather small data set and therefore inappropriate for use with this method. Partial least squares is used when factors are many and highly co-linear. Furthermore, partial least squares does not provide calculations to understand the underlying relationship among the variables. However, because it is used to construct predictive models we attempted to use it in this context.

Canonical correlation analysis provided support for the regression analysis findings that DNA fingerprinting data has the power to predict quantity of cichoric acid **3** and tetraene **1** and **2** in lines of *Echinacea*. The list of the 22 most significant DNA predictors is provided in Table 1, whereas the remaining 210 DNA variables out of the total 232 are useless. These 22 variables, however, are all needed together for prediction purposes.

The Mantel test lent support to the association between the DNA data and the chemical data. A low, although positive, correlation between the two data sets

Table 1
Thirty-six cultivated lines of *E. purpurea* and 16 selected wild-harvested *Echinacea* species (marked with "sb" as a suffix of the accession name) were analyzed for their cichoric acid and dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z* tetraenoic acid isobutyl amide content[a]

Accession	Cichoric acid **3** (ppm)	Dodeca- 2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutyl amide **1** and **2** (ppm)
BSG-L	10841	3696.4
ASG-79	1296	802.2
AS-203	1269	5703.5
BSG-118	5692	2802.3
ASG-118	14240	4286.0
BSG-15	5551	483.2
ASG-94B	2812	3406.2
BSGL-273	13742	1337.7
ASGL-226	19344	295.7
ASGL-97	13275	1410.9
BSG-285	21037	2009.4
ASG-2741	8801	1153.2
ASGL-45	5552	516.9
BSGM-43	22083	2850.2
BSGL-202	7351	2819.8
ASG-135	1364	5662.8
EP-2(sb)	787.8	196.8
EP-5(sb)	626	5710.0
BS-191	6883	5916.8
BSC-11A	22526	3677.0
ASG-153	10432	1049.1
BS-203	4941	846.7
BSG-125	1385	2260.8
ASG-142	855	1898.7
ASG-001	16255	1462.3
ASG-203	3644	3732.7
BSG-11C	2412	3178.9
ASG-286	8231	2484.3
ASG-289	22053	2191.9
ASGL-296	6104	72.23
BSG-44	12722	14299.3
ASG-86	11418	12303.7
BS-184	9204	3006.9
ASG-94	13363	2161.7
ASG-118b	8625	3421.1
ASG-151	3998	3298.1
BSG-77A	4965	1472.9
EA-4 (sb)	1523	1045.0
ANGST-05	0	3676.1
EA-6 (sb)	1228	2011.3
E-1 (sb)	23693	2794.0
EPA-10 (sb)	3117.1	2985.0
EPA013 (sb)	2680	0.0
EPA-3 (sb)	2509	3086.0
EPP-5 (sb)	11808	4537.6
EPN-3 (sb)	12221	331.6
EPP-7 (sb)	304.7	3302.8
ES-1 (sb)	1575	1696.5
ES-5 (sb)	4320	381.7
ES-6 (sb)	6538	336.1
E-3 (sb)	2297	3190.6
EPA-1 (sb)	619.0	2983.5

[a] Notation of wild-harvested samples' tentative identification: EA = *E. angustifolia*, EP = E. *purpurea;* EPA = *E. pallida*; EPN = *E. paradoxa* var. *neglecta* ; EPP = *E. paradoxa* var. *paradoxa*; ES, E = *E. simulata*.

that was observed needs to be discussed. The low correlation is an overall, single figure, correlation. By itself, it does not detect the association between the individual and comparable pair of observations in two data sets. Furthermore, there is considerable variance in the association, as can be seen in the regression analysis, especially portrayed in Figs. 1 and 2. The chemical data, although predictable from the DNA data, was not associated in a one to one fashion, which can be explained by the temporal phytochemical variation during the life cycle of the plant. In addition, tissue specific variation as well as experimental error may explain the deviations within the association.

Efforts to correlate phytochemistry with DNA data are at a preliminary stage for medicinal plants. However, in food crops, the quantitative traits loci technique has already been successfully applied to mapping of the flavonoid maysin in corn, leading to the identification of candidate genes affecting maysin concentration (Byrne et al., 1998). From the present studies, using a limited number of *Echinacea* samples we conclude that AFLPs may be used to estimate the expected accumulation of cichoric acid **3** and tetraene **1** and **2** in *Echinacea*. However, further efforts to standardize growth, processing, and other extrinsic factors which influence secondary metabolism are necessary to assure a more accurate quantitative prediction of phytochemicals by DNA fingerprinting.

Table 2
Squared multiple correlations between the DNA data and the first canonical variables of the phytochemical data[a]

Primer combination and band size	Cichoric acid **3**	Tetraene **1** and **2**
D12-440	0.2727	0.3070
G10-222	0.0536	0.0834
H12-154	0.0881	0.0885
H10-140	0.0032	0.0303
H10-245	0.0756	0.0889
H10-207	0.0453	0.0573
H10-315	0.0376	0.0553
D12-198	0.0028	0.0335
H12-182	0.2071	0.2193
D10-185	0.0026	0.0461
G10-100	0.0043	0.0133
H10-110	0.0001	0.1312
H10-375	0.0018	0.0045
H10-215	0.0067	0.0077
D12-383	0.0005	0.0711
D10-088	0.0111	0.1852
G10-214	0.0066	0.0570
H10-325	0.0017	0.0193
D12-295	0.0132	0.1984
D10-270	0.0044	0.2331
H10-142	0.0082	0.0892
H10-119	0.0248	0.0639

[a] Primer pair combinations: D10 = Eco-ACC/Mse-CCG, D12 = Eco-ACC/Mse-CGC, G10 = Eco-AGC/Mse-CCG, H10 = Eco-AGG/Mse-CCG, H12 = Eco-AGG/Mse-CGC. Band size is provided after the dash in base pairs.

Work is currently under way with a broader sample consisting of wild germplasm and with the knowledge of our inherent limitations over the control of phytochemical metabolism.

4. Experimental

4.1. Plant material

Rootstocks (2 year old) of *Echinacea purpurea* (L.) Moench were obtained from Trout Lake Farm LLC, Trout Lake, Washington, USA. These were cloned accessions developed from elite plants showing both agronomic and chemical variation in their commercial germplasm and have been in cultivation for 25 years, since collection of the original seed in South Dakota. Additional root samples of *Echinacea* spp. were taken from wild plants collected in the midwestern US. Voucher specimens have been deposited at the Department of Agriculture Ottawa herbarium (DAO). All rootstocks were transplanted and grown in a greenhouse under uniform conditions of 25°C temperature, 16:8 L:D (day length and night period) (Table 1).

4.2. AFLP data collection

Approximately 100 mg leaf material from each single *Echinacea* plant was used to extract genomic DNA using the CTAB procedure of Doyle and Doyle (1987). The DNA was dissolved in low TE (10 mM Tris–HCl, 0.1 mM EDTA) pH 8.0. The purity of the DNA was first estimated using spectrophotometric A_{260}/A_{280} readings (Spectronic 1201, Milton-Roy). The concentration was then measured with a fluorescence assay using 33258 Hoechst fluorochrome (Cesarone et al., 1979) in a Dyna Quant™ 200 mini-fluorometer (Hoefer Pharmacia Biotech) (Anon, 1995). The DNA was diluted with sterile water to a final working concentration of 100 ng/μL. The AFLP reactions were performed according to the description in Vos et al. (1995). For each sample a total of 500 ng genomic DNA was double digested with an Mse1 and EcoR1 combination at 37°C for 2 h. The enzymes were then deactivated at 70°C for 15 min. The products were then diluted 10-fold in TE buffer. The diluted products were then ligated to the end-specific adapters, as in Vos et al. (1995). A pre-selective PCR amplification was performed using primers complementary to each of the two adapters containing an additional base. The resulting templates were diluted 1:50 in TE buffer then subjected to a selective amplification with primers differing from the pre-selective ones by having three additional bases. The following primer pair combinations were used for the selective PCR amplications, Eco-ACC/Mse-CCG, Eco-ACC/Mse-CGC, Eco-AGC/Mse-CCG, Eco-AGG/Mse-CCG, Eco-AGG/Mse-CGC, the three base pair indicated as a suffix of the enzyme specific adapter. The Eco-primer, was radioactively end-labeled with ^{33}P and T4 kinase. The PCR reaction conditions were optimized for each primer combination. In general, the first 10 cycles consisted of a touchdown PCR as follows, on an MJ200: 94°C for 60 s, 65–56°C for 60 s (drop 1°C each cycle), 72°C for 90 s; this was followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s. An equal volume of formamide dye (98% formamide, 10 mM EDTA, 0.1% bromophenol blue, xylene cyanol) was added. The samples were heated for 3 min at 90°C and placed on ice for loading the samples on the gel. Separation of amplified DNA fragments was done by electrophoresis on 5% denaturing polyacrylamide gels (Sambrook et al., 1989, 1: 6.37). Gels were then fixed, dried and exposed onto X-ray films (Kodak diagnostic film X-Omat™). The DNA fingerprints were visualized on a light box, scored as 1 or 0 and manually recorded on a spreadsheet (not shown).

4.3. Chemistry data collection

4.3.1. Sample preparation

Dried plant samples (aerial parts) were milled to powder (40 mesh). A portion of each powdered sample (~0.5 g) was extracted three times in 8 ml fresh 70% ethanol using ultrasound (5 min), followed by centrifugation and collection of the supernatants. The supernatants were pooled, the extract volume was adjusted to 25 ml, and 1.5 ml was filtered through a 0.22 μm PTFE membrane prior to injection of 5 μl into the HPLC system.

4.3.2. HPLC analysis

The instrumentation used was a Beckman HPLC system consisting of Autosampler, Module 502, Solvent delivery system Module 126, Photodiode array detector Module 168, and System Gold software (version 8.10). Separation was achieved on a 5 μm LiChrospher 100 RP-18, 125×4.6 mm cartridge column (EM Sciences) with 5 μm LiChrospher 100RP18, 4×4.6 mm guard cartridge (EM Sciences). Mobile phases for the cichoric acid **3** analysis consisted of: A (50 μmol/l NaH_2PO_4, pH 4.5) and B: 1% 0.1 N H_3PO_4 in CH_3CN. The linear gradient used was: 0–25% B in 20 min, 25–0% in 1 min followed by equilibration for 7 min. The mobile phase for the lipophilic components consisted of A: Water and B: CH_3CN. The linear gradient used was 40–80% B in 30 min, 80–40% in 1 min, and equilibration for 9 min. Flow rate was 1 ml/min. Detection wavelengths were 320 nm, versus 450 nm reference (hydrophilic components) and 210 nm, versus 450 nm reference (lipophilic components). Quantitation was based on response factors calculated from injection of known amounts of standard materials. Standards were isolated and identified as

described in Bauer et al. (1988) and Bauer and Remiger (1989). In the hydrophilic analyses, cichoric acid eluted at about 8.0 min. In the lipophilic analyses, dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutyl amide **1** and **2** eluted at 14.2 min.

4.4. Data analysis

The AFLP data set and the chemical data set were analyzed with SAS® procedures (SAS Institute Inc., SAS System for Windows™ release 6.12). The AFLP data were coded 1 for band present, 0 for band absent for five primer pair combinations. In all, 232 DNA markers for each of the 52 plants itemized in Table 1 were recorded (not shown). The chemical data are shown (Table 1).

4.4.1. Regression analysis

A regression analysis was carried out using PROC REG for each primer combination separately using the STEPWISE model selection method, the *X* variables (independent) being the binary variables for each primer combination and the *Y* variables (dependent) being the two chemical variables. The STEPWISE selection was performed to keep only those variables that are significant based on their *F*-statistic ($P < 0.15$ which is also the default set in SAS®). At the end all the variables that remained in each primer combination after the stepwise selection were combined, and submitted to a final and similar regression procedure to yield those variables to be kept for subsequent analyses. The *Y* variables must be quantitative as in the phytochemical data, but the *X* variables may be binary (Rawlings, 1988) as in the DNA data which was drawn from the AFLP fingerprints. A final regression procedure, also using the stepwise selection model, was carried out with the selected chemical variables. Then, a separate plot for each chemical was produced for the actual versus the predicted chemical quantities by the DNA fingerprints, for each individual plant. A curve of best fit was drawn through the scatter plot, using the PLOTIT SAS® macro. The upper bounds and lower bounds of a 95% confidence interval for each individual prediction was computed and plotted.

The residuals, calculated as the actual minus the predicted values, were also plotted against the predicted values to assess, along with the Student residuals, that the model with stepwise selection is adequate. An adequate model is one in which none or very few observations have a value of 2 or above.

In an attempt to optimize the linear relationship between the actual and the predicted, the same analysis was repeated with the dependent variables, i.e. the phytochemical variables, transformed with the log transformation and with various values of the power transformation.

4.4.2. Partial least square

The same data were analyzed by the partial least squares method (see Garthwaite, 1994) using the PLS procedure in SAS/STAT®. Partial least squares involves the use of variables, (i.e. DNA polymorphism in our case) to predict other variables, (i.e. the phytochemical quantities).

4.4.3. Canonical correlation

The data were also subjected to a canonical correlation analysis, using PROC CANCORR in SAS/STAT®, primarily in order to examine the results of the canonical redundancy analysis. In other words, to observe if the set of canonical variables of the DNA is a good predictor of the set of chemical variables.

4.4.4. Mantel tests

The DNA fingerprints, i.e. the presence/absence data matrix, and the chemical data matrix containing the quantitative observations of cichoric acid and tetraene, were each subjected to the computation of pairwise distances between the individual plant samples. Gower (1971) distances were obtained for each data matrix using a macro in SAS®. The two distance matrices were then subjected to a Mantel (1967) test using MxComp in NTSYSpc (Rohlf, 1999) in order to test the hypothesis that there is no association between the two data sets. A rejection would signify that there is an association between the chemical data and the DNA data.

All the computations for the analyses above were performed on an NT4.0 workstation using the SAS® system for windows release 6.12 (SAS Institute) and all plots were drawn with SAS/graph®.

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Biosynthesis of the bicycloalternarenes, mixed terpenoids of *Alternaria alternata*

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Abstract

By incorporation of [2-^{13}C]-mevalonate, [1-^{13}C]-acetate and [1-^{13}C]-glucose we could reveal that the phytopathogenic fungus *Alternaria alternata* biosynthesized the mixed terpenoids bicycloalternarenes via the classic mevalonate pathway. The polyketid pathway does not participate in the biosynthesis of bicycloalternarenes, because there is no incorporation of [^{13}C]-acetate into the C-ring of these compounds. The labelling pattern in this nonterpenoid part of bicycloalternarenes after feeding with [1-^{13}C]-glucose and [U-$^{13}C_6$]-glucose, respectively, allows the assumption that metabolites of the shikimate pathway are involved. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Alternaria alternata*; Fungi imperfecti; Biosynthesis; Bicycloalternarenes; Isoprenoids

1. Introduction

In the middle of the last century the most important biochemical pathways both in primary and secondary metabolism were elucidated. However, surprising and unexpected results were occasionally reported. The discovery of an alternative pathway in the biosynthesis of isoprenoids is one such event (for reviews see Eisenreich et al., 1998; Lichtenthaler, 1999).

In feeding experiments again and again experimental data were not in agreement with the well-known mevalonate pathway found up to now in all organisms. The research groups of Rohmer and Arigoni discovered a novel biochemical sequence leading to the isoprenic units dimethylallylpyrophosphate and isopentenylpyrophosphate (Rohmer et al., 1993; Schwarz and Arigoni, 1999). Glyceraldehyde-3-phosphate and pyruvate were found as precursors in this non-mevalonate terpenoid biosynthesis (Rohmer et al., 1996). An important intermediate is 1-deoxy-D-xylulose-5-phosphate (Arigoni et al., 1997; Schwender et al., 1997). Therefore, this alternative biosynthesis is also called DOXP pathway. It was found that many bacteria use this pathway, whereas higher plants operate both the mevalonate and the DOXP pathway. On the other hand fungi including yeasts are probably unable to produce terpenoids with the DOXP route (Disch and Rohmer, 1998).

Recently, we reported the isolation and structure elucidation of the bicycloalternarenes (BCAs) which are biosynthesized by the phytopathogenic fungus *A. alternata* (Liebermann et al., in press). Our further interest was focused on the biosynthesis of these mixed terpenoids. Two questions should be answered. Which of the two pathways is used by *A. alternata*? Are the BCAs meroterpenoids biosynthesized by a combination of the polyketid and terpenoid pathways?

2. Results and discussion

At present, one of the most important methods to elucidate biosynthetic pathways is the evaluation of the labelling pattern observed after feeding with ^{13}C-precursors. Before carrying out such investigations we tested the incorporation rate of ^{14}C-labelled precursors added at the beginning of the fermentation. Table 1

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Table 1
Incorporation of radiactively labelled precursors in metabolites of *A. alternata*

Precursor	Specific incorporation rate (%)[a] in			
	BCA 6	BCA 2	BCA 1	Ergosterol
[1-^{14}C]-Acetate	0.064/0.22	0.072/0.25	0.057/0.20	0.13/0.31
[2-^{14}C]-Mevalonate	0.15/1.06	0.17/1.21	–	0.66/3.67

[a] The first value is calculated only at mmol C, the second additional in consideration of the known biosynthesis pathway.

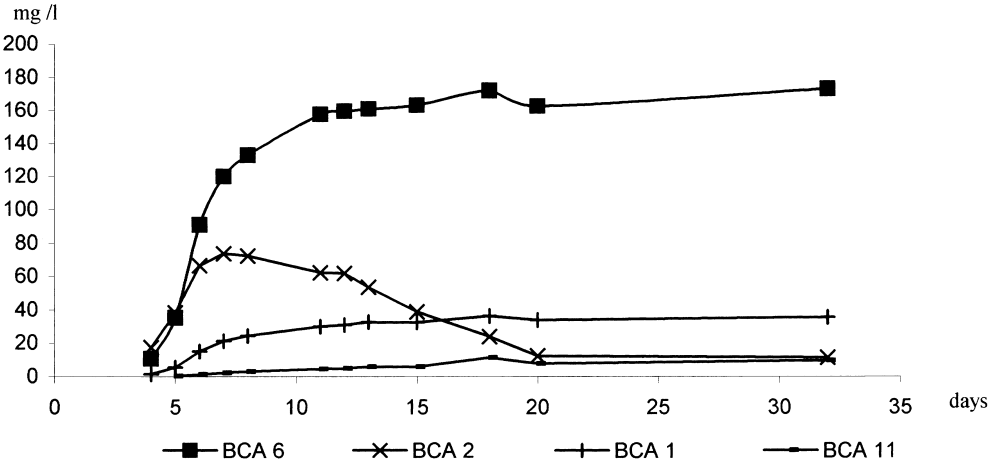
shows that the specifc incorporation rates of acetate and mevalonate both into BCAs and into ergosterol, which was used for comparison, were at a reasonable level. The better incorporation of mevalonate compared with acetate already points to the use of the classic mevalonate pathway by *A. alternata*. When, at the beginning of the cultivation, we add fluvastatin (20 mg/100 ml) as an inhibitor of the 3-hydroxy-3-methylglutaric-CoA-reductase, mycelium growth was nearly completely inhibited. However, a concentration of 2 mg/100 ml did not have a negative influence either on mycelium growth or on production of the BCAs.

In contrast to the BCAs the assignment of chemical shifts of ^{13}C-NMR spectra to the carbons of the related tricycloalternarenes (TCAs) is well-known (Liebermann et al., 1997; Nussbaum et al., 1999). Therefore, in the incorporation experiments with ^{13}C-precursors (see below) TCAs corresponding to the BCAs were used to measure the labelling pattern. For that purpose a selected BCA was isolated and converted to a pair of TCAs (Liebermann et al., 2000), one of them was isolated by HPLC and the ^{13}C-enrichment in this compound was recorded.

Biosynthesis of the BCAs starts relatively early (see Scheme 1), that is on the fourth day after inoculation, therefore, the ^{13}C-precursors were already added at the beginning of the cultivation. In the incorporation experiments with [2-^{13}C]-mevalono-lactone and [1-^{13}C]-acetate each BCA 6 via TCA 6b was used for measurement. The absolute ^{13}C-abundances were obtained from the analysis of well separated ^{13}C-satellite signals of ^{1}H at C-atom 8 in the ^{1}H-NMR spectra and in consideration of an unlabelled ^{13}C-NMR spectrum of TCA 6b. In the case of [1-^{13}C]-D-glucose BCA 2 via TCA 2b was used. The ^{13}C-enrichment was calculated by comparison with a ^{13}C-NMR spectrum of unlabelled TCA 2b under identical conditions.

A. alternata metabolized glucose and acetate simultaneously (Hänel et al., 1985). Normally, 40 g of glucose and 3.7 g of sodium acetate per 1000 ml culture medium were used as carbon source in the fermentation to produce the BCAs. But these compounds were also biosynthesized in sufficient amounts without acetate or by a decrease of glucose to 25 g/l, so that we could modify the culture medium in the ^{13}C-feeding experiments in order to get useful results. However, in the case of incorporation of [1-^{13}C]-acetate the relative amount of the labelled substance was too high leading to an excessive enrichment and additional disturbing couplings. It is likely, that acetate is preferentially used during BCA biosynthesis. Nevertheless, the results of the incorporation experiments were clear.

Results of the incorporation of [2-^{13}C]-mevalonolactone, [1-^{13}C]-acetate and [1-^{13}C]-glucose are summarized in Table 2 and Fig. 1. Undoubtedly the ^{13}C-labelling pattern after incorporation of [2-^{13}C]-mevalonolactone proves the existence of the classic mevalonate pathway supported by the pattern after incorporation of [1-^{13}C]-acetate. But these results do not exclude a possible crosstalk contribution via the alternative route. In plants such crosstalks between both pathways were reported (Eisenreich et al., 1998). However, the pattern which originated from feeding with [1-^{13}C]-glucose (Lichtenthaler, 1998) confirms that BCAs were only



Scheme 1. Kinetics of BCAs production.

biosynthesized by the mevalonate route. This is recognizable from the ^{13}C level of C4, C8 and C12 being in the same range like the other unlabelled atoms in the terpenoid part of TCA 2b. So far, these results are in accordance with the finding that fungi are unable to produce isoprenoids using the DOXP route (Disch and Rohmer, 1998).

After feeding with [1-^{13}C]-acetate no increased ^{13}C-labelling is found in the C-ring of the corresponding TCA. Therefore, we conclude that the polyketid pathway is not involved in the biosynthesis of BCAs (TCAs) and these compounds cannot be denoted as meroterpenoids.

Incorporation of [1-^{13}C]-glucose leads to a very interesting result. Additional labeling of C-16 and C-18 can be explained by the involvement of 3-deoxy-D-arabino-heptulosonic acid. By the equilibrium state between fructose-1,6-bisphosphate, dihydroxyaceton phosphate and glyceraldehyde 3-phosphate [1-^{13}C]-glucose will be converted via glycolysis to [1,6-^{13}C]-fructose-1,6-bisphosphate on the one hand and further to [3-^{13}C]-phosphoenolpyruvate on the other hand. Via reversible reactions in the pentose phosphate cycle [4-^{13}C]-erythrose-phosphate originates from [1,6-^{13}C]-fructose-1,6-bisphosphate (via [1,6-^{13}C]-fructose-6-phosphate). As is known, tetrose and phosphoenolpyruvate react in the first step of the shikimate pathway to 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP). ^{13}C-labelling would be expected in 3,7-positions. A hypothetical pathway of this compound and a sesquiterpene precursor to the BCAs is conceivable, leading to the observed labelling of C-16 and C-18 (Fig. 2). To our knowledge a biosynthetic connection between the mevalonate pathway and such metabolites of the shikimate pathway have not been reported so far (for reviews see Bentley, 1990; Herrmann and Weaver 1999).

In order to verify this hypothesis we fed *A. alternata* with a mixture of [U-$^{13}C_6$]-glucose and unlabelled glucose. ^{13}C–^{13}C couplings should provide information on the mode of assembly and rearrangements of the carbon skeleton. Such investigations were successful carried out recently in the biosynthesis elucidation of several secondary metabolites (Bacher et al., 1999; Eichinger et al., 1999).

Results of [U-$^{13}C_6$]-glucose incorporation into BCAs (TCAs) are summarized in Table 3 and Fig. 3 (for TCA 6a only). Incorporation of intact carbon units are recognizable by corresponding ^{13}C–^{13}C couplings. In the case of breaking of C–C bonds and recombination of fragments an intensive dilution by the unlabelled compounds takes place leading to the lack of such couplings. The coupling pattern in the terpenoid units of TCA 6a and 6b can be explained doubtless by the intact incorporation of [1,2-$^{13}C_2$]-acetate. Following the mevalonate pathway, contiguously ^{13}C atoms are present in C1/C2, C3/C4, C6/C6′, C7/C8, C10/C10′, and C11/C12. The ^{13}C–^{13}C coherence was confirmed by 2D INADEQUATE

Table 2
Incorporation of [2-^{13}C]-mevalono-lactone (1) and [1-^{13}C]-acetate (2) in TCA 6b, and [1-^{13}C]-glucose (3) in TCA 2b

C-atom	^{13}C (ppm)	% ^{13}C (1)	% ^{13}C (2)	^{13}C [ppm]	% ^{13}C (3)
1	26.5	1.50	1.16	68.6	**10.0**
2	73.1	1.23	**30.1**	135.2	2.06
2′	22.9	**6.64**	1.10	13.7	**12.5**
3	78.2	1.37	0.81[a]	125.3	**12.8**
4	29.1	1.24	**35.9**	24.8	1.82
5	31.8	**6.67**	–[b]	34.6	**12.7**
6	31.9	1.26	**41.3**	31.0	1.96
6′	19.4	1.38	1.13	20.1	**13.4**
7	150.3	1.43	1.07	149.9	**10.1**
8	120.0	1.26	**40.6**	119.9	1.77
9	45.0	**6.92**	1.23	45.0	**11.9**
10	88.6	1.33	**38.3**	88.7	2.08
10′	23.4	1.36	1.09	23.1	**12.8**
11	46.6	1.37	1.26	46.4	**10.2**
12	15.1	1.34	**38.7**	14.8	2.64
13	105.2	1.30	1.71	104.9	1.26
14	172.9	1.26	1.64	172.8	1.39
15	27.7	1.28	1.65	27.7	1.46
16	29.5	1.33	1.56	29.4	**10.6**
17	71.0	1.23	1.50	70.9	1.36
18	197.6	1.29	0.91[a]	197.7	**9.9**

[a] Additional coupling in the noise.
[b] Not clear determined, sitting on the tailing of C-6.

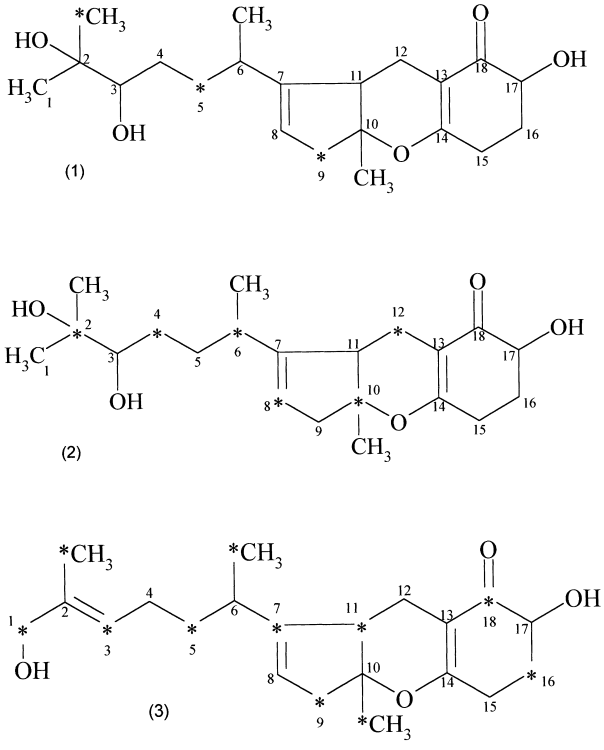


Fig. 1. ^{13}C-labelling patterns after incorporation of [2-^{13}C]-mevalono-lactone (1) and [1-^{13}C]-acetate (2) in TCA 6b, and [1-^{13}C]-glucose (3) in TCA 2b (see Table 2). * Position with increased ^{13}C-level.

NMR spectra. The fact that no ^{13}C–^{13}C long-range coupling was detected additionally confirms the mevalonate pathway. Such couplings point at the biosynthesis via 1-deoxy-D-xylulose-5-phosphate (Eichinger et al., 1999).

To understand the labeling pattern in the nonterpenoid part one has to take in consideration that a parent BCA can be converted to a pair of TCAs (Liebermann et al., in press). Due to the keto-enol tautomerism of the 1,3-diketone structure in the six-membered ring of the BCAs and because of the free rotation of this ring, different labeling patterns appear in both TCAs.

The intact ^{13}C pairs (C14/C15 in TCA 6a, C18/C17 in TCA 6b) are in congruence with the hypothetical pathway

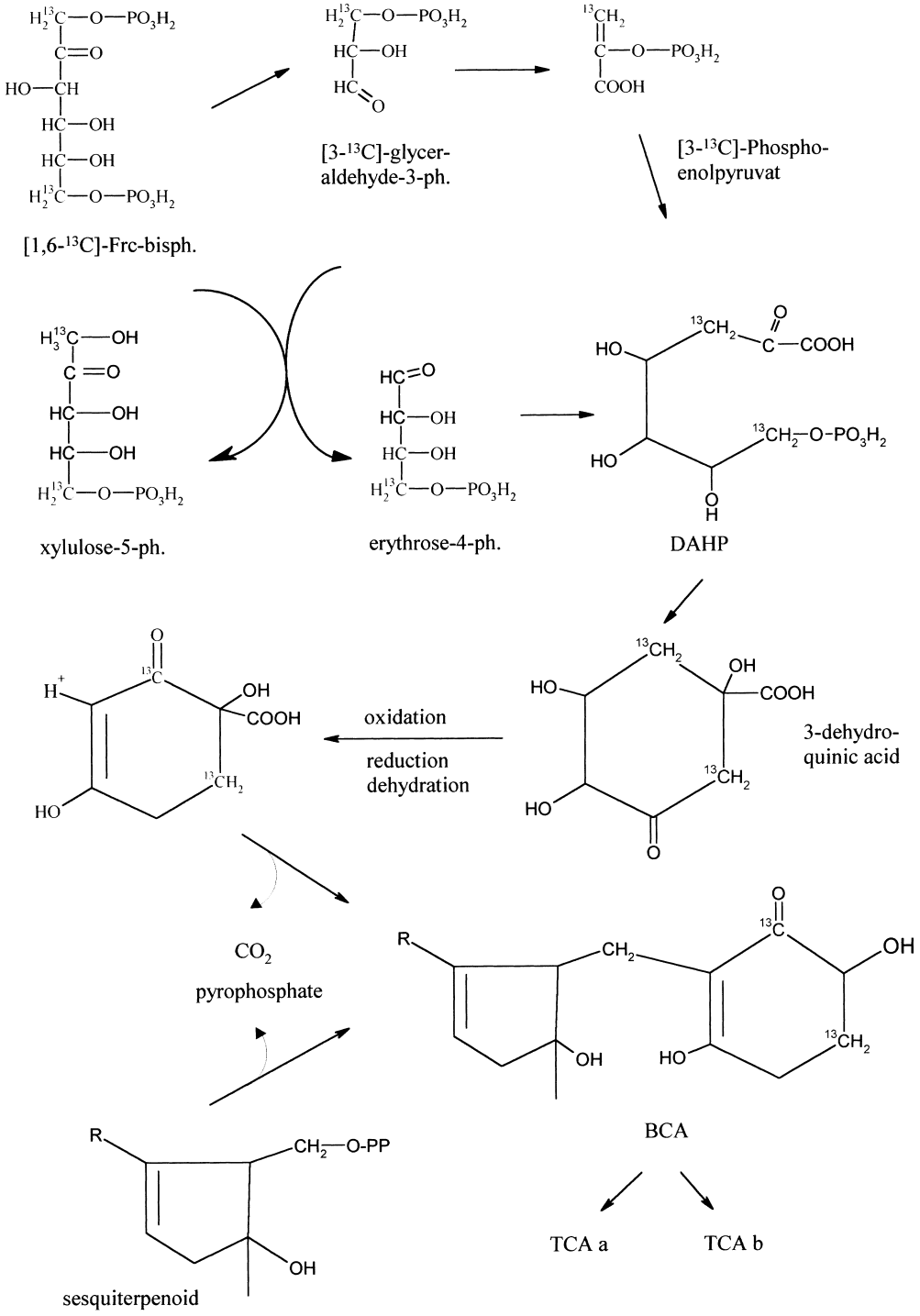


Fig. 2. Hypothetical pathway for BCA biosynthesis. ^{13}C-Labelling of the nonterpenoid part after feeding of [1-^{13}C]-glucose is pictured, labelling of the sesquiterpenoid part is left out.

Table 3
Incorporation of [U-$^{13}C_6$]-glucose[a] in TCA 6b (1) and TCA 6a (2)

	(1)				(2)			
C-Atom	^{13}C (ppm)	% ^{13}C[b]	% ^{13}C^{13}C[c]	J_{CC} (Hz)	^{13}C (ppm)	% ^{13}C[b]	% ^{13}C–^{13}C[c]	J_{CC} (Hz)
1	26.5	5.7	65 (2)	40	26.5	5.4	65 (2)	40
2	73.1	5.6	65 (1)	40	73.1	5.2	65 (1)	40
2′	22.9	5.3	–		22.9	5.1	–	
3	78.2	5.4	65 (4)	39	78.1	4.9	65 (4)	39
4	29.1	5.6	64 (3)	39	29.5	4.7	64 (3)	39
5	31.8	5.1	–		31.8	5.0	–	
6	31.9	5.7	65 (6′)	34	31.9	4.4	66 (6′)	34
6′	19.4	5.8	64 (6)	34	19.4	5.2	65 (6)	34
7	150.3	5.8	65 (8)	70	150.3	5.0	65 (8)	70
8	120.0	5.6	66 (7)	70	120.0	5.0	66 (7)	70
9	45.0	5.5	–		45.0	5.4	–	
10	88.6	6.2	65 (10′)	42	88.6	5.1	64 (10′)	42
10′	23.4	5.7	66 (10)	42	23.4	5.6	67 (10)	42
11	46.6	5.9	65 (12)	34	46.6	5.2	65 (12)	34
12	15.1	5.8	65 (11)	34	15.1	5.2	66 (11)	34
13	105.2	6.5	25 (14)	70	106.2	5.5	27 (18, 17[e])	56, 12[e]
							2 (18)	56
14	172.9	6.3	44 (15),	46	171.9	4.8	72 (15)	49
			22 (13,15)	70, 46				
			3 (13)	71				
15	27.7	6.0	[d]		66.5	5.1	72 (14)	49
16	29.5	5.1	69 (15)	32.5	28.8	5.5	75 (17)	32
17	71.0	5.1	72 (18)	40	33.6	5.5	46 (18, 16)	40, 32
							25 (18, 16, 13[e])	40, 32, 12[e]
							8 (16)	32
18	197.6	5.3	73 (17)	40	197.0	5.1	48 (17)	40
							20 (13, 17)	56, 40
							2 (13)	56

[a] (4%, diluted with unlabelled glucose).
[b] ^{13}C Abundance obtained from ^{13}C satellite analysis of H-8.
[c] Percentage coupling calculated as percentage of total signal for a given carbon atom involved in ^{13}C–^{13}C coupling, carbon coupled to the respective index carbon are in parentheses.
[d] Spectrum higher order (not analyzed).
[e] $^{2}J_{CC}$.

(see Fig. 2). Therefore, firstly one expects also an uninterrupted incorporation of the erythrose unit. The following results are well-defined only in the spectrum of TCA 6a (C13/18/17/16 part). The labelling pattern and the INADEQUATE experiment show a strong coherence of three ^{13}C atoms and a diminished additional coupling to C13 (Fig. 3). This result can be explained by the

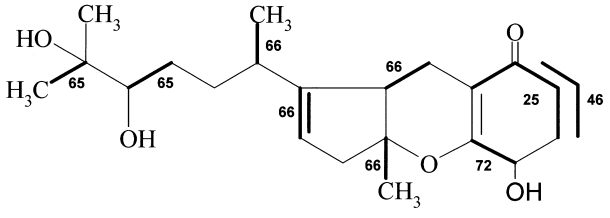


Fig. 3. Incorporation of [U-$^{13}C_6$]-glucose[a] in TCA 6a (2), [a](4% w/w, diluted with unlabelled glucose); bold bars: carbon atoms involved in ^{13}C–^{13}C coupling, percentage coupling calculated as percentage of total signal (see Table 3).

equilibrium state between fructose-1,6-bisphosphate, dihydroxyaceton phosphate and glyceraldehyde 3-phosphate (see above). By the dilution of unlabelled compounds the C3/C4 atoms in fructose-1,6-bisphosphate and, as a result, the C1/C2 atoms in erythrose-4-phosphate are only slightly doubly ^{13}C labelled. The isotopomer pattern found in the nonterpenoid part of BCA(TCA) is comparable with those reported for other shikimate derived metabolites (Werner et al., 1997). Furthermore, we can exclude the existence of any symmetrical intermediate, such as benzoic acid or derivates thereof, in the hypothetical pathway of BCAs.

Results of the incorporation experiments with [U-$^{13}C_6$]-glucose strongly confirm that the shikimate pathway is involved in the biosynthesis of BCAs, but further proofs will be necessary. Feedings of *A. alternata* with [^{13}C]-erythrose labelled in different positions will be performed, but the shuffling of the label via the pentose phosphate pathway has to take into account. Therefore, a retrobiosynthetic ^{13}C NMR study (with the

labeling pattern of tyrosine) could definitively verify the incorporation results in the nonterpenoid unit.

The labelling pattern in the nonterpenoid part of BCAs (TCAs) after feeding with [1-^{13}C]-glucose and [U-$^{13}C_6$]-glucose unequivocally rules out an involvement of seduheptulose-7-phosphate formed by iterative C_2 fragment transfer in a pentose phosphate pathway found in the biosynthesis of carba sugars (Degwert et al., 1986; Höfs et al., 2000).

3. Experimental

The strain and cultivation of *A. alternata* were described elsewhere (Nussbaum et al., 1999), a supplementation of the culture medium with 3.7 g sodium acetate per litre is possible. Isolation of BCAs and conversion to the corresponding TCA pairs is described in Liebermann et al. (in press). Separation of the TCAs 6a/b and 2a/b was carried out by isocratic HPLC in MeOH-H_2O (13:7) and (3:1), respectively (Nussbaum et al., 1999). ^{1}H and ^{13}C NMR were recorded at 400 and 100 MHz, respectively, using a Bruker DRX 400 spectrometer (5 mm probe head with a z-gradient). ^{1}H, ^{13}C and 2D INADEQUATE experiments were performed at 27°C using standard Bruker software (XWINNMR 2.6).

Ergosterol was isolated from the fresh mass of *A. alternata* mycelium, 20 g and 200 ml EtOH were refluxed for 2 h at 95°C. The remainder was washed three times with 50 ml EtOH. The pooled EtOH extracts were hydrolysed by refluxing with 1 N KOH for 1 h at 95°C. After cooling, the mixture was extracted four times with 50 ml *n*-hexane. After evaporation of the solvent the remainder dissolved in MeOH, was purified by solid phase extraction over a 6 ml mini column with silica gel RP 18. The methanolic passage was evaporated, the back was dissolved in EtOH and purified by HPLC, conditions: Hibar Lichrosorb RP-18, 250 × 25 mm, 7 μm, guard column 30 × 16 mm, 10 μm, flow rate 9 ml/min, MeOH–H_2O (19:1), detection 282 nm, elution time ergosterol 100 min.

Feeding with ^{14}C precursors: altogether 15 mg in 5 × 100 ml culture medium (without acetate), [1-^{14}C]-acetate (sp. act 3.14–3.66 × 10^6 Bq/mmol), [2-^{14}C]-mevalonate (sp. act 8.33–15.2 × 10^6 Bq/mmol) opened from the lactone by addition of equivalent amounts NaOH.

Feeding of ^{13}C precursors: [2-^{13}C]-mevalono-lactone, opened by addition of equivalent amounts NaOH, 99% ^{13}C, altogether 500 mg in 15 × 100 ml culture medium (40 g glucose/l, without acetate); [1-^{13}C]-acetate, 99% ^{13}C, altogether 10 g in 15 × 100 ml culture medium (26.6 g glucose/l); [1-^{13}C]-D-glucose, altogether 42 g (33% ^{13}C) in 14×100 ml culture medium (without acetate); [U-$^{13}C_6$]-D-glucose (99% ^{13}C), 4% (w/w) diluted with unlabeled glucose, altogether 50 g in 15 × 100 ml culture medium (without acetate).

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Distribution of 8-oxygenated leaf-surface flavones in the genus *Ocimum*

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Abstract

Nine species of *Ocimum* (Lamiaceae) were surveyed for leaf-surface flavonoids by means of HPLC with diode array detection and atmospheric pressure chemical ionisation (APCI) mass spectrometry. The analysis revealed the presence of 23 different flavones, most of which were identified by comparing their UV and mass spectra with those of standards. Almost all taxa investigated contained flavones methoxylated in the 6- and 8-positions, such as nevadensin, xanthomicrol and gardenin B. The same taxa also produced flavones methoxylated in the 6-position but hydroxylated in the 8-position, including isothymusin (5,8,4′-trihydroxy-6,7-dimethoxyflavone), pedunculin (5,8-dihydroxy-6,7,4′-trimethoxyflavone) and a new flavone, 5,7,8-trihydroxy-6,4′-dimethoxyflavone, which was given the trivial name pilosin. This compound was isolated from *O. americanum* var. *pilosum* and also detected as a minor constituent in *O.*×*citriodorum* leaf extracts. Its molecular structure was elucidated by means of NMR spectroscopy. 8-Oxygenated flavones were absent only from *O. lamiifolium*. APCI mass spectrometry of the flavonoids revealed that the product ions formed by collision induced dissociation of the protonated molecule provided structural information about the substitution pattern of the A-ring. The chemotaxonomic and biogenetic implications of the results are discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Ocimum*; Lamiaceae; Basil; Chemotaxonomy; APCI mass spectrometry; 8-Hydroxylated external flavones; 5,7,8-Trihydroxy-6,4′-dimethoxyflavone

1. Introduction

Ocimum L. is a member of the Lamiaceae, subfamily Nepetoideae. The delimitation of the genus has been revised recently to include *Becium* and *Erythrochlamys*, and the taxon now comprises approximately 65 species (Paton et al., 1999). Many of these are highly aromatic, and several are economically important as essential oil producers, medicinal herbs and food flavourings, notably *O. basilicum* L. Some species have been reported to show insecticidal activity (e.g. Stein et al., 1988). The taxonomy of the group is difficult, partly due to the cultivation, selection and hybridisation of useful species by humans. Morphological characters are not always sufficient to accurately delimit each taxon, so characters from other disciplines such as anatomy and chemistry are a useful addition for taxonomic studies.

Flavonoids have been used extensively in chemotaxonomic studies of the Lamiaceae (Tomás-Barberán and Gil, 1992). Many species in this family produce both flavonoid glycosides in their vacuoles and flavonoid aglycones on the plant surface (Tomás-Barberán and Wollenweber, 1990). These aglycones, which are often highly methoxylated, frequently possess characteristic UV spectra and can be identified by means of high performance liquid chromatography with diode array detection (HPLC-DAD) of crude extracts when appropriate standards are available for comparison (Grayer et al., 1996). This avoids the need for time-consuming isolations to be carried out for every component and, as only small amounts of plant material are required, the procedure is ideal for chemotaxonomic surveys.

Previously, 16 different external flavonoids have been reported from species of *Ocimum*. Nevadensin and salvigenin were found in *O. canum* Sims (= *O. americanum* L.) (Xaasan et al., 1980) and hispidulin was also detected in this species (Tomás-Barberán and Wollenweber, 1990). In *O. basilicum*, nevadensin and salvigenin are the major flavones, and these co-occur with many minor external

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flavonoids, including cirsiliol, cirsilineol, eupatorin, apigenin, acacetin, genkwanin, apigenin 7,4′-dimethyl ether, cirsimaritin, ladanein and gardenin B (Grayer et al., 1996). Other workers have reported xanthomicrol from the same species (Fatope and Takeda, 1988). An unstable 8-hydroxylated flavone, isothymusin (5,8,4′-trihydroxy-6,7-dimethoxyflavone), was reported by Grayer and Veitch (1998) in *O. grandiflorum* Lam. (= *Becium grandiflorum* Pic. Serm.). Isothymusin also has been detected recently in *O. gratissimum* L., together with cirsimaritin, xanthomicrol and, additionally in one cultivar, luteolin (Grayer et al., 2000; Vieira et al., 2000). Most 8-hydroxylated flavones can be distinguished readily from other flavones on the basis of their UV spectra, the most characteristic feature being a strong band III with λ_{max} at approximately 300 nm. However, as these flavonoids tend to be unstable in solution at room temperature, extracts need to be investigated soon after preparation (Grayer and Veitch, 1998).

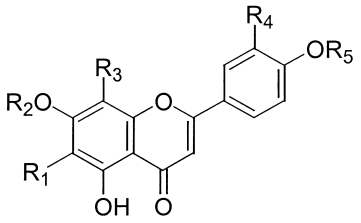
In the present study, surface extracts of nine different species of *Ocimum* have been surveyed for 8-hydroxylated and other external flavones using HPLC-DAD. Extracts were also analysed by HPLC with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) to aid flavonoid identification and to investigate whether product ion spectra generated by collision induced dissociation (CID) could provide additional features useful for the identification of surface flavonoids. A major compound from the surface extract of *O. americanum* var. *pilosum* with an unusual UV spectrum was isolated and identified as a new flavonoid using NMR spectroscopy.

2. Results and discussion

2.1. Identification of the external flavonoids

Flavones were obtained from *Ocimum* species by briefly washing intact, fresh leaves with dichloromethane. This indicated that these compounds were external and not vacuolar. Prolonged extraction of freeze-dried whole leaves in diethyl ether yielded the same sets of compounds, and HPLC analysis confirmed that the proportions of the flavones in the extracts of fresh and dried leaves were very similar. Among the extracts of these *Ocimum* species 23 flavonoids were detected, 15 of which were identified by comparison of their HPLC retention times, UV spectra and APCI mass spectra with those of standards. The flavonoids identified in this way were apigenin (**1**), genkwanin (**2**), acacetin (**3**), apigenin 7,4′-dimethyl ether (**4**), luteolin (**5**), ladanein (**6**), cirsimaritin (**10**), salvigenin (**11**), cirsiliol (**12**), cirsilineol (**13**) and/or eupatorin (**14**), isothymusin (**17**), nevadensin (**19**), xanthomicrol (**21**) and gardenin B (**22**) (Table 1; compounds **1–5** not shown). Both cirsilineol and eupatorin were identified previously in extracts of *O. basilicum* (Grayer et al., 1996), but the analytical methods used in the present study did not separate them (they exhibited virtually the same retention times, UV and mass spectra).

One major flavone in *O. americanum* var. *pilosum* (**16**) had a UV spectrum atypical of commonly encountered flavonoids (Fig. 1). A broad, high-intensity band III at ca. 300 nm indicated that it might have a free hydroxyl group in the 8-position (Grayer and Veitch, 1998). Band II consisted only of a slight shoulder at 287 nm, whereas a weak band I was observed at 332 nm, a feature typical of flavones without 3′-hydroxylation and methoxylated in the 4′-position. Positive ion APCI-MS gave a protonated molecule [MH^+] at m/z 331, which was compatible with the M_r of 330 required for a flavone with three OH and



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	H	H
2	H	Me	H	H	H
3	H	H	H	H	Me
4	H	Me	H	H	Me
5	H	H	H	OH	H
6	OH	Me	H	H	H
8	OH	Me	H	OMe	Me
9	OMe	H	H	H	Me
10	OMe	Me	H	H	H
11	OMe	Me	H	H	Me
12	OMe	Me	H	OH	H
13	OMe	Me	H	OMe	H
14	OMe	Me	H	OH	Me
15	OMe	Me	H	OMe	Me
16	OMe	H	OH	H	Me
17	OMe	Me	OH	H	H
18	OMe	Me	OH	H	Me
19	OMe	H	OMe	H	Me
20	OMe	H	OMe	OMe	Me
21	OMe	Me	OMe	H	H
22	OMe	Me	OMe	H	Me
23	OMe	Me	OMe	OMe	Me

Table 1
HPLC retention times, UV absorption maxima and APCI-mass spectral data for 5,6,7- or 5,6,7,8-substituted external flavones from *Ocimum* species

	A-ring substitution pattern and name of flavone	R_t (min)[a]	UV λ_{max} (nm)	MH^+ (m/z)	Relative intensities of fragments in CID spectra				
					$[MH-15]^+$	$[MH-30]^+$	$[MH-33]^+$	$[MH-48]^+$	$[MH-61]^+$
5,6-diOH-7-OMe flavones									
6	5,6-diOH-7,4′-OMe (ladanein)[b]	21.9	285, 334	315	100				
7	5,6,4′-triOH-7,3′-diOMe or 5,6,3′-triOH-7,4′-diOMe[c]	18.5	285, 345	331	100				
8	5,6,-diOH-7,3′,4′-triOMe[c]	20.0	285, 342	345	100				
5,7-diOH-6-OMe flavones									
9	5,7-diOH-6,4′-diOMe (pectolinarigenin)[c]	23.5	275, 333	315	100				
5-OH-6,7-diOMe flavones									
10	5,4′-diOH-6,7-diOMe (cirsimaritin)[b]	21.2	275, 336	315	100		95		2
11	5-OH-6,7,4′-triOMe (salvigenin)[b]	24.3	275, 333	329	100		90		2
12	5,3′,4′-triOH-6,7-diOMe (cirsiliol)[b]	19.6	273, 348	331	100		75		6
13	5,4′-diOH-6,7,3′-triOMe (cirsilineol)[b]	21.2	273, 346	345	100		84		7
14	5,3′-diOH-6,7,4′-triOMe (eupatorin)[b]	21.3	273, 344	345	100		80		6
15	5-OH-6,7,3′,4′-tetraOMe[c]	22.5	273, 343	359	100		86		2
5,7,8-triOH-6-OMe flavones									
16	5,7,8-triOH-6,4′-diOMe (pilosin)[d]	20.7	287sh, 299, 332	331	100				
5,8-diOH-6,7-diOMe flavones									
17	5,8,4′-triOH-6,7-diOMe (isothymusin)[b]	19.9	284sh, 304, 333sh	331	100		20		1
18	5,8-diOH-6,7,4′-triOMe (pedunculin)[c]	22.7	282sh, 304, 330sh	345	100		40		2
5,7-diOH-6,8-diOMe flavones									
19	5,7-diOH-6,8,4′-triOMe (nevadensin)[e]	22.9	281, 294sh, 332	345	100	28	20		
20	5,7-diOH-6,8,3′,4′-tetraOMe[c]	21.4	281, 343	375	100	20	18		
5-OH-6,7,8-triOMe flavones									
21	5,4′-diOH-6,7,8-triOMe (xanthomicrol)[b]	22.6	281, 294sh, 334	345	100	60	50	18	8
22	5-OH-6,7,8,4′-tetraOMe (gardenin B)[b]	25.6	283, 294sh, 330	359	100	80	56	20	10
23	5-OH-6,7,8,3′,4′-pentaOMe[c]	24.2	281, 342	389	97	100	50	30	12

[a] For solvent system, see Section 3.
[b] Identification by means of spectral (UV, MS) and chromatographic comparison with standards.
[c] Identification on the basis of UV and mass spectra.
[d] Identification by NMR and MS.
[e] Identification by NMR and spectral and chromatographic comparison with standards.

two OMe substituents. Compound **16** was isolated from a diethyl ether extract of *O. americanum* var. *pilosum* by semipreparative HPLC, yielding quantities sufficient for NMR spectroscopy. Nevadensin (**19**) was also isolated from the same plant source for comparative purposes. The ^{1}H NMR spectrum of **16** was similar to that of **19**, and indicated that the A-ring was fully substituted and the B-ring substituted only at the 4′-position. Two resonances characteristic of methoxyl substituents were present at δ 3.78 and 3.87 in the spectra of both compounds, while an additional methoxyl resonance was present at δ 3.88 in the spectrum of **19**. The location of the methoxyl substituents in **16** was established using 1D XSROESY experiments and comparison with analogous data obtained for **19** (Gradwell et al., 1997). Site selective excitation of the exchangeable resonance at δ 12.75 (5-OH) in the ^{1}H NMR spectrum of **19** gave ROE connectivities to resonances at δ 6.86 (H-3) and 3.78 only, establishing the latter as that of 6-OCH_3. Similarly, site selective excitation at δ 7.15 (H-3′,5′) gave a strong ROE connectivity to the methoxyl resonance at δ 3.87, establishing it as that of 4′-OCH_3. In the case of δ 8.02 (H-2′,6′), site selective excitation gave a strong ROE connectivity to δ 6.86 (H-3), and a weaker connectivity to the remaining methoxyl resonance at δ 3.88, which corresponds to 8-OCH_3. Site selective excitation of the exchangeable resonance at δ 12.49 (5-OH) in **16** gave ROE connectivities to resonances at δ 6.81 (H-3) and 3.78 only, establishing the latter as that of 6-OCH_3. Similarly, site selective excitation at δ 7.13 (H-3′,5′) gave a strong ROE connectivity to the methoxyl resonance at δ 3.87, establishing it as that of 4′-OCH_3. In contrast, the only ROE connectivity detected on site selective excitation of δ 8.12 (H-2′,6′) was to δ 6.81 (H-3). Additional information was obtained by comparing HSQC spectra of the two compounds. The ^{13}C NMR resonance assignments for 6-OCH_3 and 4′-OCH_3 in both compounds were identical, as expected, with a characteristic downfield shift of approximately 5 ppm for the methoxyl carbon at the 6-position compared to that at

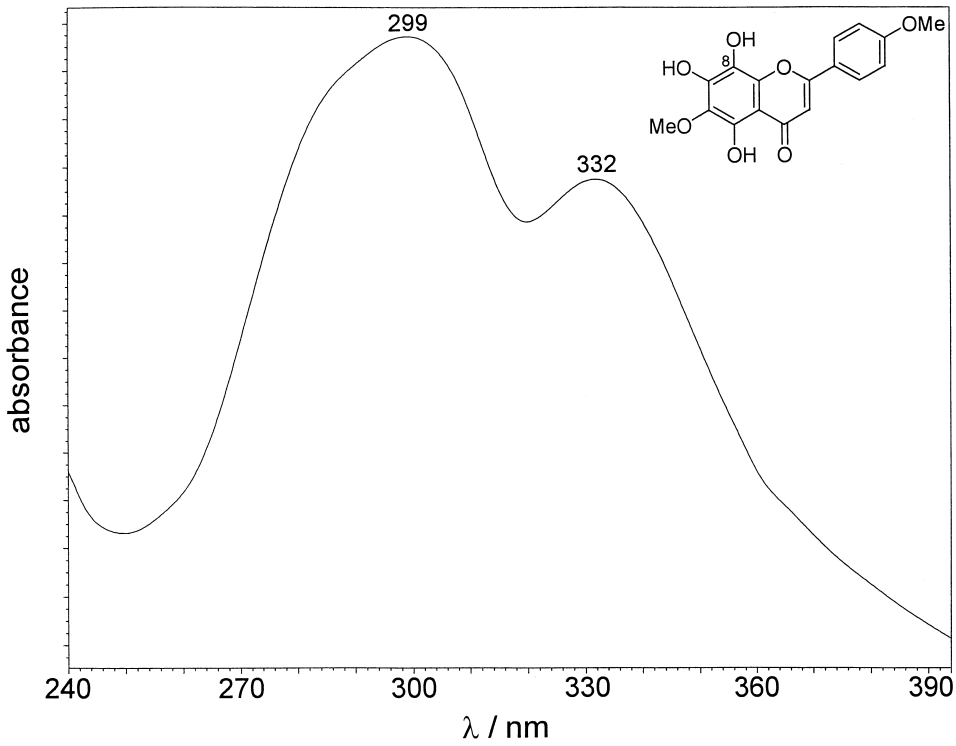


Fig. 1. UV absorption spectrum of pilosin obtained by HPLC/DAD of an extract of *O. americanum* var. *pilosin* (for conditions, see Section 3).

the 4′-position. The additional 8-methoxyl carbon of **19** at δ 60.8 is also downfield shifted with respect to the 4′-methoxyl carbon (δ 55.2), but to a slightly greater extent than the corresponding ^{13}C resonance of the 6-methoxyl carbon (δ 59.7). These results confirmed the identity of **16** as 5,7,8-trihydroxy-6,4′-dimethoxyflavone, a new flavonoid aglycone to which the trivial name of pilosin was given.

The remaining flavones, with the exception of **20**, were identified from their UV spectra obtained by HPLC/DAD, and from the pseudomolecular ion determined by APCI LC-MS. APCI-MS gave protonated molecules $[MH]^+$ of the minor flavones **7** and **8** from *O. lamiifolium* at m/z 331 and 345, respectively, indicating that **7** was a flavone with three OH and two OMe substituents and **8** a flavone with two OH and three OMe substituents. The λ_{max} of band II was 285 nm in both compounds, indicating a free 6-hydroxyl group. This indicates that the three OMe substituents of **8** are at the 7-, 3′- and 4′-positions. The λ_{max} of band I of 342 nm in **8** agrees with 3′,4′-dimethoxy substitution of the B-ring. Therefore **8** is likely to be 5,6-dihydroxy-7,3′,4′-trimethoxyflavone. The two OMe substituents of **7** could be located at the 7- and 3′-, 7- and 4′-, or 3′- and 4′-positions. The λ_{max} of band I of **7** at 345 rather than 342 nm indicates that the B-ring has only one OMe and one OH group, and therefore that the second OMe group should be at the 7-position (in general, methylation of the 7-OH substituent does not affect the UV spectrum of flavones). Therefore, compound **7** is likely to be either 5,6,4′-trihydroxy-7,3′-dimethoxyflavone or 5,6,3′-trihydroxy-7,4′-dimethoxyflavone (UV spectroscopy with NaOH as a shift reagent can distinguish between these two B-ring substitution patterns [Mabry et al., 1970], but could not be applied because **7** was not obtained pure). These identifications of **7** and **8** are consistent with the A-ring substitution pattern of the major flavone present in *O. lamiifolium*, ladanein (5,6-dihydroxy-7,4′-dimethoxyflavone). The protonated molecule of flavone **9** was at m/z 315, indicating that it possessed two OH and two OMe substituents. The λ_{max} of **9** was 275 nm for band II and 333 nm for band I, indicating that it was a 6-methoxyapigenin derivative substituted in the 4′-position. Therefore, **9** was identified as 5,7-dihydroxy-6,4′-dimethoxyflavone (pectolinarigenin). Similarly, the MS data for **15** indicated that it was a monohydroxy-tetramethoxyflavone ($[MH]^+ = m/z$ 359) and the UV data suggested that it was a derivative of 6-methoxy-luteolin ($\lambda_{max} = 273$, 343 nm). This makes it likely that **15** is 5-hydroxy-6,7,3′,4′-tetramethoxyflavone. In the same way, **23** was tentatively identified as 5-hydroxy-6,7,8, 3′,4′-pentamethoxyflavone ($[MH]^+ = m/z$ 389, $\lambda_{max} =$ 281, 342). APCI-MS in positive mode of the flavone **18** showed it to be substituted by two hydroxyl and three methoxyl groups ($[MH]^+ = m/z$ 345). Its UV spectrum (see Table 1) was very similar to that of isothymusin (5,8,4′-trihydroxy-6,7-dimethoxyflavone, **17**, $[MH]^+ =$ m/z 331). Methylation of the 5- or 8-hydroxyl changes the UV spectrum of a flavone considerably, but methylation of the 4′-hydroxyl only has a minor effect. Therefore

18 is likely to be the 4′-methyl ether of isothymusin, pedunculin. Collision induced dissociation spectra (see below and Table 1) supported these tentative identifications of **15**, **18** and **23**.

2.2. Structural information provided by collision induced dissociation (CID) spectra

APCI LC-MS provided not only the pseudomolecular ion (MH^+) for each flavone but also its CID spectrum by means of sequential mass spectrometry (MS-MS). These CID spectra appeared to be indicative of the A-ring methoxylation pattern in 5,6,7- and 5,6,7,8-oxygenated flavones, as shown in Table 1. The CID spectra of the five 5-hydroxy-6,7-dimethoxyflavones for which standards were available (**10–14**), all showed the loss of a methyl radical (15 amu) from the protonated molecule together with another prominent ion at $[MH\text{-}33]^+$, due to loss of both a methyl radical and a water molecule, and a minor ion at $[MH\text{-}61]^+$ (loss of one methyl radical, water and CO). The 5,8-dihydroxy-6,7-dimethoxyflavone, isothymusin, showed a similar fragmentation profile, but the product ion at $[MH\text{-}33]^+$ was at a lower intensity. The protonated molecule of nevadensin (which has a 5,7-dihydroxy-6,8-dimethoxy substitution pattern in the A-ring) produced both the $[MH\text{-}15]^+$ and $[MH\text{-}33]^+$ fragments after CID, and also one at $[MH\text{-}30]^+$ (loss of two methyl radicals), whereas the 5-hydroxy-6,7,8-trimethoxyflavones, xanthomicrol and gardenin B, yielded five fragments: $[MH\text{-}15]^+$, $[MH\text{-}30]^+$, $[MH\text{-}33]^+$, $[MH\text{-}48]^+$ and $[MH\text{-}61]^+$. The $[MH\text{-}48]^+$ ion can be rationalised as the loss of two methyl radicals and water. Thus, by use of CID spectra in combination with the pseudomolecular ion, structural assignments could be proposed for **20** (see Table 1). The similarity of the CID spectrum of **20** to that of **19** (nevadensin) suggests that the A-ring substitution of **20** is also 5,7-dihydroxy-6,8-dimethoxy. Furthermore, as the $[MH^+]=m/z$ 375 indicates that **20** is a dihydroxytetramethoxyflavone and the λ_{max} of band I of the UV spectrum suggests oxygenation at the 3′- and 4′-positions, **20** was tentatively identified as 5,7-dihydroxy-6,8,3′,4′-tetramethoxyflavone.

Stevens et al. (1999) also studied CID spectra of methoxylated flavonoids by APCI-MS, but achieved a more extensive fragmentation of the compounds by using a triple quadrupole mass spectrometer rather than the ion trap instrument used in the present study. In the triple quadrupole instrument, fragmentation of the precursor ion generates product ions that tend to undergo further collisions and thus additional fragmentation, producing a cascade effect. This results in a CID spectrum that contains both second and third generation fragments (Lopez et al., 1999). In contrast, in an ion trap instrument product ions are generated directly from the parent ion and not from any intermediate products, so that a simpler spectrum is obtained.

2.3. Distribution of external flavones in species of *Ocimum*

Table 2 shows the occurrence of the major flavones in the 12 accessions studied, which represent nine species of *Ocimum*. Quantities of flavonoids were estimated by integration of the appropriate HPLC peaks (see Section 3), and percentages were calculated in relation to the total amount of flavonoids in each accession. Flavones lacking 6- and 8-oxygenation such as apigenin and luteolin and their methyl ethers (compounds **1–5**), were present in small amounts in most taxa. These may function as precursors of the 6- and 6,8-oxygenated flavones that

Table 2
Composition (%) and amounts of external flavones in diethyl ether extracts of freeze-dried leaves of *Ocimum* species[a]

Species/variety	Accession number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**10/13/14**[b]	**11**	**12**	**16**	**17**	**18**	**19**	**21**	**22**	**23**	Total flavonoid (mg/g dry wt)
O. americanum L. var. *americanum*	BI 6445									8							49		43	0.1
O. americanum var. *pilosum* (Willd.) Paton	BI 6419	tr	tr	tr	5	tr	tr			tr	15		16		tr	57		tr		14.6
O. americanum cv. Sacred	BI 6442						14			63				23						0.04
O. basilicum L.	BI 2755		tr	tr	5		tr			3	37				30	19		4		0.6
O.×citriodorum Vis.	BI 6446	tr	tr	tr	4	3	3			5	38	tr	tr			41		2		3.7
O. grandiflorum Lam.	BI 6418									40				60						2.5
O. kilimandscharicum L.	BI 6420	tr	tr			5				14	77	2						tr		0.5
O. lamiifolium Hochst. ex Benth.	BI 6424						91	7	2											0.2
O. minimum L.	BI 6443				5					2	75					19				0.1
O. minimum L.	BI 6444	tr			9					2	75				7	tr		5		0.3
O. selloi Benth.	BI 6421		2							11	13			4	29			41		0.9
O. tenuiflorum L.	BI 6447	6								24	18			6				46		0.03

[a] Compounds **9**, **15** and **20** were detected in trace amounts in one plant species only and are excluded from the table. They occurred in the following accessions: **9** and **20** in *O.×citriodorum* BI 6446 and **15** in *O. americanum* var. *americanum* BI 6445. Percentages are an average of two determinations. tr = trace (1% or less).

[b] As **10**, **13** and **14** co-eluted using HPLC/DAD, only their combined amount and percentage could be determined.

accumulate in the plants. The most common hydroxylation/methoxylation pattern of the A-ring of external flavones in the *Ocimum* species studied was 5-hydroxy-6,7-dimethoxy. Compounds with this substitution pattern (e.g. salvigenin **11**) occurred, often in high concentrations, in all species apart from *O. lamiifolium*. On the other hand, 5,6-dihydroxy-7-methoxyflavones such as ladanein **6**, were accumulated only by *O. lamiifolium*. A 5,7-dihydroxy-6,8-dimethoxyflavone, nevadensin **19**, was a major compound in four taxa (*O. americanum* var. *pilosum, O. basilicum, O.×citriodorum* and one accession of *O. minimum*). 5-Hydroxy-6,7,8-trimethoxyflavones (e.g. xanthomicrol **21** and gardenin B **22**) were detected in all taxa apart from *O. lamiifolium* and *O. grandiflorum*, but were major flavonoids in only three accessions. 8-Hydroxylated flavones (5,8-dihydroxy-6,7-dimethoxy- and 5,7,8-trihydroxy-6-methoxyflavones) such as isothymusin **17**, and pilosin **16**, were detected in the majority of taxa, but only occurred in high concentrations in five accessions.

2.4. Chemotaxonomic and biogenetic considerations

According to Tomás-Barberán and Wollenweber (1990), 5-hydroxy-6,7-dimethoxyflavones are extremely common in the Lamiaceae, and 5-hydroxy-6,7,8-trimethoxyflavones and 5,6-dihydroxy-7-methoxyflavones also occur frequently. In contrast, 5,7-dihydroxy-6,8-dimethoxyflavones (e.g. nevadensin) have only been reported in the Lamiaceae from the genus *Ocimum*. It is therefore likely that all taxa containing these compounds (*O. americanum* var. *pilosum, O. basilicum, O.×citriodorum, O. minimum*) are closely related. Similarly, the presence of 8-hydroxylated surface flavones (isothymusin **17**, pedunculin **18** and pilosin **16**) in the Lamiaceae has only been noted in *Ocimum*, although these compounds may have been overlooked in previous flavonoid surveys of the family due to their relative instability. Isothymusin, pedunculin and pilosin are likely to be the precursors of the corresponding 8-methyl ethers xanthomicrol, gardenin B and nevadensin, respectively, with which they often co-occur in *Ocimum* species. These 8-methyl ethers are more stable chemically. It would be useful to survey other genera known to produce 8-methoxylated external flavones for the corresponding 8-hydroxylated derivatives. Alternatively, chemically unstable surface flavones with an 8-hydroxyl group may be stored in some plants as the more stable 8-*O*-glycosides, as is the case in *O. grandiflorum* where isothymusin was found together with the 8-*O*-glucoside (Grayer and Veitch, 1998). The immediate precursor of pilosin is likely to be its 8-deoxy derivative 5,7-dihydroxy-6,4′-dimethoxyflavone (pectolinarigenin), which was detected in trace amounts in *O.×citriodorum*, a taxon that also produces pilosin and nevadensin. Similarly, the immediate precursors of isothymusin and pedunculin are probably the 8-deoxy derivatives cirsimaritin and salvigenin, respectively, compounds which always accompanied the 8-hydroxylated flavones in the plant material investigated.

In terms of B-ring substitution patterns, most external flavones found in the genus *Ocimum* are only oxygenated at the 4′ carbon, and oxygenation at both 3′ and 4′ carbons is rare. In most taxa examined the 4′-hydroxyl groups of the major flavones were methylated, e.g. in *O. americanum* var. *pilosum, O. basilicum, O.×citriodorum, O. kilimandscharicum, O. lamiifolium, O. minimum* and *O. selloi* (nevadensin, salvigenin, ladanein and gardenin B), whereas they were free in only few accessions, e.g. *O. americanum* 'Sacred' and *O. grandiflorum* (xanthomicrol, cirsimaritin and isothymusin). The only major flavone oxygenated in both 3′- and 4′-positions was compound **23**, tentatively identified as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, which was detected in *O. americanum* var. *americanum*. Compounds **13** and **14**, also oxygenated in the 3′- and 4′-positions, were not major constituents in *Ocimum*, although taken together, compounds **10**, **13**, and **14** were among the most abundant constituents in some species (see Table 2). However, according to the UV spectral maxima (band I at 336 nm rather than 343 or 344 nm) and LC–MS analysis of the co-eluting peak (a more intense ion at *m/z* 315, due to **10**, than at *m/z* 345, due to **13** or **14**), compound **10**, which lacks 3′-oxygenation, is the major compound in these cases rather than **13** or **14**.

2.5. Qualitative and quantitative variation of external flavonoids in Ocimum

Table 2 shows that the amount of external flavonoids produced by the various accessions studied varied greatly, from 0.03 mg/g dried leaves in *O. tenuiflorum* BI 6447, to more than 14 mg/g in *O. americanum* var. *pilosum* BI 6419, a factor of ×500. Table 2 also illustrates the considerable infraspecific variation in surface flavonoids found in the three accessions of *O. americanum* studied, both in qualitative and quantitative terms. As noted previously, very high quantities of flavones were found in the accession studied for *O. americanum* var. *pilosum*, whereas the accessions examined for var. *americanum* and cultivar 'Sacred' contained very low quantities of surface flavonoids. To ensure that any quantitative differences in flavonoid content among the taxa were not due to environmental factors, all plants had been grown under the same conditions. Leaves were always harvested after a dry period of at least several days. In addition, some plants were sampled several times with long intervals in between to evaluate whether external flavonoid levels fluctuated, but no significant changes were recorded. Similar infraspecific quantitative differences in external flavone content as found in *O. americanum* were detected previously in *O. gratissimum*, and these correlated with essential oil profiles (Vieira et al.,

2000). Accessions of this species that were rich in monoterpenoid essential oils produced high concentrations of surface flavonoids, whereas accessions that produced mainly phenylpropanoid essential oils contained very low levels of external flavones. It may be that a similar correlation between essential oils and surface flavonoids exists in *O. americanum*.

During a previous investigation into the infraspecific variation in external flavonoids in *O. basilicum*, only small quantitative differences between the 16 accessions studied were found (Grayer et al., 1996). The accession of *O. basilicum* examined in this study differs from those described previously by containing pedunculin. However, at the time of the earlier study, the compound now known to be pedunculin was not recognised as a flavonoid because of its unusual UV spectrum, and was not included in the results. Re-evaluation of the data shows that pedunculin occurs in trace amounts in about half of the accessions of *O. basilicum* studied, and is accumulated in two accessions. During the present investigations, pedunculin was also detected in one out of two accessions studied of *O. minimum* (BI 6443), whereas this compound was absent from the other accession, BI 6444, which contained nevadensin instead (Table 2).

It is interesting to note that the total amount of flavonoids in *O.×citriodorum* and the percentages of nevadensin and pilosin are intermediate between those of *O. basilicum* and *O. americanum* var. *pilosum*, as *O.×citriodorum* is thought to be a hybrid between *O. basilicum* and *O. americanum* (Paton and Putievsky, 1996).

3. Experimental

3.1. Flavonoid standards

Standards of **1–5**, **12** and **14** were obtained from Apin Chemicals Ltd. Small amounts of **6**, **10**, **11**, **13**, **19**, **21**, and **22** for chromatographic and UV spectral comparison were kindly provided by Professor E. Wollenweber, and **17** was obtained from a freshly prepared extract of *O. grandiflorum* leaves (Grayer and Veitch, 1998).

3.2. Plant material

Ocimum species were grown for several months under glasshouse conditions at the Royal Botanic Gardens, Kew, after germination of the seeds in early spring. In the summer the young plants were transferred outside to expose them to natural light. Plants were harvested for chemical investigation after growing outside for approx. 2 months and after a dry period. Two flowering stems of each accession were pressed to supply herbarium vouchers. A few fresh leaves of the plants were extracted immediately, while the remaining leaves were detached from the stems, frozen at −20°C and later freeze-dried. Voucher specimens have been deposited in the Herbarium, Royal Botanic Gardens, Kew (accession numbers are given in Table 2).

3.3. Extraction of plant material and isolation of flavones

For the flavonoid survey, 1.5 g of fresh leaves of each accession were rinsed briefly with 30 ml of dichloromethane to investigate whether external flavonoids were present. For the purposes of quantitation, 0.5 g of freeze-dried leaves of the same accessions were extracted twice for 24 h with 15 ml of diethyl ether. The combined ether extracts were left to evaporate in a fume cupboard, the dried extracts were each dissolved in 1.0 ml of 80% aqueous MeOH, and these solutions were examined by analytical HPLC. For the isolation of pilosin (16) and nevadensin (19), 14 g of freeze-dried leaves of *Ocimum americanum* var. *pilosum* were extracted in 400 ml diethyl ether for 1 h. The extract was decanted and the plant material re-extracted with 300 ml diethyl ether. The combined extracts were evaporated at room temperature, the residue was dissolved in 4 ml of 80% MeOH and from this solution **16** and **19** were isolated by semi-preparative HPLC.

3.4. Analytical and semi-preparative HPLC

The HPLC system consisted of a Waters LC 600 pump and 996 photodiode array detector. LiChrospher 100RP-18 (5 μm) columns were used; 4.0 mm (i.d.)×250 mm for analytical work and 10.0 mm (i.d.)×250 mm for semi-preparative isolations. Gradient profiles based on two solvents, denoted A and B, were employed. For analytical work, A was 2% aqueous HOAc and B was MeOH–HOAc–H_2O, 18:1:1, and for semipreparative work A was H_2O and B was MeOH. Initial conditions were 75% A, 25% B, with a linear gradient reaching B = 100% at $t = 20$ min. This was followed by isocratic elution (B = 100%) to $t = 24$ min, after which the programme returned to the initial solvent composition. Column temperature was maintained at 30°C, and flow rates of 1.0 and 4.5 ml min^{-1} were used for analytical and semiprepared HPLC, respectively. For analytical HPLC of crude extracts all samples were injected (40 μl) by autosampler and run consecutively, in order to ensure that retention times were comparable.

3.5. Estimation of flavonoid content

A standard solution was prepared of 0.05% cirsiliol (Apin), and 40 μl of this solution and dilutions thereof were analysed by HPLC in order to prepare a standard curve. This was used to estimate the amount of each flavonoid present in the HPLC traces of the extracts by integration of the corresponding peaks at the λ_{max} of each compound. In these calculations it was assumed that the

molar extinction coefficient of cirsiliol was comparable to that of the other flavones analysed. Total flavonoid amounts in mg per gram dried leaves in each extract were calculated by adding the amounts of the individual flavonoids together, whereas composition percentages of the individual flavonoids were calculated by dividing their amounts by the total amount of flavonoids.

3.6. APCI LC-MS

For APCI LC-MS, chromatography was performed in a similar manner to analytical HPLC except the concentration of acetic acid in the mobile phases was 1%. Mass spectra were recorded using a quadrupole ion trap mass spectrometer (Finnigan LCQ) with the sample being ionised by an APCI source using a vaporiser temperature of 550°C, sheath and auxillary nitrogen flow pressures of 80 and 20 psi respectively, a capillary temperature of 150°C and a needle current of 5 μA. The mass spectrometer was controlled by Xcalibur 1.0 software (Finnigan) which was programmed to record the first order mass spectra and then the CID spectra of three or four of the most intense ions in each first order spectrum by means of the dynamic exclusion facility. CID spectra were obtained by prior isolation of the parent ion in the trap (isolation width 5 amu) and then applying a collision energy of 45% (without wide band activation). Dynamic exclusion allowed the CID spectra of co-eluting compounds to be recorded automatically by the instrument without prior knowledge of their molecular masses.

3.7. NMR spectroscopy

NMR spectra were acquired using a Varian 500 MHz instrument. Samples were dried carefully, dissolved in DMSO-d_6 and referenced to the residual solvent resonance at δ_H 2.50 ppm or δ_C 39.50 ppm as appropriate. All experiments were carried out at 37°C.

3.8. Pilosin (5,7,8-trihydroxy-6,4′-dimethoxyflavone) (**16**)

Yellow-brown amorphous solid. UV (MeOH) λ_{max} (nm): 287sh, 299, 332. LC/APCI-MS (positive mode): m/z 331 $[MH]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.49 (1H, *s*, 5-OH), 8.12 (2H, *d*, $J=8.5$ Hz, H-2′,6′), 7.13 (2H, *d*, $J=8.5$ Hz, H-3′,5′), 6.81 (1H, *s*, H-3), 3.87 (3H, *s*, 4′-OCH_3), 3.78 (3H, *s*, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) (assignment of non-quaternary C atoms from HSQC): δ 128.3 (C-2′,6′), 114.4 (C-3′,5′), 102.6 (C-3), 59.8 (6-OCH_3), 55.3 (4′-OCH_3).

3.9. Nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone) (**19**)

Yellow powder. UV (MeOH) λ_{max} (nm): 281, 294sh, 332. LC/APCI-MS (positive mode): m/z 345 $[MH]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.75 (1H, *s*, 5-OH), 8.02 (2H, *d*, $J=8.8$ Hz, H-2′,6′), 7.15 (2H, *d*, $J=8.8$ Hz, H-3′,5′), 6.86 (1H, *s*, H-3), 3.88 (3H, *s*, 8-OCH_3), 3.87 (3H, *s*, 4′-OCH_3), 3.78 (3H, *s*, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) (assignment of non-quaternary C atoms from HSQC): δ 127.9 (C-2′,6′), 114.5 (C-3′,5′), 102.9 (C-3), 60.8 (8-OCH_3), 59.7 (6-OCH_3), 55.2 (4′-OCH_3).

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Phytoecdysteroids in the genus *Asparagus* (Asparagaceae)

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Abstract

Phytoecdysteroids, plant steroids which are analogues of invertebrate steroid hormones, probably contribute to the deterrence of phytophagous invertebrate predators. They also seem to possess antimicrobial activity and several pharmaceutical and medicinal benefits have been ascribed to them. Here, we present a survey of seeds of 16 species of the genus *Asparagus* (Asparagaceae), including the crop species *A. officinalis*, for ecdysteroid agonists (including phytoecdysteroids) and antagonists. Seven species were found to contain ecdysteroids with levels ranging from just detectable (*A. racemosus* and *A. sarmentosus*) to relatively high (*A. laricinus*). RP-HPLC/RIA/bioassay has been used to separate positive extracts of four species (*A. falcatus, A. laricinus, A. ramosissimus* and *A. scandens*) and analyse the ecdysteroid profiles. The identities of the major ecdysteroids were confirmed by NP-HPLC. Seeds of *A. officinalis* do not contain detectable levels of ecdysteroids, but leaves, stems and roots contain low levels (detectable by RIA). This indicates that *A. officinalis* retains the genetic capacity to synthesise ecdysteroids and that future strategies could be developed for enhanced protection of asparagus spears through elevated ecdysteroid levels. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Asparagus*; Asparagaceae; Agonist; Antagonist; Asparagus; Chemotaxonomy; Ecdysteroid; Moulting hormone; Steroid

1. Introduction

The Asparagaceae is a monogeneric Family (Brummitt, 1992), which was previously included within the Liliaceae. The genus *Asparagus* comprises ca. 100 species (w^3-TROPICOS, 2000) and consists of herbs, shrubs and vines which are widespread in the Old World. Commercially, the most important member of the genus is *A. officinalis* (vegetable asparagus), the "spears" of which form a high value crop in several parts of Europe, North America and Asia. The major pest species are *Crioceris* spp (asparagus beetles), which infest asparagus wherever it is cultivated (Hill, 1987). As such, new methods for the control of invertebrate and microbial pests on this crop are sought. One area with good prospects for the control of invertebrate pests is the identification of ecdysteroid agonists or antagonists which disrupt steroid hormone action in the pest species (Dinan, 1995; Hsu et al., 1997; Dinan et al., 1999a). Certain plant species contain steroidal analogues of the ecdysteroids, which are referred to as phytoecdysteroids (Lafont et al., 1991). All known phytoecdysteroids appear to have agonistic activity at the insect ecdysteroid receptor complex (Dinan et al., 1999b). Members of several other classes of secondary plant product, including cucurbitacins, withanolides and stilbenoids, antagonise 20-hydroxyecdysone action (Dinan et al., 1999a). Phytoecdysteroids may also have chemotaxonomic value, since the presence and profile of the compounds may be diagnostic of the species (Dinan et al., 1998; Dinan et al., 2000a). We present here a survey of 18 seed samples representing 16 species in the genus *Asparagus* for ecdysteroid agonist and antagonist activities, an examination of the ecdysteroid profiles in four ecdysteroid-containing species and the distribution in plants of *A. officinalis*. Recently, three ecdysteroids (20-hydroxyecdysone, calonysterone and blechnoside B) have been isolated from *A. dumosus* and 20-hydroxyecdysone was shown to be responsible for the antimicrobial activity of extracts of this species (Ahmad et al., 1996; Khaliq-uz-Zaman et al., 2000). Thus, ecdysteroids would appear to have wide-ranging allelopathic value. Several medicinal

Abbreviations: E, ecdysone; 20E, 20-hydroxyecdysone; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NP, normal-phase; polB, polypodine B (5β; 20-dihydroxyecdysone); ponA, ponasterone A (25-deoxy-20-hydroxyecdysone); RIA, radioimmunoassay; RP, reversed-phase.

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and pharmaceutical benefits have also been ascribed to them (Sláma and Lafont, 1995).

2. Results and discussion

2.1. Ecdysteroid agonist and antagonist activities in seed extracts

None of the 18 samples tested positive for ecdysteroid antagonistic activity. However, seed extracts of seven of the species are positive in the ecdysteroid agonist assay and/or the ecdysteroid-specific radioimmunoassays (Table 1). The five samples positive in both the agonist bioassay and the RIAs almost certainly contain phytoecdysteroids. The RIAs assess the amount of material structurally related to ecdysone, while the B_{II} assay detects ecdysteroid-like biological activity. Thus, seeds of *A. laricinus* appear to contain relatively large amounts of ecdysteroids. *A. scandens* contains moderate amounts and *A. falcatus* and *A. ramosissimus* have low levels of ecdysteroid, as assessed by RIA, but the bioassay indicates that *A. falcatus, A. laricinus* and *A. scandens* possess similar activities. *A. hybridus, A. racemosus, A. ramosissimus* and *A. sarmentosus* contain very low levels of phytoecdysteroids. Analysis of parts of growing plants of *A. officinalis* revealed low levels of ecdysteroids as detected by RIA. This indicates that *A. officinalis* possesses the genetic capacity to produce ecdysteroids, but that the biosynthetic pathway is not activated to any great extent in this species. This implies that, should ecdysteroids prove to be effective pest control agents, asparagus could be genetically modified to enhance ecdysteroid levels without the need to introduce the genes for the biosynthetic enzymes.

2.2. Ecdysteroid profiles in seed extracts

2.2.1. A. laricinus

RIA data indicated that *A. laricinus* seeds possessed the highest levels of phytoecdysteroids among the species tested. Separation of a portion of the extract (1 μg ecdysone equivalents, corresponding to 5.3 mg seeds) by RP-HPLC (Fig. 1) indicated the presence of one major UV-absorbing, agonist bioassay-positive and RIA-positive peak with the same retention time as 20-hydroxyecdysone (Rt = 16.7 min). Only this major peak contributes to the bioassay activity, but RIA-positive material is found from 13 to 22 min, indicating that the minor UV-absorbing peaks in this region (mainly those at 14.0, 14.7 and 20.8 min) are also ecdysteroids. The RIA and bioassay profiles are not altered by hydrolysis

Table 1
Ecdysteroid agonist and antagonist activities in methanolic extracts of *Asparagus* spp (seed extracts unless otherwise stated)

	RIA[b]		Bioassay[c]	
Species[a]	Black	DBL-1	Agonist	Antagonist
Asparagus asparagoides[1]	–	–	–	–
Asparagus densiflorus var. *myersii*[2]	–	–	–	–
Asparagus falcatus[1]	1.83	5.80	+(+)	–
Asparagus hybridus[1]	0.21	0.21	+	–
Asparagus laricinus[1]	25.1	188.3	++	–
Asparagus officinalis[1]	–	–	–	–
leaves	0.27	0.23	C–	C–
stems	0.30	–	–	–
roots	0.18	0.36	C–	C–
Asparagus plumosus nanus[2]	–	–	–	–
Asparagus pseudoscaber[2]	–	–	–	–
Asparagus racemosus[1]	0.53	0.15	–	–
Asparagus ramosissimus[1]	1.24	4.66	+	–
Asparagus sarmentosus[1]	–	0.26	–	–
Asparagus scandens[2]	+[d]	34.4	++	–
Asparagus schoberioides[3]	–	–	–	–
Asparagus setaceus[1] (= *A. plumosus*)	–	–	–	–
Asparagus sprengeri[2] (= *A. densiflorus*)	–	–	–	–
Asparagus subulatus[1]	–	–	–	–
Asparagus verticillatus[2]	–	–	–	–
Asparagus virgatus[1]	–	–	–	–

[a] Source: [1]B&T World Seeds, Olonzac, France; [2]Chiltern Seeds, Ulverston, UK; [3]Seed Guild, Lanark, UK.

[b] Results expressed in μg ecdysone equivalents/g; – not detectable.

[c] Biological activity in the B_{II} bioassay was determined on 10 μl aliquots: – not active as the neat extract; + active as a neat extract; ++ active as a 10-fold dilution and +++ active as a 100-fold dilution. C– signifies cytotoxic as a the neat extract, but not at 10- or 100-fold dilutions.

[d] RIA-positive, but amount not quantified.

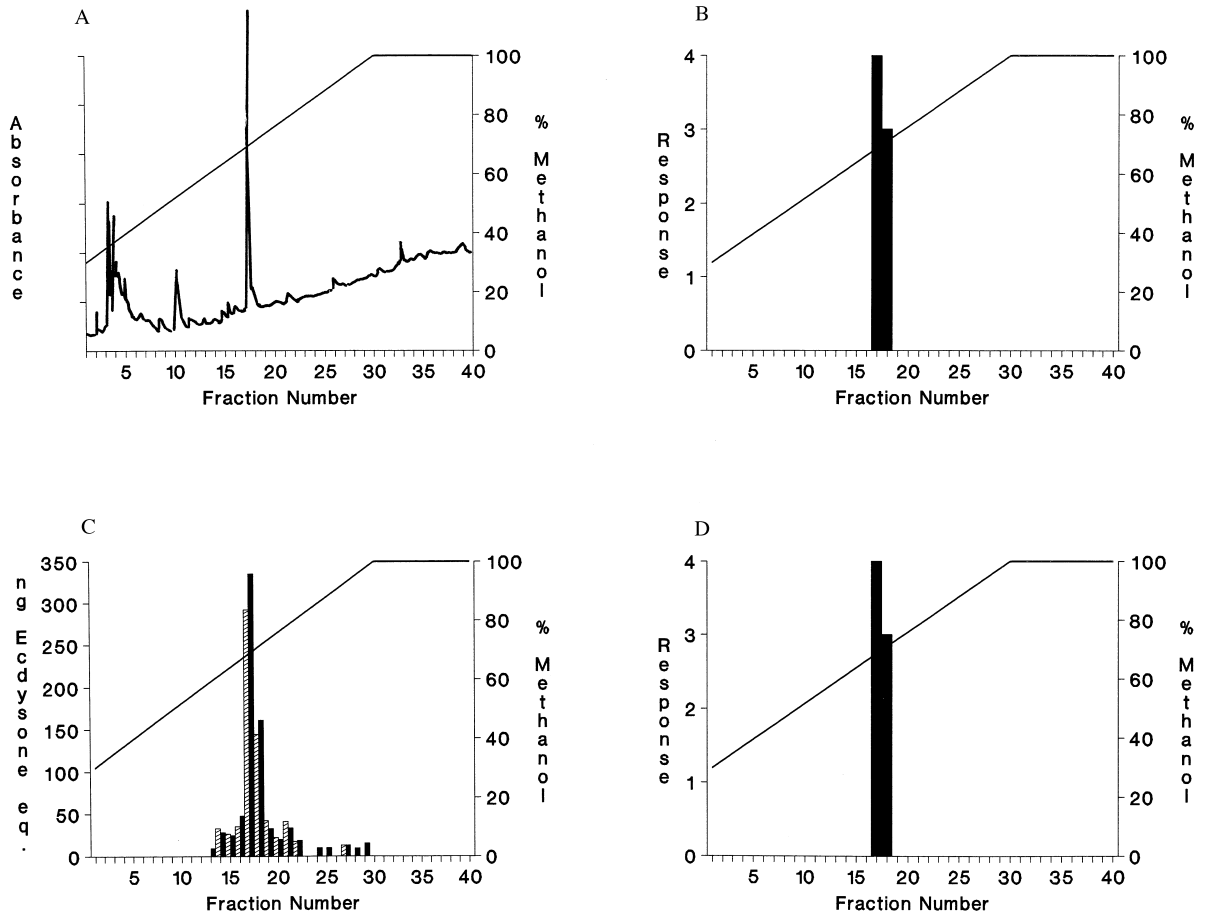


Fig. 1. RP-HPLC/RIA/bioassay of a seed extract of *Asparagus laricinus*. A portion of the seed extract (equivalent to 1 μg ecdysone equivalents with the DBL-1 antiserum) was separated on a C_{18} analytical column (Spherisorb ODS-2; 25 cm×4.6 mm i.d.; 5 μm particle size) with a MeOH/H_2O gradient at 1 ml/min and monitored at 242 nm (panel A). Fractions of 1 min duration were collected and aliquots subjected to enzymic hydrolysis, RIA (panel B; hatched columns = RIA without prior hydrolysis, solid columns = RIA after hydrolysis; NB: for each fraction, the column without hydrolyis is presented to the left of the column with hydrolysis) and agonist bioassay [panel C: without hydrolysis (2 μl aliquots); panel D: after hydrolyis (20 μl aliquots, equivalent to 2 μl before hydrolysis)]. Under these conditions, reference 20-hydroxyecdysone (20E), polypodine B (polB), inokosterone and ecdysone (E) elute in fractions 17, 17, 18 and 20, respectively.

with *Helix* enzymes, demonstrating that hydrolysable ecdysteroid conjugates are not present in this extract. The height of the peak at 16.7 min corresponds to 1.3 μg 20E, which, allowing for the cross-reactivity factor for 20E with the DBL-1 antiserum (1.3; Dinan, 1995), is what would be expected from 1 μg ecdysone equivalents if 20E accounted for the vast majority of the RIA-positive material in the extract. However, since several other ecdysteroids are known to co-elute with 20E on C_{18} reversed-phase HPLC with methanol/water solvent systems, the identity of the material in the major peak was examined on a NP-HPLC system (Fig. 2). This gave two readily detectable UV-, RIA- and bioassay-positive peaks at 6.6 and 9.8 min, coeluting with polB and 20E, respectively. The UV-peak areas signify a molar ratio of 1:11 (polB:20E). The enhanced sensitivity of detection of the polB peak in the bioassay is in accord with the greater biological activity of polB over 20E (Dinan et al., 1999b). In addition, there is a barely detectable UV-absorbing peak at 5.5 min, which is detected by RIA (fractions 11 and 12) and much more prominently by bioassay. The identity of this minor ecdysteroid with significant biological activity is at present unknown.

2.2.2. *A. scandens*

The seed extract of *A. scandens* (1 μg ecdysone equivalents = 29 mg seeds) separates on the RP-gradient system to give three major UV-absorbing peaks (14.5, 15.1 and 16.1 min) and a series of minor peaks between 12 and 20 min (Fig. 3). RIA-positive material is found throughout this region, so almost certainly most of these minor peaks correspond to ecdysteroids. However, most of the RIA-positive material elutes in fraction 17, corresponding to the peak at 16.1 min (20E). Biological activity also associated with fraction 17 (Fig. 3c). The height of the UV-peak in fraction 17 corresponds to 1.3 μg 20E. Separation of the material from this peak on the NP-gradient system (data not shown) gave two UV-absorbing,

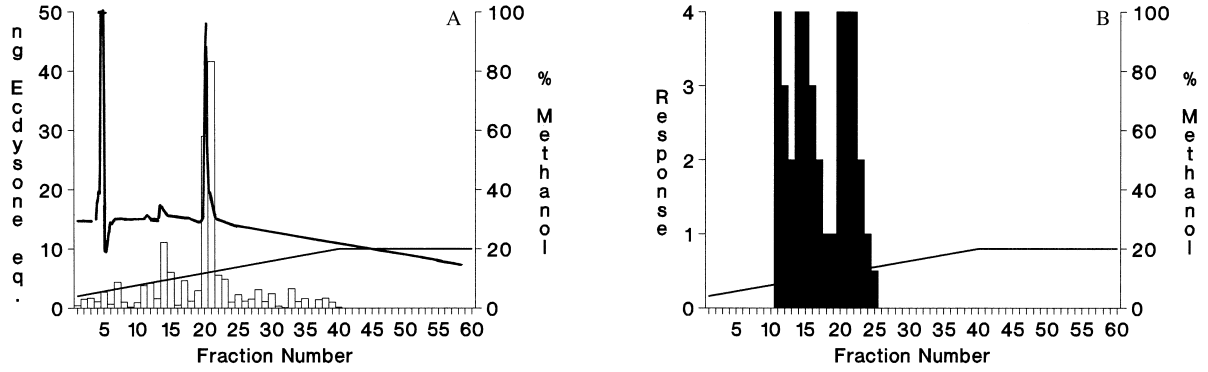


Fig. 2. RP-HPLC/RIA/bioassay of the material co-chromatographing with 20-hydroxyecdysone on RP-hplc (fractions 17 and 18) from a seed extract of *Asparagus laricinus*. The sample was separated on an Apex II Diol analytical column (15 cm×4.6 mm i.d.; 5 μm particle size) with a linear MeOH/dichloromethane gradient (4–20%) at 1 ml/min over 20 min, followed by 10 min at 20% MeOH and monitored at 242 nm (panel A). Fractions of 30 s duration were collected and aliquots subjected to RIA (panel A) and agonist bioassay (panel B [20 μl aliquots]). Under these conditions, reference polypodine B (polB), ecdysone (E), 20-hydroxyecdysone (20E) and inokosterone elute in fractions 14, 17, 20 and 21, respectively.

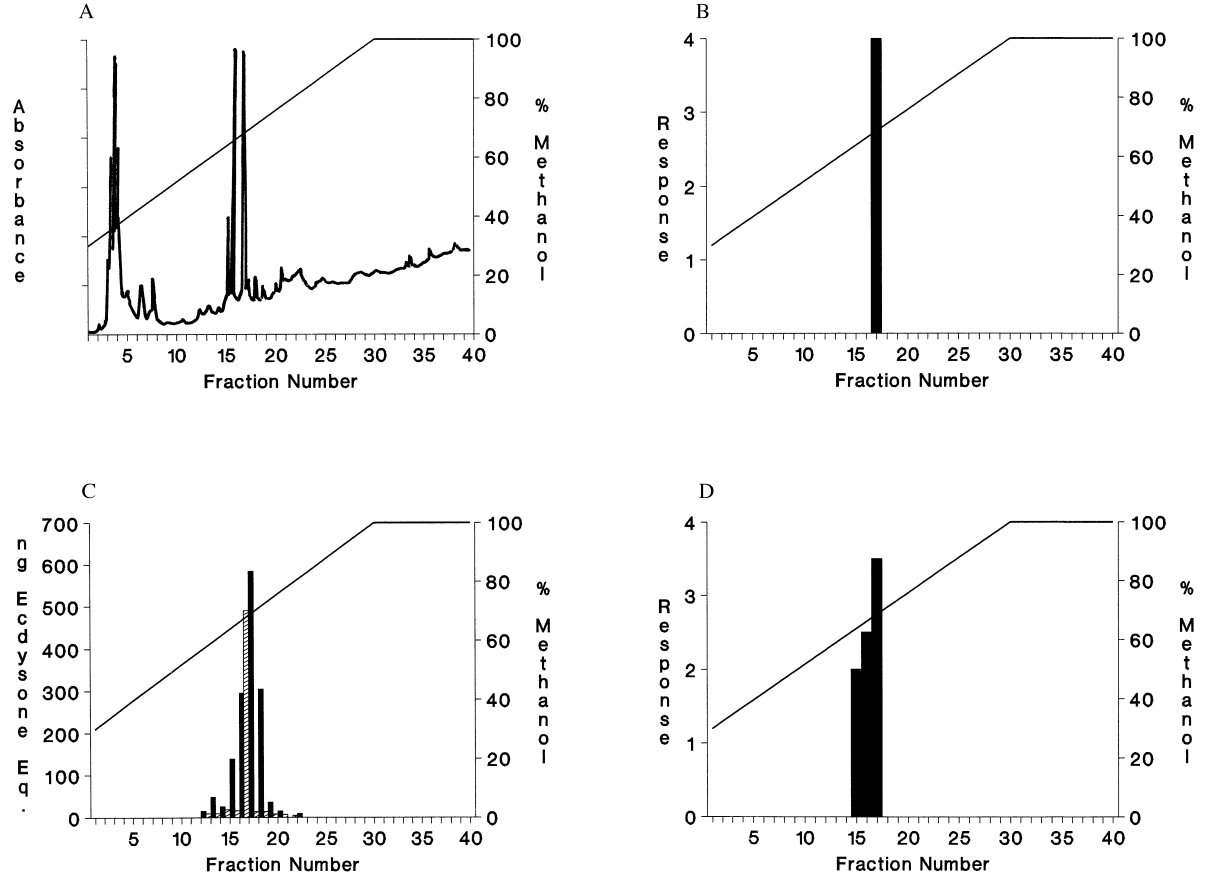


Fig. 3. RP-HPLC/RIA/bioassay of a portion of the seed extract of *Asparagus scandens* (1 μg ecdysone equivalents with the DBL-1 antiserum). Legend as for Fig. 1.

RIA- and bioassay-positive peaks, at the retention times of 20E and polB (molar ratio 20E/polB = 1.6). Enzymic hydrolysis of the RP-gradient fractions (Fig. 3b) significantly enhances the amount of RIA-positive material in fractions 13, 15, 16 and 18 (corresponding to the UV-absorbing peaks at 12.1/12.5, 14.5, 15.1 and 17.4 min, respectively), demonstrating the presence of ecdysteroid conjugates in these fractions. This is also supported by the biological activity of fractions 15 and 16 generated by enzymic hydrolysis (Fig. 3d). Since the polarity of

these fractions is similar to 20E, it is probable that the conjugates are glycosides, differing in the genin or the position of glycosylation. The conjugates eluting before 20E have similar chromatographic behaviour in this system to 20E 2-, 3-, 22- and 25-monoglucosides (Harmatha and Dinan, 1997). The conjugate peak in RP fraction 13 has the same retention time as 20E 22-glucoside, but the amount available was too small for further characterisation. The peak at 14.4 min (RP fraction 15) has the retention time of 20E 25-glucoside. The material released by hydrolysis of RP fraction 15 elutes in fraction 17 (20E/polB) on the RP-gradient, while the unhydrolysed material gives two peaks on the NP-gradient (15.7 and 19.2 min). The latter corresponds to 20E 25-glucoside, while the former probably corresponds to polB 25-glucoside. The peak at 15.1 min (RP fraction 16) corresponds to 20E 3-glucoside. The conjugated material in fraction 16 gives one UV- and RIA-positive (after hydrolysis) peak on the NP-gradient system at 15.7 min, which is in accord with 20E 3-glucoside; no evidence was obtained for the presence of polB 3-glucoside. The material released by hydrolysis of RP fraction 18 is readily detected by RIA, but not by bioassay, which indicates that the genin is not 20E and is probably ecdysone (which is very readily detected by the DBL-1 antiserum [Dinan, 1995], but has low biological activity [Dinan et al., 1999b]). On the NP-gradient, the conjugated material in RP fraction 18 separates into two peaks (15.7 and 18.2 min), indicating that the peak at 17.4 min is probably a mixture of E 3-glucoside and 5β-hydroxyecdysone 3-glucoside. Thus, in order of decreasing importance, the seed extract of *A. scandens* contains 20E 3-glucoside, 20E, polB, 20E 25-glucoside, polB 25-glucoside, E 3-glucoside (?), 5-hydroxyecdysone 3-glucoside (?) and 20E 22-glucoside(?). Definitive identification of these ecdysteroids by NMR spectroscopy must await the isolation of adequate amounts from a greater mass of seeds.

2.2.3. *A. falcatus and A. ramosissimus*

The much lower levels of RIA-positive material in these extracts do not permit such extensive analysis of the ecdysteroids present. However, the sensitivities of the RIA and bioassay detection methods allow the ecdysteroid profiles to be compared when only 50 ng ecdysone equivalents (corresponding to ca. 9 mg seeds) are separated (Fig. 3). The extract of *A. falcatus* possessed a high biological activity relative to the level of RIA-positive material (Table 1). After RP-HPLC, one major peak (fraction 17) of RIA- and bioassay-positive material is observed co-eluting with 20E. Enzymic hydrolysis releases further RIA-positive material in fractions 14–16, 18 and 21, similar to the situation seen with *A. scandens*. When the RIA-positive material from fraction 17 is separated on a normal-phase system, it gives one peak of RIA- and bioassay-positive material with the same retention time as 20E. Although there is no RIA-evidence for the presence of polB in this sample, a peak of biological activity corresponds to the retention time of polB, indicating that a low amount of this ecdysteroid is present (data not shown). The extract of *A. ramosissimus* (Fig. 4) gives three main RIA-positive peaks (fractions 17, 20 and 24), of which two (fractions 17 and 24) are biologically active. The former co-elutes with 20E and the latter with ponasterone A. This identification is supported by the high biological activities of these two ecdysteroids (Dinan et al., 1999b) Enzymic hydrolysis enhances the levels of RIA positive material in these peaks, suggesting that these peaks probably comprise mixtures of the parent ecdysteroid (20E or ponA; RIA- and bioassay positive) and a co-eluting conjugate (e.g. the 3β-xyloside [Sarker et al., 1997]; much less RIA- and bioassay positive). The retention time and low biological activity of the material in fraction 20 is compatible with it being ecdysone, but this requires further substantiation.

2.3. *Chemotaxonomic significance*

The levels of secondary metabolites present in a plant often vary between the various tissues of the plant and according to the stage of development. In this context, ungerminated seeds probably provide the most comparable source material for chemotaxonomic studies. The seeds of the species of *Asparagus* examined here differ considerably in terms of both the levels of ecdysteroids present and their ecdysteroid profiles. The combination of methods used here together with their high sensitivities has permitted the selective quantification and substantial identification of ecdysteroids in very small samples, even when, as in the cases of *A. falcatus* and *A. ramosissimus*, the total levels present are rather low. The ecdysteroid profile of *A. laricinus* is rather straightforward. In addition to containing the highest levels of ecdysteroids, seeds of this species contain predominantly 20E and polB, plus small amounts of several minor ecdysteroids; there is no evidence for the presence of ecdysteroid conjugates. Seeds of *A. scandens* contain a complicated mixture of free ecdysteroids and ecdysteroid glucosides, with the ecdysteroid conjugates accounting for the majority of ecdysteroids present. The ecdysteroid profile for *A. falcatus* is similar to that of *A. scandens*, but the total levels of ecdysteroids are far lower. The profile in seeds of *A. ramosissimus* is different again, with the emphasis on free ecdysteroids, although some conjugates are present. Thus, ecdysteroids could be useful chemotaxonomic markers for this genus.

The taxonomy of *Asparagus* has been the subject of considerable debate. In addition to discussion whether the genus should be included within the Liliaceae or form a separate Family (Asparagaceae), it has been proposed that the genus be divided into separate genera (Obermeyer, 1983; 1984) or subgenera (Clifford and Conran,

1987). Bailey (1944) divided the genus into four sections. However, Malcomber and Sebsebe (1993) concluded that there was not adequate justification for division of the genus into four sections and they proposed that *Asparagus* is the only genus in the Family, with two subgenera (*Myrsiphyllum* and *Asparagus*). Fellingham and Meyer (1995), in considering southern African species of *Asparagus*, preferred not to accept the existence of subgenera. If one considers the distribution of phytoecdysteroid-containing species in relationship to the various divisions and subdivisions which have been proposed, no clear associations with any proposed subdivisions occurs, since ecdysteroid positive and negative species are found in both *Asparagus* and *Myrsiphyllum*.

A more recent phylogeny based on restriction mapping of chloroplast DNA of 10 species (eight of which were tested here for ecdysteroid content) divides the species into two clusters (Lee et al., 1997). Both the positive species (*A. falcatus* and *A. scandens*) occur in the larger cluster (eight species), while both the species in the other cluster (*A. plumosus* and *A. virgatus*) are ecdysteroid-negative. However, the larger cluster also contains ecdysteroid-negative species. Further, of two very closely related species identified by this approach, one (*A. falcatus*) is ecdysteroid-positive and the other (*A. sprengeri*) is ecdysteroid-negative.

The presence of ecdysteroids in *A. dumosus* has been reported previously (Ahmad et al., 1996; Khaliq-uz-Zaman et al., 2000). In the Liliaceae, within which *Asparagus* is often included, ecdysteroids have been detected in *Lloydia serotina* (Liliaceae: Dinan et al., 2000b). More generally among monocotyledonous plants, species in several other genera accumulate ecdysteroids: *Agapanthus* spp (Alliaceae: Savchenko et al., 1997), *Allium* (Alliaceae: Matsuoka et al., 1969), *Blandfordia* spp (Blandfordiaceae: Sarker et al., 1996), *Briza* (Gramineae: Dinan, 1995; Savchenko et al., 1998), *Butomus* (Butomaceae: Matsuoka et al., 1969), *Paris* (Trilliaceae: Matsuoka et al., 1969; Novoselskaya et al., 1981; Singh and Thakur, 1982), *Trillium* (Trilliaceae: Matsuoka et al., 1969), *Trisetum* (Gramineae: Sarker et al., 1998) and *Xerophyllum* (Melanthaceae: Alison et al., 1997). However, too few species have been examined to discern a pattern to the distribution. Our survey of extracts of the seeds of 2454 randomly-selected plant species, using the same RIA and bioassay methodology as applied to the *Asparagus* samples (L. Dinan, T. Savchenko, P. Whiting, 2000 unpublished), sampled 278 monocotyledonous species of which 5 (1.8%) contained significant levels of phytoecdysteroids (clearly detectable by RIA and agonist bioassay) and a further 27 (9.7%) contained low levels detected by at least one of the antisera, but remaining negative in the agonist bioassay. Thus, phytoecdysteroid containing species appear to be as common among the monocots, if not slightly more common, as they are among higher plants in general (Dinan, 1998).

3. Experimental

3.1. Plant material

Seeds were purchased either from Chiltern Seeds Ltd (Ulverston, Cumbria, UK), B&T World Seeds sarl (Olonzac, France) or The Seed Guild (Lanark, UK). Voucher specimens are kept in the Department of Biological Sciences, University of Exeter.

3.2. Preparation of plant material and extraction

Seeds were ground in a pestle and mortar and samples (25 mg) were extracted three times with MeOH (1 ml) at 55°C for 3 h. The combined extracts were diluted with H_2O (1.3 ml) and partitioned against hexane (2×2 ml). The aqueous methanol phase was used for RIA, bioassays and HPLC. Other plant parts were freeze-dried for 4 days and then processed as described for the seeds.

3.3. Bioassays for ecdysteroid agonist and antagonist activities

Bioassays were performed as described previously (Clément et al., 1993). The concentration of 20-hydroxyecdysone used in the antagonist assay was 5×10^{-8} M. The potencies of a large range of ecdysteroids in this bioassay have been determined (Dinan et al., 1999b).

3.4. Radioimmunoassays

Radioimmunoassays using ecdysteroid-specific antisera were carried out using the previously described method (Dinan, 1992). The antisera used were DBL-1 and Black (generously donated by Professor Jan Koolman, Universität Marburg, Germany). The cross-reactivities of these antisera for a number of ecdysteroids have been determined (Dinan, 1995). The concentration of ecdysteroids (in μg ecdysone equivalents/g) was determined for each positive extract by plotting the RIA responses of five known aliquots (in duplicate) against the volume and determining the gradient of the fitted straight line.

3.5. HPLC

General HPLC conditions have been described previously (Dinan, 1988). Two standardised separation systems were used: a RP(C_{18})-gradient system and a NP(diol)-gradient system. These systems are described in detail elsewhere (Dinan et al., 2000a) and summarised in the legends to Figs. 1 and 2.

3.6. Enzymic hydrolysis

Portions of the HPLC fractions (100 μl of RP-fractions and 50 μl of NP-fractions) were dried and then

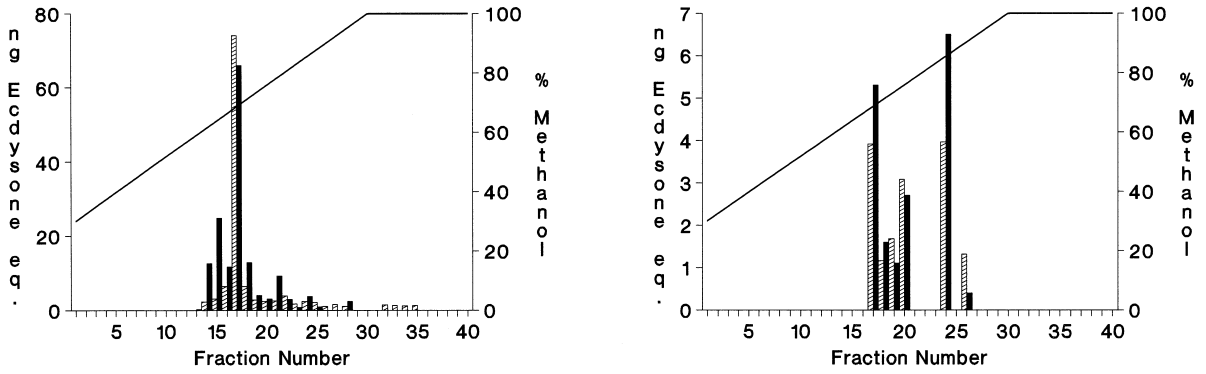


Fig. 4. RP-HPLC/RIA of portions of seed extracts (50 ng ecdysone equivalents with the DBL-1 antiserum) of *Asparagus falcatus* (panel A) and *A. ramosissimus* (panel B). HPLC conditions as for Fig. 1.

200 μl of a solution of *Helix pomatia* enzymes (Sigma Type I; 10 mg/ml in 0.1 M acetate buffer, pH 5.4) were added to each and incubated at 37°C for 48 h. The incubation was stopped by the addition of 800 μl ethanol and, after cooling at 4°C for several hours, the precipitated protein was pelleted by centrifugation in a microfuge (13,000×***g***, 30 s). Suitable aliquots of the supernatant were used for RIA and bioassay. The above conditions permit qualitative assessment for the presence of hydrolysable conjugates, but do not necessarily provide complete hydrolysis of all forms of ecdysteroid conjugates (Whiting and Dinan, 1988).

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A species-selective allelopathic substance from germinating sunflower (*Helianthus annuus* L.) seeds

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Abstract

From the exudate of germinating sunflower (*Helianthus annuus* L.) seeds was isolated a stereoisomer of diversifolide, 4, 15-dinor-3-hydroxy-1(5)-xanthene-12,8-olide (designated sundiversifolide) as determined by analysis of its IR, APCI-, ESI- and HR–MS and ^{13}C and ^{1}H NMR spectra. This substance inhibited shoot and root growth of cat's-eyes by about 50% at a concentration of 30 ppm. It also showed species-selective activity on the shoot and root growth of tested plants. When cat's-eyes seeds were incubated together with sunflower seeds, the cat's-eyes growth was inhibited. Furthermore, it was detected from an extract of river sand when sunflower seeds were incubated on the sand. These results indicate that sundiversifolide has an allelopathic function in sunflower plants. © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Helianthus annuus* L.; Compositae; Allelopathic substance; Growth inhibitor; Sundiversifolide; 4,15-Dinor-3-hydroxy-1(5)-xanthene-12,8-olide

1. Introduction

Plants release chemical compounds, which affect germination or growth of different plant species growing in their vicinity, into the environment. This phenomenon is defined as "allelopathy". Hasegawa et al. (1992) isolated an allelopathic substance that showed a potent stimulating activity in the cockscomb hypocotyl growth test from the mucilage of germinating cress (*Lepidium sativumm* L.) seeds, and Kushima et al. (1998) isolated a plant-selective allelopathic substance exuded from germinating watermelon (*Citrullus vulgaris* Schrad) seeds, suggesting that allelopathy occurs during seed germination. It has been well-known that the sunflower (*Helianthus annuus* L.) seedlings show a strong allelopathic function and that several norsesquiterpenes and ionone type bisnorsesquiterpenes, etc., have been isolated and identified as allelopathic substances from sunflower leaves (Macias et al., 1998,1999). However, allelopathy in sunflower during seed germination has not yet been studied. In this study, the isolation and identification of the bio-active substance(s) from the exudate of germinating sunflower seeds and their biological activity as allelopathic substance(s) were examined.

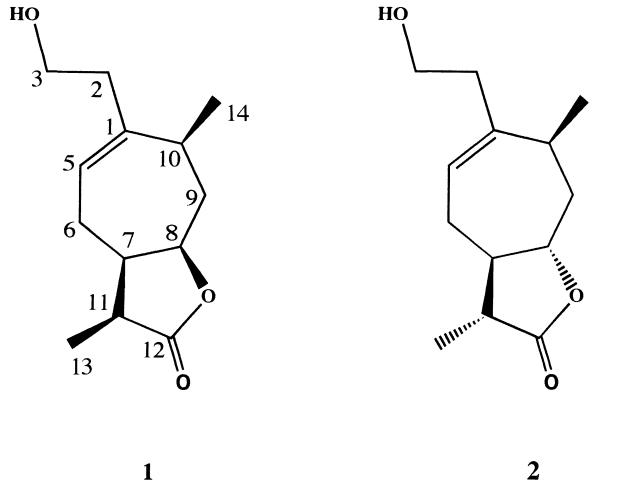
2. Results and discussion

A new plant growth-inhibiting substance, sundiversifolide (**1**) (Fig. 1), was isolated from the exudate of germinating sunflower seeds. Sundiversifolide (**1**) has a molecular formula of $C_{13}H_{20}O_3$ as determined by APCI-MS [m/z 224(M^+)], ESI-MS [m/z 224 (M^+)] and HR-EIMS [m/z 224.1409 (M^+), Δ−0.2 mmu] in conjunction with the ^{1}H and ^{13}C NMR spectral data

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(Table 1). The IR absorptions at 3450 and 1770 cm^{-1} suggested the presence of a hydroxy group and a γ-lactone (δ_{C-12} 181.9), respectively. Table 1 shows the ^{13}C and ^{1}H NMR spectral data for **1**. The ^{1}H NMR spectrum showed the presence of a primary alcohol (δ_{H-3} 3.51–3.63, δ_{C-4} 61.2), one olefin (δ_{H-5} 5.54, δ_{C-1} 143.7, and δ_{C-5} 125.3), and two secondary methyl groups (δ_{H-13} 1.11, $_{H-14}$ 1.15 and δ_{C-13} 11.0, $_{C-14}$ 21.9). The ^{13}C NMR spectrum showed the presence of 13 carbons including two oxygenated carbons [a methylene (δ_{C-3} 61.2) and a methine (δ_{C-8} 82.6)]. The structure of **1** was determined by detailed analysis of one and two dimensional NMR spectra, and the relative stereochemistry of **1** was clarified by examination, the NOE difference spectra; the NOE results suggest the γ-lactone ring junction should be fused *cis*, because of NOE enhancements between H-7 and H-6. Furthermore, NOE enhancements between H-8 and H-11, and between H-8 and H-10 were observed. Thus, the structure of sundiversifolide (**1**) was clearly established as depicted in the formula, 4,15-dinor-3-hydroxy-1(5)-xanthene-12,8-olide. Diversifolide (**2**), a stereoisomer of **1** (Fig. 1), has already been isolated from *Tithonia diversifolia*. (Kuo and Lin, 1999), but its biological activity has not yet been reported. In the present study, the biological activity of **1** was studied using the shoot and root growth tests of cat's-eyes seedlings (Fig. 2). The shoot and root growth of cat's-eyes seedling test is useful for screening bio-active substances, because it has a high sensitivity to such substances (Node et al., 2000). **1** inhibited shoot and root growth of cat's-eyes by about 50% at a concentration of 30 ppm, although **1** promoted root growth at lower



1 **2**

Fig. 1. Chemical structure of 4,15-dinor-3-hydroxy-1(5)-xanthene-12,8-olide (sundiversifolide) (**1**) and diversifolide (**2**).

Table 1
^{13}C and ^{1}H NMR spectral data of **1** (100 and 400 MHz in CD_3OD)

	σC	σH
1	143.7	
2	41.2	2.13 *m*
		2.34 *m*
3	61.2	3.51–3.63 *m*
4		
5	125.3	5.54 (*br.dd*, *J* = 9.2, 2.6 Hz)
	22.9	1.91 (*ddd*, *J* = 15.7, 12.5, 2.6 Hz)
6		2.20 *m*
7	43.5	2.76 *m*
8	82.6	4.70 (*ddd*, *J* = 9.5, 6.2, 6.2 Hz)
9	38.2	2.05 *m*
10	34.1	2.40
11	40.2	2.90 (*dq*, *J* = 7.4, 7.4 Hz)
12	181.9	
13	11.0	1.11 (*d*, *J* = 7.4 Hz)
14	21.9	1.15 (*d*, *J* = 7.3 Hz)
15		

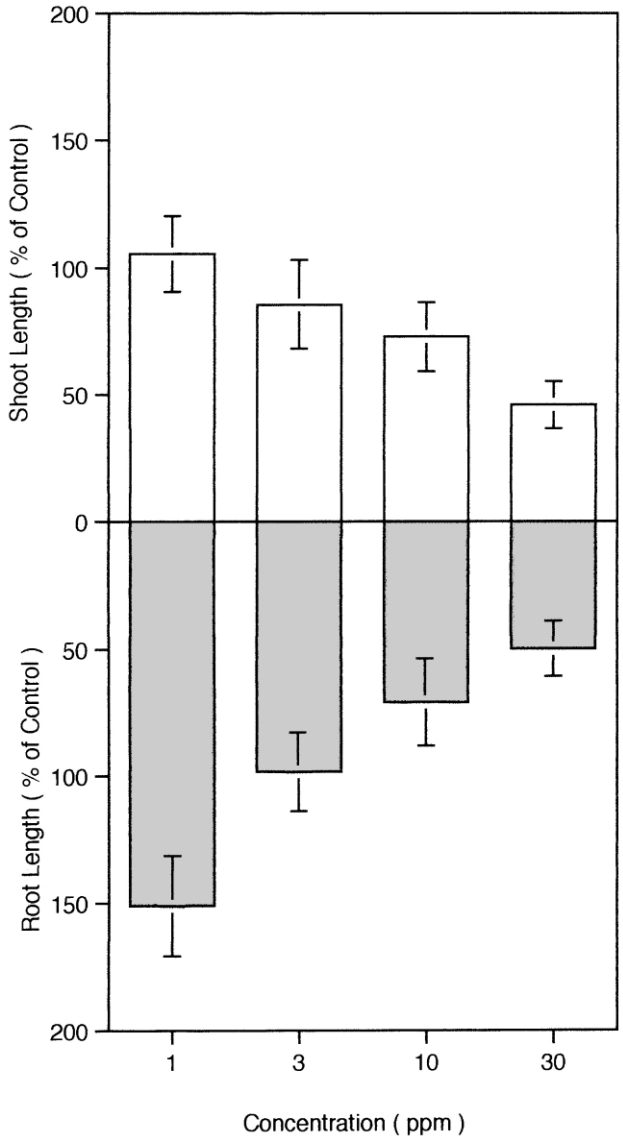


Fig. 2. Effect of isolated substance from the exudates of germinating sunflower seeds on the shoot (white) and root (grey) growth of cat's-eyes seedlings. Each value is the average of 14 measurements; bars indicate average±S.E.

concentrations (Fig. 2). **1** did not affect growth of cat's-eyes at lower concentrations. **1** also showed a species-selective activity on the shoot and root growth of the tested plants (Fig. 3). Among the six species of plants examined, tomato growth was inhibited most (Fig. 3). This substance also inhibited growth of crabgrass and barnyard grass, an interesting phenomenon because they are serious weeds all over the world. The degree of inhibition by **1** in these cases is not as high, but **1** may nevertheless become a bio-weed killer, even though the inhibition of crabgrass and barnyard grass growth was not as strong as that for tomato. On the other hand, **1** did not influence the shoot growth of sunflower and cockscomb seedlings. Yamamoto et al. (1999) reported on the allelopathy of serious weeds, such as barnyard grass, where exudates from plant roots at an early seedling development stage showed species-selective activity and inhibited shoot growth of tested plants except for barnyard grass. This indicates that if **1** has an allelopathic function, then the results of suggest that this is in self-defense against attack by other species of plants, especially at an early seedling development stage. However, in the case of sunflower roots, **1** inhibited its growth remarkably at a concentration of 30 ppm. This phenomenon may be related to sensitivity to the plant density.

We examined cat's-eyes growth when incubated together with sunflower seeds to confirm whether **1** is

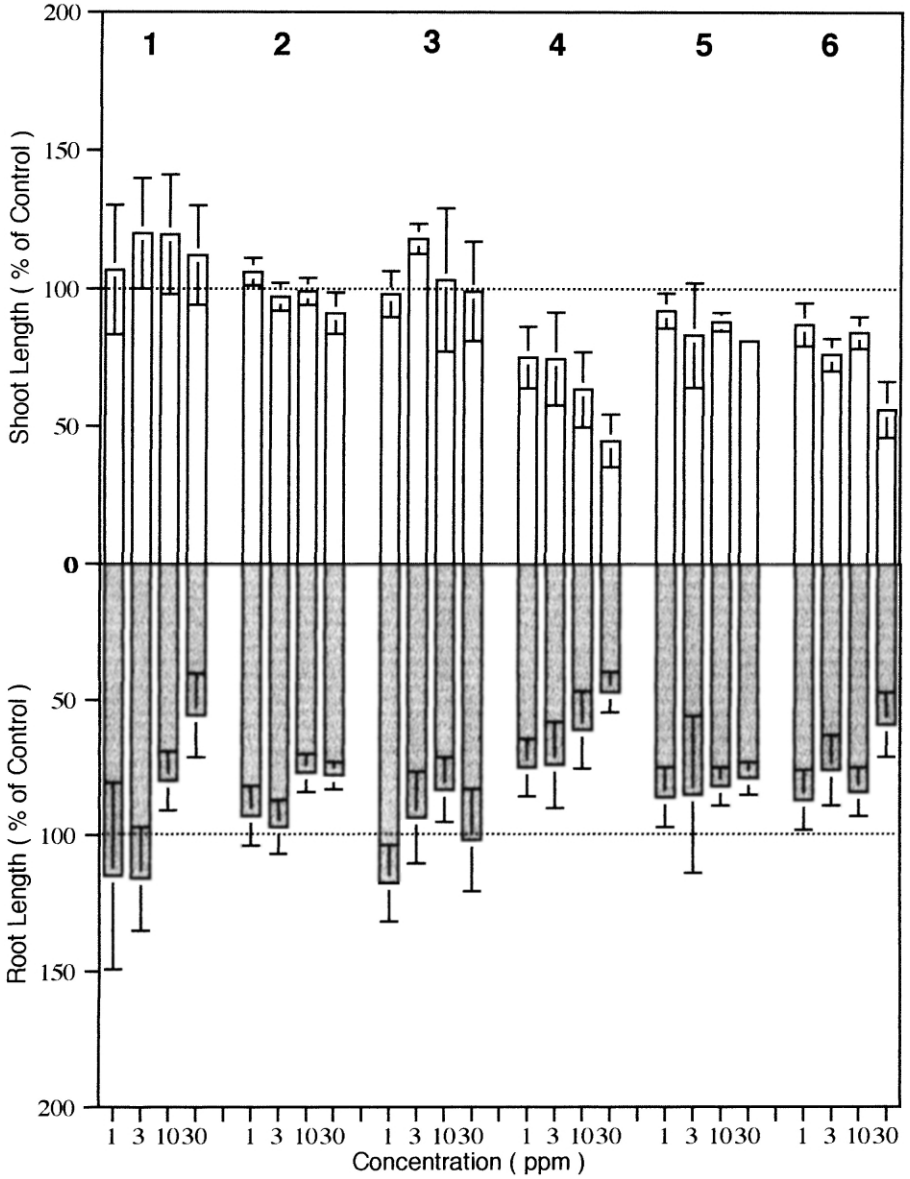


Fig. 3. Effect of isolated substance from the exudates of germinating sunflower seeds on the shoot (white) and root (grey) growth of plants tested. 1, sunflower; 2, lettuce; 3, cockscomb; 4, tomato; 5, crabgrass; 6, barnyard grass. Each value is the average of 14 (2–6) or 8 (1) measurements; bars indicate average±S.E.

allelopathic or not (Fig. 4). When sunflower seeds and cat's-eyes seeds were incubated together on moist river sand in a Petri dish, the growth of cat's-eyes was lower than that of the control. On the other hand, inter-cropping did not influence sunflower growth. To analyze for the existence of **1** in the river sand through exudation during sunflower seed incubation, fifty sunflower seeds were incubated on moist river sand for 1 day. After removal of the seeds, the river sand was rinsed with water, with the aqueous extract then analysed for **1**. Its existence was confirmed by HPLC and by ESI^+-LC-mass analysis; HPLC analysis confirmed the existence of **1** based on the retention time of **1**, and by mass spectral analysis (224[M^+]). Thus, **1** is an allelopathic substance of sunflower.

In this study, **1** is reported for the first time to be an allelopathic substance in sunflower, and it is interesting that **1** did not inhibit shoot growth of sunflower itself. Since congeners of **1** are often found in the Compositae (Mabry and Wagenits, 1990), this may mean that these have allelopathic effects in Compositae.

The results in this study indicate that **1** exuded from germinating sunflower seeds plays some important roles in the species-selective allelopathy in sunflower, and additional studies in progress will examine the effects of other sesquiterpene lactones or some chemicals from this source.

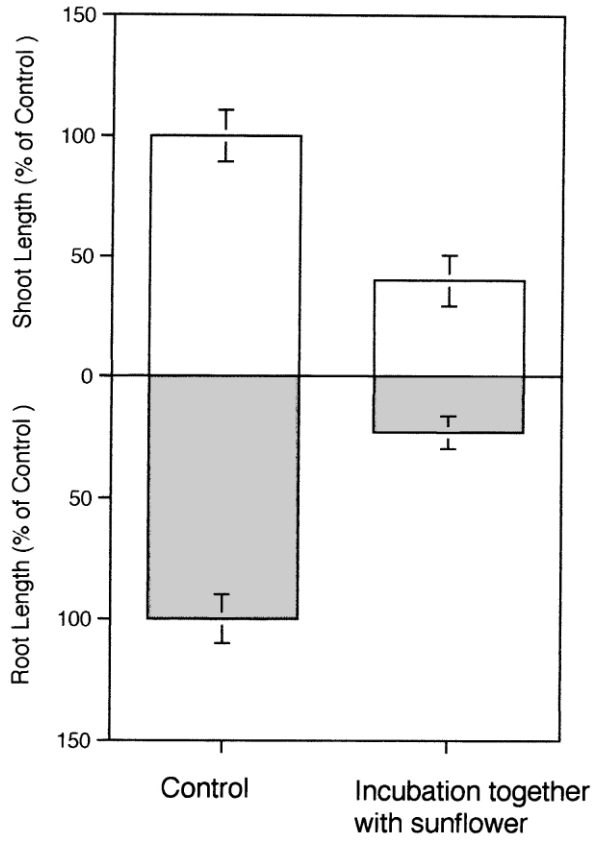


Fig. 4. Cat's-eyes growth, shoot (white) and root (grey), incubated on moist river sand (control) or together with sunflower on moist river sand in the dark at 25°C for 5 days; bars indicate average±S.E.

3. Experimental

3.1. Extraction and isolation

Sunflower (*Helianthus annuus* L.) seeds (15 l) were soaked in distilled-deionized water in large trays, then cultured for 24 h at 25°C in the dark. The culture medium was filtered to remove the seeds, and the exudate was evaporated to dryness in vacuo at 38°C. The concentrates were fractionated into acetone-soluble and insoluble fractions. The inhibitory activities of the fractions were determined using the cat's-eyes (*Veronica persica* Poiret) shoot and root growth test.

Inhibitory activity was detected in the acetone-soluble fraction. This fraction was applied to a silica gel column (3.5×36 cm) of (Wakogel C-200, Wako) which was eluted with an *n*-hexane- ethyl acetate gradient by increasing the ethyl acetate conc. in a series of 20% steps(200 ml per step) and finally with MeOH (600 ml). Inhibitory activities were found between 60 and 100% ethyl acetate in *n*- hexane fractions. The active fraction was further purified by TLC [Silica gel 70 F254, Wako; chloroform:acetic acid (95:5)], and the active zone (Rf 0–0.33) was further purified by HPLC (TSK gel ODS-80 Ts, ϕ7.8 × 300 mm, TOSOH, H_2O- MeOH, 1 : 1, v/v, 1.0 ml / min, 214 nm detector). The inhibitory activity was detected in the Rt 35–45 min fraction. This active fraction was purified again by HPLC under the same conditions. Finally, the active fraction (Rt 40–43 min) was purified by HPLC (TSK gel ODS- 80 Ts, ϕ4.6 × 250 mm, TOSOH, H_2O- MeOH, 3 : 2, v/v, 0.8 ml / min, 214 nm detector). The active eluate was evaporated to dryness (1.4 mg). The isolated substance was identified by using $APCI^+$-LC- and ESI^+-LC-mass, IR and ^1H- and ^{13}C- NMR spectral analyses.

Sundiversifolide (**1**) colorless oil, IR (film) ν_{max} cm^{-1}: 3450, 1770, 1655, APCI-MS *m*/*z*: 224 (M^+), ESI-MS *m*/*z*: 224 (M^+), HR-MS *m*/*z*: 224.1409 (M^+), calcd for $[C_{13}H_{20}O_3]^+$ 224.1411. ^1H and ^{13}C NMR spectral data are shown in Table 1.

3.2. Bioassays of compound

Seven seeds of lettuce (*Lactuca sativa*), cockscomb (*Celosia cristata*), tomato (*Lycopersicon esculentum*), crabgrass (*Digitaria ciliaris*), barnyard grass (*Echinochloa crus-galli*) and cat's-eyes, or four seeds of sunflower were placed in a 2.7 or 3.3 cm Petri dish containing the test solution dissolved in water and incubated in the dark at 25°C for 4–5 days, with shoot and root lengths of each plant species measured.

3.3. Cat's-eyes growth test incubated together with sunflower

River sand (100 ml) was washed with tap water and rinsed with ion exchanged water, with the washed sand then dried (140°C). The sand was next placed in a sterilized 9 cm Petri dish with 10 ml sterilized water, and ten presterilized (1% v/v NaOCl sol, 0.5 h) seeds of sunflower and of cat's-eyes were placed on the sterilized sand together and incubated for 5 days in the dark at 25°C; after this period, the hypocotyl and root lengths were measured.

3.4. Extraction of **1** from the exudate

In the extraction from river sand, 50 sterilized sunflower seeds in 200 ml distilled deionized water in a sterilized 25 cm Petri dish containing 300 ml sterilized river sand, was incubated for 1 day at 25°C in the dark. The culture medium was then filtered with filter paper and the filtrate was concentrated to dryness. The sand was washed with distilled water three times. The concentrate was purified by a silica gel Sep-pak cartridge (Waters) with an *n*-hexane-ethyl acetate solvent system by increasing the ethyl acetate concentration. The 50–100% fraction was further purified by C_{18} Sep-pak cartridge (Waters) with a water-methanol solvent system by increasing the methanol concentration. The 10–50% fraction was analyzed by HPLC (TSK gel ODS-80Ts, ϕ 4.6×250mm, TOSOH, H_2O–MeOH, 3:2, v/v, 0.8 ml min^{-1}, 214 nm detector), ESI^+-LC-mass (capillary, 3.5 kvolts; cone voltage, 30 V; source temp., 150°C; Symmetry C_{18}, 2.1×150 mm, Waters, 100% MeOH-0.5% formic acid, 2:3 v/v, 0.2 ml min^{-1}) and established the presence of **1** by HPLC retention time and mass spectral fragmentation.

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A computer-assisted approach for chemotaxonomic studies — diterpenes in Lamiaceae

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Abstract

This paper describes a new computer approach for chemotaxonomic studies. The methodology employed enables the search for chemical substructures as taxonomic descriptors using an expert system built with plant natural products. The operation of the system was tested with diterpenes as taxonomic markers in Lamiaceae. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Diterpenes; Lamiaceae; Chemotaxonomy; Computer-assisted chemotaxonomy

1. Introduction

Diterpenes are a class of secondary metabolites with a large variety of structures (skeletons) and are found mainly in higher plants. The interest in the isolation of these compounds is growing due to their biological activity, ecological function, use as templates for synthesis and taxonomic function (Hanson, 1995, 1996, 1997, 1998).

Among the Angiospermae, the families in which most of the diterpenes are found are Asteraceae (Seaman et al., 1990) and Lamiaceae (Menezes and Kaplan, 1993). The frequent occurrence of certain diterpene skeletons in the Angiospermae has been used, together with the presence of other secondary metabolite classes, in chemotaxonomic and evolution studies (Figueiredo et al., 1995; Fraga et al., 1995; Menezes et al., 1995). Previous works dealing with the occurrence of diterpenes in the Lamiaceae suggested that these compounds could be used in phytogeographic studies (Cole, 1992; Esquivel et al., 1996) and to differentiate varieties of a single species (Kubo et al., 1982).

The diterpenes had been used as chemotaxonomic markers at infra- and supra-genus levels. However, only an expert system developed exclusively for taxonomy would be able to handle the huge amount of chemical and botanical data, perform the statistical analysis and study the probability of occurrence of structural requirements (chemical substructures) in large taxons.

SISTEMAT (Gastmans et al., 1990a, 1990b; Emerenciano et al., 1993) is an expert system developed in Brazil with two purposes: to help the process of structure elucidation and to perform chemotaxonomic studies (Fromanteau et al., 1993; Emerenciano et al., 1994; Macari et al., 1994/1995; Alvarenga et al., 1997; Rodrigues et al., 1997). The main difference between SISTEMAT and other expert systems [DENDRAL (Lindsay et al., 1980), DARC-EPIOS (Carabedian et al., 1988), etc.] is the storage of the isolation source (family, genus and species) from the compounds included in the database.

As the system was developed in the 1980s, time in which the computer HDs were small, SISTEMAT has also build-in a complex data compressing system. The database is built by encoding the chemical structures, using a comprehensive method and, at the same time, user friendly (Gastmans et al., 1990a,b; Emerenciano et al., 1993). In this way, all structural information of a chemical group can be recovered, such as skeleton type, positions bearing functional groups, stereochemistry, etc. For these reasons, SISTEMAT demonstrated to be a powerful tool in chemotaxonomy since it enables identification correlation between structural characteristics and the botanical source(s).

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The search for substructures (parts of a structure), modified positions and functional groups are performed by a complex mechanism in SISTEMAT. As an example, the system permit the search which species within *Ajuga* contain a specific diterpene, such as clerodane, bearing an oxygenated function at a given position.

This paper aims to present, through the use of SISTEMAT, an outline on the structural aspects of diterpenes isolated from Lamiaceae species, correlating the skeletons with the genus and the substitution patterns and/or maintenance of the functional groups of the skeletons in the genus. This work also addresses the inclusion of some Verbenaceae genera (*Clerodendrum, Peronema, Caryopteris, Callicarpa, Pygmaeopremna, Vitex, Premna, Tectona, Pityrodia* and *Cyanostegia*) in the Lamiaceae, proposed in 1992 by Cantino based on the distribution of diterpenes (Cantino, 1992; Cantino et al., 1992).

2. Methodology

2.1. Database

The occurrence of diterpenes in the Lamiaceae was extracted from data published in the literature from widespread journals indexed in Chemical Abstracts until December 1997. The data obtained from the literature search was then used to build a database with 2428 botanical occurrences with the structures coded by the SISTEMAT methods (Gastmans et al., 1990a,b; Emerenciano et al., 1993). Initially, the entries were limited to December 1997 because the data published from 1998 up to the present will be used for validation of the database.

According to Cantino (1992) the genus *Plectranthus* is a synonym for *Coleus* and *Isodon* for *Rabdosia*. In this paper, we have kept the names of the genera as they appear in the publications found in the literature search. In this way, the information stored in the database can be employed for chemosystematic studies.

One of the entries stored in the database was the skeleton name. A skeleton was considered different every time a different carbon–carbon bond appeared, such as the cleavage of a C–C bond or a carbon loss afforded new skeletons. The skeletons were grouped according to the ring cyclisation pattern. Although the differences in the ring fusion stereochemistry were not considered for the classification, compounds which presented both *cis* and *trans* ring fusion were entered separately in the database.

2.2. The search in the database

The first program employed showed the number of occurrences for each species stored in the database. The species are listed and grouped by genus. The example below (Table 1) pointed out 82 occurrences of diterpenes for the *Ajuga* genus, distributed among 16 species.

Another program analysed the number of compounds grouped by skeleton. This information allowed to build a skeleton map for the diterpene found in Lamiaceae, coded from S-1 and S-91 (Fig. 1).

Following these parameters, the occurrence number of the substances was grouped by skeleton for each genus (Tables 2 and 3).

SISTEMAT has also a third program used for the identification of structural changes in the skeletons. This program was employed to determine the positions, in a specific skeleton that are always oxidised or those that never undergo oxidation. The positions that remained unchanged kept the same atomic arrangement from the original skeleton (Fig. 1) and those that were oxidised presented a functional group (double bond, hydroxyl, etc.). The analysis was performed for skeletons with more than 10 occurrences in a genus.

An example of the use of this program in a low taxonomic hierarchy is shown in Table 4. In this table, each line corresponds to a position in the clerodane skeleton (S-19, Fig. 1) and each column represents a carbon type. For the 74 clerodane diterpenes found in *Ajuga*, 44 substances did not show oxidation at C-1, always found as a methylene group. However, for 29 substances C-1 was found as a tertiary carbon, which indicated, for these cases, oxidation (formation of a C–O bond, because no other heteroatom was listed in the Lamiaceae database). Only in one case a non-aromatic sp^2 carbon was found in this position suggesting a carbonyl group in C-1. The sum of the values presented in any of the table lines gives the total amount of substances

Table 1
Diterpene occurrences for each *Ajuga* species

Family: Lamiaceae; Genus: *Ajuga*	
Species	Occurrence number
A. chamaepitys-chia	3
A. pseudo-iva	2
A. nipponensis	10
A. chamaepitys	8
A. reptans	2
A. ciliata-villosior	10
A. decumbens	10
A. iva	4
A. pantantha	2
A. salicifolia	2
A. macrosperma	5
A. genevensis	3
A. remota	3
A. parviflora	11
A. lupulina	4
A. orientalis	1
A. australis	2
Total	82

found in the retrieved taxon. Furthermore, the tables displayed positions in the skeletons that were not oxidised. As an example, in Table 4 is demonstrated that in *Ajuga* species 74 substances were isolated with a clerodane skeleton. These diterpenes retained the substitution pattern of this skeleton (S-19) at positions C-7, C-8,

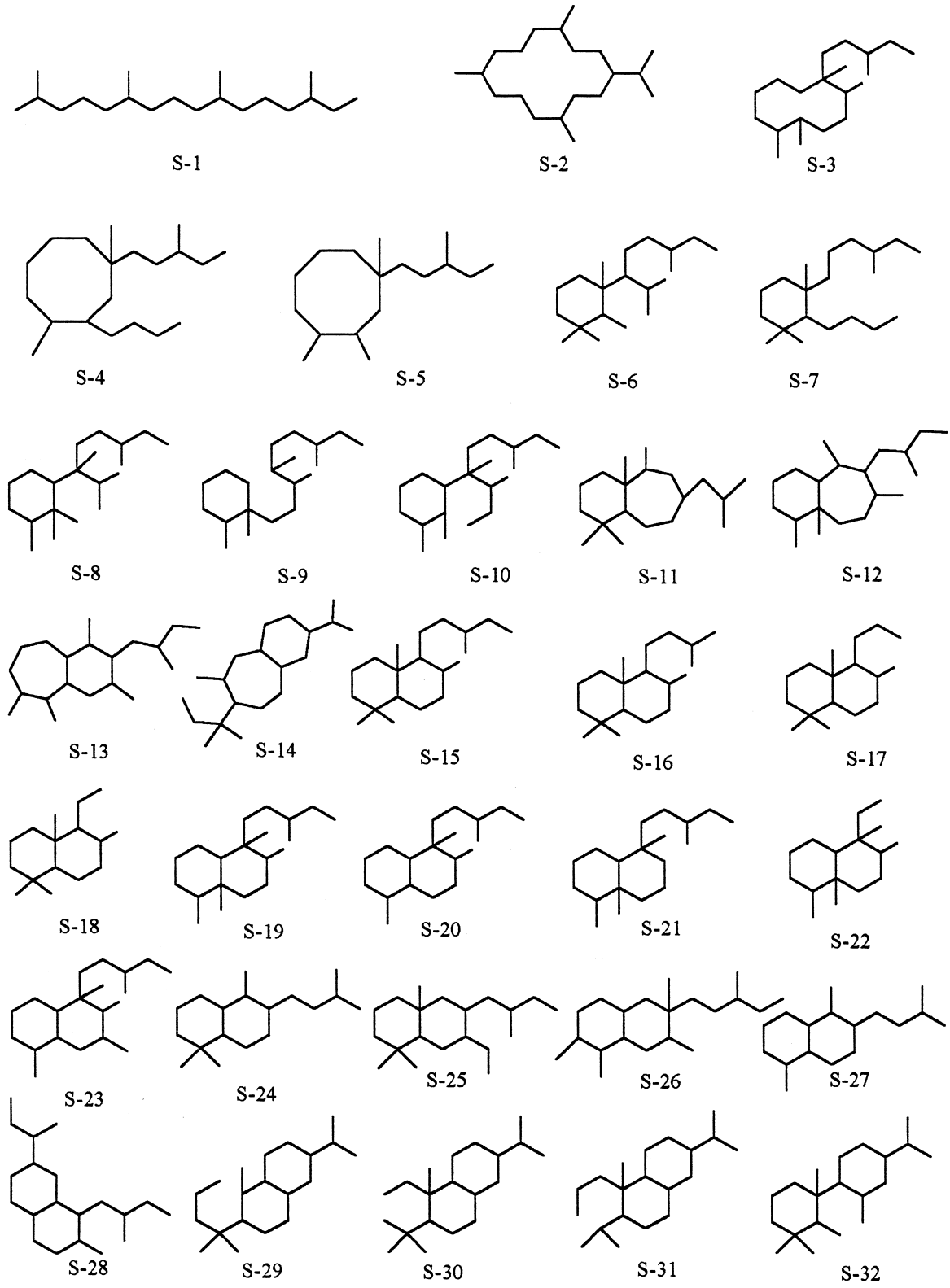


Fig. 1. Diterpenes skeletons in Lamiaceae *(continued on next page)*.

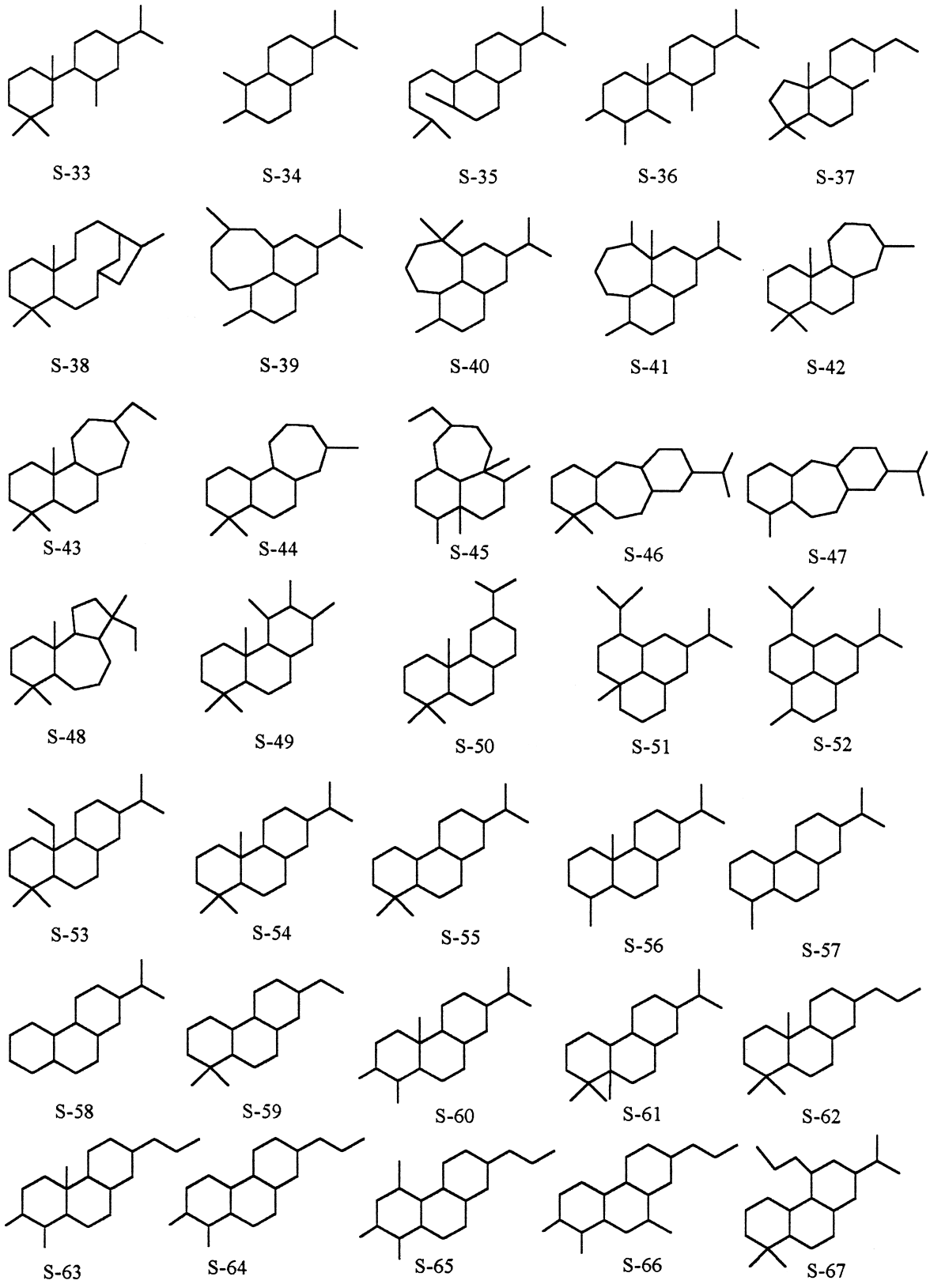


Fig. 1 *(continued)*.

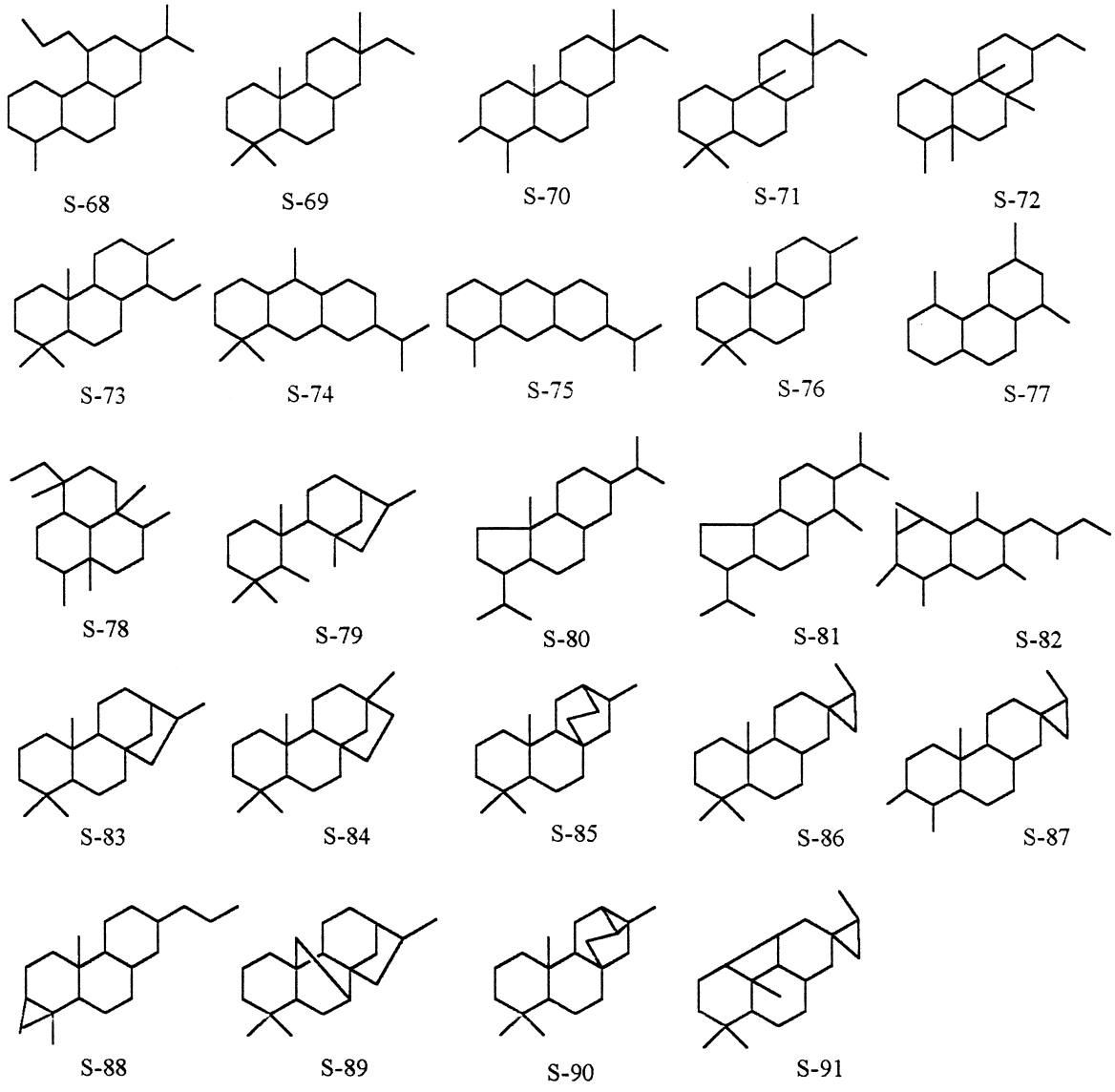


Fig. 1 *(continued)*.

C-10, C-17 and C-20, while at position C-6 was always a C–H differently from S-19 in which it appears as a CH_2.

A similar procedure was followed for other skeletons and genera in order to search for chemical patterns, i.e. to point out the positions that undergo modifications (oxidation) or those that remain unchanged. This program allows the search for similarities among the diterpenes produced in the different genera.

3. Results

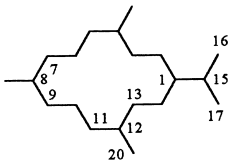
The database analysis showed that the Lamiaceae diterpenes can be divided in 91 skeletons (Fig. 1) and that some genera produce few diterpenes or are not thoroughly investigated (Table 2).

The genera that presented more than 10 diterpenes (Table 3) were arbitrarily chosen for a detailed structure analysis. The skeletons analysed were those occurring more than 10 times in the same genus.

3.1. Chemical patterns for Lamiaceae diterpenes

3.1.1. Cembranes (S-2)

In the Lamiaceae, this skeleton occurs in the *Anisomeles* genus. SISTEMAT pointed out that in all cembranes isolated in this genus the positions 8, 12 and 15 are always sp^2 carbons. Double bonds can be formed with C-7 or C-9 for C-8, C-11 or C-13 for C-12, and C-16 or C-17 for C-15. The positions 1 and 20 (CH and CH_3, respectively) remained unchanged, always appearing with the same substitution pattern as in the original skeleton.



Cembrane (S-2)

3.1.2. Labdanes (S-15)

Labdane diterpenes were found in 20 genera. In the *Ballota* genus, besides the original quaternary carbons at positions 4 and 10, the CH_2 carbons at 1, 2, 11 and 12, the CH at 5 and the methyl groups at 17 and 20 were never oxidised, preserving the substitution pattern described for the original skeleton.

All *Coleus* labdanes were derived from manoyl oxide, i.e. labdanes skeletons with an epoxide bridge at C-8 and C-13. They always possessed a carbonyl group at C-11 and a double bond between C-14 and C-15. The carbons at 2, 3, 5, 12, 16, 17, 18, 19 and 20 remained unchanged when compared with the original labdane skeleton.

Table 2
Distribution of diterpene skeletons among the Lamiaceae genera with less than 10 occurrences

Genus (number of species)[a]	Skeletons (occurrence)
Aeollanthus (1)	S-54 (1)
Agastache (1)	S-55 (1)
Amaracus (2)	S-69 (5)
Callicarpa (3)	S-19 (2); S-54 (1); S-69 (1); S-83 (2)
Caryopteris (2)	S-19 (6); S-62 (1)
Cleonia (1)	S-69 (1)
Cyanostegia (1)	S-19 (1)
Dauphinea (1)	S-54 (2)
Englerastrum (1)	S-83 (8)
Fuerstia (1)	S-54 (1)
Galeopsis (2)	S-7 (2); S-15 (6); S-25 (1)
Horminum (1)	S-54 (1)
Hoslundia (1)	S-54 (3); S-61 (1)
Hyptis (3)	S-54 (4); S-69 (1)
Lasiocorys (1)	S-15 (1)
Leucas (3)	S-1 (1); S-15 (2); S-69 (1)
Lycopus (1)	S-69 (1)
Meriandra (1)	S-54 (2); S-55 (2)
Orthosiphon (1)	S-69 (4)
Pogostemon (2)	S-1 (1); S-73 (8)
Peronema (1)	S-19 (7)
Pygmaeopremna (1)	S-30 (2); S-54 (1); S-61 (2); S-74 (1)
Pityrodia (1)	S-19 (1)
Rosmarinus (1)	S-54 (8); S-55 (1)
Roylea (1)	S-15 (3)
Satureja (1)	S-15 (1); S-69 (3); S-70 (3)
Tectona (1)	S-15 (1)
Tetradenia (1)	S-54 (1); S-69 (2)
Vitex (2)	S-15 (3)
Zhumeria (1)	S-15 (1); S-35 (1); S-74 (1)

[a] Number of species in which diterpenes were reported.

Lagochilus labdanes possessed an epoxide bridge between carbon 9 and 13, forming the so-called grindelane derivatives. Carbons at positions 3, 15, 16 and 18 (equatorial methyl group at C-4) are also always oxidised. The other carbons were preserved, displaying the original substitution pattern as the original skeleton. The labdanes isolated from *Leonotis* and *Leonurus* presented similar characteristics, carbons at 1, 2, 11, 12 and 18 (equatorial methyl group at C-4) were never oxidised and C-9 was always bound to oxygen. In *Leonotis*, position 3 was always found as a CH_2 group. In *Leonurus*, position 5 is always a CH and C-20 was never oxidised (Table 5).

Table 3
Distribution of diterpene skeletons among the Lamiaceae genera with more than 10 occurrences in total

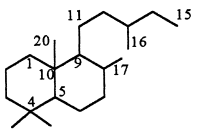
Genus (number of species)	Skeletons (occurrence)
Ajuga (16)	S-19 (82)
Anisomeles (3)	S-2 (15)
Ballota (8)	S-6 (1); S-7 (1); S-15 (26); S-43 (2)
Clerodendrum (8)	S-19 (11); S-54 (1); S-62 (1); S-63 (4)
Coleus (9)	S-15 (20); S-29 (1); S-32 (2); S-36 (1); S-46 (1); S-54 (31); S-56 (1); S-62 (2); S-63 (4); S-86 (16); S-87 (5); S-91 (1)
Isodon (20)	S-54 (3); S-79 (23); S-83 (76)
Lagochilus (2)	S-15 (10)
Leonotis (6)	S-15 (20)
Leonurus (5)	S-15 (21); S-19 (6); S-21 (1)
Lepechinia (5)	S-46 (2); S-54 (15)
Marrubium (8)	S-15 (18)
Nepeta (4)	S-15 (1); S-54 (10); S-69 (17)
Plectranthus (19)	S-29 (2); S-32 (1); S-51 (1); S-54 (98); S-62 (24); S-63 (13); S-64 (2); S-65 (2); S-66 (1); S-80 (2); S-83 (8); S-86 (17); S-87 (12)
Premna (4)	S-14 (1); S-15(2); S-19 (2); S-54 (2); S-69 (3); S-76 (1)
Rabdosia (28)	S-38 (11); S-54 (10); S-69 (3); S-79 (59); S-83 (168); S-89 (2)
Salvia (91)	S-3 (1); S-8 (1); S-9 (3); S-10 (4); S-11 (1); S-12 (3); S-13 (3); S-15 (7); S-17 (2); S-18 (5); S-19 (77); S-24 (2); S-27 (3); S-28 (1); S-30 (1); S-31 (2); S-32 (8); S-33 (5); S-34 (1) S-35 (33); S-39 (4); S-40 (5); S-41 (3); S-42 (1); S-44 (2); S-45 (6); S-46 (19); S-47 (2); S-48 (1); S-50 (2); S-52 (4); S-53 (1); S-54 (275); S-55 (37); S-57 (16); S-58 (1); S-59 (1); S-60 (3); S-61 (1); S-67 (1); S-68 (1); S-69 (25); S-74 (2); S-75 (1); S-76 (3); S-77 (1); S-78 (4); S-81 (1); S-82 (1); S-83 (5)
Scutellaria (13)	S-19 (98)
Sideritis (40)	S-1 (2); S-15 (71); S-16 (1); S-18 (1); S-37 (1); S-54 (1); S-69 (1); S-71 (6); S-83 (130); S-84 (35); S-85 (14); S-90 (15)
Solenostemon (2)	S-54 (6); S-86 (9); S-87 (13)
Stachys (5)	S-15 (1); S-19 (10); S-49 (5); S-83 (4)
Teucrium (58)	S-4 (3); S-5 (2); S-10 (1); S-15 (1); S-19 (296); S-20 (67); S-22 (2); S-23 (1); S-26 (2); S-54 (1); S-62 (10); S-63 (2); S-72 (4); S-88 (2)

Table 4
Structural data from diterpenes with a clerodane skeleton (S-19) isolated from *Ajuga* species stored in the database[a]

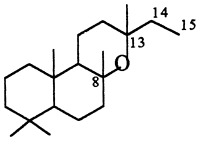
Position	CH_3	CH_2	CH	C	CH_2=	CH=	C=	HCT	CT	HC[a]	C[a]
C-1	0	44	29	0	0	0	1	0	0	0	0
C-2	0	53	21	0	0	0	0	0	0	0	0
C-3	0	45	29	0	0	0	0	0	0	0	0
C-4	0	0	2	70	0	0	2	0	0	0	0
C-5	0	0	0	74	0	0	0	0	0	0	0
C-6	0	0	74	0	0	0	0	0	0	0	0
C-7	0	74	0	0	0	0	0	0	0	0	0
C-8	0	0	74	0	0	0	0	0	0	0	0
C-9	0	0	0	74	0	0	0	0	0	0	0
C-10	0	0	74	0	0	0	0	0	0	0	0
C-11	0	40	34	0	0	0	0	0	0	0	0
C-12	0	47	27	0	0	0	0	0	0	0	0
C-13	0	0	34	0	0	0	40	0	0	0	0
C-14	0	30	0	0	0	44	0	0	0	0	0
C-15	0	13	17	0	0	4	40	0	0	0	0
C-16	0	40	34	0	0	0	0	0	0	0	0
C-17	74	0	0	0	0	0	0	0	0	0	0
C-18	0	70	0	0	2	0	2	0	0	0	0
C-19	2	72	0	0	0	0	0	0	0	0	0
C-20	74	0	0	0	0	0	0	0	0	0	0

[a] Atom in an aromatic ring; T triple bond.

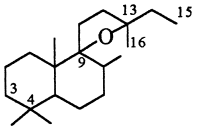
For *Marrubium* and *Sideritis* did not have positions consistently substituted with oxygen or double bonds. In *Marrubium* the positions 1, 2, 5, 7, 11, 17, 18 and 20 were never oxidised. In *Sideritis*, the positions 1, 5, 9, 11 and 20 were conserved (Table 5).



Labdane (S-15)



Manoyl Oxide



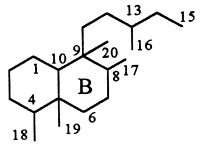
Grindelane

3.1.3. Clerodanes (S-19)

The presence of clerodanes was reported in 13 Lamiaceae genera to date. The substances isolated from *Scutellaria* and *Ajuga* presented some similarities: C-6 is bound to an oxygenated function and conserved the original skeleton at the positions 10, 17 and 20. In *Ajuga*, carbons 7 and 8 were never oxidised, while in *Scutellaria* oxidation in these position may occur (Table 6).

The compounds isolated from *Salvia* and *Teucrium* also presented similarities. In these species, C-13 was sp^2 hybridised forming a double bond with C-14 or C-16. All the other carbons were oxidised in *Salvia*. In *Teucrium* C-17 was never oxidised and C-19 was always oxygenated.

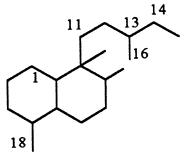
The database demonstrated that *Stachys* preserved the positions 1, 6, 8, 10, 11, 12, 16, 17, 18, 19 and 20 and position 13 was always oxidised. In *Clerodedrum* the positions 1, 7, 8, 10, 12, 17 and 20 were never oxidised.



Clerodane (S-19)

3.1.4. 19-Norclerodanes (S-20)

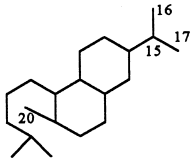
The loss of C-19 was only observed in the genus *Teucrium*, yielding the 19-norclerodanes. In this group, only positions 1 and 11 were preserved. The position 12 was always oxygenated and C-13 presented a double bond with C-14 or with C-16.



19-Norclerodane (S-20)

3.1.5. 4,5-Seco-20(10→5)abeoabietanes (S-35)

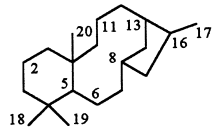
This skeleton, derived from abietane (S-54), was found in *Zhumeria* (only one occurrence) and in *Salvia*. The compounds isolated from *Salvia* could be oxidised at any position, except for 15, 16 (or 17) and 20 which maintain the same functional groups as the original skeleton.



4,5-seco-20(10→5)*abeo*abietane (S-35)

3.1.6. 8,9-Secokauranes (S-38)

This group was reported in *Rabdosia*. In all reported compounds, carbons at 2, 5, 6, 11, 12, 13, 18, 19 and 20 maintain the same substitution pattern as the original skeleton. The 8,9-secokauranes isolated in this genus always bear a carbonyl group at C-9 and C-15, a double bond between C-16 and 17 and oxygen linkage at C-7.



8,9-secokaurane (S-38)

3.1.7. Icetexanes (S-46)

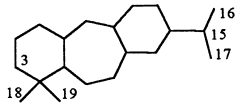
This skeleton, also derived from abietane (S-54), was found only in three Lamiaceae genera: *Coleus* (one occurrence), *Lepechinia* (two occurrences) and *Salvia*.

Table 5
Substitution pattern determined for labdanes in some Lamiaceae genera[a]

Carbon number[b]	Genus						
	Ballota	*Coleus*	*Lagochilus*	*Leonotis*	*Leonurus*	*Marrubium*	*Sideritis*
C-1	CH_2		CH_2	CH_2	CH_2	CH_2	CH_2
C-2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	
C-3		CH_2	CHOR	CH_2			
C-5	CH	CH	CH		CH	CH	CH
C-6			CH_2				
C-7			CH_2			CH_2	
C-8		EP (13)	CH				
C-9			EP (13)	COR	COR		CH
C-11	CH_2	C=O	CH_2	CH_2	CH_2	CH_2	CH_2
C-12	CH_2	CH_2	CH_2	CH_2	CH_2		
C-13		EP (8)	EP (9)				
C-14		=CH	CH_2				
C-15		$=CH_2$	CH_2OR				
C-16		CH_3	CH_2OR				
C-17	CH_3	CH_3	CH_3		CH_3	CH_3	
C-18		CH_3	CH_2OR	CH_3	CH_3	CH_3	
C-19		CH_3	CH_3				
C-20	CH_3	CH_3	CH_3		CH_3	CH_3	CH_3

[a] CH_3 (methyl group), CH_2 (methylene group), CH (tertiary carbon), EP (epoxy group), C=O (carbonyl group);=(double bond); R (hydrogen or another group).
[b] C-4 and C-10 are not listed in the table because these are quaternary carbons that remained unchanged.

Icetexanes isolated from *Salvia* were not oxidised at positions 3, 15, 16, 17 and 18 (equatorial methyl).



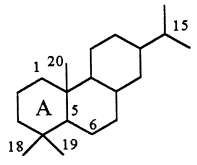
Icetexane (S-46)

3.1.8. Abietanes (S-54)

Abietane is the skeleton with the highest occurrence and most widespread in Lamiaceae. The compounds produced by *Salvia* and *Rabdosia* are the most oxidised. In *Salvia* abietanes any carbon can be oxidised while in *Rabdosia* only carbon 5 was not oxidised (Table 7).

Plectranthus abietanes were not oxidised at carbons 1 and 20. In *Coleus*, the carbons preserved were 1, 2, 15, 18 (equatorial methyl) and 19 (axial methyl).

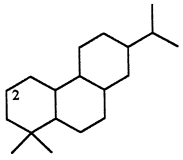
The abietanes found in *Lepechinia* and *Nepeta* are the least oxidised. In both genera, the isopropyl group (side-chain) remained unchanged. In *Lepechini*a, besides the side-chain, the carbons in the A ring were conserved. *Nepeta* abietanes were not oxidised at carbons 2, 5, 6, 19 (axial methyl at C-4) and 20 (Table 7).



Abietane (S-54)

3.1.9. 20-Norabietanes (S-55)

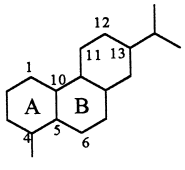
This skeleton occurred in four Lamiaceae genera but was most frequently found in *Salvia*. The compounds isolated in this genus were oxidised at any position except at C-2.



20-Norabietane (S-55)

3.1.10. 19,20-Dinorabietanes (S-57)

This skeleton was only found in *Salvia*. In these substances C-11, C-12 and C-13 were sp^2 hybridised. The SISTEMAT programs also indicated that all 19,20-Dinorabietanes from *Salvia* possessed carbons 5 and 10 as part of an aromatic ring. This constant pattern was not found for carbons 1, 2, 3 and 4 and neither for 6, 7, 8 and 9. These findings allow us to conclude that for some substances, the A ring is aromatic whereas in some other examples the B ring is aromatic.



19,20-Dinorabietane (S-57)

Table 6
Substitution pattern determined for clerodanes in some Lamiaceae genera[a]

Carbon number[b]	Genus					
	Ajuga	*Clerodendrum*	*Salvia*	*Scutellaria*	*Stachys*	*Teucrium*
C-1		CH_2			CH_2	
C-6	CHOR			CHOR	CH_2	
C-7	CH_2	CH_2				
C-8	CH	CH			CH	
C-10	CH	CH		CH	CH	
C-11					CH_2	
C-12		CH_2			CH_2	
C-13			=C		COR	=C
C-14			=CH			=CH
C-16					CH_3	
C-17	CH_3	CH_3		CH_3	CH_3	CH_3
C-18					CH_3	
C-19					CH_3	CH_2OR
C-20	CH_3	CH_3		CH_3	CH_3	

[a] See Table 5.
[b] Carbons 2, 3 and 4 are not listed because they not present a constant substitution pattern. Carbons 5 and 9 are quaternary carbons.

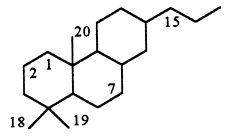
Table 7
Positions conserved in the abietane skeleton for some Lamiaceae genera[a]

Carbon number[b]	Genus				
	Coleus	*Lepechinia*	*Nepeta*	*Plectranthus*	*Rabdosia*
C-1	CH_2	CH_2		CH_2	
C-2	CH_2	CH_2	CH_2		
C-3		CH_2			
C-5		CH	CH		CH
C-6			CH_2		
C-15	CH	CH	CH		
C-16		CH_3	CH_3		
C-17		CH_3	CH_3		
C-18	CH_3				
C-19	CH_3	CH_3	CH_3		
C-20			CH_3	CH_3	

[a] See Table 5.
[b] Carbons 7, 8, 9, 11, 12, 13 and 14 are not listed because they did not displayed a constant substitution pattern. Carbons 4 and 10 are quaternary carbons.

3.1.11. 17(15→16)Abeoabietanes (S-62)

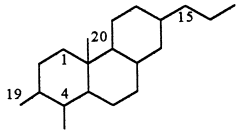
This skeleton was found in five Lamiaceae genera. In the *Plectranthus* genus, these diterpenes were preserved at positions 1, 2, 15, 18 (equatorial methyl group) and 20, while in *Teucrium* this same feature was observed for carbons 1, 2, 18, 19 and 20. In the latter, however, carbon 7 was always oxidised possessing a carbonyl group for all substances examined.



17(15→16)*abeo*abietane (S-62)

3.1.12. 17(15→16)Abeotriptolidanes (S-63)

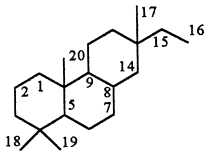
This skeleton was found only in *Clerodendrum*, *Coleus*, *Plectranthus* and *Teucrium* species. The substances isolated from *Plectranthus* maintained positions 1, 15, 19 and 20 as in the original skeleton and carbon 4 was always sp^2 hybridised.



17(15→16)*abeo*triptolidane (S-63)

3.1.13. Pimaranes (S-69)

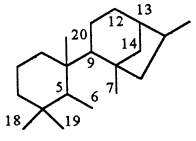
This skeleton was found in 14 genera. All the pimaranes isolated from *Nepeta* and *Salvia* had a double bond between C-15 and C-16. Additional double bonds were found in *Nepeta* pimaranes between carbons 7(8), 8(9) or 8(14). In these substances the equatorial methyl group (C-18) was bound to an oxygen. The substances from both genera were not oxidised at C-17, C-20 and C-19 (axial methyl at C-4). The compounds found in *Salvia* preserved positions 1, 2, 5, 11 and 12 and in *Nepeta* in 2, 3, 5, 6, 11 and 12.



Pimarane (S-69)

3.1.14. Fujinanes (S-79)

This skeleton is a 6,7-secokaurane which occurred specifically in *Rabdosia* and *Isodon*. The positions 5, 9, 12 and 13 were preserved in both genera. Additionally, carbons 2 and 14 were not oxidised in *Rabdosia* and in *Isodon* the equatorial methyl (C-18) was also preserved. The fujinanes had at C-7 a carboxyl group that may also be present as an ester and those from *Isodon* always had an oxygen linkage at C-20.

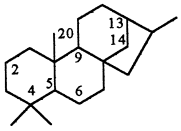


Fujinane (S-79)

3.1.15. Kauranes (S-83)

The occurrence of kauranes was reported for 8 Lamiaceae genera. The compounds isolated from *Isodon* and *Rabdosia* are highly oxidised. In *Isodon* C-5 and C-9 were preserved, while *Rabdosia* maintained only C-5 as in the original skeleton.

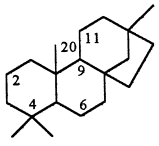
The kauranes found in *Sideritis* were less oxidised preserving position 2, 5, 6, 9, 13, 14, 19 (axial methyl at C-4) and 20.



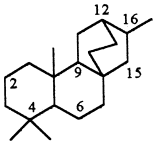
Kaurane (S-83)

3.1.16. Beyeranes (S-84), atisanes (S-85) and trachylobanes (S-90)

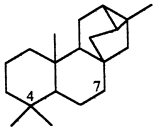
In the Lamiaceae, these three skeletons were found exclusively in *Sideritis*. The axial methyl (C-19) attached to C-4 was not oxidised. The beyeranes were preserved at positions 2, 5, 6, 9, 11 and 20. The atisanes always had an oxygen bound at C-16 but were not oxidised at carbons 2, 3, 5, 6, 9, 12, 15 and 20. The trachylobanes were always oxidised at C-7, having carbon–oxygen bonds, whereas the other positions (except C-18) were as in the original skeleton.



Beyerane (S-84)



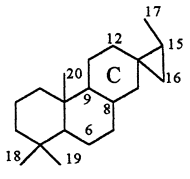
Atisane (S-85)



Trachylobane (S-90)

3.1.17. 13(16)-Cyclopropylabietane (S-86)

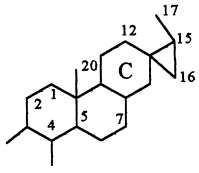
This skeleton is derived from abietane and was reported as being present in *Coleus*, *Plectranthus* and *Solenostemon*. The substances isolated from *Coleus* and *Plectranthus* displayed some similarities. In both genera was detected an oxygen atom bound at positions 6 and 12, formation of a carbonyl at C-11 and C-14, and a double bond between C-8 and C-9. The substitution pattern from the original skeleton was retrieved at positions 1, 2, 5 and 20. The cyclopropylabietanes from *Plectranthus* were additionally preserved at 15, 16, 17 and 18 while those isolated from *Coleus* presented an oxygen attached to C-7.



13(16)-Cyclopropylabietane (S-86)

3.1.18. 13(16)-Cyclopropyltriptolidane (S-87)

This skeleton was also found in *Coleus*, *Plectranthus* and *Solenostemon*. The compounds isolated from *Plectranthus* and *Solenostemon* were similar. In both genera, the substances presented oxygen bound at positions 6 and 12, formation of a carbonyl at C-11 and C-14 and a double bond between C-8 and C-9. The original skeleton was maintained at positions 1, 5 and 20 and carbon 4 was a sp^2 hybridised in all substances. The structures reported in *Plectranthus* were also preserved at C-15, C-16 and C-17, but had an oxygen bound to C-7. The substances from *Solenostemon* were never oxidised at C-2.



13(16)-Cyclopropyltriptolidane (S-87)

4. Discussion

The discussion is based on the 13 skeletons that were most frequently isolated from Lamiaceae, corresponding to 87.8% of the structures described. The distribution of these skeletons in all Lamiaceae genera, according to Cantino (1992), grouped by subfamilies is presented in Table 8.

The results indicate that the majority of the labdanes (S-15) occur in the subfamily Lamioideae, representing 42.5% of the total diterpenes isolated from this subfamily. *Sideritis*, also in the Lamioideae, was different from the other genera frequently accumulating kaurane diterpenes (S-83). Moreover, this genus was the only one in the family to accumulate beyeranes, atisanes and trachylobanes.

The subfamily Nepetoideae was characterised for the majority of abietane diterpenes (S-54) and its derivatives (S-35, S-55, S-62, S-86 and S-87). These substances represented 47.7% of the total diterpenes reported in this subfamily. In this subfamily the majority of structures was found in *Salvia*, *Plectranthus* (and *Coleus*) and *Isodon* (and *Rabdosia*). *Salvia* presented the highest number of species studied and the highest occurrence number distributed among 50 diterpene skeletons.

The *Isodon* (*Rabsodia*) diterpenes were mainly from the kaurane (S-83) and fujinane (S-79). The major feature observed for the kauranes from both genera was the high oxidation level, only C-5 in *Rabdosia* and C-5 and C-9 in *Isodon* were preserved.

Plectranthus (*Coleus*) was characterised by the accumulation of abietanes (S-54), 17(15→16)*abeo*abietanes (S-62), 13(16)-cyclopropylabietanes (S-86) and 13(16)-cyclopropyltriptolidanes (S-87). Considering only the

Table 8
Occurrence number of the major diterpene skeletons isolated from Lamiaceae[a]

Subfamily	Genus	Skeletons												
		S-15	S-19	S-20	S-35	S-54	S-55	S-62	S-69	S-79	S-83	S-84	S-86	S-87
Lamioideae	*Ballota*	26												
	Leonotis	20												
	Lasiocorys	1												
	Leucas	2							1					
	Galeopsis	6												
	Lagochilus	10												
	Leonurus	21	6											
	Marrubium	18												
	Sideritis	71				1			1		130	35		
	Roylea	3												
	Stachys	1	10								4			
Viticoideae	*Callicarpa*		2			1			1		2			
	Premna	2	2			2			3					
	Pygmaeopremna					1								
	Vitex	3												
Chloanthoideae	*Cyanostegia*		1											
	Tectona	1												
	Pityrodia		1											
Scutellarioideae	*Scutellaria*		98											
Ajugoideae	*Ajuga*		82											
Teucrioideae	*Teucrium*	1	296	67		1		10						
	Peronema		7											
	Clerodendrum		11			1		1						
	Caryopteris		6					1						
Nepetoideae	*Salvia*	7	77		33	275	37		25		5			
	Coleus	20				31		2					16	5
	Plectranthus					98		24			8		17	12
	Lepechinia					15								
	Nepeta	1				10			17					
	Rabdosia					10			3	59	168			
	Isodon					3				23	76			
	Solenostemon					6							9	13
	Aeollantus					1								
	Amaracus								5					
	Fuerstia					1								
	Horminum					1								
	Hoslundia					3								
	Hyptis					4			1					
	Meriandra					2	2							
	Cleonia								1					
	Englerastrum										8			
	Lycopus								1					
	Rosmarinus					8	1							
	Orthosiphon								4					
	Dauphinea					2								
	Tetradenia					1			2					
	Satureja	1							3					
	Agastache						1							
	Zhumeria	1			1									

[a] According to Cantino who included some Verbenaceae species (Cantino et al., 1992).

diterpene accumulation, one can observe a proximity within the subfamily between *Plectranthus* (*Coleus*) and *Solenostemon*, because these are the only genera to accumulate 13(16)-cyclopropylabietanes (S-86) and 13(16)-cyclopropyltriptolidanes (S-87). This proximity is also verified by the same substitution pattern for these skeletons in the C ring and an oxygenated function at C-6 for both genera.

The subfamilies Ajugoideae and Scutellarioideae presented many similarities concerning the structural char-

acteristics of the diterpenes. Both subfamilies produced only clerodanes (S-19). The substitution pattern observed for this skeleton was almost the same in the B ring, only C-7 and C-8 were different in all substances isolated from species of both subfamilies.

Some of the Verbenaeceae genera included by Cantino (1992) in Lamiaceae (*Clerodrendrum*, *Peronema*, *Caryopteris*, *Callicarpa*, *Pygmaeoprenna*, *Vitex*, *Premna*, *Tectona*, *Pityrodia* and *Cyanostegia*) had few reports of diterpenes in the literature. However, all diterpenes reported for these genera presented the same skeletons reported for the Lamiaceae genera.

Cantino (1992) placed the Verbenaceae genera *Clerodendrum*, *Peronema* and *Caryopteris* in the Lamiaceae subfamily Teucrioideae, together with *Teucrium*. Analysing the diterpene production this placement seemed coherent. All these genera accumulated the same diterpene skeletons, mainly clerodanes (S-19), as observed for *Teucrium*. This genus was the only one in Lamiaceae to produce 19-norclerodanes.

The subfamilies Viticoideae and Lamioideae produced the same type of diterpene skeletons which might indicate some proximity between both subfamilies. However, it is important to remark that Viticoideae only had a low occurrence of diterpenes (26).

5. Conclusion

The database was built only with the names of genera and species, and some characteristics as local variations, season of harvesting and the plant part studied were not included. These characteristics could also be inserted in the database which would allow additional types of analysis and comparisons.

The results presented in this paper can be discussed under several points of view and be modified as more species are studied. However, the general features of diterpene chemistry did not change as the database entries increased. The research groups involved in the isolation of natural products of some genus have already confirmed some of these results, at the genus level.

The research in chemotaxonomy demands the handling of several types of data. As shown in this paper, the expert system SISTEMAT was able to analyse relationships between chemical structure and taxonomic group. These results, besides the application to chemotaxonomy, can be used also for research focussing structure determination because they point to the class of natural products (diterpenes) that can be isolated, the major skeletons and the positions that are most likely oxidised.

The distribution of the diterpene skeletons in some subfamilies showed an agreement with the botanical classification, i.e. Lamioideae produced mainly labdanes (S-15); Scutellarioideae, Ajugoideae and Teucrioideae produced clerodanes (S-19) and Nepetoideae contained mainly abietanes (S-54) and kauranes (S-83).

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Effects of temperature on polyunsaturation in cytostatic lipids of *Haslea ostrearia*

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Abstract

Unusual chemicals produced by the 'blue oyster' diatom, *Haslea ostrearia,* include the water-soluble blue pigment marennine and numerous polyunsaturated sesterterpene oils or haslenes. Aqueous extracts of the alga exhibit in vitro and in vivo activities against human lung cancer cells and anti-HIV effects. Here we report that three haslenes also demonstrate in vitro cytostatic action against a human lung cancer cell line. The most active haslene is the most unsaturated and unsaturation in the haslenes increases with increasing algal growth temperature. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Haslea ostrearia*; Bacillariophycane; Diatoms; Isoprenoid alkenes; Haslenes; Highly branched isoprenoids; Temperature; Unsaturation; Cancer; Cytostatic

1. Introduction

Haslenes are unsaturated C_{25} highly branched isoprenoid (HBI) hydrocarbons which apparently originate from relatively few species of diatomaceous algae (Volkman et al., 1994; Sinninghe Damsté et al., 1999; Belt et al., 2000). Nonetheless, they are widely distributed and sometimes abundant in aquatic environments and, for instance, are found in sediments from the USA, UK and mainland Europe (Robson and Rowland, 1986; Rowland and Robson, 1990) and even Antarctica (Johns et al., 1999), where they are thought to originate from diatoms living on sea-ice (Nichols et al., 1988).

Laboratory culturing experiments under axenic conditions (Wraige et al., 1999) have shown that one biological producer of haslenes is *Haslea ostrearia,* which is a large pennate diatom (Round et al., 1990). Other diatom sources include other *Haslea* species (Massé et al., in press), *Rhizosolenia setigera* (Volkman et al., 1994; Sinninghe Damsté et al., 1999) and *Pleurosigma intermedium* (Belt et al., 2000) although these have yet to be shown to produce haslenes under axenic conditions. The structures and configurations of many of the haslenes from *H. ostrearia* have been established previously by spectroscopic studies (e.g. Belt et al., 1996; Johns et al., 1999; Wraige et al., 1999). Since several haslenes have been obtained pure in milligram to gram quantities, and given the anti-cancer and anti-HIV activities of some other chemicals produced by *H. ostrearia* (Carbonnelle et al., 1999; Bergé et al., 1999), we decided to determine whether the haslenes also exhibited bioactivity.

Activity *was* observed but varied for dienes, trienes and tetraenes. We therefore examined some factors controlling unsaturation (cf. Wraige et al., 1998). Temperature exhibited the major effect, unsaturation increasing with increasing temperature.

2. Results and discussion

Milligram quantities of pure hasla-6(17),23-diene (**I**), 22*R* and 22*RS*-hasla-6(17),9,23-trienes (**II**) and

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hasla-6(17),9,13,23-tetraene (**III**) were isolated from pastes obtained from large-scale cultures of *H. ostrearia* as reported previously (e.g. Johns et al., 1999; Wraige et al., 1999). We then measured the concentration of each pure haslene in aqueous dimethylsulfoxide solution required to reduce the growth of an evolving, human non-small-cell bronchopulmonary carcinoma cell line (Roussakis et al., 1991) in vitro by 50% in 72 h (IC_{50}) compared to untreated controls (Mosmann, 1983). This is a widely accepted preliminary screening measure of in vitro cytotoxic activity (Suffness and Pezzuto, 1991). For comparison with other studies, we also measured the IC_{50} of the tetraene (compound **III**) against a non-evolving murine cancer cell line (P_{388}) (Suffness and Pezzuto, 1991). Haslenes with three and four degrees of unsaturation were able to produce an effect (Table 1).

Cytocidal activities of chemicals are not uncommon in in vitro tests of marine natural products (e.g. Patterson et al., 1991) but at some concentrations the haslenes exhibited only anti-proliferative or cytostatic effects on the growth of the NSCLC-N6 cell line without cytocidal activity. That is, the growth of the cancer cells was slowed or halted but the cells were not killed. Further specificity was indicated by an observed effect of the triene on the NSCLC-N6 cell line (Roussakis et al., 1991) whereas there was no such effect on a clone of this line. It would be interesting to isolate larger quantities for more refined in vivo bioactivity tests and particularly to examine the effects of more unsaturated haslenes such as the pentaenes and hexaenes. We therefore investigated the culture conditions necessary for production of polyunsaturates.

Unsaturation in the so-called *normal* (unbranched) lipids of plants, which are principally esters of fatty acids, usually increases with decreasing growth temperature. This is thought to be a protective response to chilling and to involve regulation of properties such as membrane fluidity and stability (reviewed by Quinn et al., 1989). Essentially the principle is that *cis*-double bonds decrease the melting point of a normal lipid and, therefore, organisms living in low temperature environments require more unsaturated *normal* lipids to preserve fluidity. This general inverse relationship with temperature apparently also extends to the *normal* lipids of some algae. Thus lipid unsaturation in the widespread sedimentary *n*-alkenones derived from some prymnesiophyte algae has been widely used as a proxy measure of palaeo-sea surface temperatures for palaeoclimatic studies (e.g. Eglinton et al., 1992). However, there are numerous exceptions to this common relationship where *normal* lipids possess more than two double bonds (e.g. Farkas et al., 2000 and references therein) and neither is it known what the effect of the *trans*-double bonds, which usually occur in acyclic isoprenoids such as haslenes, would have on melting point and fluidity. All haslenes isolated thus far (e.g. Belt et al., 1994) and even the parent saturated haslane (Robson and Rowland, 1986) are oils even at room temperature.

In preliminary experiments with *H. ostrearia* cultures, we noted considerable diversity in haslene distributions as revealed by gas chromatography–mass spectrometry (GC–MS; Wraige et al., 1997). Since salinity appeared to exhibit no appreciable effect on unsaturation (Wraige et al., 1998), we investigated the effect of growth temperature on haslene distributions. Small-scale batch cultures of *H. ostrearia* were grown in the laboratory in replicate in a controlled atmosphere in modified Provosali media at constant salinity and irradiance intensity at temperatures of 5, 15 and 25±0.5°C. A stock of the alga used as inocula for these experiments was grown at 15±1°C for multiple generations. Lipid extracts were obtained by solvent extraction and examined by GC–MS (Johns et al., 1999; Wraige et al., 1999).

Whilst growth from the inoculum was slow to initiate at 5°C, requiring about 10 days to reach the logarithmic phase (Fig. 1a) the only haslenes produced between 10 and 27 days, were the 6(17), 23 and 5, 23 dienes (Figs. 2 and 3, compounds **I** and **IV**) the structural characterizations of which, we have reported previously (e.g. Belt et al., 1994; Johns et al., 1999). A re-equilibration thus occurred from the corresponding triene isomer distributions, which dominated the inoculum grown at 15°C. However, cell growth had not reached the stationary phase by 27 days (ca. 170,000 cell ml^{-1}) so we cannot rule out the possibility that more unsaturated haslenes could be produced in later growth, though we consider it unlikely. Indeed, monitoring of a single growth cycle of the alga at 15°C, revealed faster growth than at 5°C, probably partially due to prior acclimation of the stock culture at this temperature and in this case

Table 1
Cytostatic activity of individual haslenes to cell line NSCLC-N6 established from a primary epidermoid carcinoma of human origin (Roussakis et al., 1991) assayed by the methods of Mosmann (1983)

Haslene	Structure number (Fig. 2)	IC_{50}[b] (μg ml^{-1})	Cancer cell line[a] (in vitro)
Hasla-6(17), 23-diene	**I**	> 30 (Inactive)	NSCLC-N6 (Human lung)
22*R*-Hasla-6(17), 9,23-triene	**II**	13.4	NSCLC-N6 (Human lung)
22*RS*-Hasla-6(17), 9,23-triene	**II**	14.4	NSCLC-N6 (Human lung)
Hasla-6(17),9,13, 23-tetraene	**III**	3.8	NSCLC-N6 (Human lung)
Hasla-6(17),9,13, 23-tetraene	**III**	< 3.3	P_{388} (Mouse)

[a] The NSCLC-N6 cell line derives from a human non-small-cell bronchopulmonary carcinoma (moderately differentiated, rarely keratinizing, classified as T2NOMO).

[b] IC_{50} is the concentration of haslene required to reduce the cancer cell concentration by 50% compared to untreated controls after 72 h.

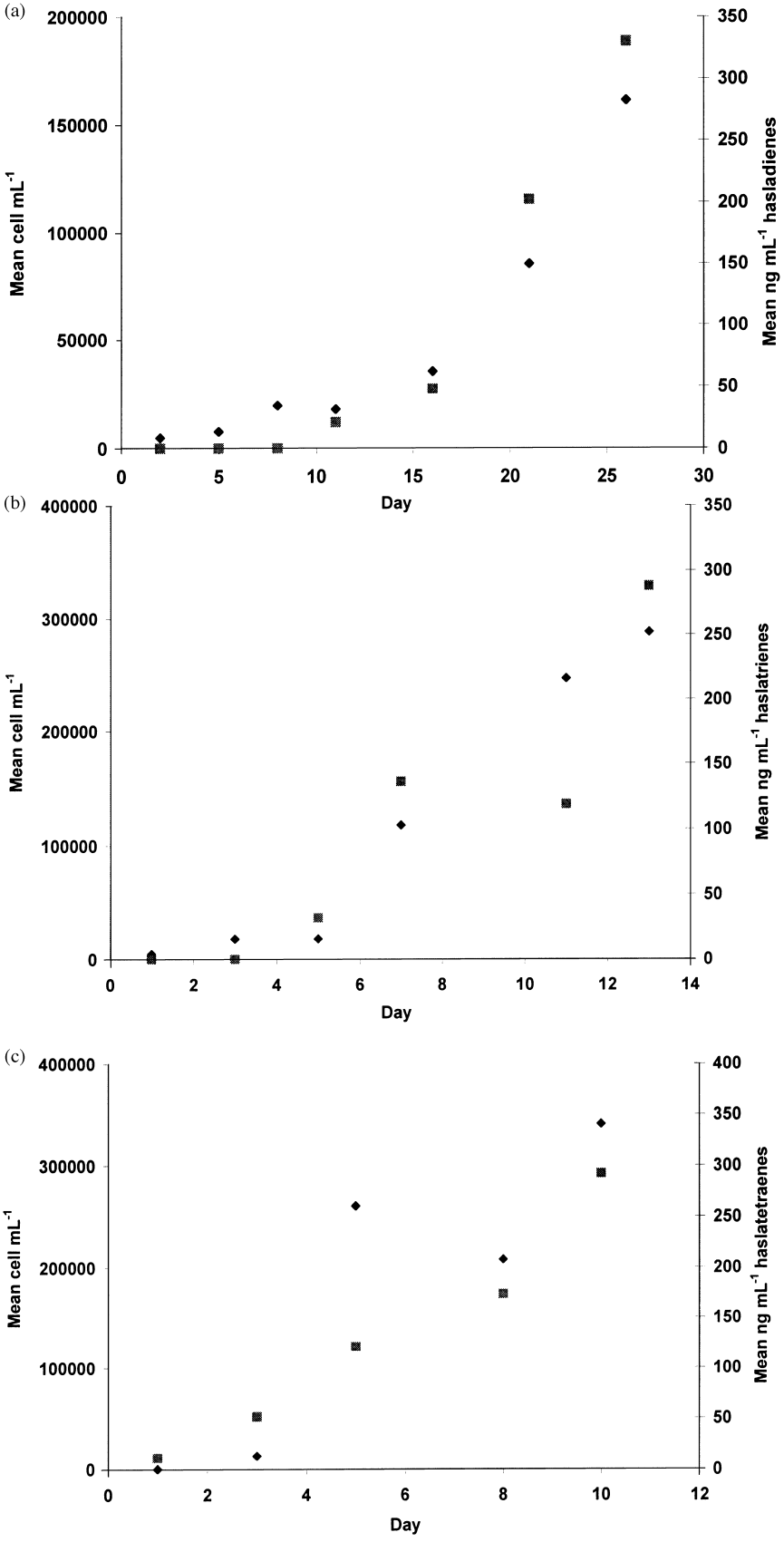


Fig. 1. Mean ($n=4$) cell concentration (◆) and total haslene production (■) in *H. ostrearia* at (a) 5°C (hasla-5,23-diene plus 6(17),23 diene), (b) 15°C (hasla-5,9,23-triene plus 6(17),9,23 triene), (c) 25°C (hasla-5,9,13,23-tetraene plus 6(17),9,13,23 tetraene).

at a cell concentration of ca. 170,000 cell ml^{-1} at day 7 (Fig. 1b), the hasla-trienes (compounds **II** and **V**) were dominant (Fig. 3), as they were in the inoculum. Between day 5 and day 11, tetraenes (compounds **III** and **VI**) were also detected (Fig. 3). At 25°C, production of successively more polyunsaturated analogues was observed, including production of tetraenes by day 3 and additionally, pentaene production from day 8 (ca. 170,000 cell ml^{-1}; Figs. 1c and 3). Thus, there is a significant relationship between culture temperature (and perhaps growth phase) and haslene unsaturation, together with a quite rapid equilibration of haslene distributions with temperature. No such effect was observed on the haslapentaene **VII** produced by *R. setigera* (Sinninghe Damsté et al., 1999).

Opportunities to examine the effects of phenotypic variables on unsaturation in algal isoprenoids do not often arise. Acyclic isoprenoids (presumably including the haslenes) are initially biosynthesised as polyunsaturated species via isopentenyl diphosphate and dimethylallyl diphosphate, unlike *n*-fatty acids which are mainly desaturated after production (Quinn et al., 1989). Although many algae produce polyunsaturated isoprenoid alkenes such as squalene, lycopene, phytoene and other carotenes, the efficiency of the cyclisation of squalene to sterols and of the desaturation of phytoene to other carotenoids means that significant accumulation of unsaturated hydrocarbons is usually not observed in micro-organisms. Significant amounts of partially saturated squalenes are found in some archaebacteria, such as the anaerobic, thermophilic methanogen, *Methanobacterium thermoautotrophicum* and the variations in unsaturation have been suggested to allow internal proton regulation (Tornabene et al., 1979). However, it has also been argued that the effects of hydrogen flux in the corresponding ether lipids would outweigh those in the hydrocarbons and nothing conclusive is known.

Determination of whether the biosynthesis of the haslenes is *via* the mevalonate or non-mevalonate route may be helpful in elucidating their location and functions (cf. Rohmer et al., 1996).

Culturing of *H. ostrearia* should seemingly be conducted at the higher growth temperatures used herein (e.g. 25°C) if the production of the tetra- and pentaunsaturated haslenes is to be maximised for further bioactivity testing with cancer cells.

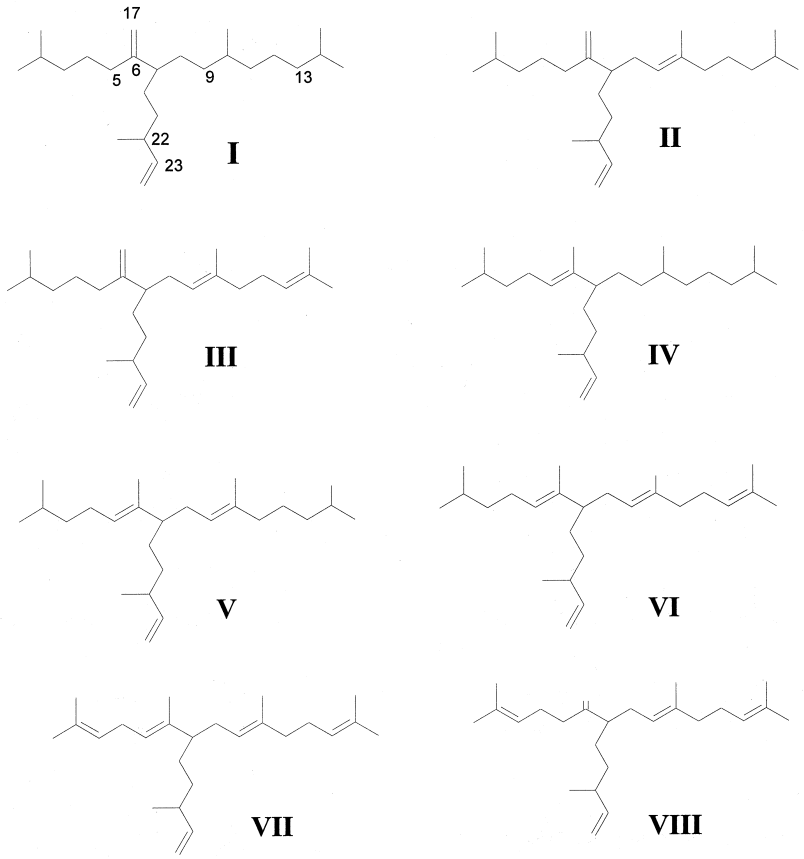


Fig. 2. Structures of haslenes produced by *Haslea ostrearia* at different culture temperatures. Structures were established previously by NMR spectroscopy and other techniques applied to milligram quantities of pure haslenes isolated from larger batch cultures of *H. ostrearia* (e.g. Belt et al., 1994, 1996; Johns et al., 1999; Wraige et al., 1999).

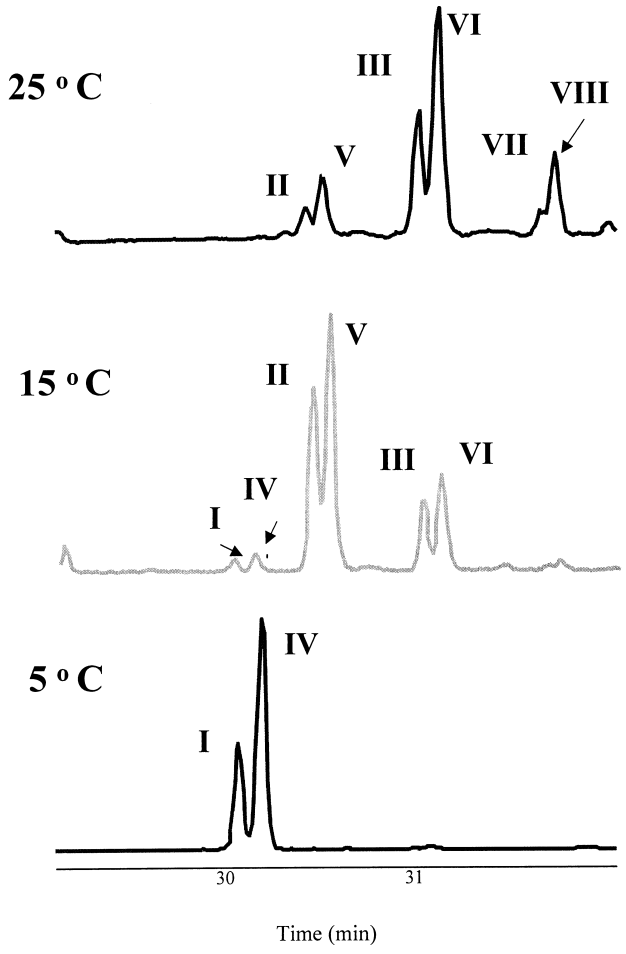


Fig. 3. Partial gas chromatograms illustrating the distributions of haslenes in *H. ostrearia* cultures grown at 5 (day 27), 15 (day 7) and 25°C (day 10). Structures of the haslenes are shown in Fig. 2. Identities were confirmed by comparison of retention indices and mass spectra with authenticated compounds. Gas chromatography–mass spectrometry conditions: HP 5890 GC-MSD 12 m HP- Ultra 1 column, 50–300 at 5°C 40 eV.

3. Experimental

3.1. Cytostatic tests

The cytostatic activity of individual haslenes (**I–III**) to cell line NSCLC-N6 established from a primary epidermoid carcinoma of human origin (Roussakis et al., 1991) was assayed by the method of Mosmann (1983). The cells were cultured in RPM1 1640 medium with 5% fetal calf serum, to which was added 100 µl of penicillin, 100 µg streptomycin ml^{-1} and 2 mM glutamine, at 37°C in a 5% CO_2 atmosphere.

Experiments were performed in conditions of continuous exposure, in solution in water + 5% dimethylsulphoxide on microtiter plates (2×10^5 cells ml^{-1}). Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product by live mitochondria. Three concentrations were tested in duplicate. Cell growth was estimated at day 0 and day 3 (72 h) and for investigation of cytostatic and anti-proliferative effects also at 24 and 48 h.

3.2. Algal cultures

H. ostrearia was isolated from oyster ponds of the Baie de Bourgneuf (France). Cultures were grown in quadruplicate in 250 ml Erlenmeyer flasks at 5, 15 and 25°C with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. They were incubated in a modified ES 1/3 Provasoli medium at salinities of ca. 32 per mil under 100 µmol photons m^{-2} s^{-1} irradiance. Samples were harvested by filtration. The structures of the haslenes isolated from larger batch cultures were established previously by NMR spectroscopy (e.g. Belt et al., 1994; 1996; Johns et al., 1999; Wraige et al., 1999).

3.3. Instrumental

GC–MS was performed using a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector fitted with a 12 m (0.2 mm i.d.) fused silica capillary column (HP-1 Ultra stationary phase). Auto-splitless injection and helium carrier gas were used. The gas chromatograph oven temperature was programmed from 40 to 300°C at 5° min^{-1} and held at the final temperature for 10 min. Mass spectrometer operating conditions were; ion source temperature 250° and 70 eV ionisation energy. Spectra (35–500 Daltons) were collected using Hewlett Packard Chemstation™ software.

Acknowledgements

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Membrane disruption and enzyme inhibition by naturally-occurring and modified chacotriose-containing *Solanum* steroidal glycoalkaloids

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Abstract

Naturally-occurring 3β-*O*-chacotriosides of solasodine (solamargine), of its 22*S*, 25*S* isomer tomatidenol (β-solamarine), and of solanidine (chaconine), as well as ring E- and F-modified derivatives of solamargine were prepared and assayed in order to assess the relevance of aglycone structural features to membrane-disruption and enzyme-inhibitory activities of the related glycoalkaloids. A ring E-opened dihydro-derivative of solasodine (the chacotrioside of dihydrosolasodine A) did not bind to cholesterol, stigmasterol or ergosterol in vitro, disrupt PC/cholesterol liposomes or mammalian erythrocytes, or inhibit acetylcholinesterase in vitro. It did not synergise with the solatrioside of dihydrosolasodine A or solasonine (nor did solamargine with dihydrosolasodine A solatrioside) in haemolysis tests. The ring F modified derivative, *N*-nitrososolamargine, did not inhibit acetylcholinesterase in vitro, but lysed liposomes at $\geqslant$150 μM and pH 7. Increasing the pH to 8 (but not 9) further enhanced disruption. The combination of *N*-nitrososolamargine and solasonine did not cause any disruption of liposomes. β-Solamarine showed no anti-acetylcholinesterase activity in vitro at up to 100 μM, but disrupted liposomes at 75 and 150 μM, although not to the extent caused by solamargine or chaconine. In combination with both the (inactive) solatriosides, solasonine and solanine, 75 μM β-solamarine produced synergistic effects, with liposome disruption greater than 150 μM β-solamarine alone. β-Solamarine, solamargine and chaconine showed similar haemolytic activity. β-Solamarine synergised with the solatriosides solasonine and solanine in disrupting erythrocytes. Preliminary structure–activity relationships were evaluated for the active chacotriosides in an attempt to define the scope and limitations of this model study. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Solanum* spp.; Solanaceae; Membrane disruption; Enzyme inhibition; Structure–activity relationships; Glycoalkaloids; Chaconine; β-Solamarine; Solamargine; *N*-nitroso-solasodine glycosides; Dihydrosolasodine A glycosides

1. Introduction

Steroidal glycoalkaloids are found mainly in the genus *Solanum* (and the related genus *Lycopersicon*) (Schreiber, 1968; Ripperger and Schreiber, 1981; Ripperger, 1998) and are of interest from both ecological and human health viewpoints. In wild species, they are thought to be an important component of the plant's chemical armoury against herbivores and microbial pathogens (Roddick, 1996). In cultivated species, of which potato, tomato and eggplant are the most prominent, the defensive role of glycoalkaloids is more equivocal, mainly because of the emergence of counter-adapted pests or pathogens commonly associated with large-stand growth of genetically-uniform plants (Roddick, 1996). The potential and actual toxicity particularly of the potato glycoalkaloids has on-going implications for aspects of food safety (Smith et al., 1996) and possibly other health-related matters (e.g. post-anaesthetic recovery times — Krasowski et al., 1997). Solasodine glycosides have been used for treating skin cancers (Cham et al., 1991; Cham, 1994).

The biological activity of the *Solanum* glycoalkaloids derives mainly from two properties — (a) the ability to complex with membrane 3β-hydroxy sterols and disrupt membrane integrity and function, and (b) inhibition of

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acetylcholinesterase (an important enzyme in nerve impulse transmission) (Roddick, 1996) and butyrylcholinesterase (an enzyme of uncertain function but possibly protective against specific toxins — Cooper, 1994). Glycoalkaloids have been isolated from more than 350 species, most of which contain more than one compound (Schreiber, 1968; Ripperger and Schreiber, 1981; Ripperger, 1998). In many species, 'paired' glycoalkaloids are present, i.e. two compounds based on a common aglycone but differing in their carbohydrate moiety (Fig. 1). With regard to membrane-disruptive activity, chacotriose-based glycoalkaloids are normally highly active whereas solatriose-based compounds show lower or no activity. There is evidence too that such 'paired' glycoalkaloids may interact synergistically (Roddick and Rijnenberg, 1987; Roddick et al., 1988; Fewell and Roddick, 1993; Fewell et al., 1994). Synergisms may also occur between solatriose- and chacotriose-based glycoalkaloids containing a different aglycone (Roddick et al., 1990). Against acetylcholin esterase, both members of a glycoalkaloid 'pair' appear equally inhibitory and do not synergise (Roddick, 1989).

The most widely studied chacotriose-based glycoalkaloid is chaconine (Fig. 1), the major glycoalkaloid of the potato. In contrast, little or nothing is known of the potency of chacotriosides based on other aglycones. Given the evident key role of the aglycone structure, it seemed of interest to explore in more detail the extent to which modifications in rings E and F influence the disruptive/inhibitory properties of the glycosides. To this end, a set of five model chacotriosides was selected: in addition to the commercially-available chaconine, four related compounds (vide infra) were isolated from different *Solanum* plants and/or were synthesised, all with the same A/B rings functionality, including the C5–C6 double bond (Fig. 1). The relative activity of these compounds was then determined in standard membrane lysis and enzyme inhibition tests, to assess the consequences of each of the structural modifications, and hence the importance of: (a) the type of indolizidine (E/F rings junction) moiety (spirosolane vs solanidane series); (b) the *R* and/or *S* configuration at C22 and C25 (solamargine vs β-solamarine); (c) the opening of the ether bridge in ring E (dihydrosolasodine A chacotrioside [DSAC] vs solamargine); and (d) the availability of the unshared electron pair on the ring F nitrogen atom (solamargine vs *N*-nitrososolamargine) to form bioactive iminium ion intermediates, in the manner envisaged for other biological systems (Kim et al., 1996). The solatriosides solasonine, dihydrosolasodine A solatrioside (DSAS) and *N*-nitrososolasonine (Fig. 1) were also prepared and tested for comparison purposes.

Aglycone (R^1 = H)	*Glycoalkaloid* (R^1 = *Chacotriose*)	(R^1 = *Solatriose*)
Solanidine (22*R*, 25*S*)	Chaconine (C)	Solanine (S)
Tomatidenol (22*S*, 25*S*)	β-Solamarine (βSr)	α-Solamarine (αSr)
Solasodine (22*R*, 25*R*)	Solamargine (Sm)	Solasonine (Ss)
Dihydrosolasodine A (22*S*, 25*R*)	Dihydrosolasodine A chacotrioside (DSAC)	Dihydrosolasodine A solatrioside (DSAS)
N-Nitrososolasodine (22*R*, 25*R*)	*N*-Nitrososolamargine (NO-Sm)	*N*-Nitrososolasonine (NO-Ss)

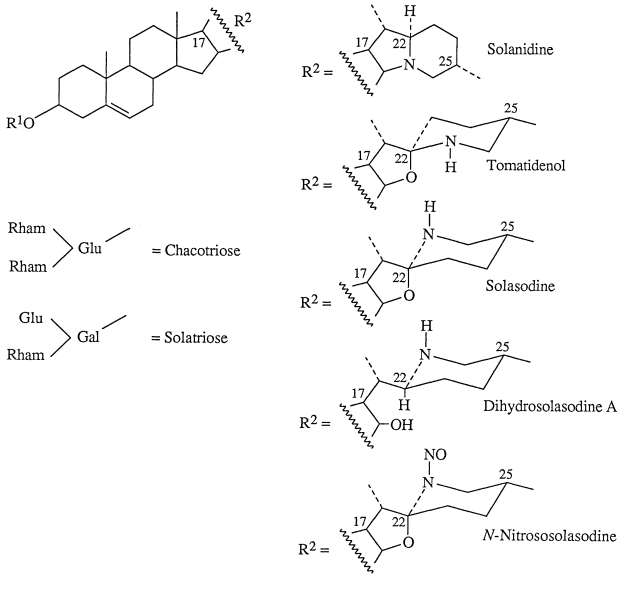


Fig. 1. Structures of glycoalkaloids and aglycones.

2. Results

DSAC failed to exhibit activity in any of the tests conducted (data not shown). These included disruption of PC/cholesterol liposomes at concentrations up to 500 μM and over the pH range 7–9, lysis of porcine erythrocytes, and inhibition of acetylcholinesterase in vitro. DSAC was incapable of binding to cholesterol, stigmasterol or ergosterol in vitro and did not synergise with DSAS or with the solatriose-based parent glycoalkaloid solasonine in haemolysis tests. In addition, solamargine did not synergise with DSAS in such tests.

N-Nitrososolamargine caused slight disruption of PC/cholesterol liposomes at 150 μM (but not at 75 μM) and pH 7 which increased with increasing concentration (Fig. 2). Increasing the pH to 8 slightly enhanced disruptive activity, but at pH 9 activity was the same as at pH 7 (Fig. 2). The combination of *N*-nitrososolamargine and solasonine did not cause any disruption of liposomes (although the combination of solamargine and solasonine produced a marked synergism). *N*-Nitrososolamargine did not inhibit acetylcholinesterase activity in vitro (Fig. 3).

The activity of β-solamarine was compared with that of solamargine and of chaconine. β-Solamarine and solamargine showed no anti-acetylcholinesterase activity

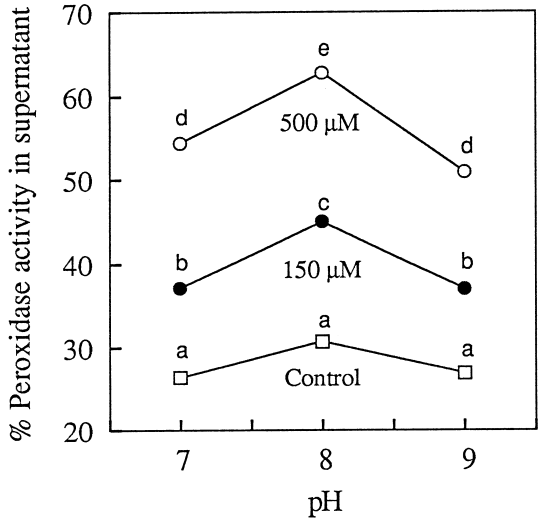


Fig. 2. Effect of *N*-nitrososolamargine concentration on peroxidase release from PC/cholesterol liposomes incubated at different pH for 1 h at room temp. Each point is the mean of five repicates. Treatments with a different/the same letter code are/are not significantly different from each other, respectively.

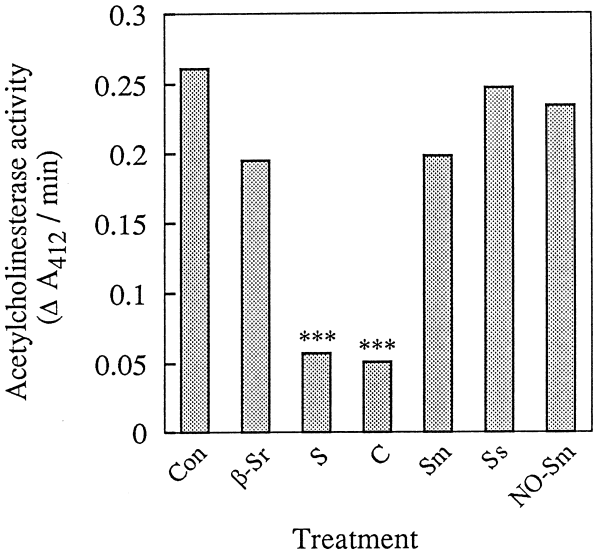


Fig. 3. Effects of different glycoalkaloids on bovine acetylcholinesterase activity in vitro. Con = control, β-Sr = β-solamarine, S = solanine, C = chaconine, Sm = solamargine, Ss = solasonine, No-Sm = *N*-nitrososolamargine. All treatments were 100 μM at pH 7. Each treatment consisted of five replicates. *** = significantly different from the control at $p = 0.001$.

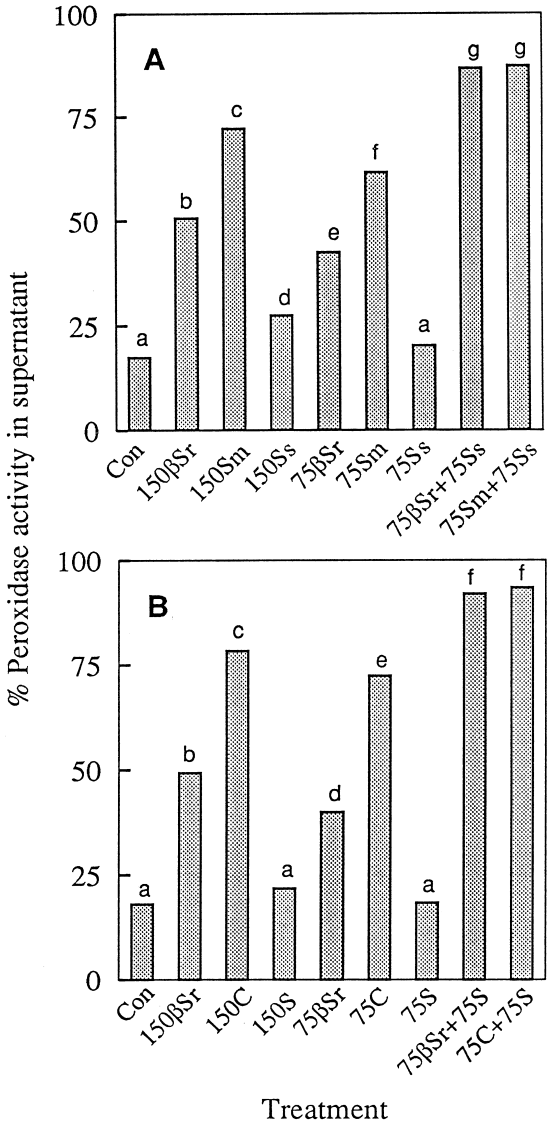


Fig. 4. Effects of individual glycoalkaloids and glycoalkaloid combinations on peroxidase release from PC/cholesterol liposomes incubated for 1 h at room temp. A = tomatidenol and solasodine glycosides; B = tomatidenol and solanidine glycosides. βSr = β-solamarine, Sm = solamargine, Ss = solasonine, C = chaconine, S = solanine. Values preceding glycoalkaloid abbreviations represent the concentration in μM. Each treatment consisted of five replicates. Within each group (A and B), treatments with a different/the same letter code are/are not significantly different from each other at $p = 0.05$.

in vitro at a concentration of up to 100 μM, but chaconine was highly inhibitory (Fig. 3). Of the solatriosides solasonine and solanine, the former was without effect and the latter highly inhibitory (Fig. 3). On the other hand, PC/cholesterol liposomes were disrupted by β-solamarine at 75 and 150 μM (Fig. 4A and B), although not to the extent caused by solamargine (Fig. 4A) and chaconine (Fig. 4B). The solatriosides, solasonine and solanine were inactive at 75 μM (cf. the control), but in combination with 75 μM β-solamarine (and discounting the effects of the control) both produced a synergistic effect, with disruption greater than 150 βM β-solamarine alone (Fig. 4A and B). Synergisms also occurred between 75 μM solasonine and solamargine and between 75 μM solanine and chaconine (Fig. 4A and B).

The dose–response haemolytic activities of β-solamarine, solamargine and chaconine are shown in Fig. 5. Although the same batch of human blood was used,

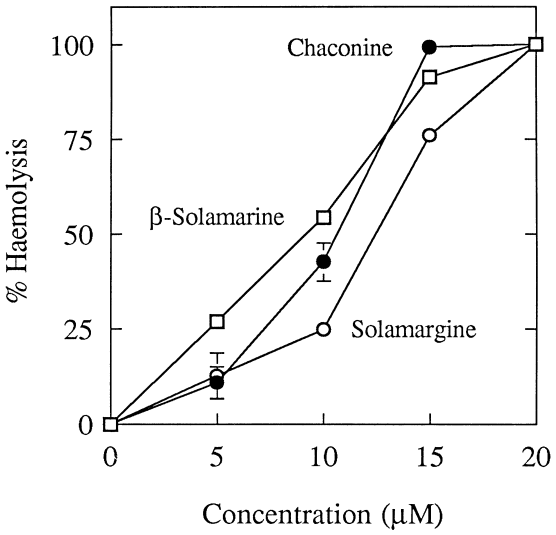


Fig. 5. Effects of β-solamarine, chaconine and solamargine on haemolysis of human erythrocytes in vitro. Erythrocytes were incubated with glycoalkaloids at 37°C for 2 h and haemolysis determined by haemocytometer counts. Each glycoalkaloid was tested at a different time. Each value is based on the mean cell count in five random fields of vision in each of three replicate aliquots. Vertical bars represent 2× the standard error and are shown only where they exceed the size of the symbol.

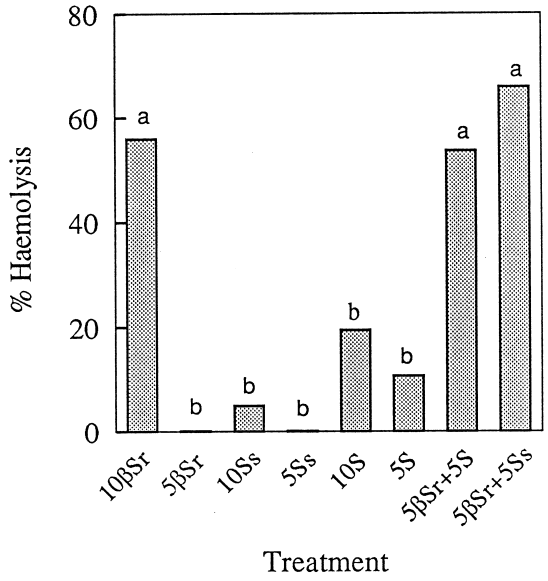


Fig. 6. Effects of individual glycoalkaloids and glycoalkaloid combinations on haemolysis of human erythrocytes in vitro. Erythrocytes were incubated with glycoalkaloids at 37°C for 2 h and haemolysis determined by haemocytometer counts. βSr = β-solamarine, S = solanine, Ss = solasonine. Values preceding glycoalkaloid abbreviations represent the concentration in μM. Each value is based on the mean cell count in five random fields of vision in each of three replicate aliquots. Treatments with a different/the same letter code are/are not significantly different from each other at $p = 0.05$.

availability of compounds necessitated each compound being tested at a different time (i.e. not within the same experiment). This would tend to hinder comparisons of relative activity. The data nevertheless suggest that β-solamarine, solamargine and chaconine show comparable haemolytic activity. β-Solamarine interacted synergistically with the solatriosides solasonine and solanine (Fig. 6). At the concentration tested (5 μM), all three compounds were inactive individually, but the combined treatments produced erythrocyte lysis comparable with 10 μM β-solamarine.

3. Discussion

Opening of ring E of the aglycone in the solasodine glycoalkaloids alters the rigid furanopiperidine system of rings E/F, apparently enhances the product basicity (Ripperger, 1992) and nullifies the inhibitory activity characteristically associated with these glycoalkaloids, such as in vitro sterol-binding, pH-dependent disruption of membranes and synergistic interactions with complementary triosides. The fact that DSAC caused no lysis at 500 μM, further suggests that the structural modification had eliminated the surfactant properties of this steroidal glycoside. Failure to inhibit acetylcholinseterase in vitro is not surprising since neither solamargine nor solasonine possess this property (Fig. 3; Roddick, 1989). The aglycone itself (dihydrosolasodine A, or solaverbascine) occurs in leaves of *Solanum verbascifolium* L. as glycosides of yet undisclosed structure, from which it has been isolated by hydrolysis (Adam et al., 1980). Incidentally, chacotriosides (of the related spirosolane aglycones, solaverols) have also been found in *S. verbascifolium* (Yamashita et al., 1990).

As with DSAC, the lack of anti-cholinesterase activity of *N*-nitrososolamargine is not unexpected. Severe curtailment of the liposome-lytic activities of *N*-nitrososolamargine (compared with solamargine; Roddick et al., 1990) is conceivably a consequence of nitrosation, since the unshared electron pair on the nitrogen atom of the spiro-aminoacetal moiety of the aglycone would be unavailable for formation of bioactive iminium ion species (Kim et al., 1996). An additional effect might arguably arise from elimination of the pH-related ionization changes associated with the ring nitrogen (and normally seen in chaconine and solamargine), even though the (marginal) pH effect on liposomes over the range 7–9 was similar to that for chaconine and solamargine (Roddick and Rijnenberg, 1986; Roddick et al., 1992). In fact, very little is understood about pH effects on glycoalkaloid activity, although pH-related efficacy apparently cannot be fully explained by protonation of the ring nitrogen (Arneson and Durbin, 1968). It is not known if the lack of a synergistic interaction with solasonine derives from the reduced (sub-threshold) activity of *N*-nitrososolamargine or from the structural modification altering molecular interactions with solatriosides.

The inability of β-solamarine to inhibit acetylcholinesterase in vitro, together with similar lack of effect of solasonine and solamargine but significant inhibition by solanine and chaconine, suggest that, in solatriosides and chacotriosides, the fused indolizidine ring (in the potato glycoalkaloids) is a prerequisite for such action whereas the spirosolane ring system does not lead to inhibition of this enzyme. On the other hand, inhibition by the spirosolane glycoalkaloid α-tomatine, although not as strong as solanine or chaconine (Roddick, 1989), points to contributions from other structural features, probably in an interactive capacity with the heterocyclic nitrogen. Tomatine differs from β-solamarine in lacking a C5–C6 double bond and possessing a lycotetraose rather than a chacotriose moiety, both of which features are reputedly important for biological activity (Schreiber, 1957). However, the findings that demissine (C5–C6 saturated solanidine + lycotetraose) and commersonine (solanidine + commertetraose) were much more inhibitory than tomatine (Bushway et al., 1987) indicate that the configuration of the nitrogen-containing E/F ring(s) is a more important determinant of anti-cholinesterase activity than the carbohydrate moiety. Available information suggests that the biological activity of glycoalkaloids also varies widely depending on the test/target system employed (e.g. membrane, enzyme, etc.). Thus claims that C5–C6 unsaturation is important in teratogenesis (Gaffield and Keeler, 1994) and the size of the carbohydrate moiety in membrane lysis (Keukens et al., 1992) and insect antifeedant activity (Sinden et al., 1980) may be of little relevance to cholinesterase inhibition.

The marked liposome- and erythrocyte-disruptive properties of β-solamarine are consistent with findings of earlier work with other chacotriose glycoalkaloids (Roddick and Rijnenberg, 1986; Roddick et al., 1988, 1990, 1992). The conclusion from the liposome study (Fig. 4A and B) that β-solamarine < solamargine ≈chaconine and from other studies that solamargine = chaconine (Roddick et al., 1990), indicate that liposome-lytic activity is equivalent in solanidane and 22*R*, 25*R* spirosolane glycoalkaloids but less in 22*S*, 25*S* spirosolanes. Whilst the difference between β-solamarine and solamargine can be specifically attributed to ring F structure, it is not yet clear if this is due to the configuration of the imino group, or of the C25 methyl, or both. On the other hand, the haemolysis data (Fig. 5) suggest that this rank order of activity may differ for erythrocyte membranes, which may be more affected by β-solamarine than solamargine. Strictly, the experimental protocol used does not allow such a conclusion regarding haemolytic potency to be drawn with conviction (see Results section) and additional work is required to check this.

The synergisms between β-solamarine and the solatriose-based solasonine and solanine in disruption of liposomes and erythrocytes (Figs. 4A, B and 6) are in keeping with previous findings (Roddick et al., 1990) and confirm that this interaction is reliant on a complementation between solatriose and chacotriose glycoalkaloids, irrespective of the aglycone. Insufficient β-solamarine was available to check its interaction with chacotriosides such as chaconine or solamargine, but on the basis of earlier observations (Roddick et al., 1990), an additive rather than a synergistic effect would be expected. In summary, the structural features implicated in activity in our assays — as far as the aglycone moiety is concerned — are an intact ring E, the E/F rings arrangement present in the spirosolane and solanidane derivatives, and a pair of unshared electrons on the nitrogen atom in ring F. However, a full appraisal of the factors influencing the membrane-disruption- and enzyme-inhibitory activity of *Solanum* glycoalkaloids has yet to be made.

α- and β-Solamarine occur naturally in a number of *Solanum* species, but are not as widespread as solasodine-based glycoalkaloids. In *S. dulcamara* L., *S. macrocarpum* L. and *S. schimperianum* Hochst., they are present alongside solasonine and solamargine, whilst in *S. curtilobum* Juz. et Buk., *S. brachycarpum* (Correll) Correll and *S. tuberosum* L. var. Kennebec they co-occur with solanine and chaconine (Schreiber, 1968; Ripperger and Schreiber, 1981; Ripperger, 1998). Members of all three pairs of glycoalkaloids were detected in *S. dulcamara* (De Vincenzi et al., 1996) and *S. dasyphyllum* Schum. and Thonn. (Adesina, 1985). Thus the possibility exists of synergisms within and between different glycoalkaloid types under natural conditons. Human and livestock poisonings and fatalities have been reported from ingestion of *S. dulcamara,* but the exact cause of death was not established (Cooper and Johnson, 1984).

More specifically, β-solamarine is able to mobilise glucocorticoids from the adrenals of rats (Daróczy and Hernádi, 1971) and inhibit sarcoma 180 development in mice (Kupchan et al., 1965), as do also solamargine and solasonine (Cham and Daunter, 1990a). The latter also displayed significant cytotoxic activity in vitro against a variety of cancer cell lines (Daunter and Cham, 1990; Lin et al., 1990; Nakamura et al., 1996). The view has been expressed that the binding of glycoalkaloids to tumour cells might be mediated through their rhamnosyl moiety (Cham & Daunter 1990b; Nakamura et al., 1996) which recognises endogenous lectins of cell receptor sites. Indeed, the β-chacotriosyl moiety (with two rhamnose units) was found to bind more effectively to cell receptor sites than the β-solatriosyl moiety (with one rhamnose unit) (Nakamura et al., 1996; Blankemeyer et al., 1998; Chataing et al., 1998). These results recall the anti-Walker 256 carcinoma activity of the rhamnose-containing mono-and di-glycosides of solasodine, solaplumbinin and solaplumbin (Singh et al., 1974) and raise the question of anticancer activity in the major potato glycoalkaloids (especially chaconine

which contains two rhamnose units), which, surprisingly, has not yet been addressed. Another potentially important issue which still remains to be resolved is whether glycoalkaloids synergise in terms of anticancer activity.

In other bioassays, no azasteroid-type activity could be demonstrated for solasodine, tomatidenol and their corresponding glycosides against larvae of the tobacco hornworm, *Manduca sexta*. However, the inhibitory effect of solamargine on larvae of the red flour beetle, *Tribolium castaneum,* was completely abolished by addition of cholesterol or sitosterol, while solasonine was not affected by either sterol (Weissenberg et al., 1998). This is in agreement with previous in vitro studies showing much reduced sterol-binding capacity by the latter glycoalkaloid compared with the former (Tschesche and Wulff, 1964; Roddick et al., 1992).

The observation that many *Solanum* species including the major crop plants potato and eggplant produce two glycoalkaloids, one of them more potent than the other in terms of membrane disruption, has led to speculation by Friedman and McDonald (1999) about their origins, as related to their possible contribution to disease resistance in plants. Two alternative hypotheses were advanced: (1) the plant produces initially only one glycoalkaloid but, as an adaptive response to acquired immunity of the pathogen over time, later elaborates a second one (by modification of the carbohydrate moiety) which is biologically more active; (2) both glycoalkaloids are formed at the same time in sub-optimal quantities, but disease resistance is maximized by the observed synergistic interaction between the two glycoalkaloids. Assessment of the role of glycoalkaloids in disease resistance may have implications in food toxicology and processing.

4. Experimental

4.1. Chemicals and materials

Unless otherwise stated, all fine biochemicals were purchased from Sigma Chemicals, Poole, UK. β-Solamarine, solasonine, solamargine and DSAC and DSAS were prepared from *Solanum dulcamara* leaves and/or *Solanum khasianum* berries (Weissenberg, 1982) and purified by CC on alumina, followed by repeated crystallization till homogenous on TLC. Their identity and purity were ascertained by FAB-MS, ^{1}H- and ^{13}C-NMR and by comparison with literature data (Schreiber, 1968; Ripperger and Schreiber, 1981). *N*-Nitrososolamargine and *N*-nitrososolasonine were prepared according to a reported procedure (Bite and Shabana, 1974). α-Solanine was a gift from Prof. Dr K. Schreiber, Institute of Plant Biochemistry, Halle, Germany and was re-crystallised from EtOH before use. Erythrocytes were prepared either from pig or human blood. Pig blood was collected fresh at a local abbatoir into heparinised containers. Certified human blood (type O, rhesus positive) was obtained from the Royal Devon and Exeter Hospital Transfusion Unit, Exeter, UK in sealed containers. The blood was just past its expiry date for transfusion, but the erythrocytes appeared normal when viewed under the microscope and responded similarly to glycoalkaloids to what had been found previously. All blood samples were stored at 4°C.

4.2. Preparation and treatment of erythrocytes

Methods for both pig and human blood were as previously described (Roddick et al., 1988). Blood was washed 2× with 9× its volume of Pi-buffered saline (0.02 M $Na_2HPO_4.2H_2O$, 0.005 M KH_2PO_4, 0.12 M NaC1) pH 7.4 (centrifuging at 400 ***g*** for 10 min between washes), after which erythrocytes were resuspended in 50× their original volume of Pi-buffered saline. Three millilitres of diluted erythrocyte suspension were mixed with 1 ml of glycoalkaloid solution prepared in Pi-buffered saline and the mixture incubated at 37°C for 2 h. Erythrocyte counts were made using a haemocytometer at ×400 magnification and % haemolysis determined by reference to erythrocyte counts in glycoalkaloid-free controls.

4.3. Preparation and treatment of liposomes

Details of methods have been reported previously (Roddick and Rijnenberg, 1986). Fifty milligrams of egg yolk phosphatidylcholine (PC, type VII-E), 10 mg cholesterol and 2 mg stearylamine were dissolved in 20 ml $CHCl_3$/MeOH (9/1) and the solution evaporated to dryness at room temperature. Wall-deposited lipids were brought into suspension in 1.5 ml of a solution of 10 mg/ml horseradish peroxidase (type II) and 30 mg/ml bovine serum albumin in 0.2 M Na_2HPO_4/NaH_2PO_4 buffer pH 7.2 by agitation with glass beads. The suspension was sonicated for six 20 s intervals at 26 kHz, diluted to 5 ml with 0.05 M Tris–HCl buffer, pH 7.2 and centrifuged at 50,000 ***g*** for 30 min at 4°C. The pellet was washed twice in Tris–HCl (centrifuging as before) and the final pellet suspended in 1 ml Tris–HCl. Glycoalkaloids were prepared as a 2 mM solution in 2 mM HCl. A 0.3 ml aliquot of this solution was mixed with 50 μl of liposome suspension and 3.65 ml Tris–HCl, pH 7.2 and incubated for 1 h at 25°C. Following centrifugation at 50,000 ***g*** for 30 min at 4°C, the supernatant was decanted and the pellet was resuspended in 4 ml Tris–HCl. The extent of liposome disruption was assessed by determining, spectrophotometrically, relative peroxidase activity in the supernatant and the pellet. The assay was based on oxidation of pyrogallol (2.5 ml of a 25 mM solution in 10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0)

by H_2O_2 (1%, v/v)-derived oxygen at room temperature. The rate of change of absorbance at 420 nm was used as an index of peroxidase activity. For assay of peroxidase in liposome pellets, distilled water in the reaction mixture (0.225 ml) was replaced by 0.025% (v/v) Triton X-100 to disrupt the liposome membrane.

4.4. Estimation of acetylcholinesterase inhibition

A spectrophotometric method (Roddick, 1989) was employed. The following were mixed in a quartz cuvette — 2.8 ml 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0; 200 μl glycoalkaloid in 2 mM HCl; 20 μl of 0.075 M acetylthiocholine iodide, 100 μl Pi buffer (pH 7) containing 0.01 M 5,5-dithiobis-2-nitrobenzoic acid and 1.5 mg/ml $NaHCO_3$ 50 μl (5 units/ml) aq. acetylcholinesterase (type XII;-S; from bovine erythrocytes). After 3 min at room temperature, absorbance was measured at 412 nm and enzyme activity expressed as ΔA_{412}/min.

4.5. Replication and statistical analyses

All treatments comprised between three and five replicates as indicated in legends and all experiments were repeated at least once. Statistical significance was determined using one-way or two-way analysis of variance and *t*-tests on untransformed means.

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Furanocoumarins from *Dorstenia gigas*

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Abstract

A series of linear and angular prenylated furanocoumarins and a benzofuran derivative were isolated from leaves and twigs of *Dorstenia gigas* (Moraceae), a plant occurring endemically on Socotra Island (Yemen). The structures were elucidated by spectroscopic methods (NMR, MS, UV) and chemical derivatization. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Dorstenia gigas*; Moraceae; Furanocoumarins; Benzofuran derivative

1. Introduction

The genus *Dorstenia* (Moraceae) consists of approximately 170 mostly tropical species (Barthlott, 1998). Very few of them are phytochemically investigated. All *Dorstenia* species are assumed to contain furanocoumarins with phototoxic effects (Frohne and Pfänder, 1997). A study of light-activated toxins of the Moraceae revealed the occurrence of phototoxins in all investigated *Dorstenia* species. The phototoxicity was most likely due to the presence of psoralen and bergapten (Swain and Downum, 1990). Furanocoumarins, mainly psoralen, bergapten, isobergapten, pimpinellin and isopimpinellin, were previously isolated from the Latin American species *Dorstenia contrajerva* (Terreaux et al., 1995; Tovar-Miranda et al., 1998), *D. brasiliensis* (Kuster et al., 1994), *D. asaroides* (Vilegas et al., 1997a), *D. bryoniifolia* (Pozetti, 1988; Vilegas et al., 1997b), *D. bahiensis* (Vilegas et al., 1997b), *D. cayapiaa, D. heringerii* (Vilegas et al., 1994), *D. excentria*, *D. drakena* and *D. lindeniana* (Rojas-Lima et al., 1999) as well as the African species *D. psilurus* (Nagijui et al., 1998) and *D. barnimiana* (Woldu et al., 1988). Furthermore, phytochemical studies of the genus afforded the occurrence of a cardenolide (Casagrande et al., 1974), triterpenes (Vilegas et al., 1997a,b), fatty acids (Pozetti et al., 1988), steroids (Kuster et al., 1994; Vilegas et al., 1997b), different benzofurans (Woldu et al., 1988; Ngadjui et al., 1999b) and several prenylated and geranylated flavonoids (Abegaz et al., 1998; Ngadjui et al., 1998, 1999a,b,c; Tsopmo et al., 1998).

Several *Dorstenia* species are used in folk medicine, mainly against skin diseases, due to the content of furanocoumarins which exhibit a broad range of biological activities. Photosensitizing effects have been reported for bacteria, fungi, mammalian cells, DNA viruses and bacteriophages (Murray et al., 1982).

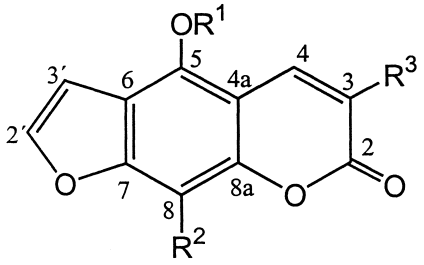
As part of our work on isolation and identification of constituents of Yemenian plants we investigated the leaves and twigs of *Dorstenia gigas*. *D. gigas* is a succulent shrub growing endemically on steep limestone and granit cliffs at Socotra island (Miller and Cope, 1996). In this paper we report the isolation and structural characterization of eleven new furanocoumarins and one benzofuran derivative, along with nine known furanocoumarins and several phytosterols.

2. Results and discussion

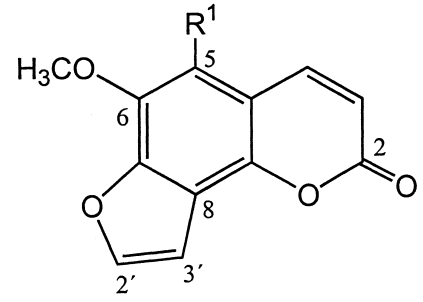
Chromatographic separation of the *n*-heptane extract afforded the known linear furanocoumarins dimethoxychalepensin (**1**), isoimperatorin (**2**), furopinnarin (**3**), swietenocoumarin B (**4**), 5-methoxy-8-geranyloxypsoralen (**5**), cnidilin (**6**) and additionally the new compounds **7**, **8**, **15**, **20** and **21** (Fig. 1). Their structures were

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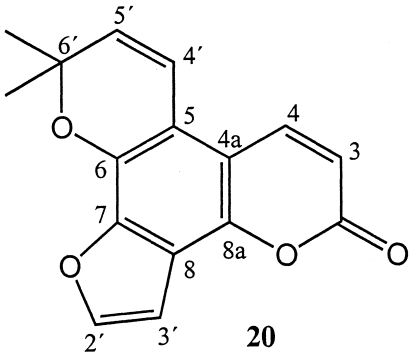
0031-9422/01/$ - see front matter © 2001 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(00)00419-2



Compound	R^1	R^2	R^3	R^4
1	CH_3	OCH_3	e	-
2	a	H	H	-
3	CH_3	e	H	-
4	CH_3	a	H	-
5	CH_3	g	H	-
6	a	OCH_3	H	-
7	CH_3	H	a	-
8	CH_3	OCH_3	a	-
9	c	H	H	H
10	CH_3	O-c	H	H
11	CH_3	c	H	H
12	CH_3	H	c	H
13	CH_3	OCH_3	c	H
14	CH_3	f	H	-



Compound	R^1	R^4
15	a	-
16	c	H
17	c	Glc
18	d	-
19	b	-



20

Residues

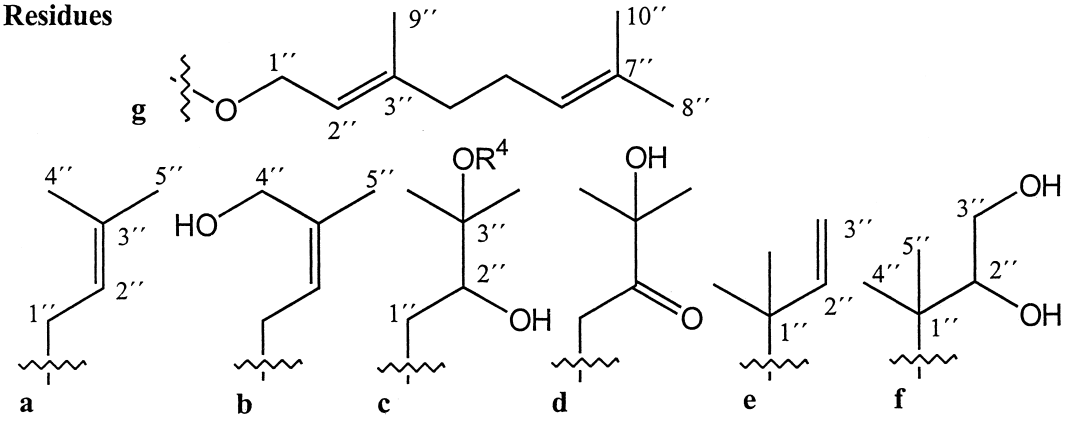


Fig. 1. Furanocoumarins of *Dorstenia gigas*.

elucidated on the basis of spectral data and some chemical transformations as well as by comparison with data reported in the literature.

The only hitherto known natural source of dimethoxychalepensin (**1**) were callus cultures of *Ruta graveolens* (Reinhard et al., 1971), its synthesis was reported by Hernández-Galán et al. (1993). However, the published spectral data of the synthesized compound (UV, ^{1}H NMR) differ from our results. We obtained compound **1**, one of the main furanocoumarins from *D. gigas*, as yellow needles, mp 132–134°C from *n*-hexane/EtOAc. NMR (Tables 1 and 2), MS, UV and IR spectra are in complete accordance with the structure of dimethoxychalepensin (**1**).

Compound **2** exhibiting spectral data of a linear prenyl ether substituted furanocoumarin was identified as isoimperatorin, previously detected in several Apiaceae species (Lee and Soine, 1969a).

The UV absorption of compounds **3**, **4**, **5** and **6** indicated linear furanocoumarins, the ^{1}H NMR spectra documented, in addition to different prenyl residues, the presence of one methoxy group in each case. For

Table 1
^{1}H NMR data [δ, multiplicity (J)] of furanocoumarins measured in $CDCl_3$[a]

	Linear furanocoumarins							Angular furanocoumarins					
H	**1**	**7**	**8**	**11**	**12**	**13**	**14**	**15**	**16**	**17**[a]	**18**	**19**	**20**
3	–	–	–	6.25 *d* (9.6)	–	–	6.28 *d* (9.9)	6.40 *d* (9.9)	6.36 *d* (9.9)	6.37 *d* (9.9)	6.42 *d* (9.9)	6.43 *d* (9.9)	6.43 *d* (9.8)
4	7.99 *s*	7.88 *td* (1.4/0.6)	7.84 *t* (1.4)	8.26 *d* (9.6)	8.09 *br s*	8.05 *br s*	8.18 *d* (9.9)	7.93 *d* (9.9)	8.10 *d* (9.9)	8.35 *d* (9.9)	7.71 *d* (9.9)	8.02 *d* (9.9)	7.98 *d* (9.8)
8	–	7.14 br s	–		7.14 br s	–	–	–	–	–	–	–	–
2′	7.61 *d* (2.5)	7.58 *d* (2.5)	7.61 *d* (2.5)	7.79 *d* (9.6)	7.59 *d* (2.5)	7.62 *d* (2.2)	7.61 *d* (2.5)	7.66 *d* (2.2)	7.65 *d* (2.2)	7.87 *d* (2.2)	7.69 *d* (2.2)	7.66 *d* (2.2)	7.67 *d* (2.2)
3′	6.99 *d* (2.5)	7.00 *dd* (2.5/1.0)	6.98 *d* (2.5)	7.22 *d* (2.5)	7.02 *dd* (2.5/1.0)	6.99 *d* (2.2)	6.99 *d* (2.5)	7.12 *d* (2.2)	7.10 *d* (2.2)	7.11*d* (2.2)	7.16 *d* (2.2)	7.12 *d* (2.2)	7.10 *d* (2.2)
OMe-5	4.18 *s*	4.26 *s*	4.15 *s*	4.25 *s*	4.27 *s*	4.17 *s*	4.26 *s*						
-6 or-8	4.16 *s*		4.16 *s*			4.16 *s*		4.10 *s*	4.19 *s*	4.16 *s*	4.14 *s*	4.16 *s*	
1″ A	5.100 *dd*[b] (17.7/1.1)	3.26 *br d* 2 H (7.2)	3.27 *br d* 2 H (7.2)	3.21 *dd* (14.0/3.3)	2.88 *dd* (14.3/2.1)	2.89 *dd* (14.3/1.9)	–	3.67 *br d* 2 H (6.6)	3.22 *dd* (13.9/2.2)	3.27 *dd* (13.8/2.1)	4.26 *s* 2 H	3.75 *br d* 2 H (7.3)	
1″ B	5.104 *dd*[b] (−10.4/1.1)			3.13 *dd* (14.0/9.9)	2.58 *dd* (14.3/10.0)	2.58 *dd* (14.3/10.2)			2.99 *dd* (13.9/10.3)	3.04 *dd* (13.9/10.3)			
2″	6.220 *dd*[b] (17.7/-10.4)	5.34 *br t* (7.2)	5.35 *br t* (7.2)	3.80 *dd* (9.9/3.3)	3.71 *dd* (10.0/2.1)	3.72 *dd* (10.2/1.9)	4.62 *dd* (8.2/3.6)	5.07 *br t* (6.6)	3.57 *ddd* (10.3/4.2/2.2)	3.62 *dd* (10.3/2.1)	–	5.25 *br t* (7.3)	
4″	1.51 *s*	1.72 *br s*	1.74 *br s*	1.30 *s*	1.33 *s*[d]	1.33 *s*[d]	1.76 *s*[d]	1.84 *br s*	1.38 *s*[d]	1.41 *s*	1.57 *s*	4.34 *s* 2 H	
5″	1.51 *s*	1.81 *br s*	1.81 *br s*	1.30 *s*	1.29 *s*[d]	1.29 *s*[d]	1.74 *s*[d]	1.70 *br s*	1.36 *s*[d]	1.41 *s*	1.57 *s*	1.79 *d* (1.4)	

Further signals: **14**: 3.65-3.57 *m* 2 H (H-3″)
16: 2.51 *d* (4.2, OH-2″), 2.18 *br s* (OH-3″)
17: glucosyl residue: 4.58 *d* (7.8, H-1‴), 3.23 *dd* (9.1/7.8, H-2‴), 3.38 *t* (8.9, H-3‴), 3.30[c] (H-4‴), 3.28[c] (H-5‴), 3.85 *dd* (12.0/2.1, H-6‴A), 3.66 *dd* (12.0/5.4, H-6‴B)
20: 6.74 *d* (10.1, H-4′), 5.81 *d* (10.1, H-5′), 1.56 *s* 6 H (Me)

[a] Measured in CD_3OD.
[b] δ and J values determined by ABX spin system simulation/iteration.
[c] Chemical shift of HSQC correlation peak.
[d] Signals may be exchanged.

Table 2
^{13}C NMR data (δ) of furanocoumarins measured in $CDCl_3$

	Linear furanocoumarins									Angular furanocoumarins					
C	**1**	**5**[a]	**6**[a]	**7**[a]	**8**	**11**[a,b]	**12**	**13**[a]	**14**	**15**[a]	**16**	**17**[b]	**18**	**19**	**20**
2	159.2	160.5	160.5	162.2	161.3	163.6	163.4	162.6	160.6	160.8	160.8	163.0	160.4	160.6	160.9
3	131.5	112.8	112.8	124.9	125.2	112.4	122.6	123.0	112.2	114.0	113.9	113.8	114.6	114.3	114.3
4	133.2	139.4	139.4	133.7	133.7	141.7	137.1	137.0	139.7	142.0	142.2	145.6	141.1	141.6	139.9
4a	107.9	107.5	107.5	107.1	108.4	106.8	106.8	108.1	107.7	112.9	113.6	115.4	113.4	112.6	109.7
5	143.9	144.5[c]	144.4	148.8	143.4	149.4	149.0	143.6	148.3	125.9	123.2	126.4	117.4	124.5	112.7
6	114.5	114.5	114.5	112.7	114.9	114.0	112.7	114.9	114.6	140.0	140.4	142.0	140.2	139.6	135.7
7	149.2	150.9	150.8	157.4	148.8	159.3	157.8	149.2	157.2	149.3	148.5	150.3	148.1	148.9	146.1
8	127.6	126.8	126.9	93.5	128.0	107.8	93.5	127.9	112.8	117.0	117.6	118.3	118.4	117.3	118.2
8a	143.0	144.4[c]	144.4	151.6	142.6	151.5	151.8	142.8	150.6	144.8	144.5	145.4	144.3	144.8	142.8
2′	144.9	145.1	145.1	144.5	144.8	146.5	144.7	144.9	144.2	145.1	145.2	147.1	145.4	145.2	146.2
3′	105.0	105.1	105.0	104.9	104.9	106.4	105.0	105.0	104.2	104.5	104.5	104.9	104.6	104.5	104.8
OMe-5	60.7	60.8		60.1	60.9	61.0	60.0	60.8	60.8						
OMe-6/8	61.6		60.8		61.7			61.7		61.1	60.9	61.3	60.7	61.2	
1″	40.6	70.2	70.4	29.1	29.2	26.7	34.2	34.2	42.7	24.3	27.7	28.4	32.8	24.0	117.1 (4′)
2″	145.5	119.5	119.8	119.6	119.5	78.5	77.3	77.2	76.8	122.6	79.3	79.3	211.3	125.3	131.3 (5′)
3″	112.1	143.1	139.7	135.0	135.0	74.0	72.9	72.9	63.9	132.7	73.1	81.5	76.4	135.7	77.1 (6′)
4″	26.2	39.6	18.0	17.9	18.0	25.9[c]	26.2[c]	26.3[c]	26.0[c]	18.0	26.0[c]	23.5[c]	26.9	61.7	
5″	26.2	26.4	25.8	25.8	25.9	25.1[c]	23.9[c]	24.0[c]	24.1[c]	25.6	24.0[c]	22.6[c]	26.9	21.7	

Further signals: **5**: 123.8 (6″), 131.7 (7″), 25.6 (8″), 16.5 (9″), 17.6 (10″)
17: glucosyl residue: 98.4 (1‴), 75.4 (2‴), 78.2 (3‴), 71.7 (4‴), 77.8 (5‴), 62.8 (6‴)
20: 27.4 (Me)

[a] Assignments are based on comparison with other furanocoumarins and with literature data.
[b] Measured in CD_3OD.
[c] Signals may be exchanged.

identification of the substances the position of the methoxy function in every compound was determined from the observed NOE effects.

Compound **3**, obtained as light pink plates from *n*-hexane/EtOAc, was characterized as furopinnarin, a substance first isolated from roots of *Ruta pinnata* (Reyes and Gonzáles, 1970).

According to ^1H NMR and UV data compound **4** was identified as swietenocoumarin B. Until now this compound was found only in the bark of the Indian Rutaceae *Chloroxylon swietenia* (Bhide et al., 1977). Its MS fragmentation pattern was in accordance with the proposed structure, too.

Compounds **5** and **6** gave no molecular ion in their EIMS. The base peak arose at *m/z* 232 indicating a linear furanocoumarin with one methoxy substituent and a second substituent connected with a C–O juncture. However, their ESI–MS afforded the $[M+H]^+$ ions at *m/z* 369 and 301, respectively. On consideration of ^1H NMR, UV and IR spectra compound **5** was identified as 5-methoxy-8-geranyloxypsoralen, described previously as a component of *Komarovia anisospermum* (Sokolova et al., 1976). Compound **6** is identical to cnidilin. The structures of compounds **5** and **6** were further supported by ^{13}C NMR data (Table 2).

Compound **7** ($[M]^+$ *m/z* 284.1049, $C_{17}H_{16}O_4$) showed UV absorption maxima (λ_{max} nm 220, 241 sh, 249, 261, 269, 316) characteristic for linear furanocoumarins monoxygenated at C-5 (Lee and Soine, 1969a,b). Its ^1H NMR spectrum (Table 1) displayed the typical coupled furan protons, a signal for H-4, an aromatic methoxy group and a C-prenyl group. The appearance of H-3′ as double doublet ($J=2.5$, 1.0 Hz) indicated the presence of hydrogen at C-8 (Steck and Mazurek, 1972). The signal of H-4 appeared as double triplet according to its long-range couplings with H-8 ($J=0.6$ Hz) and H_2-1″ ($J=1.4$ Hz). According to the spectral data **7** is assigned the structure of 5-methoxy-3-(3-methyl-but-2-enyl)-psoralen.

The ^1H NMR data and the MS fragmentation pattern of **8** ($[M]^+$ *m/z* 314.1145, $C_{18}H_{18}O_5$) were similar to **7** but revealed the occurrence of one additional methoxy group. In comparison with the ^1H NMR spectrum of **7** the H-8 signal and the long-range coupling of H-3′ disappeared indicating a substitution at C-8. The UV absorption spectrum (λ_{max} nm 222, 242, 249, 271, 313) was typical for 5,8-dioxygenated psoralens. In the NOE difference spectrum 5-OMe showed a strong NOE with H-3′ and a weaker one with H-4, whereas irradiation of 8-OMe did not give any NOE. The OMe-protons at C-5 and C-8 exhibited HMBC correlations to C-5 and C-8, respectively. Long-range correlations between H_2-1″ and C-2, C-3, C-4 and NOE correlations of H_2-1″ with H-4 prove the position of the prenyl substituent at C-3. Thus, compound **8** was characterized as 5,8-dimethoxy-3-(3-methyl-but-2-enyl)-psoralen.

Compound **15** showed a molecular ion peak at *m/z* 284 like **7** and its elemental composition obtained by HR-EIMS agrees with the molecular formula $C_{17}H_{16}O_4$. Its ^{1}H NMR spectrum (Table 1) exhibited in addition to furanocoumarin signals an aromatic methoxy group and a C-prenyl group. The UV spectrum of compound **15** (λ_{max} nm 221, 252, 304) was distinctly different to those of **7** and **8** and indicated an angular structure which is supported by the downfield shift of the H-2′ ^{1}H NMR signal compared to **7** and **8**. Due to the observed NOESY correlations, for example between H-4 and H_2-1″ and H-2″, the structure was established as 6-methoxy-5-(3-methyl-but-2-enyl)-angelicin.

Compound **20** gave the molecular ion $[M]^+$ at *m/z* 268 and its molecular formula was determined as $C_{16}H_{12}O_4$. Its ^{1}H NMR spectrum displayed besides furanocoumarin signals the geminal methyl groups (δ 1.56) and two doublets (δ 6.74 and 5.81, $J = 10.1$ Hz) belonging to vicinal pyran ring protons. NOE irradiation of H-4 caused an enhancement of H-4′ suggesting the pyran ring annelation at position 5 and 6. The assumed structure was proved by HSQC and HMBC experiments. Long-range correlations of H-4′ with C-5, C-6, C-7, C-4a, C-5′ and C-6′, and between H-5′ and C-5, C-6′ and the methyl groups confirmed the constitution. Compound **20** represent a new structural type and was named dorstegin.

The EIMS of **21** showed a molecular ion at *m/z* 254 corresponding to an elemental composition of $C_{16}H_{14}O_3$. In its ^{1}H NMR spectrum signals arose for eight aromatic protons, four of them belonging to a *para*-substituted benzol (δ 7.16 and 6.78, $J = 8.5$ Hz) and another three to an aromatic three spin system: a doublet at δ 7.32 ($J = 8.5$ Hz), an *ortho* and *meta* coupled double doublet at δ 6.81 ($J = 8.5/2.2$ Hz) and a *meta* coupled signal at δ 6.96 ($J = 2.2$ Hz). Additional resonances were assigned to an aromatic methoxy group and an isolated CH_2-group. Compound **21** showed hydroxyl absorption in the IR. The presence of one hydroxy group was confirmed by the formation of a trimethylsilyl ether exhibiting the molecular ion at *m/z* 326 obtained by GC–MS. The MS fragmentation pattern (Fig. 2A) suggested two linked aromatic parts: a methoxybenzofuran moiety (*m/z* 161) and a hydroxy benzyl residue (*m/z* 107, fragment a). After silylation the fragment *m/z* 107 is shifted to *m/z* 179 (GC–MS) proving the localisation of the hydroxy function. On the basis of NOE difference (Fig. 2B), HMBC and COSY long-range experiments the structure of **21** was determined as 2-(*p*-hydroxy-benzyl)-6-methoxy-benzofuran. Especially important were the observed HMBC correlations from H_2-1′ to C-2, C-3, C-2′, C-3′/7′ and between the OMe-protons and C-6 as well as COSY correlations from H_2-1′ to H-3, H-3′ and H-7′ and between H-3 and H-7. Compound **21** showed antifungal activity in the *Cladosporium* bioassay.

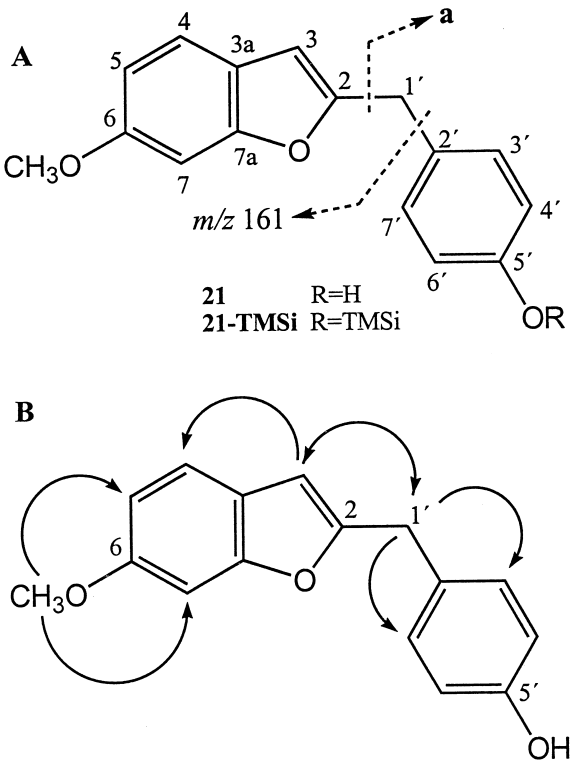


Fig. 2. (A) MS fragmentation and (B) NOE correlations of **21**.

Furthermore, the phytosterols sitosterol, stigmasterol, campesterol and stigmastanol were isolated from the *n*-heptane extract and identified as their acetates by GC–MS.

Ten more polar furanocoumarins were identified in the ethylacetate extract: the known compounds oxypeucedaninhydrate (**9**), byakangelicin (**10**), swietenocoumarin F (**11**) and seven new substances (**12**, **13**, **14**, **16**, **17**, **18**, **19**), including three structural isomers of swietenocoumarin F.

UV absorption and chemical shift of the furanocoumarin protons of **9** appeared nearly identical to those of **2** suggesting a close structural relationship between both compounds. **9** was optically active ($[\alpha]_D$ +13.9°). According to the obtained spectral data **9** was identified as oxypeucedaninhydrate known from several species belonging to Apiaceae and Rutaceae.

^{1}H NMR and MS spectra of compound **10** revealed the occurrence of one additional methoxy group compared to **9** and was characterized as (+)-byakangelicin known from Apiceae and Rutaceae species.

Swietenocoumarin F (**11**) was previously isolated as a racemate from the bark of *Chloroxylon swietenia* belonging to the Rutaceae (Bhide et al., 1977). However, compound **11** isolated from *D. gigas* exhibited optical activity ($[\alpha]_D$ −17°). All recorded spectral data were consistent with the assumed structure.

UV absorption and ^{1}H NMR signals (Table 1) belonging to the furanocoumarin rings of compound **12**

were very similar to those from **7** indicating a linear furanocoumarin with O-substitution (OMe) at C-5 and a C-substituent at C-3. Compound **12** gave the molecular ion $[M]^+$ at *m/z* 318 and its molecular formula was determined as $C_{17}H_{18}O_6$ by HR-EIMS. The ^1H resonances assigned to the side chain and the observed key ions in the EIMS are in accordance with a 3-methyl-2,3-dihydroxybutyl residue. HMBC correlations between H-1″A/B and C-2, C-3 and C-4 as well as the NOE correlation of H-4 and H-1″A/B confirm the position of the side chain at C-3. The methoxy protons showed a strong NOE with H-3′ and a weak one with H-4 and HMBC correlations to C-5. On the basis of these data compound **12** was characterized as 5-methoxy-3-(3-methyl-2,3-dihydroxybutyl)-psoralen.

Compound **13** ($[M]^+$ at *m/z* 348, $C_{18}H_{20}O_7$) differs from **12** just by the presence of one additional methoxy group. The absence of a signal for H-8 and the downfield shift of C-8 gave evidence that this methoxy moiety is attached at C-8. This is further supported by the UV spectrum of **13** which is typical for linear 5,8-dioxygenated furanocoumarins. These data are consistent with the structure 5,8-dimethoxy-3-(3-methyl-2,3-dihydroxybutyl)-psoralen. Examination of the most polar ethyl acetate fraction by LC–MS revealed a not further characterized glycoside of **13** as a minor component ($[M+H]^+$ at *m/z* 511 and a key ion at 349 in the ESI–MS). A psoralen derivative with a 3-methyl-2,3-dihydroxybutyl residue at C-3 (elemiferone) was so far only known from *Amyris elemifera* (Rutaceae), however, this compound lacks methoxy substituents (Philip and Burke, 1985).

The molecular formula of compound **14** was deduced as $C_{17}H_{18}O_6$ ($[M]^+$, *m/z* 318) from high resolution EIMS. **14** exhibited an UV absorption characteristic of C-5 monoxygenated linear furanocoumarins indicating a methoxy substituent at this position. This was supported by a strong NOE between the OMe-protons and H-3′ and a weak one with H-4. The HMBC correlations of H_3-4″ and H_3-5″ with C-8, C-1″ and C-2″ clearly indicated an oxidized 1,1-dimethylallyl group attached at C-8. Furopinnarin (**3**), the corresponding non-oxidized form, was isolated from the *n*-heptane extract. Both compounds displayed nearly identical UV spectral data. The presence of two vicinal hydroxyls in the side chain of **14** was confirmed by the formation of a methylboronate. The resulting molecular ion at *m/z* 342 was detected by GC–MS. The MS fragmentation pattern of **14** gave further evidence for the assumed constitution of the side chain. While the fragment observed at *m/z* 287 ($C_{16}H_{15}O_5$) documented the elimination of the terminal CH_2OH unit, the base peak at *m/z* 257 ($[M-61]^+$, $C_{15}H_{13}O_4$) was formed by loss of $C_2H_5O_2$. Anisocoumarin D, a dihydrofuranocoumarin with an oxidized 1,1-dimethylallyl group attached at C-3, isolated from *Clausena anisata* exhibited a similar fragmentation with the base peak at $[M-61]^+$ (Ngadjui et al., 1989).

Compounds **16**, **17**, **18** and **19** showed the typical UV spectrum of angular furanocoumarins. Compound **16** ($[M]^+$ at *m/z* 318, $C_{17}H_{18}O_6$), the main component of the ethyl acetate extract, represented a structural isomer of swietenocoumarin F (**11**). ^1H NMR and EIMS fragmentation of **11** and **16** revealed similar results indicating the presence of a 3-methyl-2,3-dihydroxybutyl residue. The vicinal hydroxy groups were confirmed by formation of the corresponding methylboronate which gave the molecular ion at *m/z* 342. Long-range correlations of H-1″A/B with C-4a, C-5, C-6 and C-2″ as well as NOE correlations between H-4 and H-1″A/B indicated the attachment of this side chain at C-5. The OMe-protons exhibited HMBC correlations to C-6 and NOE correlations with H-1″A/B, H-2″ and H-2′. According to the observed spectral data the structure of **16** was established as 6-methoxy-5-(3-methyl-2,3-dihydroxybutyl)-angelicin.

NMR and MS data indicated that compound **17** ($[M]^+$ at *m/z* 480, $C_{23}H_{28}O_{11}$) is a monoglycoside of **16**. Hydrolysis of **17** by 3 N HCl revealed the aglycon 16 and glucose. Both compounds were identified by co-chromatography with reference substances. The NMR signals of **17** were typical for a β-glucoside ($J_{1''',2'''} = 7.8$ Hz). The position of the glucosyl moiety was deduced from HMBC and NOESY experiments. HMBC correlation of H-1‴ and C-3″ as well as the NOESY correlation between H-1‴ and one methyl group prove the attachment of glucose at the tertiary 3″-hydroxyl group. **17** was identified as 6-methoxy-5-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methyl-butyl]-angelicin.

The molecular ion of **18** at *m/z* 316 ($C_{18}H_{16}O_6$) was two mass units lower than that of **16** suggesting a dehydrated compound. The HMBC correlations of H_2-1″ ($\delta = 4.26$ s, 2H) and H_3-4″/5″ ($\delta = 1.57$ s, 6H) with a carbon at δ 211.3 indicate a keto function at C-2″. Evidently, oxidation at C-2″ lead to the formation of 6-methoxy-5-(3-hydroxy-2-oxo-3-methyl-butyl)-angelicin. The keto function at C-2″ could be confirmed by the reaction with methoxyamine. The resulting methoxime derivative (*m/z* 345) was detected by GC–MS.

High resolution mass spectrometry of compound **19** established the molecular formula as $C_{17}H_{16}O_5$ ($[M]^+$, *m/z* 300). The ^1H and ^{13}C NMR spectra of **19** showed a close resemblance to those of **15**. However, whereas in **15** signals of two methyl groups occurred (CH_3-4″, CH_3-5″), in **19** the signals of one methyl group were replaced by signals belonging to a CH_2OH-group (δ ^1H 4.34, δ ^{13}C 61.7). As proved by a NOE between H-2″ ($\delta = 5.25$) and the methyl group at δ 1.79, the methyl group is in *cis* position to H-2″ whereas the CH_2OH-group is in *trans* position. The structure is supported by the MS fragmentation pattern showing the degradation of the side chain: *m/z* 269 $[M-CH_2OH]^+$, *m/z* 229

$[M-C_4H_7O]^+$, *m/z* 216 $[M-C_5H_8O]^+$. After trimethylsilylation no molecular ion was observed, but a prominent fragment at *m/z* 282 resulting from the loss of TMSiOH. According to the recorded spectral data **19** was characterized as 6-methoxy-5-(4-hydroxy-3-methyl-but-2-enyl)-angelicin. Previously the isolation of a coumarin bearing the 4-hydroxy-3-methyl-but-2-enyl residue at position C-8 (arnottinin) was reported from the xylem of *Xanthoxylum arnottianum*. The ^{1}H NMR data reported of its side chain (Igutchi et al., 1975) are identical with our results for the residue of **19**.

The furanocoumarins found in *D. gigas* display a complex structural diversity not comparable to the compounds identified in previously investigated *Dorstenia* species. A special feature of the *D. gigas* furanocoumarins seems the incorporation of prenyl units either as 1,1-dimethylallyl or 3-methyl-but-2-enyl groups in oxidized or nonoxidized form. Prenylation at C-8 lead to the formation of the observed angular structures. An additional heterocyclic ring was formed in compound **20** by the interaction of a prenyl substituent at C-5 and oxygen at C-6. The variety of furanocoumarins detected in other species of the genus *Dorstenia* is restricted to linear and angular structures with methoxy substituents. An exception represents compound **22** found in *D. contrajerva* (Terreaux et al., 1995), *D. brasiliensis* (Kuster et al., 1994), *D. cayapiaa* (Llabres and Baiwir, 1992) and *D. bahiensis* (Vilegas et al., 1997a,b). However, the occurrence of prenylated flavonoids seems common for several species.

The furanocoumarin pattern of *D. gigas* exhibit similarities to that of some Rutaceae and Apiaceae species. Prenylated furanocoumarins are widely distributed in both families. However, oxidation of the side chain is more frequent in Rutaceae. Furthermore, 1,1-dimethylallyl substitution at C-3 as found in 1 is rare and has been detected only in Rutaceae (Murray et al., 1982).

3. Experimental

3.1. General methods

CC: silica gel (Merck, 40–63 μm) and Sephadex LH 20 (Fluka). Prep. TLC: precoated silica gel plates 60 F_{254}, 20×20 cm, thickness 0.5 or 1 mm (Merck). Mp: uncorr., crystallization from *n*-hexane/EtOAc. $[\alpha]_D$: Jasco TIP 1000 Polarimeter, solvent MeOH. IR: Bruker IFS 28. UV: Kontron Uvicon 940, solvent MeOH. NMR, 1D: Varian Unity 500 spectrometer, 499.83 MHz (^{1}H) and 125.7 MHz (^{13}C) or VARIAN GEMINI spectrometer 300, 300.24 MHz (^{1}H) and 75.5 MHz (^{13}C), solvent $CDCl_3$ (unless otherwise reported), 2D: (GHMBC, GHSQC, NOESY, ^{1}H–^{1}H-COSY) Varian Unity 500 spectrometer, 499.83 MHz, solvent $CDCl_3$. Chemical shifts were referenced to internal TMS ($\delta=0$, ^{1}H) and $CDCl_3$ ($\delta=77.0$, ^{13}C) or CD_3OD ($\delta=49.0$, ^{13}C), respectively. HR-EIMS (resolution 7000) and EIMS (DIS): 70 eV, AMD 402, AMD Intectra. GC–MS: Voyager (Thermo Quest CE Instruments), EI 70 eV; source temp. 200°C; column DB-5MS (15 m×0.25 mm, 0.25 μm film thickness), injection temp. 250°C, interface temp. 300°C, carrier gas He, flow rate 1.2 ml/min, splitless injection, column temperature program: initial temperature 60°C for 1 min, raised to 110°C at a rate of 25 grd/min, hold for 1 min, then raised to 300°C at a rate of 10 grd/min and hold on this temperature for 30 min; positive ion ESI–MS: TSQ 7000 (Finnigan, electrospray voltage 4.5 kV, heated capillary temperature 220°C; sheath gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system, equipped with a RP18-column (4 μm, 1×100 mm, Ultrasep), HPLC conditions: gradient system from H_2O–CH_3CN 4:1 (each of them contained 0.2% HOAc) to 1:9 within 15 min, then hold on this value for 10 min; flow rate 70 μl min^{-1}. All mass spectra are averaged and background substracted. Derivatization: Methylboronation was carried out as described by Takatsuto et al. (1982). Bioassay: Antifungal activity was tested according to Gottstein et al. (1984).

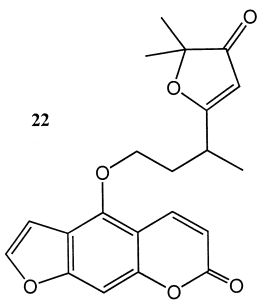
3.2. Plant material

Leaves and twigs of *D. gigas* Schweinf. ex Balf. were collected at Socotra Island, Yemen, in February 1999 and identified by Dr. A.G. Miller. A voucher specimen is deposited at the Herbarium of the Royal Botanic Garden in Edinburgh (UK).

3.3. Extraction and purification

The dried and powdered plant material (330 g) was extracted exhaustively with 90% EtOH. The combined extracts were reduced to the aq. phase and partitioned successively between *n*-heptane (33.4 g), EtOAc (34.0 g) and *n*-BuOH (12.0 g) and evaporated to dryness.

The residue of the *n*-heptane extract was chromatographed on a silicia gel column using a *n*-hexane–EtOAc gradient system to give several fractions which were combined according to TLC monitoring. Three coumarin containing fractions eluted with 15–25% EtOAc



were further processed. Compound **1** (331 mg) crystallized from the fraction eluted first. The remaining mother liquor was separated repeatedly on Sephadex LH-20 eluting with MeOH. The coumarin containing fraction gave sterols by crystallization in MeOH. Further repeated CC on silicia gel using *n*-hexane–EtOAc, *n*-hexane–$CHCl_3$ and *n*-hexane–Et_2O mixtures and following separation by prep. HPLC afforded compounds **2** (6 mg), **7** (4 mg), **8** (9 mg) and **15** (14 mg). The post coumarin fraction from Sephadex LH-20 gave by prep. TLC ($CHCl_3$–MeOH, 97:3) compound **21** (8 mg).

The fraction eluted from silicia gel with 20% EtOAc was successively rechromatographed on Sephadex in MeOH and silicia gel using *n*-hexane–EtOAc to afford compounds **3** (44 mg) and **5** (79 mg). Combination of prep. TLC and prep. HPLC gave compound **4** (4 mg).

Repeated column chromatography of the following fraction on Sephadex LH-20, silicia gel and purification by prep. HPLC yielded **20** (27 mg) and **6** (29 mg).

5.7 g of the EtOAc extract were subjected to CC on silicia gel eluting with solvents of increasing polarity (*n*-hexane–EtOAc, EtOAc–MeOH). The first fractions contained the furanocoumarins found in the *n*-heptane phase and were not further processed. The following fraction gave after work-up (CC on Sephadex LH-20 and silicia gel, prep HPLC) the compounds **18** (2 mg) and **19** (2.5 mg). Elution with 70% EtOAc afforded 774 mg of **16**. Separation of the next two fraction by repeated CC and prep. TLC on silicia gel and prep. HPLC resulted in the isolation of **9** (19 mg), **10** (8 mg), **12** (4 mg), **13** (8 mg), and **11** (32 mg) and **14** (5 mg), respectively. The more polar fraction yielded after work-up the furanocoumarin glycoside **17** (210 mg). Compound **17** was also the main component of the *n*-BuOH extract.

3.3.1. Dimethoxychalepensin (*1*)

Yellow needles, mp 132–134°C. EIMS *m/z* (rel. int.): 314.1148 $[M]^+$ (100, calc. for $C_{18}H_{18}O_5$ 314.1156), 299 (58), 284 (29), 269 (26), 259 (30), 241 (9), 229 (12), 225 (6), 213 (10), 128 (5), 69 (6), 57 (6). IR ν_{max}^{KBr} cm^{-1}: 3406, 3126, 3080, 2946, 1714 (C=O), 1600, 1483, 1343, 1296, 1273, 1171, 1195, 1148, 1121, 1075, 874, 784, 735; UV λ_{max} nm (log ε): 222 (4.44), 241 (4.15), 249 (4.15), 271 (4.32), 313 (4.15), UV λ_{min} nm (log ε): 238 (4.14), 245 (4.14), 254 (4.10), 288 (3.89). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

3.3.2. Isoimperatorin (*2*)

White needles, mp 90°C. ^1H NMR and UV specta are identical to those reported by Lee and Soine (1969a,b); EIMS is equivalent to data displayed by Beier et al. (1994).

3.3.3. Furopinnarin (*3*)

Light pink plates, mp 127–128°C. UV λ_{max} nm (log ϵ): 224 (4.45), 143 sh, 251 (4.26), 266 (4.28), 270 sh, 310 (4.15), UV λ_{min} nm (log ϵ): 237 (4.17), 257 (4.21), 282 (3.77). ^1H NMR, IR and EIMS agree with reference data (Reyes and Gonzáles, 1970).

3.3.4. Swietenocoumarin B (*4*)

Yellowish crystals, mp 144°C. ^1H NMR and UV spectra are equivalent to those reported by Bhide et al. (1977). EIMS *m/z* (rel. int.): 284 (78), 269 (100), 254 (8), 241 (30), 225 (11), 216 (15), 213 (7), 201 (6), 186 (9), 186 (9), 171 (5), 141 (5), 115 (10), 77 (6), 63 (5).

3.3.5. 5-Methoxy-8-geranyloxypsoralen (*5*)

Green oil. ^1H NMR, UV and IR are in accordance with data reported by Sokolova et al. (1976). ^{13}C NMR: Table 2.

3.3.6. Cnidilin (*6*)

Yellowish crystals. ^1H NMR, UV and EIMS spectra are in accordance with data described by Lee and Soine (1969a). ^{13}C NMR: Table 2.

3.3.7. 5-Methoxy-3-(3-methyl-but-2-enyl)-psoralen (*7*)

Yellowish solid. EIMS *m/z* (rel. int.): 284.1040 $[M]^+$ (46, calc. for $C_{17}H_{16}O_4$ 284.1049), 269 (23), 253 (14), 241 (18), 229 (100), 214 (12), 201 (4), 186 (6), 177 (4), 164 (3), 152 (2), 128 (3), 115 (3), 77 (2), 63 (1), 51 (3). UV λ_{max} nm (log ε): 220 (4.26), 241 sh, 249 (4.06), 261 (4.03), 316 (4.02), UV λ_{min} nm (log ε): 239 (4.04), 256 (4.01), 266 (4.01), 279 (3.64). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

3.3.8. 5,8-Dimethoxy-3-(3-methyl-but-2-enyl)-psoralen (*8*)

Yellowish crystals, mp 104–106°C. EIMS *m/z* (rel. int.): 314.1145 $[M]^+$ (100, calc. for $C_{18}H_{18}O_5$ 314.1156), 299 (29), 283 (23), 271 (17), 259 861), 256 (9), 244 (9), 229 (6), 201 (6), 173 (4), 143 (4), 119 (6), 69 (14); IR ν_{max}^{KBr} cm^{-1}: 3125, 2925, 2852, 1709 (C=O), 1628, 1603, 1484, 1365, 1348, 1165, 144, 1079, 1060, 875, 750. UV λ_{max} nm (log ε): 222 (4.47), 242 (4.21), 249 (4.20), 271 (4.32), 313 (4.16), UV λ_{min} nm (log ε): 238 (4.18), 245 (4.18), 255 (4.13), 288 (3.91). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

3.3.9. (+)-Oxypeucedaninhydrate (*9*)

Pale brown solid. $[\alpha]_D^{24}$ +13.9 (*c* 0.14). UV λ_{max} nm (log ε): 220 (4.31), 242 sh, 249 (4.16), 257 (4.11), 266 (4.09), 309 (4.02), UV λ_{min} nm (log ε): 235 (4.09), 255 (4.10), 264 (4.08), 309 (4.02). ^1H NMR and EIMS data are identical to those reported by Steck and Mazurek (1972) and Beier et al. (1994), respectively.

3.3.10. (+)-Byakangelicin (*10*)

Yellowish needles, mp 120°C. $[\alpha]_D^{24}$ +5.5° (*c* 0.20). ^1H NMR (Thastrup and Lemmich, 1983), UV absorption (Reisch et al., 1969) and MS fragmentation (Saiki et al., 1974) are identical to literature.

*3.3.11. (−)-Swietenocoumarin F (**11**)*

Yellowish crystals, mp 182°C (MeOH). $[\alpha]_D^{24}$ −17.0° (*c* 0.14). EIMS *m/z* (rel. int.): 318 (9), 260 (19), 229 (46), 217 (100), 201 (8), 186 (9), 171 (3), 159 (3), 145 (1), 131 (4), 115 (3), 102 (3), 77 (2), 59 (8). IR (KBr) ν_{max}^{KBr} cm^{-1}: 3413 (OH), 3122, 3073, 2977, 1696 (C=O), 1604, 1586, 1476, 1441, 1429, 1374, 1353, 1292, 1266, 1214, 1144, 1091, 1057, 989,950, 862, 827, 752. ^1H NMR: Table 1. ^{13}C NMR: Table 2. UV agree with data reported by Bhide et al. (1977).

*3.3.12. 5-Methoxy-3-(3-methyl-2,3-dihydroxybutyl)-psoralen (**12**)*

Brownish solid. $[\alpha]_D^{25}$ −21.2° (*c* 0.09). EIMS *m/z* (rel. int.): 318.1114 $[M]^+$ (4, calc. for $C_{17}H_{18}O_6$ 318.1104), 259 (100), 242 (7), 230 (51), 229 (33), 215 (13), 187 (8), 171 (2), 129 (1), 102 (2), 59 (9). ESI–MS *m/z* (rel int.): 319 $[M+H]^+$ (100), 301 (16), 229 (4). UV λ_{max} nm (log ε): 221 (4.40), 241 sh, 249 (4.20), 260 (4.18), 269 (4.18), 314 (4.16), UV λ_{min} nm (log ε): 239 (4.19), 255 (4.15), 266 (4.16), 279 (3.72). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

*3.3.13. 5,8-Dimethoxy-3-(3-methyl-2,3-dihydroxybutyl)-psoralen (**13**)*

Brownish solid. $[\alpha]_D^{21}$ −14.5° (*c* 0.08). EIMS *m/z* (rel. int.): 348.1194 $[M]^+$ (26, calc. for $C_{18}H_{20}O_7$ 348.1209), 289 (100), 272 (6), 259 (47), 245 (42), 229 (13), 217 (6), 201 (12), 187 (5), 173 (6), 89 (4), 59 (17). ESI–MS *m/z* (rel. int.): 349 $[M+H]^+$ (100), 331 (15), 259 (5). UV λ_{max} nm (log ε): 222 (4.32), 241 (4.07), 249 (4.07), 269 (4.15), 312 (4.03), UV λ_{min} nm (log ε): 238 (4.06), 245 (4.05), 254 (4.00), 287 (3.77). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

*3.3.14. 5-Methoxy-8-(1,1-dimethyl-2,3-dihydroxypropyl)-psoralen (**14**)*

Pale brown solid. $[\alpha]_D^{24}$ +14.5° (*c* 0.08). EIMS *m/z* (rel. int.): 318.1108 $[M]^+$ (1, calc. for $C_{17}H_{18}O_6$ 318.1103), 287 (6), 257 (100), 243 (10), 229 (25), 213 (6), 199 (7), 185 (5), 171 (8), 157 (3), 128 (8), 115 (6), 91 (4), 77 (4), 69 (4). ESI–MS *m/z* (rel. int.): 319 $[M+H]^+$ (100), 271 (15). UV λ_{max} nm (log ε): 222 (4.25), 145 sh, 251 (4.04), 265 (4.04), 271 sh, 310 (3.91), UV λ_{min} nm (log ε): 237 (3.99), 257 (4.00), 283 (3.61). ^1H NMR: Table 1. ^{13}C NMR: Table 2. *Methylboronate derivative*: GC–EIMS *m/z* (rel. int.): 342 (15), 257 (100), 229 (23).

*3.3.15. 6-Methoxy-5-(3-methyl-but-2-enyl)-angelicin (**15**)*

Yellowish crystals, mp 115°C. EIMS *m/z* (rel. int.): 284.1049 $[M]^+$ (100, calc. for $C_{17}H_{16}O_4$ 284.1048), 269 (22), 253 (20), 241 (11), 229 14), 216 (15), 202 (11), 187 (4), 171 (5), 115 (6), 83 (4), 69 (12). IR ν_{max}^{KBr} cm^{-1}: 3116, 2972, 2918, 2856, 1713 (C=O), 1653, 1627, 1570, 1457, 1445, 1380, 1344, 1314, 1138, 1120, 1054, 822, 753. UV λ_{max} nm (log ε): 221 (4.48), 252 (4.43), 304 (4.00), UV λ_{min} nm (log ε): 233 84.17), 277 (3.68). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

*3.3.16. 6-Methoxy-5-(3-methyl-2,3-dihydroxybutyl)-angelicin (**16**)*

White needles, mp 178–179°C. $[\alpha]_D^{26}$ +58.6° (*c* 0.11). EIMS *m/z* (rel. int.): 318.1074 $[M]^+$ (46, calc. for $C_{17}H_{18}O_6$ 318.1103), 260 (9), 229 (71), 217 (100), 199 (8), 186 (8), 149 (1), 59 (14). IR ν_{max}^{KBr} cm^{-1}: 3390 (OH), 3142, 3108, 3066, 2976, 1716 (C=O), 1624, 1572, 1472, 1447, 1426, 1392, 1374, 1345, 1317, 1280, 1263, 1141, 1118, 1059, 1009, 970, 958, 876, 843, 832, 785, 758. UV λ_{max} nm (log ε): 220 (4.46), 253 (4.46), 304 (4.03), UV λ_{min} nm (log ε): 233 (4.14), 276 (3.72). ^1H NMR: Table 1. ^{13}C NMR: Table 2. *Methylboronate derivative*: GC–EIMS *m/z* (rel. int.): 342 (67), 229 (100), 215 (51), 186 (37), 113 (92), 71 (74).

*3.3.17. 6-Methoxy-5-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methyl-butyl]-angelicin (**17**)*

Light pink solid. $[\alpha]_D^{26}$ +8.3° (c 0.135). EIMS *m/z* (rel. int.): 480 $[M]^+$ (4), 318 (12), 301 (12), 289 (7), 259 (26), 229 (100), 217 (54), 201, (14), 186 (19), 171 (11), 145 (11), 115 (11), 102 (11), 85 (11), 73 (23), 55 (25). ESI–MS *m/z* (rel. int.): 481 $[M+H]^+$(84), 319 $[M+H\text{-}162]^+$ (100). IR ν_{max}^{KBr} cm^{-1}: 3421 (OH), 2977, 2936, 1718, 1623, 1570, 1392, 1344, 1318, 1266, 1214, 1120, 1074, 880, 829, 755. UV λ_{max} nm (log ε): 220 (4.45), 252 (4.42), 303 (4.01), UV λ_{min} nm (log ε): 233 (4.12), 277 (3.72). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

*3.3.18. 6-Methoxy-5-(3-hydroxy-2-oxo-3-methyl-butyl)-angelicin (**18**)*

Pale brown solid. EIMS *m/z* (rel. int.): 316.0943 $[M]^+$ (23, calc. for $C_{17}H_{16}O_6$ 316.0947), 258 (8), 253 (5), 230 (100), 215 (68), 198 (7), 186 (8), 171 (3), 149 (5), 135 (2), 129 (3), 111 (5), 87 (9), 83 (10), 69 (15), 59 (50). UV λ_{max} nm (log ε): 218 (4.19), 252 (4.12), 301 (3.73), UV λ_{min} nm (log ε): 234 (3.98), 278 (3.58). ^1H NMR: Table 1. ^{13}C NMR: Table 2. *Methoxime derivative*: GC–EIMS *m/z* (rel. int.): 345 (24), 316 (13), 230 (65), 229 (100), 215 (39), 186 (37), 59 (72).

*3.3.19. 6-Methoxy-5-(4-hydroxy-3-methyl-but-2-enyl)-angelicin (**19**)*

Pale brown solid. EIMS *m/z* (rel. int.): 300.1015 $[M]^+$ (100, calc. for $C_{17}H_{16}O_5$ 300.0998), 282 (11), 269 (19), 260 (14), 251 (98), 239 (14), 229 (59), 223 (27), 216 (90), 201 (18), 195 (10), 186 (11), 171 (8), 145 (4), 127 (6), 115 (11), 102 (6), 95 (6), 73 (6), 59 (5), 55 (5), 51 (5). UV λ_{max} nm (log ε): 220 (4.41), 251 (4.31), 306 (3.93), UV λ_{min} nm (log ε): 235 (4.18), 283 (3.78). ^1H NMR: Table 1. ^{13}C NMR: Table 2. *TMSi derivative*. GC–EIMS *m/z* (rel. int.): 282 $[M-OTMSi]^+$ (57), 267 (22), 251 (100), 239 (19), 223 (73), 195 (66), 115 (30).

*3.3.20. Dorstegin (**20**)*

Yellow crystals, mp 145°C. EIMS *m/z* (rel. int.): *m/z* 268.0739 $[M]^+$ (21, calc. for $C_{16}H_{12}O_4$ 268.0736), 253 (100), 239 (1), 225 (11), 197 (1), 169 (1), 139 (1), 112 (6). IR ν_{max}^{KBr} cm^{-1}: 3148, 3125, 3049, 2975, 2962, 1716 (C=O), 1635, 1590, 1471, 1440, 1398, 1374, 1359, 1343, 1306, 1270, 1230, 1199, 1172, 1161, 1140, 1114, 1040, 1010, 880, 824, 764738, 693, 667, 659. UV λ_{max} nm (log ε): 205 (4.28), 240 (4.46), 273 sh, 282 (4.28), 301 (4.18), UV λ_{min} nm (log ε): 217 (4.17), 257 (3.89), 291 (4.14). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

*3.3.21. 2-(p-Hydroxy-benzyl)-6-methoxy-benzofuran (**21**)*

Red-brown solid. EIMS *m/z* (rel. int.): 254.0930 $[M]^+$ (100, calc. for $C_{16}H_{14}O_3$ 254.1048), 239 (13), 223 (4), 211 (5), 193 (1), 181 (2), 165, (6) 161 (17), 127 (6), 121 (6), 107 (4), 77 (3), 69 (3), 57 (4), 55 (3). IR ν_{max}^{CHCl3} cm^{-1}: 3595 (OH), 2941, 2835, 1625, 1514, 1491, 1440, 1291, 1272, 1148, 1107, 1029, 961, 826. UV λ_{max} nm (log ε): 205 (4.36), 249 (4.06), 287 (3.75), UV λ_{min} nm (log ε): 235 (3.90), 277 (3.69). ^1H NMR ($CDCl_3$) δ (J): 7.32 H-4 (*d*, 8.5), 7.16 H-3′/7′ (*d*, 8.4), 6.96 H-7 (*br d*, 2.2), 6.81 H-5 (*dd*, 8.5/2.2), 6.78 H-4′/6′ (*d*, 8.4), 6.27 H-3 (*br s*), 3.99 H_2-1′ (*br s*), 3.82 OMe (*s*). ^{13}C NMR ($CDCl_3$) δ: 157.3 C-6, 157.2 C-2, 155.9 C-7a, 154.3 C-5′, 130.1 C-3′/7′, 129.7 C-2′, 122.1 C-3a, 120.4 C-4, 115.4 C-4′/6′, 111.2 C-5, 102.8 C-3, 95.9 C-7, 55.7 6-OMe, 34.1 C-1′. *TMSi derivative*. GC–EIMS *m/z* (rel. int.): 326 $[M]^+$ (100), 311 (34), 295 (20), 253 (10), 237 (25), 179 [a] (5), 165 (22), 161 (57), 155 (49), 148 (26), 118 (21).

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Tetrahydroisoquinoline-monoterpene and iridoid glycosides from *Alangium lamarckii*

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Abstract

From the water soluble fraction of the dried fruits of *Alangium lamarckii*, four tetrahydroisoquinoline-monoterpene glycosides, 6-*O*-methyl-*N*-deacetylisoipecosidic acid, 7-*O*-methyl-*N*-deacetylisoipecosidic acid, 6,7-di-*O*-methyl-*N*-deacetylisoipecosidic acid and 6″-*O*-α-D-glucopyranosyl-6-*O*-methyl-*N*-deacetylisoipecosidic acid, and an iridoid glycoside, 6′-*O*-α-D-glucopyranosylloganic acid, were isolated, together with six known compounds. The structures of the previously unknown compounds were determined by spectroscopic and chemical means. The significance of these glucosides in the biogenesis of *Alangium* alkaloids is discussed; 6-*O*-methyl-*N*-deacetylisoipecosidic acid was also chemically converted into 10-*O*-demethylprotoemetine and dihydroisoalangine.
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Keywords: *Alangium lamarckii*; Alangiaceae; Fruits; Structure elucidation; Tetrahydroisoquinoline-monoterpene glycosides; *N*-Deacetylisoipecosidic acids; Alkaloids; Ipecoside; Iridoid glycoside; 6′-*O*-α-D-glucopyranosylloganic acid

1. Introduction

Previous phytochemical investigations of *Alangium lamarckii* belonging to the Alangiaceae, which has been used as Indian folk medicine, resulted in the isolation of diverse *Alangium* alkaloids (Fujii and Ohba, 1998; Itoh, et al., 2000). We have recently isolated from the *n*-BuOH-soluble fraction of the fruits of *A. lamarckii* numerous tetrahydroisoquinoline-monoterpene glycosides, which were closely related to the *Alangium* alkaloids (Itoh, et al., 1994, 1995, 1996, 1997, 1998). In a continuation of our phytochemical studies on the fruits of *A. lamarckii*, we have examined the constituents of the water-soluble fraction and have isolated five new glycosides. We report here the structure elucidation of these new compounds and discuss the possibility of intermediacy of the isolated glycosides with a carboxyl group in the biogenesis of *Alangium* alkaloids.

2. Results and discussion

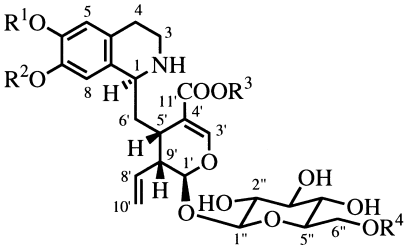
The H_2O-soluble fraction of the dried fruits of *A. lamarckii* was separated by a combination of chromatographic procedures to afford five new compounds **1**–**5**, along with five known glycosides, 6-*O*-methyl-*N*-deacetylipecosidic acid (**6**) (Tanahashi et al., 2000), loganic acid (**7**) (Shoeb, et al., 1975), sweroside (Inouye et al., 1966), secologanoside (Calis and Sticher, 1984), benzyl alcohol xylopyranosyl(1→6)glucopyranoside (Otsuka et al., 1990), and a known alkaloid, cephaeline (Wiegrebe et al., 1984).

Compound **1** was isolated as a colorless crystalline solid, mp 192–194°, $[\alpha]_D$ −203° and was recognized as an isomer of 6-*O*-methyl-*N*-deacetylipecosidic acid (**6**), $C_{25}H_{33}NO_{11}$, from its HR-SIMS. It showed UV maxima at 230, 285 and 292 sh nm and IR bands at 3394, 1641 and 1518 cm^{-1}. Its ^1H NMR spectrum exhibited signals for two acetal protons at δ 4.79 (*d*, $J = 8.0$ Hz) and 5.75 (*d*, $J = 9.0$ Hz), a vinyl group at δ 5.22 (*dt*, $J = 10.5$ and 1.5 Hz), 5.30 (*dt*, $J = 17.5$ and 1.5 Hz) and 5.84 (*ddd*, $J = 17.5$, 10.5 and 7.5 Hz), an olefinic proton at δ 7.53 (*s*), a methoxyl group at δ 3.83 (*s*) and two aromatic protons at δ 6.69 (*s*) and 6.74 (*s*) along with the

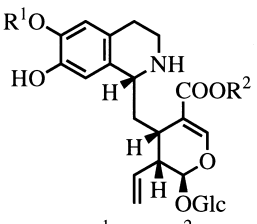
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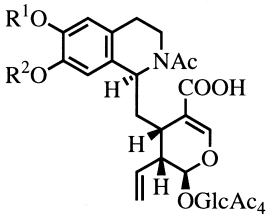
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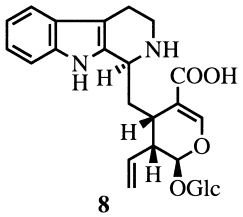
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2: R^1=R^3=R^4=H, R^2=Me
3: R^1=R^2=Me, R^3=R^4=H
4: R^1=Me, R^2=R^3=H, R^4=α-Glc
14: R^1=R^2=R^4=H, R^3=Me
19: R^1=R^2=R^3=R^4=H



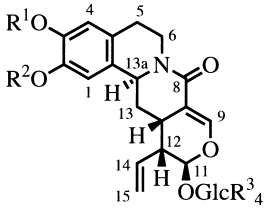
6: R^1=Me, R^2=H
17: R^1=H, R^2=Me
20: R^1=R^2=H



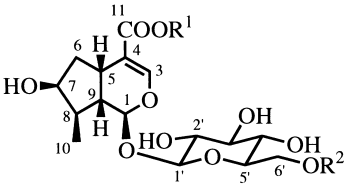
10: R^1=Me, R^2=Ac
12: R^1=Ac, R^2=Me



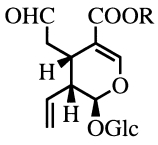
8



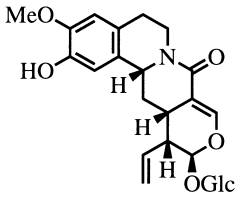
9: R^1=Me, R^2=R^3=Ac
11: R^1=R^3=Ac, R^2=Me
15: R^1=Me, R^2=R^3=H



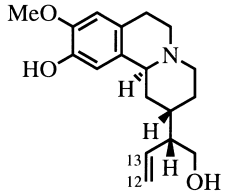
5: R^1=H, R^2=α-Glc
7: R^1=R^2=H
13: R^1=Me, R^2=α-Glc



16: R=Me
18: R=H



21



22
24: 12,13-dihydro

signals arising from a β-glucopyranosyl moiety. These spectral features were closely related to those of **6**, but the ^{13}C NMR spectra differed significantly in the chemical shifts of C-1, C-3, C-3′, C-4′, C-5′, and C-6′. The location of the methoxyl group at C-6 was deduced by the correlation between H-5 and OMe in a NOESY experiment. These findings, together with biogenetic considerations, suggested **1** to be a C-1 epimer of **6**. This suggestion was supported by comparison of the ^{13}C NMR spectral data of **1** with those of strictosidinic acid (**8**) (Subhadhirasakul et al., 1994). Final confirmation was given by acetylation of **1** to isoalangiside pentaacetate (**9**) (Itoh et al., 1995) and 6-*O*-methylisoipecosidic acid pentaacetate (**10**). Accordingly, **1** was established as 6-*O*-methyl-*N*-deacetylisoipecosidic acid.

The second new glucoside **2** has the same molecular formula as **1**. Its ^{1}H and ^{13}C NMR spectral features were closely similar to those of **1** except for the signals arising from the aromatic ring system. The chemical shifts of C-1, C-3, C-3′, C-4′, C-5′, and C-6′ suggested that **2** should possess the same stereochemistry at C-1 as **1**. The placement of the methoxyl group at C-7 was ascertained by a cross-peak between H-8 and OMe in the NOESY spectrum. Acetylation of **2** gave 3-*O*-demethyl-2-*O*-methylisoalangiside pentaacetate (**11**) (Itoh et al., 1995) and 7-*O*-methylisoipecosidic acid pentaacetate (**12**). Thus, glucoside **2** was characterized as 7-*O*-methyl-*N*-deacetylisoipecosidic acid.

Glucoside **3** displayed the molecular formula, $C_{26}H_{35}NO_{11}$, from its HR-SIMS. Its ^{1}H and ^{13}C NMR spectral features showed that **3** has a structure closely related to **1** and **2**, but with an additional methoxyl group. Its NOESY experiments showed that two methoxyl groups were located at C-6 and C-7. Therefore, **3** was deduced to be 6,7-di-*O*-methyl-*N*-deacetylisoipecosidic acid.

The spectral features of glycoside **4** were also similar to those of **1**. Its HR-SI mass spectrum showed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 684.2518 consistent with a molecular formula $C_{31}H_{43}NO_{16}$, which was $C_6H_{10}O_5$ more than that of **1**, suggesting the presence of an additional hexose in **4**. The ^{1}H and ^{13}C NMR spectra showed, besides the signals due to a 6-*O*-methyl-*N*-deacetylisoipecosidic acid moiety, the signals for an α-glucosyl unit (Tables 1 and 3). The location of the second glucose unit at C-6″ of 6-*O*-methyl-*N*-deacetylisoipecosidic acid (**1**) was shown by the downfield shift of C-6″ and the upfield shift of C-5″ as compared with those of **1**, as well as the cross-peaks between H_2-6″ and C-1‴ and between H-1‴ and C-6″ in its HMBC spectrum. Furthermore, the chemical shifts of the carbon signals arising from the glycosyl moiety of **4** were in good agreement with those of 6′-*O*-α-D-glucopyranosylalan-

Table 1
^{1}H NMR spectral data of **1**–**4** in CD_3OD at 500 MHz

H	**1**			**2**			**3**			**4**		
1	4.18	*brt*	(8.0)	4.21	*dd*	(11.5, 3.5)	4.23	*dd*	(11.5, 4.0)	4.16	*dd*	(10.5, 5.0)
3	3.20	*ddd*	(12.5, 9.5, 5.0)	3.19	*ddd*	(12.5, 9.5, 5.0)	3.21	*ddd*	(12.5, 10.0, 5.0)	3.17	*ddd*	(12.5, 10.5, 4.0)
3	3.47	*dt*	(12.5, 5.0)	3.45	*dt*	(12.5, 5.0)	3.49	*dt*	(12.5, 5.0)	3.47	*ddd*	(12.5, 5.5, 4.0)
4	2.89	*dt*	(16.5, 5.0)	2.84	*dt*	(17.0, 5.0)	2.91	*dt*	(17.0, 5.0)	2.87	*dt*	(16.5, 4.0)
4	3.06	*ddd*	(16.5, 9.5, 5.0)	3.01	*ddd*	(17.0, 9.5, 5.0)	3.08	*ddd*	(17.0, 10.0, 5.0)	3.06	*ddd*	(16.5, 10.5, 5.5)
5	6.74	*s*		6.61	*s*		6.77	*s*		6.74	*s*	
8	6.69	*s*		6.83	*s*		6.87	*s*		6.80	*s*	
1′	5.75	*d*	(9.0)	5.84	*d*	(9.5)	5.84	*d*	(9.5)	5.69	*d*	(9.0)
3′	7.53	*s*		7.53	*s*		7.52	*s*		7.49	*s*	
5′	2.86	*td*	(8.0, 5.0)	2.88	*ddd*	(12.0, 5.0, 4.0)	2.87	*dt*	(12.0, 4.5)	2.83	*dt*	(10.5, 5.0)
6′	2.09	*brt*	(8.0)	2.07	*ddd*	(14.5, 11.5, 4.0)	2.07	*ddd*	(14.5, 11.5, 4.5)	2.08	*ddd*	(14.5, 10.5, 5.0)
6′	2.09	*brt*	(8.0)	2.22	*ddd*	(14.5, 12.0, 3.5)	2.24	*ddd*	(14.5, 12.0, 4.0)	2.12	*ddd*	(14.5, 10.5, 5.0)
8′	5.84	*ddd*	(17.5, 10.5, 7.5)	5.85	*ddd*	(17.5, 10.5, 7.5)	5.85	*ddd*	(17.5, 10.5, 7.5)	5.84	*ddd*	(17.5, 10.5, 7.5)
9′	2.65	*dddt*	(9.0, 7.5, 5.0, 1.5)	2.65	*dddt*	(9.5, 7.5, 5.0, 1.5)	2.65	*dddt*	(9.5, 7.5, 4.5, 1.5)	2.64	*dddt*	(9.0, 7.5, 5.0, 1.0)
10′	5.22	*dt*	(10.5, 1.5)	5.21	*dt*	(10.5, 1.5)	5.20	*dt*	(10.5, 1.5)	5.22	*dt*	(10.5, 1.0)
10′	5.30	*dt*	(17.5, 1.5)	5.30	*dt*	(17.5, 1.5)	5.29	*dt*	(17.5, 1.5)	5.29	*dt*	(17.5, 1.0)
1″	4.79	*d*	(8.0)	4.78	*d*	(8.0)	4.79	*d*	(8.0)	4.81	*d*	(8.0)
2″	3.19	*dd*	(9.0, 8.0)	3.19	*dd*	(9.0, 8.0)	3.19	*dd*	(9.0, 8.0)	3.21	*dd*	(9.0, 8.0)
3″	3.40	*t*	(9.0)	3.39	*t*	(9.0)	3.39	*t*	(9.0)	3.41	*t*	(9.0)
4″	3.25	*dd*	(10.0, 9.0)	3.22	*dd*	(9.5, 9.0)	3.21	*dd*	(9.5, 9.0)	3.34	*brt*	(9.5)
5″	3.35	*ddd*	(10.0, 6.5, 2.0)	3.35	*ddd*	(9.5, 7.0, 2.0)	3.34	*ddd*	(9.5, 7.0, 2.0)	3.56	*ddd*	(9.5, 6.0, 2.0)
6″	3.65	*dd*	(12.0, 6.5)	3.62	*dd*	(12.0, 7.0)	3.61	*dd*	(11.5, 7.0)	3.85	*dd*	(11.0, 2.0)
6″	3.94	*dd*	(12.0, 2.0)	3.94	*dd*	(12.0, 2.0)	3.94	*dd*	(11.5, 2.0)	3.90	*dd*	(11.0, 6.0)
OMe	3.83	*s*		3.84	*s*		3.80	*s*		3.83	*s*	
OMe							3.82	*s*				
1‴										4.88	*d*	(3.5)
2‴										3.41	*dd*	(9.5, 3.5)
3‴										3.69	*t*	(9.5)
4‴										3.36	*t*	(9.5)
5‴										3.66-3.71	*m*	
6‴										3.66-3.71	*m*	
6‴										3.79	*m*	

giside (Itoh et al., 1997). Thus, glycoside **4** was characterized as 6″-*O*-α-D-glucopyranosyl-6-*O*-methyl-*N*-deacetylisoipecosidic acid.

The last new glycoside **5**, $C_{22}H_{34}O_{15}$, afforded methyl ester **13** on treatment with diazomethane. The ^{1}H and ^{13}C NMR spectral features of **5** closely resembled those of loganic acid (**7**), except for the signals arising from an additional α-glucosyl unit. The linkage of the α-glucose unit to C-6′ of the loganic acid moiety was determined by the glycosylation shifts of C-6′ and C-5′, along with its HMBC and NOESY correlations. Accordingly, glycoside **5** was elucidated to be 6′-*O*-α-D-glucopyranosylloganic acid.

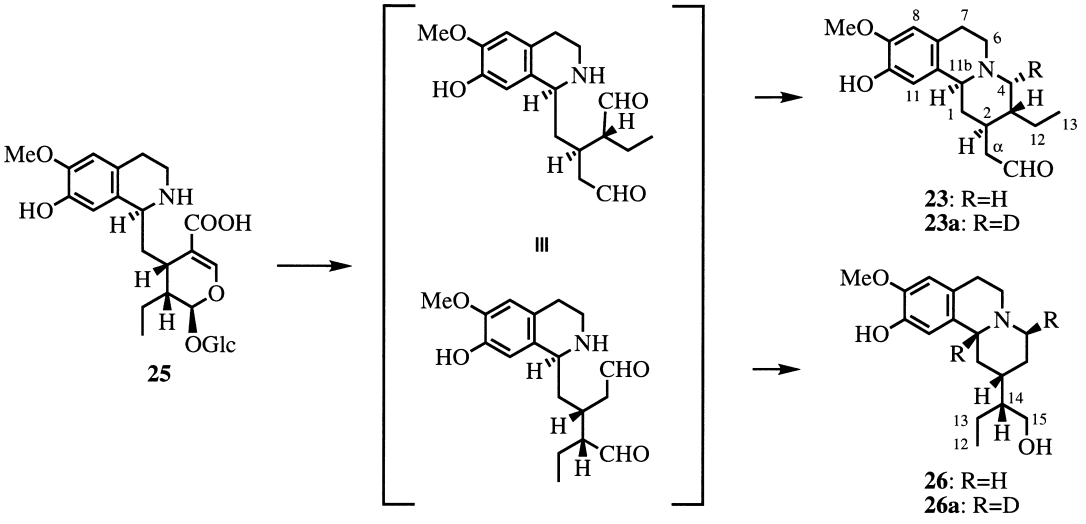
Previous biosynthetic studies demonstrated that *Alangium* alkaloids represented by cephaeline were biosynthesized from a postulated intermediate *N*-deacetylisoipecoside (**14**) (Nagakura et al., 1978). We have recently isolated isoalangiside (**15**), 3-*O*-demethyl-2-*O*-methylisoalangiside and methylisoalangiside possessing the C-13a*S* configuration as the first glucosides with the same stereochemistry as *N*-deacetylisoipecoside (**14**) and *Alangium* alkaloids, supporting the proposed biosynthetic scheme (Itoh et al., 1995). Compounds **1**–**4** possessing amino and carboxyl groups were classified into the second group of glycosides with the same stereochemistry as the *Alangium* alkaloids. Indeed both radiolabelling studies (Nagakura et al., 1978; Battersby et al., 1978) and recent enzymatic studies demonstrated that secologanin (**16**) is condensed with dopamine in a Pictet-Spengler manner to form two epimers, *N*-deacetylisoipecoside (**14**) and *N*-deacetylipecoside (**17**) (De-Eknamkul et al., 1997). However, the present results implied an alternative plausible pathway, where secologanic acid (**18**), rather than secologanin (**16**) is condensed with dopamine to form *N*-deacetylisoipecosidic acid (**19**) and *N*-deacetylipecosidic acid (**20**). Secologanic acid (**18**) has not so far been isolated from the fruits of *Alangium lamarckii*, but a major product of the plant material is loganic acid (**7**), which was found to be converted to **18** in *Catharanthus roseus* (Guarnaccia et al., 1974), suggesting the intermediacy of secologanic acid (**18**). *N*-Deacetylisoipecosidic acid (**19**) could be further methylated to **1**, **2** and **3**, while *N*-deacetylipecosidic acid (**20**) may be transformed to alangiside (**21**) via **6**. Supposing 6- and/or 7-*O*-methylated *N*-deacetylisoipecosidic acids could suffer hydrogenation of the vinyl group and deglucosylation

Table 2
^{1}H NMR spectral data of **5** and **13** in CD_3OD at 500 MHz

H	**5**			**13**		
1	5.17	*d*	(5.0)	5.17	*d*	(5.0)
3	7.38	*d*	(1.0)	7.39	*d*	(1.5)
5	3.10	*qd*	(8.0, 1.0)	3.11	*brq*	(8.0)
6	1.65	*ddd*	(14.0, 8.0, 5.0)	1.61	*ddd*	(14.0, 8.0, 4.5)
6	2.24	*ddd*	(14.0, 8.0, 1.5)	2.22	*ddd*	(14.0, 8.0, 1.5)
7	4.17	*td*	(5.0, 1.5)	4.07	*td*	(4.5, 1.5)
8	1.88	*dqd*	(8.0, 7.0, 5.0)	1.87	*dqd*	(8.0, 7.0, 4.5)
9	2.00	*td*	(8.0, 5.0)	1.99	*td*	(8.0, 5.0)
10	1.09	*d*	(7.0)	1.09	*d*	(7.0)
1′	4.68	*d*	(8.0)	4.67	*d*	(8.0)
2′	3.22	*dd*	(9.0, 8.0)	3.21	*dd*	(9.0, 8.0)
3′	3.34–3.40	*m*		3.33–3.39	*m*	
4′	3.34–3.40	*m*		3.33–3.39	*m*	
5′	3.53	*ddd*	(9.0, 5.5, 2.0)	3.53	*ddd*	(9.0, 5.5, 2.0)
6′	3.77	*dd*	(11.0, 2.0)	3.77	*dd*	(11.0, 2.0)
6′	3.92	*dd*	(11.0, 5.5)	3.91	*dd*	(11.0, 5.5)
1″	4.84	*d*	(3.5)	4.84	*d*	(3.5)
2″	3.37	*dd*	(9.5, 3.5)	3.33–3.39	*m*	
3″	3.62	*dd*	(9.5, 9.0)	3.61	*t*	(9.5)
4″	3.30	*t*	(9.0)	3.29	*t*	(9.5)
5″	3.63	*ddd*	(9.0, 5.0, 2.0)	3.62	*ddd*	(9.5, 5.0, 2.0)
6″	3.67	*dd*	(11.0, 5.0)	3.67	*dd*	(11.5, 5.0)
6″	3.79	*dd*	(11.0, 2.0)	3.79	*dd*	(11.5, 2.0)
OMe				3.68	*s*	

followed by spontaneous decarboxylation, recyclization and reduction, we could reasonably account for the co-occurrence in this plant of demethylprotoemetinols (Ali et al., 1982) with different substitution patterns in the aromatic ring A. Through a different manner of recyclization after deglucosylation, **1** might be converted to alangine (**22**), which has recently been isolated from this plant material (Itoh et al., 2000).

To rationalize our proposal, the chemical transformation of **1** to 10-*O*-demethylprotoemetine (**23**) and dihydroalangine (**24**) was conducted. Glucoside **1** was subjected to catalytic hydrogenation to yield dihydro-6-*O*-methyl-*N*-deacetylisoipecosidic acid (**25**) which was hydrolyzed with 5% HCl and then treated with $NaBH_3CN$ at pH 6.5–7.0 to afford 10-*O*-demethylprotoemetine (**23**) and dihydroisoalangine (**26**). The structure of **26** was confirmed by comparing its spectroscopic data with those of alangine (**22**) (Itoh et al., 2000). The NMR spectra of **26** indicated its structural similarity to **22** except that a vinyl group was replaced by an ethyl group in **26**. However, when their ^{1}H and ^{13}C NMR spectral data were inspected there were remarkable dif-



Scheme 1. Chemical conversion of **25** to **23** and **26**.

Table 3
^{13}C NMR spectral data of **1–4** and **6** in CD_3OD at 125 MHz

C	1	2	3	4	6
1	53.8	54.0	53.9	53.9	57.5
3	41.0	40.8	40.8	41.4	39.1
4	26.1	26.0	26.2	26.2	25.9
4a	124.0	125.4	126.0	124.4	123.9
5	112.7	116.3	113.1	112.8	112.4
6	149.0	147.7	150.4[a]	149.0	149.1
7	147.0	148.6	150.0[a]	146.7	146.9
8	113.5	110.6	110.9	114.0	114.1
8a	125.9	124.7	125.5	125.9	126.0
1′	96.8	96.7	96.7	97.0	96.8
3′	153.2	153.3	153.1	152.9	150.2
4′	113.7	113.6	113.9	114.4	116.2
5′	33.7	34.0	34.1	33.8	35.0
6′	36.7	36.8	36.8	36.3	38.7
8′	136.5	136.6	136.6	136.5	136.2
9′	46.0	46.0	46.0	46.2	46.0
10′	119.0	118.9	118.9	119.2	120.2
11′	175.6	175.7	176.0	175.9	175.0
1″	100.3	100.3	100.3	100.4	100.2
2″	74.8	74.8	74.8	74.8	74.8
3″	78.0	78.1	78.1	78.1	78.0
4″	71.7	71.9	71.9	71.9	71.7
5″	78.5	78.6	78.6	76.5	78.4
6″	62.9	63.1	63.1	67.9	62.8
OMe	56.4	56.8	56.5	56.4	56.4
OMe			56.9		
1‴				99.8	
2‴				73.7[b]	
3‴				75.4	
4‴				71.6	
5‴				73.8[b]	
6‴				62.6	

[a,b] Values with the same letters are interchangeable.

ferences in the chemical shifts of H-1ax, H-6ax, and H-11b, as well as C-1, C-2, C-3, C-4, C-6, C-7, C-11a, and C-11b. The chemical shifts of **26** were comparable with those of the corresponding signals of 3-*epi*-antirhine rather than those of antirhine (Kan-Fan et al., 1986). In addition, the presence of Bohlmann bands in its IR spectrum suggested a *trans*-quinolizidine ring junction in analogy with 3-*epi*-antirhine. These spectral data led us to assume that **26** must be a C-11b epimer of **24**. This assumption was supported by the NOE interactions between H-2 and H-4ax, H-2 and H-11b, H-4ax and H-11b, H-6ax and H-11b, and H-4ax and H-6ax. In the ^1H and ^{13}C NMR spectra of **26**, some of the signals were accompanied by minor signals, which might be ascribed to a C-14 epimer formed through tautomerism of the C-15 aldehyde.

The formation of dihydroisoalangine (**26**) was an unexpected result since dihydroalangine (**24**) should be formed from **1** if the stereochemistry of **1** was retained. The inversion of C-11b was demonstrated by reduction with $NaBD_3CN$ to afford [4-^2H]-10-*O*-demethylprotoemetine (**23a**) and [4,11b-$^2H_2$]-dihydroisoalangine (**26a**). This result showed the possibility that **26** might be formed from **25** via 1,4,5,11b-tetradehydro derivative.

Although an unexpected epimerization was observed, these chemical transformations supported the possibility that glycosides with a carboxyl group, which are favorable for decarboxylation, could be significant intermediates in the biosynthesis of the alkaloids in this plant.

3. Experimental

3.1. General

UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and IR spectra on a Shimadzu FTIR-8200 spectrophotometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter and CD spectra on a Shimadzu-AVIV 62 A DS circular dichroism spectrometer. ^1H (500, 300 or 200 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on Varian VXR-500, Varian Gemini-300 or Varian Gemini-200 spectrometers with TMS as an internal standard. MS and HRMS were obtained with a Hitachi M-4100 mass spectrometer. Glycerol or 3-nitrobenzyl alcohol was used for SIMS and HR-SIMS as the matrix. MPLC was carried out with Wakogel LP-40 C18. TLC was performed on precoated Kieselgel $60F_{254}$ plates (Merck).

3.2. Plant material

The dried fruits of *Alangium lamarckii*, collected in India, were purchased from Mikuni, Osaka, Japan, in 1992. A voucher specimen (KPFY-921) is deposited in our laboratory.

3.3. Extraction and isolation

Dried fruits (4.5 kg) of *A. lamarckii* were extracted with hot MeOH and the extracts were fractionated as described previously (Itoh et al., 1995). A part (110.2 g) of the residue (556.1 g) from the H_2O layers was fractionated on reversed-phase MPLC. Elution with H_2O–MeOH mixtures of the indicated MeOH content gave 5 frs, 1 (0%, 86.4 g), 2 (0%, 7.28 g), 3 (5%, 2.06 g), 4 (10%, 2.79 g), and 5 (20–100%, 5.97 g). A portion (20.0 g) of fr. 1 was purified by DIAION HP-20 CC with MeOH–H_2O (1:9–3:7) and prep. HPLC (μBondasphere 5 μ C18-100 Å, MeOH-H_2O, 3:7) to afford **1** (154 mg), **2** (70.5 mg), **5** (15.3 mg), **7** (1.18 g), and secologanoside (11.3 mg). Frs. 2–4 were submitted to prep. HPLC (μBondasphere 5 μ C18-100 Å, MeOH–H_2O, 3:7), respectively. Fr. 2 yielded **1** (844 mg), **2** (274 mg), and **7** (3.74 g); fr. 3: **1** (483 mg), **3** (5.8 mg), **4** (3.7 mg), **6** (5.5 mg), **7** (0.8 mg), secologanoside (53.9 mg), cephaeline (4.9 mg), benzyl alcohol xylopyranosyl(1→6) glucopyranoside (6.8 mg), and sweroside (9.9 mg); fr. 4: **1** (7.3 mg), **3** (107 mg) and **6** (324 mg). Fr. 5 was not examined.

3.4. 6-O-Methyl-N-deacetylisoipecosidic acid (*1*)

Colorless crystalline solid, mp 192–194° (MeOH). $[\alpha]_D^{22}$ −203° (*c* 1.1, H_2O). UV λ_{max}^{MeOH}nm (log ε): 230 (4.14), 285 (3.58), 292 sh (3.52). IR ν_{max}^{KBr} cm^{-1}: 3394, 1641, 1518, 1379. ^1H NMR: Table 1. ^{13}C NMR: Table 3. CD λ_{max}^{MeOH}nm (Δ ε): 230 (−13.9), 286 (−1.0). Negative ion SIMS *m/z*: 522 $[M-H]^-$, 342, 176. Negative ion HR-SIMS Found 522.1982 $[M-H]^-$; $C_{25}H_{32}NO_{11}$ requires 522.1976. NOESY: H-5/OMe; H-5/H-4 (δ 2.89); H-8/H_2-6′; H-8/H-1.

3.5. 7-O-Methyl-N-deacetylisoipecosidic acid (*2*)

Colorless crystalline solid, mp 250–252° (MeOH). $[\alpha]_D^{24}$ −201° (*c* 0.2, H_2O). UV λ_{max}^{MeOH} nm (log ε): 229.5 (4.12), 285.5 (3.54), 292 sh (3.54). IR ν_{max}^{KBr} cm^{-1}: 3316, 1636, 1518, 1396. ^1H NMR: Table 1. ^{13}C NMR: Table 3. Negative ion SIMS *m/z*: 522 $[M-H]^-$. Negative ion HR-SIMS Found 522.1992 $[M-H]^-$; $C_{25}H_{32}NO_{11}$ requires 522.1976. NOESY: H-8/OMe; H-8/H-6′ (δ 2.22).

3.6. 6,7-Di-O-methyl-N-deacetylisoipecosidic acid (*3*)

Colorless crystalline solid, mp 198–201° (MeOH). $[\alpha]_D^{26}$ −205° (*c* 0.5, H_2O). UV λ_{max}^{MeOH} nm (log ε): 233 (4.20), 279 sh (3.51), 284 (3.54), 287 sh (3.52). IR ν_{max}^{KBr} cm^{-1}: 3344, 1632, 1520, 1377. ^1H NMR: Table 1. ^{13}C NMR: Table 3. CD λ_{max}^{MeOH} nm (Δ ε): 228 (-19.1), 284 (-1.0). Negative ion SIMS *m/z*: 536 $[M-H]^-$, 356. Negative ion HR-SIMS Found 536.2145 $[M-H]^-$; $C_{26}H_{34}NO_{11}$ requires 536.2133. NOESY: H-5/OMe (δ 3.80); H-8/OMe (δ 3.82); H-5/H-4 (δ 2.91); H-8/H-6′ (δ 2.24); H-8/H-1.

3.7. 6-O-Methyl-N-deacetyl-6″-O-α-D-glucopyranosyl-isoipecosidic acid (*4*)

White powder. $[\alpha]_D^{30}$ −177° (*c* 0.3, H_2O). UV λ_{max}^{MeOH} nm (log ε): 228.5 (4.12), 285 (3.54), 292 sh (3.46). IR ν_{max}^{KBr} cm^{-1}: 3385, 1645, 1522, 1385. ^1H NMR: Table 1. ^{13}C NMR: Table 3. Negative ion SIMS *m/z*: 684 $[M-H]^-$, 522, 176. Negative ion HR-SIMS Found 684.2518 $[M-H]^-$; $C_{31}H_{42}NO_{16}$ requires 684.2505. NOESY: H-5/OMe; H-5/H-4 (δ 2.87); H-8/H-1; H-8/H-6′ (δ 2.08); H-1′/H-1″; H-6″ (δ 3.85)/H-1‴. HMBC: H-1′ to C-1″; H-1″ to C-1′; H-1″ to C-5″; H_2-6″ to C-1‴; H-1‴ to C-6″.

3.8. 6′-O-α-D-Glucopyranosylloganic acid (*5*)

White powder. IR ν_{max}^{KBr} cm^{-1}: 3415, 1686, 1638. ^1H NMR: Table 2. ^{13}C NMR: Table 4. Negative ion SIMS *m/z*: 537 $[M-H]^-$, 375, 213. Negative ion HR-SIMS Found 537.1827 $[M-H]^-$; $C_{22}H_{33}O_{15}$ requires 537.1820. NOESY: H-1/H-1′; H-1′/H-3′; H-1′/H-5′; H_2-6′/H-1″. HMBC: H-1 to C-1′; H-1′ to C-1; H_2-6′ to C-1″; H-1″ to C-6′.

Table 4
^{13}C NMR spectral data of **5** and **13** in CD_3OD at 125 MHz

C	5	13
1	98.3	98.4
3	152.0	152.2
4	114.3	113.9
5	32.4	32.4
6	42.7	42.7
7	75.0	75.0
8	42.3	42.4
9	46.6	46.5
10	13.7	13.7
11	171.0	169.5
1′	100.4	100.4
2′	74.7	74.7
3′	78.1	78.1
4′	71.6[a]	71.6[c]
5′	76.7	76.7
6′	67.9	67.9
1″	100.1	
2″	73.6[b]	73.6[d]
3″	75.3	75.3
4″	71.7[a]	71.7[c]
5″	73.7[b]	73.7[d]
6″	62.6	62.6
OMe		51.6

[a–d] Values with the same letter are interchangeable.

3.9. Acetylation of *1*

Compound **1** (50.6 mg) was acetylated with Ac_2O in pyridine and the crude acetate was purified by prep. TLC with $CHCl_3$–MeOH (19:1) to afford isoalangiside pentaacetate (**9**) (20.6 mg) and 6-*O*-methylisoipecosidic acid pentaacetate (**10**) (14.8 mg). Isoalangiside pentaacetate (**9**) was identified with an authentic sample (^1H NMR, UV, IR, SIMS and $[\alpha]_D^{25}$ −120° (*c* 1.5, $CHCl_3$)). 6-*O*-Methylisoipecosidic acid pentaacetate (**10**): $[\alpha]_D^{26}$ −54° (*c* 1.1, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε): 220 sh (4.30), 278.5 (3.46). IR ν_{max}^{KBr} cm^{-1}: 1759, 1624, 1512, 1371. ^1H NMR ($CDCl_3$, 200 MHz): δ 1.94[1], 2.00 (3H, each *s*, Ac), 2.00, 2.03 (6H, each *s*, 2×Ac), 2.06, 2.12[1] (3H, each *s*, Ac), 2.17, 2.24[1] (3H, each *s*, Ac), 2.29 (3H, *s*, Ac), 3.72 (1H, *m*, H-5″), 3.79 (3H, *s*, OMe), 4.14 (1H, *brd*, *J* = 12.0 Hz, H-6″), 4.31 (1H, *dd*, *J* = 12.0, 4.5 Hz, H-6″), 4.86[1], 4.90 (1H, each *d*, *J* = 8.0 Hz, H-1″), 5.01 (1H, *brt*, *J* = 8.5 Hz, H-2″), 5.11 (1H, *brt*, *J* = 9.0 Hz, H-4″), 5.23 (1H, *brt*, *J* = 9.0 Hz, H-3″), 5.15–5.33 (3H, *m*, H-1′, H_2-10′), 5.52 (1H, *dt*, *J* = 18.0, 9.0 Hz, H-8′), 6.66, 6.84 (2H, each *s*, 2×ArH), 7.43, 7.53[1] (1H, each *d*, *J* = 1.5 Hz, H-3′). Positive ion SIMS *m/z*: 776 $[M+H]^+$, 331, 220.

3.10. Acetylation of *2*

Conventional acetylation of **2** (25.7 mg) and subsequent purification by prep. TLC ($CHCl_3$–MeOH, 19:1)

[1] Represents signals due to the rotomer in the ^1H NMR spectral data of **10** (Itoh et al., 1991).

afforded 3-*O*-demethyl-2-*O*-methylisoalangiside pentaacetate (**11**) (9.1 mg) and 7-*O*-methylisoipecosidic acid pentaacetate (**12**) (9.4 mg). 3-*O*-Demethyl-2-*O*-methylisoalangiside pentaacetate (**11**) was identified with an authentic sample [^{1}H NMR, UV, IR, SIMS and $[\alpha]_D^{27}$ −136° (*c* 0.92, $CHCl_3$)]. 7-*O*-Methylisoipecosidic acid pentaacetate (**12**): ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.94, 1.98[1] (3H, each *s*, Ac), 2.01, 2.03 (6H, each *s*, 2×Ac), 2.07, 2.13[1] (3H, each *s*, Ac), 2.15, 2.25[1] (3H, each *s*, Ac), 2.29 (3H, *s*, Ac), 3.74 (1H, *m*, H-5″), 3.79 (3H, *s*, OMe), 4.14 (1H, *brd*, *J*=12.0 Hz, H-6″), 4.31 (1H, *dd*, *J*=12.0, 4.5 Hz, H-6″), 4.86[1], 4.90 (1H, each *d*, *J*=8.0 Hz, H-1″), 5.00 (1H, *brt*, *J*=8.5 Hz, H-2″), 5.11 (1H, *brt*, *J*=9.0 Hz, H-4″), 5.23 (1H, *brt*, *J*=9.0 Hz, H-3″), 5.22–5.36 (3H, *m*, H-1′, H_2-10′), 5.52 (1H, *dt*, *J*=17.0, 9.0 Hz, H-8′), 6.75, 6.76 (2H, each *s*, 2×ArH), 7.42, 7.52[1] (1H, each *d*, *J*=1.5 Hz, H-3′). Positive ion SIMS *m*/*z*: 776 $[M+H]^+$, 331, 220.

3.11. Methylation of 5

A solution of **5** (13.3 mg) in H_2O (0.5 ml) and MeOH (1.0 ml) was treated with CH_2N_2–Et_2O, and further purified by prep. HPLC (H_2O–MeOH, 6:4) to afford **13** (4.5 mg). $[\alpha]_D^{29}$ −0.3° (*c* 0.36, H_2O). UV λ_{max}^{MeOH} nm(log ε): 235.5 (4.02). IR ν_{max}^{KBr} cm^{-1}: 3384, 1695, 1636. ^{1}H NMR: Table 2. ^{13}C NMR: Table 4. Negative ion SIMS *m*/*z*: 551 $[M-H]^-$, 227. Negative ion HR-SIMS Found 551.1989 $[M-H]^-$; $C_{23}H_{35}O_{15}$ requires 551.1977. NOESY: H-1/H-1′; H-6′ (δ 3.77)/H-1″; H-1′/H-5′. HMBC: H-1″ to C-6′; H_2-6′ to C-1″; H-1 to C-1′; H-1′ to C-1; OMe to C-11.

3.12. Catalytic hydrogenation of 1

A solution of **1** (100 mg) in EtOH (30 ml) was hydrogenated over PtO_2 (50 mg) at room temperature for 3 h in hydrogen. The catalyst was filtered and the filtrate was concentrated in vacuo. The residue (98.6 mg) was recrystallized from MeOH to afford dihydro-6-*O*-methyl-*N*-deacetylisoipecosidic acid (**25**) (59.1 mg, 59%) as a colorless crystalline solid. Further prep. HPLC purification (μBondasphere 5 μ C18-100 Å, MeOH–H_2O, 3:7) of the mother liquor yielded **25** (13.7 mg, 14%). mp 195–196°C (decomp. MeOH). $[\alpha]_D^{20}$ −206° (*c* 1.0, H_2O). UV λ_{max}^{MeOH} nm (log ε): 231 (4.14), 285.5 (3.57), 292 sh (3.50). IR ν_{max}^{KBr} cm^{-1}: 3394, 1643, 1518, 1381. ^{1}H NMR (CD_3OD, 300 MHz): δ 1.00 (3H, *t*, *J*=7.0 Hz, H_3-10′), 1.33 (1H, *dqd*, *J*=15.0, 7.0, 4.0 Hz, H-8′), 1.78 (2H, *m*, H-8′, H-9′), 1.93 (1H, *ddd*, *J*=14.0, 11.0, 5.0 Hz, H-6′), 2.02 (1H, *ddd*, *J*=14.0, 11.0, 4.0 Hz, H-6′), 2.84–2.94 (2H, *m*, H-4, H-5′), 3.22 (1H, *dd*, *J*=9.0, 8.0 Hz, H-2″), 3.41 (1H, *t*, *J*=9.0 Hz, H-3″), 3.52 (1H, *ddd*, *J*=12.0, 5.0, 3.5 Hz, H-3), 3.65 (1H, *dd*, *J*=11.5, 6.0 Hz, H-6″), 3.83 (3H, *s*, OMe), 3.93 (1H, *dd*, *J*=11.5, 2.0 Hz, H-6″), 4.13 (1H, *dd*, *J*=11.0, 4.0 Hz, H-1), 4.78 (1H, d, *J*=8.0 Hz, H-1″), 5.54 (1H, d, *J*=9.0 Hz, H-1′), 6.70 (1H, *s*, H-8), 6.75 (1H, *s*, H-5), 7.52 (1H, s, H-3′). Negative ion SIMS *m*/*z*: 524 $[M-H]^-$, 362, 176. Negative ion HR–SIMS Found 524.2139 $[M-H]^-$; $C_{25}H_{34}NO_{11}$ requires 524.2133.

3.13. Conversion of 25 into 10-O-demethylprotoemetine (23) and dihydroisoalangine (26)

A solution of **25** (63.9 mg) in 5% HCl (6 ml) was heated for 5 h at 90°C and the cooled reaction mixture was adjusted to pH 6.5–7.0 with 5% $NaHCO_3$, diluted with MeOH (50 ml) and treated with $NaBH_3CN$ (75 mg). The reaction mixture was stirred for 18 h at room temperature. After removal of MeOH in vacuo, the reaction mixture was basified with 14% NH_4OH and extracted with $CHCl_3$. The washed and dried $CHCl_3$ layers were concentrated in vacuo to leave a residue (37.7 mg), which was subjected to prep. TLC ($CHCl_3$–MeOH–NH_4OH, 90:9:1), giving rise to 10-*O*-demethylprotoemetine (**23**) (5.0 mg, 14%) and dihydroisoalangine (**26**) (21.7 mg, 59%). **23**: $[\alpha]_D^{24}$ −21° (*c* 0.89, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε): 225 sh (3.84), 286 (3.54). IR ν_{max}^{KBr} cm^{-1}: 3394, 1720, 1611, 1593, 1514. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.92 (3H, *t*, *J*=7.5 Hz, H_3-13), 1.13 (1H, *ddq*, *J*=14.0, 9.0, 7.5 Hz, H-12), 1.31 (1H, *brq*, *J*=12.0 Hz, H-1ax), 1.54 (1H, *m*, H-3), 1.58 (1H, *dqd*, *J*=14.0, 7.5, 3.0 Hz, H-12), 1.95 (1H, *m*, H-2), 2.10 (1H, *m*, H-4ax), 2.32 (1H, *ddd*, *J*=17.0, 8.0, 2.0 Hz, H-α), 2.34 (1H, *m*, H-1eq), 2.54 (1H, *m*, H-6), 2.64 (1H, *brd*, *J*=16.0 Hz, H-7), 2.70 (1H, *ddd*, *J*=17.0, 3.5, 1.0 Hz, H-α), 3.01 (1H, *m*, H-6), 3.09–3.20 (3H, *m*, H-7, H-4eq, H-11b), 3.85 (3H, *s*, OMe), 6.55 (1H, *s*, H-8), 6.73 (1H, s, H-11), 9.86 (1H, *dd*, *J*=2.0, 1.0 Hz, CHO). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 11.0 (C-13), 23.7 (C-12), 29.0 (C-7), 35.8 (C-2), 38.0 (C-1), 41.2 (C-3), 48.0 (C-α), 52.5 (C-6), 55.9 (OMe), 61.0 (C-4), 62.4 (C-11b), 110.7, 110.9 (C-8, C-11), 125.5 (C-7a), 130.0 (C-11a), 143.7 (C-10), 145.1 (C-9), 202.4 (CHO). EIMS *m*/*z*: 303 $[M]^+$, 302, 274, 191, 177. HR-EIMS Found 303.1815 $[M]^+$; $C_{18}H_{25}NO_3$ requires 303.1836. **26**: $[\alpha]_D^{22}$ +71° (*c* 0.76, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε): 227 sh (3.82), 286 (3.58), 292 sh (3.52). IR ν_{max}^{KBr} cm^{-1}: 3358, 2933, 2812, 2754, 1612, 1593, 1514. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.92[2], 0.93 (3H, each *t*, *J*=7.5 Hz, H_3-12), 1.24 (1H, *q*, *J*=12.0 Hz, H-1ax), 1.32 (2H, *m*, H-13, H-14), 1.45 (1H, *m*, H-13), 1.53 (1H, *m*, H-3ax), 1.65 (1H, *brd*, *J*=13.0 Hz, H-3eq), 1.74 (1H, m, H-2), 2.16[2], 2.19 (1H, each brd, *J*=12.0 Hz, H-1eq), 2.32, 2.33[2] (1H, each *td*, *J*=12.0, 2.0 Hz, H-4ax), 2.48 (1H, *td*, *J*=11.5, 4.0 Hz, H-6ax), 2.63 (1H, *brd*, *J*=15.5 Hz, H-7eq), 2.97 (1H, *ddd*, *J*=11.5, 6.0, 1.5 Hz, H-6eq), 3.03 (2H, *m*, H-4eq, H-11b), 3.11 (1H, *ddd*, *J*=15.5, 11.5, 6.0 Hz, H-7ax), 3.62 (2H, *m*, H_2-15), 3.82 (3H, s, OMe), 6.54 (1H, *s*, H-8), 6.75[2], 6.77

[2] Represents signals due to the rotomer in the ^{1}H and ^{13}C NMR spectral data of **26** and **26a**

(1H, each *s*, H-11). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 12.1, 12.2[2] (C-12), 20.5[2], 20.6 (C-13), 28.2[2], 28.6 (C-3), 29.0 (C-7), 34.6, 34.8[2] (C-1), 36.8 (C-2), 47.1 (C-14), 52.4 (C-6), 55.8 (OMe), 56.7 (C-4), 62.5 (C-15), 62.8 (C-11b), 110.8 (C-8), 111.0, 111.1[2] (C-11), 125.7 (C-7a), 130.8 (C-11a), 143.6 (C-10), 145.2 (C-9). EIMS *m*/*z*: 305 $[M]^+$, 304, 232, 230, 191, 177. HR-EIMS Found 305.1998 $[M]^+$; $C_{18}H_{27}NO_3$ requires 305.1992.

3.14. Conversion of **25** into [4-^{2}H]-10-O-demethylprotoemetine (**23a**) and [4,11b-$^2H_2$]-dihydroisoalangine (**26a**) with $NaBD_3CN$

A solution of **25** (10.0 mg) in 5% HCl (1 ml) was heated for 5 h at 90°C and the cooled reaction mixture was adjusted to pH 6.5–7.0 with 5% $NaHCO_3$, diluted with MeOH (10 ml) and treated with $NaBD_3CN$ (12 mg). The reaction mixture was stirred for 23 h at room temperature and worked-up in the same way as for **23** and **26** to yield [4-^{2}H]-10-*O*-demethylprotoemetine (**23a**) (0.1 mg) and [4,11b-$^2H_2$]-dihydroisoalangine (**26a**) (1.4 mg). **23a**: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.92 (3H, *t*, J = 7.5 Hz, H_3-13), 1.12 (1H, *m*, H-12), 1.31 (1H, *m*, H-1ax), 1.58 (2H, *m*, H-3, H-12), 1.95 (1H, *m*, H-2), 2.33 (2H, *m*, H-1eq, H-α), 2.50–2.70 (2H, *m*, H-6, H-7), 2.70 (1H, *ddd*, J = 17.0, 3.5, 1.0 Hz, H-α), 2.95–3.28 (4H, *m*, H-6, H-7, H-4eq, H-11b), 3.85 (3H, *s*, OMe), 6.55 (1H, *s*, H-8), 6.73 (1H, *s*, H-11), 9.86 (1H, *dd*, J = 2.0, 1.0 Hz, CHO). EIMS *m*/*z*: 304 $[M]^+$, 303, 275, 192, 177. **26a**: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.94[2], 0.95 (3H, each *t*, J = 7.0 Hz, H_3-12), 1.25–1.84 (7H, *m*, H-1ax, H_2-13, H-14, H_2-3, H-2), 2.22 (1H, *m*, H-1eq), 2.52 (1H, *td*, J = 11.5, 4.0 Hz, H-6ax), 2.64 (1H, *brd*, J = 15.5 Hz, H-7eq), 3.01 (1H, *brdd*, J = 11.5, 6.0 Hz, H-6eq), 3.05 (1H, *brs*, H-4eq), 3.14 (1H, *ddd*, J = 15.5, 11.5, 6.0 Hz, H-7ax), 3.67 (2H, *m*, H_2-15), 3.85 (3H, *s*, OMe), 6.55 (1H, *s*, H-8), 6.779[2], 6.784 (1H, each *s*, H-11). EIMS *m*/*z*: 307 $[M]^+$, 234, 193, 178.

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Flavonoids and phenylpropanoid derivatives from *Campanula barbata*

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Abstract

Four new phenylpropanoid derivatives, barbatosides A–D, and a new catechin, barbatoflavan, were isolated from the whole plant of *Campanula barbata* L. (Campanulaceae) and identified as wahlenbergioside-3′-*O*-glucoside, wahlenbergioside-3′-*O*-(2‴-(*p*-methoxycinnamoyl))-glucoside, wahlenbergioside-3′-*O*-(4‴-(*trans*-*p*-coumaroyl))-glucoside, wahlenbergioside-3′-*O*-(4‴-(*cis*-*p*-coumaroyl))-glucoside and 3-acetyl-5-methoxy-7,3′,4′-trihydroxy-8-*O*-glucoside-flavan-3-ol, respectively, by spectroscopic methods. In addition, four flavonols were isolated and identified as kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside. Barbatoflavan demonstrated scavenging properties towards the DPPH radical. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Campanula barbata*; Campanulaceae; Radical scavenging activity; Flavonoids; Phenylpropanoid derivatives; Barbatosides A–D; Barbatoflavan

1. Introduction

In the course of our search for new antioxidants from higher plants, particular attention has been given to plants growing at high altitude (> 2000 m). Such species have to face more intense UV radiation and may respond to the resulting oxidative stress by producing a greater diversity of antioxidant molecules.

Campanula barbata L. (Campanulaceae) is a hairy plant of height 10–40 cm, growing in the alpine regions of central and boreal Europe. To our knowledge, no previous investigation has been done on its constituents.

In a TLC autographic assay with the methanol extract of *C. barbata* whole plant, compounds were observed which reduced the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Takao et al., 1994; Cuendet et al., 1997).

2. Results and discussion

The methanolic extract of the whole plant was fractionated by a combination of CC on silicagel, medium-pressure liquid chromatography (MPLC) and gel filtration on Sephadex LH-20 to provide compounds **1–9** (see Experimental).

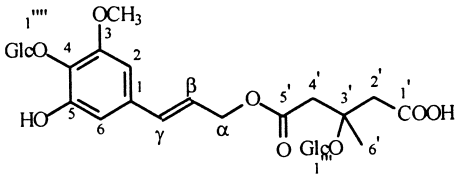
Compounds **1**–**4** were identified as kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside by comparison of their spectroscopic data (^{1}H - and ^{13}C -NMR, UV, FAB-MS) with literature values (Markham et al., 1978).

Compound **5**, named barbatoside A, was obtained as a white amorphous powder. Its UV spectrum showed absorption at λ_{max} 221 and 266 nm and suggested the presence of an aromatic system conjugated with an unsaturated side chain. Reaction with Godin reagent (Godin, 1954) gave a grey-blue colour. The ES–MS presented a signal at *m*/*z* 687, corresponding to $[M+Na]^+$. A MS/MS experiment on this quasimolecular ion gave two fragments at *m*/*z* 525 $[(M+Na)-162]^+$ and 363 $[(M+Na)-324]^+$ indicating the loss of two hexosyl groups. According to ES–MS, ^{13}C and DEPT NMR data, the molecular formula was deduced to be $C_{28}H_{40}O_{18}$. In the ^{1}H NMR spectrum, the presence of a *trans*-propenyl alcohol was indicated by a pair of olefinic signals at δ 6.55 (1H, *d*, $J = 16.0$ Hz), 6.23 (1H, *dt*, $J = 16.0$, 6.5 Hz), and a methylene doublet at δ 4.71 (2H, *d*, $J = 6.5$ Hz). Two *meta*-coupled aromatic protons of the 3,4,5-trisubstituted phenyl residue

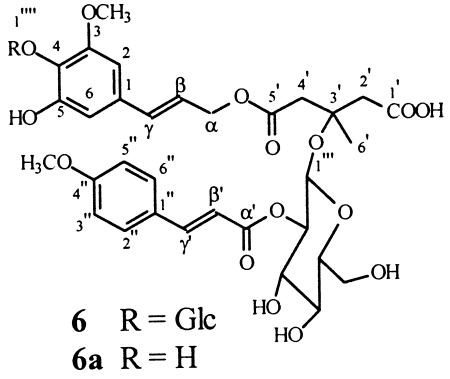
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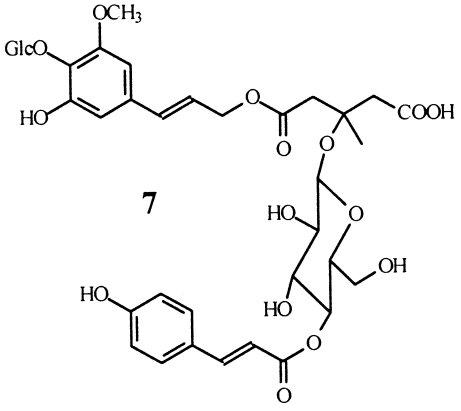
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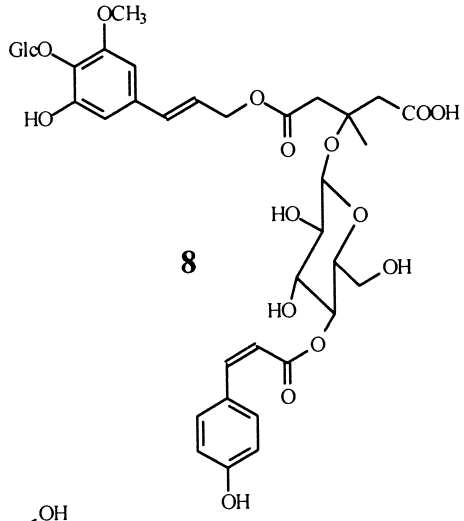
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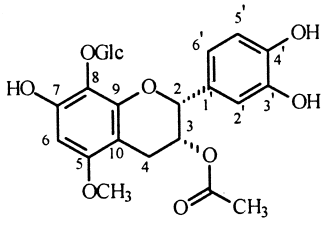
6 R = Glc
6a R = H



7



8



9

appeared at δ 6.61 (2H, *d*, $J = 3.0$ Hz). A three-proton singlet at δ 3.84 was characteristic of a methoxyl group placed on the aromatic nucleus. The remaining resonances, a methyl signal at δ 1.51 (3H, *s*), the four protons at δ 2.99 (1H, *d*, $J = 15.5$ Hz), 2.91 (1H, *d*, $J = 15.5$ Hz) and 2.85 (2H, *d*, $J = 6.5$ Hz) corresponding to two methylenes, together with the carbonyl signal at δ 172.46 in the ^{13}C NMR spectrum, demonstrated the presence of an ester derivative of a 3-hydroxyl-3-methylglutarate moiety (HMG) (Mizutani et al., 1988). The diacid moiety was found to be bound to C-α through an ester linkage, by comparing the ^{1}H NMR shifts of the methyleneoxy protons H_2-α with those reported for esterified derivatives (Mizutani et al., 1988; Yuda et al., 1990). Sugar moieties were identified as two glucoses, by comparing ^{13}C NMR data with that of the literature (Harborne and Mabry, 1982). This information suggested that **5** was a "tangshenoside type" phenylpropanoid derivative (Mizutani et al., 1988; Yuda et al., 1990). Comparison of the spectral data of **5** with those of tangshenoside I (Mizutani et al., 1988) revealed that there was only one methoxyl group in compound **5** instead of two (in positions C-3 and C-5) in the case of tangshenoside I. A similar compound, without the glucosyl moiety in position 3′, is known as wahlenbergioside (Ma et al., 1997). A GHMBC experiment confirmed the two glucose and methoxyl group positions. The stereochemistry of the HMG moiety was not established. Thus, compound **5**, named barbatoside A, is wahlenbergioside-3′-*O*-glucoside.

The reaction of **6** with Godin reagent gave a grey-blue colour and the first part of its UV spectrum showed the same absorption peaks as that of compound **5**. This suggested that **6** was also a "tangshenoside type"

phenylpropanoid derivative. The ES–MS spectrum gave a signal at m/z 823 and another at m/z 483, corresponding to the pseudomolecular ion $[M-H]^-$ and to the loss of the *trans*-propenyl alcohol moiety linked to a 3,4,5-trisubstituted phenyl residue $[(M-H)\text{-}340]^-$, respectively. In the ^1H and ^{13}C NMR spectra, all signals corresponding to compound **5** were present. However, the GHSQC spectrum showed that the C-2‴ was shifted downfield and thus this position was esterified. The substituent at C-2‴ was identified as *trans*-*p*-methoxycoumaric acid. The *trans* configuration was determined from the coupling constant $J_{\beta'\gamma'}$. Treatment with β-glucosidase cleaved the glucose moiety located at HO-C-4 to provide **6a**. The presence of a second glucose residue was demonstrated by acidic hydrolysis of **6a** and by TLC comparison of the sugar with an authentic sample. Thus, compound **6**, named barbatoside B, is wahlenbergioside-3′-*O*-(2‴-(*p*-methoxycinnamoyl))-glucoside.

The UV spectrum of **7** was almost the same as that of **6**. Moreover, this substance also had a grey-blue colour when sprayed on TLC with Godin's reagent. This suggested a structural relationship between **6** and compound **7**. A molecular mass of 810 amu was deduced from the ES–MS spectrum which exhibited a quasimolecular ion $[M+Na]^+$ at m/z 833. Moreover, the peak at m/z 493 corresponded to the loss of the *trans*-propenyl alcohol moiety linked to a 3,4,5-trisubstituted phenyl residue $[(M+Na)\text{-}340]^+$. The ^1H and ^{13}C NMR spectra of **7** were very similar to those of **6**, with the exception of the signal for the methoxyl group OCH_3–C-4″, which was missing in **7**. After enzymatic hydrolysis of **7** with β-glucosidase followed by acid hydrolysis, it was possible to conclude the presence of two glucosyl moieties, as in **6**. However, the *p*-coumaroyl group was linked at position C-4 of the C-3′ sugar because of the downfield shift observed for this proton in the ^1H NMR spectrum. Thus, compound **7**, named barbatoside C, is wahlenbergioside-3′-*O*- (4‴-(*p*-coumaroyl))- glucoside (*trans* configuration).

Compound **8** was separated from **7** by semi-preparative HPLC. Compound **7** and **8** showed the same UV and MS spectra. However, in **8**, the *p*-coumaroyl moiety was found to possess a *cis* configuration, from the ^1H NMR signals of H-β and H-γ which appeared as two *d* with a coupling constant of 12.5 Hz. Thus, compound **8**, named barbatoside D, is wahlenbergioside-3′-*O*-(4‴-(*p*-coumaroyl))-glucoside (*cis* configuration).

Phenylpropanoid derivatives with structures similar to compounds **5**–**8** have already been found in the genera *Codonopsis*, *Lobelia* and *Wahlenbergia* in the Campanulaceae family (Ishimaru et al., 1991; Lee and Ko, 1992; Ma et al., 1997). On the other hand, none possessed the *p*-coumaroyl moiety attached to the glucose at position C-3′, as in compounds **6**–**8**.

The APCI–MS spectrum of **9** showed a molecular mass of 524 amu. A fragment observed at m/z 481 $[(M-H)\text{-}42]^-$ corresponded to the loss of an acetyl moiety. The loss of a hexosyl group led to the peak at m/z 361 $[(M-H)\text{-}162]^-$. The elimination of both the sugar and the acetyl moieties explained the detected fragment at m/z 319 $[(M-H)\text{-}204]^-$. From the ^1H and ^{13}C NMR data, the molecular formula was established to be $C_{24}H_{28}O_{13}$. The ^1H NMR spectrum showed three signals in the lowfield region at δ 6.75 (1H, *d*, $J=8.0$ Hz), 6.80 (1H, *dd*, $J=8.0$, 1.5 Hz) and 7.06 (1H, *d*, $J=1.5$ Hz) indicative of a 3,4-dihydroxyphenyl moiety attached at C-2. Other signals at δ 6.16 (1H, *s*), 5.38 (1H, *br. s*), 5.01 (1H, *s*), 2.96 (1H, *dd*, $J=17.5$, 4.5 Hz), 2.84 (1H, *dd*, $J=17.5$, 2.0 Hz) corresponded to a chromane substituted in positions 3, 5, 7 and 8 (Adinolfi et al., 1989; Sakar et al., 1993). The remaining signals were attributed to a methoxyl group at δ 3.79 (3H, *s*), to a methyl at δ 1.87 (3H, *s*), and to a glucosyl moiety. The ^{13}C NMR spectrum allowed confirmation of the catechin skeleton (Bennini et al., 1993). A signal at δ 172.01 for the carbonyl group of the acetyl moiety, together with a signal for the methyl group at δ 20.77 was observed. The linkage position of the acetyl moiety was at C-3 because of the downfield shift of this carbon compared with an unsubstituted C-3 skeleton (Nonaka et al., 1983). A GHMBC experiment placed the methoxyl group in position C-5 and showed the glucosyl moiety to be linked to the hydroxyl at C-8.

Shifts obtained in the ^1H and ^{13}C NMR spectra for positions C-2 and C-3 indicated that the stereochemistry of **9** was that of epicatechin (Shen et al., 1993; Harborne, 1994). Thus, compound **9**, named barbatoflavan, is 3-acetyl-5-methoxy-7,3′,4′-trihydroxy-8-*O*-glucoside-flavan-3-ol.

Compounds **5**–**9** were tested in a spectrophotometric assay for free radical scavenging activity, using DPPH. Quercetin and BHT [2,6-di(*tert*-butyl)-4-methylphenol] were used as reference compounds. Wahlenbergioside derivatives **5**–**8** were inactive. On the other hand, **9** had an activity similar to that of quercetin (Table 3).

Antioxidant properties of catechins have been the subject of several studies due to their contribution to the antioxidant properties of black and green teas (Serafini et al., 1994). Green tea beverages have demonstrated protective effects against cardio-vasucular and liver diseases (Imai and Nakachi, 1995). Catechins were also found useful as food preservatives. These compounds prevent the formation of toxic oxygen species and peroxides in food (Nakayama et al., 1994).

3. Experimental

3.1. General

TLC: Silica gel 60 F_{254} sheets (Merck); $CHCl_3$–MeOH–H_2O (65:35:5). Open column chromatography (CC): Sephadex LH-20 (Pharmacia) and silica gel

Table 1
^{1}H NMR spectral data of compounds **5**, **6**, **7** and **8** (measured in CD_3OD). Attributions are supported by GHSQC and GHMBC data[a]

Assignment	**5**	**6**	**7**	**8**
H-2	6.61 (1H, *d*, *J* = 3.0)	6.56 (1H, *d*, *J* = 2.0)	6.63 (1H, *d*, *J* = 1.5)	6.63 (1H, *d*, *J* = 1.5)
H-6	6.61 (1H, *d*, *J* = 3.0)	6.54 (1H, *d*, *J* = 2.0)	6.61 (1H, *d*, *J* = 1.5)	6.61 (1H, *d*, *J* = 1.5)
OCH_3	3.84 (3H, *s*)	3.83 (3H, *s*)	3.85 (3H, *s*)	3.85 (3H, *s*)
H_2-α	4.71 (2H, *d*, *J* = 6.5)	4.60 (2H, *d*, *J* = 6.0)	4.73 (2H, *d*, *J* = 6.5)	4.73 (2H, *d*, *J* = 6.5)
H-β	6.23 (1H, *dt*, *J* = 16.0, 6.5)	6.14 (1H, *dt*, *J* = 16.0, 6.0)	6.26 (1H, *dt*, *J* = 16.0, 6.5)	6.26 (1H, *dt*, *J* = 16.0, 6.5)
H-γ	6.55 (1H, *d*, *J* = 16.0)	6.42 (1H, *d*, *J* = 16.0)	6.57 (1H, *d*, *J* = 16.0)	6.57 (1H, *d*, *J* = 16.0)
Ha-2′	2.99 (1H, *d*, *J* = 15.5)	2.86 (1H, *m*)	2.89 (1H, *m*)	2.89 (1H, *m*)
Hb-2′	2.85 (1H, *d*, *J* = 6.5)	2.86 (1H, *m*)	2.89 (1H, *m*)	2.89 (1H, *m*)
Ha-4′	2.91 (1H, *d*, *J* = 15.5)	2.86 (1H, *m*)	2.98 (1H, *m*)	2.98 (1H, *m*)
Hb-4′	2.85 (1H, *d*, *J* = 6.5)	2.86 (1H, *m*)	2.98 (1H, *m*)	2.98 (1H, *m*)
H-6′	1.51 (3H, *s*)	1.52 (3H, *s*)	1.50 (3H, *s*)	1.50 (3H, *s*)
H-2″	–	7.50 (1H, *d*, *J* = 8.5)	7.47 (1H, *d*, *J* = 9.0)	7.67 (1H, *d*, *J* = 9.0)
H-3″	–	6.90 (1H, *d*, *J* = 8.5)	6.81 (1H, *d*, *J* = 9.0)	6.75 (1H, *d*, *J* = 9.0)
H-5″	–	6.90 (1H, *d*, *J* = 8.5)	6.81 (1H, *d*, *J* = 9.0)	6.75 (1H, *d*, *J* = 9.0)
H-6″	–	7.50 (1H, *d*, *J* = 8.5)	7.47 (1H, *d*, *J* = 9.0)	7.67 (1H, *d*, *J* = 9.0)
OCH_3	–	3.80 (3H, *s*)	–	–
H-β′	–	6.39 (1H, *d*, *J* = 16.0)	6.36 (1H, *d*, *J* = 16.0)	5.79 (1H, *d*, *J* = 12.5)
H-γ′	–	7.65 (1H, *d*, *J* = 16.0)	7.66 (1H, *d*, *J* = 16.0)	6.90 (1H, *d*, *J* = 12.5)
H-1‴	4.60 (1H, *d*, *J* = 8.0)	4.87 (1H, *d*, *J* = 7.5)	4.68 (1H, *d*, *J* = 8.0)	4.68 (1H, *d*, *J* = 8.0)
H-2‴	3.17 (1H, *dd*, *J* = 9.0, 8.0)	4.79 (1H, *t*, *J* = 7.5)		
H-3‴	3.28 (1H, *m*)	3.59 (1H, *t*, *J* = 9.0)	3.65 (1H, *t*, *J* = 9.5)	3.65 (1H, *t*, *J* = 9.5)
H-4‴	3.28 (1H, *m*)		4.81 (1H, *m*)	4.81 (1H, *m*)
H-5‴	3.39 (1H, *m*)			
Ha-6‴	3.82 (1H, *dd*, *J* = 12.0, 2.5)	3.85 (1H, *dd*, *J* = 12.0, 2.0)	3.58 (1H, *d*, *J* = 12.0)	3.58 (1H, *d*, *J* = 12.0)
Hb-6‴	3.63 (1H, *dd*, *J* = 12.0, 4.5)	3.67 (1H, *dd*, *J* = 12.0, 4.5)		
H-1⁗	4.68 (1H, *d*, *J* = 8.0)	4.68 (1H, *d*, *J* = 7.5)	4.68 (1H, *d*, *J* = 8.0)	4.68 (1H, *d*, *J* = 8.0)
H-2⁗	3.49 (1H, *m*)			
H-3⁗	3.45 (1H, *m*)		3.42 (1H, *t*, *J* = 9.0)	3.42 (1H, *t*, *J* = 9.0)
H-4⁗	3.43 (1H, *m*)			
H-5⁗	3.28 (1H, *m*)			
Ha-6⁗	3.82 (1H, *d*, *J* = 12.0)	3.80 (1H, *dd*, *J* = 12.0, 2.5)	3.79 (1H, *dd*, *J* = 12.0, 2.5)	3.79 (1H, *dd*, *J* = 12.0, 2.5)
Hb-6⁗	3.73 (1H, *dd*, *J* = 12.0, 4.5)	3.72 (1H, *dd*, *J* = 12.0, 4.5)	3.72 (1H, *dd*, *J* = 12.0, 4.5)	3.72 (1H, *dd*, *J* = 12.0, 4.5)

[a] Proton coupling constants (*J* in Hz), signal multiplicities and integrations are in parentheses.

(63–200 μm, Merck). Medium-pressure liquid chromatography (MPLC): home-packed LiChroprep-RP-18 column (15–25 μm, 460×36 mm i.d., Merck). Anal. HPLC: Hewlett Packard 1050 instrument equipped with a photodiode array detector; Nucleosil RP-18 column (7 μm; 250×4 mm i.d., Macherey–Nagel); MeCN–H_2O (5:95→50:50 in 30 min, 0.05% CF_3COOH, 1 ml/min). UV: Shimadzu UV-160A and Perkin-Elmer Lambda-3 spectrophotometers. Mp: Mettler FP-80/82 hot-stage apparatus; uncorrected. $[\alpha]_D^{20}$: Perkin-Elmer 241 polarimeter. ^{1}H and ^{13}C NMR: Varian Inova 500 spectrometer; δ in ppm rel. to TMS; *J* in Hz; carbon multiplicities from DEPT experiments. FAB–MS: Finnigan-MAT TSQ-700 triple-stage quadrupole instrument. ES–MS, APCI–MS: MAT-LCQ ion trap instrument.

3.2. Plant material

The whole plant of *Campanula barbata* L. was collected in La Creusaz above Les Marecottes, Valais, Switzerland, in July 1996. A voucher specimen (No. 96169) has been deposited at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland.

3.3. Extraction and isolation

The dried whole plant (430 g) was ground and extracted at room temp. successively with CH_2Cl_2 (3×1500 ml) and MeOH (3×1500 ml) to yield 14 and 72 g of extract, respectively. A portion (50 g) of the MeOH extract was subjected to CC on silica gel eluted with $CHCl_3$–MeOH (9:1–1:1) to provide 15 fractions (1–15). Fraction 4 contained pure kaempferol-3-*O*-glucoside (**1**; 45 mg). Fraction 6 was purified by gel filtration on Sephadex LH-20 MeOH–H_2O (1:1) to yield **9** (68 mg). Fraction 7 was also separated by filtration on Sephadex LH-20 MeOH to give kaempferol-3-*O*-rutinoside (**2**; 140 mg) and quercetin-3-*O*-glucoside (**3**; 65 mg). Two successive filtrations on Sephadex LH-20 (MeOH and MeOH–H_2O 1:1) were made on fraction 8 to obtain fractions $8c_{1-7}$ and $8d_{1-6}$. $8c_6$ and $8d_4$ were then mixed and separated by semi-preparative HPLC (RP-18, MeCN–H_2O 27:73, flow rate 4 ml/min) affording **7** (3.5 mg) and **8** (1.5 mg). **6** (15 mg) was obtained after separation of fraction 10 by MPLC (RP-18, MeCN–H_2O 15:85). Fraction 12 was separated by MPLC (RP-18, MeOH–H_2O 20:80) to yield 13 fractions. Fractions

12e and 12i contained pure compounds **5** (120 mg) and **4** (quercetin-3-*O*-rutinoside; 180 mg), respectively.

3.4. Reduction of 2,2-diphenyl-1-picrylhydrazyl (2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl; DPPH) radical

Thirty microlitres of a soln. containing the compound to be tested and 200 μl of MeOH were added to 50 μl of a 0.02% MeOH soln. of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage of activity was calculated.

3.5. Enzymatic hydrolysis of 3, 6 and 7

One milligramme of compound with 1 mg of β-glucosidase (Sigma G-0395) in 2 ml of buffer (NaOAc 0.5 M), adjusted to pH 5 with acetic acid, was left at room temp. for 14 h; the reaction mixt. was partitioned successively with AcOEt and *n*-BuOH. The compound without the terminal glucose was obtained in the butanolic fraction.

Table 2
^{13}C NMR spectral data of compounds **5**, **6**, and **7** (measured in CD_3OD). Attributions are supported by GHSQC and GHMBC data

Carbon no.	5	6	7
1	135.05	135.11	135.12
2	108.86	108.92	108.91
3	154.20	154.22	154.28
4	135.12	135.17	135.21
5	151.84	151.88	151.94
6	103.48	103.44	103.48
OCH_3	56.75	56.75	56.75
α	66.06	65.89	66.09
β	124.17	124.47	124.19
γ	134.89	134.32	134.99
2′	44.33	44.59	44.28
3′	77.59	77.82	77.68
4′	44.33	44.59	44.28
5′	172.46	171.86	172.45
6′	24.80	24.19	24.85
1″	–	128.39	127.15
2″	–	130.98	131.26
3″	–	115.43	116.85
4″	–	163.11	161.44
5″	–	115.43	116.85
6″	–	130.98	131.26
OCH_3	–	55.88	–
α′	–	167.93	168.58
β′	–	116.47	114.83
γ′	–	146.23	147.21
1‴	98.22	96.57	98.36
2‴	75.04	75.18	75.24
3‴	77.65	76.39	75.72
4‴	71.43	71.62	72.45
5‴	77.89	77.88	75.97
6‴	62.75	62.71	62.52
1⁗	106.69	106.79	106.77
2⁗	75.28	75.35	75.35
3⁗	77.51	77.68	77.68
4⁗	70.78	70.83	70.85
5⁗	78.24	78.33	78.34
6⁗	62.05	62.08	62.09

3.6. Acid hydrolysis of 2, 4, 6 and 7

Two milligrammes of pure compound (**2** and **4**), or the residue obtained in the butanolic extract after enzymatic hydrolysis (**6** and **7**), in 10 ml 2 N HCl aqueous solution was heated at 100°C for 3 h. The reaction mixture was diluted with water (5 ml) and successively extracted with AcOEt and BuOH. A TLC with organic fractions was run with reference substances to identify the aglycone. The aqueous phase, containing free sugars, was neutralised with $NaHCO_3$ to pH 6, then dried. The residue was checked for sugars by TLC (comparison with authentic samples) using AcOEt–MeOH–H_2O–HOAc (65:15:15:20) and anisidine phthalate as the detection reagent. The presence of glucose (**2**, **4**, **6** and **7**) and rhamnose (**2** and **4**) was confirmed.

3.7. Wahlenbergioside-3′-O-glucoside (barbatoside A; 5)

Amorphous white powder. Mp 115–118°C. $[\alpha]_D^{20}$ −24° (MeOH, *c* 0.3). UV λ_{max} (MeOH) nm (log ε): 221 (4.38), 266 (4.06). ES–MS (positive ion mode) *m*/*z* 687.3 $[(M+H)+Na]^+$, 525.1 $[(M+Na)\text{-}162]^+$, 363.1 $[(M+Na)\text{-}324]^+$. ^{1}H and ^{13}C NMR : Tables 1 and 2.

3.8. Wahlenbergioside- 3′-O-(2‴-(p-methoxycinnamoyl))-glucoside (barbatoside B; 6)

Amorphous white powder. Mp 124–125°C. $[\alpha]_D^{20}$ −40 (MeOH, *c*0.1). UV λ_{max} (MeOH) nm (log ε): 222 (4.69), 271 (4.43), 309 (4.33). ES–MS (negative ion mode) *m*/*z* 823.1 $[M\text{-}H]^-$, 483 $[(M-H)\text{-}340]^-$. ^{1}H and ^{13}C NMR: Tables 1 and 2.

3.9. Wahlenbergioside-3′-O-(4‴-p-coumaroyl)-glucoside (trans) (barbatoside C; 7)

Amorphous white powder. Mp 121–122°C. UV λ_{max} (MeOH) nm (log ε): 222 (5.71), 266 (4.48), 314 (4.32). ES–MS (positive ion mode) *m*/*z* 833.0 $[(M+H)+Na]^+$, 493 $[(M+Na)\text{-}340]^+$. ^{1}H and ^{13}C NMR: Tables 1 and 2.

Table 3
Percentage of DPPH radical reduction in a spectrophotometric assay caused by compound **9** at different concentrations. Measurement at 517 nm, determination after 30 min

Concentration (μM)	Quercetin	BHT	9
80.0	97.1	41.3	95.9
40.0	96.5	29.5	89.7
20.0	94.9	14.9	49.9
10.0	53.7	8.1	26.5
5.0	25.8	3.3	16.2
2.5	15.3	3.3	8.8

3.10. *Wahlenbergioside-3′-O-(4‴-*p*-coumaroyl)-glucoside* (cis) *(barbatoside D;* ***8****)*

Amorphous white powder. ^{1}H NMR: Table 1.

3.11. *3-Acetyl-5-methoxyl-7,3′,4′-trihydroxyl-8-O-glucoside-flavan-3-ol (barbatoflavan;* ***9****)*

Amorphous cream powder. Mp 143–145°C. $[\alpha]_D^{20}$ −78 (MeOH, *c* 0.2). UV λ_{max} (MeOH) nm (log ε): 230 (sh) (4.02), 281 (3.54). APCI–MS (negative ion mode) *m/z* 523.1 $[M-H]^-$, 481.3 $[(M-H)-42]^-$, 361.4 $[(M-H)-162]^-$, 319.5 $[(M-H)-204]^-$. ^{1}H NMR (CD_3OD) : 7.06 (1H, *d*, *J* = 1.5 Hz, H-2′); 6.80 (1H, *dd*, *J* = 8.0, 1.5 Hz, H-6′); 6.75 (1H, *d*, *J* = 8.0 Hz, H-5′); 6.16 (1H, *s*, H-6); 5.38 (1H, br. *s*, H-3); 5.01 (1H, *s*, H-2); 4.68 (1H, *d*, *J* = 7.5 Hz, H-1″); 3.79 (3H, *s*, OCH_3); 3.76 (2H, *m*, H-6″); 3.39–3.48 (3H, non resolved, H-2″, H-3″, H-4″); 3.24 (1H, *m*, H-5″); 2.96 (1H, *dd*, *J* = 17.5, 4.5 Hz, Ha-4); 2.84 (1H, *dd*, *J* = 17.5, 2.0 Hz, Hb-4); 1.87 (3H, *s*, CH_3). ^{13}C NMR (CD_3OD): 172.01 (CO); 153.33 (C-7); 152.55 (C-5); 149.43 (C-9); 146.01 (C-4′); 145.98 (C-3′); 130.92 (C-1′); 128.68 (C-8); 119.04 (C-6′); 115.98 (C-5′); 115.01 (C-2′); 106.66 (C-1″); 101.16 (C-10); 93.79 (C-6); 78.38 (C-2); 78.00 (C-5″); 77.66 (C-3″); 75.75 (C-2″); 71.13 (C-4″); 69.33 (C-3); 62.51 (C-6″); 56.83 (OCH_3); 26.62 (C-4); 20.77 (CH_3).

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Alkaloids from *Crinum moorei*

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Abstract

Thirteen alkaloids were isolated from *Crinum moorei* two of which are new. These are 3-[4′-(8′-aminoethyl)phenoxy] bulbispermine and mooreine. The structures of the new alkaloids were determined by spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Crinum moorei*; Amaryllidaceae; Alkaloids; 3-[4′-(8′-Amino ethyl)phenoxy] bulbispermine; Mooreine

1. Introduction

Crinum moorei (Amaryllidaceae) is one of 21 *Crinum* species found in southern Africa (Verdoorn, 1973). *Crinum* species are used by Zulu healers against swelling of the body and for urinary tract problems (Hutchings et al., 1996). We report here on the isolation of the new compounds 3-[4′-(8′-aminoethyl)phenoxy] bulbispermine **1**, and **2** which we have designated as mooreine, systematic name [1,3]dioxolo [4,5-j]pyrrolo [3,2,1-de] phenanthridinum, 1,2,4,5,12b, 12c-hexahydro-2-hydroxy-12c-methoxy (Tables 1 and 2). Also isolated from this species were the known compounds crinine, undulatine (Viladomat et al., 1995a), 3-*O*-acetylcrinine (Campbell et al., 1998), powelline, cherylline (Kobayashi et al., 1984), crinamidine, epibuphanisine, epivittatine (Viladomat et al., 1995b), 1-epideacetyl-bowdensine (Viladomat et al., 1996), lycorine, and 1-*O*-acetyllycorine (Evidente et al., 1983).

2. Results and discussion

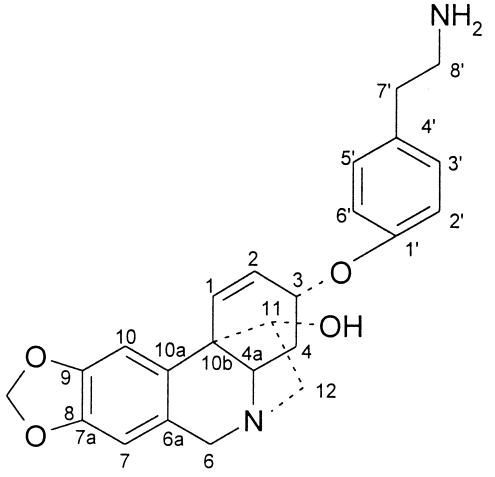
The ^{1}H NMR of **1** showed the typical singlets associated with H-7, H-10, and the methylenedioxy group at 6.86, 6.53 and 5.87, respectively. From the HETCOR and COSY analysis, the resonance of H-3 and H-11 could be pinpointed at δ 4.40 and 3.95, respectively. The first is clearly coupled to H-1 and H-2 and also to the methylene group at C-4 while the second is coupled to the methylene group at 3.32. These were the only likely positions to which the tyramine residue- whose presence was clearly indicated by the A_2X_2 system present at δ 6.73 and δ 7.05 — could be attached. HMBC and NOESY were not helpful and no connectivities were observed between H-11 or H-3 and either C-6′ and C-2′ or C-7′ and C-8′. Also no NOE effects were observed between protons on the basic ring and the phenethyl moiety. Acetylation of the compound resulted in a downfield shift of both H-3 and H-11 to around 5.48 and 4.97 ppm, respectively. This indicates that acetylation occurred at both positions which further proved by the molecular ion of the acetate of 371 mass unit with the loss of 60 and 43 mass units, respectively. The possibility of the compound being a mixture of two compounds was rejected by developing bulbispermine and tyramine on a tlc using the same solvent system used for the purification of 1. Tyramine has R_f value of 0.67 while bulbispermine R_f value of 0.59. From the above acetylation results, it is clear that the compound undergone degradation at some stage during storage or acetylation. NOE studies did not reveal much useful information but did show a good correlation between H-11 *exo* and H-12 *exo*. Construction of an accurate model using the NOE information showed, however, that the 3- and 11-positions were not sterically crowded

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and equally exposed to attack by a tyramine moiety. In these circumstances, the tyramine residue was attached at the more reactive allylic C-3. In addition, computer simulation programme ^{1}H NMR data, using Advanced Chemistry Development programme (ACD) from Aldrich 1998, supported the attachment at C-3, as indicated in Table 3. It is suggested that the linkage to C-3 is via the phenolic group of the tyramine moiety. This is based on the observation that in both plicamine and secoplicamine (both bonded through N), C-8′ resonates in the ^{13}C spectrum at 49.0 and 50.0 ppm, respectively (Ünver et al., 1999) whereas in 1 the relevant chemical shift is at 44.0 ppm. In addition, the shift of the ethylene group in authentic tyramine (36.7 and 43.4 for C-7′ and C-8′, respectively) correspond closely to that observed for the corresponding group in compound 1. Furthermore, acetylation of tyramine at both C-1 and NH_2 showed only



Compound **1**

Table 1
^{1}H NMR data (CD_3OD) for compound **1**, and bulbispermine

	1	Bulbispermine
H-2′, H-6′	7.05 *d* (*J* = 8.5)	
H-7	6.86 *s*	6.83 *s*
H-3′, H-5′	6.73 *d* (*J* = 8.6)	
H-10	6.53 *s*	6.48 *s*
H-2	6.24 *dd* (*J* = 2.3, 10.3)	6.23 *dd* (*J* = 2.2, 10.35)
H-1	6.05 *dd* (*J* = 1.2, 10.3)	6.02 *d* (*J* = 10.35)
OCH_2O	5.87 *s*	5.86 *s*
H-3	4.40 *m*	4.3d *dd* (*J* = 2,4,8)
H-6α	4.27 *d* (*J* = 16.9)	4.22 *d* (*J* = 16.9)
H-11	3.95 *dd* (*J* = 3.6,7)	3.93 *dd* (*J* = 2.9, 7)
H-6α	3.72 *d* (*J* = 16.9)	3.68 *d* (*J* = 16.9)
H-12		3.41 *dd* (1H, *J* = 7, 13.69)
H-12′	3.32 *m* (2H)	3.32 *m* (1H)
H-4a	3.32 *m*	3.20 *m*
2H-8′	2.90 *t* (*J* = 7)	
2H-7′	2.70 *t* (*J* = 7)	
2H-4	2.02 *m*	2.03 *m*

Table 2
^{13}C NMR data (CD_3OD) for compound **1**, and bulbispermine

C no.	**1**	Bulbispermine
C-1′	157.4 *s*	
C-9	148.4 *s*	148.3 *s*
C-8	147.9 *s*	147.9 *s*
C-10a	137.7 *s*	137.7 *s*
C-1	137.4 *d*	137.4 *d*
C-2′, C-6′	131.0 *d*	
C-4−	130.9 *s*	
C-6a	127.1 *s*	127.1 *s*
C-2	125.2 *d*	125.2 *d*
C-3′, C-5′	116.7 *d*	
C-10	108.1 *d*	108.1 *d*
C-7	104.5 *d*	104.5 *d*
OCH_2O	102.4 *t*	102.5 *t*
C-11	81.3 *d*	81.3 *d*
C-3	68.6 *d*	68.6 *d*
C-4a	67.7 *d*	67.6 *d*
C-12	63.9 *t*	64.0 *t*
C-6	61.8 *t*	61.7 *t*
C-10b	51.7 *s*	51.6 *s*
C-8′	44.0 *t*	
C-7′	37.9 *t*	
C-4	34.7 *t*	34.7 *t*

Table 3
Computer simulation programme ^{1}H NMR chemical shifts of Protons at positions 3 and 11 for bulbispermine and bulbispermine with the tyramine attached either to C-3 or C-11

Proton no.	Bulbispermine	Bulbispermine with tyramine moiety attached to	
		C-3	C-11
3-H	4.37	4.68	4.21
11-H	3.91	3.91	5.12

Table 4
^{1}H NMR and ^{13}C NMR (CD_3OD) for compound **2**

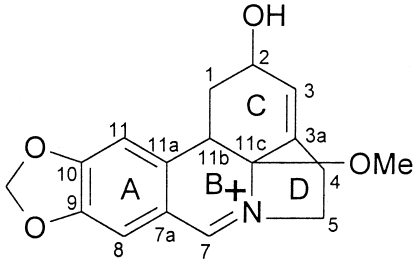
Atom no.	^{13}C	^{1}H
9	158.5 *s*	–
10	152.8 *s*	–
7	142.1 *d*	9.36 *s*
11a	139.3 *s*	–
7a	134.2 *s*	–
3a	128.4	–
3	127.6 *d*	6.45 *m*
8	106.1 *d*	7.63 *s*
OCH_2O	105.6 *t*	6.38
11	100.8 *d*	7.77 *s*
11c	79.4 *s*	–
2	72.0 *d*	4.61 *m*
11b	68.9 *d*	5.31 *m*
5	57.1 *t*	5.00 *m* (2H)
1	54.8 *t*	5.49 *ddd* (0.69, 3.8, 5.3)
		5.48 *ddd* (0.46, 1.14, 5.3)
4	26.5 *t*	3.32 *m* (2H)
OMe	48.0	3.32 *s* (3H)

0.15 ppm dowfield shift of protons at C-2′ and C-6′ compared to 0.25 ppm downfield shift of protons at C-3′ and C-5′. Protons attached to C-8′ also shifted downfield by 0.28 ppm. Chemical shifts of C-2′, C-6′ remained unaffected while that of 3′, 5′ were shifted downfield by 6 ppm.

The compound gave no molecular ion in its HRMS. However, it showed a fragment ion at 286.1058 ($C_{16}H_{16}NO_4$) which is the molecular ion of bulbispermine after loss of the tyramine moiety.

Compound **2** under normal conditions of electron bombardment showed no molecular ion. However, using an electrospray instrument it exhibited a molecular ion at m/z 300. This corresponds to the proposed molecular formula of C_{17} $H_{18}NO_4$ The ^{1}H NMR and ^{13}C NMR (Table 4) for ring A, B, D closely resemble those reported for the anhydrolycorinium ion (Petitt et al., 1984). The ^{1}H NMR showed four singlets at 9.36, 7.77, 7.63 and 6.38 assigned to H-7, H-11, H-8 and the methylenedioxy, respectively. It also showed multiplets at 6.45, 5.31, 5.00, 4.61 and 3.32 assigned to H-3, H-11b, H-5, H-2 and H-4, respectively. The two *ddd* at 5.49 and 5.48 were assigned to the two protons at position 1. The multiplicities of the peaks relating to the aliphatic portion of the molecule were completely assigned by COSY analysis. It showed a correlation between H-2 (δ 4.61) and the one proton multiplet of H-3 at δ 6.41 and the ddd of H-1β and H-1α at 5.49 and 5.48. In addition, there was a correlation between the *ddd* of both protons at position 1 and that of H-11b at δ 5.31. The assignment of the signal at 5.31 to H-11b was supported by the long range coupling between the signal at 5.31 and that of H-11 (7.77). The deshielding of C-5 and C-4 protons is due to their α- and β-positions with respect to the nitrogen of the salt (Bastida et al., 1992).

The ^{13}C NMR showed four methine sp^2 carbons in the aromatic region at 142, 127, 105 and 101 ppm assigned to C-7, C-3, C-8, and C-11. The chemical shifts of the six quaternary carbons C-3a, C-7a, C-9, C-10, C-11a and C-11c are shown in Table 4. The placement of the methoxy group on C-11c was based on the chemical shift of this carbon atom and was in good agreement with predictions for ^{13}C chemical shifts derived from a commercially available modelling program. Lack of material and marked instability prevented a more detailed analysis of the compound and the proposed structure should be regarded as tentative.



compound **2**

3. Experimental

NMR spectra were recorded in $CDCl_3$ and CD_3OD using TMS as internal standard at 500 and 200 MHz for ^{1}H and 50 and 125 MHz for ^{13}C. Chemical shifts are recorded in δ units and coupling constants (*J*) in Hz. Mass spectra were recorded on a Kratos MS 80 RF double-focussing magnetic sector instrument at 70 eV. Silica gel Merck (230–400 mesh) was used for VLC . Silica gel 60 F_{254} analytical and prep. TLC (2 mm) were used for additional separation. Spot detection by UV light (254) and Dragendorff's reagent.

3.1. Plant material

Crinum moorei (Hook f.) plants were obtained in December 1997 from Green Goblin Nursery, Durban, South Africa and their identity confirmed by Dr. T.J. Edwards, School of Botany and Zoology, University of Natal Pietermaritzburg. A voucher specimen (Elgorashi2 NU) was deposited in the University of Natal Herbarium, Pietermaritzburg.

3.2. Extraction and isolation

The dried and powdered non flowering whole plants (62.5 g) were extracted with Petr. ether (60–80°) and 95% EtOH for 40 h each using a Soxhlet apparatus. The ethanolic extract was evaporated under reduced pressure and the residue was treated with 4% aq. HOAc. The aqueous acidic solution was filtered. The solution was basified with NH_4OH to pH 9.5 after removal of neutral material with Et_2O. The basified solution was extracted with Et_2O, EtOAc, and *n*-BuOH to give fraction A, B, C, respectively (Ghosal et al., 1983).

Fractions A and B were combined and kept overnight in MeOH at room temp. to give lycorine as a powder (81 mg). The remaining crude extract subjected to VLC on silica gel eluting with $CHCl_3$ and then with $CHCl_3$ enriched gradually with MeOH up to 50% MeOH to give five fractions. Fraction I was developed on PLC (2 mm) using $CHCl_3$–MeOH (9:1) which were developed again using $CHCl_3$–Et_2NH (20:1), $CHCl_3$–Et_2NH (40:1), benzene–MeOH (9:1) to give epibuphanisine (56 mg), 1-*O*-acetyllycorine (32 mg), undulatine (11 mg) and 3-*O*-acetylcrinine (8 mg).

Fraction II was developed on TLC using $CHCl_3$–Et_2NH (20:1) to give two bands which were developed further on $CHCl_3$–Et_2NH (40:1) twice to give epivittatine (53 mg), cherylline (35 mg) and $CHCl_3$–MeOH (10:1) to give crinamidine (7 mg). Fractions III, IV, V were developed on PLC using $CHCl_3$–Et_2NH (20:1) to

give powelline (20 mg), crinine (36 mg), and 1-epideacetylbowdenisine (16 mg).

Fraction C was subjected to VLC using silica gel eluting with $CHCl_3$ enriched gradually with MeOH to give two fractions. Fraction I was developed on TLC using $CHCl_3$–MeOH (2:1) to give compound 2 (7 mg). Fraction II was developed on PLC (2 mm) using $CHCl_3$–CH_2Cl_2–EtOH–MeOH (7:7:7:4) and NH_3 vapour to give compound **1** (11 mg).

3.2.1. Conversion of tyramine to its acetate

Twenty-nine mg of tyramine were heated with equal volumes of pyridine and acetic anhydride at 60°C for half an hour and kept overnight at room temp. to give tyramine-diacetate (39 mg). ^{1}H NMR (CD_3OD): 7.26 *d* (8.4, H-2′ and H-6′), 7.04 *d* (8.4, H-3′ and H-5′), 3.41 *t* (7.5, 2H-8′), 2.81 *t* (7.5, 2H-7′), 2Me (*s*, 2.02 and 1.93). ^{13}C NMR (CD_3OD): 173.2 *s* and 171.4 *s* (2 O$\underline{C}$OCH_3), 150.7 *s* (C-1′), 130.7 *d* (C-2′ and C-6′), 138.9 *s* (C-4′), 122.6 *d* (C-3′ and C-5′), 41.9 *t* (C-8′), 35.7 *t* (C-7′), 22.5 and 20.9 *q* (2Me).

3.2.2. Tyramine

^{1}H NMR (CD_3OD): 7.11 *d* (8.42, H-2′ and H-6′), 6.79 *d* (8.42, H-3′ and H-5′), 3.13 *t* (7.14, 2H-8′), 2.88 *t* (7.09, 2H-7′). ^{13}C NMR (CD_3OD): 157.2 *s* (C-1′), 130.7 *d* (C-2′ and C-6′), 130.9 *s* (C-4′), 116.4 *d* (C-3′ and C-5′), 43.4t (C-8′), 36.7 *t* (C-7′)

3.2.3. Compound *1*

Amorphous compound (11 mg)$[\alpha]_D^{29} + 53.3°$ ($CHCl_3$, *c*, 0.045) ^{1}H and ^{13}CNMR (see Tables 1 and 2). GC–MS 70 eV, *m*/*z* (% rel. int.): 286 (< 1%) 258 (100), 248 (26), 186 (18), 129 (9), 115 (14), 107 (11), 44 (35), 43 (56), 42 (17).

3.2.4. Compound *2*

Amorphous compound (7 mg) ^{1}H and ^{13}C NMR (see Table 4), electrospray (% rel. int.): 300 [M^+, 5), 292 (5), 284 (100), 266 (5).

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PHYTOCHEMISTRY

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Book reviews

Allelopathy Update. Volume 1, International Status, Volume 2, Basic and Applied Aspects
Edited by S.S. Narwal, Science Publishers, Enfield, New Hampshire, USA, 1999. 335 pp. and 348 pp. ISBN 1-57808-063-0, £91.00 the set

Allelopathy, defined as hostile biochemical interactions between one higher plant and another, has been a controversial area of plant science which has taken a long time to develop. Relatively few good examples have been discovered in the field where there is unequivocable evidence supporting the harmful effect of one plant species on its neighbour. There is the case of the walnut tree and its allelochemical juglone. There are also the shrubby plants of the California chapparal and of the Florida scrub community with their terpenoids and phenolics investigated by Muller, Fischer and others. This early work was reviewed by Elroy Rice in his textbook first published in 1974 and revised later in 1984. Since then, there have been few publications dealing with allelopathy, but none has provided a general overview. The present two volumes are intended to update Rice's classic book and cover in part the research carried out from the 1980s up to the turn of the century.

The first of these two volumes is essentially a geographical survey of allelopathic research country by country. It is a curious concept since allelopathic interactions do not confine themselves to country boundaries. Unfortunately, too, there is a serious gap in that much of the best research is carried out in North America but the editor was unable to persuade any scientists from those countries to participate. The UK, Germany and France are also incidentally missing. The European contributions come from the Czech Republic, Greece, Hungary, Italy, Poland, Portugal and Spain. Allelopathic research in Asia is centred on India, Iraq, Japan, Jordan, Korea, Mauritius, Philippines, while the South American chapters are restricted to Brazil and Costa Rica. Not surprisingly, these chapters are of a variable quality and deal with a number of topics of marginal relevance to the phenomenon of allelopathy. Agricultural problems and the effects of weeds on crop plants dominate. Work in progress is illustrated in tabular form and each chapter has an extensive reference list.

The second volume contains 16 chapters which deal with allelopathic interactions in grassland, desert and temperate systems, with some emphasis on the effects of weedy species on crop plants. There are two chapters on insect resistance in plants which seem out of place here. the main problem with many of the recent experiments reported in volume 2 is the relatively uncritical assumption that allelochemicals reported in one plant species are able to survive in the soil long enough to exert an effect on a neighbour or that the chemical effect on growth or seed germination occurs in the appropriate receiver species. Reliance is still placed on the inhibition of lettuce seed, in spite of the fact this is rarely relevant to the field situation. My conclusion from scanning these contributions is that the standard of phytochemistry is of a good quality, but that there is still a serious lack of critical assessment of the biological and microbial aspects of these potentially interesting allelopathic interactions.

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Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization
H.R. Lerner (Ed.). Marcel Dekker, New York, Basel, 1999, 730 pp., ISBN 0-8247-0044-9

The book entitled *Plant Responses to Environmental Stresses from Phytohormones to Genome Reorganization*, edited by H.R. Lerner, provides an excellent overview of the mechanisms of plant stress response. A wide

range of different mechanisms that play a central role in the adaptation processes of plant to different types of stresses are described, including transitional physiological and biochemical changes, variations in plant hormone levels, transcriptional and post-transcriptional regulation and even reorganization of the genome.

The book illustrates how many different processes lead to the creation of a new physiological mode in which the plant can more quickly adapt to stress. Chapter 3 deals with the adaptation of plant cell cultures to stress factors, while chapters 5 and 6 concentrate on similar responses in bacteria and cyanobacteria. Exploration of adaptation mechanisms in procaryotes is useful as these are important as model organisms. However, the parallels between pro- and eukaryotic processes have not always been brought into sharp focus.

Chapter 4 provides a very good background for understanding basic rules of stress managment in eukaryotes by transcriptional regulation, including the role of signal integration networks and transcription factors.

The other chapters give a many-faceted description of the genetic, biochemical and physiological processes in higher plants.

Chapters 7–9 present an interesting look at the effects of stress on epigenetic stability. This kind of holistic approach to understanding biological processes is of ever increasing importance. Chapters 10 and 11 continue in the same vein, exploring the mechanisms of evolution in plants, one of the most dynamic fields in modern biology.

In both "red" and "green" biology, the origin and effect of oxidative stress and its influence on the genome, is of great interest. Chapter 11 gives a good summary of the latest data in this field. However, in my opinion, the role of bacterial and fungal plant pathogens as a source of active oxygen has not been adequately dealt with.

The role of plant hormones, especially ABA, in the regulation of gene expression during plant stress (chapters 13–15) is extremely well described. Chapter 14 summarizes the newest data on the molecular mechanisms of ABA-dependent and ABA-independent gene regulation in response to wounding, oxidative stress, low temperature, drought, CO_2 concentration, interaction with pathogens and high salt concentations.

One of the most characteristic responses of plants to conditions of stress is the development of chlorosis. This reflects changes in photosynthetic capacity due to variation in the expression of photosynthetic genes. Possible signals involved in the regulation of these genes are discussed (chapter 21).

In summary, this book can be presented as an excellent tool for both students and scientists involved in plant research on physiological, biochemical, genetic or molecular genetic levels.

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Book reviews

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Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization
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The book entitled *Plant Responses to Environmental Stresses from Phytohormones to Genome Reorganization*, edited by H.R. Lerner, provides an excellent overview of the mechanisms of plant stress response. A wide

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Agmatine deiminase from maize shoots: purification and properties

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Abstract

Agmatine deiminase was purified to a specific activity of 537 nkat/mg protein using an improved procedure. The recovery was 47% and the enzyme was homogeneous and remarkably stable. The molecular mass of the enzyme as determined by gel filtration was 75 kDa, and SDS-PAGE suggests that the enzyme is a hetero climer composed of subunits of 43.5 and 44 kDa. The K_m for agmatine was 12 μM and arcaine was shown to be a potent competitive inhibitor of the enzyme, with a K_i of 3.3 μM. The enzyme does not have either putrescine synthase activity or the activities of its components ornithine and putrescine transcarbamylase. These results distinctly demonstrate that agmatine deiminase is different from putrescine synthase. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Zea mays; Maize; Agmatine deiminase; Putrescine synthase; Enzyme purification

1. Introduction

Polyamines are synthesized from arginine and ornithine in higher plants (Smith, 1990). In the arginine pathway, agmatine deiminase (EC 3.5.3.12) hydrolyzes agmatine to *N*-carbamoylputrescine and ammonia (Smith, 1969). Agmatine deiminase has been purified to homogeneity from maize shoots (Yanagisawa and Suzuki, 1981), rice (Chaudhuri and Gosh, 1985), and soybean (Park and Cho, 1990). On the other hand, putrescine synthase has been purified to homogeneity from grass pea (Srivenugopal and Adiga, 1981) and cucumber (Prasad and Adiga, 1986). Putrescine synthase has activities similar to those of agmatine deiminase, putrescine transcarbamylase, ornithine transcarb amylase, and carbamate kinase in a single polypeptide, and converts agmatine and ornithine to ammonia, putrescine, and citrulline in the presence of inorganic phosphate. Prior to this study, little was known about the kinetics of agmatine deiminase, although it was reported in studies with soybean that this enzyme was a monomer similar to putrescine synthase. However, it was uncertain whether agmatine deiminase was putrescine synthase itself or clearly different from putrescine synthase.

In this paper, the characteristics of agmatine deiminase purified from maize shoots by an improved procedure were examined, and compared with those of agmatine deiminase and putrescine synthase from other plants.

2. Results and discussion

2.1. Agmatine deiminase assays

Agmatine deiminase activity was previously determined by measuring the ammonia released from agmatine in Conway microdiffusion units with Nessler reagent (Yanagisawa and Suzuki, 1981); however, this procedure is insensitive. Moreover, ammonia is released from agmatine by Cu-containing amine oxidase in higher plants, and the estimation of agmatine deiminase activity by measuring the ammonia released from agmatine with extracts including Cu-containing amine oxidase is not accurate. Cu-containing amine oxidase has been detected in several species of leguminous and dicotyledonous plants (Suzuki et al., 1990), and recently in monocotyledonous plants including maize (Cogoni et al., 1990; Suzuki and Hagiwara, 1993). Therefore,

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agmatine deiminase activity was determined by measuring *N*-carbamoylputrescine by the diacetyl monoxime method (Boyde and Rahmatullah, 1980). When measuring the enzyme activity of plant extracts and in an early purification step that involved both agmatine deiminase and Cu-containing amine oxidase, 1 mM sodium diethyldithiocarbamate was added to the reaction mixture. This reagent is a potent inhibitor of Cu-containing amine oxidase and did not influence either the enzyme activity or the colorimetric reaction.

2.2. Effect of dark and light on activities of agmatine deiminase from maize shoots

Agmatine deiminase activities of maize shoots grown for 7 days in the dark and light at 25°C were 0.25 and 0.28 nkat/shoot, respectively. No significant difference was found between the enzyme activities of maize shoots grown in the dark and light for 7 days.

2.3. Purification of agmatine deiminase

Agmatine deiminase from maize shoots grown for 7 days in the dark was purified by a DE52 batchwise method, DE52 column chromatography, Sephadex G-100 column chromatography, and agmatine-epoxy-Sepharose 6B column chromatography. The purification procedure was largely the same as that described previously (Yanagisawa and Suzuki, 1981), but with the following major modifications: 1 mM phenylmethyl sulfonyl fluoride (PMSF) and EDTA were added to the extraction buffer; and the affinity adsorbent was changed from agmatine–succinic acid-hexamethylenediamine-Sepharose 4B to agmatine-epoxy-Sepharose 6B. The enzyme was purified to a specific activity of 537 nkat/mg protein, and obtained in 47% yield (Table 1). The enzyme was previously purified to a specific activity of 230 nkat/mg protein and lost 30% of its original activity after 3 days at 4°C (Yanagisawa and Suzuki, 1981). However, the enzyme purified with the improved procedure was stable for two months at 4°C without any loss of activity. The increase in specific activity and the outstanding stability of the enzyme suggests that the enzyme purified with the improved procedure has not been modified during purification.

2.4. Kinetics of agmatine deiminase

The enzyme reactions were linear with respect to time and enzyme concentrations under standard assay conditions. The optimal pH of the enzyme purified by the improved procedure was 6.5, with 85% of the maximum activity at pH 6.0 and 8.0. The enzyme showed a Michaelis–Menten-type saturation curve. A K_m for agmatine of 12 μM was obtained from double-reciprocal plots (Fig. 1). It has been reported that the K_m values for agmatine of the enzymes previously purified from maize shoots (Yanagisawa and Suzuki, 1981) and soybean axes (Park and Cho, 1990) were 0.19 and 5 mM, respectively. The high K_m values reported previously might be due to an inaccurate estimation of the enzyme activity and the long reaction time. The difference in the specificity of agmatine against agmatine

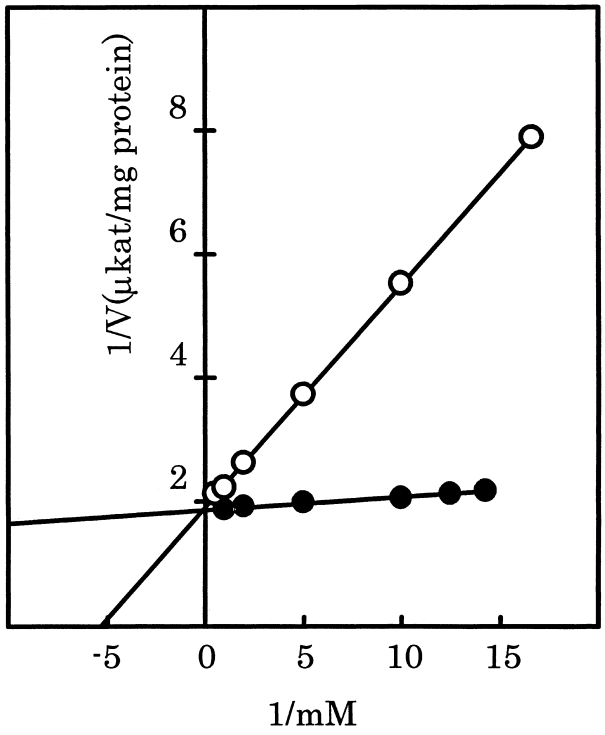


Fig. 1. Competitive inhibition of agmatine deiminase from maize shoots by arcaine. Control (closed circles), in the presence of 50 μM arcaine sulfate (open circles): 50 ng of the enzyme was used.

Table 1
Purification of agmatine deiminase from maize shoots

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
1. Crude extract	603	107	0.178	1	100
2. Eluate from DE52	90.5	92.9	1.03	5.75	86.4
3. Eluate from DE52 column	18.8	87.1	4.63	26	81
4. Eluate from Sephadex G-100 column	2.61	68.2	26.2	147	63.5
5. Eluate from affinity column	0.0944	50.7	537	3010	47.2

deiminase and putrescine synthase could not be determined, since the K_m for agmatine of putrescine synthase has not yet been reported. Arcaine, an analogue of agmatine, is reportedly a potent inhibitor of the enzyme (Smith, 1969; Yanagisawa and Suzuki, 1981), and this inhibition is competitive, with a K_i for arcaine of 3.3 μM (Fig. 1). It is unclear whether agmatine deiminase purified from soybean and rice and putrescine synthase would also be inhibited by arcaine.

2.5. Subunit structure of agmatine deiminase

The molecular mass of enzyme purified by the improved procedure was determined to be 75 kDa by the gel filtration method with Sephadex G-100 column chromatography. SDS-PAGE of the enzyme showed very close polypeptides of 43.5 and 44 kDa (Fig. 2). Sugar moieties were not found in the enzyme as evidenced by the phenol sulfuric acid method. These results suggest that the enzyme is a hetero dimer, although those from maize (Yanagisawa and Suzuki, 1981) and rice (Chaudhuri and Gosh, 1985) were reported to be dimers, and that from soybean was reported to be a monomer (Park and Cho, 1990).

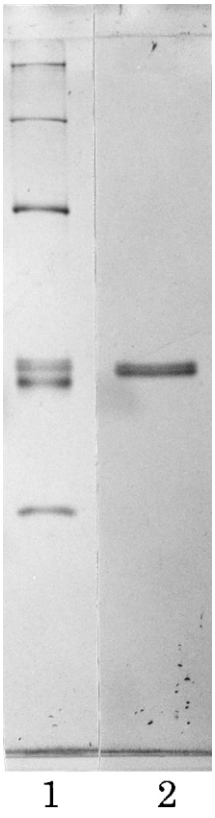


Fig. 2. SDS-PAGE of agmatine deiminase from maize shoots. Line 1, agmatine deiminase with standard proteins: myosin (200 kDa), β-galactosidase (116 kDa), bovine albumin (66.3 kDa), aldolase (42.4 kDa), and carbonic anhydrase (30 kDa); line 2, agmatine deiminase.

2.6. Putrescine synthase activity

Putrescine synthase, purified to homogeneity from grass pea (Srivenugopal and Adiga, 1981) and cucumber (Prasad and Adiga, 1986), exhibited agmatine deiminase activity of 0.39 and 0.73 nkat/mg protein, respectively. This enzyme was highly unstable: grass pea putrescine synthase lost its component activities within 48 h at 4°C after purification. Agmatine deiminase purified from maize did not have the activities of putrescine synthase (citrulline formation), putrescine transcarbamylase (*N*-carbamoylputrescine formation), or ornithine transcarbamylase (citrulline formation).

These results distinctly demonstrate that agmatine deiminase differs from putrescine synthase.

3. Experimental

3.1. Chemicals

N-Carbamoylputrescine was prepared by the method of Smith and Garraway (1964). Further purification of *N*-carbamoylputrescine by Amberlite CG-50 Na^+ (type 2) column chromatography to remove *N*,*N*′-dicarbamoylputrescine and putrescine contamination was carried out as described by Akamatsu et al. (1978). *N*-Carbamoylputrescine (mp 185°C) was finally recrystallized from EtOH. Agmatine sulfate and arcaine sulfate were purchased from Sigma Chemicals. All other chemicals were of analytical reagent grade. Agmatine-epoxy-Sepharose 6B was prepared by coupling agmatine sulfate (0.2 g) to epoxy-activated Sepharose 6B (7 g) in 50 ml of 0.1 M Na_2CO_3 at room temperature for 24 h.

3.2. Plant materials

Seeds of maize (*Zea mays* L. cv. Goldencross Bantam T51) were soaked in water for 6 h at 25°C and then germinated in moist vermiculite in the dark or continuous light (ca 3000 lux, at plant level). The excised shoots were sterilized with 0.1% benzalkonium chloride for 10 min and thoroughly washed with deionized water.

3.3. Plant extracts

To examine the effect of dark and light on enzyme activity, the shoots (ca 5 g) grown in the dark and light were homogenized in a chilled mortar with 10 ml of 50 mM K Pi buffer pH 6.5 containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 1 mM PMSF (buffer A). The homogenate was centrifuged at 15,000 *g* for 20 min. The supernatant was dialyzed against 500 ml of 50 mM K Pi buffer pH 6.5 containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA (buffer B), changed three times at

8–12 h intervals. The dialyzate was centrifuged at 15,000 *g* for 20 min. The supernatant was assayed for enzyme activity.

3.4. Enzyme assays

Enzyme activity was assayed by measuring *N*-carbamoylputrescine with the diacetyl monoxime method of Boyde and Rahmatullah (1980). The standard assay mixture, consisting of 50 μmol of K Pi buffer, pH 6.5, 2 μmol of agmatine sulfate, 1 μmol of sodium diethyldithiocarbamate (in the case of plant extracts and the enzymes purified through steps 1–3 below) and enzyme solution in a total volume of 1 ml, was incubated for 30 min at 30°C. The reaction was initiated by adding the enzyme and terminated by the addition of 0.1 ml 50% TCA. After denatured protein was removed by centrifugation at 1500 *g* for 15 min (this treatment was omitted for the final purified enzyme), 3 ml of the chromogenic solution was added to 0.5 ml of the supernatant in a 10 ml screw tube. The samples were mixed vigorously and then boiled at 100°C for 5 min. After cooling to room temperature in the dark, the absorbance of the samples was measured at 530 nm. Protein was determined according to the method of Bensadoun and Weinstein (1976) with BSA as a standard.

3.5. Purification

Step 1. The shoots (ca 450 g) grown for 7 days in the dark were homogenized in a blender with 900 ml of buffer A. The homogenate was squeezed through a nylon mesh (74 μm) and centrifuged at 15,000 *g* for 20 min. The supernatant was used for the next step. Some (ca 5 ml) of the supernatant was dialyzed as before and the enzyme activity was measured.

Step 2. The supernatant from step 1 was stirred with DE52 equilibrated with buffer B (supernatant/DE52, 1 l/50 g) for 1 h, and then the DE52 was recovered in a Buchner funnel. After washing with 0.5 l of buffer B, the DE52 was stirred again for 5min in 250 ml of buffer B containing 30% ammonium sulfate saturation. The DE52 was removed in a Buchner funnel and re-washed with 50 ml of buffer B containing 30% ammonium sulfate saturation. The filtrate was brought to 70% ammonium sulfate saturation and allowed to stand for 30 min. After centrifugation at 15,000 *g* for 20 min, the precipitate was dissolved with 30 ml of buffer A and then dialyzed against buffer B (three exchanges at 8–l2 h intervals). The dialyzate was centrifuged at 15,000 *g* for 20 min and the supernatant was used for the next step.

Step 3. The supernatant from step 2 was applied to a DE52 column (2.5×15 cm) equilibrated with buffer B. After washing with 200 ml of buffer B, the enzyme was eluted with a linear gradient of 150 ml of buffer B and 150 ml of 0.5 M KH_2PO_4. Active fractions were pooled and the pH was adjusted to 6.5 with 2 N KOH.

Step 4. The enzyme solution from step 3 was concentrated with a Collodion Bag 13200E (Sartorius). The concentrated enzyme was passed through a Sephadex G-100 column equilibrated with buffer B. Active fractions were pooled.

Step 5. The pH of the result of step 4 was adjusted to 8.5 with 2 N KOH. The enzyme solution was applied to an agmatine-epoxy-Sepharose GB column (1×5 cm) equilibrated with 50 mM K Pi buffer pH 8.5 containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA (buffer C). After washing with 50 ml of buffer C and then 100 ml of 0.5 M K Pi buffer pH 8.5 containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA (buffer D), the enzyme was eluted by a pulse label method with 10 ml of buffer D containing 10 mM agmatine sulfate. Active fractions were pooled and then dialyzed against 1 l of buffer B changed three times at 8–12 h intervals. The dialyzate was used as the purified enzyme and stored at 4°C.

3.6. Determination of molecular mass and SDS-PAGE

The molecular mass of the enzyme was estimated by gel filtration with a Sephadex G- 100 column (1.2×80 cm) equilibrated with 50 mM K Pi buffer pH 6.5 containing 0.1 M KCl and 5 mM 2-mercaptoethanol. The column was calibrated with BSA dimer (132 kDa), BSA (66 kDa), ovalbumin (45 kDa), and cyt. *c* (12.4 kDa) as standard proteins. SDS-PAGE was performed as described by Laemmli (1970). Proteins were visualized with a silver stain kit (Daiichi Pure Chemicals Co., Ltd).

3.7. Putrescine synthase and related enzyme activity

The assay mixture of putrescine synthase was similar to that described by Srivenugopal and Adiga (1981). Two different amounts of the purified enzyme (0.5 and 1 μg) were used. After incubation at 30°C for 2 h, the enzyme reaction was terminated by boiling for 10 min. After denatured protein was removed by centrifugation at 15,000 *g* for 20 min, the supernatant was concentrated with a rotary evaporator. Citrulline was determined by PC (*iso*-PrOH–pyridine–H_2O–HOAc = 8:8:4:1) with ninhydrin and *p*-dimethylaminobenzaldehyde reagents. The assay mixtures of ornithine transcarbamylase or putrescine transcarbamylase consisted of 50 μmol of K Pi buffer, pH 7, 2 μmol of sodium carbamoyl phosphate and ornithine or putrescine, and enzyme (0.5 and 1 μg) in a total volume of 1 ml. After incubation at 30°C for 1 h, the enzyme reactions were terminated by the addition of 0.1 ml 50% TCA. After denatured protein was removed by centrifugation at 1500 *g* for 10 min, citrulline or *N*-carbamylputrescine in the supernatant (0.5 ml) was estimated with the standard assay method.

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Purification and characterization of coclaurine *N*-methyltransferase from cultured *Coptis japonica* cells

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Abstract

S-Adenosyl-L-methionine (SAM): coclaurine *N*-methyltransferase (CNMT), which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to the amino group of the tetrahydrobenzylisoquinoline alkaloid coclaurine, was purified 340-fold from *Coptis japonica* cells in 1% yield to give an almost homogeneous protein. The purified enzyme, which occurred as a homotetramer with a native M_r of 160 kDa (gel-filtration chromatography) and a subunit M_r of 45 kDa (SDS-polyacrylamide gel electrophoresis), had an optimum pH of 7.0 and a pI of 4.2. Whereas (*R*)-coclaurine was the best substrate for enzyme activity, *Coptis* CNMT had broad substrate specificity and no stereospecificity; CNMT methylated norlaudanosoline, 6,7-dimethoxyl-1,2,3,4-tetrahydroisoquinoline and 1-methyl-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline. The enzyme did not require any metal ion. *p*-Chloromercuribenzoate and iodoacetamide did not inhibit CNMT activity, but the addition of Co^{2+}, Cu^{2+} or Mn^{2+} at 5 mM severely inhibited such activity by 75, 47 and 57%, respectively. The substrate-saturation kinetics of CNMT for norreticuline and SAM were of the typical Michaelis–Menten-type with respective K_m values of 0.38 and 0.65 mM. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Alkaloid biosynthesis; Berberine; *Coptis japonica*; Ranunculaceae; Isoquinoline alkaloid; *S*-Adenosyl-L-methionine; Coclaurine *N*-methyltransferase

1. Introduction

Berberine, a benzylisoquinoline alkaloid obtained from *Berberis* and *Coptis* species, is an important pharmaceutical alkaloid with antibacterial, stomachic and anti-inflammatory activity (Sato et al., 1994). The biotechnological importance of this alkaloid and the basic interest in elucidating the steps involved in its biosynthesis have led to the complete characterization of this pathway in plant cell cultures (Sato et al., 1993, 1994; Kutchan, 1998). Berberine is derived from L-tyrosine via 13 different enzyme reactions involving an *N*-methyltransferase (NMT) (Frenzel and Zenk, 1990a; Wat et al., 1986), three *O*-methyltransferases (OMTs) (Rueffer et al., 1983; Muemmler et al., 1985; Frenzel and Zenk, 1990b; Sato et al., 1993, 1994), a hydroxylase (Loeffler and Zenk, 1990), a berberine bridge enzyme (Steffens et al., 1984), a methylenedioxy ring-forming enzyme (Rueffer and Zenk, 1985) and a tetrahydroprotoberberine oxidase (Yamada and Okada, 1985; Galneder et al., 1988). Only norcoclaurine 6-*O*-methyltransferase (6OMT) (Rueffer et. al., 1983; Sato et al., 1994), 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) (Frenzel and Zenk, 1990b) and (*S*)-scoulerine 9-*O*-methyltransferase (SMT) (Sato et al., 1993) have been highly purified and characterized.

The enzymological characterization of secondary metabolism and the biotechnological application of these biosynthetic enzymes should prove useful for the biotransformation of fine chemicals. One difficulty in purifying the enzymes in the berberine biosynthetic pathway is that they have similar enzymological properties due to the similarity of their reaction mechanisms and substrates (Sato et al., 1994; Morishige et al., 2000). Recent isolation of the cDNAs of methyltransferases and the investigation of their expression in *Escherichia coli* have provided further information about OMTs (Frick and Kutchan, 1999; Morishige et al., 2000). However, there is little information available on coclaurine *N*-methyltransferase (CNMT), a unique *N*-methyltransferase

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in berberine biosynthesis (Fig. 1) (Frenzel and Zenk, 1990a). CNMT catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to coclaurine with the formation of *N*-methylcoclaurine. So far, of the *N*-methyltransferases involved in secondary plant metabolism, only the putrescine *N*-methyltransferase in nicotine biosynthesis has been well characterized, and its cDNA has been isolated (Hibi et al., 1994). Characterization of the CNMT in berberine biosynthesis should help us understand the molecular aspects of NMT in secondary metabolism, especially alkaloid biosynthesis. We report here the purification and characterization of CNMT from cultured *Coptis japonica* cells and compare it with OMTs in berberine biosynthesis. Its broad substrate specificity and biotechnological applications are discussed.

2. Results and discussion

2.1. Purification of coclaurine N-methyltransferase (CNMT)

An outline of the purification of coclaurine *N*-methyltransferase (CNMT) from cultured *Coptis* cells is shown in Table 1. The enzyme was enriched 343-fold and gave a yield of 1% after ammonium sulfate fractionation, Phenyl Sepharose, Q-Sepharose, Mono Q anion-exchange and Mono P column chromatographies of the crude extract. Preliminary experiments showed that chromatography of the crude enzyme extracts of *Coptis* cells in 20 mM Tris–HCl buffer on a Q-Sepharose column gave two distinct active fractions. Since the activity of the first CNMT fraction was only 25% that of the second fraction, the latter was used for further purification. Although final isoelectric focusing chromatography on a Mono P column considerably decreased the recovery of CNMT activity, this step was essential for removing contaminating OMTs and purifying the enzyme to near homogeneity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and silver staining showed that the fraction purified through the Mono P column gave only two protein bands which had similar mobilities (silver staining) (Fig. 2). Whereas it was not possible to separate these proteins further, careful analysis by SDS-PAGE and enzyme assay

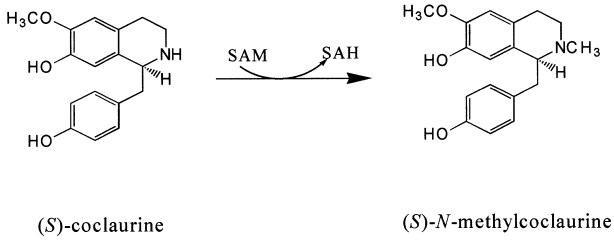


Fig. 1. Reaction catalyzed by coclaurine *N*-methyltransferase (CNMT). SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

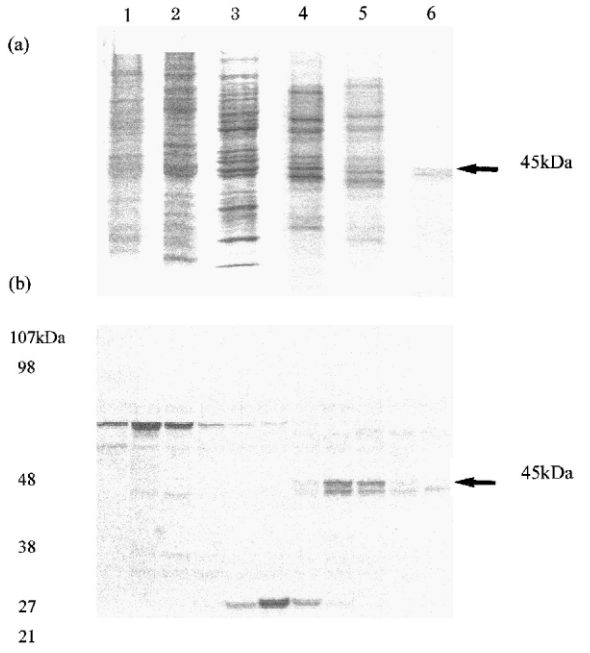


Fig. 2. SDS-PAGE analysis of purified coclaurine *N*-methyltransferase. (a) Analysis of proteins at various stages of purification. Fractions from each purification step were separated by SDS-PAGE and then stained with Coomassie Brilliant Blue: Lane 1, crude extract; Lane 2, 30–50% $(NH_4)_2SO_4$; Lane 3, Phenyl Sepharose; Lane 4, Q-Sepharose; Lane 5, Mono Q; Lane 6, Mono P column purified fraction. (b) Analysis of purified coclaurine *N*-methyltransferase by SDS-PAGE. Fractions were separated from the Mono P column, analyzed by SDS-PAGE in 10% polyacrylamide gel and made visible by silver staining.

Table 1
Purification of coclaurine *N*-methyltransferase from *Coptis japonica* cells[a]

Purification step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification fold	Yield (%)
Crude extract	512	428	0.83	1.0	100
30–50% Ammonium sulfate	151	240	1.59	1.91	54
Phenyl Sepharose	65.1	369	5.66	6.81	82
Q-Sepharose	13.2	204	15.4	18.6	45
Mono Q	0.76	34.4	45.3	54.5	8
Mono P	0.02	5.7	285	343	1.2

[a] Two hundred grams of cultured *Coptis* cells were homogenized for purification of the enzyme.

indicated that the larger 45-kDa protein corresponded to CNMT activity (data not shown). Since the CNMT purified in the Mono P column showed no *O*-methyltransferase activity and no positive protein band was detected by Western blotting with the anti-*Coptis* 4′OMT and SMT antibodies, we used this fraction as the purified CNMT fraction (data not shown).

To our knowledge, this is the first study in which coclaurine *N*-methyltransferase has been obtained in high purity and characterized. Since four methyltransferases (4′OMT, 6 OMT, SMT and CNMT) with similar reactions and substrates are involved in berberine biosynthesis (Rueffer et al., 1983; Muemmler et al., 1985; Frenzel and Zenk, 1990a,b; Sato et al., 1993, 1994; Morishige et al., 2000), it is not easy to purify each to homogeneity. In fact, preparation of homogenous 4′OMT and 6OMT required the heterologous expression of enzymes in *E. coli* (Morishige et al., 2000). In our study, also, 4′OMT and SMT could not be separated from CNMT during the early purification steps, even though their primary structures differ considerably (Takeshita et al., 1995; Morishige et al., 2000).

2.2. CNMT characterization

SDS-PAGE analysis showed that the molecular mass of the CNMT monomer is 45 kDa, whereas gel-filtration by Superose 12 column chromatography showed that the molecular mass of native CNMT was approximately 160 kDa. These results suggest that the native enzyme is a homotetramer. Chromatofocusing of CNMT on a Mono P column gave an isoelectric point (pI) of 4.2, and examination of the pH-dependence of enzyme activity showed that the highest activity was at pH 7.0, whereas 50% of the activity was at pH 6.0 and 9.0 (data not shown).

Examination of the effects of metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} at 5 mM) and potential inhibitors (EDTA, iodoacetamide, *p*-chloromercuribenzoate at 1 or 5 mM) on CNMT activity indicated that the enzyme did not require a divalent cation and the addition of EDTA did not inhibit activity, whereas the addition of Ca^{2+} at 5 mM slightly increased activity by 25%. The addition of some divalent cations (i.e. Co^{2+}, Cu^{2+} or Mn^{2+}), however, severely inhibited enzyme activity (by 75, 47 and 57%, respectively). Whereas some *O*-methyltransferase activities are inhibited by the addition of SH-directed inhibitors, e.g., *p*-chloromercuribenzoate and iodoacetamide at 5 mM, these chemicals did not inhibit CNMT activity.

2.3. Substrate specificity of CNMT

The substrate specificity of CNMT was examined for a wide range of isoquinoline alkaloid substrates (Fig. 3).

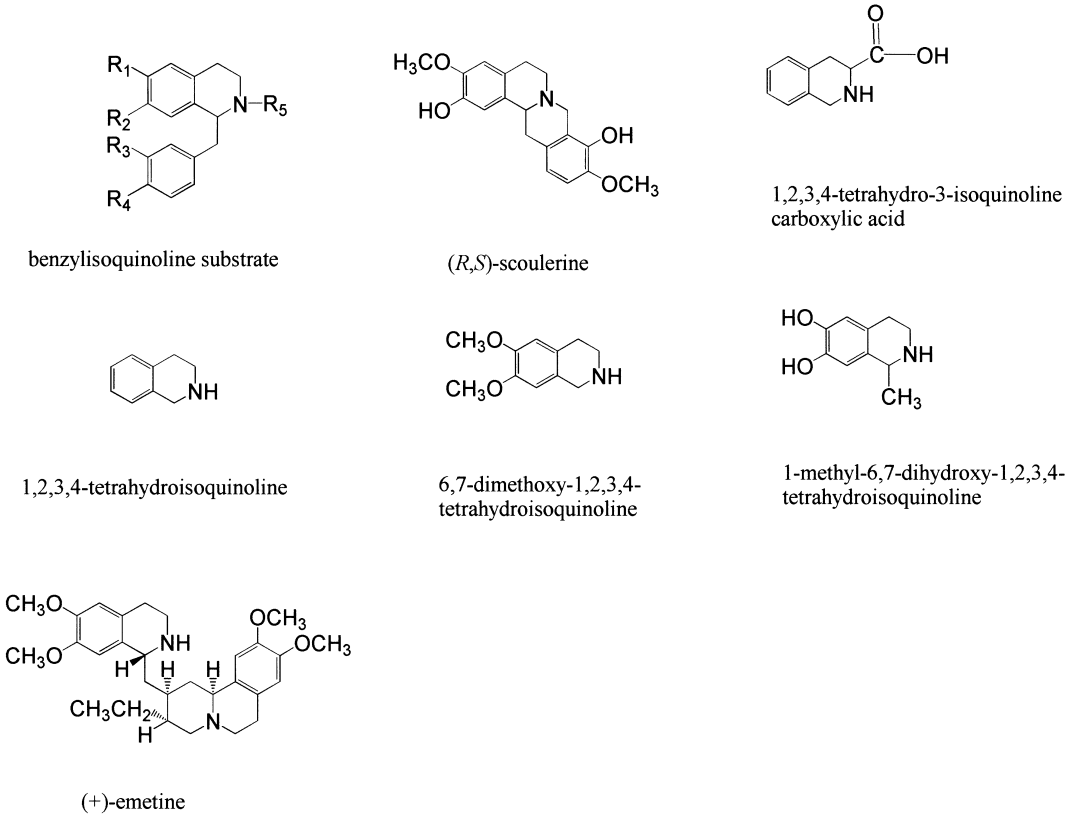


Fig. 3. Structures of isoquinoline alkaloids and chemicals tested as CNMT substrates. The benzylisoquinoline substrates in Table 2 are coclaurine, norreticuline, norlaudanosoline and 6-*O*-methylnorlaudanosoline.

The incorporation of radioactivity from *S*-adenosyl-L-[methyl-^{3}H] methionine into alkaloid products was used to measure enzyme activity, since the radioactivities measured corresponded to those of HPLC measurement for several substrates (data not shown). When (*S*)-coclaurine at 1 mM was chosen as the control substrate (i.e. the relative incorporation was taken as 100%) for *Coptis* CNMT, the respective relative activities with (*R*)-coclaurine, norreticuline, norlaudanosoline and 6-*O*-methylnorlaudanosoline were 122, 55, 48 and 38% (Table 2). Interestingly, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was also a good substrate, whereas 1,2,3,4-tetrahydroisoquinoline was not methylated. *Coptis* CNMT transferred the methyl group to a fairly broad range of isoquinoline substrates; i.e. from simple dimethoxy-tetrahydroisoquinoline to more complicated benzylisoquinoline substrates. The similar relative activities of (*R*)-coclaurine and (*S*)-coclaurine suggests that *Coptis* CNMT was not stereospecific. However, the lack of methylation activity with 4-*O*-methyldopamine, tryptamine, histamine and purine suggests that the isoquinoline ring is important for the *Coptis* CNMT reaction (data not shown).

Coptis CNMT has a substrate specificity similar to that of *Berberis* NMT (Frenzel and Zenk, 1990a), in that both enzymes show no stereospecificity and have relatively broad substrate specificity for (*R*)- and (*S*)-coclaurine, norreticuline and 6-*O*-methylnorlaudanosoline. *Coptis* CNMT also *N*-methylates norlaudanosoline, whereas *Berberis* NMT does not. These results indicate that *Coptis* CNMT has a much broader substrate specificity; *O*-methylation of the isoquinoline moiety of the benzylisoquinoline substrate has little effect on *Coptis* CNMT activity. The fact that even 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was methylated, as well as norreticuline, suggests that the benzyl moiety of norreticuline is not essential for the CNMT reaction. However, the fact that there was no CNMT activity with emetine indicates that replacement of this benzyl moiety with a large group makes this compound non-functional as a substrate. The lack of methylation activity with 1,2,3,4-tetrahydroisoquinoline suggests that hydroxylation of the isoquinoline ring is important for the *Coptis* CNMT reaction. The methylation of 1-methyl-6-7-dihydroxy-1,2,3,4-tetrahydroisoquinoline supports this idea.

2.4. Kinetics

Although (*R*)-coclaurine was the best substrate, we also determined the enzyme characteristics of CNMT with norreticuline, the most readily available substrate, and its similar product formation. CNMT from *Coptis* cells showed Michaelis–Menten-type kinetics for norreticuline and SAM (Fig. 4). Fig. 4 shows that *Coptis* CNMT followed a sequential bi-substrate mechanism (ordered bi bi or rapid equilibrium random bi bi), but further experiments on product inhibition are needed to determine the actual mechanism. The kinetic constants of CNMT (K_m and V_{max}) were calculated from double-reciprocal plots. The V_{max} of CNMT was estimated from Lineweaver–Burk double-reciprocal plots, and the respective apparent K_m values for norreticuline and *S*-adenosyl-L-methionine (SAM) were 0.38 and 0.65 mM (data not shown).

Although the K_m values of *Coptis* CNMT for norreticuline (0.38 mM) and SAM (0.65 mM) are somewhat larger than those of *Berberis* CNMT (ca.0.2 mM for (*R*)-norreticuline and 0.04 mM for SAM) and *Sanguinaria* NMT (0.02 mM for (*R,S*)-tetrahydroberberine and 0.012 mM for SAM) (Frenzel and Zenk, 1990a, O'Keefe and Beecher, 1994), the *Coptis* CNMT values are still smaller than those of 6OMT (2.23 mM for (*R,S*)-norlaudanosoline and 3.95 mM for SAM) (Sato et al., 1994). Enzymological characterization of the purified CNMT protein should be beneficial in the future use of this enzyme with other *O*-methyltransferases to convert various isoquinoline derivatives into *N*-methylated products by enzymological biotransformation; e.g., from norlaudanosoline to reticuline.

Table 2
Substrate specificity of purified *Coptis* coclaurine *N*-methyltransferase[a]

Substrate	Relative activity	R1[b]	R2	R3	R4	R5
(*R*)-Coclaurine	122	OMe	OH	H	OH	H
(*S*)-Coclaurine	100	OMe	OH	H	OH	H
(*R,S*)-Norreticuline	55	OMe	OH	OH	OMe	H
(*R,S*)-Norlaudanosoline	48	OH	OH	OH	OH	H
(*R,S*)-6-*O*-Methylnorlaudanosoline	38	OMe	OH	OH	OH	H
(*R,S*)-Scoulerine	0					
6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline	39					
1,2,3,4-Tetrahydroisoquinoline	0					
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	10					
1,2,3,4-Tetrahydro-3-isoquinolinecarboxylic acid	0					
(+)-Emetine	0					

[a] The enzyme reaction mixture was incubated for 1 h at 30°C; total volume 50 μl containing 100 mM potassium phosphate (pH 7.0), 2.5 mM sodium ascorbate, 1 mM ^{3}H-SAM and 20 μl of the purified Mono-P fraction.

[b] See Fig. 3 for the numbering system.

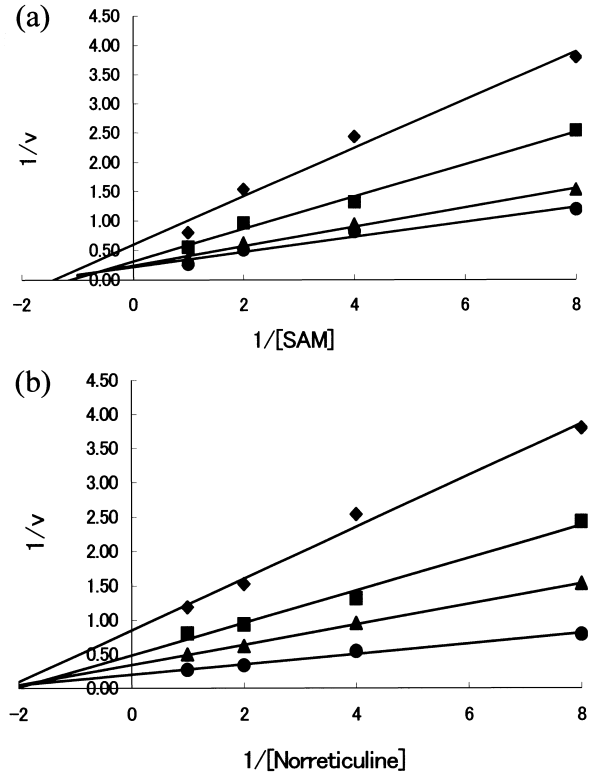


Fig. 4. Double reciprocal plots of CNMT activity. (a) Plots of 1/v versus 1/[SAM] at various constant concentrations of norreticuline. (b) Plots of 1/v versus 1/[norreticuline] at various constant concentrations of SAM. ◆, ■, ▲ and ● respectively represent 0.125, 0.25, 0.5 and 1.0 mM of these substrates.

3. Experimental

3.1. Plant material

Cultured *Coptis japonica* cells with high berberine productivity were used (Sato and Yamada, 1984). Cells were subcultured every 3 weeks on a rotary shaker (ca. 100 rpm) in the dark in Linsmaier-Skoog liquid medium containing 10 μM naphthalene acetic acid and 0.01 μM 6-benzyladenine as described elsewhere (Sato and Yamada, 1984), and 14-day-old cultured cells were used for enzyme purification. Harvested cells were immediately frozen in liquid nitrogen and stored at −80°C until use.

3.2. Chemicals

(*R,S*)-1-[(3,4-Dihydroxyphenyl)methyl]-1,2,3,4-tetrahydro-6,7-isoquinolinediol (norlaudanosoline or tetrahydropapaveroline), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, 1,2,3,4-tetrahydroisoquinoline, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide and 1,2,3,4-tetrahydro-3-isoquinoline carboxylic acid hydrochloride were purchased from Aldrich. (*R*)-Coclaurine and (*S*)-coclaurine were gifts from Dr. N. Nagakura, Kobe Women's College of Pharmacy. [Methyl-^{3}H] *S*-adenosyl-L-methionine (SAM) was purchased from NEN^{TM} Life Science, and the other alkaloids were gifts from Mitsui Petrochemical Industries Ltd. The other chemicals used were of the highest purity available.

3.3. Enzyme assay and protein determination

CNMT enzyme activity was measured by HPLC or by the incorporation of [methyl-^{3}H] into the product from [methyl-^{3}H] SAM. Enzyme activities during purification were analyzed by HPLC. The standard assay solution consisted of 0.1 mM norreticuline, 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM sodium ascorbate, 1 mM SAM and 20 μl of the enzyme solution. Norreticuline was selected as the standard substrate because of its availability and similarity of product formation to that of coclaurine, the native substrate. The assay mixture was incubated at 30°C for 60 min, after which the reaction was stopped by adding 50 μl of methanol. After protein precipitation at 10,000×*g* for 10 min at 4°C, the amount of reticuline formed was determined by the following HPLC separation: mobile phase, 22% acetonitrile and 1% acetic acid; column, LiChrosper 100RP-18 (250×4 mm, Cica Merck); flow rate, 1.0 ml/min; detection, at 280 nm.

Transfer of the [methyl-^{3}H] group of [methyl-^{3}H] SAM was investigated for the enzymological characterization of CNMT. The standard assay solution consisted of 1 mM norreticuline, 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM sodium ascorbate, 1 mM [methyl-^{3}H] SAM (1.5 MBq/μmol) and 20 μl of the purified enzyme fraction; total volume 50 μl. This mixture was incubated at 30°C for 20 min, after which the reaction was terminated by adding 150 μl of Na_2CO_3 (0.5 M) and 400 μl of isoamylalcohol. After vigorous shaking, the mixture was centrifuged at 10,000×*g* for 5 min at room temperature, and 150 μl of the organic phase was then used to determine the radioactivity. An assay mixture without the enzyme fraction was used as the control. The radioactivities incorporated corresponded to the activities measured by HPLC assay.

Protein concentration was determined with a Bio-Rad protein assay kit according to the manufacturer's protocols.

3.4. Purification of coclaurine N-methyltransferase

All operations were performed at 0–4°C, and all the buffers contained 20 mM β-mercaptoethanol and 10% glycerol unless stated otherwise. Typically, about 200 g of frozen cells were homogenized in 450 ml 0.2 M Tris–HCl buffer (pH 7.5) in a Waring blender at top speed for 2 min, and then sonicated for 20 min and filtered. The filtrate was centrifuged at 10,000×*g* for 50 min. Part of the supernatant which was desalted through an NAP-10 column (Pharmacia) was designated the crude

extract. The enzyme which precipitated between 30 and 50% $(NH_4)_2SO_4$ saturation was dissolved in 60 ml of the extraction buffer and then centrifuged. The protein solution obtained was desalted in a PD-10 column (Pharmacia) and then applied to a Phenyl Sepharose CL-4B column (Pharmacia, 50 ml of bed volume) that had been equilibrated with a starting buffer (200 mM Tris–HCl (pH 7.5) containing 30% $(NH_4)_2SO_4$). After the column had been washed sufficiently with the same buffer to remove any remaining yellow alkaloids, CNMT was eluted with 120 ml of a linear gradient (30–0%) of $(NH_4)_2SO_4$ solution. The CNMT fractions were then subjected to ion-exchange chromatography in a Q-Sepharose FF column (Pharmacia, 50 ml of bed volume) that had been pre-equilibrated with 200 mM Tris–HCl buffer (pH 7.5). After the column was washed with the same buffer, CNMT was eluted with a linear gradient (0–0.5 M) of NaCl solution (120 ml). Active fractions were collected, desalted and purified in an FPLC system with a Mono Q column (HR 5/5, Pharmacia) that had been pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.5). CNMT was then eluted stepwise with a gradient (0–0.35 M) of NaCl solution (40 ml). The active fractions were combined and chromatofocused in a Mono P column (HR 5/20, Pharmacia) in an FPLC system: starting buffer; 25 mM Bis Tris–IDA (iminodiacetic acid), pH 7.1, eluting buffer; 10% Polybuffer 74 adjusted to pH 4 by IDA. The pHs of the eluted fractions were immediately adjusted with 100 mM Tris–HCl (pH 7.5). The purified enzyme was kept at −20°C in 50% glycerol. Since repeated freezing and thawing markedly reduced CNMT activity, thawed samples were not repeatedly frozen. CNMT was active after 1 year at −20°C when frozen together with 50% glycerol. All FPLC steps were done at room temperature.

3.5. Characterization of coclaurine N-methyltransferase

The CNMT fraction purified on the Mono P column was used for further enzymological characterization. Effects of pH, metal ions and chemicals on CNMT activity were determined with norreticuline as the substrate. Divalent cations were added as their chloride salts. The optimum pH of CNMT activity was determined with the following buffers at concentrations of 100 mM: 2-(*N*-morpholino)ethane sulfuric acid (MES) (pH 5.4, 6.2 and 6.6), potassium phosphate (pH 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8), *N*-2-hydroxyethypiperazine-N-2-ethane sulfonic acid (HEPES) (pH 6.5, 7.0, 7.5, 8.0 and 8.5), Tris–HCl (pH 7.5, 8.0, 8.5 and 9.0), glycine-NaOH (pH 9.0 and 10.0).

3.6. Molecular weight determination

The subunit molecular weight of CNMT was determined by SDS-PAGE (10%) analysis with molecular weight standards (Bio-Rad). The native molecular weight of CNMT was determined by gel filtration chromatography on a calibrated Superose 12 column (HR 10/30, Pharmacia).

3.7. Kinetic properties

Kinetic constants for CNMT activity were determined for various amounts of norreticuline and SAM with 0.2 μg of purified CNMT in a 50 μl reaction mixture. Kinetic data were fitted to the standard equation.

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Identification of phytochelatin-related peptides in maize seedlings exposed to cadmium and obtained enzymatically in vitro

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Abstract

An analytical strategy based on the sensitivity of electrospray tandem mass spectrometry following a simplified and reproducible sample preparation procedure was evaluated for the determination of Cd-induced phytochelatins (PC) and related peptides in four maize varieties. In addition to the three known families of PC (PC, desGly-PC and iso-PC(Glu)) that were observed, novel PC and desGly-PC homologues lacking the N-terminal γ-linked Glu were isolated from maize root extracts for the first time. Additionally the complete sequence of iso-PC_3(Glu) was determined by tandem mass spectrometry. Peptides obtained in vivo and in vitro as the result of the reaction of glutathione with the enzyme phytochelatin synthase were compared. Minor forms detected from in vitro reactions include compounds with intramolecular or intermolecular disulfide bonds resulting from the oxidation of SH groups, phytochelatin homologues lacking the N-terminal γ-linked Glu, and new PC-related peptides with a Cys–Cys motif. Since peptides lacking a γGlu residue could be generated as artifacts in electrospray mass spectrometry, the application of capillary electrophoresis with online electrospray mass spectrometry allowed the separation and detection of such peptides as endogenous molecules present in planta and as products of in vitro reactions. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Zea mays; Maize seedlings; Cadmium; Electrospray mass spectrometry; Phytochelatin-related peptides

1. Introduction

Phytochelatins (PC) are metal-binding peptides which are enzymatically synthesized from glutathione (GSH). These peptides have been shown to be formed in plants by various metals such as cadmium, copper and several other heavy metals (Rauser, 1995; Zenk, 1996).

In addition to PCs which possess the typical (γGlu–Cys)$_n$–Gly ($n=2$–11) (Grill et al., 1986) sequence, several variant structures have also been detected. Homophytochelatins (iso-PC(βAla), (γGlu–Cys)$_n$–βAla, $n=2$–7) were first isolated from plants of the tribe Phaseoleae (Fabaceae) (Grill et al., 1986; Klapheck et al., 1995) and isophytochelatins (Ser) (iso-PC(Ser), (γGlu–Cys)$_n$–Ser, $n=2$–4) have been detected in species of the family Poaceae (Klapheck et al., 1994). A homologue of glutathione with C-terminal linked Glu instead of Gly and isophytochelatins (Glu) (iso-PC(Glu), (γGlu–Cys)$_n$–Glu, $n=2$–3) were isolated from maize plants exposed to cadmium (Meuwly et al., 1993, 1995). Additionally, desglycine phytochelatins (des Gly–PC, (γGlu–Cys)$_n$), which lack the C-terminal amino acid residue were first discovered in maize (Bernhard and Kägi, 1987). The last PC-related peptides identified are isophytochelatins (Gln) (iso-PC(Gln), (γGlu–Cys)$_n$–Gln, $n=3$–4), which were isolated from hairy roots of horseradish exposed to cadmium (Kubota et al., 2000).

The classical approach used for the determination of PC is based on reversed-phase chromatography with online derivatization of the sulfhydryl groups with Ellman's reagent (DTNB) and spectrophotometric detection at 410 nm. The non-specific nature of this detection technique only allows the different thiol peptides in plants to be identified on the basis of their retention time in RP HPLC. In our case, this method is used as a first step to determine the presence and quantity of PC-related peptides in Cd-exposed maize root extracts.

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However, the DTNB reaction is the only quantitative method for reduced PCs and their homologues in chromatography; using on-line electrospray MS/MS after RP HPLC is not yet feasible partly due to the low concentration of the eluted compounds. Additionally, the majority of mass spectrometric data available for iso-PCs in the literature were obtained for the DTNB-derivatized compounds after a fastidious sample preparation procedure. MS/MS spectra obtained for the derivatized compounds show an incomplete fragmentation pattern and poor sensitivity (Meuwly et al., 1995).

Since the levels of PC homologues present in plants exposed to heavy metals are often rather low, the procedure used for the isolation of metal-binding peptides has to be simplified to avoid the loss of minor forms. The objective of this work was to develop a sensitive and reproducible method for the isolation and characterization of PC-related peptides in plants exposed to Cd. The strategy is based on the specificity and sensitivity of electrospray tandem mass spectrometry following the sample preparation procedure.

In this context, we attempted to characterize the three families of thiol peptides already isolated from maize (PC, desGly–PC and iso-PC(Glu)) by Meuwly et al. (1995) and to determine the presence of other PC homologues present at trace levels. Mass spectrometry was also applied for the determination of the minor forms in the post-reaction mixture of GSH with the enzyme phytochelatin synthase (PC synthase) to compare thiol peptides generated by in vivo and in vitro reactions.

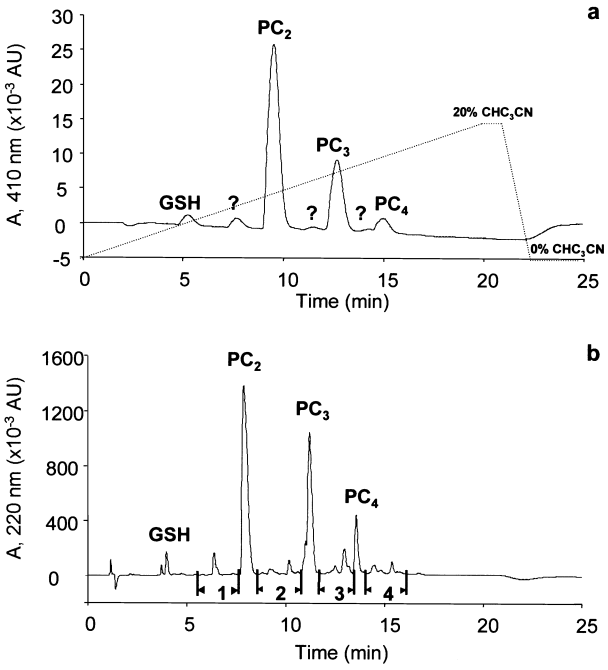


Fig. 1. Reversed-phase chromatograms for the mixture resulting from in vitro reaction of GSH with the enzyme phytochelatin synthase; (a) 10 μg injected, postcolumn derivatization with DTNB and detection at 410 nm; (b) 500 μg injected, UV detection at 220 nm. The fractions 1–4 were collected for MS/MS characterization as indicated. The gradient profile is indicated in % CH_3CN by the dotted line.

2. Results and discussion

2.1. Characterization of minor species in the mixture resulting from in vitro enzymatic reaction

2.1.1. Isolation and characterization of minor species by electrospray MS (ESI MS)

The in vitro enzymatic reaction of GSH with the enzyme PC synthase yields a mixture of PCs. In our previous work the electrospray mass spectrum obtained revealed the presence of major signals at molecular masses of 540, 772 and 1004, corresponding to PC_2, PC_3 and PC_4, respectively, in the proportion 10:5:3 (Vacchina et al., 1999). In addition, several other peaks were detected that had not been identified up to now or that were attributed to fragmented PC formed during the electrospray ionization process. A reversed-phase chromatogram obtained after postcolumn derivatization with Ellman's reagent (Fig. 1a) of this mixture shows the presence of minor thiol group-containing compounds. Sharper TNB peaks with greater resolution can be generated by using a lower HPLC flow rate and a more concentrated DTNB solution added at a slower flow rate allowing for a smaller reaction loop to give a residence time of about 1.2 min (Meuwly et al., 1993). The optimized procedure adopted in this work aimed first at finding conditions allowing the separation of unidentified minor peaks from PCs using online UV detection at 220 nm (Fig. 1b).

The procedure used to isolate the minor forms was based on the collection of the intermediate fractions in the chromatogram of the concentrated PC mixture (500 μg injected) (Fig. 1b). Four different fractions (collected as indicated in Fig. 1b) were concentrated by rotary evaporation and lyophilization.

Fig. 2 shows mass spectra obtained for the four different fractions (1–4) in the MS mode. These spectra are composed primarily of protonated molecules $[M+H]^+$ which allow the identification of each form according to its mass. However, doubly charged ions are observed for peptides with molecular masses larger than 1000 Da. Three different categories of compounds have been tentatively identified on the basis of their molecular mass: (i) reduced and oxidized forms of phytochelatins (compounds with an intramolecular or intermolecular S–S bond); (ii) phytochelatin homologues lacking the N-terminal γ-linked Glu and (iii) PC-related peptides with a Cys–Cys motif.

The first category of compounds include the oxidized forms of PCs and desGly–PCs which elute before their corresponding reduced PC in the reversed-phase chromatogram of the PC mixture. Fig. 2a shows the presence of the peaks at m/z 481 and 538 corresponding to

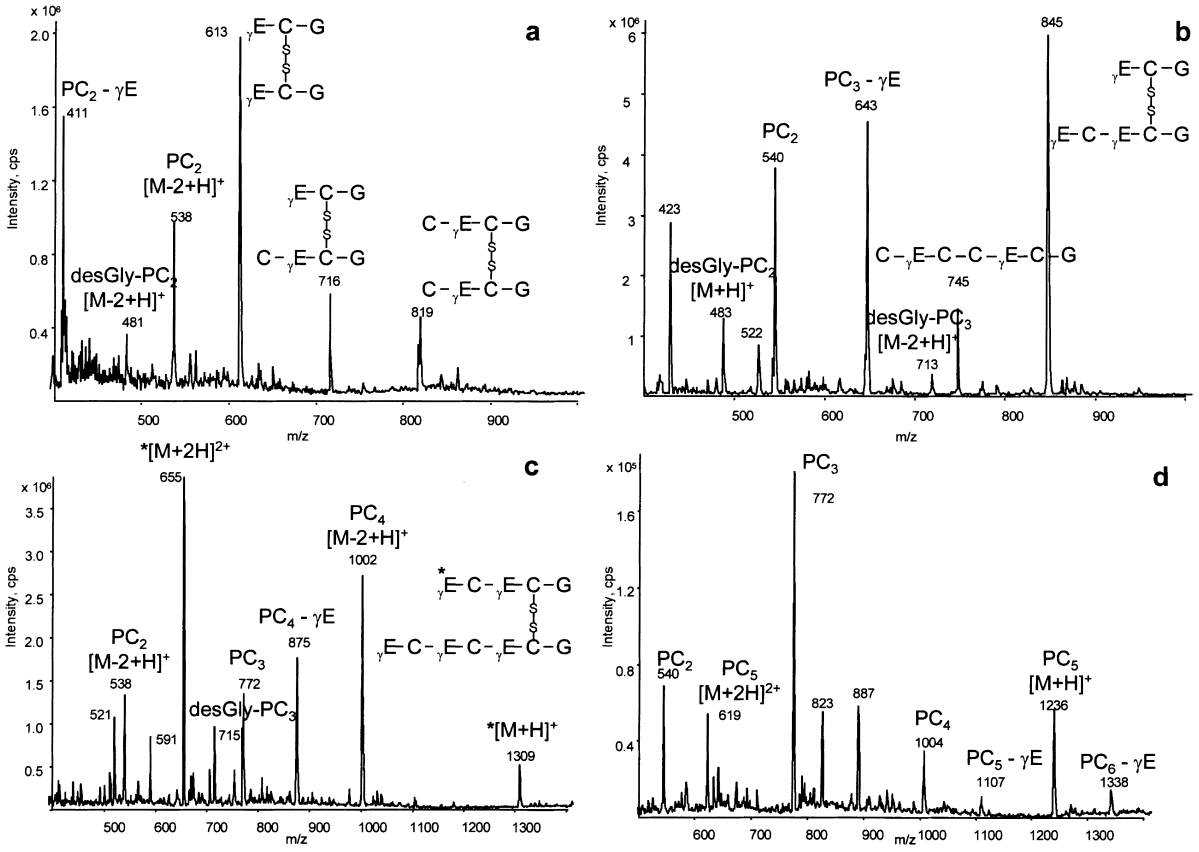


Fig. 2. Electrospray mass spectra of fractions collected in reversed-phase HPLC of PC mixture resulting from in vitro enzymatic reaction (Fig. 1b). (a) Fraction 1; (b) fraction 2; (c) fraction 3; (d) fraction 4.

oxidized desGly–PC_2 and PC_2, respectively. The peaks at m/z 713 (Fig. 2b) and 1002 (Fig. 2c) correspond to the oxidized forms of desGlyPC_3 and PC_4, respectively. The presence of PC_2 and PC_3 in their reduced forms in fractions 2 and 3 (Fig. 2b–c) is due to the tailing of these compounds leading to their inadvertant collection. In fraction 4 (Fig. 2d), the presence of high concentrations of PC_2 and PC_3 might be explained by the formation of an intermolecular disulfide bond (the hydrophobicity of the resulting compound is close to PC_5) that is cleaved during the ionization process. Fig. 2d clearly shows the presence of peaks at m/z 1236 and 619 corresponding to the singly and doubly charged molecular ions of PC_5, respectively. The compound is present only at trace levels compared to other PC (with $n=2$–4) and is observed only after preconcentration of the sample.

In Fig. 2a, the most intense peak at m/z 613 can be attributed to the oxidized glutathione (GSSG). The peaks at m/z 716 (m/z 613 + 103) and 819 (m/z 716 + 103) (Fig. 2a) are tentatively attributed to the molecular ions of compounds with an intermolecular disulfide bond between two thiol peptides; m/z 716 and 819 correspond to the compounds formed from GSH and Cys–(γGlu–Cys)–Gly and two molecules of Cys–(γGlu–Cys)–Gly, respectively. These observations confirm the presence of phytochelatin homologues resulting from the cleavage of the N-terminal γ-linked Glu. In a similar fashion, the peaks at m/z 845 (Fig. 2b) and 1309 (Fig. 2c) can be obtained as the result of oxidation by establishing an S–S bond between GSH and PC_2, and between PC_2 and PC_3, respectively. The peaks observed at m/z 1309 and 655 in Fig. 2c correspond to the singly and doubly charged ions of the same form.

Concerning the PC-related peptides lacking the γ-linked Glu (abbreviation: PC–γGlu), two different mechanisms may be proposed to explain their formation. The peaks at m/z 411, 643, 875, 1107 and 1338 (Fig. 2a–d) may be the heaviest y-ion fragments of PC_2, PC_3, PC_4, PC_5 and PC_6, respectively, generated during the electrospray ionization process. Alternatively, they may originate from enzymatic cleavage of γGlu. γ-Glutamyl transpeptidases (γGtases) are the only enzymes known to hydrolyse the unique N-terminal amine bond of glutathione (Martin and Slovin, 2000). Two different observations support the explanation that PC–γGlu peptides are formed by an enzymatic process. The peptides in question are found in the fraction preceding the corresponding PC peak in the chromatogram, and therefore it is unlikely they represent artifacts of PC fragmentation in the mass spectrometer. The absence of PC_3 in its oxidized or reduced form in fraction 2 (Fig. 2b), which contains PC_3–γGlu, further supports this reasoning.

In Fig. 2b, the presence of an intense peak at m/z 745 suggests a compound with a Cys–Cys peptide-bond which may be produced by the in vitro reaction. The mass difference (+102 Da) compared with the peak m/z 643 (PC_3–γGlu) is tentatively explained by an additional cysteine in the sequence (+103 Da) and the loss of 1 Da. The latter could be obtained by a rearrangement between the two adjacent Cys in the sequence. Oxidation leading to a disulfide bond seems unlikely since the spatial configuration does not allow an S–S bond to be established between adjacent Cys residues. As well, an oxidized compound with an intramolecular S–S bond between two proximal Cys would be observed at m/z 744.

2.1.2. Confirmation of signal identity by electrospray MS/MS (ESI MS/MS)

In the MS/MS mode, the protonated molecular ions are fragmented by collision induced dissociation (CID) with inert gas molecules leading to the acquisition of the mass spectra of fragment ions. Peptides fragment primarily at the amine bonds to produce a "ladder" of sequence ions. The charge can be retained on the amino terminus (type b-ion) or on the carboxy terminus (type y-ion). CID spectra of the major peaks discussed above are shown in Fig. 3.

Fig. 3a, d and g show the MS/MS spectra of signals at m/z 411, 643 and 875, which correspond to the molecular ions of PC_2–γGlu, PC_3–γGlu and PC_4–γGlu, respectively. The presence of the heaviest type y-ion fragment corresponding to the fragmentation of the N-terminal Cys confirms the sequence of the predicted peptide. At this stage it is impossible to determine the origin of the observed PC–γGlu forms. The fragmentation pattern of such PC homologues (PC–γGlu) is identical to the second generation of fragment ions obtained from the heaviest type y-ions of the corresponding PC. In the case of PC_5–γGlu and PC_6–γGlu, the incomplete fragmentation pattern of the peaks at m/z 1107 and 1338, respectively (spectra not shown), do not allow us to confirm the sequence of the peptides by the presence of the heaviest type y-ion. This is due to the low concentration of peptides in fraction 4 (Fig. 2d) and the incomplete fragmentation obtained in the case of peptides of 10 or more amino acid residues.

In the case of PCs in their oxidized form, the intramolecular bond between the sulfhydryl groups of proximal Cys leads to an incomplete fragmentation pattern even when the collision gas and kinetic energy of ions are adjusted. This problem was encountered by Meuwly et al. (1995) for three thiol peptides families (PC, desGly–PC and iso-PC(Glu)), the current work confirms this and extends it to several related compounds. After prolonged reduction of the PCs, the sulfhydryl-reduced form can be obtained and characterized in MALDI mass spectrometry (Schmöger et al., 2000). However, the addition of strong reducing agent such as $NaBH_4$ suppresses the signal in electrospray ionization and cannot be used for the purpose of peptide sequencing in ESI MS/MS. Fig. 3h shows the mass spectrum obtained for the fragmentation of the peak at m/z 1236 corresponding to the molecular ion of PC_5 in its reduced form. The fragmentation pattern allowed the peptide sequence to be confirmed despite the incomplete series of type-y and-b fragment ions that is obtained for reduced peptides with molecular masses larger than 1000 Da.

The MS/MS spectrum of the molecular ion at m/z 613 (cf. Fig. 2a) is shown in Fig. 3c, which corresponds to oxidized glutathione (GSSG). While the fragmentation pattern shows the majority of the expected fragments, the absence of a fragment at m/z 307 can be ascribed to the difficulty of cleaving the intermolecular S–S bond established between two glutathione molecules. In the case of the other oxidized peptides observed in Fig. 2 (m/z 716, 819, 845 and 1309) the complexity of the mass spectra obtained due to several cleavage possibilities in MS/MS (spectra not shown) makes the interpretation more difficult.

In Fig. 3e, the fragmentation of the peak at m/z 745 (cf. Fig. 2b) allowed the elucidation of the complete sequence of the Cys–Cys sequence-containing peptide. On the basis of the proposed formula, almost all the observed masses can be correlated to the predicted type-y and-y fragments ions. In the type-b series, the successive loss of 103 Da (mass of one cysteine) and 102 Da (cysteine with the loss of 1 Da) leads to the observed ions b_3 and b_4, respectively. In a similar fashion, the successive loss of 103 Da and 102 Da leading to the y_3 and y_4 ions confirms the peptide bond between two Cys. The heaviest y fragment (y_6) allowed us to confirm the N-terminal linked Cys. MS/MS data lead to the following conclusions: (i) the peptide observed is under its reduced form since in its oxidized form an incomplete fragmentation pattern would be observed; (ii) a Cys–Cys peptide bond is clearly confirmed by the type-b and y-ions and (iii) the successive loss of 103 Da and 102 Da in the fragmentation process can only be explained by a rearrangement between the two adjacent Cys residues in the sequence. To our knowledge no similar thiolate peptide has been isolated up to now. At this stage we can make the hypothesis concerning a transpeptidation reaction between two molecules of PC_2–γGlu respectively leading to the observed compound. The same enzyme might be involved in the reaction starting by the cleavage of the C-terminal linked Gly of PC_2–γGlu the energy being transferred to establish a Cys–Cys peptide bond between the two peptides.

2.2. Determination of phytochelatins and related peptides in maize root extracts

2.2.1. RP HPLC of plant extracts with postcolumn derivatization

Reversed-phase chromatograms of Cd-exposed and control maize root extracts with thiol-specific DTNB

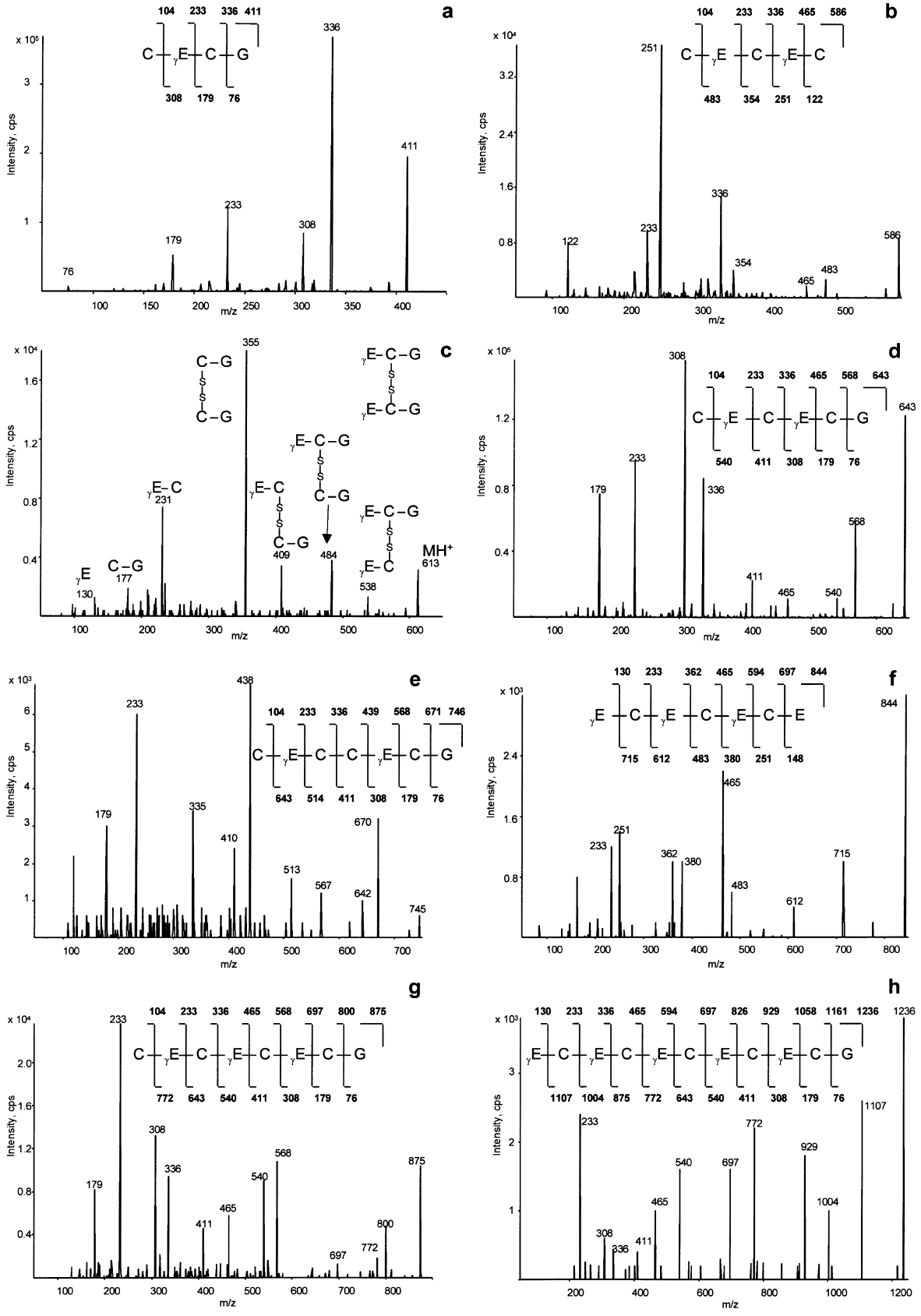


Fig. 3. Electrospray MS/MS spectra of peaks corresponding to PC and related peptides detected in the PC mixture resulting from in vitro enzymatic reaction and/or in Cd-exposed maize root extracts. (a) *m/z* 411, PC_2–γE; (b) *m/z* 586, desGly–PC_3–γE; (c) *m/z* 613, oxydized glutathione (GSSG); (d) *m/z* 643, PC_3–γE; (e) *m/z* 745, peptide with Cys–Cys bond; (f) *m/z* 844, iso-PC_3(Glu); (g) *m/z* 875, PC_4–γE; (h) *m/z* 1236, PC_5.

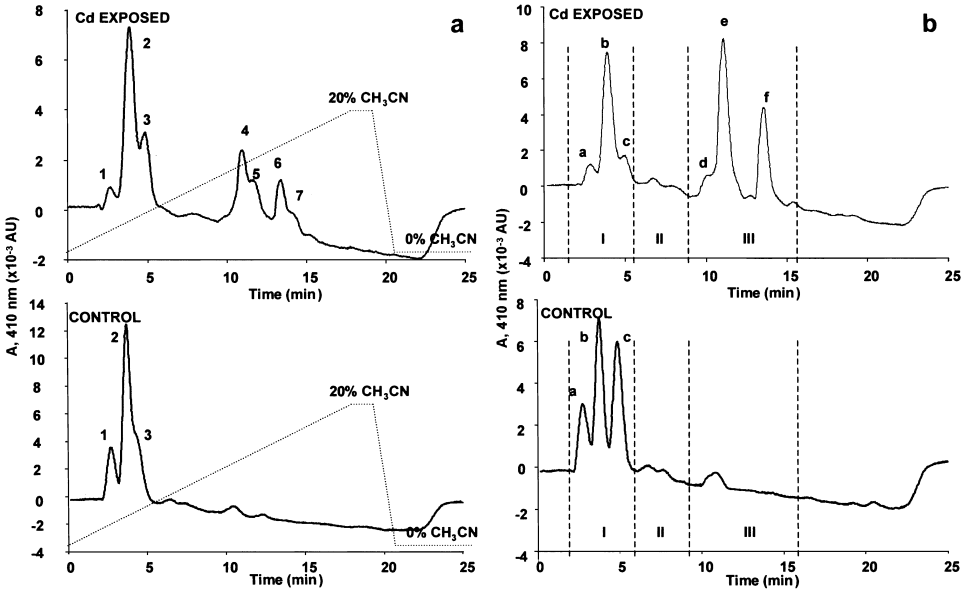


Fig. 4. Reversed-phase chromatograms for Cd-exposed (upper) and control (lower) maize roots extracts generated by postcolumn derivatization with DTNB and detection at 410 nm. Maize roots of were exposed to $Cd(NO_3)_2$ 100 μM for 7 days. 100 μl extract was injected. The gradient profile is indicated in% CH_3CN by the dotted line. (a) *Zea mays* cv. 'Boss', 1 — Cys, 3 — GSH, 4 — PC_3, 5 — desGly–PC_3, 6 — PC_4, 7 — desGly–PC_4; (b) *Zea mays* cv. 'Zwerge', a — Cys, c — GSH, d — iso-PC_3(Glu), e — PC_3, f — PC_4. Vertical dashed lines indicated how the fractions (I–III) were collected (with UV detection) for MS/MS characterization.

postcolumn reaction are shown in Fig. 4. For three maize varieties (Fig. 4a for *Zea mays* cv. 'Boss') the chromatograms show two major peaks (4 and 6) at 11.0 and 13.5 min corresponding to PC_3 and PC_4, respectively (chromatographic data not shown). The peaks 4 and 6 are each followed by a minor unresolved peak. On the basis of the mass spectrometric data previously obtained for the intermediate fractions collected in the chromatogram of the PC enzymatic mixture, peak 5 can be tentatively attributed to desGly–PC_3 which elutes after PC_3. The identity of the peak was investigated by treating PC_3 (obtained as described in the Materials and methods section) with the enzyme carboxypeptidase A which is known to hydrolyse the C-terminal linked Gly of phytochelatins (unpublished data). Fig. 5a depicts the chromatogram obtained for the post-reaction mixture after 2 h incubation with postcolumn derivatization and spectrophotometric detection at 410 nm. The peaks 1 and 2 (in Fig. 5a) fit with the peak 4 and 5 of the chromatograms obtained for maize samples (Fig. 4a), respectively. The peaks were collected in the same chromatographic conditions but with online detection at 220 nm (chromatographic data not shown). In these conditions, the two resolved peaks 1 and 2 were characterized in ESI MS/MS and were identified as PC_3 and desGly–PC_3 (spectrum in Fig. 5b), respectively.

For the last maize variety (*Zea mays* cv. 'Zwerge' in Fig. 4b) the chromatographic profile shows two major peaks (e and f), the first one being preceded by a minor peak which may be attributed to an iso-PC (peak d). However, the identity of the eluted compounds can only

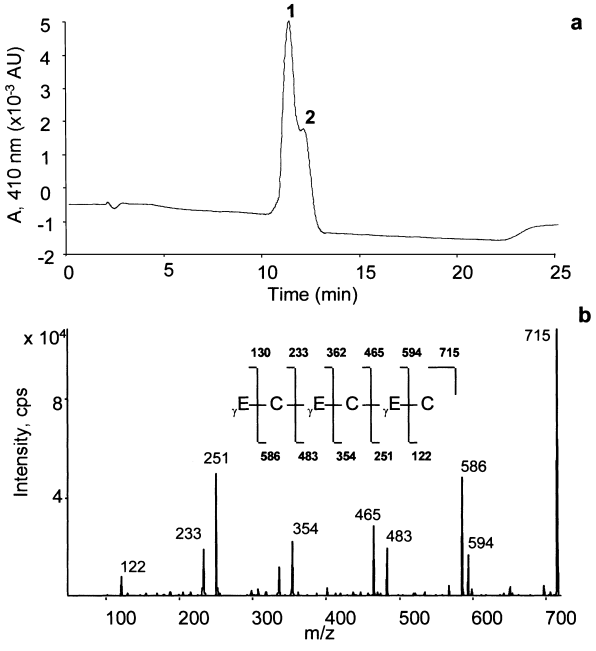


Fig. 5. Peak identification in a reversed-phase chromatogram of the post-reaction mixture of a purified PC_3 standard with the enzyme carboxypeptidase A. (a) 2 h incubation at 25°C, postcolumn derivatization with DTNB and visible detection at 410 nm; (b) tandem MS spectrum of isolated peak 2. Peaks 1 and 2 were assigned to PC_3 and desGly–PC_3, respectively.

be confirmed by mass spectrometry. Peaks 1 and 3 (Fig. 4a) or a and c (Fig. 4b) were attributed to cysteine and glutathione, respectively, on the basis of the retention time of the corresponding standard. Peak 2 (Fig. 4a)

or b (Fig. 4b) is probably due to impurites present in the plant extract.

The concentrations of PC were determined based on a GSH calibration curve, after correction for derivatization efficiencies with DTNB (as chain length of PC increased, while derivatization efficiency decreased). Derivatization efficiencies obtained in our previous work (unpublished data) are of 81, 77 and 50% for PC_2, PC_3 and PC_4, respectively. In the case of maize samples, we assumed for calculation that the derivation efficiencies are the same for desGly–PC_n, iso–PC_n and its corresponding PC_n form. Concentrations of PC-related peptides were 0.42, 0.20, 0.45 and 0.82% by dry wt of roots for *Zea mays* cv. 'Boss', 'Consul', 'Popcorn neuform' and 'Zwerge', respectively.

2.2.2. Strategy for the isolation of the eluted species in RP HPLC

The use of ESI MS/MS to characterize phytochelatin peptides eluting from a reversed-phase column encountered a number of problems due to the low concentration of the compounds and the elution of salts present in the sample extract. For these reasons, an off-line procedure was developed for the characterization of the PC-related peptides using ESI MS/MS.

The procedure, described in detail in the Materials and methods, was adapted from Grill et al. (1991). Significant changes were the use of a 10 fold more concentrated extract for injection on the reversed-phase analytical column and detection at 220 nm (chromatograms not shown). In Fig. 4 (chromatograms obtained with postcolumn derivatization and detection at 410 nm) the dashed lines indicate how fractions were collected.

Fractions II and III collected in the middle of the RP HPLC chromatogram are salt free. Consequently, the freeze-dried material was directly redisolved in acidic solution for ESI MS analysis. Fraction I contained high concentrations of salts which are only partially eliminated during the preconcentration step with aqueous solution (0.1% TFA in water for 5 min) preceding the linear gradient of acetonitrile to elute the peptides. For this reason fraction I did not give any significant ionization signal for peptides in ESI MS.

2.2.3. Characterization of the eluted species by ESI MS/MS

ESI MS/MS was applied to the fractions II and III collected in the chromatograms of maize extracts (Fig. 4). The low concentrations levels in fraction II did not allow us to detect PC_2 by mass spectrometry. In the spectra of fractions III, several minor peaks are observed that can be attributed to peptides that may be naturally present in plants or to Cd-induced peptides. To discriminate among Cd-induced and non-induced peptides, the mass spectrum of the control fraction prepared in the same way was subtracted. ESI mass spectra of the fractions III before and after subtraction of the control (acquired in the same conditions) are shown in Fig. 6a and b for *Zea mays* cv. 'Zwerge' and 'Boss', respectively.

For *Zea mays* cv. 'Zwerge' (Fig. 6a) the peaks at m/z 715, 772, 947 and 1004 correspond to the molecular ions of desGly–PC_3, PC_3, desGly–PC_4 and PC_4, respectively. The spectrum also shows clearly the presence of the peak at m/z 844 that is attributed to the molecular ion of iso-PC_3(Glu). Meuwly et al. (1993; 1995) identified the TNB-derivatized homologue of glutathione with C-terminal α-linked Glu (γ-Glutamylcysteinylglutamic acid) and iso-PC(Glu) in maize seedlings using mass spectrometry. These authors used a time consuming combination of post-column derivatization of thiolate peptides separated in RP HPLC followed by a second chromatographic step for the purification of the derivatized compounds. The major disadvantages of this method are the poor performance obtained in MS/MS for the resulting compounds due to: (i) the high mass of the derivatized compounds leading to incomplete fragmentation in the CID mode; (ii) the poor sensitivity obtained for derivatized compounds and (iii) the incomplete fragmentation pattern due to the formation of intramolecular S–S bond between proximal Cys residues in the sequence that could arise during initial derivatization or subsequent purification. To our knowledge, the complete fragmentation profile of the original iso-PC_3(Glu) has not been obtained up to now in MS/MS. In our work, the sequence of this form was confirmed by fragmenting the molecular ion in the CID mode (Fig. 3f). The complete series of y-type ion was observed and a good sensitivity was obtained. Peaks at m/z 643 and 875 (Fig. 6a) are attributed to PC_3–γGlu and PC_4–γGlu that have already been observed in vitro for the mixture resulting from enzymatic reaction of GSH with PC synthase. MS/MS spectra of peaks at m/z 643 and 875 are identical to those shown in Fig. 3d and g, respectively. In the same way, the peak m/z 586 is attributed to desGly-PC_3- γGlu and the sequence was confirmed by MS/MS (Fig. 3b). Resulting compounds likely due to the enzymatic hydrolysis of the N-terminal γ-linked Glu of desGly–PC are only observed in vivo. We also attempted to fragment the unknown minor peaks (m/z 551, 567, 729, 891) (Fig. 6a) but the fragmentation pattern did not correspond to any PC homologue.

For the three other maize varieties (*Zea mays* cv. 'Boss', 'Consul' and 'Popcorn neuform'), only PC and desGly–PC (with $n = 3$ and 4) were detected as the major thiolate peptides in the collected fractions. Thus, mass spectrometric data confirm the chromatographic profile obtained in Fig. 4a (for *Zea mays* cv. 'Boss') and the attribution of the observed peaks 5 and 7 to desGly–PC_3 and desGly–PC_4, respectively. The mass spectrum in Fig. 6b was obtained from fraction III collected for *Zea mays* cv. 'Boss' and shows the presence of additional minor peaks. The peak at m/z 875 was attributed

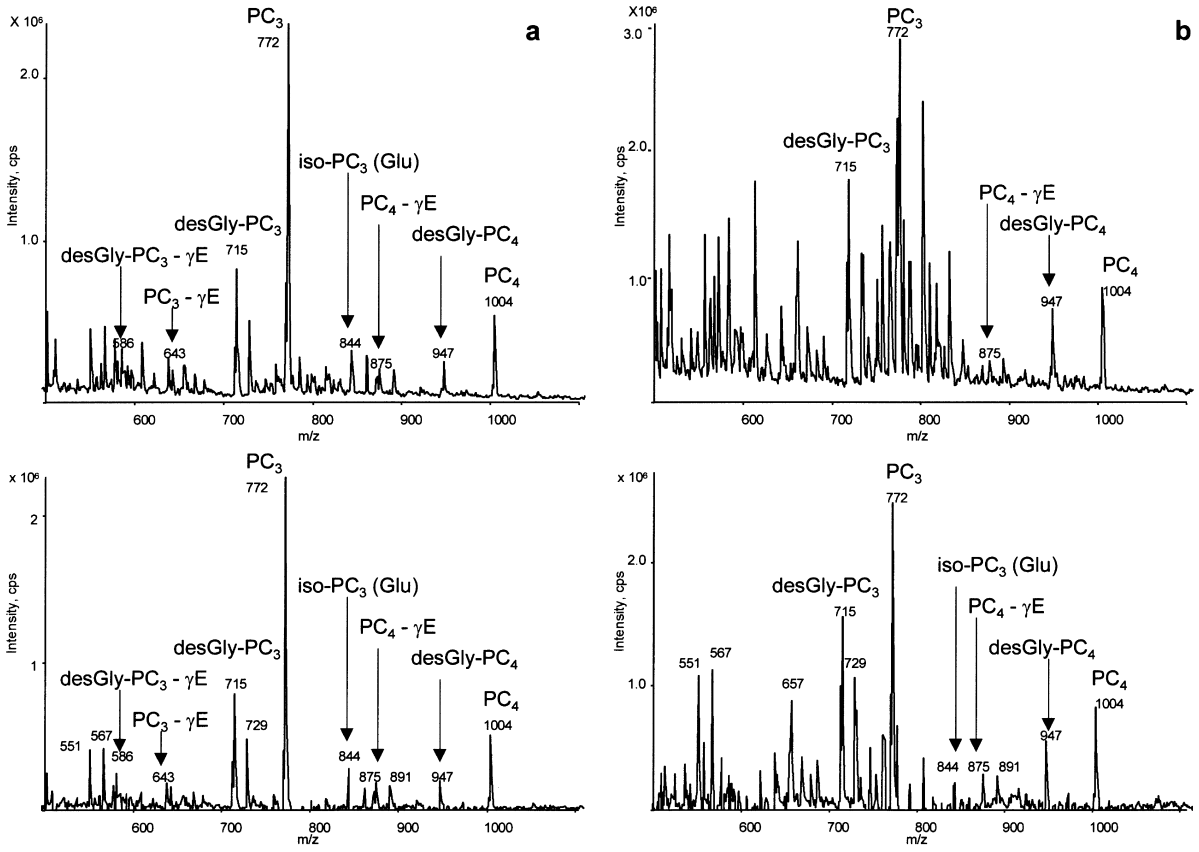


Fig. 6. Electrospray mass spectra of fractions collected in reversed-phase HPLC–UV of Cd-exposed maize root extracts (upper) and after subtraction of the control (lower). (a) Fractions III of Fig. 4b for *Zea mays* cv. 'Zwerge'; (b) fractions III of Fig. 4a for *Zea mays* cv. 'Boss'.

to PC_4–γGlu. The peak at *m*/*z* 844 was revealed after subtraction of the control and was tentatively attributed to iso-PC_3(Glu) even if the low intensity of the peak does not allow its formal identification in MS/MS.

2.3. Separation of PC-related peptides by capillary electrophoresis with online identification by ESI MS

2.3.1. PC obtained in vitro from enzymatic reaction

The coupling of capillary electrophoresis (CE) with ESI MS was utilized to adddress the question of whether the presence of homologous phytochelatins lacking the N-terminal γGlu are present as endogenous peptides in vitro and as products of in vivo reactions, or whether they represent artifacts generated in ESI MS.

In the case of the mixture synthesised in vitro, the conditions were optimized to obtain a maximum resolution for PC_2, PC_3 and PC_4 using UV detection at 220 nm (data not shown). Because of the problem of compatibility of the electrolyte with the electrospray ionization process, the addition of EDTA (Kubota et al., 1998) or micelle-forming surfactants (such as SDS) to the buffer cannot be used for peptide separation. Consequently, the only parameters to be optimized were the lengths of the capillary, the pH and concentration of the buffer, the injection mode and the voltage applied for the separation. Although CE with UV detection was shown to give an excellent separation for PCs, the resolution is poorer in the case of ESI MS detection. The optimization of the CE–ESI MS interface was described in our previous work (Mounicou et al., 2000).

For the coupling CE–ESI MS, a wide mass range was first selected for signal acquisition and further reduced to focus on significant intense peaks and to increase the signal to noise ratio. The electrophoregram obtained at the optimized separation conditions with ESI MS detection is shown in Fig. 7a. The separation of PC_3 (*m*/*z* 772) from PC_3–γGlu (*m*/*z* 643) was achieved, leading to the conclusion that the latter is produced enzymatically. This type of compound is likely obtained from PC as the result of enzymatic hydrolysis of the N-terminal γ-linked Glu. The enzyme preparation isolated from *Silene cucubalus* suspension cell culture (Grill et al., 1989) and used to produce the PCs in vitro may contain other enzymatic activity in addition to the predominant PC synthase.

2.3.2. Fractions isolated from maize extracts

The conditions previously optimized were applied to the separation of PC-related peptides for the fractions III collected in the RP HPLC chromatogram of maize extracts (cf. Fig. 4). For the experiments in CE, the

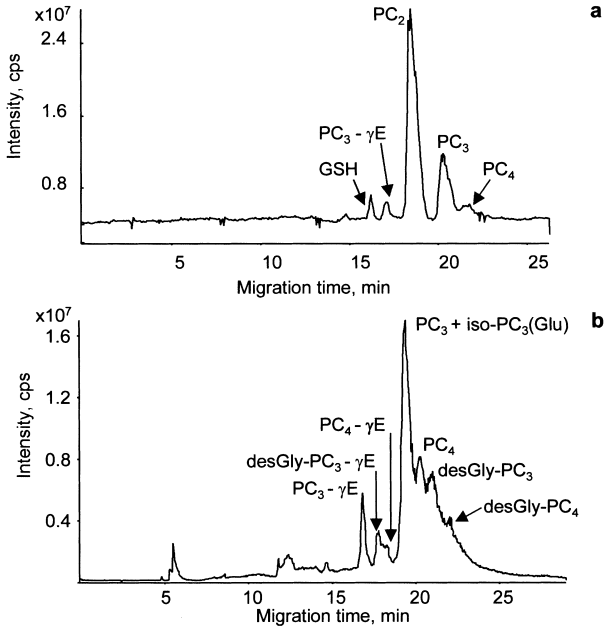


Fig. 7. Electrophoregrams with electrospray MS detection of PC and related peptides. (a) PC mixture resulting from enzymatic reaction (500 μg ml^{-1}); (b) fraction III collected in RP HPLC chromatogram of Cd-exposed maize root extract (*Zea mays* cv. 'Zwerge', as indicated in Fig. 4b). Conditions: capillary length, 95 cm; electrolyte, 5 mM ammonium acetate buffer pH 4.0; injection, 5 psi (4 s); separation, voltage (25 kV) + pressure (0.4 psi). Masses selected for acquisition: *m/z* 308, 483, 540, 586, 643, 715, 772, 844, 875, 947, 1004; width: ±4 Da; step size: 0.05 Da; Dwell time: 5 ms.

lyophilized material was dissolved in a minimum volume of buffer solution since less than 1 μl is injected on the separation capillary. An electrophoregram obtained for fraction III of *Zea mays* cv. 'Zwerge' is shown in Fig. 7b. The analysis was performed in 30 min and the separation of PC_3 and PC_4 and their corresponding desglycyl peptides (desGly–PC_3 and desGly–PC_4) was partially achieved. The identical migration time of PC_3 and iso-PC_3(Glu) can be attributed to a weak difference in the isoelectric points between the two peptides.

Additionally, the peptides desGly–PC_3–γGlu, PC_3–γGlu and PC_4–γGlu (*m/z* 586, 683 and 875) are well separated from their corresponding PC and desGly–PC leading to the conclusion that these compounds are formed enzymatically.

3. Conclusion

In conclusion, we have characterized two new thiol peptide families Cys–$(\gamma Glu–Cys)_n$-Gly (with $n = 1–5$) and Cys–$(\gamma Glu–Cys)_n$ ($n = 2$) the last being detected only in planta. The known iso-PC_3(Glu) present in maize exposed to Cd has been completely sequenced in MS/MS. Furthermore, a Cys–Cys bond-containing peptide has been identified as a minor product from the in vitro enzymatic reaction from GSH. We also have shown the high potential of tandem mass spectrometry for elucidating the structure of minor PC forms originally present in a complex matrix. Future work will focus on the characterization of the thiol peptides present as Cd-binding complexes which are involved in metal detoxification in plants.

4. Experimental

4.1. Phytochelatins resulting from in vitro enzymatic reaction

In vitro synthesis of phytochelatins from glutathione with the enzyme PC synthase isolated from *Silene cucubalus* suspension cell culture was carried out according to the method of Grill et al. (1991). The specific activity of the purified enzyme is 463 pkat / mg of protein (1 kat = the amount of enzyme that catalyses a reaction rate of 1 mol of substance per s) (Grill et al., 1989). 2000–10 000 pkat of the enzyme were incubated at 25°C with 1 mM GSH and 0.8 mM $Cd(NO_3)_2$ in 1.2 l of buffer solution (pH 8.0). An amount of 0.02% of NaN_3 was added to retard bacterial growth.

Proteins were precipitated from the resulting solution by the addition of $(NH_4)_2SO_4$ to reach a concentration of 85% followed by a centrifugation at 8000 *g* for 30 min. Phytochelatins were precipitated from the supernatant by the addition of 20 ml of 1 M $Cd(NO_3)_2$ solution. The precipitated mixture of phytochelatins was centrifuged and washed twice with water. The washed precipitate was stable for several months when stored at −20°C.

The precipitated phytochelatins were dissolved in 3.6 M HCl and separated by semi-preparative RP HPLC using elution with a concentrated gradient of CH_3CN in water. A pool of fractions was collected and CH_3CN was rotavaporated. The fractions were lyophilized and freeze-dried material stored at −80°C.

The heart-cut fractions corresponding to individual phytochelatins (PC_2, PC_3 and PC_4) were prepared in the same way as previously described to produce compounds used as standards.

4.2. Plant material and culture conditions

Seeds of *Zea mays* cv. 'Boss', 'Consul', 'Zwerge' and 'Popcorn neuform' were germinated and grown in sand at controlled temperature (22°C). 15 days after planting, the roots were exposed to 100 μM Cd ($Cd(NO_3)_2$) solution in water. After 7 days of exposure, samples were collected. Plant roots were excised from the rest of the plant and rinsed with deionized water. They were frozen in liquid nitrogen and stored at −20°C. The same protocol was used for non-exposed plants used as control samples for RP HPLC and mass spectrometric experiments.

4.3. Instrumentation

Chromatographic separations were performed using a Model K-1001 HPLC pump (KNAUER, Berlin, Germany) equiped with a UV detector (KNAUER K-2600). Injections were made with an autosampler (KNAUER K-3800) with a 100 μl injection loop (RHEODYNE, Cotati, CA, USA).

Electrospray MS/MS experiments were performed using an API 300 (PE SCIEX, Thornhill, ON, Canada) triple quadrupole mass spectrometer. BioToolBox software was used for the interpretation of fragmentation patterns and the elucidation of peptide sequences.

Capillary electrophoresis experiments were carried out with a BECKMANN P/ACE 2200 instrument (BECKMANN Instruments, Inc., Fullerto, CA, USA). For the CE-ESI MS interfacing, a make-up flow was supplied by means of a HARVARD Model 55111 syringe pump (HARVARD Apparatus, Southnatick, MA, USA).

4.4. Materials

HPLC separations were carried out using a reversed-phase Eurospher-100 C_{18} (particle size 10 μm, pore size 300 Å) column (250×4 mm id) equipped with a precolumn (10×4 mm id) (KNAUER, Berlin, Germany). Capillary electrophoresis separations were carried out in an uncoated fused silica capillary (BECKMANN Instruments, Inc., Fullerto, CA, USA) of length 95 cm, 75 μm internal diameter and 150 μm external diameter.

Analytical reagents were purchased from SIGMA-ALDRICH (Steinheim, Germany) and MERCK (Darmstadt, Germany). CH_3CN and MeOH were of LC grade and TFA and HOAc of spectroscopic grade. Water purified using a Milli-Q system (MILLIPORE, Bedford, MA, USA) was used. The enzyme carboxypeptidase A (from bovine pancreas) was purchased from SIGMA-ALDRICH (Steinheim, Germany).

4.5. Analytical procedures

4.5.1. Sample preparation from plants

For the determination of PC and related peptides in RP HPLC with postcolumn derivatization, the procedure described by Grill et al. (1991) was used. Phytochelatin assays of plant tissue were performed by freezing the material in liquid nitrogen followed by grinding with a mortar and pestle. The sample (400 mg of tissue powder) was mixed with alkaline solution (400 μl 1 N NaOH solution containing 1 mg ml^{-1} $NaBH_4$) and 100 μl water and the suspension was sonicated by 10 pulses of 5 s each at maximal energy setting. The cell debris were subsequently sedimented by centrifugation at 10 000 *g* at 4°C for 5 min. Proteins were precipitated from the supernatant (500 μl) by the acidification with 100 μl 3.6 N HCl solution and sample incubation on ice for 5 min. The precipitated proteins were further sedimented by centrifugation at 10 000 g at 4°C for 5 min and the supernatant (600 μl) was filtered (0.45 μm) before injection on the reversed-phase column.

The procedure for isolation of PC-related peptides for characterization in ESI MS/MS analysis was adapted to concentrate species in the maize root extracts. H_2O was used as an extractant instead of alkaline solution. 1 g of tissue powder was mixed with 1 ml water and the suspension sonicated. The debris were sedimented by centrifugation and the supernatant lyophilized to concentrate PC in the extract. The dried residue was resuspended in 100 μl alkaline solution, 25 μl H_2O was added and the solution acidified with 25 μl 3.6 N HCl solution before incubation on ice. The precipitated proteins were eliminated by centrifugation. The 10 fold more concentrated supernatant (150 μl) was filtered before injection in the RP HPLC for the isolation of PC-related peptide fractions.

4.5.2. Postcolumn derivatization RP HPLC analysis of PCs and related peptides

PC and related peptides were separated on a reversed-phase C_{18} column and the postcolumn derivatization of the sulfhydryl groups with Ellman's reagent (DTNB, 5,5′-dithiobis[2-nitrobenzoic acid]) and spectrophotometric detection was carried out according to Grill et al. (1991). An 100 μl aliquot was injected on the column. The separation included a step of on-column preconcentration of the sample (5 min) with 0.1% TFA in water followed by a linear increase of CH_3CN in the eluent to 20% in 18 min. The flow rate was set at 2 ml min^{-1}. The derivatization was accomplished by mixing the eluate with the reagent solution (DTNB) using a KNAUER postcolumn pump at a flow rate of 2 ml min^{-1}. The mixture is passed through a reaction loop (5.0 ml capacity) at room temperature. A better derivatization efficiency can be reached by elevating the temperature of the postcolumn reaction loop to 37°C (Meuwly et al., 1993). Absorbance at 410 nm was monitored to measure the derivatized thiolate peptides.

4.5.3. Isolation of PC and related peptides for characterization in ESI MS/MS

To collect fractions for the characterization of peptides by ESI MS/MS the chromatographic conditions previously described were used but with UV detection at 220 nm. The eluate containing CH_3CN was collected. The organic solvent was removed by rotary evaporation and the aqueous residue was freeze-dried and stored at −20°C before analysis. The dried residue was further dissolved in 200 μl of 60 mM HOAc in 30% MeOH (for ESI MS/MS analysis) or in 50 μl 5 mM NH_4OAc buffer pH 4.0 (for CE with online ESI MS detection).

4.5.4. ESI MS/MS operating conditions

The triple quadrupole mass spectrometer can be operated in the MS mode for the determination of the molecular mass of molecular ions of peptides and in the MS/MS configuration to elucidate peptide sequences.

In the MS operating mode, the first quadrupole Q1 was swept over a given mass range and Q3 was operated in rf-only mode. The orifice potential was 40 V, the ion spray voltage was 4100 V and the ion multiplier potential was 2400 V. The total spectra of peptides were acquired first in the range 300–1500 Da using a 10 ms dwell time and a 1 Da step size during 10 scans. The mass range was further reduced to focus on interesting compounds using a 5 ms dwell time and 0.5 Da step size for accurate mass measurement.

In the MS/MS mode, Q1 is used as a filter to transmit the molecular ion to the collision chamber (Q2) where it is fragmented by energy transfer with gas molecules. Fragments ions are analysed by Q3 to provide a mass spectrum; the product ion scan mode was selected for data acquisition. The mass of Q1 was fixed and Q3 was swept over a given mass range to determine the ions that result from the fragmentation of the precursor molecular ion. The collision gas was nitrogen and the collision energies used were in the range 20–50 eV depending on the mass of the peptides and to obtain optimum fragmentation (Vacchina et al., 1999).

4.5.5. Capillary electrophoresis and CE–ESI MS interface

The separation capillary was pre-conditioned with 0.5 M NaOH and flushed with water for 30 min. NH_4OAc buffer was selected as an electrolyte because of it's widely shown compatibility with electrospray ionization (Mounicou et al., 2000). Neutral and acidic pH (6–7) conditions were investigated and the optimium separation was obtained for the PC enzymatic mixture at pH 4.0. The buffer concentration was kept as low as possible to minimize the signal suppression in the electrospray source and the Joule heating. A concentration of 5 mM was found to assure sufficient conductivity and resulted in the current of 7 μA for a potential of 25 kV. The PC mixture obtained enzymatically in vitro was used as standard mixture to optimize the separation of PC_2, PC_3 and PC_4. Samples were injected applying a pressure of 5 psi for 4 s. After running each sample, the CE capillary was regenerated using a 0.1 M NaOH flush for 2 min and milli-Q H_20 for 2 min, followed by 2 min of flushing with the electrolyte (running buffer solution) to equilibrate the surface.

For the CE–ESI MS coupling, the design described by Corr and Anacleto (1996) was used. The electrophoretic capillary was threaded through the ion-spray needle to terminate 1 mm from the end of the sprayer. A sheath-flow was supplied coaxially to merge with the separation capillary flow just before the orifice. The makeup flow of 2 μl min^{-1} (60 mM HOAc in 30% MeOH, pH 3) was supplied by means of a Harvard Model 55111 Syringe pump. The potential applied to the ion-spray was 4.1 kV. The resulting potential difference across the separation capillary was 20.9 kV. An electrospray nebulizer gas flow of 1.0 l min^{-1} was used for all experiments.

For data acquisition, a large mass range was first selected (range 300–1400 Da) using a 2 ms dwell time and a 1 Da step size. To improve the signal to noise ratio, defined masses were further selected to focus on major peaks of interest (for thiolate peptides already observed in direct MS analysis) using a 5 ms dwell time and 0.05 Da step size.

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Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves

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Abstract

Accumulation of 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc) was induced in maize leaves by treatment with $CuCl_2$, chitopentaose, penta-*N*-acetylchitopentaose, or jasmonic acid (JA). The accumulation of HDMBOA-Glc was accompanied by a decrease in level of 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc). When the leaf segments were treated with JA in the presence of [Me-$^2H_3$]L-methionine, the label was efficiently incorporated into HDMBOA-Glc, while no incorporation into DIMBOA-Glc or HMBOA-Glc was detected, suggesting the conversion of constitutive DIMBOA-Glc to HDMBOA-Glc by methylation at the 4-position. Levels of endogenous JA and its leucine conjugate transiently increased prior to the accumulation of HDMBOA-Glc in leaf segments treated with $CuCl_2$ and chitopentaose. The lipoxygenase inhibitor ibuprofen suppressed the accumulation of HDMBOA-Glc induced by $CuCl_2$ treatment, and the reduced accumulation of HDMBOA-Glc was recovered by addition of JA. These findings suggested that JA functions as a signal transducer in the induction of HDMBOA-Glc accumulation. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Zea mays*; Poaceae; Maize; Benzoxazinone; Jasmonic acid; Induction

1. Introduction

1,4-Benzoxazin-3-ones (Bxs), major secondary metabolites found in Poaceous plants such as wheat, maize and rye, occur as glucosides, and are implicated in the resistance of plants to pathogens and insects (Niemeyer, 1988). Biological activities of the glucosides are weaker than the corresponding aglycones (Corcuera et al., 1985; Givovich and Niemeyer, 1995). Upon disintegration of tissues by infection or insect attack they are thought to be rapidly hydrolyzed to produce antifeeding and antimicrobial aglycones. Recently, β-glucosidases specific for Bx glucosides have been found to occur in non-autotrophic wheat and rye (Sue et al., 2000a,b), and in non-autotrophic and autotrophic maize (Ebisui et al., 1998; Oikawa et al., 1999).

The predominant Bx species found in maize is 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc) (Niemeyer, 1988). Its demethoxy analogue, 2-(2,4-dihydroxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIBOA-Glc), and the lactam glucoside, 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HMBOA-Glc), are found in smaller amounts (Tipton et al., 1967). Fully methoxylated 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc) and 8-methoxy DIMBOA-Glc (DIM_2BOA-Glc) were isolated as very minor components (Hofman and Hofmanová, 1970, 1971).

Changes in contents of Bxs following infection by pathogenic fungi, insect feeding and artificial damage have been investigated in maize and wheat plants (Gutierrez et al., 1988; Niemeyer et al., 1989; Morse et al., 1991; Weibull and Niemeyer, 1995). In these studies, the changes in DIMBOA level or total Bx content were examined. There has been only one report of induction of accumulation of HDMBOA-Glc (Bücker

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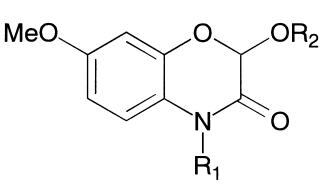
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and Grambow, 1990). When wheat plants were infected with the stem rust fungus, the content of HDMBOA-Glc increased only in a moderately resistant cultivar. Here, we examined changes in the contents of Bxs in maize leaves after treatment with various compounds that are known to elicit defense reactions in other Poaceous plant systems. In addition, to address the mechanism underlying the induced changes in Bx contents, we investigated the action of jasmonic acid (JA).

2. Results

2.1. Effects of treatment with various compounds on Bx contents in maize leaf segments

Segments obtained from the third leaf of 10-day-old maize seedlings were floated on solutions containing various compounds for 48 h, and changes in amount of Bxs were measured. The chemical structures of the investigated Bxs are shown in Fig. 1. Floating on the solutions of 0.75 mM chitopentaose [$(GlcN)_5$] and penta-*N*-acetylchitopentaose [$(GlcNAc)_5$] caused an increase in the level of HDMBOA-Glc (1.6 and 1.9 μmol g^{-1} fr. wt, respectively) and a corresponding decrease in that of DIMBOA-Glc (Fig. 2). $CuCl_2$ (0.5 mM) and JA (50 μM) resulted in the marked accumulation of HDMBOA-Glc, to a level of about 5 μmol g^{-1} fr. wt which was about 20 times that observed in the intact unchallenged leaf. A corresponding decrease in the level of DIMBOA-Glc was also detected after treatment with $CuCl_2$ and JA. Application of 1 mM ethephon and 0.75 M sucrose caused an increase in HDMBOA-Glc and a decrease in DIMBOA-Glc to an extent similar to that of 0.75 mM $(GlcN)_5$ and $(GlcNAc)_5$ respectively. Wounding did not affect the Bx contents. Although data are not shown, 1 mM aminocyclopropancarboxylic acid (ACC) exerted an effect similar to 1 mM ethephon. Other chemicals examined included abscisic acid, indole acetic acid, zeatin, gibberellin A_3 and salicylic acid at 50 μM, and NaCl at 0.75 M; however, these showed no detectable differences. Segments treated with $(GlcN)_5$ and $CuCl_2$ contained 0.1–0.2 μmol g^{-1} fr. wt of HMBOA and DIMBOA. Since necrotic damage was observed in these segments at the cut ends, but not in those treated with $(GlcNAc)_5$, the amounts of the aglycones were thought to be formed by more extensive hydrolysis of the corresponding glucoside.



	R_1	R_2
DIMBOA	OH	H
DIMBOA-Glc	OH	Glc
HMBOA	H	H
HMBOA-Glc	H	Glc
HDMBOA-Glc	OMe	Glc

Fig. 1. Structures of benzoxazinones and their glucosides.

We next investigated the effects of concentrations of $(GlcN)_5$, $CuCl_2$ and JA (Fig. 3). JA induced the accumulation of HDMBOA-Glc at the lowest concentration, followed by $CuCl_2$ and $(GlcN)_5$. The increase in levels of HDMBOA-Glc and the decreases in that of DIMBOA-Glc were detectable at 0.1 and 0.05 mM and reached a saturation level at 0.75 and 0.5 mM for $(GlcN)_5$ and $CuCl_2$ treatment, respectively. The change in Bx composition was detected following treatment with 0.002 mM JA, and the levels of HDMBOA-Glc increased along with the JA concentration up to 0.2 mM. DIMBOA and HMBOA-Glc levels were increased slightly with increasing concentrations of $(GlcN)_5$ and $CuCl_2$, and a small amount of HMBOA was found in segments treated with higher concentrations of these chemicals.

Fig. 4 shows the effects of duration of treatment on HDMBOA-Glc accumulation. In leaf segments treated with 0.5 mM $CuCl_2$ or 50 μM JA, the amounts of HDMBOA-Glc increased steeply to reach a plateau 48 h after treatment (about 5 μmol g^{-1} fr. wt), and remained at this level thereafter until the end of the experimental period (96 h). Although the maximal level of HDMBOA-Glc in 0.5 mM $(GlcN)_5$-treated leaves was about one quarter of that in leaves treated with $CuCl_2$ or JA, the manner of accumulation of HDMBOA-Glc was substantially the same.

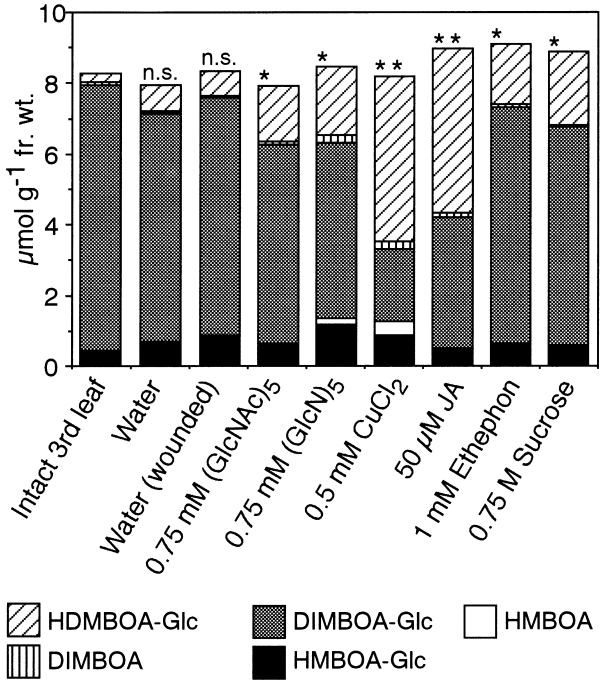


Fig. 2. Effects of treatment with various compounds on benzoxazinone contents in leaf segments. The segments were prepared from the third leaf of 10-day-old maize, and floated for 48 h on solutions containing various compounds as indicated. Differences in levels of HDMBOA-Glc between intact and treated leaves were tested by the Kruskal–Wallis test followed by a Dann's multiple-comparison test; n.s., not significant; * $P < 0.05$, ** $P < 0.01$.

2.2. Involvement of JA in changes in Bx content

Since JA induced the accumulation of HDMBOA-Glc most effectively among the compounds tested, we hypothesized that JA plays a role in signal transduction as suggested in other plant systems (Creelman and Mullet, 1997). To examine this possibility, the changes in level of endogenous JA were measured in $CuCl_2$- and $(GlcN)_5$-treated leaf segments. Before treatment, the levels of JA in leaf segments were very low (0.03 nmol g^{-1} fr. wt or less). JA levels started to increase immediately after treatment with $CuCl_2$, reaching a maximum level (0.3 nmol g^{-1} fr. wt) 6 h after treatment, and then decreased rapidly to about 0.04 nmol g^{-1} fr. wt (Fig. 5A). We also found similar changes in the leucine conjugate of JA (JA-Leu, Schmidt et al., 1990), the maximum level being about half that of JA (Fig. 5B). In $(GlcN)_5$-treated tissues, JA and JA-Leu appeared as in $CuCl_2$-treated segments, reaching a maximum level at 2 h, and then decreased to a barely detectable level. Their maxima occurred about 4 h earlier than in the $CuCl_2$-treated tissues. The maximum level of each was about one quarter of those in the $CuCl_2$-treated segments.

We also investigated the effects of ibuprofen (Ib), a compound that has been shown to inhibit lipoxygenase and hence biosynthesis of JA (Staswick et al., 1991), to examine the potential involvement of JA in the accumulation of HDMBOA-Glc following treatment with $CuCl_2$. This compound alone showed little effect at 1 mM, whereas it reduced the production of HDMBOA-Glc induced by 0.5 mM $CuCl_2$ to a level about one-third of that in tissues treated with $CuCl_2$ alone (Fig. 6). When JA (50 μM) was added to the solution of $CuCl_2$ (0.5 mM) and Ib (1 mM), the accumulated amount of HDMBOA-Glc increased to a level as high as that in the $CuCl_2$-treated segments.

The effects of the translational inhibitor cycloheximide (Cx) were also examined (Fig. 6). When Cx was applied at 20 μM in the presence of $CuCl_2$, the level of HDMBOA-Glc remained as low as that in the control segments. There was little increase in the accumulated amount of HDMBOA-Glc when JA (50 μM) was added to the solution of $CuCl_2$ (0.5 mM) plus Cx (20 μM) (Fig. 6). On the other hand, the amounts of DIMBOA increased following treatment with the mixture of $CuCl_2$, Cx and JA. This is considered to be related to

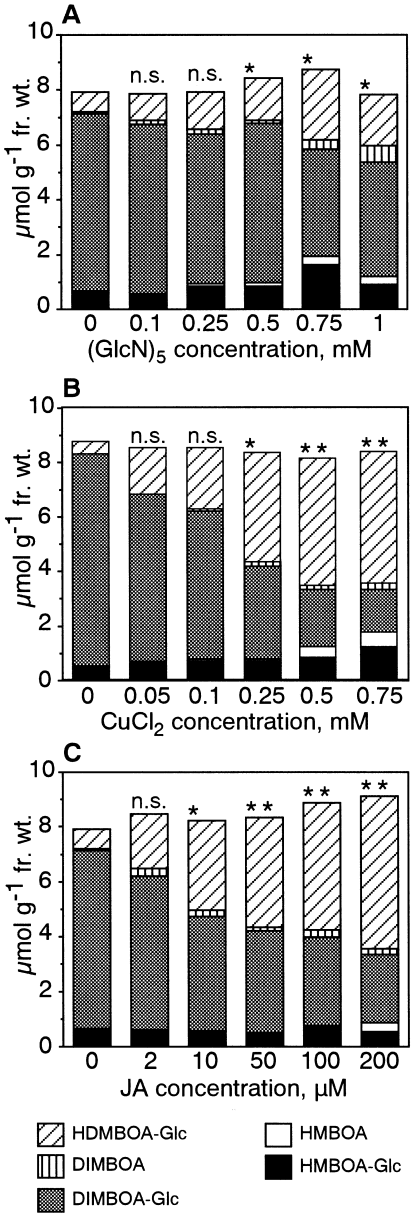


Fig. 3. Effects of concentration of chitopentaose [$(GlcN)_5$, (**A**)], $CuCl_2$ (**B**), and jasmonic acid (JA, **C**) on benzoxazinone contents in maize leaf segments. Benzoxazinone contents were determined after 48 h incubation with the different chemicals. Differences in levels of HDMBOA-Glc between leaves treated with water (showed as 0 mM or 0 μM) and inducers were tested by the Kruskal–Wallis test followed by a Dann's multiple-comparison test; n.s.. not significant; * $P < 0.05$, ** $P < 0.01$.

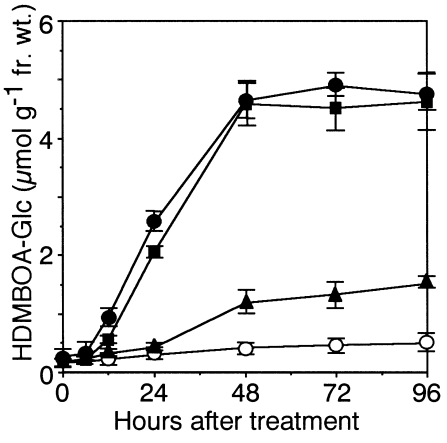


Fig. 4. Time course of accumulation of HDMBOA-Glc in leaf segments treated with 50 μM jasmonic acid (●), 0.5 mM $CuCl_2$ (■), 0.75 mM chitopentaose (▲), and water (○). Error bars indicat standard deviations of five replicates.

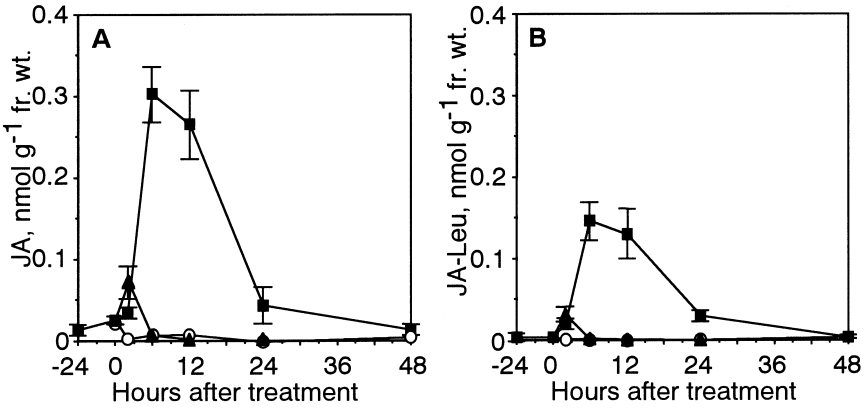


Fig. 5. Time course of accumulation of jasmonic acid (JA, **A**) and its leucine conjugate (JA-Leu, **B**) in leaf segments treated with 0.5 mM $CuCl_2$ (■), 0.75 mM chitopentaose (▲), and water (○). Error bars indicate standard deviations of five replicates.

the action of JA applied exogenously because no increase in levels of DIMBOA was observed following treatment with the mixture of $CuCl_2$ and Cx. The effect of exogenous JA that does not involve protein synthesis may have become visible in the presence of Cx.

2.3. Administration of [Me-$^2H_3$]L-methionine

To investigate the origin of the HDMBOA-Glc that accumulated after treatment with JA, [Me-$^2H_3$]L-methionine was administered to the leaf segments in the presence of 50 μM JA. The incorporation of deuterium atoms was analyzed by ion-spray LC/MS. For HDMBOA-Glc, the pseudomolecular ion containing three deuterium atoms was clearly observed at *m*/*z* 391 in addition to pseudomolecular ion at *m*/*z* 388, demonstrating incorporation of the methyl group from [Me-$^2H_3$]L-methionine. On the other hand, the deuterium atoms were scarcely incorporated into HMBOA-Glc or DIMBOA-Glc (Table 1).

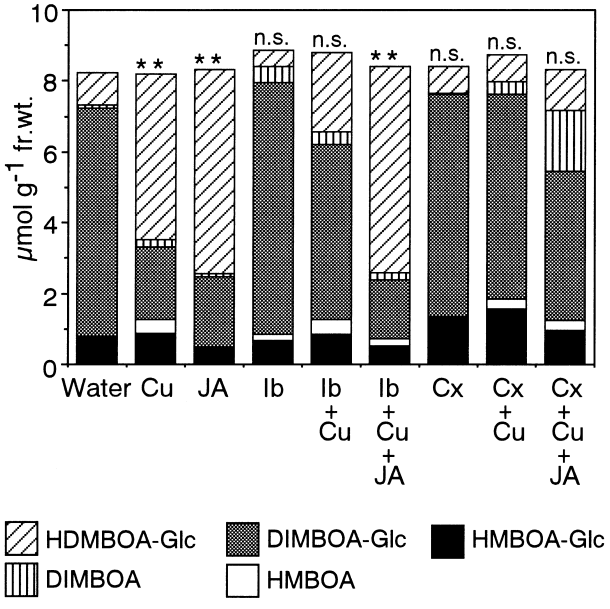


Fig. 6. Effects of ibuprofen (Ib) and cycloheximide (Cx) on accumulation of HDMBOA-Glc induced by $CuCl_2$. Ib (1 mM) and Cx (20 μM) were administered to leaf segments treated with water, 0.5 mM $CuCl_2$, or 0.5 mM $CuCl_2$ plus 50 μM jasmonic acid (JA). Differences in HDMBOA-Glc in water and treated leaves were tested by Kruskal–Wallis test followed by a Dann's multiple-comparison test; n.s., not significant; ** $P < 0.01$.

3. Discussion

In the present study, we found that maize leaves accumulated HDMBOA-Glc when treated with $(GlcNAc)_5$, $(GlcN)_5$ or $CuCl_2$. Chitin and chitosan fragments have been demonstrated to induce defense reactions in many plants including Poaceous species. Production of oat phytoalexins, avenanthramides, is effectively induced by treatment with these compounds (Bordin et al., 1991). In addition, accumulation of phytoalexins in rice suspension (cell) cultured cells (Nojiri et al., 1996) and lignification in wheat leaves (Barber et al., 1989) are induced by treatment with chitin fragments. Fungal cell walls are largely composed of chitin and glucan (Bartnicki-Garcia, 1968; Mendgen et al., 1985). Fragments released from chitin by hydrolytic enzymes have been suggested to be recognized by plant cells as

Table 1
Incorporation of deuterium atoms into benzoxazinones from [Me-$^2H_3$]L-methionine. Maize leaf segments were treated with JA (50 μM) in the presence of [Me-$^2H_3$]L-methionine (1 mM)

Benzoxazinone	Amount (nmol)	Labeled compound (%)	Incorporation (%)
HMBOA-Glc	80	0.37	0.01
DIMBOA-Glc	210	n.d.[a]	n.d.
HDMBOA-Glc	490	39	6.4

[a] n.d., Not detectable.

elicitors (Boller, 1995). The induction of phytoalexin production by heavy metal ions has also been shown in numerous species, although their mode of action has not been clarified. In particular, $CuCl_2$ was the most effective elicitor for phytoalexin production in rice leaves (Kodama et al., 1988). These findings suggested that the accumulation of HDMBOA-Glc may be part of the defense mechanisms in maize.

Accumulation of HDMBOA-Glc has been suggested to be involved in the defense system in wheat (Bücker and Grambow, 1990). HDMBOA-Glc accumulated in leaves only in certain combinations between moderately resistant wheat cultivars and a race of the stem rust fungus. These previous authors suggested that HDMBOA-Glc plays a role in defense reactions as a phytoalexin in wheat. The observation that HDMBOA-Glc accumulated in infected wheat leaves may support the defensive role of HDMBOA-Glc in maize.

HDMBOA, produced by hydrolysis of the corresponding glucoside, spontaneously decomposes to give MBOA, and the speed of decomposition is faster than that of other Bxs (Grambow et al., 1986). MBOA itself has a number of biological activities (Virtanen et al., 1957; Elnaghy and Linko, 1962), but intermediate species or other degradation products may also be important for inhibiting pathogens, as in the case of DIMBOA, another precursor of MBOA (Woodward et al., 1978b). HDMBOA-Glc may have a role in the plant defense system as part of a rapid response to invasion.

In addition to $(GlcNAc)_5$, $(GlcN)_5$ and $CuCl_2$, treatment with low concentrations of JA cause accumulation of HDMBOA-Glc. In the $CuCl_2$-treated segments, JA and JA-Leu levels transiently increased preceeding accumulation of HDMBOA-Glc. In $(GlcN)_5$-treated tissue, increased levels of JA and JA-Leu were low corresponding to the low accumulation rate of HDMBOA-Glc. Furthermore, the lipoxygenase inhibitor Ib impeded accumulation of HDMBOA-Glc in $CuCl_2$-treated tissues, and addition of JA restored production of HDMBOA-Glc. These findings suggest that the inducers trigger the biosynthesis of JA, as has been reported previously in a few species of plants (Creelman and Mullet, 1997), and JA evoked HDMBOA-Glc biosynthesis. Cycloheximide inhibited the production of HDMBOA-Glc almost completely, and JA did not restore the reduced production, indicating that translation of new protein is involved in this process.

Jasmonate-induced accumulation of secondary metabolites has been reported in numerous plants. The activity of JA has been mainly reported to be related to defense reactions in the Poaceae. Barley leaf segments treated with methyl jasmonate have been reported to accumulate 4-coumaroyl putrescine and 4-coumaroyl agmatine (Lee et al., 1997). The accumulation of these compounds has also been demonstrated in leaves (von Röpenack et al., 1998) and roots (Peipp et al., 1997) infected with fungi, although endogenous levels of JA were not measured after infection. In rice leaves and suspension cultured cells, treatment with elicitors caused transient accumulation of JA prior to accumulation of phytoalexins (Nojiri et al., 1996; Rakwal et al., 1996). These observations suggest that JA has a general role in the defense processes in the Poaceae.

The levels of accumulation of HDMBOA-Glc and the accompanying decrease in DIMBOA-Glc level were almost identical. When maize leaf segments were fed with [Me-$^2H_3$]L-methionine in the presence of JA, deuterium atoms were effectively incorporated into HDMBOA-Glc, but were scarcely detectable in either DIMBOA-Glc or HMBOA-Glc. These findings indicate that HDMBOA-Glc is produced by methylation of constitutive DIMBOA-Glc at the 4-position, but not by de novo biosynthesis, because the 7-methoxy groups of DIMBOA-Glc and HMBOA-Glc have been shown to originate from methionine (Reimann and Byerrum, 1964).

4. Experimental

4.1. Plant material and chemicals

Seeds of *Zea mays* cv. Snowdent 108 were purchased from Yukijirushi Seeds and Plants Co., Sapporo, Japan. [Me-$^2H_3$]L-Methionine was purchased from Isotec Inc., USA, chitopentaose and penta-*N*-acetylchitopentaose were from Seikagaku Kogyo, Tokyo, Japan, and other chemicals were from Wako Pure Chemical Industries, Osaka, Japan.

4.2. Preparation of benzoxazinones

Authentic DIMBOA-Glc, HMBOA-Glc and HDMBOA-Glc were extracted from maize seedlings with MeOH containing 2% HOAc, and purified by the method described previously (Nakagawa et al., 1995). The identities of the compounds were confirmed by ^1H NMR, MS and UV spectroscopy in reference to the data reported in the literature (Hofman and Hofmanová, 1970; Tipton et al., 1967; Woodward et al., 1978a; Lyons et al., 1988). The aglycones DIMBOA and HMBOA were prepared by hydrolysis of the corresponding glucosides using almond β-glucosidase (Sigma), and 6-methoxybenzoxazolinone (MBOA) was obtained by heating DIMBOA, according to the method described previously (Nakagawa et al., 1995).

4.3. Seedling culture

Maize seeds were immersed in conc. H_2SO_4 for 3 min to facilitate germination, and washed several times with sterilized water. Seeds were then sown in vermiculite

and incubated at 25°C with a 12 h period of illumination under fluorescent lamps (15 W m^{-2}). Germination occurred 42–48 h after sowing, the first leaf began to emerge from the sheath 72–86 h after germination, and the plant height was 20–25 cm 10 days after germination.

4.4. Treatment with chemicals

One leaf segment (about 2 cm along the longitudinal axis) excised from the third leaves of 10-day-old seedlings was floated on a soln. (1 ml) containing the chemical of interest in a Petri dish (4 cm i.d.). A segment was taken from one plant. For wounding, a bundle of sewing needles (about 1 cm in diameter) was used. Incubation was performed at 25°C with a 12 h period of illumination from fluorescent lamps (15 W m^{-2}). More than five replicates were performed for each treatment. Data of HDMBOA-Glc in Figs. 2, 3 and 6 were processed to evaluate significant differences in the values by a Kruskal–Wallis test followed by a Dann's multiple comparison test.

4.5. Analysis of benzoxazinones in plant material

Leaf segments were frozen in liquid N_2 and ground to a powder, followed by extraction with MeOH containing 2% HOAc. The extract was filtered through a disposable membrane filter (LCR13-LG, Millipore) and analyzed by HPLC (Shimadzu 10Avp) with a 4.6×150 mm metal-free Wakosil II 5C18 HG column (Wako). A two-solvent system was used to generate the mobile phase: solvent A was HOAc–H_2O (1:1000) and solvent B was MeCN. The flow rate was 0.8 ml/min. The mobile phase at the initiation of each run was A:B at a ratio of 92.5:7.5. After injection, the ratio was held for 20 min, and then a 20 min linear gradient from 92.5:7.5 to 85:15 (A:B) and another 5 min linear gradient from 85:15 to 60:40 (A:B) were applied. Elution of compounds was monitored at 280 nm.

4.6. Quantification of jasmonic acid

Extraction and quantification were carried out according to the method of Tamogami and Kodama (1998) with slight modifications. Leaf segments were frozen in liquid N_2 and ground to a powder, followed by extraction with 70% MeOH. Then, dihydrojasmonic acid (DHJA) was added to the extract as an internal standard. The extracts were filtered and concentrated to a small volume in vacuo. After adjusting the pH of the residual soln. to 3 with 1 N HCl, the soln. was extracted three times with Et_2O. The organic phase was washed with water, dried (Na_2SO_4) and concentrated in vacuo. The residue was dissolved in MeOH and subjected to turbo-ion-spray LC/MS/MS analysis. Negative ion-spray ionization mass spectra were obtained using a Perkin Elmer Sciex API-300 (ion-spray voltage: −3800 V, orifice voltage: −25 V, nebulizer gas: air, curtain gas: N_2) combined with HP 1100 HPLC system equipped with a 2.1×150 mm Inertsil ODS-2 column. The column was eluted with MeCN–H_2O–HCO_2H (600:400:1) at a flow rate of 0.2 ml/min. The parent and product ions were monitored at m/z 209/59 (JA), 322/129 (JA-Leu) and 211/59 (DHJA).

4.7. Administration of inhibitors

Leaf segments were floated on a soln. of 0.5 mM $CuCl_2$ containing either the lipoxygenase inhibitor ibuprofen (1 mM) or translational inhibitor cycloheximide (20 μM) in the presence or absence of 50 μM JA. After 48 h incubation, the contents of benzoxazinones in the segments were determined as described above.

4.8. Administration of [Me-$^2H_3$]L-methionine

Leaf segments were floated on 1 mM [Me-$^2H_3$]L-methionine soln. in the presence of 50 μM JA. After incubation for 48 h at 25°C, the segments were extracted with MeOH containing 2% HOAc and filtered. The filtrates were subjected to ion-spray LC/MS analysis. Positive ion-spray ionization mass spectra were obtained using a Perkin Elmer Sciex API-165 (ion-spray voltage: 5000 V, orifice voltage: 30 V, nebulizer gas: air, curtain gas: N_2) combined with a Shimadzu 10A HPLC system. HPLC conditions were the same as those described above. Percentages of compounds containing deuterium atoms in individual benzoxazinones were calculated from the ion intensity in the $[M+H]^+$ peak region.

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Visible light-dependent degradation of long-chain alkenes in killed cells of *Emiliania huxleyi* and *Nannochloropsis salina*

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Abstract

Photosensitized degradation rates of phytoplanktonic *n*-alkenes under visible light exposure were determined in laboratory experiments. Killed cells of *Emiliania huxleyi* and *Nannochloropsis salina* were used as source of biogenic alkenes. In *E. huxleyi* killed cells, minor C_{31} and C_{33} *n*-alkenes were strongly photodegraded, while the major C_{37} and C_{38} *n*-alkenes appeared particularly recalcitrant towards photochemical processes. This particular photochemical recalcitrance has been attributed to the chemical structure and localization of these hydrocarbons in the cells. Most of the *n*-alkenes of *N. salina* were strongly photodegraded in killed cells. The photodegradation of phytoplanktonic alkenes showed apparent second-order kinetics with respect to light exposure, and the half-life doses obtained logically decrease with increasing number of double bonds in these compounds. These results strongly suggest that significant amounts of phytoplanktonic *n*-alkenes must be photodegraded in the euphotic zone of the oceans during senescence. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Emiliania huxleyi*; *Nannochloropsis salina*; Long-chain *n*-alkenes; Photodegradation; Visible light; Killed cells

1. Introduction

In healthy cells of phytoplankton the primary route for energy conversion from the excited chlorophyll singlet state (^{1}Chl) goes via the fast photochemical reactions of photosynthesis (Foote et al., 1970). In dead phytoplanktonic cells this pathway is obviously not functional; thus accelerated production of the longer living triplet state (^{3}Chl) (by intersystem crossing) and of toxic oxygen species (singlet oxygen, hydrogen peroxide, superoxide ion and hydroxyl radical) (by reaction of ^{3}Chl with ground state oxygen) (Rontani, 1999) might be expected. The rate of formation of these potentially damaging species can then exceed the quenching capacity of the photoprotective system of the cells and photodegradation can occur (Merzlyak and Hendry, 1994). Chlorophylls would tend to remain associated with other hydrophobic cellular compounds, such as membrane lipids, in phytodetritus (Nelson, 1993). The photooxidative effect of chlorophyll sensitization can be strongly amplified within such a micro-environment; moreover, the lifetime of singlet oxygen ($^1O_2$) produced from sensitizers in a lipid-rich hydrophobic micro-environment could be longer and its potential diffusive distance greater, than if produced by sensitizers in an aqueous solution (Suwa et al., 1977).

It has been previously demonstrated that chlorophylls (Nelson, 1993), the chlorophyll phytyl chain (Rontani et al., 1995), carotenoids (Nelson, 1993), sterols (Rontani et al., 1997a) and monounsaturated fatty acids (Rontani, 1998) could be quickly photodegraded in senescent or dead phytoplanktonic cells. In contrast, some other lipids such as *n*-alkenones appeared to be relatively protected from photochemical damages in senescent cells of *Emiliania huxleyi* (Rontani et al., 1997b).

The present paper examines the rates of light-dependent degradation of long-chain *n*-alkenes in senescent cells of the Prymnesiophycea *Emiliania huxleyi* and the Eustigmatophycea *Nannochloropsis salina*.

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2. Results and discussion

2.1. Photodegradation of the hydrocarbon fraction of E. huxleyi CS-57

C_{31} alkadienes, C_{33} alkadi- tri and tetraenes and C_{37} and C_{38} alkatrienes have been reported to be present in different strains of *E. huxleyi*, although their distributions show significant differences between strains (Volkman et al., 1980; Conte et al., 1995). Recently, it was established that the hydrocarbon fraction of *E. huxleyi* CS-57 was mainly composed of straight chain C_{37} and C_{38} alkenes with two, three or four double bonds depending on growth temperature (Grossi et al., 2000). C_{31} dienes and C_{33} diene, trienes and tetraene were also present but in smaller amounts. At 20°C, the C_{37} and C_{38} tetraenes are lacking and C_{31} and C_{33} *n*-alkenes account in total for 9% of the total hydrocarbons.

In order to determine the photodegradation rates of these alkenes, we irradiated dead cells of *E. huxleyi* CS-57 for different times with two Osram Fluora lamps providing a spectral distribution of light between 400 and 700 nm (well suited for photobiological processes) and an irradiance (as Photosynthetically Active Radiation (PAR)) of 113 μmol m^{-2} s^{-1}. Sterile dark controls carried out in parallel showed no significant degradation of

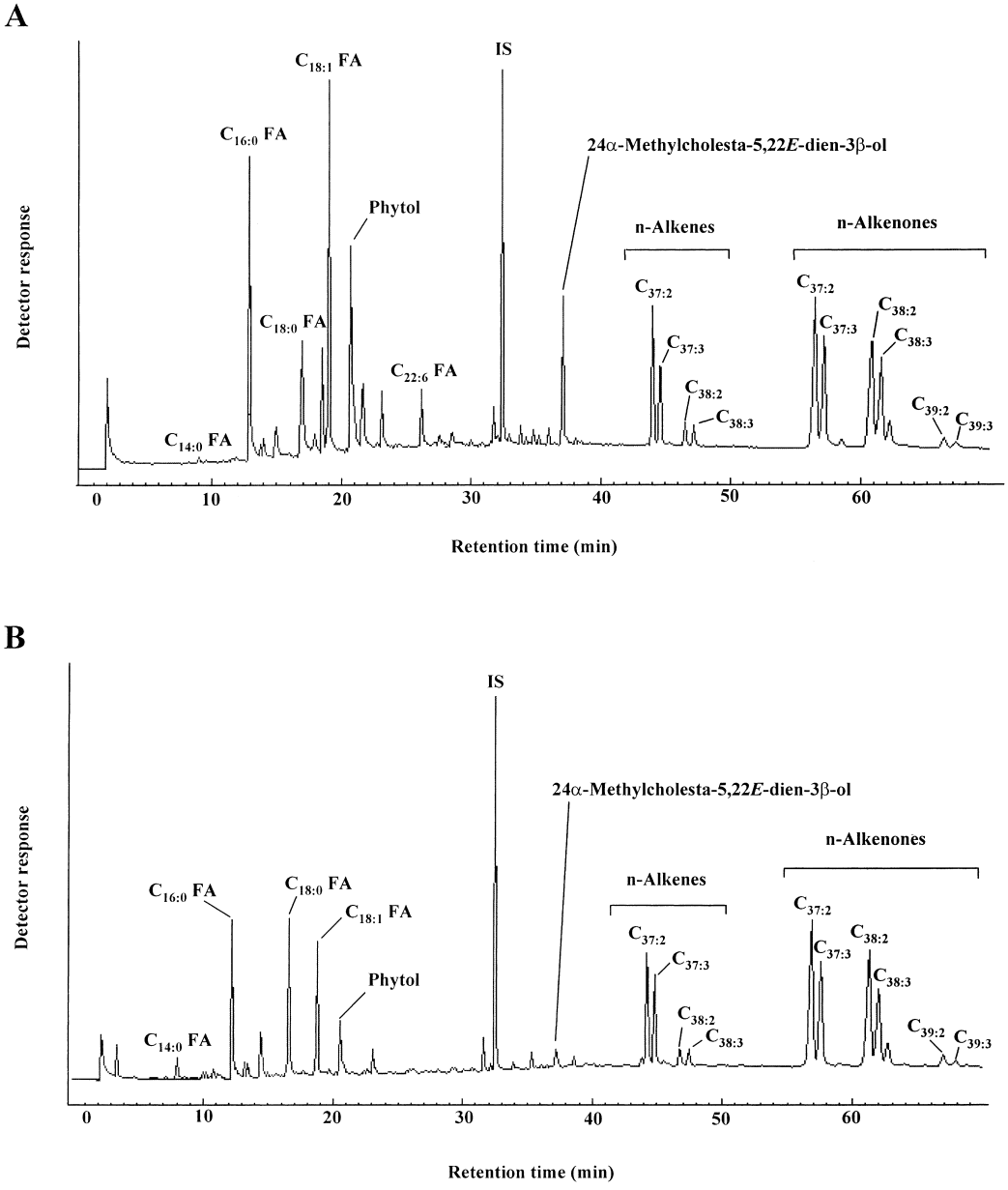


Fig. 1. Gas chromatograms (polar BPX-50 capillary column) of the unsaponifiable fractions obtained after exposition of *Emiliania huxleyi* CS-57 broken cells at 20°C for 20 days to different light regimes: (A) without irradiation under sterile conditions and (B) after exposure to 194 mol m^{-2}. IS = Internal standard (triacontane).

lipids. After photodegradation of all sensitizer substances (e.g. chlorophylls and phaeopigments), the photosensitized degradation processes stopped in irradiated flasks. At this time, a significant portion of 24α-methylcholesta-5,22*E*-dien-3β-ol, phytol and unsaturated fatty acids was photodegraded, while C_{37} and C_{38} *n*-alkenes and *n*-alkenones were practically unchanged (Fig. 1). These results are in good agreement with the poor photochemical reactivity of alkenones previously observed in killed cells of another strain of *E. huxleyi* (Rontani et al., 1997b). We found no evidence of a significant light-dependent degradation of C_{37} and C_{38} *n*-alkenes during these experiments. In contrast, C_{31} and C_{33} *n*-alkenes were quickly degraded during irradiation. These differences of photoreactivity imply distinct biological syntheses and/or functions for these two groups of hydrocarbons in *E. huxleyi* cells. This is in good agreement with the distinct quantitative and qualitative evolutions with varying growth temperatures of C_{31} and C_{33} alkenes on the one hand, and of C_{37} and C_{38} alkenes on the other hand, previously observed (Grossi et al., 2000). First-order kinetics generally adequately describe the photodegradation of lipids in phytodetritus (Nelson, 1993; Rontani et al., 1998) and the rate constants are obtained by the slopes of the regression lines determined as $\ln(C/Co) = -k_1 D$ (where C is the concentration at the time of sampling, Co is the initial concentration and D is the light dose). For the C_{31} and C_{33} *n*-alkenes, the experimental points obtained deviate significantly from a linear dependence. In contrast, plots of 1/[alkene] against light exposure present a relatively good linear fit (Fig. 2, Table 1). These results suggest that the photodegradation of C_{31} and C_{33} *n*-alkenes is a second-order process, which fits the equation $-\mathrm{d[alkene]}/\mathrm{d}D = k_2[\mathrm{alkene}]^2$. The half-life doses obtained logically decrease with the increasing number of double bonds of these compounds (Table 1).

Table 1
Apparent second-order rate constants (k_2)[a] and half-life doses ($D_{1/2}$)[b] for light-dependent degradation of minor *n*-alkenes of *Emiliania huxleyi*

n-Alkene	k_2 (μmol^{-1} 1 mol^{-1} m^2)	Regression r^2	*n*	$D1/2$ (mol m^{-2})
$C_{33:2}$	0.32	0.62	5	51.1
$C_{33:3}$	0.81	0.92	7	16.6
$C_{33:4}$	10.70	–	2	1.9
$C_{31:2}$	0.35	0.98	10	9.4

[a] The rate constants represent the slopes of regression lines determined as $1/C = k_2.D$, where C =concentration at the time of sampling and D =cumulative light dose (mol m^{-2}).
[b] $D_{1/2} = 1/(C_0.k_2)$, where C_0 =initial concentration.

Interestingly, the stereochemistry of the internal double bonds in C_{31} and C_{33} *n*-alkenes has been established to be *cis*, while C_{37} and C_{38} alkenes internal double bonds exhibit a *trans* geometry (Rieley et al., 1998; Grossi et al., 2000). The photochemical recalcitrance of C_{37} and C_{38} *n*-alkenes could thus be partly attributed to the *trans* geometry of their internal double bonds. The *trans* configuration is known to be seven fold less reactive towards singlet oxygen than the *cis* geometry (Hurst et al., 1985). However, these stereochemical considerations are not sufficient to fully explain the differences of photoreactivity observed between these *n*-alkenes.

Chlorophylls or phaeopigments, which are potential sensitizers of photoreactions, tend to remain associated with hydrophobic cellular material, such as membrane

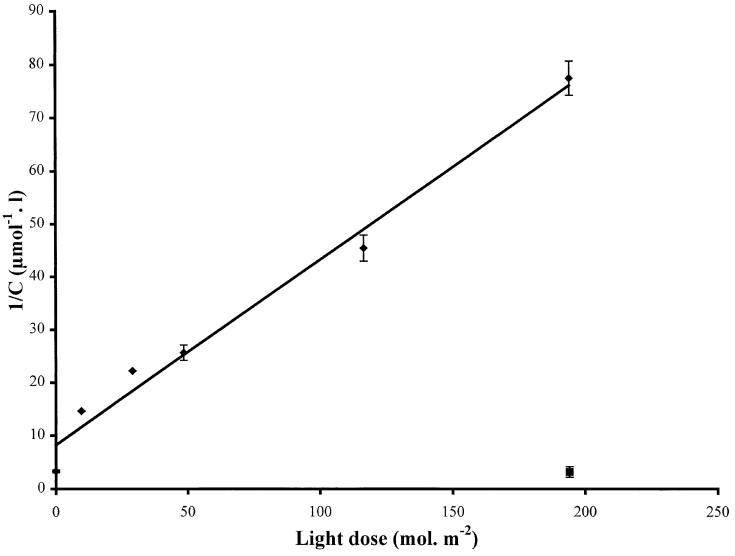


Fig. 2. Fit to apparent second-order kinetics for the photodegradation of *Emiliania huxleyi* CS-57 $C_{31:2}$ alkene. Square symbols represent values for dark control samples shown at the equivalent time points as samples from the parallel illuminated treatment. Irradiance for the light treatment was 113 μmol m^{-2} s^{-1}.

lipids, in phytodetritus (Nelson, 1993). As important components of the membranes of *E. huxleyi*, 24α-methylcholesta-5,22*E*-dien-3β-ol, phytol and unsaturated fatty acids are in fact quickly photodegraded in broken cells (Fig. 1). On the basis of their fast light-dependent degradation, C_{31} and C_{33} alkenes seem also to be membrane components. In contrast, the recalcitrance of C_{37} and C_{38} alkenes and alkenones towards photodegradation processes suggests a localization of these compounds elsewhere than in cellular membranes. This hypothesis is in good agreement with the results of Conte and Eglinton (1993), who failed to detect alkenones in membranes of *E. huxleyi* after cell fragmentation experiments. This very important aspect requires further investigation, since data concerning the localisation, the biological functions and the mode of synthesis of these compounds are still lacking.

2.2. Photodegradation of the hydrocarbon fraction of *N. salina*

It was previously established that the hydrocarbon fraction of *N. salina* was mainly composed of saturated and (poly)unsaturated (up to four double bonds) hydrocarbons ranging from C_{14} to C_{31}, the major compounds being the C_{25}, C_{27} and C_{29} alkenes (Gelin et al., 1997). Gas chromatography/mass spectrometry (GC/MS) analysis of the hydrocarbon fraction of our strain of *N. salina* confirmed these results (Fig. 3A). These authors have also determined the position of the double bonds of some of these alkenes by means of dimethyl disulfide (DMDS) treatment (Vincenti et al., 1987). It appeared that in the monoenes, the double bond is at the terminal position and in the dienes, they are predominantly at the terminal and ω^{17} positions. It is interesting to note that the electron impact (EI) mass spectra of the two isomeric $C_{27:3}$ alkenes **9** and **10** present a very strong base peak at m/z 79, which strongly suggests the presence of conjugated systems.

Irradiation of dead cells of *N. salina* resulted in a strong modification of the hydrocarbon fraction (Fig. 3B). We could not find evidence of a significant light-dependent degradation of monounsaturated hydrocarbons; this result can be attributed to the terminal position of the double bond in these compounds, which is poorly reactive towards singlet oxygen (Hurst et al., 1985). In contrast, di, tri-, and tetraenes were strongly photodegraded during irradiation. Plots of 1/[alkene] against light exposure present a very good linear fit (Fig. 4, Table 2). As in the case of *E. huxleyi*, the photodegradation of the *n*-alkenes of *N. salina* seems to be a second-order process, which fits the equation $-\mathrm{d[alkene]}/\mathrm{d}D = k_2[\mathrm{alkene}]^2$. The half-life doses obtained logically decrease with increasing number of double bonds of these compounds (Table 2). The determination of the position and the stereochemistry of all the internal double bonds would be necessary in order to be able to explain the differences of photoreactivity observed between alkenes with the same unsaturation degree (e.g. between compounds **6** and **12**). We can also note that the alkatrienes **9** and **10**, which seem to possess conjugated systems are not faster photodegraded than the non-conjugated alkatriene **5** (Table 2).

It was previously demonstrated that photoprocesses involving singlet oxygen play a key role during the visible light-induced degradation of the chlorophyll phytyl chain (Rontani et al., 1995), of sterols (Rontani et al., 1997a) and of mono-unsaturated fatty acids (Rontani, 1998) in senescent phytoplanktonic cells. In the case of the photodegradation of phytoplanktonic long-chain alkenes, no explanation can be given concerning the photoreaction involved, since we failed to identify sufficiently informative photoproducts.

2.3. In situ photodegradation of the different phytoplanktonic alkenes and its consequences

It is generally considered that approximately 95% of the total particulate organic matter consists of particles <20 μm having settling velocities lesser than 1 m d^{-1} (McCave, 1975). Consequently, the bulk of the organic particulate matter has a considerably long residence time within the euphotic layer. Assuming a surface daily irradiance of 60 mol m^{-2} (PAR) [a value representative of mid-latitude waters under a clear summer day (Nelson, 1993)], it clearly appears that C_{25}-C_{33} phytoplanktonic alkenes (half-life doses ranging from 2 to 350 mol m^{-2}) may be significantly photodegraded in the upper portion of the euphotic zone during senescence. Some of the more reactive alkenes (trienes and tetraenes) (half-life doses ranging from 2 to 17 mol m^{-2}) may be photodegraded even near the limit of the euphotic layer

Table 2
Apparent second-order rate constants (k_2) and half-life doses ($D_{1/2}$) for light-dependent degradation of *n*-alkenes of *Nannochloropis salina*

Code[c]	*n*-Alkene	k_2 (μmol^{-1} 1 mol^{-1} m^2)	Regression r^2	n	$D_{1/2}$ (mol m^{-2})
2	$C_{25:2}$	ND*	–	–	ND[a]
3	$C_{27:2}$	0.018	0.98	10	328
6	$C_{27:2}$	0.009	0.96	10	95
5	$C_{27:3}$	0.249	0.97	12	7
9 and 10	$C_{27:3}$ (conjugated?)	0.124	0.96	8	8
7	$C_{27:4}$	ND[b]	–	2	ND[b]
8	$C_{28:2}$	0.025	0.99	10	342
12	$C_{29:2}$	0.0037	0.99	10	297
14	$C_{29:3}$	0.199	0.98	12	10

[a] Not determined (coelution with a contaminant).
[b] Not determined (photodegradation too fast).
[c] These numbers refer to peaks in Fig. 3.

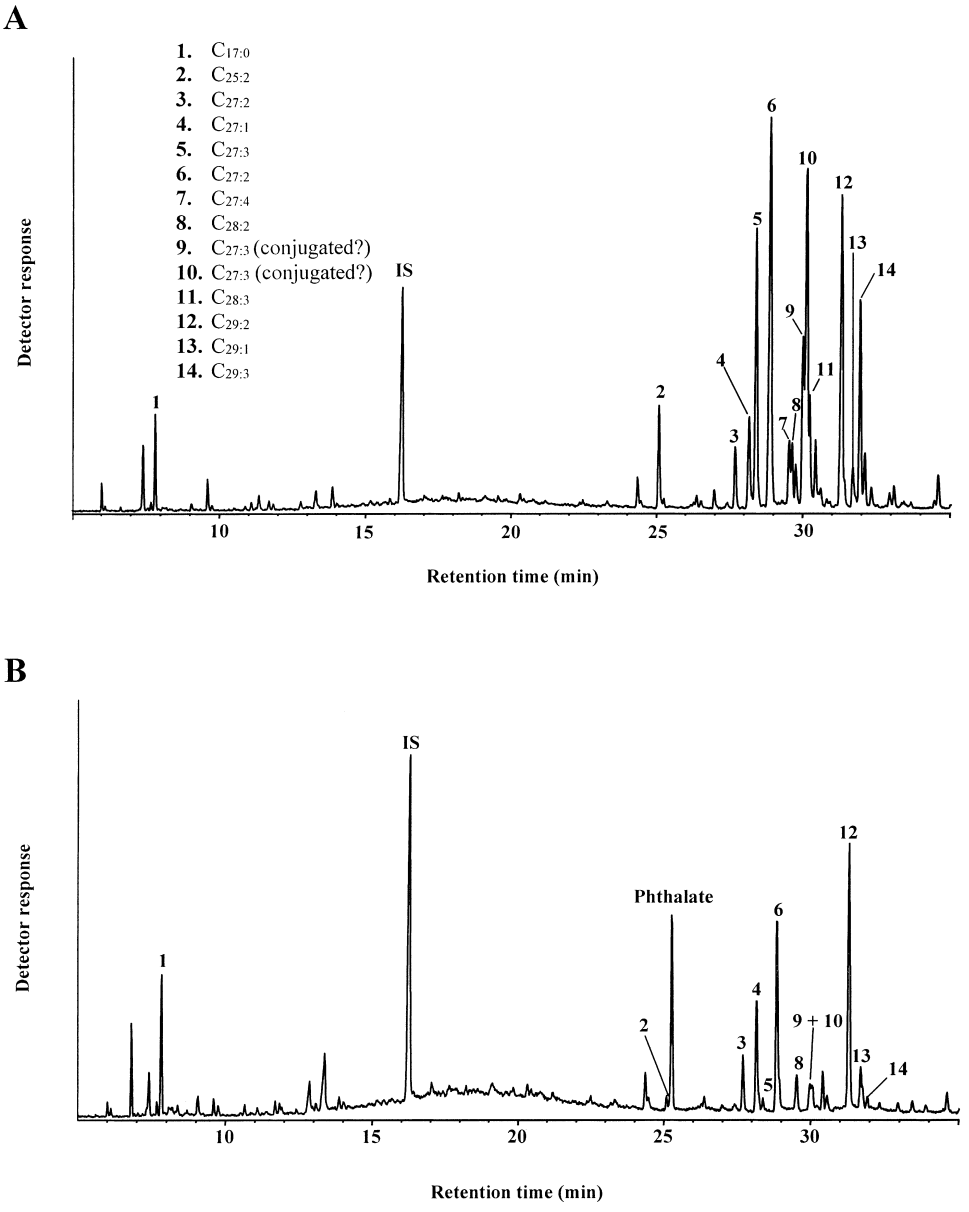


Fig. 3. Total ion chromatograms (HP5-MS capillary column) of the hydrocarbon fractions obtained after exposition of *Nannochloropsis salina* CCAP849/4 broken cells at 20°C for 18.9 days to different light regimes: (A) without irradiation under sterile conditions and (B) after exposure to 185 mol m^{-2}. IS = internal standard (heneicosane).

(daily irradiance 0.6 mol m^{-2}). However, under such low light conditions, particle sinking or biologically mediated degradation may have a greater impact on alkene concentration than light-dependent degradation processes (Vernet and Mitchell, 1990).

These results are important, since at present we have an imperfect understanding of the degradation rates and mechanisms, and even of the fundamental parameters which determine reaction pathways and kinetics of biogenic organic matter in the marine environment. In particular, it is generally considered that the magnitude of photochemical oxidation of organic matter in the upper oceans needs to be assessed (Water Column Working Group Report, 1992).

Due to the linear relationships to temperature obtained for the ratio of the sum of C_{37} and C_{38} alkenes versus the total hydrocarbons of *E. huxleyi* CS-57, it was previously suggested that this ratio may occasionally be useful in paleoclimate studies (Grossi et al., 2000). Unfortunately, we observed a significant change of this ratio during the irradiation of killed cells of *E. huxleyi* CS-57 at 20°C (0.925–0.994), this increase being due to the specific recalcitrance of C_{37} and C_{38} alkenes towards photochemical processes. The strong differences of photoreactivity observed between C_{37} and C_{38} alkenes and the other hydrocarbons of *Emiliania huxleyi* may thus represent a serious hindrance for the use of their ratio in paleoclimate studies.

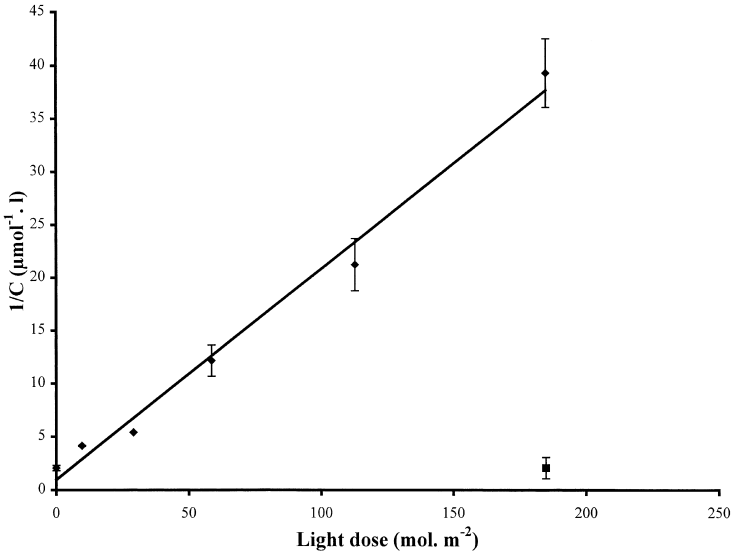


Fig. 4. Fit to apparent second-order kinetics for the photodegradation of *Nannochloropsis salina* CCAP849/4 $C_{29:3}$ alkene. Square symbols represent values for dark control samples shown at the equivalent time points as samples from the parallel illuminated treatment. Irradiance for the light treatment was 113 µmol m^{-2} s^{-1}.

Wakeham et al. (1991) previously observed in the Black Sea decreasing concentrations of C_{31}–C_{33} alkenes with increasing depth, while C_{37}–C_{39} alkenes persisted both in the water column and in the sediment. These authors invoked selective degradation to explain the observed depth profiles. The strong differences of photoreactivity of these two groups of *n*-alkenes observed in the present work well support this hypothesis.

3. Experimental

3.1. Biological material

Emiliania huxleyi strain CS-57 was obtained from the CSIRO (Australia) collection of living microalgae and *Nannochloropsis salina* strain CCAP849/4 was donated to us by the Netherlands Institute for Sea Research (NIOZ). These two strains were grown in 500 ml f/2 medium (Guillard and Ryther, 1962) at 20°C using two 30 W fluorescent lamps (Osram, Fluora) and a 12 h light and dark cycle. The cultures were harvested by centrifugation (8000 *g*) during the stationary phase for maximum yield. Prior to experiments the concentrated cells were taken through several freeze-thaw cycles to provide some disruption of cellular structure (Nelson, 1993).

3.2. Photodegradation experiments

Broken phytoplanktonic cells were distributed in Pyrex flasks containing 50 ml of supernatant to which had been added 1 ml of a 0.1 M solution of mercuric chloride (to avoid bacterial growth). The flask contents were irradiated for various durations (with magnetic stirring) using the same lamps as those used for growth at 20°C. Irradiance [as Photosynthetically Active Radiation (PAR)] was measured using a Licor LI 1000 data logger equipped with an LI 1935A spherical quantum sensor. Dark controls were carried out in parallel.

3.3. Treatment of phytoplanktonic cells

After filtration on GF/F (Whatman) paper, the filter was directly saponified with 1 N KOH in MeOH/H_2O (1:1) for 2 h (after addition of an internal standard). After cooling, the unsaponifiable lipids were extracted with dichloromethane (3×20 ml), dried over anhydrous Na_2SO_4, filtered and concentrated by means of rotary evaporation. The organic residues obtained from *E. huxleyi* were directly derivatized and analysed, while those obtained from *N. salina* were first chromatographed over a wet packed (*n*-hexane) column (20×1 cm) of silicagel (0.5% H_2O). The hydrocarbon fraction was eluted with 80 ml of *n*-hexane.

3.4. Derivatization

The residues obtained from *E. huxleyi* were taken up in 400 µl of a mixture of pyridine and bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Supelco) (3:1, v/v) and allowed to silylated at 50°C for 1 h. Following evaporation to dryness under nitrogen, the residue was taken up in hexane or ethyl acetate and analysed by gas chromatography (GC) and gas chromatography/electron impact mass spectrometry (GC/EIMS).

3.5. GC and GC/EIMS analyses

GC analyses were performed with a Girdel series 30 gas chromatograph equipped with a Ross injector and a flame ionization (FID) detector. Different chromatographic conditions were used: (A): 30 m×0.25 mm (i.d.) fused capillary column coated with 50% phenyl silicone (BPX50) (0.25 μm film thickness) (SGE); oven temperature programmed from 150 to 300°C at a rate of 5°C min^{-1}. (B): 30 m×0.25 mm (i.d.) fused capillary column coated with 5% phenyl silicone (HP-5MS) (0.25 μm film thickness) (Hewlett Packard); oven temperature programmed from 150 to 300°C at a rate of 5°C min^{-1}. N_2 was used as carrier gas (1 bar) and the injector and detector temperatures were 290 and 300°C respectively.

GC/EIMS analyses were carried out with a HP 5890 series II plus gas chromatograph connected to a HP 5972 mass spectrometer. The following operative conditions were employed: 30 m×0.25 mm (i.d.) capillary column coated with HP-5MS (Hewlett Packard) (film thickness, 0.25 μm); oven temperature programmed from 60 to 130°C at 30°C min^{-1} and then from 130 to 300°C at a rate of 4°C min^{-1}; carrier gas (He) pressure was maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 to 1.5 bar at a rate of 0.04 bar min^{-1}; injector (on column with a retention gap) temperature was 50°C and electron energy 70 eV. The source temperature was 170°C and the cycle time, 1.5 s.

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Silicon and heavy metal tolerance of higher plants

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Abstract

The heavy metal tolerant *Cardaminopsis halleri,* grown on Zn and Cu polluted soil, showed electron dense metal containing precipitates (Zn, Cu, Sn, Fe, Al) on the leaf surface, in the intercellular spaces (Zn, Cu, Sn), the cell walls and the cell wall thickenings of the xylem vessels (Zn, traces of Cu and Fe). Large amounts of Zn were measured in the vacuoles, the main storage compartment for this metal in *Cardaminopsis*. The cytoplasm and nuclei contained small precipitates, including mainly Zn and Si. As shown by ESI Zn was colocalized with Si in these structures. The EEL-spectra of the cytoplasmic precipitates corresponded with the spectra of Zn-silicate. Besides Zn-silicate, electron translucent structures in the cytoplasm were identified as SiO_2 by their EEL spectra. It was concluded that in the cytoplasm of *Cardaminopsis* Zn is transiently accumulated as silicate, being slowly degraded to SiO_2. Zn is translocated into the vacuole and accumulated in an unknown form. A second Si and Zn-uptake mechanism was found, excluding a membrane and cytoplasm passage. Pinocytotic vesicles, formed by the plasmamembrane and the tonoplast, enable a direct translocation of Si and Zn from extracellular compartments into the vacuole. The formation of Zn-silicate is part of the heavy metal tolerance mechanism and may be responsible for the amelioration of the Zn toxicity in *Cardaminopsis*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Cardaminopsis halleri*; Heavy metal tolerance; Silicon; Zinc; Copper

1. Introduction

Silicon is not considered to be an essential element for higher plants (Epstein, 1999). However, there is increasing evidence that Si is not only a cell wall incrustation, responsible for the rigidity of leaves in monocots, but is also involved in physiological processes (see review Hodson and Evans, 1995; Epstein, 1999). Metal toxicities in several species have been shown to be ameliorated by Si: Mn toxicity in *Oryza* (Horiguchi, 1988) and *Phaseolus* (Horst and Marschner, 1978) and Al toxicity in *Zea mays* (Barceló et al.,1993), *Hordeum* (Hammond et al., 1995), *Pinus* (Emadian and Newton, 1989), *Picea* (Hodson and Wilkins, 1991), *Sorghum* (Galvez et al., 1987; Hodson and Sangster, 1993) and soybean (Baylis et al., 1994). Similar observations have been made in fish (Birchall et al., 1989). Concluding from a colocalisation of Si and Al in *Picea* needles, *Sorghum, Triticum* and *Pinus* roots it was suggested that the formation of extracellular insoluble Al/Si compounds is responsible for the amelioration effects (Hodson and Sangster, 1993; Turnau et al., 1993). However, in other experiments no Al was detected in cellular Si-precipitates (Hodson and Bell, 1986; Hodson and Sangster, 1990). Precipitation of the metals in extracellular compartments does not seem to be a general cause for the observed Si effects.

In monocots Si is accumulated up to a level of 15% (dry wt basis) and 34% in particular organs (Ernst et al., 1995), without any relation to a heavy metal tolerance of the species (Neumann et al., 1999). Dicotyledons are rather poor Si-accumulators (most dicots less than 1% of the dry wt). However in some species (e.g. *Minuartia verna, Silene vulgaris, Thlaspi coerulescens, Viola calaminaria, Pisum sativum dgl, brz*) Si-deposits were found not only in cell walls and intercellular spaces, but also in the cytoplasm and the vacuoles (Neumann et al, 1997a; Bringezu et al., 1998). These Si-accumulating dicotyledons are heavy metal tolerant or mutants, defective in heavy metal uptake (Becker et al., 1999). The final product of Si-accumulation in dicotyledons is SiO_2, which arises from Ca- or Sn-silicate, forming water containing soft crystal-like structures (Bringezu et al., 1998; Lichtenberger and Neumann, 1996; 1997; Neumann et al., 1997a,b). A colocalization of Si with heavy metals was excluded (Neumann et al., 1997a; Bringezu et al., 1999).

The aim of this study was to investigate the function of Si in heavy metal tolerance by using a heavy metal tolerant plant, grown on a polluted natural location.

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2. Results and discussion

2.1. Cardaminopsis — ultrastructure

Leaves of *Cardaminopsis* were collected on a middle aged slag-heap, heavily polluted with Zn (21,000 mg/kg) and Cu. Some remarkable differences with the common ultrastructure of leaves, especially dark and electron translucent precipitates in different compartments, were observed. A survey of some of the typical structures is given in Figs. 1 and 2. The surface of the leaves was covered with electron dense crystals (Fig. 1A). Intercellular spaces of the leaf parenchyma frequently contained dark needle-shaped precipitates, often surrounding electron

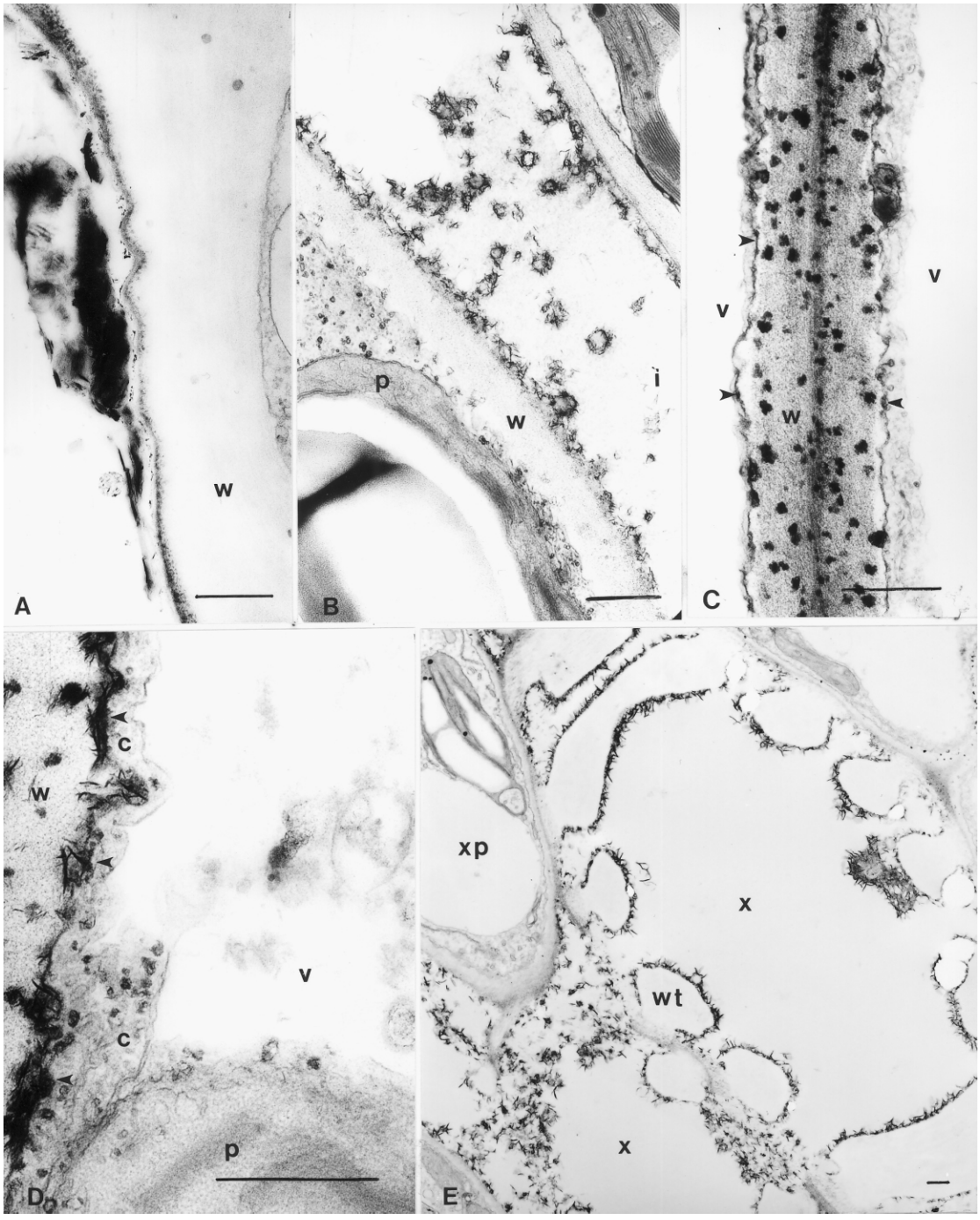


Fig. 1. Ultrastructure of the leaves of *Cardaminopsis*.(A) Precipitates on the leaf surface; (B) precipitates in the intercellular space; (C) dark precipitates in the cell wall; (D) electron dense precipitates between the wall and the plasmamembrane (→); (E) dark precipitates on the cell wall thickenings of xylem vessels; c-cytoplasm, i-intercellular space, p-plastid, w-cell wall, wt-cell wall thickenings, v-vacuole, x-xylem vessel, xp-xylem parenchyma cell; A,B×30000, C×37500, D×60000, E×9500; Bar = 0.5 μm.

translucent structures (Fig. 1B). Similar electron dense precipitates occurred inside the cell walls, especially of the epidermal and bundle parenchyma cells (Fig.1C) and between the cell walls and the plasma membrane (Fig. 1D). The cell wall thickenings of the xylem vessels were also covered with dark needle-shaped crystals (Fig. 1E). The most striking structures in both the cytoplasm and the nuclei were small precipitates (cytoplasm ~30–50 nm; nucleus ~5–10 nm), located without a surrounding membrane inside the cytoplasm and the nuclei, respectively (Fig. 2A,B). Many of the precipitates consisted of an electron dense border region and an electron translucent central part. Unusual invaginations into the vacuoles were observed in all leaf parenchyma cells. In contrast to the known pinocytotic vesicles the invaginations in *Cardaminopsis* were formed by the plasmamembrane and the tonoplast and included a small border of cytoplasm (Fig. 2C). The vesicles containing electron dense material of extracytoplasmic origin (Fig. 2D), may be cut off discharging their content into the vacuole (Fig. 2E).

2.2. Localization of heavy metals (EDX)

As in many other metal-tolerant plants (Neumann et al., 1995, 1997a; Bringezu et al., 1999) metals were excreted by *Cardaminopsis* leaves and cover their surface (Fig. 1A). The precipitates consisted of Zn-, Cu-, Sn-,

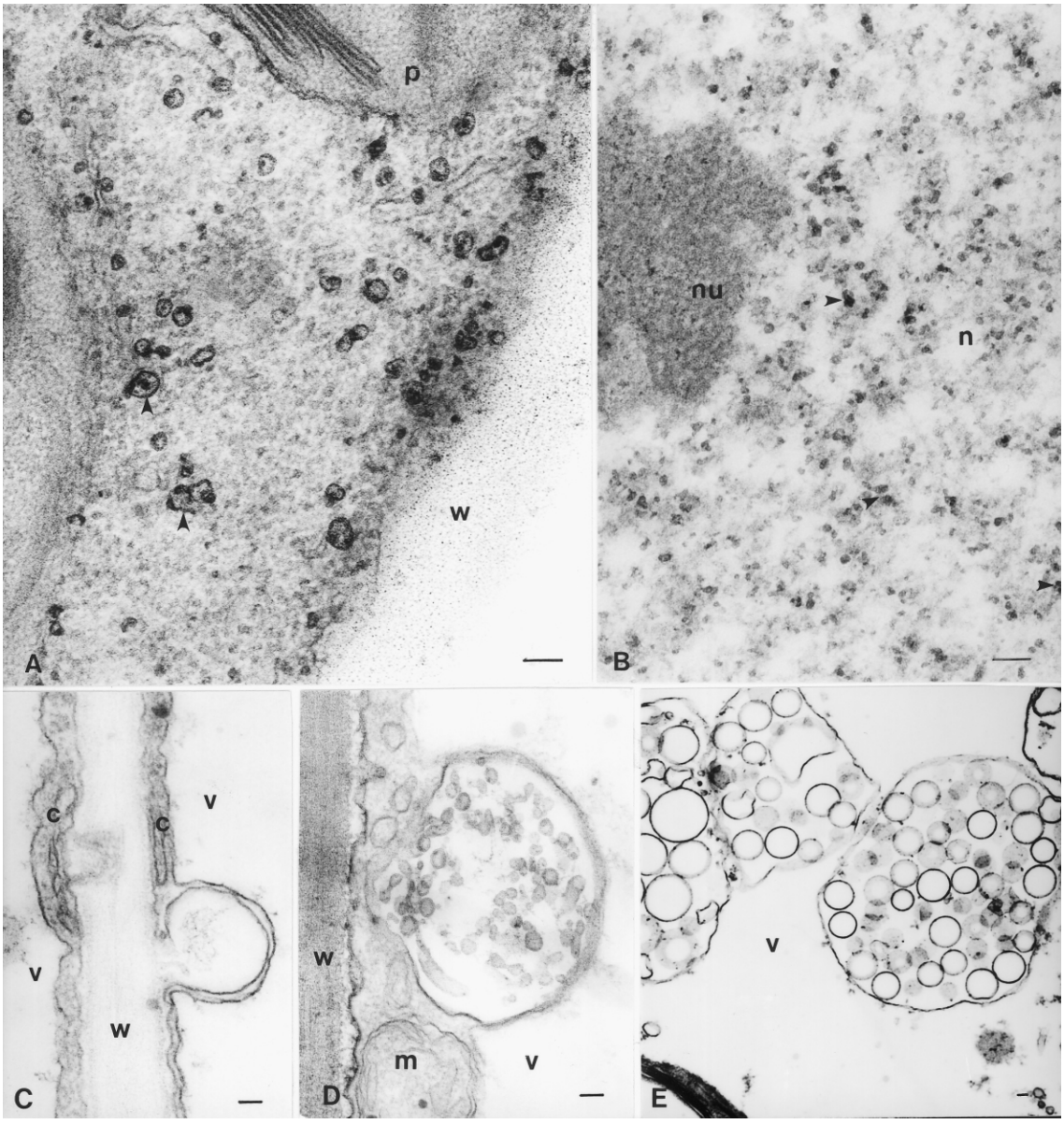


Fig. 2. Ultrastructure of the leaves of *Cardaminopsis*. (A) Dark bordered precipitates in the cytoplasm (→); (B) dark bordered precipitates in the nucleus (→); (C) invaginations into the vacuole; (D) electron dense material in a vacuolar vesicle; (E) vesicles inside the vacuole; c-cytoplasm, m-mitochodria, n-nucleus, nu-nucleolus, p-plastid, v-vacuole, w-cell wall; A,B×75,000, C,D×47,000, E×20,000; Bar = 0.1 μm.

Fe-, and Al-salts of variable composition (Fig. 3A). Often thick electron translucent layers, identified by EEL-spectroscopy as SiO_2 (see: Lichtenberger and Neumann, 1996; 1997), covered the epidermal cell walls (data not shown). The dark precipitates in the intercellular spaces (Fig. 1B) contained Si, Zn, Cu, and Sn (Fig. 3B). The largest heavy metal concentrations in the cell walls were found in the outer epidermal walls (Fig. 3C), but the anticlinal epidermal cell walls and the walls of the parenchyma cells also contained heavy metals of a similar composition. The dark precipitates on the cell walls of the xylem vessels (Fig. 1E) contained Zn and traces of Cu and Fe (Fig. 3D). The EDX-spectra of cytoplasm and nuclei (data not shown) were very similar, showing mainly high Zn and Si peaks (Fig. 3E). However, the vacuoles are the main storage compartment for Zn (Fig. 3F). Cu was found mostly in extracellular compartments. Besides the electron dense precipitates, which contain heavy metals and Si, electron translucent structures were found in the intercellular spaces (Fig. 1B), in the cytoplasm (Fig. 4A) and vacuoles. The EDX spectra of these structures showed only Si (Fig. 4B).

2.3. Elemental mapping (ESI) and binding states of the heavy metals (EELS)

EDX-analysis in the transmission mode allows spot analysis on ultrathin sections, but not the representation of the spatial distribution of the elements. Moreover the resolution is limited by the spot diameter used. These disadvantages are overcomed by the use of inelastically scattered electrons, specific for the element of interest, for image formation (ESI). The corresponding spectra include information on the charge of an element and its binding partners (EELS).

Electron dense precipitates in the cell wall (Fig. 5A), in the intercellular space (Fig. 5B), in the cytoplasm (Fig. 5C) and nuclei (data not shown) contained Zn and

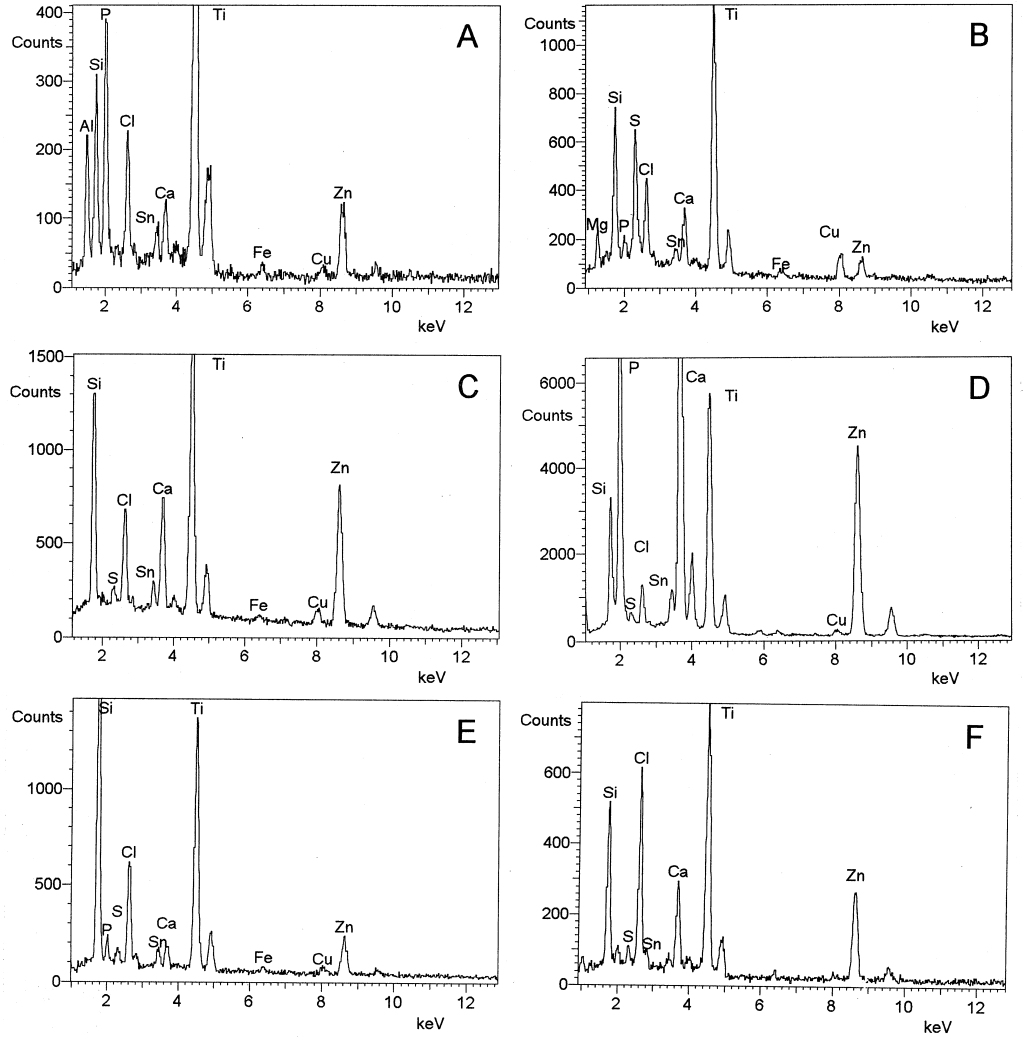


Fig. 3. EDX-spectra from leaves of *Cardaminopsis*. (A) Excreted precipitates on the leaf surface; (B) dark precipitates in the intercellular space; (C) epidermal cell wall; (D) precipitates on the cell wall thickenings of xylem vessels; (E) cytoplasm; (F) vacuole; the Ti-peaks are caused from the grids.

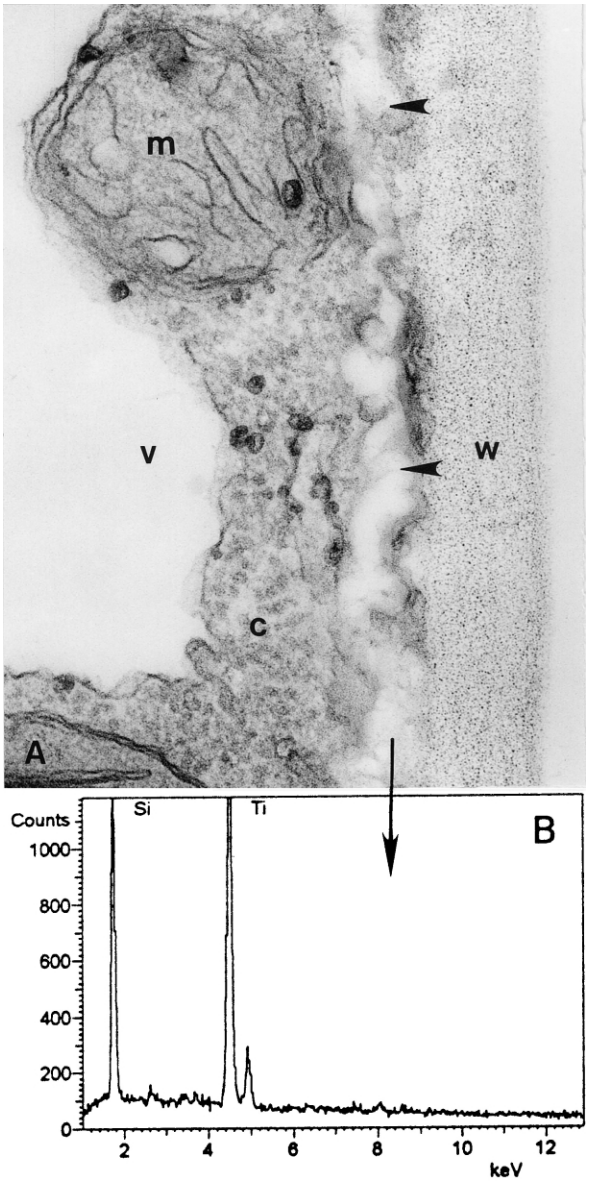


Fig. 4. Electron translucent precipitates in leaves of *Cardaminopsis*. (A) Electron translucent precipitate (→) in the cytoplasm; (B) corresponding EDX spectrum; c-cytoplasm, m-mitochondria, v-vacuole; A×75,000; Bar = 0.1 μm.

Si, indicated by the light blue color, due to the overlapping of the green (Si) and blue (Zn) ESI images. EEL-spectra of these structures are characteristic for Zn-silicate, as shown earlier by a comparison with standard spectra (Fig. 6) and corresponding quantumchemical calculations (Lichtenberger and Neumann, 1996; Bringezu et al., 1999). Electron translucent structures in the cytoplasm containing only Si, as shown by the green color in the ESI image (Fig. 5C), were identified as SiO_2 by their EEL-spectra (Fig. 7). The specificity of the spectra was confirmed by comparison with standard spectra and by quantumchemical calculations (Bringezu et al., 1999; Lichtenberger and Neumann, 1996; Neumann et al., 1997a,b, 1999). A colocalization of Zn and Si was also shown in vacuolar vesicles (Fig. 5D). Sometimes only Si was detected in these structures (Fig. 5E). Si-containing precipitates were also found inside the vacuole on the tonoplast membrane (Fig. 5F).

Heavy metals are covalently bound or chelated during their transport through the cytoplasm. In contrast to experiments with cell cultures (Grill et al., 1985) in *Cardaminopsis* leaves we have not found phytochelatins, which are thought to be responsible for chelating metals in the cytoplasm. This is not surprising, because in other metal-tolerant species, grown on heavy metal polluted soil as well, likewise no phytochelatins could be detected, and Zn is not able to form thermodynamically stable complexes with phytochelatins (Leopold et al., 1999). The ultrastructural and analytical data suggest a role for Si during the transport of Zn into the vacuole. In *Cardaminopsis* Zn is precipitated as silicate in the cytoplasm. The Zn-silicate, a transient storage compound for the metal, is slowly degraded to SiO_2 (Schwieger et al., 1987). SiO_2 precipitates in the cytoplasm (Fig. 5C) are the result of this process. While Si is precipitated in the cytoplasm, Zn is translocated into the vacuole. The binding partner inside the vacuole is not known so far. EDX spectra gave no hint for an inorganic binding partner, organic acids may be responsible for the accumulation inside the vacuole (Mathys, 1977; Godbold et al., 1984; Salt et al., 1999).

Besides this uptake mechanism a second route without membrane and cytoplasm passage exists. In parenchyma leaf cells of *Cardaminopsis* unusual invaginations into the vacuole occur (Fig. 2C,D). These vesicles contain electron dense material, identified as Zn-silicate (Fig. 5D), which is taken up from extracellular compartments. The membranes of the vesicles are digested inside the vacuole and the Zn-silicate is degraded. The resulting SiO_2 is detectable as a small layer covering the tonoplast (Fig. 5F).

The formation of silicates as part of the metal tolerance mechanism does not seem to be restricted to Zn. The amelioration of Al and Mn toxicity by Si was observed earlier (Horst and Marschner, 1978; Galvez et al., 1987; Horiguchi, 1988; Emadian and Newton, 1989; Hodson and Wilkins, 1991; Barceló et al., 1993; Hodson and Sangster, 1993; Baylis et al., 1994; Hammond et al., 1995) and a normalization of Cu-mediated growth inhibition by Si in cell cultures of *Silene* and tomato (Neumann, unpublished results) may be based on the same effect.

It seems that this mechanism is also realized in other heavy metal tolerant, SiO_2 accumulating species. The final product of this process is SiO_2 and it depends on the heavy metal concentration in the leaves whether it is possible to detect the metal-silicates in the cytoplasm. In the heavy metal tolerant and SiO_2 accumulating *Silene cucubalis* and *Minuartia verna*, grown on a slightly Zn-polluted location, no Zn-silicate was detectable (Neumann et al., 1997a; Bringezu et al., 1999). In contrast these species grown on the same, highly Zn-polluted place as

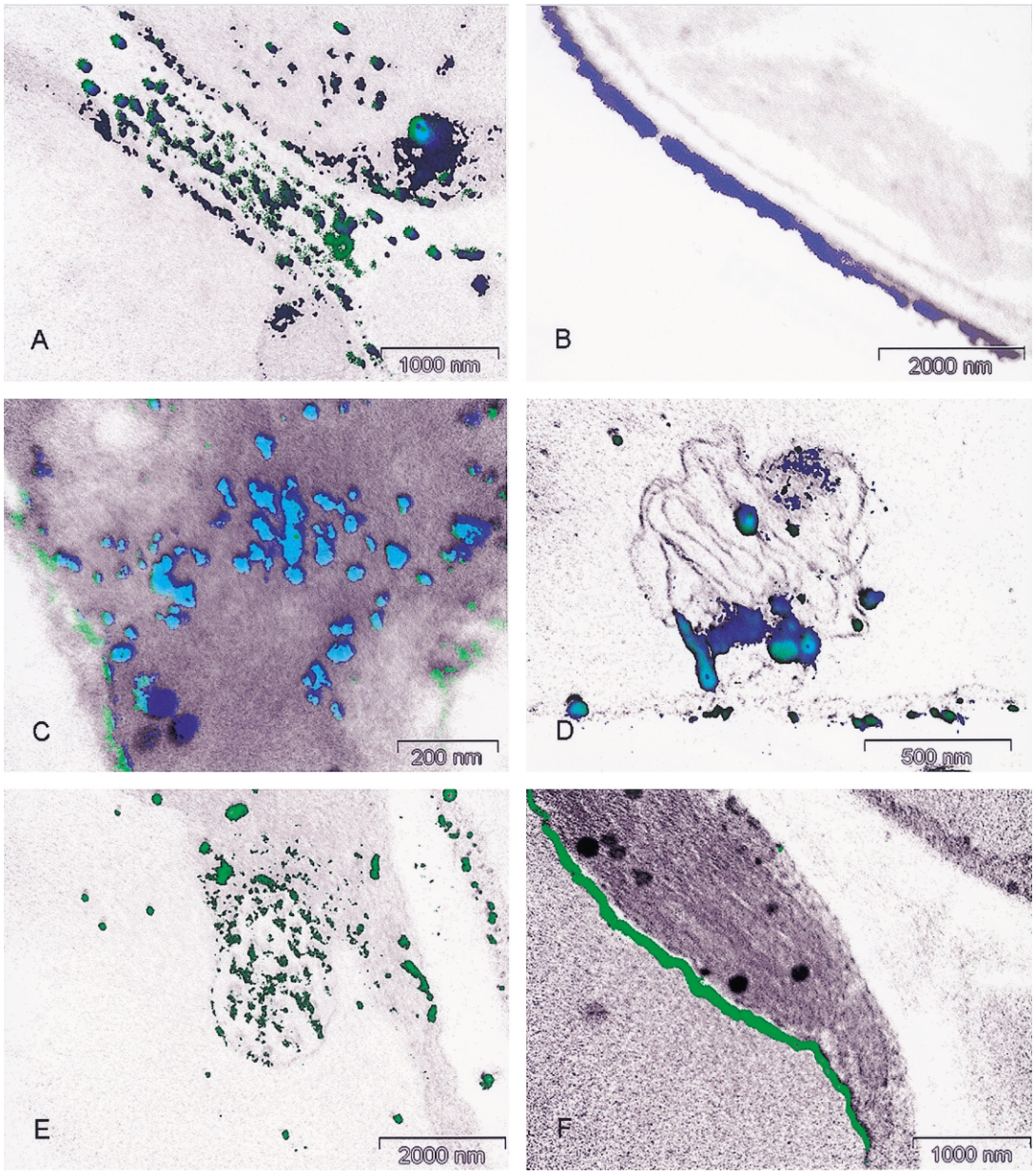


Fig. 5. Elemental mapping (ESI) from leaves of *Cardaminopsis*. A colocalization of Zn and Si is shown by overlying the zero loss image with element specific images, differing in the color (Si-green, Zn-blue; the colocalization of both elements is shown by a light blue color); (A) cell wall; (B) intercellular space; (C) cytoplasm, note the Si-containing precipitates near the plasmamembrane (→) and the tonoplast (→); (D) vesicle in the central vacuole with Zn-silicate; (E) vesicle in the central vacuole containing only Si; (F) vacuolar SiO_2 deposit on the tonoplast; c-cytoplasm, i-intercellular space, p-plastid, v-vacuole, w-cell wall.

Cardaminopsis, showed also Zn and Si containing cytoplasmic precipitates. So the assistance of Si at the Zn-tolerance seems not to be restricted to *Cardaminopsis*, but to be of more general importance.

3. Experimental

3.1. Plant material

Cardaminopsis halleri (L.) Hay. was collected from a middle aged Cu/Zn slag-heap (Langelsheim, Harz mountains, Germany). Small pieces of the leaves of these material were used for fixation.

3.2. Electron microscopy

Fixation and embedding have been described earlier (Neumann et al., 1995). Briefly, for ultrastructural investigation the glutaraldehyde/OsO_4-fixed material was embedded in ERL. For analytical electron microscopy the material was chilled in liquid propane (JFD 030, Balzers, Lichtenstein), freeze substituted in acetone (CSauto, Leica, Bensheim, Germany) and embedded in ERL. Ultrathin

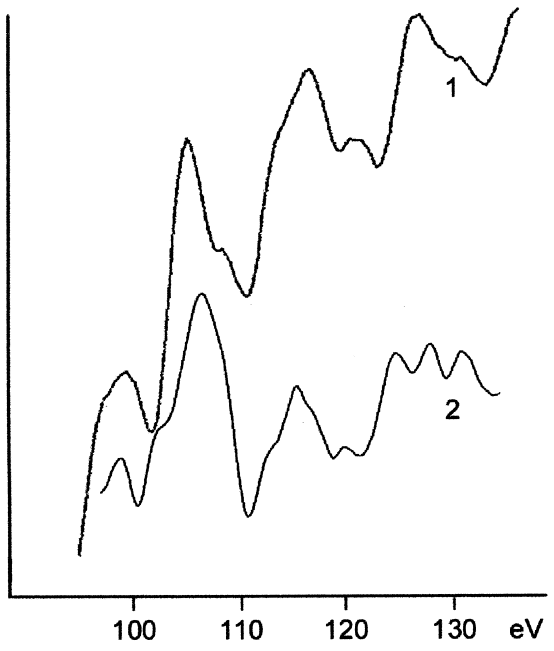


Fig. 6. EEL-spectra of the Si–$L_{2,3}$ edge. Zn-silicate standard (1); cytoplasmic precipitate of *Cardaminopsis* (2) (see Fig. 3A, 6C).

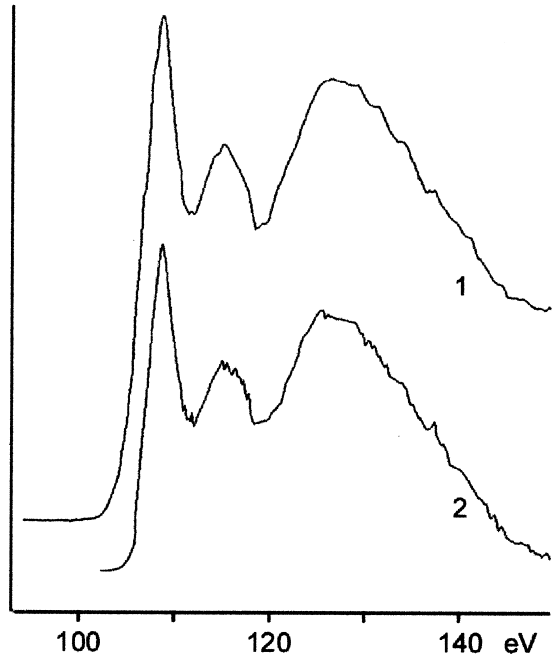


Fig. 7. EEL-spectra of the Si–$L_{2,3}$ edge. SiO_2 standard (1); vacuolar precipitate of *Cardaminopsis* (2) (see Fig. 6F).

sections were cut with a diamond knife and investigated with a transmission electron microscope (EM912 OMEGA, LEO Oberkochen, Germany) equipped with an incolumn energy filter and an EDX-system (Link exIII, Oxford Instruments, High Wycombe, Bucks, UK).

3.3. Energy dispersive analysis of X-rays (EDX)

Sections of freeze substituted leaves (~200 nm) were collected on Ti-grids without supporting film and analysed with a 100 nm spot at 80 KeV. The Ti peaks in the spectra are caused by the grids.

3.4. Electron energy loss spectroscopy (EELS)

EEL spectra were obtained using the OMEGA filter of the EM912 with an experimentally tested resolution of 1.2 eV at 80 kV. Spectra were recorded with a TV camera and processed with an image processing system (ANALYSIS 3.0).

3.5. Electron spectroscopic imaging (ESI)

For ESI 50–70 nm thick sections on Cu-grids without supporting film were used. Images were recorded with a SIT-TV camera with an energy resolution of ~ 5eV and processed with an image processing system (ANALYSIS 3.0, SIS Software GmBH, Münster, Germany). The background was subtracted with the 2-window method. For colocalization the ESI images of the corresponding elements were computer assisted colored and overlaid with the zero loss image.

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Occurrence of the insecticidal 16,17-didehydro-16(*E*)-stemofoline in *Stemona collinsae*

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Abstract

The occurrence of two alkaloids, 16,17-didehydro-16(*E*)-stemofoline and its isomer at C-4, 16,17-didehydro-4(*E*)-16(*E*)-stemofoline, were found together with a known insecticidal compound, stemofoline, in *Stemona collinsae*. The 16,17-didehydro-16(*E*)-stemofoline displayed higher insecticidal and antifeedant activities against the diamondback moth larvae than stemofoline. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Stemona collinsae*; Stemonaceae; Insecticidal activity; Alkaloid; Didehydrostemofoline

1. Introduction

The root extract of *Stemona collinsae* Craib (Stemonaceae) distributed in Southeast Asia had been reported to possess insecticidal properties (Burkill, 1966). While three rotenoids, stemona acetal, stemonal and stemonone have been isolated from this plant (Shiengthong et al., 1974), the major insecticidal constituents of this plant are unknown. The purpose of this study was to identify the insecticidal constituents in *S. collinsae*.

2. Results and discussion

By repeated chromatography, three compounds (**1–3**) were isolated from a methanol extract of the dried root of *S. collinsae* collected in Thailand. The levels of these three compounds in the dried root were determined to be 30, 76 and 7 mg/kg by HPLC analysis, respectively. The spectral data for compound **1** were in good agreement with those of stemofoline isolated from the root of *S. japonica* (Irie et al., 1970).

The HR-EIMS of compound **2** $[\alpha]_D^{18}$ +230° (MeOH, $c=0.74$); m.p. 172–174°C (recrystallized from MeOH); UV λ_{max}^{MeOH} nm (log ε): 294 (4.41); IR ν_{max}^{KBr} cm^{-1}: 2950, 1740 1690, 1620, 1460 and 1360, gave the molecular ion peak at m/z 385.1900 ($C_{22}H_{27}NO_5$, calcd. for 385.1889). In the ^1H NMR spectrum of **2** (Table 1) which was very close to that of **1**, two olefinic proton signals at 5.50 ppm (*d*, $J=15.6$ Hz) and 5.78 ppm (*dt*, $J=15.6$ and 6.4 Hz), respectively, were observed in place of multiplet signals at 1.5–1.6 ppm due to H-16 and H-17 in **1**. Thus the structure of compound **2** was determined to be 16,17-didehydro-16(*E*)-stemofoline as shown in Fig. 1.

Similar spectral data of the minor compound **3** to those of **2**, $[\alpha]_D^{18}$ +130° (MeOH; $c=0.01$), EIMS; m/z 385 ($C_{22}H_{27}NO_5$); UV λ_{max}^{MeOH} nm (log ε): 295 (4.34); IR ν_{max}^{KBr} cm^{-1}: 2950, 1740, 1690, 1620, 1460 and 1360, suggested that this compound is a geometrical isomer of **2**. In the ^1H NMR spectrum of **3**, only the downfield shift of the methyl proton signal (1.46 ppm) of C-21 from that (1.38 ppm) of **2** was significantly observed. A model experiment clearly verified that compound **3** was converted into **2** by irradiation by light. These data led us to

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propose the structure of **3** to be the geometrical isomer at C-4, 16,17-didehydro-4(*E*)-16(*E*)-stemofoline (Fig. 1). The possibility that **2** was an artifact derived from **3** could not be ruled out.

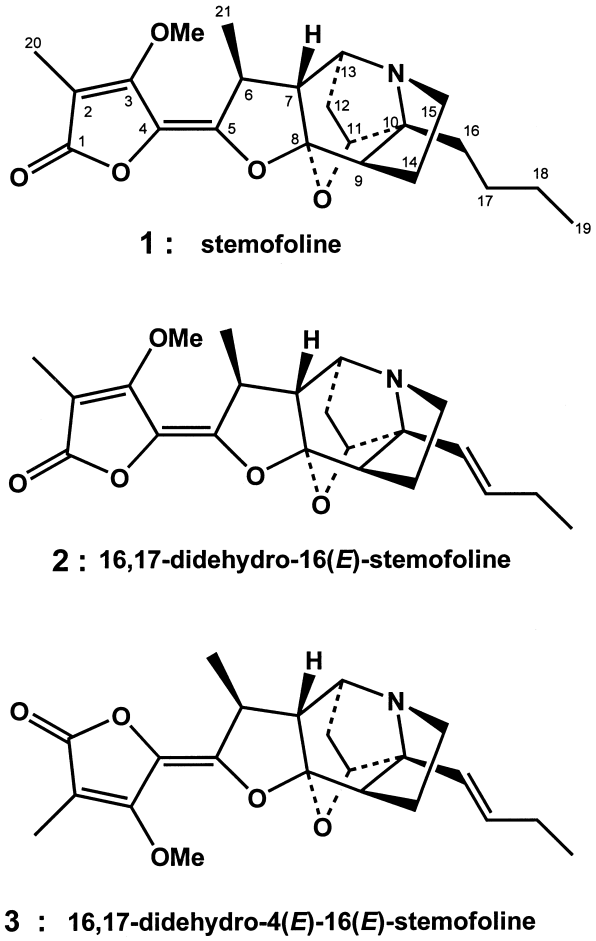


Fig. 1. Structures of compounds **1–3** isolated from *Stemona collinsae*.

Insecticidal and antifeedant activities of **1** and **2** were examined using the 3rd instar larvae of the pyrethroid-resistant diamondback moth, a pest to various vegetable crops. The activities of **3** were not tested because of sample limitation. As shown in Table 2, compound **2** showed LD_{50} value for the larvae mortality after **2** days at 0.63 μg/cm^2 and complete antifeedant activity at a dose of 2.5 μg/cm^2 after 4 days. Delaney and Wilkins (1995) reported that the LD_{50} of rotenone against 3rd instar larvae of diamondback moth after **3** days was 2.0 μg/cm^2. Hence, the activity of compound **2** must be stronger than that of rotenone. The activities of **1** were significantly weaker than those of **2**. The insecticidal activity of stemofoline and related alkaloids, stemospironine and stemonine, against 4th instar silkworm larvae has been reported by Sakata et al. (1978). The activity of stemofoline was 10^4-fold higher than those of stemospironine and stemonine. In contrast, all of these compounds were reported to be inactive against the 5th instar larvae of cabbage armyworms. On the basis of these observations, insecticidal activity of 16,17-didehydro-16(*E*)-stemofoline (**2**) was evaluated to be strongest among the stemofoline-related alkaloids so far isolated from the plants of genus *Stemona*.

Table 2
Insecticidal activities for compounds **1** and **2**[a]

Compound	Antifeedant[b] dose (μg/cm^2)				Mortality[c] LD_{50} (μg/cm^2)
	10	2.5	0.625	0.157	
1	A	B	C	C	5.50
2	A	A	B	B	0.63

[a] Data in a single test because of sample limitation.
[b] Antifeedant activity after 4 days; A: 0% of feeding area (FA); B: 0 < FA < 90%; C: 90 < FA < 100; D: FA = 100%.
[c] Mortality after 2 days.

Table 1
^{1}H NMR spectral data (400 MHz, $CDCl_3$) for compounds **1–3**

Position	Compound **1**	Compound **2**	Compound **3**
H-6	ca. 3.1 (*m*)	ca. 3.1 (*m*)	ca. 3.1 (*m*)
H-7	1.7–2.0 (*m*)	1.7–2.0 (*m*)	1.7–2.0 (*m*)
H-9	2.70 (*d*, *J* = 6.4)	2.84 (*d*, *J* = 5.8)	2.86 (*d*, *J* = 6.1)
H-11	4.25 (*br, s*)	4.20 (*br, s*)	4.22 (*br, s*)
H_2-12	1.7–2.0 (*m*)	1.7–2.0 (*m*)	1.7–2.0 (*m*)
H-13	3.49 (*br, s*)	3.50 (*br, s*)	3.52 (*br, s*)
H_2-14	1.7–2.0 (*m*)	1.7–2.0 (*m*)	1.7–2.0 (*m*)
H_2-15	ca. 3.1 and ca. 3.0 (*m*)	ca. 3.1 and ca. 2.95 (*m*)	ca. 3.2 and ca. 3.0 (*m*)
H_2-16 (or H-16)	1.5–1.6 (*m*)	5.50 (*d*, *J* = 15.6)	5.50 (*d*, *J* = 15.6)
H_2-17 (or H-17)	1.5–1.6 (*m*)	5.78 (*dt*, *J* = 15.6, 6.4)	5.78 (*dt*, *J* = 15.6, 6.4)
H_2-18	1.5–1.6 (*m*)	ca. 2.05 (*m*)	ca. 2.05 (*m*)
H_3-19	0.91 (3H, t, *J* = 7.0)	0.99 (3H, *t*, *J* = 7.3)	0.99 (3H, *t*, *J* = 7.9)
H_3-20	2.07 (*s*)	2.07 (*s*)	2.07 (*s*)
OMe	4.13 (*s*)	4.14 (*s*)	4.13 (*s*)
H_3-21	1.37 (3H, *d*, *J* = 6.4)	1.38 (3H, *d*, *J* = 6.4)	1.46 (3H, *d*, *J* = 6.7)

3. Experimental

3.1. General

The following instruments were used; Mp: Yanaco micro melting point apparatus (Yanaco Seisakusho Co. Ltd, Japan), OR: Jasco J-5 polarimeter, IR: Shimadzu model 435, UV: Shimadzu UV-200, ^{1}H NMR: JEOL JX 400 NMR (400 MHz, in $CDCl_3$ and TMS as an internal standard), MS: JEOL-DX 300, and HPLC: Hitachi 665-A.

3.2. Extraction and isolation

Dried root (500 g) of *S. collinsae* was ground, and extracted with methanol (1 l) for 2–3 weeks at room temperature. The methanol extract (1.5 g) was partitioned between EtOAc (150 ml×2) and water (200 ml), and the EtOAc solubles (1.3 g) were subjected to silica gel chromatography, this being eluted stepwise with 20% increasing amounts of EtOAc in toluene, and then with MeOH. The fraction eluted with MeOH was again subjected to silica gel chromatography eluted with $CHCl_3$/MeOH to give two active fractions, F-1 (100% $CHCl_3$ eluate) and F-2 (2% MeOH eluate). Compound **2** (27 mg) was obtained by repeated recrystallization from F-1. Purification of F-2 by HPLC (Capcell Pak C_{18} (150×6 mm), 60% MeOH in 0.1 M [$(NH_4)_2CO_3$]) gave compound **1** (t_R = 11.6–13 min, 12 mg) and **3** (t_R = 14–16 min, 1.4 mg) along with **2** (t_R = 10–11.5 min, 1.5 mg).

Compound **2**: 16,17-didehydro-16(*E*)-stemofoline), colorless crystals (MeOH), m.p. 172–174°C; $[\alpha]_D^{18}$ +230° (MeOH, c = 0.74); EIMS at 70 eV, m/z (% rel. int.): 385 $[M]^+$ (45), 230 (13), 202 (35), 188 (8), 174 (6), 160 (100), 146 (8), 134 (15), 120 (12), 106 (15), 91 (10), 83 (18), 69 (15); HR-EIMS (probe) 70 eV: $[M]^+$ m/z 385.1900 ($C_{22}H_{27}NO_5$, requires 385.1889); UV λ_{max}^{MeOH} nm (log ε): 294 (4.41); IR ν_{max}^{KBr} cm^{-1}: 2950, 1740 1690, 1620, 1460 and 1360; ^{1}H NMR (see Table 1).

Compound **3**: 16,17-didehydro-4(*E*)-16(*E*)-stemofoline, a colorless glassy resin, $[\alpha]_D^{18}$ +130° (MeOH; c = 0.01); $C_{22}H_{27}NO_5$; EIMS at 70 eV: $[M]^+$ m/z (% rel. int.): 385 $[M]^+$ (60), 356 (12), 238 (15), 202 (37), 160 (100), 145 (7), 134 (10), 106 (10), 91 (12), 83 (16), 69 (10); UV λ_{max}^{MeOH} nm (log ε): 295 (4.34); IR ν_{max}^{KBr} cm^{-1}: 2950, 1740, 1690, 1620, 1460 and 1360; ^{1}H NMR (see Table 1).

3.3. Photo-isomerization of *3* to *2*

A mixture of **2** and **3** (1:9) was used for a photo-isomerization experiment because of the instability of compound **3**. This mixture (1 mg) in MeOH was allowed to stand under fluorescent light at 27°C. The compositional ratios of the mixture after 24, 48 and 120 h were determined by HPLC. Compound **3** was converted into **2** by 14, 19 and 39% after 24, 48 and 120 h, respectively. During the experiment, the sum of the amounts of compounds **2** and **3** remained constant.

3.4. Insecticidal tests

Insecticidal activities were tested by the method reported previously (Ohigashi et al., 1991) with slight modification. Ten 3rd instar larvae of diamondback moth (*Plutella xylostella*) were released in a Petri-dish placing three cabbage leaf disks (2 cm in diameter) treated with a definite dose of test compound on both surfaces. The mortality was assessed after 2 days, and the antifeedant effect was determined by the rate of non-feeding area to the whole [(a total area of non-consumed leaf part/ total area of test leaf)×100] after 4 days.

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Sequestration of phenanthroindolizidine alkaloids by an Asclepiadaceae-feeding danaid butterfly, *Ideopsis similis*

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Abstract

3-Demethyl-14α-hydroxyisotylocrebrine *N*-oxide was isolated along with three homologous isotylocrebrine type alkaloids from the imagines and pupae of *Ideopsis similis* reared on the host plant, *Tylophora tanakae*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Tylophora tanakae*; Phenanthroindolizidine alkaloid; Isotylocrebrine; *Ideopsis similis*; Danaid butterfly; Asclepiadaceae

1. Introduction

An important and intriguing aspect in insect–plant interactions can be found in selective uptake and storage of particular plant secondary metabolites by phytophagous insects. This phenomenon, designated ''sequestration'' is an outcome of evolutionary associations between insects and phytochemicals through diverse ecological and biochemical processes, and is one of the major subjects of chemical ecology. A number of insects, especially moths and butterflies, sequester various compounds of dietary origin or from non-host plants pharmacophagously (Duffey, 1980; Nishida, 1995). In danaid butterflies, cardenolides have often been implicated in chemical defense (Brower et al., 1982; Brower, 1984), and pyrrolizidine alkaloids in both chemical defense and pheromone production (Schneider et al., 1975; Eisner and Meinwald, 1987).

In the South-western Islands of Japan, *Ideopsis similis* specializes on an asclepiad plant, *Tylophora tanakae* Maxim., which has already been reported to contain phenanthroindolizidine alkaloids (PIAs) (Abe et al., 1995). Some of the PIAs present in this plant are known to act as oviposition stimulants for the butterfly (Honda et al., 1995a) and to exhibit cytotoxic activities to a variety of cancer cells (Abe et al., 1998). We now report of the isolation and identification of 3-demethyl-14α-hydroxyisotylocrebrine and its *N*-oxide, together with minor alkaloids from the imagines and pupae of *I. similis*.

2. Results and discussion

During the investigation of the ingredients from *Tylophora tanakae*, we previously isolated fourteen phenanthroindolizidine alkaloids including two known ones, tylophorine and isotylocrebrine, and their structures were determined (Abe et al., 1995, 1998). We also examined the alkaloid pattern in the cultivated plant, in which isotylocrebrine type alkaloids (3,4,6,7-tetrasubstituted) were present as a major group (Fig. 1), while tylophorine type alkaloids (2,3,6,7-tetrasubstituted) were predominant in the wild plant collected in the South-western Islands (Abe et al., 1998). When the imagines and pupae of *I. similis* were treated to obtain the $CHCl_3$ soluble fractions in the alkaline condition, respectively, the possibility of the presence of PIAs was suggested by the HPLC analysis, while two Dragendorff's reagent (D)-positive spots were observed in TLC. The alkaloid fractions of imagines and pupae showed similar patterns (Fig. 2) and several peaks seemed to be duplicated with PIAs having close retention time in HPLC as in the leaves of cultivated plants (Fig. 1).

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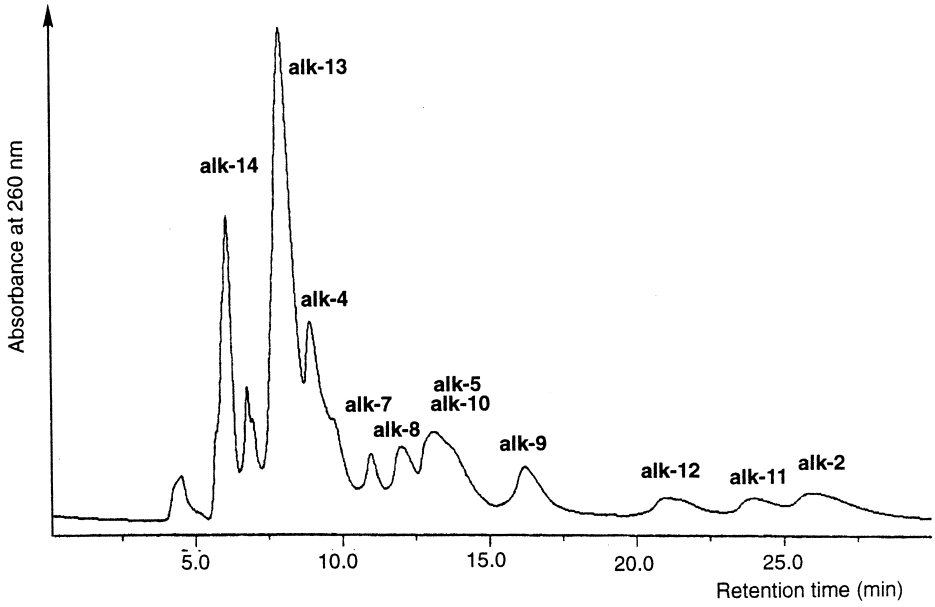


Fig. 1. HPLC chromatogram for alkaloid fraction of *T. tanakae* (Abe et al., 1998). alk-2: tylophorine, alk-4: 3-demethylisotylocrebrine, alk-5: isotylocrebrine *N*-oxide, alk-7: 3-demethyl-14α-hydroxyisotylocrebrine *N*-oxide, alk-8: 6-demethyltylocrebrine, alk-9: tylophorinine *N*-oxide, alk-10: 7-demethyltylophorine, alk-11: tylophorine *N*-oxide, alk-12: 7-demethyltylophorine *N*-oxide, alk-13: 3,6-didemethylisotylocrebrine, alk-14: 3,6-didemethyl-14α-hydroxyisotylocrebrine.

Each alkaloid fraction was subjected to silica gel column chromatography to obtain **1** along with minor alkaloids (**2–4**) and **5**. Based on the ^{1}H NMR spectrum of **1**, the presence of three methoxy groups, two *ortho*-coupled protons (δ 7.32, 8.01, $J=9$ Hz), two *para*-coupled protons (δ 7.11 and 9.30), and one proton linked to the hydroxylated carbon (δ 5.35, d, $J=3$ Hz) was observed, suggesting **1** to be a phenanthroindolizidine alkaloid. Doublet signals assignable to C-9 methylene protons at the neighbouring location to *N*-atom were observed in the lower chemical shifts (δ 4.82 and 5.34), suggesting that **1** formed an *N*-oxide. The locations of three methoxy residues were finally confirmed to be at C-4, 6, 7 by the NOE experiment, in which the correlations were observed between H-14 (δ 5.35)/H-1 (δ 8.01), H-5 (δ 9.30)/two methoxy groups (δ 3.89 and 4.09), and H-8 (δ 7.11)/and another methoxy group (δ 4.08). Since a $[M+1]^+$ peak suggested a molecular formula to be $C_{23}H_{25}O_6N$, and HPLC and TLC also afforded the identical retention time and R_f value with the authentic alkaloid, **1** was assigned as 3-demethyl-14α-hydroxyisotylocrebrine *N*-oxide.

Alkaloid **2** was further purified on a silica gel column to give a single spot on TLC. All signals in the ^{1}H NMR spectrum of **2** were assignable as 3-demethyl-14α-hydroxyisotylocrebrine, and HPLC and TLC of **2** were in good agreement with those of the authentic sample. The $[M+1]^+$ peak was also observed at *m/z* 396. The structure of **3** was assigned to be isotylocrebrine *N*-oxide based on the ^{1}H NMR spectrum and the correlations in NOE as in **1**, and a $[M]^+$ peak was observed at *m/z* 409. The locations of four methoxy substituents in the phenanthrene moiety were confirmed by cross peaks between H-8/7-OCH_3, H-8/H-9 and H-5/4-OCH_3, 6-OCH_3 in the NOESY spectrum.

Alkaloid **4** was suggested to be a didemethylisotylocrebrine homologue, based on the presence of two methoxy groups along with *ortho*-coupled protons (δ 7.21, 8.15, $J=9$ Hz) and *para*-coupled protons (δ 7.08, 7.95). The signal of H-1 proton (δ 8.15) showed a NOE to H-14β at δ 5.35. Since H-9 was observed at the lower field (δ 4.88, 5.33) as those of **1** and **3**, **4** was considered to be an *N*-oxide. The H-5 signal was observed at δ 7.95, i.e. a resonance not as low as those in the other 4-methoxy alkaloids, **1**, **2** and **3**, and its cross peak was only to the 6-methoxy group in the NOESY spectrum. The H-2 signal showed no correlation with any methoxy groups. Thus, the structure of **4** was determined to be 3,4-didemethyl-14α-hydroxyisotylocrebrine *N*-oxide.

In the quantitative analysis of **1**, the approximate amount was estimated as 5.8 μg per adult and 14.5 μg per pupa, based on the comparison of the peak area with that of the known amount of **1**. The amount of **2** was estimated as 4.8 μg per adult and 3.5 μg per pupa, respectively.

The polar substance, designated **5** in Fig. 2, was noted as a sharp peak in the HPLC chromatogram, as well as by another D-positive spot using TLC. Although **5** seems to contain twelve carbons based on the ^{13}C NMR spectrum, its structure requires further investigation.

Our results definitely demonstrate that *I. similis* sequesters and stores specific PIAs of host-plant origin

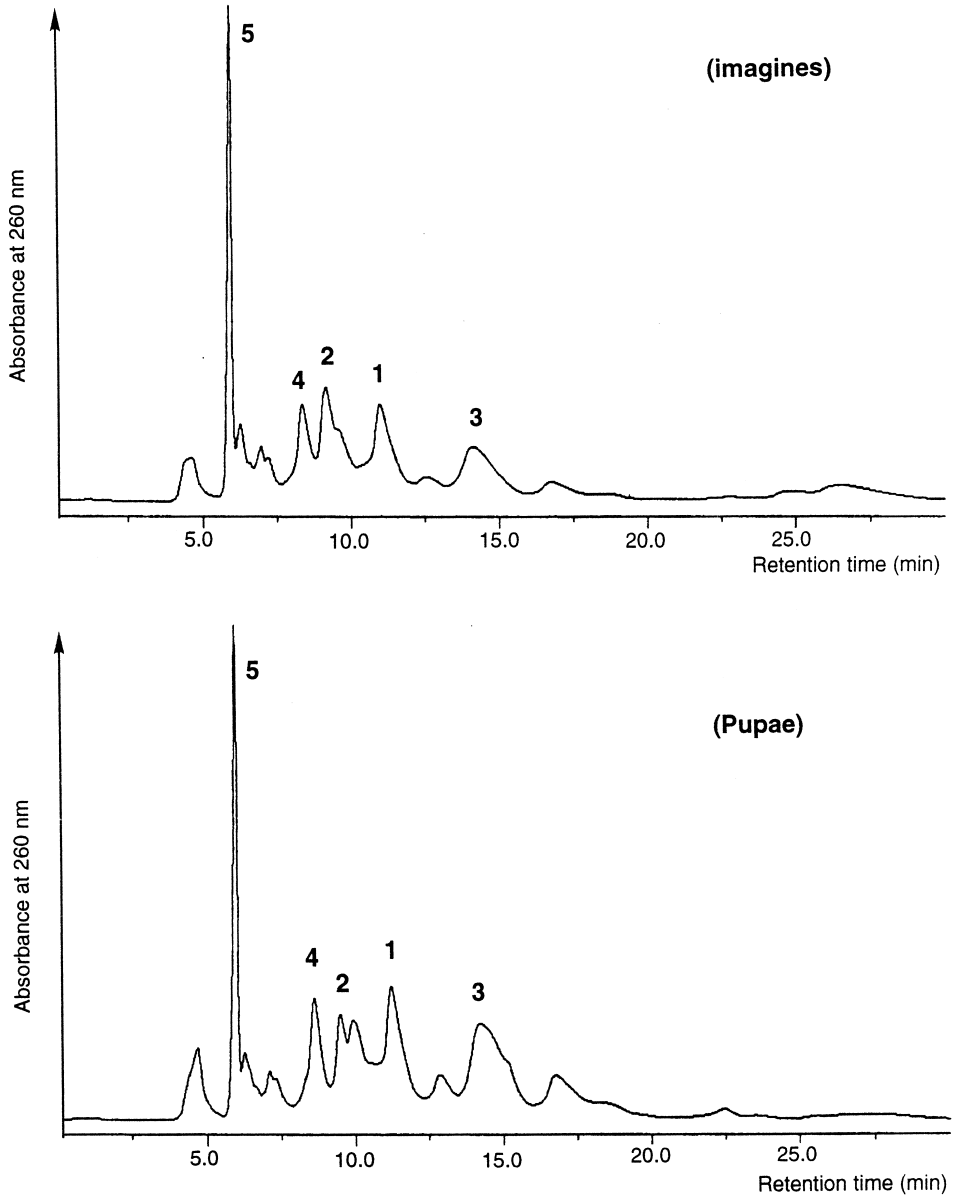
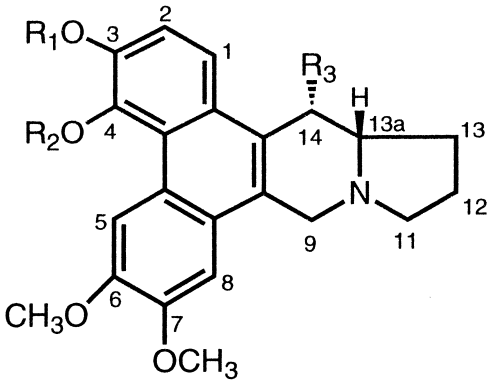


Fig. 2. HPLC chromatograms for alkaloid fractions of *I. similis*.

during their larval stage. Since tylocrebrine, one of the phenanthroindolizidine alkaloids, is known to inhibit protein biosynthesis (Govindachari and Viswanathan, 1978) and we also described its cytotoxic potency using five cell lines (Abe et al., 1998), they are highly likely to serve as defensive compounds against predators. Male adults of *I. similis* as well as other danaid butterflies are attracted to pyrrolizidine alkaloid-containing plants presumably to acquire pyrrolizidine alkaloids and to produce dihydropyrrolizines that are secreted from the male abdominal hairpencils and the alar scent organs (Honda et al., 1995b). It is, therefore, feasible that adults of this butterfly are dually defended by both PIAs and pyrrolizidine alkaloids. Defensive efficacy of PIAs against predatory enemies is now under investigation.

It should be noted that the alkaloids isolated from the insect body were limited to isotylocrebrine homologues including **1–4**, among the alkaloids so far known, such as isotylocrebrine, tylophorine, tylophorinine, tylocrebrine and septicine types. As described before, the plant alkaloids show different patterns according to the growing locations or conditions. The presence of only isotylocrebrine homologues in the insects is considered to be due to the host plant. Alkaloid **4** is not known from *Tylophora* species thus far. The examination of the alkaloids in the host plant which was used for larval rearing is to be studied to confirm the origin of **4**, as well as the structure determination of **5**. To the best of our knowledge, this is the first report of sequestration of PIAs by an insect.



1 : R_1=H, R_2=CH_3, R_3=OH, *N*-oxide
2 : R_1=H, R_2=CH_3, R_3=OH
3 : R_1=R_2=CH_3, R_3=H, *N*-oxide
4 : R_1=R_2=H, R_3=OH, *N*-oxide

Chart 1.

3. Experimental

3.1. General

^{1}H NMR: 500 MHz, HPLC: column: Capcell Pak C_{18} (Shiseido) (SG 120 type, 4.6 mm i.d. ×250 mm), solvent: 25% CH_3CN–HOAc (2000:1), flow rate, 0.6 ml/min, detection: UV absorption at 260 nm. Solvent system for silica gel column chromatography: solvent 1 $CHCl_3$–MeOH–H_2O (7:1:1.6∼7:2:1.2, bottom layer), solvent 2 EtOAc–MeOH–H_2O (8:1:1.2∼6:1:1.2, top layer).

3.2. Insects and extraction of PIAs from the imagines

The offspring of female adults of *I. similis* collected in Yaeyama Islands were used for PIA analyses. Larvae were reared on potted host plants, *T. tanakae* in a greenhouse at 22–25°C under a natural photoperiod. Extraction of PIAs from the adults was carried out by soaking fresh specimens (81 individuals, within 24 h of eclosion in 1996) in MeOH (500 ml) at room temperature for 3 months. The samples collected in 1994 (112 individuals) and 1995 (20) were treated in the same manner, and the whole MeOH solutions were combined and concentrated in vacuo. The extract was suspended in benzene and partitioned with 5% HOAc (benzene extract: fraction 1: 723 mg, oily). The HOAc layer was partitioned with $CHCl_3$ (fraction 2: 860 mg, oily). The HOAc layer was then treated with 5% NH_4OH, and again partitioned with $CHCl_3$ (fraction 3: 129 mg). After $CHCl_3$ partition, the water layer was extracted with *n*-BuOH (fraction 4: 222 mg). Fraction 3 showed a positive reaction on TLC by Dragendorff's reagent. Slightly positive staining was observed in fraction 4 but none in fractions 1 and 2. Fr 3 was subjected to successive silica gel column chromatography with solvents 1 and 2 to afford **1–4** and **5**.

3.3. Isolation of the alkaloids from the pupae

A total of 88 pupae reared in 1999 (average weight: 668 mg/pupa), soaked in MeOH (170 ml) within 24 h of larva-pupal ecdysis, were subsequently homogenized in MeOH to extract PIAs and treated in the same manner as of the imagines (Fr 1, 790 mg; Fr 2, 420 mg; Fr 3, 193 mg; Fr 4, 183 mg). After column chromatography, alkaloids **1–4** and **5** were isolated.

3.4. 3-Demethyl-14α-hydroxyisotylocrebrine N-oxide (*1*)

^{1}H NMR ($CDCl_3$-CD_3OD) δ: 3.75 (1H, *q*, *J*=9 Hz, H-11a), 3.89 (3H, *s*, 4-OCH_3), 3.90 (1H, *br t*, *J*=9 Hz, H-11b), 4.08 (3H, *s*, 7-OCH_3), 4.09 (3H, *s*, 6-OCH_3), 4.82, 5.34 (1H each, *d*, *J*=15 Hz, H-9), 5.35 (1H, *d*, *J*=3 Hz, H-14β), 7.11 (1H, *s*, H-8), 7.32 (1H, *d*, *J*=9 Hz, H-2), 8.01 (1H, *d*, *J*=9 Hz, H-1), 9.30 (1H, *s*, H-5). NOEs: H-1/H-14β, 4-OCH_3/H-5, H-5/6-OCH_3, 7-OCH_3/H-8, H-8/H-9. HR-FABMS *m*/*z*: 412.1761, calcd for $C_{23}H_{25}O_6N$+ H 412.1760.

3.5. 3-Demethyl-14α-hydroxyisotylocrebrine (*2*)

^{1}H NMR ($CDCl_3$–CD_3OD) δ: 3.60, 4.20 (1H each, *d*, *J*=15 Hz, H-9), 3.88 (3H, *s*, 4-OCH_3), 4.07 (3H, *s*, 7-OCH_3), 4.12 (3H, *s*, 6-OCH_3), 5.04 (1H, *br s*, H-14β), 7.20 (1H, *s*, H-8), 7.40 (1H, *d*, *J*=9 Hz, H-2), 8.09 (1H, *d*, *J*=9 Hz, H-1), 9.28 (1H, *s*, H-5). FABMS *m*/*z*: 396 $[M+1]^+$.

3.6. Isotylocrebrine N-oxide (*3*)

^{1}H NMR (CD_3OD) δ: 3.76 (3H, *s*, 3-OCH_3), 3.85 (3H, *s*, 6-OCH_3), 4.030, 4.033 (3H each, *s*, 4, 7-OCH_3), 4.97, 5.13 (1H each, *d*, *J*=15 Hz, H-9), 7.16 (1H, *s*, H-8), 7.26 (1H, *d*, *J*=9 Hz, H-2), 7.81 (1H, *d*, *J*=9 Hz, H-1), 9.27 (1H, *s*, H-5). NOEs: 4-OCH_3/H-5, H-5/6-OCH_3, 7-OCH_3/H-8, H-8/H-9. FABMS *m*/*z*: 409 $[M]^+$.

3.7. 3,4-Didemethyl-14α-hydroxyisotylocrebrine N-oxide (*4*)

^{1}H NMR (CD_3OD) δ: 4.06 (3H, *s*, 7-OCH_3), 4.09 (3H, *s*, 6-OCH_3), 4.88, 5.33 (1H each, *d*, *J*=15 Hz, H-9), 5.35 (1H, *br s*, H-14β), 7.08 (1H, *s*, H-8), 7.21 (1H, *d*, *J*=9 Hz, H-2), 7.95 (1H, *s*, H-5), 8.15 (1H, *d*, *J*=9 Hz, H-1). NOEs: H-1/H-14β, H-5/6-OCH_3, 7-OCH_3/H-8. FABMS *m*/*z*: 380 $[M-H_2O+1]^+$.

3.8. Quantitative estimation of *1* and *2*

One tenth of fraction 3 (imagines and pupae), 3-demethyl-14α-hydroxyisotylocrebrine *N*-oxide (0.13 mg)

and 3-demethyl-14α-hydroxyisotylocrebrine (0.09 mg) were dissolved in MeOH (2.0 ml), respectively. Each solution (20 μl) was subjected to HPLC and the amounts of **1** and **2** were estimated by the comparison of peak area.

3.9. Alkaloid **5**

^{13}C NMR (CD_3OD) δ: 11.4 (*q*), 14.3 (*q*), 24.0 (*t*), 25.0 (*t*), 30.1 (*t*), 31.6 (*t*), 40.2 (*d*), 69.2 (*t*), 129.9 (*d*), 132.4 (*d*), 133.6 (*s*), 169.4 (*s*).

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Fragrance volatiles of developing and senescing carnation flowers

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Abstract

Thirteen major volatiles of the carnation flower fragrance signature have been identified by GC/MS. Of these, ten, hexanal, *(2E)*-hexenal, 1-hexanol, 2-hexanol, 3-hexen-1-ol, nonanal, benzaldehyde, benzyl alcohol, benzyl benzoate and caryophyllene, were quantified. The steady-state levels of these ten volatiles change independently as the flowers develop and senesce, suggesting that their synthesis is developmentally regulated. In addition, the chemical composition of the fragrance signature in naturally senesced flowers proved to be very different from that for flowers that had been induced to senesce prematurely by treatment with ethylene. Thus, senescence-related changes in carnation floral scent appear not to be directly regulated by ethylene. From cellular fractionation studies, it is evident that all of the volatiles, except 2-hexanol, are present in both membranous and cytosolic compartments, suggesting that their synthesis is membrane-associated and that they subsequently partition into the cytosol in accordance with partition coefficients. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Dianthus caryophyllus*; Caryophyllaceae; Carnation; Fragrance signature; Subcellular localization; Senescence; Secondary lipid metabolites; Benzenoids; Caryophyllene; C_6-Aldehydes and alcohols; Ethylene

1. Introduction

The fragrance signature of flowers is a composite of volatile chemicals in specific stoichiometric concentrations (Knudsen et al., 1993). Typically, these compounds are fatty acid derivatives, benzenoids, phenylpropanoids and isoprenoids, and have low polarity, slight water solubility, high vapour pressure and high lipophilicity (Knudsen et al., 1993; Ohloff, 1994). Many flower fragrances are pleasant to the human sensory system and have potential application as components of perfume (Rees, 1991).

Although numerous volatile components of floral scent have been identified, the biochemistry of flower fragrance formation is not well understood. Generally, flower buds do not have a scent, and the characteristic fragrance of a flower appears during anthesis as the petals open. For example, flower fragrance compounds are formed from precursor compounds in the flower buds of *Jasminum polyanthum* upon treatment with a crude enzyme preparation from opening flowers (Watanabe et al., 1993). This suggests that the final biosynthetic reactions are developmentally regulated and only occur as the flowers begin to open. Watanabe et al. (1993) have proposed that the enzymes mediating these terminal reactions are either newly induced or activated during flower opening. As well, Loughrin et al. (1992) have reported that the volatile components of *Nicotiana suaveolens* flowers increase dramatically post-anthesis.

It has also been proposed that precursors of floral scent are formed in leaves and transported through the phloem to flowers (Ackermann et al., 1989). In addition, some volatiles, in particular the C-6 aldehydes and alcohols comprising green note, are common elements of floral and leaf scent (Knudsen et al., 1993; Gardner, 1995). These compounds are produced through the lipoxygenase pathway from free linoleic and linolenic acids derived from glyceride lipids. This pathway is well characterized for leaves. Specifically, linoleic and linolenic acid are converted to their respective 13-hydroperoxides by lipoxygenase, and these in turn are hydrolyzed by hydroperoxide lyase forming hexanal and *(3Z)*-hexenal, respectively. *(3Z)*-Hexenal can be isomerized to *(2E)*-hexenal, and the C-6 aldehydes can all

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be reduced to their corresponding alcohols by alcohol dehydrogenase (Gardner, 1995). Recent evidence indicates that hydroperoxide lyase is associated with plastid envelope membranes in leaves (Bleé and Joyard, 1996). This has not been demonstrated in petals, although subcellular fractionation studies with carnation flowers have indicated that green note volatiles are present in the cytosol but originate from membranes (Hudak and Thompson, 1997).

In the present study, 13 major volatiles of the carnation flower signature have been identified by GC/MS. Through fractionation studies, it has been demonstrated that the volatiles are present in both membranous and cytosolic compartments, that many of them are synthesized within membranes and that their synthesis is developmentally regulated.

2. Results and discussion

2.1. Steady-state levels of fragrance volatiles

Ten volatiles were identified as components of the carnation flower fragrance signature by GC/MS of purged petal homogenates. In each case, the identity of the compound was confirmed using the internal mass spectrum library of the detector and also by spiking with an authentic standard. The complement of volatiles included fatty acid derivatives, specifically the aldehydes, hexanal, *(2E)*-hexenal and nonanal, and the alcohols, 1-hexanol, 2-hexanol and 3-hexen-ol, as well as the benzenoids, benzaldehyde, benzyl alcohol and benzyl benzoate, and caryophyllene, an isoprenoid. Three additional benzoids, hexyl benzoate, 3-hexen-1-ol benzoate and methoxypropenylphenol (most likely eugenol (2-methoxy-4-(2-propenyl)phenol)) were also identified by GC/MS, but were not further analyzed.

Steady-state levels of the ten volatiles for which authentic standards were available were quantified in petal homogenates of flowers at various stages of post-harvest development to determine whether there are changes in stoichiometric concentrations of the fragrance components as the flowers open and senesce. Flowers were selected for volatile analysis at four stages: Stage I (flower bud still partially closed with the petals extending 2 cm beyond the sepals); Stage II (fully open flowers with yellow tinted centers); Stage III (fully open flowers without yellow tinted centers) and Stage IV (senescing flowers showing pronounced petal-inrolling) as defined by Mayak et al. (1981). The ten volatiles comprised distinctly different proportions of the fragrance signature, and these proportions changed significantly as the intact flowers opened and began to senesce (Fig. 1). The benzenoids, in particular benzaldehyde and benzyl alcohol, and caryophyllene, an isoprenoid, were much more abundant than the fatty acid derivatives at all stages of flower development. Specifically, benzaldehyde, benzyl alcohol and caryophyllene had peak values at different stages of flower development of 38.9, 14.1 and 33.1 μg/g fr. wt of tissue, respectively (Fig. 1b). Among the benzenoids, only benzyl benzoate was present at low concentrations ($<$ 1 μg/g fr. wt of tissue) (Fig. 1b). By contrast, all of the fatty acid derivatives accounted for lesser proportions of the fragrance note. Hexanal, *(2E)*-hexenal, 2-hexanol and nonanal were present at concentrations of <1 μg/g fr. wt of tissue, and 1-hexanol, 3-hexen-1-ol and nonanal at concentrations of <5 μg/g fresh weight of petal tissue (Fig. 1a).

Of particular interest is the finding that the levels of the individual volatiles changed dramatically and independently during flower development. For example, at stage II of development when the flower is fully open and the fragrance signature is organoleptically full and strong, only 5 of the volatiles, hexanal, *(2E)*-hexenal, benzaldehyde, benzyl benzoate and caryophyllene, are present at peak levels (Fig. 1a, b and c). This suggests that the fatty acid derivatives, hexanal and *(2E)*-hexenal, and benzyl benzoate contribute significantly to the full fragrance signature of the carnation flower despite being present at comparatively low concentrations.

As the flowers begin to senesce, their fragrance weakens and changes qualitatively, becoming less organoleptically pleasant. In keeping with this, steady-state levels of 5 of the volatiles, *(2E)*-hexenal, 2-hexanol, benzaldehyde, benzyl benzoate and caryophyllene, decline to comparatively low levels by stage IV of flower development when the edges of the petals have begun to inroll (Fig. 1a, b and c). Petal inrolling is the first morphological manifestation of carnation flower senescence (Mayak et al., 1981). Other volatiles, specifically hexanal, and 3-hexen-1-ol, show little change in abundance during flower development (Fig. 1a), whereas still others, specifically 1-hexanol and benzyl alcohol, show increased abundance between stages II and IV of development (Fig. 1a, b and c).

It is thus apparent that the steady-state levels of components of the carnation petal fragrance are independently regulated during flower development. The patterns of change in concentration proved to be distinguishable for each of the 10 major volatiles analyzed. This complex pattern of developmental regulation results in changes in the stoichiometry of the volatile complement that presumably engender subtle alterations in fragrance during opening of the flower as well as the more dramatic changes accompanying petal senescence.

2.2. Subcellular localization of fragrance volatiles

Cell fractionation studies revealed that all of the volatiles but one, 2-hexanol, are present in membranes

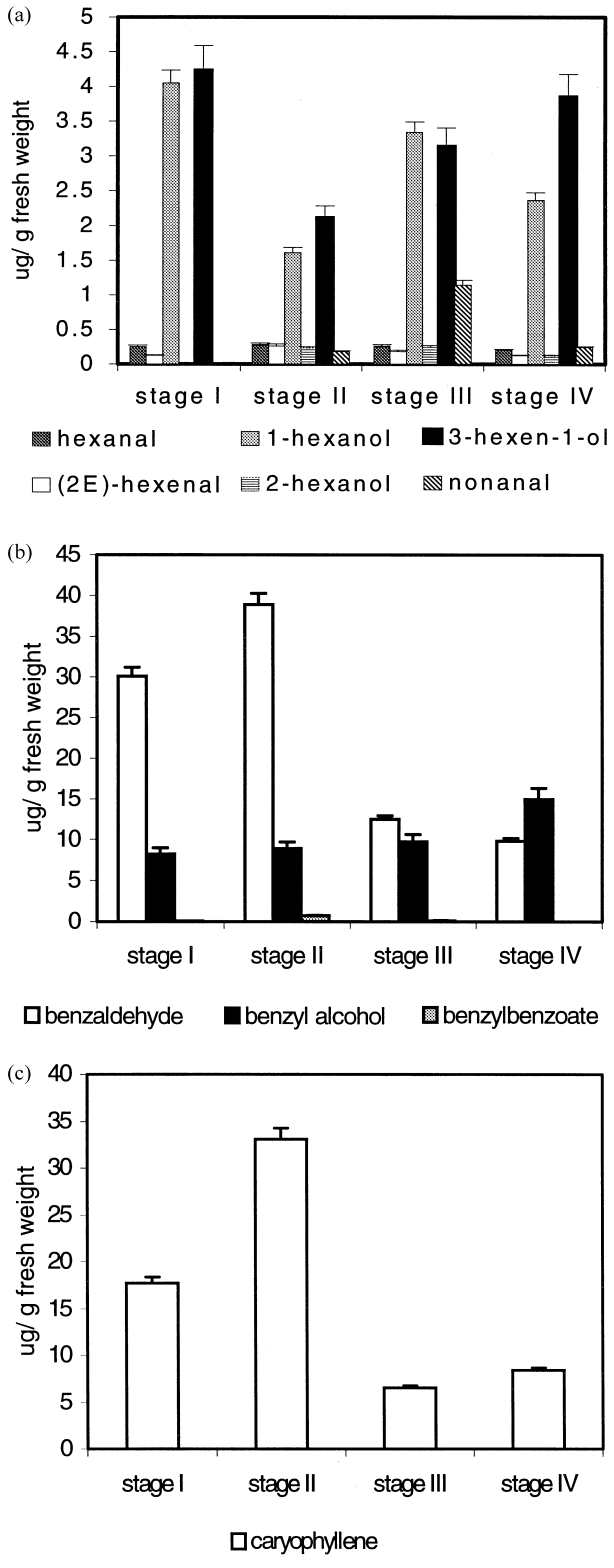


Fig. 1. Changes in the levels of carnation petal fragrance volatiles during post-harvest development of cut flowers. The volatiles were purged from homogenates. (a) Fatty acid derivatives: hexanal; *(2E)*-hexenal; 1-hexanol; 2-hexanol; 3-hexen-1-ol; nonanal. (b) Benzenoids: benzaldehyde; benzyl alcohol; benzyl benzoate. (c) Caryophyllene. Values are means±S.E. for $n=3$.

as well as in the cytosol (Figs. 2, 3 and 4). Levels of each volatile were measured by purge and trap for a total membrane fraction obtained by centrifuging the homogenate at 250,000×*g* for 2 h and in the corresponding membrane-free supernatant (cytosol) for flowers at all four stages of development. The *(2E)*-hexenal detected by purge-trapping has been shown to be a mixture of *(3Z)*-hexenal, the immediate product of the action of hydroperoxide lyase on linolenic acid hydroperoxide, and *(2E)*-hexenal (Riley and Thompson, 1998). *(3Z)*-hexenal is thermally unstable and chemically converted to *(2E)*-hexenal by the high temperature of 350°C required for purging (Ohloff, 1994).

Data for volatiles that are fatty acid derivatives are given in Fig. 2. Four of these volatiles, hexanal, *(2E)*-hexenal, 1-hexanol and 3-hexen-1-ol, are C-6 aldehydes and alcohols that collectively comprise green note, a herbaceous aroma that is well characterized for leaf tissue (Gardner, 1995). Levels of membranous hexanal and *(2E)*-hexenal are comparatively low in the petals of stage I flowers, rise to reach a peak by stage II of development and then decline again by stage IV as the flowers begin to senesce (Fig. 2a and b). By contrast, levels of membranous C-6 alcohols, 1-hexanol and 3-hexen-1-ol, increase between stages II and III of development (Fig. 2c and e), which is consistent with the fact that they are metabolically derived from hexanal and *(3Z)*-hexenal, respectively.

The finding that these green note volatiles are present in membranes is consistent with previous reports that enzymes mediating their formation are membrane-associated. Hexanal and *(3Z)*-hexenal are formed from linoleic and linolenic acids, respectively, by the sequential actions of lipoxygenase and hydroperoxide lyase, which are thought to be associated with plastid envelope membranes (Bleé and Jayard, 1996). 1-Hexanol and 3-hexen-1-ol are formed from hexanal and *(3Z)*-hexenal by alcohol dehydrogenase, and *(2E)*-hexenal is formed from *(3Z)*-hexenal by an alkenal isomerase (Gardner, 1995). It has been proposed that alcohol dehydrogenase and alkenal isomerase are also associated with plastid envelope membranes (Bleé and Joyard, 1996). Accordingly, since green note volatiles are present in both the membranous and cytosolic compartments of carnation petals, it can be presumed that they are formed in membranes and subsequently partition into the cytosol in accordance with partition coefficients. Indeed, challenging the cytosolic fraction with endogenous linoleic and linolenic acids did not result in increased hexanal and *(2E)*-hexenal levels, respectively, whereas levels of these volatiles did increase in the membranous fraction upon addition of exogenous linoleic and linolenic acids (data not shown). Furthermore, for stage II flowers the levels of membranous hexanal and *(2E)*-hexenal are higher than can be accounted for by simple equilibration between the membranous and cytosolic compartments.

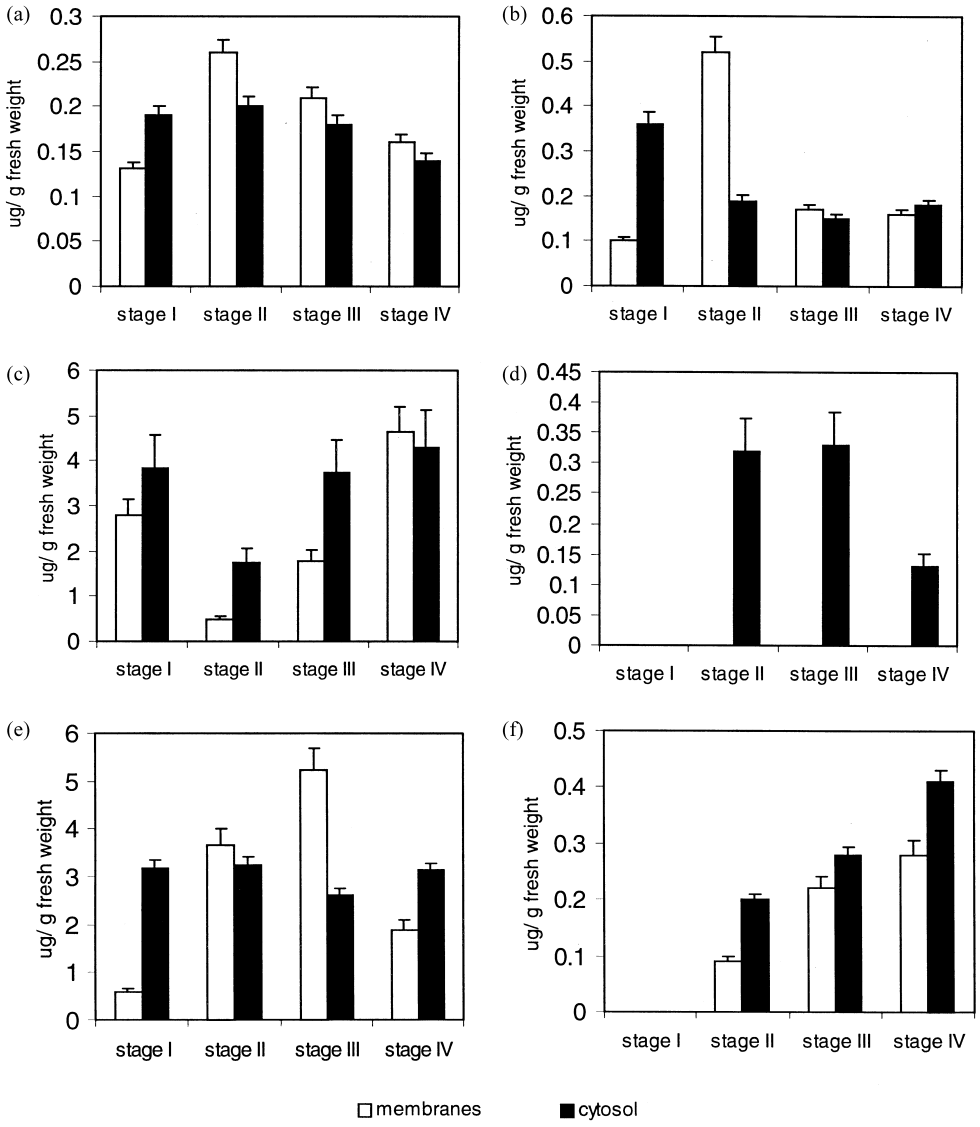


Fig. 2. Levels of fragrance volatiles derived from fatty acids in cytosol and membrane fractions isolated from petals of carnation flowers at various stages of development. (a) Hexanal; (b) *(2E)*-hexenal; (c) 1-hexanol; (d) 2-hexanol; (e) 3-hexen-1-ol; (f) nonanal. Values are means±S.E. for $n=3$.

Specifically, membranous levels of hexanal and *(2E)*-hexenal account for 57 and 67%, respectively, of the total (membranous + cytosolic), whereas the size of the membranous pellet is estimated to be only 1% of the combined membranous and cytosolic compartments. Using a partition coefficient of 60 (Kamlet et al., 1988), only 27% of the total hexanal and 20% of the total *(2E)*-hexenal would be in the membranous compartment if the apportionment between membranes and cytosol were strictly attributable to equilibration.

These findings support the contention that at least hexanal and *(2E)*-hexenal originate from membranes. However, the possibility that there is some conversion of hexanal and *(3Z)*-hexenal to their respective alcohols and of *(3Z)*-hexenal to *(2E)*-hexenal by soluble isoforms of alcohol dehydrogenase and alkene isomerase after they partition from their membranes of origin into the cytosol is not precluded. By contrast, 2-hexanol was only found in the cytosol of carnation petals (Fig. 2D), indicating that it is isomerized, presumably from 1-hexanol, exclusively within this subcellular fraction. Moreover, it is apparent that this isomerization is not particularly active in carnation petals for the absolute values for 2-hexanol are on average only ~10% of those for 1-hexanol at all stages of flower development (Fig. 2c and d).

The volatile metabolites derived from the aromatic biosynthetic pathway, specifically benzaldehyde, benzyl alcohol and benzyl benzoate, as well as caryophyllene, an isoprenoid, are also present in membranous and cytosolic fractions from petals (Figs. 3 and 4). Benzaldehyde, benzyl alcohol and benzyl benzoate are thought

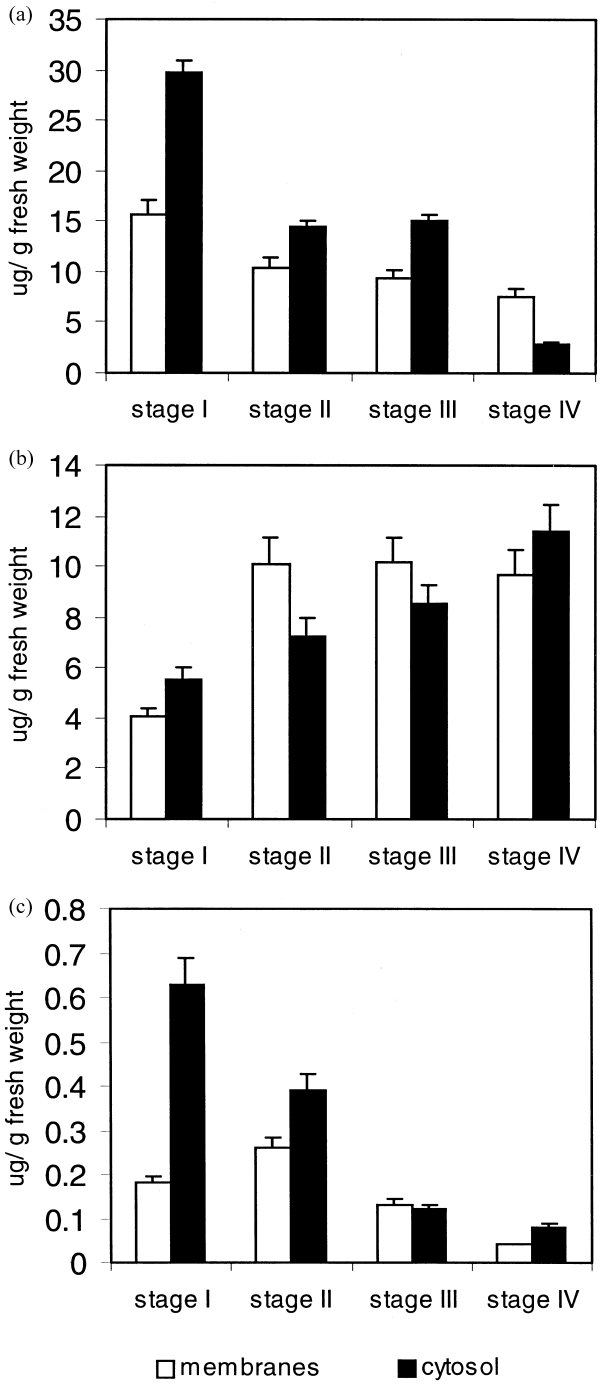


Fig. 3. Levels of benzenoid fragrance volatiles in cytosol and membranes isolated from petals of carnation flowers at various stages of development. (a) Benzaldehyde; (b) benzyl alcohol; (c) benzyl benzoate. Values are means±S.E. for $n=3$.

to be derived from benzoic acid through a three-step reaction series: reduction of the carboacid to benzaldehyde, reduction of benzaldehyde to benzyl alcohol and esterification of benzyl alcohol to form benzyl benzoate (Dey, 1989). Caryophyllene is formed from farnesylpyrophosphate by sesquiterpene cyclase (Chappell, 1995). The precise subcellular localization of these enzymes is

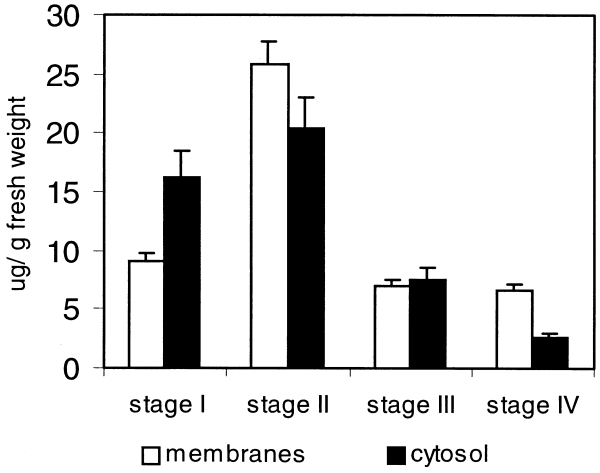


Fig. 4. Levels of the isoprenoid fragrance volatile, caryophyllene, in cytosol and membranes isolated from petals of carnation flowers at various stages of development. Values are means±S.E. for $n=3$.

not well established. However, the finding in the present study that for many stages of flower development membranous levels of these volatiles were $\geqslant$ corresponding levels in the cytosol (Figs. 3 and 4) indicates that they may be synthesized within membranes and subsequently partition out of the membrane bilayer into the cytosol.

2.3. Treatment with ethylene

Carnation flowers exhibit a climacteric-like peak in ethylene production just prior to the onset of petal-inrolling at stage IV of development (Mayak et al., 1981). Inrolling is the first morphological manifestation of petal senescence and is thought to reflect membrane leakiness attributable to changes in the chemical composition and biophysical properties of membrane bilayers. Senescence of carnation petals can also be induced prematurely by treatment of young flowers with ethylene. Indeed, young stage II flowers treated with 0.5 ppm ethylene acquire all the features, including petal-inrolling, of senescing stage IV flowers within 15 h (Mayak et al., 1981).

In the present study, flowers at stage II of development were treated with 0.5 ppm ethylene for 15 h to determine the effects of premature petal senescence on the fragrance signature. Of particular interest is the finding that although ethylene-treated stage II flowers and stage IV flowers both exhibit petal-inrolling, the stoichiometries of their fragrance signatures are distinctly different (Table 1). Steady-state levels of all of the fragrance volatiles, except hexanal and *(2E)*-hexenal, proved to be lower in ethylene-treated stage II flowers than in untreated stage II flowers, and they were also lower in the ethylene-treated flowers than in senescing stage IV flowers (Table 1). Only the C-6 aldehydes, hexanal and *(2E)*-hexenal, proved to be an exception to

Table 1
Effects of ethylene treatment on the levels of fragrance volatiles in carnation petals (purged total homogenate). Values are means for $n=3$

		μg/g fr. wt.		
		Stage II	Stage II E[a]	Stage IV
A	1-Hexanol	4.05	0.25	7.37
B	2-Hexanol	0.23	ND	0.12
C	3-Hexen-1-ol	2.11	0.60	3.85
D	Nonanal	0.18	ND	0.29
E	Caryophyllene	33.09	0.20	8.40
F	Benzaldehyde	38.90	0.40	9.80
G	Benzyl alcohol	8.90	1.70	14.94
H	Benzyl benzoate	0.70	0.02	0.02
I	Hexanal	0.28	5.19	0.20
J	*(2E)*-Hexenal	0.26	1.78	0.12

[a] E = ethylene treated.

this pattern. Levels of hexanal and *(2E)*-hexenal in the ethylene-treated flowers were higher by ~18-fold and ~35-fold, respectively, than their corresponding levels in untreated stage II flowers, and higher as well, by ~29-fold and ~18-fold, than their corresponding levels in senescing stage IV flowers (Table 1). Thus, treatment of young flowers with ethylene induces premature morphological symptoms of senescence reflecting membrane damage, but does not simulate the effects of natural senescence on the fragrance signature of the carnation flower.

Northern blot analysis indicated that the increased levels of hexanal and *(2E)*-hexenal following ethylene treatment are not attributable to up-regulation of hydroperoxide lyase, the enzyme that forms C-6 aldehydes from C-18 fatty acid hydroperoxides (Fig. 5). These experiments were conducted using a heterologous probe for hydroperoxide lyase, and mRNA was only detectable in total RNA preparations from stage II flowers that had not been treated with ethylene (Fig. 5). Accordingly, this effect of ethylene on the steady-state levels of hexanal and *(2E)*-hexenal appears to reflect either up-regulation of some other step in the C-6 aldehyde biosynthetic pathway that is rate-limiting or post-translational activation of enzyme activity. It is also apparent that senescence-related changes in carnation floral scent are not regulated directly by ethylene. The chemical composition of the fragrance signature in naturally aged flowers is very different from that for flowers that have been aged prematurely by treatment with ethylene.

3. Experimental

3.1. Chemicals

Chemicals were obtained from Sigma Chemical Co. (Mississauga, ON).

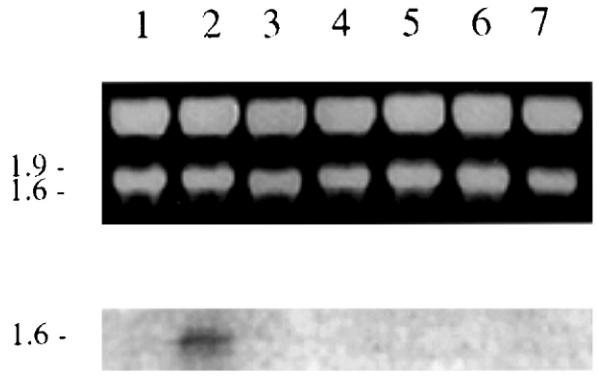


Fig. 5. Northern blot of total RNA isolated from carnation petals at various stages of development. Each lane was loaded with 10 μg of RNA. Lane 1, flowers at stage I of development; lane 2, flowers at stage II of development; lane 3, flowers at stage III of development; lane 4, flowers at stage IV of development; lane 5, stage II flowers treated with 0.5 ppm ethylene for 9 h; lane 6, stage II flowers treated with 0.5 ppm ethylene for 12 h; lane 7, stage II flowers treated with 0.5 ppm ethylene for 15 h. Upper panel, ethidium bromide-stained agarose gel. Lower panel, blot probed with full-length cDNA for hydroperoxide lyase from *Arabidopsis thaliana*. Molecular weight markers are indicated.

3.2. Plant material

Carnation flowers (*Dianthus caryophyllus* L. cv Improved White Sim, California Florida Plant, Salinas, CA) were grown as described (Hudak and Thompson, 1997). Flowers were cut at stage I of development (flower bud partially closed) and allowed to open and senesce in deionized water at 25°C under constant illumination. For some experiments, cut flowers that had reached stage II of development (Mayak et al., 1981) were placed in a chamber (135 l) and treated for 15 h with 0.5 ppm ethylene. Ethylene treatment was terminated by removing the flowers from the chamber and venting them for 1 h.

3.3. Plant homogenization and subcellular fractionation

Tissue homogenization was achieved by blending (12.5 g fr. wt) of carnation petals in 75 ml of homogenate buffer (250 mM sorbitol pH 7.4, 50 mM EPPS, 10 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 1 mM benzamadine, 5 mM amino-*n*-caproic acid and 4% (w/v) polyvinylpolypyrrolidone) at 4°C in a Sorvall Omnimixer at a setting of 5 for 45 s. This was followed by an additional blending with a Polytron Homogenizer at setting 6 for 60 s at 4°C. The resulting slurry was filtered through four-ply Veratec cheesecloth (Veratec, Walpole, MA) and centrifuged at 4°C for 2 h at 250,000 × *g* in a Beckman 60 Ti rotor to obtain total membranes (the pellet) and cytosol (the supernatant). The membranous fraction was washed by resuspension in 5 volumes of buffer (250 mM

sorbitol pH 7.4, 50 mM EPPS, 10 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 1 mM benzamadine, 5 mM amino-*n*-caproic acid) and centrifuged at 4°C for 1 h at 250 000×*g*. The resulting pellet of washed membranes was resuspended in 12.5 ml of homogenate buffer.

3.4. Volatile analysis

Fragrance volatiles were identified and analyzed by GC/MS (Bate et al., 1998). For this purpose, 5 ml of sample (homogenate, cytosol or total washed membranes) were added to 5 ml of saturated $CaCl_2$ solution and placed on ice to quench any further reaction. The samples were then purged and trapped using a Dynamic Thermal Stripper 1000 (Supelco, Bellefonte, PA) fitted with a Carbotrap 300 multi-bed thermal desorption tube (Supelco, Bellefonte, PA). This entailed placing the samples in the purge vessel, preheating them to 40°C for 5 min and purging with nitrogen for 20 min at 50°C. The emitted volatiles were trapped in the desorption tube, and the desorption tube was then dried for 10 min at 40°C. Flow rates for sparging and drying were 140 and 60 ml/min, respectively. The volatiles were desorbed for 3 min at 350°C directly into a GC/MS (HP-5890 series II GC and HP-5970 MS) using a Thermal Desorption 890 Unit (Supelco, Bellefonte, PA). The GC was fitted with a DB Wax column, 30 m×0.25 mm ID, 0.25 μm film (J&W Scientific, Brockville, ON). The oven temperature was held at 50°C for 5 min and then increased to 250°C at 6°C/min and held for 10 min. The carrier gas was helium, flowing at 39 cm/s. The MS detector was operated at a scan range of 35–150 m/z at 0.16 s/scan. The separated peaks were identified using the MS library and also by spiking the samples with authentic standards. Confirmation of the library identification of separated peaks was achieved by purging homogenate buffer containing known amounts of authentic standards and also by spiking these samples with authentic standards.

Recovery factors were obtained for each volatile by running parallel samples spiked with a known amount of authentic standard, which was added at the time of tissue homogenization. The data points reported were all corrected using these recovery factors.

3.5. RNA isolation and northern blotting

Total RNA was isolated from carnation petals at various stages of development according to Puissant and Hodebine (1990). For northern analysis, total RNA was fractionated on 1.0% formaldehyde agarose gels and immobilized on nylon membranes (ICN, Costa Mesa, CA). Immobilized RNA was hybridized with cDNA for hydroperoxide lyase from *Arabidopsis thaliana* (Accession No. T20864) (Bate et al., 1998) by incubating the nylon membranes overnight at 42°C with full-length cDNA labeled with α-^{32}P-dCTP using a random primer kit (Boehringer, Indianapolis, IN). The membranes were then washed once in 1×SSC containing 0.1% SDS at room temperature for 15 min and three times in 0.2×SSC containing 0.1% SDS at 65°C for 15 min each. Hybridization was visualized by autoradiography after an overnight exposure at −70°C.

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Anthocyanins from the scarlet flowers of *Anemone coronaria*

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Abstract

Three acylated anthocyanins were isolated from the scarlet flowers of *Anemone coronaria* 'St. Brigid Red' along with a known pigment, pelargonidin 3-lathyroside. The structures of the acylated pigments were based on a pelargonidin 3-lathyroside skeleton acylated at different positions with malonic acid. The first pigment was identified as pelargonidin 3-*O*-[2-(β-D-xylopyranosyl)-6-*O*-(malonyl)-β-D-galactopyranoside], the second was pelargonidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(methyl-malonyl)-β-D-galactopyranoside], and the third was (6″-*O*-(pelargonidin 3-*O*-[2″-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]))((4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-*O*-tartaryl)malonate. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Anemone coronaria*; Ranunculaceae; Acylated anthocyanins; Pelargonidin 3-lathyroside; Malonic, tartaric and glucosylcaffeic acid; Scarlet flower colour

1. Introduction

In 1939 Robinson and his co-workers were the first to investigate the flower colour pigments of *Anemone coronaria,* and reported that both pelargonidin and delphinidin glycosides were present in this flower (Lawrence et al., 1939). Thirty years later, Harborne determined that scarlet *A. coronaria* petals contained pelargonidin lathyroside and pelargonidin, cyanidin and malvidin glucosides which were also detected in the pollen of this species (Harborne, 1967). More recently, pelargonidin, cyanidin and delphinidin glycosides were observed in the red, magenta and blue cultivars of this species; however, their detailed structures were not elucidated (Harvey, 1971, Reynaud and Netien, 1971). Continuing our research on acylated anthocyanins in the Ranunculaceae (Saito et al., 1996, Toki et al., 1996; Özden et al., 1998; Saito et al., 1998), we have determined the structures of the pigments of scarlet flowers of *A. coronaria* 'St. Brigid Red' by using modern analytical procedures.

In this paper, we wish to report the isolation and structure determination of four anthocyanins **1**–**4**, including three new acylated pelargonidin 3-lathyrosides (Anemone Red Anthocyanins **2**–**4**) in the scarlet flowers of *A. coronaria* as well as pelargonidin 3-lathyroside **1**.

2. Results and discussion

Four major anthocyanin peaks were observed in the acetic acid extract of the scarlet flowers of *A. coronaria* 'St. Brigid Red' on HPLC. The relative frequency of their occurrence was 7.3% (pigment **1**), 35.4% (pigment **2**), 9.8% (pigment **3**) and 14.5% (pigment **4**). All four anthocyanins were isolated as orange-red powders using procedures similar to those reported previously (Saito et al., 1996; Toki et al., 1996; Saito et al., 1998).

The chromatographic and spectral properties of the anthocyanins are summarized in Table 1. Acid hydrolysis of all four pigments resulted in pelargonidin. In addition, acid hydrolysis of **1**–**4** resulted in galactose and xylose, pigments **2** and **3** resulted in malonic acid, and pigment **4** resulted in glucose, caffeic acid, malonic acid and tartaric acid. Alkaline hydrolysis of **2**–**4** produced only one deacylated anthocyanin, which was identical with pigment **1** by HPLC and TLC. The FAB mass spectra of **1** and the deacyl anthocyanin showed [M^+] 565 m/z ($C_{26}H_{29}O_{14}$) indicating that it is composed of pelargonidin with one molecule of galactose and xylose. The structure was determined by the analysis of the ^{1}H NMR spectral data (Table 2). In the ^{1}H–^{1}H-COSY spectra the proton of Gal-H2 is shifted to lower field (δ 4.07, t, $J=7.6$ Hz), and is directly correlated with the anomeric proton (δ 5.51, d, $J=7.6$ Hz) of Gal-H1 (Table 2). Therefore, xylose is attached to the OH-2 of Gal

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Table 1
Chromatographic and spectral data for Anemone red anthocyanins

Anthocyanins[a]	BAW[b]	R_f values(×100)			HPLCRt	Spectral data in 0.1% HCl–MeOH			FAB–MS
		n-BuOH-HCl[b]	1%HCl	HAc-HCl	(min)	λ_{max}(nm)	E_{acyl}/El_{vis}(%)	E_{440}/E_{vis}(%)	$[M]^+$
1	54	43	18	62	16.1	270,431,511	–	38	565
2	58	51	23	73	21.5	270,431,511	–	39	651
3	64	63	27	81	25.9	270,432,512	–	37	665
4	56	59	21	79	23.0	288,324,433,512	62	38	1107

[a] **1** Pelargonidin 3-[2-(xylosyl)-galactoside], **2** pelargonidin 3-[2-(xylosyl)-6-(malonyl)galactoside], **3** pelargonidin 3-[2-(xylosyl)-6-(methyl-malonyl)-galactoside]. **4** (6″-O-(pelargonidin 3-[2-(xylosyl)-galactosyl]))((4-glucosyl)caffeoyl)-tartaryl)malonate.
[b] See Experimental for abbreviations.

through a glycosidic bond. Galactose was confirmed to be attached to the 3-OH group of pelargonidin as evidenced from the DIFNOE measurement where a NOE was observed between H-4 of pelargonidin and H-1 of Gal (Fig. 1). Thus, pigment **1** and deacylanthocyanin are pelargonidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranoside]. The FAB mass spectra of **2**–**4** gave $[M^+]$ at 651 *m*/*z* ($C_{29}H_{31}O_{17}$) for **2**, 665 *m*/*z* ($C_{30}H_{33}O_{17}$) for **3**, and 1107 *m*/*z* ($C_{48}H_{51}O_{30}$) for **4**. These molecular formulas were confirmed by the measurements of high resolution mass spectra. In order to elucidate the structures of **2**–**4**, their ^{1}H NMR spectra were measured in CF_3CO_2D–DMSO-d_6 (1:9). By analysis of these spectra, the molecular ratios for pigment **2** was one molecule each of pelargonidin, galactose, xylose and malonic acid; **3** one molecule each of pelargonidin, galactose, xylose malonic acid and methanol; and **4** one molecule each of pelargonidin, galactose, xylose, glucose, malonic acid, tartaric acid and caffeic acid (Table 2 and Fig. 1). In their sugar moieties, three anomeric protons of galactose were assigned at δ 5.51–5.56 (*d*, *J* = 7.6 Hz, pigment **2**–**4**), in addition to three anomeric protons of xylose at δ 4.68–4.71 (*d*, *J* = 7.6–7.9 Hz, pigment **2**–**4**), and one of glucose at δ 4.81 (*d*, *J* = 7.0 Hz, pigment **4**), respectively. Furthermore, the observed large coupling constants in the sugar moieties indicated that these sugars have β-configurations (Table 2).

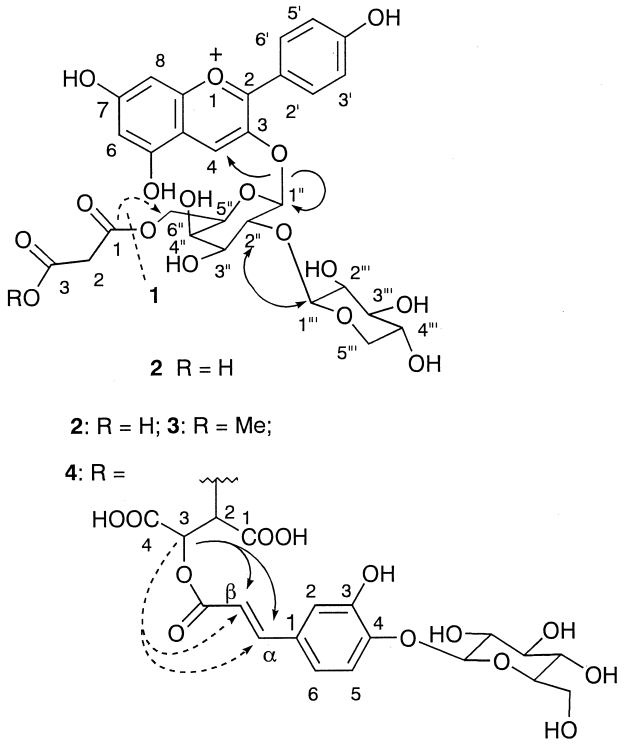


Fig. 1. Anemone red anthocyanins, **1**, **2**, **3**, **4**. Observed NOE's are indicated by arrows. Observed HMBC are indicated by dotted arrows.

2.1. Pigment 2

In the ^{1}H NMR spectra of pigment **2**, two methylene protons (-CH_2–) of Gal at δ 4.22 and 4.22 were shifted to lower field indicating that the OH-6 of Gal is bonded with malonic acid. The methylene protons of the malonic acid moiety were observed at δ 3.38 (*d*, *J* = 15.6 Hz) and 3.33 (*d*, *J* = 15.6 Hz). Observed negative DIFNOE between H-1 of xylose and H-2 of Gal indicates that its xylose is attached to the OH-2 group of Gal through a glycosidic bond (Fig. 1). Therefore, pigment **2** was determined to be pelargonidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(malonyl)-β-D-galactopyranoside], which is a new anthocyanin (Harborne and Grayer, 1988; Strack and Wray, 1994).

2.2. Pigment 3

The ^{1}H NMR spectrum of **3** was superimposed on that of **2** except for the proton signal of a methyl ester moiety at δ 3.53 (*s*, 3H) (Table 2) and, moreover, its [M +] is 655 *m*/*z*. Thus, pigment **3** was pelargonidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(methyl-malonyl)-β-D-galactropyranoside, which is a new anthocyanin. This is the first report of an acylated anthocyanin whose malonic acid moiety was esterified with methanol in plants (Harborne and Grayer, 1988, Strack and Wray, 1994).

2.3. Pigment 4

By analysis of the ^{1}H NMR spectrum of **4**, the proton signals of pelargonidin 3-[2-(xylosyl)-6-(malonyl)-galactoside] moiety, were identical with those of **2** (Table 2).

Table 2
NMR spectral data for Anemone red anthocyanins (500 and 125.78 MHz, in DMSO-d_6, TMS as internal standard, J Hz in parentheses)[a]

		4	3	2	1
	δC	δH	δH	δH	δH
Pelargonidin					
2	162.0				
3	143.2				
4	135.1	8.89 *s* *1	8.87 *s*	8.87 *s*	8.91 *s*
5	157.6				
6	102.4	6.89 *brs* *2	6.92 *d* (1.5)	6.89 *brs*	6.89 *d* (1.5)
7	168.7				
8	94.2	7.04 *brs* *3	7.05 *d* (1.5)	7.04 *brs*	7.03 *d* (1.5)
9	155.9				
10	112.0				
1′	119.2				
2′,6′	116.7	8.60 *d* (9.2)	8.61 *d* (9.2)	8.61 *d* (9.2)	8.61 *d* (9.2)
3′,5′	134.8	7.08 *d* (9.2)	7.09 *d* (9.2)	7.09 *d* (9.2)	7.09 *d* (9.2)
4′	164.7				
Caffeic acid					
1	128.5				
2	121.1	7.23 *d* (1.5)			
3	147.0				
4	147.9				
5	115.5	7.12 *d* (8.5)			
6	115.2	7.17 *dd* (1.5,8.5)			
α	114.5	6.49 *d* (15.7) *4			
β	146.5	7.59 *d* (15.9) *5			
COOH	165.8				
Malonic acid					
	165.6				
ab	40.5	3.65 *d* (16.2) *11	3.50 *d* (16.5)	3.38 *d* (15.6)	
		3.58 *d* (16.2) *11	3.45 *d* (16.2)	3.33 *d* (15.6)	
	165.2				
Tartaric acid					
1	70.4	5.67 *d* (2.7) *6			
2	71.6	5.60 *d* (2.7) *7			
1-COOH	167.0				
2-COOH	166.8				
Galactose					
1	99.8	5.55 *d* (7.6) *8	5.56 *d* (7.6)	5.55 *d* (7.6)	5.51 *d* (7.6)
2	77.5	4.09 *t* (8.2)	4.11 *t* (9.2)	4.10 *t* (8.6)	4.07 *t* (7.9)
3	68.0	3.80 *brd* (10.4)	3.80 *d* (9.6)	3.80 *dd* (3.3, 10.6)	3.77 *dd* (3.0, 9.5)
4	68.0	3.81 *brs*	3.81 *brs*	3.81 *brs*	3.80 *brs*
5	73.0	4.17 *d* (5.5)	4.17 *m*	4.18 *m*	3.56 *d* (6.0)
6a	64.5	4.25 *m*	4.22 *m*	4.22 *m*	3.48 *dd* (6.4, 11.0)
6b		4.25 *m* *9	4.23 *m*	4.22 *m*	3.80 *dd* (6.0, 11.0)
Xylose					
1	104.3	4.68 *d* (7.9) *10	4.71 *d* (7.9)	4.70 *d* (7.6)	4.67 *d* (7.9)
2	74.3	2.95 *t* (8.5)	2.96 *t* (7.9)	2.96 *t* (8.6)	2.95 *t* (8.2)
3	76.8	3.12 *t* (8.9)	3.13 *t* (8.6)	3.13 *t* (9.0)	3.12 *t* (8.9)
4	69.4	3.27 *ddd* (5.2, 8.9, 11.0)	3.30 *ddd* (5.5, 9.5, 9.8)	3.29 *ddd* (5.0, 9.0, 9.7)	3.26 *ddd* (5.5, 9.0, 10.4)
5a	66.0	3.00 *t* (11.0)	3.03 *t* (11.0)	3.03 *t* (11.0)	2.99 *t* (10.6)
5b		3.60 *m*	3.65 *dd* (5.5, 11.0)	3.64 *dd* (5.5, 11.0)	3.58 *dd* (5.5, 10.7)
Glucose					
1	101.6	4.81 *d* (7.0)			
2	73.0	3.31 *m*			
3	70.3	3.18 *t* (9.2)			

(*continued on next page*)

Table 2 (*continued*)

	4		3	2	1
	δC	δH	δH	δH	δH
4	76.0	3.35 *m*			
5	77.3	3.60 *m*			
6a	61.0	3.63 *m*			
6b		3.71 *d* (10.4)			
Methanol					
CH_3			3.53 *s*		

[a] Observed major HMBC correlations: *1: C3, C9, C5, C2, gal-C1; *2: Pel-C8, C5, C7, C10; *3: Pel-C6, C7, C9, C10; *4: Caf-C1; *5: Caf-C2, Caf-C6, Caf-CO; *6: Ta-CO(1); *7: Ta-CO(2); *8: Pel-C3; *9: Mal-CO(2); *10: Gal-C2; *11: Mal-CO(1), Mal-CO(2).

In addition to these, the proton signals of caffeic acid, glucose and tartaric acid were observed (Table 2). The two methine protons of tartaric acid were shifted to lower field at δ 5.67 (*d*, $J=2.7$ Hz) and δ 5.60 (*d*, $J=2.7$ Hz) indicating that both OH groups of tartaric acid are bound with caffeic acid and malonic acid, respectively. On alkaline hydrolysis of **4**, 4-glucosylcaffeic acid was detected by TLC and HPLC (Toki et al., 1994a, b), and its glycosyl position was confirmed by the DIFNOE spectrum (Fig. 1). The ^{13}C NMR spectroscopic signals of **4** were correlated with its proton signals, respectively, and assigned by ^{1}H–^{13}C COSY(HMQC) and HMBC spectral measurements as shown in Table 2. Moreover, linkages of sugars and acids were confirmed by means of ^{1}H–^{1}H COSY, DIFNOE and HMBC spectra (Fig. 1). Therefore, **4** was determined to be (6″-*O*-(pelargonidin 3-*O*-[2″-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl])) ((4-*O* - D - glucopyranosyl) - *trans* - caffeoyl) - *O* - tartaryl) malonate, which is a new pigment. This is the first report in which an anthocyanin contained one molecule of tartaric acid as its component in plants (Harborne and Grayer, 1988; Strack and Wray, 1994).

To date, there are nine glycosylation patterns of anthocyanidins in the flowers of Ranunculaceae (Harborne, 1967; Harborne and Grayer, 1988; Strack and Wray, 1994; Saito et al., 1996; Toki et al., 1996; Saito et al., 1998). The 3-glucosides of anthocyanidins are very common in the flowers of this family (Toki et al., 1996). However, other glycoside types are rather restricted in this family such as 3-sambubioside in *Ranunculus asiatics* (Toki et al., 1996) and *Anemone hepatica* (Reynaud and Neitien, 1971), 3-[2-glucosyl-galactoside] in *Pulsatilla cernua* (Yoshitama et al.,1998), 3-lathyroside in *Anemone coronaria* (Harborne, 1967), 3-rutinoside in *Anemonopsis macrophylla* (Harvey, 1971), 3-rhamnoside-5-glucoside in *Anemone hepatica* (Reynaud and Neitien, 1971), 3,5-diglucoside in *Aquilegia* spp and 3-sambubioside-5-glucoside in *Aquilegia* and *A. formosa* (Harborne and Grayer, 1988), 3-glucoside-7-glycoside in *Consolida armeniaca* (Saito et al., 1996) and *Delphinium hybridum* (Saito et al., 1998), and 3-rutinoside-7-glycoside in *Delphinium hybridum* (Kondo et al., 1990, 1991; Saito et al., 1998), and *Aconitum chinense* (Takeda et al., 1994). Regarding the acylation moieties of anthocyanins, it has been shown that some anthocyanidin glycosides are acylated with acids in the flowers of this family as follows: anthocyanidin 3-malonylsambubiosides in *R. asiaticus* (Toki et al., 1996), pelargonidin 3-caffeoylglucosylgalactoside in *P. cernua* (Yoshitama et al., 1998), delphinidin 3-malonylglucoside-7-poly-*p*-hydroxybenzoylglycosides in *C. armeniaca* (Saito et al., 1996), delphinidin 3-rutinoside-7-poly-*p*-hydroxybenzoylglycosides in *D. hybridum* (Kondo et al., 1990, 1991; Saito et al., 1998), and delphinidin 3-rutinoside-7-poly-*p*-hydroxybenzoylglycosides in *Aconitum chinense* (Takeda et al., 1994).

In this study, we found that the 3-lathyrose residues of pelargonidin are acylated with malonic acid in the pigments **2**, **3**, **4** of *A. coronaria*. Furthermore, both carboxyl groups of malonyl moieties in pigments **3** and **4** are bound with two OH groups such as 6-OH of a galactose unit and 2-OH of a tartaric acid in **4**, and 6-OH of a galactose unit and OH of methanol unit in **3**, respectively. The occurrence of the di-ester structures of the malonic acid moiety in anthocyanin pigments have so far been reported in the pigment of *Eichhornia crassipes* (Toki et al., 1994b) and the pigment of *Dianthus caryophyllus* (Bloor, 1998). Therefore, this is the third report of malonate diester anthocyanins in plants (Harborne and Grayer, 1988; Strack and Wray, 1994).

3. Experimental

3.1. Plant material

The tubers of *Anemone coronaria* 'St. Brigid Red' were purchased from Takii Nursery Co., Ltd, Kyoto Japan, and cultivated in the experimental farm of Minami-Kyushu University. Fresh petals were collected and dried at 45°.

3.2. Extraction and isolation

The dried petals (50 g) were extracted with 5% HOAc at room temp. overnight. The extract was adsorbed on a Diaion HP-20 column, this being with H_2O. The pigments were next eluted with MeOH–HOAc–H_2O (75:5:20). After

concn, the eluate was fractionated with Sephadex LH-20 CC using HOAc–MeOH–H_2O (1:6:12). The frs were further purified with PC (*n*-BuOH–HOAc–H_2O, 4:1:2 and 15% HOAc) and HPLC. Prep. HPLC was performed on a Hitachi 6200 system, using an Inertsil ODS-2 column (20×20 mm) with a HOAc solvent. Pigment **1** (8.5 mg), **2** (18.2 mg), **3** (3.2 mg) and **4** (11.5 mg) were obtained.

3.3. Analysis

Pigment identification was carried out by standard procedures involving deacylation with alkali, acid hydrolysis and H_2O_2 oxidation (Lawrence et al., 1939). TLC was carried out on microcrystalline cellulose (Avicel SF, Funakoshi). Solvents used for anthocyanins were BAW (*n*-BuOH–HOAc–H_2O, 4:1:5), HOAc–HCl (HOAc–HCl–H_2O, 15:3:82), 2N *n*-BuOH-HCl, 1:1 and 1% HCl, for sugars were BAW, *i*-PrOH–*n*–BuOH–H_2O (7:1:2) and PhOH–H_2O (4:1), and for acids were BAW, EtOAc–HOAc–H_2O (3:1:1) and EtOH–H_2O–NH_4OH (16:3:1). R_f values of tartaric and malonic acids were 0.64 and 0.46 in BAW, 0.63 and 0.42 in EtOAc–HOAc–H_2O, and 0.08 and 0.06 in EtOH–H_2O–NH_4OH. HPLC was performed as described previously (Toki et al., 1996; Saito et al., 1998) using an Inertsil ODS-2 column (4.6×250 mm) at 35°, with a flow rate of 0.8 ml min^{-1} and monitoring at 520 nm. R_f values of 4-glucosylcaffeic acid were 0.55 (BAW) and 0.46 (6% HOAc) ; Rt of HPLC was 9.4 (min). NMR spectra were measured in CF_3COOD-DMSO-d_6 (1:9) and recorded at 500 MHz on JEOL JMS GX-500 instrument for ^1H NMR and ^{13}C at 125.78 MHz. Chemical shifts are reported in δ values. FAB-MS were recorded in the positive mode with a magic bullet and negative mode with a glycerol matrix.

Chromatographical and spectral (NMR, UV, FABMS) data of compounds **1**–**4** were shown in Tables 1 and 2.

3.4. High-resolution FABMS

Pigment **2**: HR-FABMS calc. for $C_{29}H_{31}O_{17}$: 651.1561. Found: 651.1549. Pigment **3**: HR-FABMS calc. for $C_{30}H_{33}O_{17}$: 665.1717. Found: 665.1749. Pigment **4**: HR-FABMS calc. for $C_{48}H_{51}O_{30}$: 1107.2465. Found: 1107.2456.

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Kaempferide triglycoside: a possible factor of resistance of carnation (*Dianthus caryophyllus*) to *Fusarium oxysporum* f. sp. *dianthi*

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Abstract

A kaempferide triglycoside has been found as a constitutive component in an uninfected carnation (*Dianthus caryophyllus*) of the cultivar Novada. The chemical structure has been determined mainly by the use of spectroscopic methods, including 2D NMR experiments. It showed a strong activity in restricting fungal parasite development, which could contribute to the known ability of carnation cv. Novada to resist to *Fusarium oxysporum* f. sp. *dianthi* infection. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Dianthus caryophyllus*; Caryophyllaceae; Carnation; Kaempferide glycoside; NMR; Fungitoxic activity

1. Introduction

Fusarium oxysporum f. sp. *dianthi* represents the most important fungal parasite affecting carnation (*Dianthus caryophyllus* L., Caryophyllaceae) cultivated areas, where it causes severe crop losses all over the world (Garibaldi and Gullino, 1987). Carnation of the cultivar Novada has a commercial interest because of its high resistance to the parasite (Baayen, 1986; Baayen et al., 1988). Species of this cultivar have been, therefore, extensively studied to elucidate the mechanism of this high resistance degree at a biochemical level. An adequate explanation seems to reside in the production, after a *F. oxysporum* f. sp. *dianthi* attack, of nitrogen-containing phytoalexins effective against the pathogen (Niemann et al., 1992). These molecules have been identified as anthranilic acid derivatives, dianthalexins and dianthramides (Reinhard and Matern, 1989). However, within the tissues of the same carnation a further compound, which does not contain nitrogen and displays fungitoxic properties, has been detected but not identified (Niemann, 1992; Niemann and Baayen, 1988). Since in another highly resistant carnation cultivar a constitutive phenol is responsible for the fungitoxic properties of the tissue extract (Curir et al., 1996), the possibility that the unidentified fungitoxic compound from cv Novada is a phenol derivative could not be excluded. Therefore, a research has been undertaken to assess if Novada uninoculated tissues contain pre-formed compounds effective against *F. oxysporum* f. sp. *dianthi*, besides the already recognized nitrogen-containing phytoalexins.

As a result of such a study, herein we describe the isolation and structural elucidation of a new kaempferide triglycoside (**1**), a factor of resistance of carnation to *F. oxysporum* f. sp. *dianthi*, as evidenced by preliminary fungitoxic data.

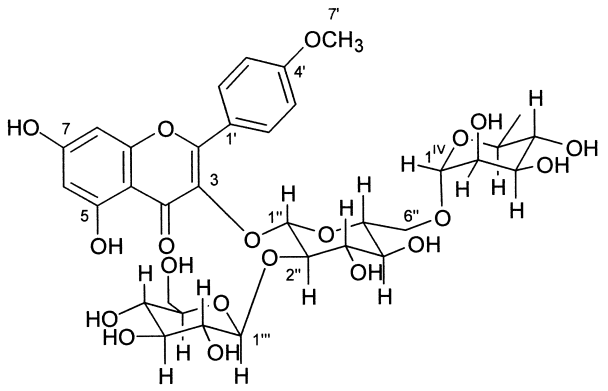
2. Results and discussion

Compound **1**, a yellow powder with $[\alpha]_D^{25} = -16°$ (MeOH; $c = 0.002$), was obtained in pure form only after repeated chromatographic steps based on C-18 reverse-phase and Sephadex LH-20 gel filtration (see Section 3). It gave a pseudomolecular ion peak at m/z 769.2170 $(M–H)^-$ in the negative-ion high-resolution FAB mass spectrum, consistent with the molecular formula $C_{34}H_{42}O_{20}$ (calc. m/z 769.2191). The UV spectrum (MeOH, Table 1) showed absorptions at λ_{max} 264, 316 and 350 nm, in accordance with a C-3 substituted flavonol

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skeleton (Mabry et al., 1970). The bathochromic shifts obtained by addition of several diagnostic reagents (Table 1) were in good agreement with the data reported for the kaempferide skeleton (Mabry et al., 1970).



1

The 500 MHz ^{1}H and ^{13}C NMR spectra (Table 2) confirmed these conclusions and, in addition, revealed that a triglycosidic chain should account for the remaining atoms of the molecular formula. The combined interpretation of these spectra was aided by the 2D HMQC spectrum, which allowed us to associate all the protons with the relevant carbon signals, and by the 2D HMBC spectrum, which was essential to interconnect the different spin systems.

In particular, two broad singlets at δ 6.24 and 6.42 in the ^{1}H NMR spectrum were attributed to H-6 and H-8, respectively, while the two 2H integrating doublets at δ 6.92 and 8.06 fitted well with data reported for H-3′ and H-2′, respectively. The singlet at δ 3.39 (3H) was assigned to the 7′-OMe group by the following series of evidences: (i) the downfield shift of the ^{13}C NMR signal of C-4′ (δ 162.8) suggested the presence of an ether linkage at that position (Bilia et al., 1993); (ii) a correlation between the methoxy protons (δ 3.39) and the signal at δ 6.92 (H-3′ and H-5′) was evident in the ROESY spectrum; (iii) the presence of a key HMBC cross peak between H-7′ and C-4′. Finally, the HMBC spectrum allowed us to unambiguously assign all the ^{13}C resonances of this aglycone.

Going on, three anomeric carbon signals at δ 101.4, 104.5 and 101.8 were identified in the ^{13}C NMR spectrum and associated with the relevant protons (δ 5.38, 4.80 and 4.50, respectively) through the HMQC experiment.

Table 1
UV spectral absorptions of **1**

λ_{max} in nm				
MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc
350 *sh*	384	400 *sh*	400 *sh*	350
316	316	350	350	272
264	272	308	306	
		270	272	

Table 2
^{13}C and ^{1}H NMR data of compound **1** in CD_3OD

Pos.	δ_C (mult.)	δ_H (mult., *J* in Hz)
2	159.0 (C)	
3	134.1 (C)	
4	180.0 (C)	
5	163.1 (C)	
6	99.8 (CH)	6.24 (*s*)
7	166.1 (C)	
8	95.0 (CH)	6.42 (*s*)
9	158.1 (C)	
10	105.1 (C)	
1′	124.0 (C)	
2′-6′	132.8 (CH)	8.06 (*d*, 8.5)
3′-5′	115.8 (CH)	6.92 (*d*, 8.5)
4′	162.8 (C)	
7′	50.5 (CH_3)	3.39 (*s*)
1″	101.4 (CH)	5.38 (*d*, 7.5)
2″	81.5 (CH)	3.77 (*t*, 7.5)
3″	77.5 (CH)	3.61[a]
4″	71.9 (CH)	3.31 (*t*, 9.5)
5″	77.7 (CH)	3.50 (*ddd*, 9.5, 5.5, 3.5)
6″	63.3 (CH_2)	3.67[a]
		3.60[a]
1‴	104.5 (CH)	4.80 (*d*, 7.0)
2‴	74.5 (CH)	3.43[a]
3‴	77.1 (CH)	3.37[a]
4‴	71.5 (CH)	3.72 (*dd*, 8.5, 7.5)
5‴	77.5 (CH)	3.61[a]
6‴	62.8 (CH_2)	3.86[a]
		3.77[a]
1^{IV}	101.8 (CH)	4.50 (*bs*)
2^{IV}	72.6 (CH)	3.62 (*bd*, 3.0)
3^{IV}	71.8 (CH)	3.74 (*dd*, 3.0, 8.5)
4^{IV}	73.8 (CH)	3.29 [a]
5^{IV}	71.5 (CH)	3.42 (*m*)
6^{IV}	17.1 (CH_3)	1.12 (*d*, 6.0)

[a] Overlapped with other signals.

Although the mid-field region of the ^{1}H NMR spectrum of **1** contained several overlapping signals, analysis of homonuclear COSY and HOHAHA 2D experiments allowed us to assign all the proton resonances of the sugar moieties. In particular, the 1D subspectra of the 2D HOHAHA experiment relative to each spin system allowed a better evaluation of most coupling constants, and this was very useful to elucidate the monosaccharide relative stereochemistry.

Hence, when the anomeric proton at δ 5.38 (H-1″) was used as a starting point, a sequence of four oxymethines and one oxymethylene (Table 2) was identified from the above spectra. The large coupling constants observed for all the protons, in accordance with axial–axial relationships, indicated the β-glucopyranose nature of this sugar. In addition, the ROESY couplings of H-1″ with H-3″ and H-5″, and of H-2″ with H-4″ further supported the above conclusion. This residue should be linked to the C-3 of the aglycone, as indicated by the key HMBC correlation peak between the anomeric proton H-1″ and C-3 (δ 134.1).

The HMBC cross peaks of H-2″ (δ 3.77) with the anomeric carbon at δ 104.5 and of H_2-6″ (δ 3.60 and 3.67) with the anomeric carbon at δ 101.8 identified position 2 and 6 of the inner glucose as glycosidic linkage sites. This was also confirmed by the spatial couplings of H-2″ with H-1‴ (δ 4.80) and of H_2-6″ with H-1^{IV} (δ 4.50), evidenced in the ROESY spectrum.

The monosaccharide linked at position 2″ has been identified as a further β-glucose basing on the same arguments as those used for the characterization of the previous sugar unit. On the other hand, the spin system starting with H-1^{IV} extended to four oxymethine and one methyl group (see Table 2). This sugar moiety was identified as a rhamnopyranose due to the axial-axial couplings H-3^{IV}/H-4^{IV} and H-4^{IV}/H-5^{IV} and the axial-equatorial relationship between H-2^{IV} and H-3^{IV}. In accordance with data reported in the literature (Sang et al., 1999), the α-anomeric configuration of this sugar was judged by the chemical shift of C-3^{IV} (δ 71.8) and C-5^{IV} (δ 71.5).

The nature of the sugar residues was confirmed by chemical analysis. To this aim, compound **1** was subjected to acid methanolysis followed by silylation with TRISIL-Z. GC analysis of the released saccharides showed a sugar composition of glucose and rhamnose in the relative ratio 2:1. Therefore, if the hexopyranoses are assumed to belong to the most commonly found series, D for glucose and L for rhamnose, the stereostructure of **1** is completely defined as kaempferide 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside.

Compound **1** exhibited a good fungitoxic activity towards *F. oxysporum* f. sp. *dianthi* conidia germination, as shown in Table 3. Its inhibitory activity is statistically appreciable, at 50 and 100 μM on potato dextrose liquid medium, throughout all the observation periods, while the effectiveness of the lowest tested dosage becomes statistically significant after 2 days of culture. However, when the fungitoxic activity is assayed on a gelled medium slab, the concentration requested to induce the inhibition of conidia germination is consistently lower: 1, 2 and 5 μM are enough to determine a visible, a clearly present or a strong inhibitory effect, respectively (Table 4).

Table 3
Fungitoxic activity of **1** towards *F. oxysporum* f. sp. *dianthi* conidia germination in liquid medium[a]

μM of **1**	% Of conidia germination	Days of observation
0	85a	1
10	70a	
50	20b	
100	0c	
0	95a	2
10	73b	
50	25c	
100	0d	
0	98a	4
10	75b	
50	30c	
100	10d	

[a] Each value is the mean of 50 observations. Values in each column and for each period of observation followed by the same letter are not significantly different for $P = 0.05$, according to the Student–Newmann–Keuls statistical test. Values were arcsin-transformed before the analysis.

The concentration of the investigated flavonoid (**1**) within the plant fresh tissues is about 3.9 μmol/g for the stem and 0.8 μmol/g for roots, these values being calculated from ε, measured at λ_{max} 347.5 in EtOH, as indicated by Mabry et al. (1970). These values fall within the range of concentrations effective in vitro. Compound **1** could therefore play a role in determining the Novada resistance ability (Curir et al., 1993; Baayen et al., 1997). To the best of our knowledge, this is the first report of a carnation constitutive flavonol glycoside with fungitoxic properties. Further investigations are required to ascertain if the biosynthesis of this constituent increases as a consequence of an infection attempt by *F. oxysporum* f. sp. *dianthi*.

3. Experimental

3.1. General experimental procedure

FABMS (glycerol matrix, CsI) were measured on a VG Prospec (FISONS) mass spectrometer. Optical rotation was determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.34$, $\delta_C = 49.0$). ^1H connectivities were determined by using COSY and HOHAHA experiments. The 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear ^1H–^{13}C connectivities were determined with 2D HMQC experiments with a BIRD pulse sequence followed by a delay of 0.5 s before each scan to suppress the signal from proton not directly bonded to ^{13}C. The interpulse delays were adjusted for an average value of $^1J_{CH}$ of 125 Hz. Two- and three-bond

Table 4
Fungitoxic activity of **1** after 4 days of observation[a]

μmol of **1**	Fungitoxic activity
0	−
1	±
2	+
5	++

[a] The compound was spotted on TLC plates, further covered by a layer of potato dextrose agar containing a *F. oxysporum* f. sp. *dianthi* conidial suspension.

heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HMBC experiments optimized for a $^{2,3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments.

3.2. Extraction and isolation

Carnation plants of the cultivar Novada were obtained by courtesy of Professor J. De Jong, CPLO-DRO Institute, Wageningen, Holland. A voucher specimen has been deposited at the Orto Botanico of Università di Torino, Italy. Leafy stems (40 cm) and plant roots were separately harvested and extracted as follows. Plant fresh tissues (100 g) were homogenized with a Waring Blender in 2 l of bidistilled H_2O; the homogenates were extracted for 6 h under N_2 atmosphere in a Soxhlet apparatus, and then filtered through cheesecloth and nylon net. The finest debris was removed by centrifugation at 3000 *g* for 0.5 h. To 1 l of clear liquid, put into a separating funnel, 100 ml diethyl ether were added and the upper phase, containing large amounts of fat, was discarded; this step was repeated at least 3 times. Then, 10 ml HCOOH per litre of water phase were added, and the solution loaded onto a glass column (100×5 cm) containing cellulose packed with EtOH-1% HCOOH; the elution was carried out using EtOH–H_2O (2:8, v/v). The hydroalcoholic fractions containing the investigated compound were pooled, concentrated under reduced pressure, loaded on a column (100×5 cm) filled with Polyamide 6 (Riedel-de Haën, Germany), and eluted with a linear gradient from H_2O to H_2O–EtOH (8:2, v/v). The hydroalcoholic fractions containing the investigated compound, pooled and concentrated as above, were re-chromatographed onto a column (50×1.5 cm) filled with silica gel 60 packed and eluted with H_2O–EtOH (linear elution gradient from 9:1 to 1:9, v/v). The further purification phase was carried out by a column (50×0.8 cm) filled with silica gel 100 C_{18}-reversed phase packed with EtOH, and loading the samples in 5% HCOOH-containing solutions. The column was first eluted with H_2O, and then with isoamyl alcohol. The alcoholic fractions were finally purified by gel-filtration through a Sephadex LH-20 column (30×1 cm) eluted with EtOH, to give **1** (15 mg).

3.3. Kaempferide 3-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (*1*)

$[\alpha]_D^{25} = -16^\circ$ (MeOH; $c = 0.002$); FABMS (negative ion): m/z 769 $[M\text{-}H]^-$; HRFABMS: found m/z 769.2170, calc. for $C_{34}H_{42}O_{20}$ m/z 769.2191. UV spectra: Table 1; ^{1}H NMR and ^{13}C NMR spectra: Table 2.

3.4. Methanolysis of *1*: sugar analysis

A solution of **1** (1 mg) in anhydrous 2M HCl in MeOH (0.5 ml) was heated at 80°C in a stoppered reaction vial for 8 h. After cooling, neutralization with Ag_2CO_3 and centrifugation, the supernatant was evaporated to dryness under N_2. The residue was trimethylsilylated with TRISIL Z (Pierce Chemical Co.) for 15 min at room temp. GLC analysis gave peaks that co-eluted with those of the authentic methyltrisilglucoside [R_t (min): 37.6 and 42.8 (2 mol)] and methyltrisilrhamnoside [R_t (min): 9.3 (1 mol)]. GLC analyses were run using a Hewlett-Packard 5890 gas chromatograph equipped with a fused-silica column SBB-I Supelco (30 m×0.32 mm; i.d. 0.25 μm film) and a dual FID detector. Column temperature: 140°C; Injector and detector temperature: 200°C

3.5. Fungal material

Fusarium oxysporum f. sp. *dianthi*, race 2, isolate P 1121, was obtained by courtesy of Professor Garibaldi, Dipartimento VA.P.R.A., Università di Torino, Italy. Fungus was sub-cultured in Petri dishes on potato dextrose agar while, to obtain conidia production, explants were kept on liquid potato dextrose medium for 1 week as a shaken culture. Mycelium was removed from the liquid medium by filtration through nylon net and glass wool and the conidia, harvested by centrifugation, were transferred into fresh liquid medium to obtain a concentration of about 1×10^7 conidia/ml.

3.6. Evaluation of the fungitoxic activity

Known amounts (1, 2 and 5 μmol, respectively) of compound **1** were dissolved in EtOH, spotted on silica gel TLC plates, 20×20 cm and dried. The TLC plates were then sprayed with a potato dextrose agar solution, containing the *F. oxysporum* f. sp. *dianthi* conidial suspension, and incubated for 4 days in a moist chamber. At the end of the incubation period, the fungitoxic activity was evaluated and expressed, according to an already published evaluation scale (Niemann et al., 1992), as — (not present), ± (just visible), + (clearly present) and ++ (strong). A further assay was performed by adding 10, 50 and 100 μM of **1**, respectively, to the liquid potato dextrose medium containing the *F. oxysporum* f. sp. *dianthi* conidia. After 1, 2 and 4 days of incubation, the percentages of germinated conidia were respectively recorded. Before the statistical analysis, to normalize the percentage data, their transformation in angular values has been performed (arcsin x).

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Bioactive constituents from gum guggul (*Commiphora wightii*)

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Abstract

Bioactivity-directed fractionation and purification afforded cytotoxic components of *Commiphora wightii*. The exudates of *C. wightii* were extracted with EtOAc and the extract was subjected to repeated column chromatography. A fraction showing cytotoxic activity was characterized as a mixture of two ferulates with an unusual skeleton by spectral and chemical methods, including by NMR, GC–MS and chemical derivatization. This fraction also showed moderate scavenging effect against 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radicals. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Commiphora wightii*; Berseracaea; Ferulates; Polyhydroxyalkenyl esters; Cytotoxicity; DPPH

1. Introduction

Commiphora wightii (Burseraceae) is a branched shrub or a small tree (2–3 m high) found in some states of India and Pakistan. Guggul, the exudate of *C. wightii*, is mentioned in the classic Ayruvedic literature as an efficacious treatment for bone fractures, arthritis, inflammation, obesity, cardiovascular disease, and lipid disorders. Guggul lipid, a mixture of lipid steroids isolated from the resin of *C. wightii* has been available in the Indian market since 1988 as a potent hypolipidemic agent (Satyavati, 1991).

With the discovery of the hypolipidemic activity of the gum resin, some chemical investigations have been reported. It was found that guggul resin is a complex mixture of various classes of chemical compounds, such as lignans, lipids, diterpenoids and steroids. From the benzene phase, a waxy solid, which is a mixture of esters (Fig. 1) based on homologous long chain tetraols and ferulic acid was identified (Satyavati, 1991). Through a more detailed study by Kumar and Dev (1987), the absolute stereochemistry of these tetraols was determined.

As part of a search for bioactive substances from natural products, it was found that the EtOAc extract of *C. wightii* showed significant in vitro cytotoxicity. The isolation and identification of the cytotoxic active constituents of this plant and their chemical structures and bioactivities are reported here.

2. Results and discussion

The MTT assay (tetrazolium dye method) in MCF-7 breast tumor cells was used to isolate the bioactive fraction from guggul gum. The EtOAc extract of guggul gum was subjected to bioassay directed fractionation and yielded three active fractions (A5, A6 and A8) on elution with a solvent mixture of $CHCl_3$/MeOH with increasing MeOH content. The combined fractions were applied to a Sephadex LH-20 column which was eluted with 95% aqueous EtOH to yield an active fraction (B3). This fraction was then applied to a silica gel column eluted with acetone/hexane (2:3) to yield an active fraction (C8). Fraction C8 was next subjected to a RP-18 column chromatography to yield a chemically homogeneous active fraction (D).

Fraction D exhibited a red fluorescent spot under UV_{365nm} light on silica gel F_{254} plates. In the ^1H NMR

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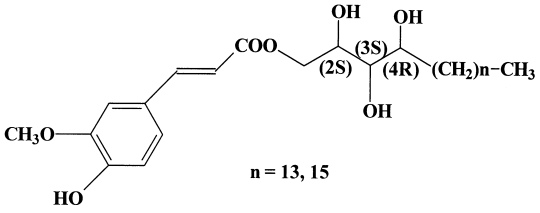
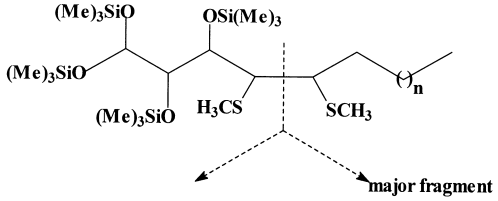


Fig. 1. Structures of known ferulates.



n = 16, major fragment: m/z 299
n = 17, major fragment: m/z 313

Fig. 2. GC–MS fragmentation diagram of tetrols.

spectrum, a group of proton signals was assigned to the feruloyl moiety [δ 7.65, *d*, (J = 15.7 Hz, H-3), δ 7.06, *m*, (H-5, 9); δ 6.91, *d*, (J = 8.0 Hz, H-8), δ 6.31, *d*, (J = 15.7 Hz, H-2), and δ 3.93, *s*, (3H, OCH_3-6)] (Katagiri et al., 1997). The signals for the aliphatic methylene groups (δ 1.24), a terminal methyl (δ 0.88, *t*, J = 6.9 Hz), and a carbinyl methylene (δ 4.37, *m*) were assignable to those of a long chain alcoholic residue. Three-oxygen substituted methine protons (δ 3.47–4.27) indicated the presence of a highly oxygenated alcohol. Comparison of the data of D with those for guggultetrol (Kumar and Dev, 1987), indicated that the only substantial difference was the presence of two olefinic proton signals (δ 5.35, *m* and δ 5.00, *m*) and two allylic proton signals (δ 2.01, *m*). Thus, D was suggested to be an unsaturated alkyl ferulate with three hydroxyl groups at C-2′, C-3′ and 4′. This proposal was supported by the ^{1}H–^{1}H COSY spectrum, which displayed correlations between H-1′ and H-2′, H-2′ and H-3′ and H-3′ and H-4′.

Alkaline hydrolysis of D yielded a mixture of two fatty alcohols. The DCI–MS data showed two molecular weights of 562 and 576, respectively. In order to quantify and characterize the alcohol in D, GC–MS and GC analyses were performed. Because of the substitution of multiple hydroxyl groups, the hydrolyzed alcohol mixture was first derivatized into TMS ethers. The double bond localization was carried out by GC–MS analysis of the corresponding bis(methylthio)-derivative vicinally substituted at the original double bond, which afforded an intense mass fragment arising from cleavage between the two carbons both substituted with a methylthio group (Francis and Veland, 1981). The resulting bis-(methylthio)-TMS ethers prepared from fraction D gave two major GC peaks, D1 and D2, which indicated that the predominant alcohols in fraction D were D1 (~81%) and D2 (~19%), respectively. In the GC–MS spectra, the peak D1 (R_f = 42.0 min) showed a fragment ion at m/z 299 (100%), which suggested the localization of bis(methylthio) substitution at C-5 and C-6. Peak D2 (R_f = 49.1 min) showed a fragment ion at m/z 313 (100%), which also suggested localization of the bis(methylthio) substitution at C-5 and C-6 (shown in Fig. 2). The double bond localization was also supported by the NMR spectra. In the ^{1}H NMR spectrum, allylic proton signals indicated that only two hydrogen atoms were attached to the double bond, while in the ^{1}H–^{1}H COSY spectrum, the allylic proton signal thus coupled with olefinic proton. In the HMBC spectrum, the double bond localization was further confirmed by correlations of H-7′ to C-5′ and C-6′, and of H-5′ to C-7′.

The geometry of the double bonds in the alcohol was determined from analysis of the ^{13}C NMR data. The spectrum of the mixture exhibited an allylic carbon signal at δ 27.7, and was reliably assigned to the methylene carbon adjacent to a *cis*-double bond in aliphatic chains. The corresponding allylic methylene carbons adjacent to a *trans*-bond are usually observed around δ 32.6 (Batchelor et al., 1974). This result suggested that the predominant alcohol in this fraction should have a *cis*-geometry. The absolute configuration of one the known ferulates (guggultetrol-18), was deduced as *D*-xylo (2S, 3S, 4R-configuration) by direct comparison with synthetic compounds (Kumar and Dev, 1987). However, it was not possible to immediately determine the absolute stereochemistry of this fraction.

Combining the above information, the alcohols obtained by hydrolysis of the ferulates were concluded to be a mixture of (*z*)-5-tricosene-1,2,3,4-tetraol and (*z*)-5-tetracosene-1,2,3,4-tetraol (Fig. 3).

For the antioxidant assays, five different concentrations of fraction D were prepared for the DPPH test (Fig. 5). The IC_{50} of fraction D was determined as 16.0 mcg/ml (28 μM). Comparatively, gallic acid and catechin are essential to the antioxidant activity of tea (Chen and Ho, 1995), and their IC_{50} values were reported as 10.6 μM and 18.6 μM, respectively (Chen et al., 1999). It was determined that the scavenging effect of fraction D on DPPH radicals was moderate compared with gallic acid and catechin. More importantly, fraction D showed a significant cytotoxic activity. Fraction D decreased cell viability in MCF-7 (breast tumor cells) and PC-3 (prostate) tumor cells. The IC_{50} in both cells

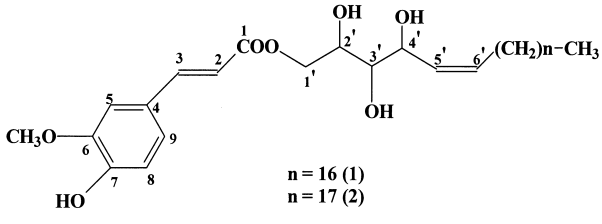


Fig. 3. Structures of the ferulate mixture.

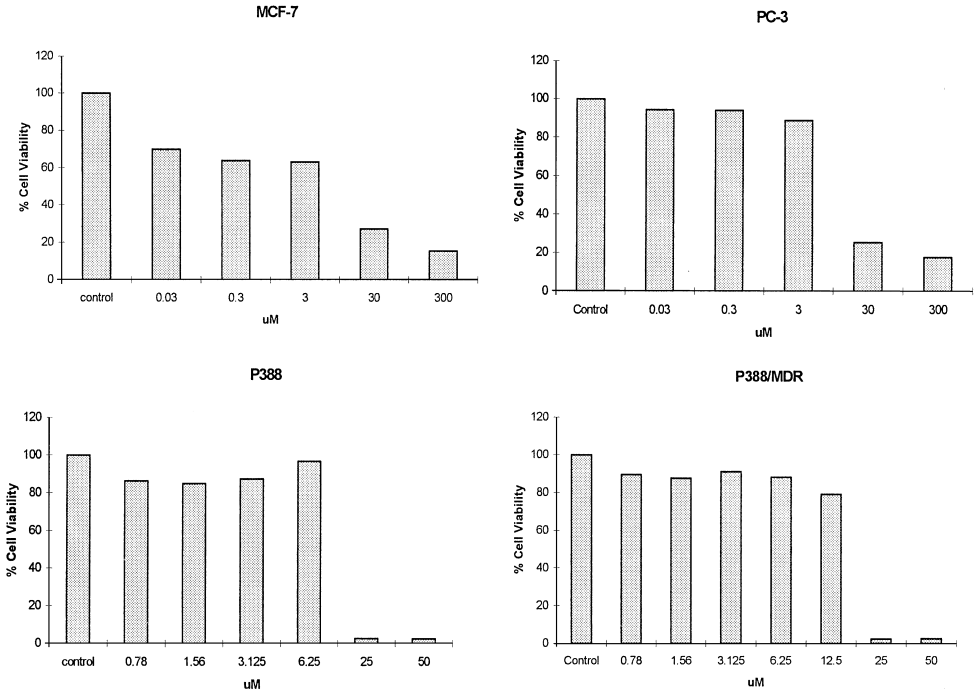


Fig. 4. Cell viability assay by MTT in MCF-7, PC-3, P388 parental, and P388 cells transfected to overexpress P-glycoprotein. Each graph represents combined data of two separate experiments performed in triplicate (±S.D.). Dried fraction D was reconstituted in 100% EtOH and all experiments included a vehicle (EtOH) control.

were 14.3 mcg/ml (25 μM). To determine if fraction D could overcome a common mechanism of tumor resistance, P388 tumor cells transfected to overexpress the multidrug resistance pump P-glycoprotein were used. Fraction D decreased cell viability with $IC_{50} < 25$ μM in both transfected (P388/MDR) and parental cell lines (Fig. 4). This fact suggested that this agent might be able to overcome P-glycoprotein mediated drug resistance.

In summary, cytotoxic activity directed fractionation led to the identification of an active fraction from *C. wightii*. The fraction was a new class of naturally occurring lipids and showed strong cytotoxic activity and some free radical scavenging activity.

3. Experimental

3.1. General

TLC was performed on Sigma-Aldrich silica gel TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by UV_{365nm} light and spraying with 10% (v/v) H_2SO_4 ethanol solution. Silica gel (130–270 mesh), Sephadex LH-20 and RP-18 (60 μm) (Sigma Chemical Co., St Louis, MO) were used for column chromatography. Potassium hydroxide, bis-(trimethylsilyl)-trifluoroacetamide, trimethylchlorosilane, dimethyl disulfide (DMDS), iodine, and sodiumbisulfite were purchased from Sigma Chemical Co. (St Louis, MO). All solvents used were purchased from Fisher Scientific (Springfield, NJ).

3.2. Instrumentation

^1H NMR and ^{13}C NMR spectra were obtained on a VXR-200 instrument and recorded in $CDCl_3$ with TMS as internal reference. Signals are reported in ppm (δ). 2D NMR spectra were recorded on a U-500 instrument (Varian Inc., Melbourne, Australia). Methanol-d_4 was used as solvent, and chemical shifts are expressed in parts per million (δ) using TMS as internal standard. GC and GC–MS analysis were performed on a HP 6890 GC, which is equipped with a HP 5973 mass selective detector. Mass spectra were obtained using the electron ionization (EI) mode at 70 eV. The oven temperature was 100°C, raised to 200°C at a rate of 2°C/min, held for 30 min, and finally raised to 280°C at 10°C/min, the injector temperature was 270°C and the detector temperature

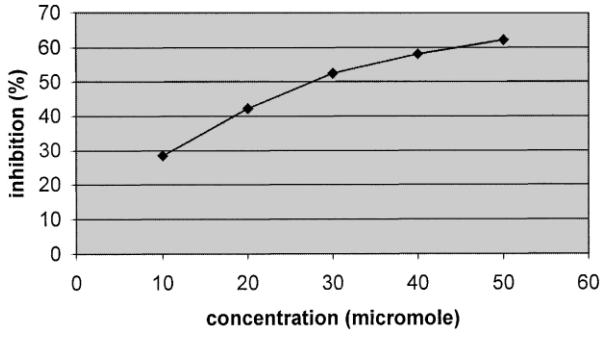


Fig. 5. Scavenging effect of fraction D from gum guggul on DPPH radicals.

was 280°C. The split ratio of sample was set at 60:1. DCI–MS data were obtained on a HP 59980 B instrument.

3.3. Plant material

The exudate of *C. wightii* was obtained from Sabinsa Co. (Piscataway, NJ) and was imported from India. This plant material was identified by Dr. Vladimir Badmaev (Sabinsa Co.).

3.4. Extraction and purification

Guggul gum (2.5 kg) was extracted with ethyl acetate at room temperature for one week. The residue was filtered and the ethyl acetate extracts were combined and concentrated under reduced pressure (850 g). A sample (100 g) was directly subjected to Si gel cc, eluted with a solvent mixture of $CHCl_3$/MeOH with increasing MeOH content and collecting 8 fractions (A).

Cytotoxic fractions A5, A6 and A8 were combined (13.6 g) and subjected to Sephadex LH-20 eluted with 95% aqueous EtOH. Four fractions (B) were collected. Among them, B3 (11 g) showed the highest level of cytotoxicity and was eluted with acetone/hexane (2:3) on the Si gel to give 9 fractions (C1–C9). The active fraction C8 (1.9 g) was then applied to a RP-18 column, using MeOH/H_2O (9:1) as eluent to afford an active fraction D (120 mg).

3.5. NMR data of D

^{1}H NMR δ ppm ($CDCl_3$, 200 MHz): δ 7.65 (1H, *d*, $J=15.7$ Hz, H-3), 7.06 (2H, *m*, H-5, 9), 6.91 (1H, *d*, $J=8.0$ Hz, H-8), 6.31 (1H, *d*, $J=15.7$ Hz, H-2), 5.35 (1H, *m*, H-5′), 5.00 (1H, m, H-6′), 4.37 (2H, *m*, H-1′), 4.27 (1H, *m*, H-2′), 3.93, (3H, *s*, OCH_3-6), 3.78 (1H, *m*, H-4′), 3.47 (1H, *m*, H-3′), 2.01 (2H, *m*, H-7′), 0.88 (3H, *t*, $J=6.9$ Hz, the terminal CH_3). ^{13}C NMR δ ppm ($CDCl_3$, 50 MHz): δ 167.2 (*s*, C-1), 148.2 (*s*, C-7), 146.8 (*s*, C-6), 146.0 (*d*, C-3), 129.9 (*s*, C-5′, 6′), 126.7 (*s*, C-4), 123.4 (*d*, C-9), 114.8 (*d*, C-8), 114.5 (*d*, C-2), 109.5 (*d*, C-5), 73.4 (*d*, C-4′), 72.2 (*d*, C-2′), 72.1 (*d*, C-3′), 66.0 (*t*, C-1′), 55.9 (*q*, OCH_3), 27.7 (*t*, C-7′), 14.1 (*q*, terminal CH_3).

3.6. Hydrolysis of fraction D

The ferulate mixture (2–4 mg) was hydrolyzed in 90% MeOH (1 ml) containing 0.4 M KOH at room temperature for 2 h. The resulting fatty alcohols were extracted with EtOAc, dried and evaporated.

3.7. Trimethylsilylated (TMS) ether derivatives

The hydrolyzed sample was derivatized to form the TMS ethers with bis-(trimethylsilyl)-trifluoroacetamide (200 μl) containing 1% trimethylchlorosilane. The reaction was completed in a Teflon-lined screw cap tube at 75°C for 30 min with a brief N_2 treatment prior to the reaction (Wang, 1998).

3.8. Determination of the position of the double bond

The double bond position was determined using the dimethyl disulfide (DMDS) additive method (Wang, 1998). Briefly, an EtOAc solution (0.1 ml) of TMS ether derivatives was dried with a nitrogen stream, incubated with DMDS (0.2 ml) containing I_2 (15 mg/ml) at 35°C for 30 min. After reduction of excess I_2 with saturated $NaHSO_3$ aqueous solution, EtOAc (0.1 ml) was added into the mixture, and after brief centrifugation, a sample (1 μl) of the upper phase was injected into the GC–MS.

3.9. Activity screening

All the fractions or extracts (20 mg) were dissolved in EtOH (10 ml) to make the concentration 0.2%. Ten microlitres of the solution was added to 10 ml system with cancer cell lines. If cell growth was 100% inhibited, the concentration of this fraction was diluted to 0.0002% for 100% inhibition. If they did, these fractions were considered as active enough for further purification.

3.10. Cytotoxicity analysis

PC-3 prostate tumor cells and MCF-7 breast tumor cells were obtained from the #18 American Type Culture Collection (ATCC, Rockville, MD). P388 mouse leukemia cells and P388 cells transformed with pHaMDR1/A retrovirus carrying an MDR1 cDNA were a gift from Dr. W. Hait (Yang et al., 1994). Cells were maintained at 37°C in an atmosphere of 5% CO_2 and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 units penicillin and 50 μg/ml streptomycin. Cells were routinely checked and found to be free of contamination by mycoplasma. Cell viability was assessed by the tetrazolium dye method (MTT) as previously described (Scudiero et al., 1988). Cells were plated in 96 well plates and incubated with various agents for 72 h. Absorbance was measured at 570 nm using a Dynatech micro plate reader.

3.11. Determination of the scavenging effect on DPPH radicals

This method was adapted from that of Chen and Ho (1995). Basically, the tested fraction (final concentrations were 10, 20, 30, 40, 50 μM, respectively), was added individually to an EtOH solution (2.5 ml) of DPPH radical (final concentration was 1.0×10^{-4} M). The mixtures were shaken vigorously and left to stand in the dark for 30 min. Thereafter, the absorbancy for the sample was measured using a spectrophotometer

(Milton Roy, Model 301) at 517 nm against blank samples without DPPH. The control sample was the DPPH solution without adding the tested sample. Each sample was duplicated in the test, and the values were averaged. The IC_{50} was obtained by extrapolation from linear regression analysis (Fig. 5).

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Inhibitors of platelet lipoxygenase from Ponkan fruit

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Abstract

An activity-guided separation for inhibitors of rat platelet 12-lipoxygenase led to the isolation of two compounds, 4-*O*-feruloyl-5-*O*-caffeoylquinic acid (IC_{50}; 5.5 μM) and methyl 4-*O*-feruloyl-5-*O*-caffeoylquinate (IC_{50}; 1.9 μM) from the peel of Ponkan fruit (*Citrus reticulata*). The complete structure of each phenolic ester was determined by NMR spectroscopy [^1H and ^{13}C NMR spectra, $^1H-^1H$ correlation spectroscopy (COSY), ^1H-detected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) spectroscopies] and other spectral methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Citrus reticulata*; Rutaceae; Ponkan; Arachidonic acid; 12-Lipoxygenase; 4-*O*-Feruloyl-5-*O*-caffeoylquinic acid; Methyl 4-*O*-feruloyl- 5-*O*-caffeoylquinate

1. Introduction

It is well known that 12-lipoxygenase (12-LO) catalyzes the initial reaction that leads to the formation of 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE) from arachidonic acid (Hamberg and Samuelsson, 1974). 12-LO metabolites are involved in atherosclerotic processes (Nakao et al., 1982), in tumor metastasis (Tang and Honn, 1994), and in activating 5-lipoxygenase (5-LO) (Yoshimoto et al., 1983; Rouzer and Samuelsson, 1986). The products of the latter reaction mediate allergic and inflammatory responses (Ford-Hutchinson et al., 1980; Morris et al., 1980). Therefore, specific inhibitors of 12-LO have the potential to prevent these diseases.

Our interest in studying 12-LO inhibitors is to chemically characterize such compounds in citrus fruit for the purpose of generating an increased commercial demand for them. Citrus plants have many kinds of bioactive compounds such as flavonoids (Horowitz and Gentili, 1977), phenylpropanoids (Fisher and Trama, 1979; Rapisarda et al., 1998), limonoids (Maier et al., 1977), carotenoids (Stewart, 1980), and so on. In terms of lipoxygenase inhibition, quercetin and certain major flavonoids were reported to be effective inhibitors of 5- and 12-LO (Hope et al., 1983; Welton et al., 1986). Flavonoids and phenylpropanoids are found in higher concentrations in the albedo, immediately under the flavedo which is the colored portion of the peel, than in either the juice vesicle or the flavedo (Nogata et al., 1994). However, most of the peel (albedo and flavedo) and rag are dried and used for animal feed after processing. Consequently, in order to expand production and consumption there is a need to develop an effective method of exploiting citrus by-products as well as understanding the bioactive compounds in citrus fruit.

We have previously reported screening for inhibitory activities of the albedo extracts of 42 species and cultivars of genus *Citrus* and those of two *Fortunella* and one *Poncirus* species against 12-LO (Nogata et al., 1996). Among the samples investigated, the extract of Ponkan fruit was shown to be one of the most effective sources of lipoxygenase inhibitors. We report herein the activity-guided isolation of two previously unreported compounds having inhibitory activities against 12-LO from the peel of Ponkan fruit and determination of their structures.

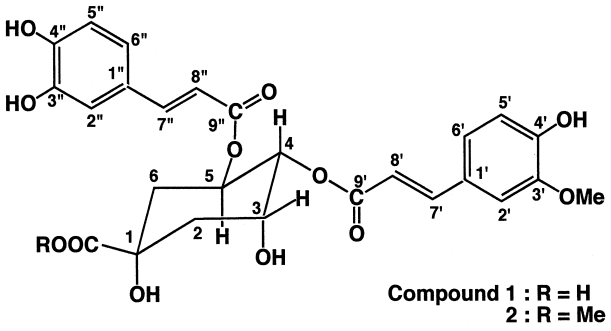
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2. Results and discussion

Dried and finely ground tissue of the Ponkan fruit was successively extracted with *n*-hexane, chloroform, ethanol and methanol. The inhibitory activity toward 12-LO was 0% for the *n*-hexane and chloroform extracts at a concentration of 100 μg/ml, and 51 and 37% for the ethanol and methanol extracts at 10 μg/ml, respectively. The ethanolic extract was subjected to Amberlite XAD-2 and Sephadex LH-20 column chromatography. The Amberlite XAD-2 and Sephadex LH-20 fraction inhibited 12-LO activity by 63 and 68%, respectively, at 10 μg/ml. Further purification of the active fraction was carried out using preparative HPLC employing linear gradient elution to give five fractions (Fr. 1–5). Inhibitory activities of Fr. 1–5 at 10 μg/ml were 44, 48, 37, 55 and 38%, respectively. Fr. 4 was subjected to HPLC with isocratic elution, and pure active compounds (**1**) and (**2**) were obtained.



Each proton and carbon signal of compound (**2**) was assigned by ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) spectroscopy (Table 1). The ^{1}H and ^{13}C NMR spectra showed that (**2**) consisted of a quinic acid moiety, a feruloyl group, and a caffeoyl group in a molar ratio of 1:1:1. The signals of the protons of H_3, H_4 and H_5 of the quinic acid moiety shifted downfield by 0.27, 1.74 and 1.56 ppm, respectively, in comparison to those of free quinic acid. When a caffeoyl group is bound to a hydroxyl group of quinic acid, the chemical shift of a proton at that position shifts 1.1–1.7 ppm downfield (Murata et al., 1995). In addition, H_3, H_4 and H_5 signals of 4,5-*O*-dicaffeoylquinic acid were reported to shift downfield by 0.34, 1.68 and 1.53 ppm (Morishita, 1984). HMBC correlations of H4 and H5 of the quinic acid moiety to carbonyl carbons of $C_{9'}$ and $C_{9''}$ indicated that the feruloyl and caffeoyl groups were connected to C_4–OH and C_5–OH, respectively. HMBC correlations of methyl protons (3.73 ppm) to the carbonyl carbon of the quinic acid moiety indicated the presence of a methyl ester group. Based on the above results and ESI-TOF-MS data, the structure of (**2**) was determined as methyl 4-*O*-feruloyl-5-*O*-caffeoylquinate. The ^{1}H and ^{13}C NMR spectral data for compound (**1**) were identical to those of (**2**) except for the methyl ester signal (3.73 ppm). Thus, the structure of (**1**) was identified to be 4-*O*-feruloyl-5-*O*-caffeoylquinic acid.

Table 1
^{1}H and ^{13}C NMR spectral data for (**2**) (in CD_3OD)

^{13}C NMR	^{1}H NMR	H coupled with C[a]
Quinic acid moiety		
1 81.1		
2 38.7	2.10,2.33 (each 1H, *m*)	
3 69.2	4.36 (1H, *m*)	H_{2eq}, H_{2ax}, H_5
4 75.0	5.13 (1H, *m*)	H_{2eq}, H_{2ax}, H_4, H_5, H_6
5 75.9	5.55 (1H, *m*)	H_6
6 38.4	2.25 (2H, *m*)	
CO 175.3		H_{2eq}, H_6, $COOCH_3$
CH_3 53.2	3.73 (3H, *s*)	
4-Feruloyl group		
1′ 127.8		$H_{5'}$,$H_{7'}$, $H_{8'}$
2′ 111.8	7.14 (1H, *d*, 1.9 Hz)	$H_{6'}$, $H_{7'}$
3′ 149.4		OCH_3, $H_{5'}$
4′ 150.8		$H_{2'}$, $H_{5'}$, $H_{6'}$
5′ 116.6	6.79 (1H, *d*, 8.2 Hz)	
6′ 124.3	7.05 (1H, *dd*, 8.1, 1.8 Hz)	$H_{2'}$, $H_{7'}$
7′ 147.7	7.66 (1H, *d*, 15.9 Hz)	$H_{2'}$, $H_{6'}$
8′ 115.2	6.39 (1H, *d*, 15.8 Hz)	$H_{7'}$
9′ 168.5		H_4, $H_{7'}$, $H_{8'}$
CH_3 56.5	3.86 (3H, *s*)	
5 Caffeoyl group		
1″ 127.6		$H_{8''}$, $H_{7''}$, $H_{5''}$
2″ 115.2	7.01 (1H, *d*, 1.9 Hz)	$H_{6''}$, $H_{7''}$
3″ 146.9		$H_{5''}$
4″ 149.8		$H_{2''}$, $H_{5''}$, $H_{6''}$
5″ 116.5	6.76 (1H, *d*, 8.3 Hz)	$H_{6''}$
6″ 123.1	6.91 (1H, *dd*, 8.2, 1.9 Hz)	$H_{5''}$, $H_{2''}$, $H_{7''}$
7″ 147.7	7.51 (1H, *d*, 15.9 Hz)	$H_{2''}$, $H_{6''}$
8″ 114.6	6.17 (1H, *d*, 15.9 Hz)	
9″ 168.0		H_5, $H_{7''}$, $H_{8''}$

[a] In HMBC spectrum.

Since caffeic acid is widely known to inhibit 12-LO (Kohyama et al., 1997), we tested its effect as a positive control to assess the relative efficacy of (**1**) and (**2**) (Table 2). Both (**1**) and (**2**) inhibited 12-LO more efficiently than caffeic acid. (**1**) was found to be slightly less potent than (**2**). This result agrees with previous studies where active compounds with a carboxyl group showed

Table 2
Effects of isolated compounds, caffeic acid, ferulic acid and 3-*O*-caffeoylquinic acid on 12-LO activity[a]

	μM[c]			
	100	10	1	0.1
(**1**)	72	56	29	0
(**2**)	74	59	42	6
Caffeic acid	69	52	21	0
Ferulic acid	n.i.[b]			
3-*O*-Caffeoylquinic acid	n.i.			

[a] Inhibition (%) relative to the control value.
[b] No inhibition under 100 μM.
[c] Each value is the mean of five replications.

a less inhibitory effect on 5- and 12-LO than their methyl ester derivatives (Koshihara et al., 1984; Nishizawa et al., 1988; Yamamoto et al., 1998). 3-*O*-Caffeoylquinic acid showed no activity toward 12-LO (Table 2) which is contradictory to the suggestion that the two adjacent hydroxyl groups on an aromatic ring enhances 12-LO inhibitory activity (Welton et al., 1986; Kohyama et al., 1997). Nishizawa et al. (1988) reported that 4,5-*O*-dicaffeoylquinic acid and 4-*O*-sinapoyl-5-*O*-caffeoylquinic acid were much more potent inhibitors than chlorogenic acid against 5-LO. These results suggest that plural esterifications of hydroxycinnamic acid with quinic acid enhance an inhibitory effect against lipoxygenase. Further studies are necessary to elucidate structure–activity relationships of hydroxycinnamoylquinic acid derivatives against 12-LO.

3. Experimental

3.1. General methods

Optical rotations were determined on a JASCO (Tokyo, Japan) DIP-360 polarimeter. The MS of the compounds were obtained on a Mariner Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA, USA) by electrospray ionization time-of-flight mass spectrometry. IR spectra were recorded on a Nicolet (Madison, WI, USA) system 550 spectrometer in KBr disks. NMR spectra were obtained on JEOL (Akishima, Japan) Lambda 500 spectrometers operating at 500.00 MHz for ^{1}H and 125.65 MHz for ^{13}C at 25°C. Chemical shift values were expressed in ppm downfield from tetramethylsilane (TMS) as an internal standard and coupling constants were expressed in Hz. Samples were dissolved in CD_3OD (99.8% D, Aldrich Chemical Company, Inc.) in a 5 mm ϕ sample tube. HPLC separations were performed with SHIMADZU (Kyoto, Japan) LC6AD pumps equipped with a SHIMADZU SPD-M10AVP diode array detector.

3.2. Plant material and chemicals

Ripe fruit of Ponkan was collected at the Okitsu Branch, National Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries. [1-^{14}C]Arachidonic acid and [^{3}H]TXB_2 were obtained from New England Nuclear (Boston, MA, USA). Arachidonic acid, caffeic acid and chlorogenic acid were from Sigma Chemical Co. (St Louis, MO, USA). 3-*O*-Caffeoylquinic acid was a gift from Dr. Ogawa, Japan International Research Center for Agricultural Sciences, Okinawa Subtropical Stations.

3.3. Assay of 12-LO activities

Rat platelets were prepared from the blood of Wister-King rats (200–300 g), which were fed normally, and assay reaction conditions were as described previously (Nogata et al., 1996). Briefly, test compounds were incubated with the sonicated platelets (2 mg protein/ml) for 5 min at 37°C before addition of [1-^{14}C]arachidonic acid (4 mM). The reaction was terminated by adding 0.5 N formic acid after incubation for 5 min at 37°C. After the products were separated by TLC, the radioactivities of 12-HETE was measured using a Fujix BAS 1000 (Fuji Photo Film, Tokyo, Japan) laser imaging system. The amount of 12-HETE produced in the reaction without test compound was designated the control value corresponding to 100%. The reaction performed in the presence of formic acid was designated as 0% production of 12-HETE.

3.4. Extraction and isolation

Lyophilized and powdered portions (550 g) of Ponkan peel were successively extracted with 2 l each of *n*-hexane, chloroform, ethanol and methanol in a Soxhlet apparatus. The ethanolic extract (112 g) was conc. under reduced pressure, applied to a column of Amberlite XAD-2 (2 kg) and eluted with increasing concentrations of methanol (in increments of 20%) in water, and finally with acetone. The combined fractions of 60 and 80% aqueous methanol (3.3 g) were conc., and passed through a Sephadex LH-20 column (1 l) equilibrated with methanol and eluted with the same solvent. For further purification, the active fraction (1.0 g) was purified by prep. HPLC on a YMC-Pak ODS-AL (20ϕ×250 mm, YMC Co., Kyoto, Japan) column at a flow rate of 5 ml min^{-1}, while monitoring from 220 to 360 nm with a photodiode array detector. The following solvent systems were used for elution: a linear gradient from 40 to 80% methanol containing 0.1% TFA in H_2O (elution for 60 min) to give five fractions, and an isocratic solvent system of 45% methanol aqueous solution containing 0.1% TFA to give (**1**) (2.16 mg, t_R 62.3 min) and (**2**) (3.11 mg, t_R 72.0 min) from Fr. 4 (12.45 mg).

3.5. 4-O-Feruloyl-5-O-caffeoylquinic acid (*1*)

Yellow amorphous solid, $[\alpha]_D^{29}$ −24.7° (*c* 0.1, MeOH); UV (ethanol) nm: λ_{max} 328, 233, 216, 206; high-resolution ESI-TOF-MS calcd for $C_{26}H_{25}O_{12}$ ([M-H]$^-$) 529.1346 found 529.1332; IR (υ_{max}) cm^{-1}: 3372, 1698, 1631, 1600, 1518, 1456, 1435, 1379, 1276, 1188, 1162, 1131, 987, 817; ^{1}H NMR (CD_3OD): δ 2.13 and 2.32 (H_{2eq} and H_{2ax}, each 1H, *m*), 2.29 (H_6, 2H, *m*), 3.84 (OCH_3, 3H, *s*), 4.39 (H_3, 1H, *m*), 5.14 (H_4, 1H, *m*), 5.64 (H_5, 1H, *m*), 6.19 ($H_{8''}$, 1H, *d*, 15.9), 6.38 ($H_{8'}$, 1H, *d*, 15.8), 6.74 ($H_{5''}$, 1H, *d*, 8.3), 6.78 ($H_{5'}$, 1H, *d*, 8.2), 6.90 ($H_{6''}$, 1H, *dd*, 8.2, 1.8), 7.00 ($H_{2''}$, 1H, *d*, 1.8), 7.04 ($H_{6'}$, 1H, *dd*, 8.2, 1.8), 7.12 ($H_{2'}$, 1H, *dd*, 1.8), 7.52 ($H_{7''}$, 1H, *d*, 15.9), 7.65 ($H_{7'}$, 1H, *d*, 15.8); ^{13}C NMR (CD_3OD):δ 38.4 (C_6), 39.4 (C_2), 56.5 (OCH_3), 69.1 (C_3), 75.8 (C_4),

76.1 (C_5), 81.1 (C_1), 111.8 ($C_{2'}$), 114.8 ($C_{8''}$), 115.2 ($C_{2''}$), 115.3 ($C_{8'}$), 116.5 ($C_{5''}$), 116.8 ($C_{5'}$), 123.1 ($C_{6''}$), 124.2 ($C_{6'}$), 127.7 ($C_{1'}$), 127.7 ($C_{1''}$), 146.8 ($C_{3''}$), 147.6 ($C_{7'}$), 147.6 ($C_{7''}$), 149.4 ($C_{3'}$), 149.7 ($C_{4''}$), 150.7 ($C_{4'}$), 168.3 ($C_{9''}$), 168.6 ($C_{9'}$), 176.8 (CO); ^1H–^1H COSY correlations (CD3OD) **2**: H_{2eq}/H_{2ax}, H_{2eq}/H_3, $H_6/H_{5'}$ $H_{2ax}/H_{3'}$ $H_4/H_{3'}$ $H_4/H_{5'}$ $H_{8''}/H_{7''}$, $H_{8'}/H_{7'}$, $H_{5''}/H_{6''}$ $H_{5'}/H_{6'}$, $H_{6''}/H_{2''}$, $H_{6'}/H_{2'}$

3.6. Methyl 4-O-feruloyl-5-O-caffeoylquinate (*2*)

Colorless amorphous solid, $[\alpha]_D^{29}$ −171.0° (*c* 0.1, MeOH); UV (ethanol) nm: λ_{max} 328, 236, 219; high-resolution ESI-TOFF-MS calcd for $C_{27}H_{27}O_{12}$ ($[M\text{-}H]^-$) 543.1502 found 543.1489; IR (ν_{max}) cm^{-1}: 3367, 1693, 1631, 1605, 1522, 1456, 1435, 1379, 1276, 1183, 1162, 1131, 987, 817; ^1H and ^{13}C NMR spectral data (CD_3OD); see Table 2.

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Soybean ethanol extract increases the function of osteoblastic MC3T3-E1 cells

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Abstract

To investigate the bioactivities of soybean, which act on bone metabolism, we studied the effect of a soybean ethanol extract on the activity of osteoblast MC3T3-E1 cells. Soy extract (0.01–0.1 g/l) dose-dependently increased survival ($P < 0.05$) and DNA synthesis ($P < 0.05$) of MC3T3-E1 cells. In addition, soy extract (0.05 g/l) increased alkaline phosphatase activity ($P < 0.05$) and collagen synthesis ($P < 0.05$) of MC3T3-E1 cells. Moreover, the anti-estrogen tamoxifen eliminated the stimulation of MC3T3-E1 cells on the proliferation, ALP activity and collagen synthesis by soy extract, indicating that the main action of the soy extract on osteoblastic MC3T3-E1 cells is similar to that of estrogen effects. Treatment with soy extract prevented apoptosis, as assessed by a one-step sandwich immunoassay and DNA gel electrophoresis studies. This effect may be associated with the activation of the estrogen receptor, since we observed soy extract-mediated survival against apoptosis was blocked by the estrogen receptor antagonist tamoxifen in cells, further supporting a receptor-mediated mechanism of cell survival. These results suggest that osteoblast function is promoted by soy extract and that the estrogen receptor is involved in the response, thereby playing an important role in bone remodeling. In conclusion, soy extract has a direct stimulatory effect on bone formation in cultured osteoblastic cell in vitro. Presumably, dietary soy products are useful in the prevention of osteoporosis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Glycine max; Leguminosae; Soybeans; Osteoblast; Proliferation; Differentiation and apoptosis

1. Introduction

Estrogen deficiency induces rapid bone loss during the first decade after menopause (Genant et al., 1989). Estrogen replacement therapy is indeed effective in preventing bone loss caused by menopause, but it is accompanied by some adverse effects, such as uterine bleeding and carcinogenesis (Black et al., 1994, Genant et al., 1989). Recent studies have focused on the development of estrogen-related compounds that selectively affect bone metabolism without exhibiting estrogenic action in the uterus (Ishimi et al., 1999). The phytoestrogens, therefore, are potentially important in the prevention of postmenopausal osteoporosis caused by estrogen deficiency.

Epidemiological studies suggest that the low incidence of osteoporosis and heart diseases caused by estrogen deficiency in Asian women is attributable to their high intake of soy foods, compared with American and Finnish women (Adlercreutz et al., 1992; Brandi, 1997; Anderson et al., 1995). It has been reported that dietary soybean proteins prevent bone loss in ovariectomized (OVX) rats (Arjmandi et al., 1996). Possible candidates for the beneficial substances present in soybeans are isoflavones, such as genistein and daidzein, because these isoflavones, show a structural similarity to estradiol (E_2) and are known as phytoestrogens (Genant et al., 1989). Although these isoflavones have been

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reported to bind to estrogen receptors and protect cell growth in breast cancer cells (Santell et al., 1997; Zava et al., 1997; Barnes, 1995; Alercreutz et al., 1995), it is less known whether these isoflavones affect bone metabolism. In addition to isoflavone effects, soy protein appears to be less hypercalciuric than animal protein and soybean in itself contains many other phytochemicals (Akiyama et al., 1987). Each of the other phytochemicals in soy have been suggested to cause beneficial effects on the risk of chronic diseases. An increasing amount of evidence suggests that soyfoods may help to reduce risk of osteoporosis.

Programmed cell death (PCD), or apoptosis, is an important determinant of the life span of cells in regenerating tissues (Stella, 1995; Polunovsky et al., 1993). This process was recently demonstrated in osteoclasts (Hughes et al., 1995; Lutton et al., 1996), and, in concert with the rate of osteoclast development, is thought to play a role in the pathophysiology of postmenopausal osteoporosis (Hughes et al., 1996). It was recently shown that osteoblastic cell lines undergo apoptosis in vitro (Hill et al., 1997; Kitajima et al., 1996) and that estrogen interferes with the apoptotic program of diverse cell systems (Holly and Wass, 1989; Centrella et al., 1992; McCarthy et al., 1989).

However, the phytochemicals that act on bone metabolism as nutrients in soybean are poorly understood. Considering the wide use of phytoestrogen in the prevention of osteoporosis, we investigated whether isoflavone rich soy phytochemical extract could enhance the function and hinder the induction of the apoptosis in the osteoblastic MC3T3-E1 cells.

2. Results

2.1. Effect of soy extract on the growth of MC3T3-E1 cells

Cell metabolic activity was greater in soy extract treatment group than in the control group (Fig. 1). The formation of MTT formazan was significantly increased dose-dependently up to 117% of the basal value when cells were treated with soy extract ($P < 0.05$).

2.2. Effect of soy extract on the DNA synthesis of MC3T3-E1 cells

Treatment with 0.05 and 0.1 g/l soy extract significantly ($P < 0.05$) stimulated MC3T3-E1 cell DNA synthesis by up to 158% of basal values (Fig. 2), consistent with the results of experiments shown in Fig. 1. The soy extract-induced increases in DNA synthesis were significantly eliminated by the presence of 10^{-7} mol/l tamoxifen, reflecting the estrogen effect of soy extract (Fig. 3).

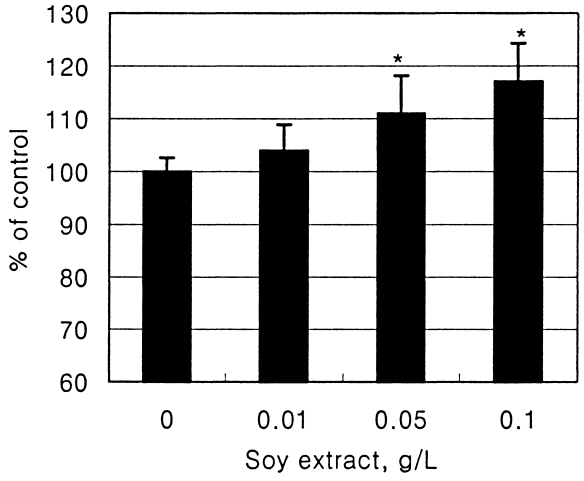


Fig. 1. Effect of soy extract on the growth of MC3T3-E1 cells by the MTT assay. MC3T3-E1 cells were cultured with vehicle or various concentrations of soy extract for 48 h. Data shown are mean±S.E.M. of the results of six cultures, expressed as a percentage of control. The control value for MTT assay was 0.753±0.008 O.D. * $P < 0.05$ compared with control.

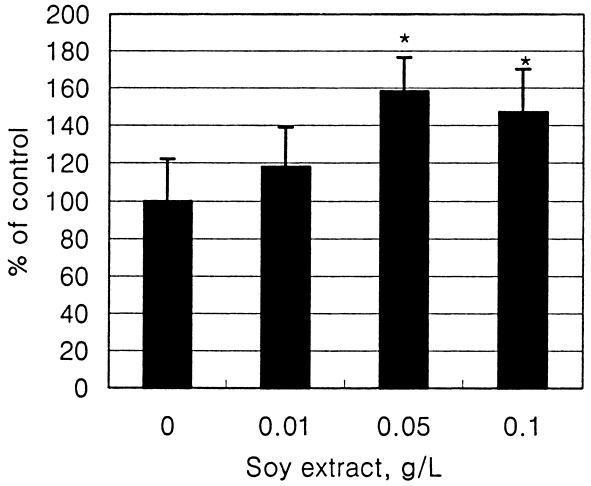


Fig. 2. Effect of soy extract on the DNA synthesis of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or various concentrations of soy extract for 48 h. Data shown are mean±S.E.M. of the results of six cultures, expressed as a percentage of control. The control value for [^{3}H]-thymidine incorporation was 3103.00±283.79 cpm. * $P < 0.05$ compared with control.

2.3. Effect of soy extract on the ALP activity of MC3T3-E1 cells

Soy extract significantly increased the ALP activity of MC3T3-E1 cells to 380% of the control activity at 0.05 g/l (Fig. 4). The soy extract-induced increases in ALP activity were clearly eliminated by the presence of 10^{-7}mol/l tamoxifen ($P < 0.05$).

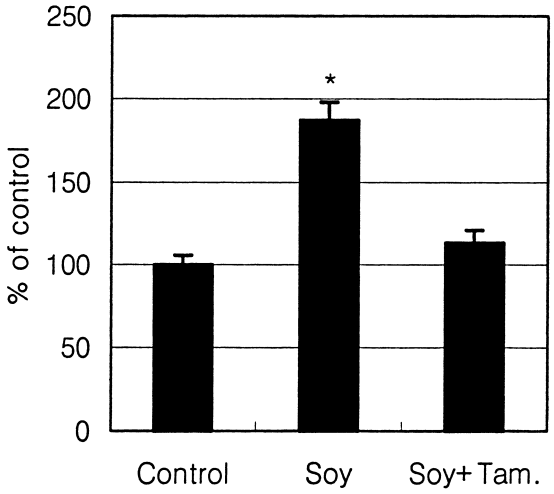


Fig. 3. Effect of tamoxifen on the proliferation of MC3T3-E1 cells in the presence of soy extract. MC3T3-E1 cells were cultured with vehicle or samples (0.05 g/l soy extract, 10^{-7} M tamoxifen) for 48 h. Data shown are mean±S.E.M. of the results of six cultures, expressed as a percentage of control. The control value for [^{3}H]-thymidine incorporation was 2607.2±148.9 cpm. *$P<0.05$ compared with control.

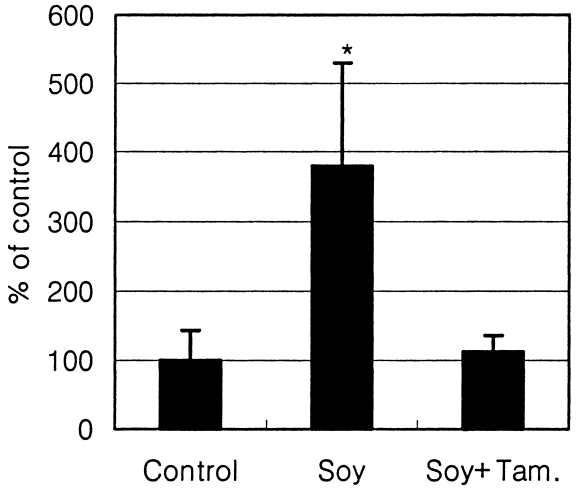


Fig. 4. Effect of soy extract on the alkaline phosphatase activity of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or samples (0.05 g/l soy extract, 10^{-7} M tamoxifen) for 48 h. Data shown are mean±S.E.M. of the results of six cultures, expressed as a percentage of control. The control value for ALP activity was $(0.51\pm0.07)\times10^3$ *p*-nitrophenyl phosphate nmol substrate cleaved per hour per 10^4 cells. *$P<0.05$ compared with control.

2.4. Effect of soy extract on the synthesis of collagenase-digestible protein (CDP) of MC3T3-E1 cells

Treatment with soy extracts at a concentration of 0.05 g/l for 48 h significantly stimulated the rate of collagen synthesis in MC3T3-E1 cells to 481% of basal values (Fig. 5, $P<0.05$). The soy extract-induced increases in the CDP synthesis were clearly eliminated by the presence of 10^{-7} mol/l tamoxifen.

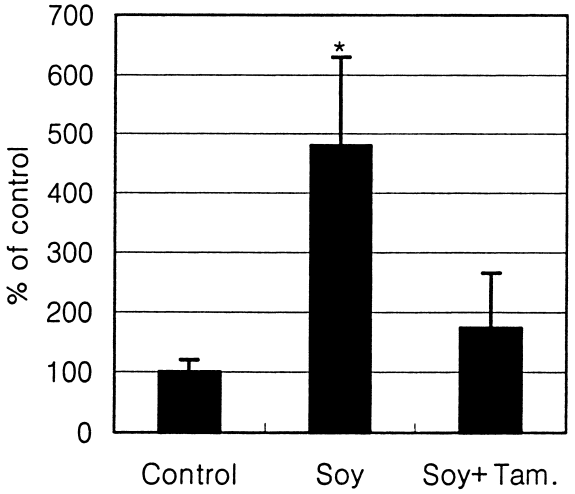


Fig. 5. Effect of soy extract on the collagen synthesis of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or samples (0.05 g/l soy extract, 10^{-7} M tamoxifen) for 48 h. Data shown are mean ±S.E.M. of the results of six cultures, expressed as a percentage of control. The control value for percentage of collagen synthesized was 1.20±0.11%. *$P<0.05$ compared with control.

2.5. Effect of soy extract for osteoblastic MC3T3-E1 cell apoptosis

DNA extracted from the serum-free culture showed extensive nuclear fragmentation, with DNA being degraded to form 200-bp ladders (Fig. 6, lane 5) whereas less degradation was observed when the cells were treated with 0.05 g/l soy extract (Fig. 6, lane 3). The soy extract-induced suppression in the osteoblast apoptosis was partially inhibited by the presence of 10^{-7} mol/l tamoxifen (Fig. 6, lane 4). No degradation was observed when the cells were cultured in 10% serum (Fig. 6, lane 2). We quantitated the apoptosis using a one-step sandwich immunoassay. MC3T3-E1 cells cultured for 48h in the absence of serum exhibited higher apoptotic index than those in the presence of 10% FBS. Addition of 0.05 g/l soy extract to MC3T3-E1 cells inhibited apoptosis stimulated by the removal of serum and the soy extract-induced suppression in the osteoblast apoptosis was blocked by the presence of 10^{-7} mol/l tamoxifen (Fig. 7).

3. Discussion

Soybeans are a rich source of phytochemicals. Many of these compounds have important beneficial effects on human and animal health. They are found in soybeans at much higher concentrations than in other common foods and are considered to be important in preventing chronic disease (Barnes et al., 1998). Possible candidates for the beneficial substances present in soybeans are isoflavones, such as genistein and daidzein. They have

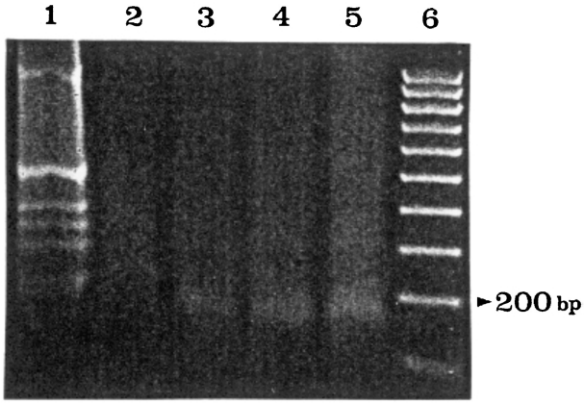


Fig. 6. Effect of soy extract for apoptosis in MC3T3-E1 osteoblastic cells detected by DNA fragmentation. MC3T3-E1 cells were cultured at a density of 10^6 cells/100 mm tissue culture dishes in the presence of 10% FBS in α-MEM. The 1 kb and 100 bp standard (lanes 1 and 6) were included as a reference. Forty-eight hours after initiation of the culture, cell monolayers were rinsed three times with serum free α-MEM and subsequently maintained for an additional 48 h in the presence of culture medium containing 10% FBS (lane 2), or 0.3% BSA (lane 5) containing 0.05 g/l soy extract (lane 3), 0.05 g/l soy extract $+10^{-7}$ mol tamoxifen (lane 4). DNA was extracted and 5–10 μg was analyzed by electrophoresis on a 1.5% agarose gel. DNA was visualized with ethidium bromide.

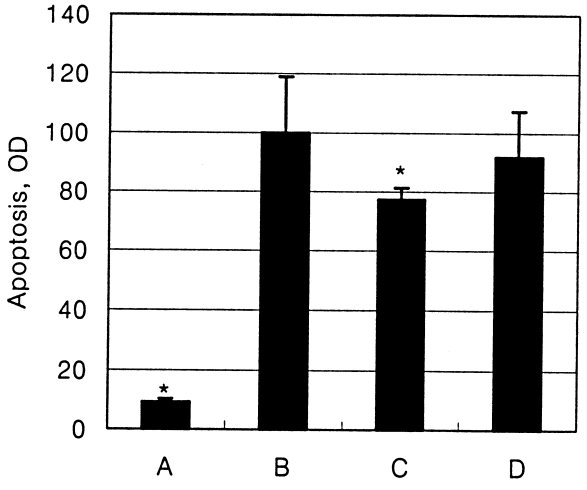


Fig. 7. Effect of soy extract on MC3T3-E1 cell apoptosis by one-step sandwich immunoassay. MC3T3-E1 cells were cultured at a density of 2×10^4 cells/well of a 24-well plate in the presence of 10% FBS in α-MEM. Forty-eight hours after initiation of the culture, cell monolayers were rinsed three times with serum free α-MEM and subsequently maintained for an additional 48 h in the presence of culture medium containing 10% FBS (A), 0.3% BSA (B) or 0.3% BSA containing 0.05 g/l soy extract (C), 0.05 g/l soy extract $+10^{-7}$ mol/l tamoxifen (D). Data shown are mean±S.E.M. of the results of six cultures. $^*P<0.05$ compared with control.

been identified as having estrogenic activity. Hence they are also commonly referred to as phytoestrogens (Barnes et al., 1998). The similarity in action between phytoestrogen and estradiol prompted us to investigate whether soybean phytochemicals might exhibit estrogenic activity in cell culture system.

The possibility that phytoestrogens may offer a natural alternative to conventional hormone replacement therapy for the prevention of bone loss due to estrogen deficiency during the menopause has led to a number of animal and clinical investigations of the effects of either soy protein or genistein on bone remodeling (Setchell and Cassidy, 1999; Anderson et al., 1995). The bone-conserving effects of soy protein containing isoflavones were also confirmed in separate studies using the ovariectomized adult rats (Arjmandi et al., 1996).

It would appear that the action of nonsteroidal phytoestrogens on bone remodeling differs from that of classical estrogens (Blair et al., 1996). The recent identification of the ERβ in bone (Kuiper et al., 1998), coupled with its ligand specificity toward phytoestrogens (Kuiper et al., 1997), may be of relevance in explaining the effects of isoflavones in this tissue. Alternatively, these effects need not be restricted to direct hormonal actions. Growth factors and cytokines play a role in regulating osteoclast activity (Manolagas and Jilka, 1995), and several of these are tyrosine kinase-mediated pathways that could conceivably be influenced by genistein. More recently it has been suggested that genistein might stimulate the production of TGFβ (Transforming growth factor β) in osteoclasts (Kim et al., 1998).

Demonstrating effects on bone density requires long-term studies, and compliance to soy foods is a major problem that must be addressed in the design of human studies. Potter et al., (1998) found significant increases in bone mineral content in postmenopausal women consuming isolated soy protein over a 6-month period compared with a diet containing casein. In a short-term study, Pansini et al. (1997) examined biochemical markers of bone turnover in postmenopausal women and showed a reduction in the urinary excretion of bone resorption markers after a diet of isolated soy protein. The incidence of osteoporosis-related fractures is lower in Asia than in most Western communities (Ho, 1996; Tobais et al., 1994), but it is difficult to discern whether this difference can be accounted for by the intake of isoflavones from soy foods, particularly because there are many other factors that can account for these epidemiologic findings. Isoflavones may to some degree inhibit osteoporosis for their estrogenic activity, but alone they may be insufficient for complete protection. In these experiments it has not been possible to separate the phytoestrogen effect from the effect of other components in the soybeans. Therefore, in this study we investigated the effect of soy phytochemical extract but not isoflavones on bone metabolism, using cell culture system.

Soy extract dose-dependently increased ALP activity and DNA synthesis. Therefore, soy extract seems to stimulate the proliferation and differentiation of osteoblasts.

In addition, soy extract increased collagen synthesis activity. These results also support the stimulatory nature of soy extract toward the function of osteoblastic cells. On the other hand, the stimulatory effects of soy extract on DNA synthesis, ALP activity and collagen synthesis were clearly blocked by the presence of 10^{-7} mol/l tamoxifen, an anti-estrogen reagent. Treatment of tamoxifen alone did not have appreciable effect (data not shown). Consistent with our finding, Sugimoto and Yamaguchi (2000) reported that daidzein-increased ALP activity in osteoblastic MC3T3-E1 cells was abolished completely by the presence of tamoxifen (10^{-7} or 10^{-6} mol/l).

These results, therefore, suggest that the effect of soy extract to increase bone formation is mediated through an estrogen-like action. This observation is in agreement with the findings of a previous study suggesting that the inhibitory effects of genistein, a soybean isoflavone, on bone resorption is mediated through an estrogen-like action (Morita et al., 1989).

Regulation of apoptosis is considered an important mechanism for controlling the number of cells available. Failure in the control of programmed cell death may become particularly relevant in pathological conditions such as degenerative diseases, where apoptosis has been identified as a key factor in disease progression or remission (Deshpande, et al. 1997; Thatte and Dahanukar, 1997; Loosdrecht et al., 1993; Mountz et al., 1994). It has been shown that estrogen promotes apoptosis in osteoclasts (Hughes et al., 1996), suggesting the development of phytoestrogens to prevent postmenopausal osteoporosis. Recent studies have demonstrated a hormonal regulation of the apoptotic program; the opposite results, reported by Hughes et al. (1996) and Spyridopoulos et al. (1997), have been observed in different cell types; steroid hormones have been frequently associated with a differential activity depending on the cell type or on the cell differentiation state. Although the molecular mechanism for this cell type- and gene-specific activity has not yet been fully clarified, one could speculate that the differential tissue distribution of the receptor cofactors, recently identified proteins that interact with steroid receptors to modulate gene transcription (Glass et al., 1997), could account for the cell-specific response to steroid hormones. In the present study, we have used the osteoblastic MC3T3-E1 cell line, as a model system to investigate whether soy phytochemical extract played a role in the manifestation of apoptosis. Our results suggest that soy extract, by interacting with estrogen receptor, prevent the onset of apoptosis. Prevention of apoptosis of osteoblast mediated by soy extract might have important consequences in the clinical use of soybeans.

We showed in the present study that soy phytochemicals has a direct stimulatory effect on bone formation in cultured MC3T3-E1 osteoblast cells in vitro. Soybean may be a useful tool in the prevention of and therapy for osteoporosis.

4. Experimental

4.1. Soy phytochemical extracts

Soy flour containing 1% isoflavones was prepared at Dr. Chung's Food Co., (Seoul, South Korea). Whole soybeans were cracked, dehulled, and flaked by standard procedures, followed by a hexane extraction to remove the majority of lipid. The defatted soy flour was extracted with aqueous ethanol (80%) and the resulting product containing carbohydrates (60–70%), fat (12%), protein (5%), ash (4%), and isoflavone (2%) was designated as soy extract.

4.2. Cell culture

Osteoblastic MC3T3-E1 cells were cultured in plastic dishes containing alpha-minimum essential medium (α-MEM, Sigma, St Louis, MO, USA) plus 10% fetal bovine serum (FBS, Sigma, St Louis, MO, USA) in a CO_2 incubator (5% CO_2 in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}, Mg^{2+} free phosphate-buffered saline (DPBS). The cells were seeded in 24-well plate (2×10^4), 6-well plate (1×10^5) or a 100-mm diameter culture dish (1×10^6) containing α-MEM supplemented with 10% FBS, cultured for 2 days to become nearly confluent. Then cells were transferred to fresh medium containing 0.3% bovine serum albumin (BSA, Sigma, St Louis, MO, USA) with soy extract or tamoxifen (Sigma, St Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO) and incubated for 48 h [final DMSO concentration $\leqslant$ 0.05% (v/v)].

4.3. Cell survival assays

MTT assay: mitochondrial function was assayed by the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St Louis, MO, USA) into an insoluble dark blue formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well of a 24-well plate was measured photometrically as previously described (Mosmann, 1983). MTT was dissolved in DPBS at a concentration of 5 g/l and sterilized by passage through a 0.22 μm filter. This stock solution was added (1 part to 10 parts medium) to each well of a 24-well tissue culture plate, and the plate was incubated at 37°C for 4 h. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates

were read on a microplate reader at a wavelength of 570–630 nm.

4.4. Cell proliferation

After the treatment with soy extract in the presence or absence of anti-estrogen tamoxifen for 48 h, the cells were labeled with 1 μCi of [^{3}H]-methyl thymidine (Amersham, Arlington Heights, IL, USA) for the last 6 h of incubation, and then were rinsed twice with DPBS and 100 g/l trichloroacetic acid (Sigma, St Louis, MO, USA). Finally, the cells were dissolved in 500 μl of 2 mol/l perchloric acid (Sigma, St Louis, MO, USA). Radioactivity was determined by scintillation counting.

4.5. Alkaline phosphatase (ALP) activity

The cells treated with soy extract in the presence or absence of tamoxifen for 48 h in 6-well plate. After washing twice with DPBS, the cells were scraped into a 1 ml of 10^{-2} mol/l Tris–HCl (pH 7.6) buffer containing 1 g/l Triton-X on ice, and centrifuged. An aliquot of the supernatant was used for the determination of ALP activity by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate.

4.6. Determination of collagen-synthesizing activity

Collagen synthesis was determined by the method of Nanes et al. with modification (Nanes et al., 1989) as indicated below. The cells were pulsed with 5 μ Ci/ml [^{3}H] proline (Amersham, Arlington Heights, IL, USA) for the last 3 h of treatment. Cell layers were rinsed with DPBS. Homogenates were collected in 5 g/l Triton X-100 (Sigma, St Louis, MO, USA), precipitated with ice-cold 100 g/l trichloroacetic acid-10 g/l tannic acid. The precipitates were acetone extracted, dried, resolubilized in 0.5 mol/l acetic acid, and neutralized with 0.5 mol/l NaOH. The amount of [^{3}H] proline incorporated into collagenase-digestible protein (CDP) and noncollagen protein (NCP) was measured using crude bacterial collagenase (Sigma, St Louis, MO, USA). Percentage of collagen synthesis (PCS) was calculated after correcting for the relative abundance of proline in CDP and NCP.

4.7. Analysis of apoptosis in cultured cells

Cells were seeded at a density of 1×10^6 cells in 100 mm tissue culture dishes. The culture conditions were the same as those described in cell proliferation assay. To demonstrate formation of oligonucleosome-sized DNA fragments during apoptosis, we used the ApopLadder EX™ (Takara Shuzo Co., Shiga, Japan) which consisted of lysis buffer including unique surfactant, enzymes, precipitant of DNA, and loading buffer. This kit enabled the selective extraction of fragmented DNA. DNA pellets were solubilized in 10^{-2} mol/l Tris–HCl (pH 8.0) and 10^{-3} mol/l EDTA. Electrophoresis was performed on a 1.5% agarose gel in the presence of 0.1 mg/l of ethidium bromide. The 1 kb and 100 bp standard were included as a reference. For quantitation of apoptosis, the cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) was used according to the manufacturer's instruction. Cells were seeded at a density of 2×10^4 cells in 24-well culture plates. The culture conditions were the same as those described in cell proliferation assay. After the incubation, the cells were lysed and intact nuclei were pelleted by centrifugation. An aliquot of the supernatant was transferred to a streptoavidin-coated well of a microtiter plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA-peroxidase were added and incubated for 2 h. During the incubation period, the anti-histone antibody bound to the histone component of the nucleosomes and simultaneously fixed the immunocomplex to the streptavidin-coated microplate via its biotinylation. Additionally, the anti-DNA-peroxidase antibody reacted with the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically with substrate.

4.8. Statistics

Experiments were carried out in six cultures and expressed as mean±S.E.M. Differences between the means were calculated using one-way analysis of variance (ANOVA) with a subsequent Duncan's multiple range test ($P<0.05$). The analysis was performed using SAS statistical software.

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Withanolide-type steroids from *Solanum cilistum*

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Abstract

Six withanolide-type steroids, named as cilistols a, b, d, q, g and f, respectively, were isolated from the leaves of *Solanum cilistum*. The major component among them, cilistol a, was characterized as (22*R*, 24*S*, 25*R*, 26*R*)-1-oxo-22, 26; 24, 25-diepoxy-17α, 26-dihydroxyergost-2,5-diene on the basis of spectroscopic properties and X-ray crystallographic analysis; the other isolated compounds had closely related structures. This is the first report of withanolide-type steroids in the *Solanum* genera. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Solanum cilistum*; Solanaceae; Withanolide-type steroid; Cilistol

1. Introduction

Our studies on the constituents of *Solanum*-genera plants have resulted in the isolation of many steroidal glycosides, such as spirostane, furostane, solasodane and solanidane glycosides, and of which were found to exhibit anti-cancer (Nakamura et al., 1996) and anti-HSV1 (Ikeda et al., 2000) activities. As a further extension of these studies, we have investigated the constituents of *Solanum cilistum* and have isolated six withanolide-type steroids which were of a different pattern from that of the steroidal glycosides so far obtained from *Solanum* plants. This paper describes the characterization of their chemical structures.

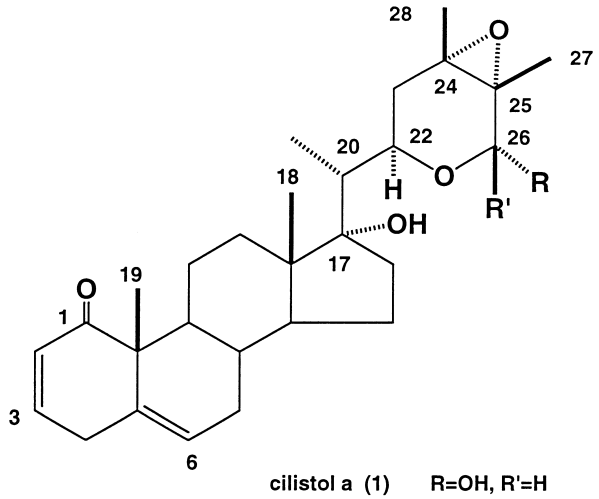
2. Results and discussion

A methanolic extract of the leaves (560 g) of this plant was extracted with hot MeOH, repeatedly, and evaporated under reduced pressure to give an extract (68.0 g), which was treated with hexane. The insoluble layer was applied to a Diaion HP-20P column, this being eluted by H_2O and 30, 50, 70 and 90% aq. MeOH, successively. The respective fractions were subsequently subjected to a variety of column chromatographic separation such as silica gel ($CHCl_3$–MeOH = 20:1→10:1, $CHCl_3$–MeOH–H_2O = 9:2:0.1), Chromatorex ODS (60%MeOH→80%MeOH→90%MeOH→MeOH), and Sephadex LH-20 (MeOH) to afford six whithanolide-type steroids, named cilistols a (**1**, 252 mg), b (**2**, 10.2 mg), d (**3**, 36.7 mg), q (**4**, 15.2 mg), g (**5**, 337 mg) and f (**6**, 167 mg).

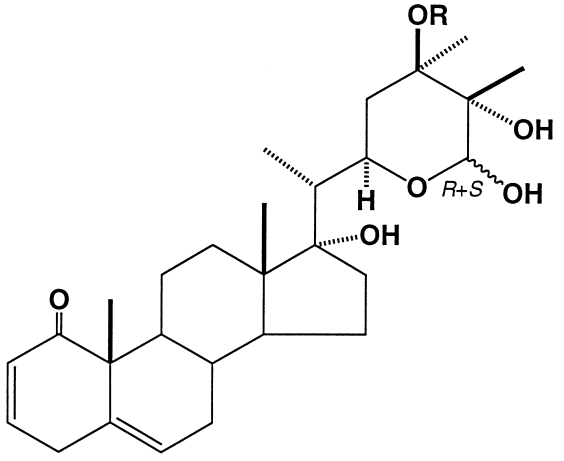
Cilistol a (**1**) was obtained as colorless plates, mp 235–236°C, $[\alpha]_D$ −17.0° (MeOH). The IR spectrum showed absorptions at 3494 cm^{-1} (OH), and at 1680 and 1660 cm^{-1} (α,β-unsaturated carbonyl group) and the UV spectrum had a λ_{max} absorption at 269.3 nm. The EIMS displayed peaks due to $[M\text{-}OH]^+$, $[C_{19}H_{23}O]^+$, and $[C_7H_{11}O_2]^+$ at *m*/*z* 439, 267 and 109, respectively, which together with the evidence of the ^{13}C NMR spectrum (Table 1, in pyridine-d_5) and elemental analysis suggested a molecular formula of $C_{28}H_{40}O_5$. The ^{13}C NMR spectrum showed 28 signals composed of one α,β-unsaturated carbonyl system (δ 203.8, 127.9 and 145.7), one tri-substituted double bond system (δ 124.9 and 136.3), one hemiacetal carbon (δ 92.8), five methyl groups (δ 10.1, 15.1, 17.4, 2×19.0), seven methylene carbons (δ 24.0, 24.1, 31.2, 32.8, 33.7, 34.0, 37.5), one oxygen bearing methine carbon (δ 67.0), four methine carbons (δ 33.7, 43.3, 44.0 and 50.6), three oxygen bearing quaternary carbons (δ 63.0, 63.1 and 85.0), two quaternary

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cilistol a (1) R=OH, R'=H
cilistol b (2) R=H, R'=OMe
cilistol d (3) R=OMe, R'=H
cilistol q (4) R=-*O*-β-D-glc · pyr, R'=H



cilistol g (5) R=H
cilistol f (6) R=Me

Table 1
^{13}C NMR spectral data (in pyridine-d_5) for cilistols a (**1**), b (**2**), d (**3**), q (**4**), (**5**) and f (**6**)

	1	2	3	4	5	6
C-1	203.8	203.9	203.9	203.3	203.9	203.9
-2	127.9	127.9	127.9	127.9	128.0	127.9
-3	145.7	145.9	145.8	145.8	145.8	145.8
-4	33.7	33.6	33.9	34.0	33.6	33.6
-5	136.3	136.3	136.3	136.3	136.4	136.3
-6	124.9	124.9	124.9	124.9	124.9	124.9
-7	31.2	31.2	31.2	31.2	31.3	31.3
-8	33.7	33.7	33.7	33.8	33.8	33.7
-9	43.3	43.3	43.3	43.3	43.4	43.3
-10	50.8	50.8	51.0	50.8	50.8	50.8
-11	37.5	37.5	37.8	37.8	37.4	37.4
-12	32.8	32.9	32.8	32.9	32.9	32.8
-13	48.1	48.2	48.2	48.3	48.3	48.2
-14	50.6	50.6	50.7	50.7	50.7	50.3, 50.6
-15	24.0	24.1	24.1	24.1	24.0	23.7, 24.0
-16	24.1	24.0	24.0	24.3	24.2	23.5, 24.1
-17	85.0	84.8	84.9	84.9	85.1, 85.3	85.0, 85.2
-18	15.1	15.1	15.1	15.1	15.3	15.2
-19	19.0	19.0	18.6	19.0	19.0	18.9, 19.0
-20	44.0	44.4	44.1	43.9	43.8, 44.2	43.2, 44.2
-21	10.1	10.0	10.2	10.2	10.5, 10.7	10.3, 10.5
-22	67.0	71.2	67.7	68.1	70.1, 74.4	69.5, 74.0
-23	34.0	33.2	33.6	33.6	41.1	38.6
-24	63.1	62.4	61.3	61.5	75.3, 77.3	75.5, 77.5
-25	63.0	61.5	61.3	61.6	74.5, 77.8	77.4, 78.6
-26	92.8	101.9	99.8	95.8	98.4, 99.1	98.0, 99.1
-27	17.4	15.2	17.3	17.1	16.2	17.3, 22.3
-28	19.0	9.2	19.0	18.9	22.6, 23.7	15.9, 17.3
24-*O*Me						50.0
26-*O*Me		56.2	55.4			
glc						
C-1				100.2		
-2				74.7		
-3				78.6		
-4				71.7		
-5				78.6		
-6				62.8		

carbons (δ 48.1 and 50.8). The ^{1}H NMR spectrum (in pyridine-d_5) displayed signals for two tertiary methyl groups (δ 0.75 and 1.21), two tertiary methyl groups adjacent to oxygen (δ 1.33 and 1.49), a secondary methyl group (δ 1.28), three olefinic protons (δ 5.45, 5.95 and 6.68). With the aid of ^{1}H–^{1}H COSY, HMQC and HMBC (Fig. 1) techniques, the plain structure for **1** was deduced. However, the relative structure could not been determined even though a NOE experiment was performed. In order to assign configurations at C-17, -22, -24, -25 and -26, a suitable crystal of **1** was chosen for X-ray crystallographic analysis. The structure is shown in Fig. 2. Consequently, the structure of **1** was established as the new compound, (22*R*, 24*S*, 25*R*, 26*R*)-1-oxo- 22, 26; 24, 25-diepoxy-17α,26-dihydroxyergost-2,5-diene.

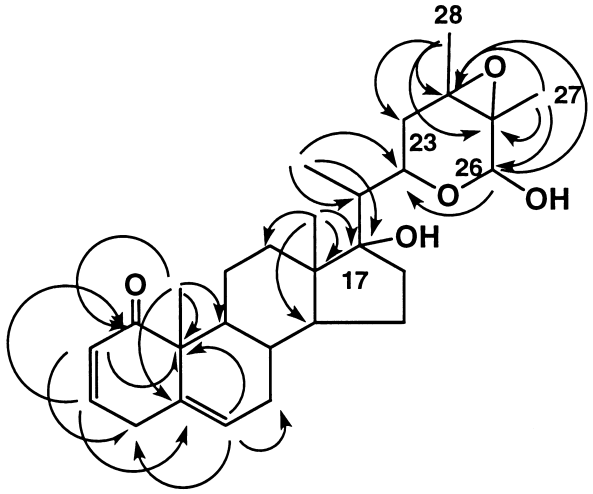


Fig. 1. Key HMBC of **1**.

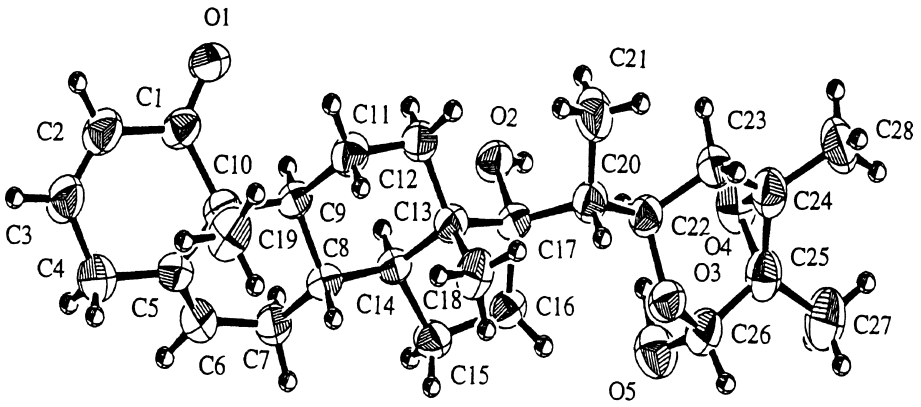


Fig. 2. ORTEP drawing of **1**.

Cilistol b (**2**) and cilistol d (**3**) were obtained as colorless plates, mp 223–225°C, $[\alpha]_D$ −11.2° (MeOH) and colorless plates, mp 236–237°C, $[\alpha]_D$ −44.0° (MeOH), respectively. Both positive FABMS showed the same peaks due to $[M+H]^+$, $[C_9H_{14}O_2]^+$, and $[C_9H_{12}O]^+$ at *m*/*z* 471, 154 and 136, respectively. The combined evidence of their FABMS, the ^{13}C NMR spectrum and elemental analysis suggested both to have the same molecular formula of $C_{29}H_{42}O_5$. Their ^1H and ^{13}C NMR spectra (Table 1, in pyridine-d_5) were closely resembled to each other and to those of **1** except the presence of a methoxyl group in **2** and **3**. Assignments of the respective signals of the ^1H and ^{13}C spectroscopic signals in **2** and **3** could be accomplished by comparing with those of **1**. Their structures were characterized to be the methyl ether derivatives of **1** and the structural difference between **2** and **3** was recognized to be the configurational isomers at C-26, since ^{13}C-chemical shift differences between **2** and **3** were observed around C-22, 26, 27. The NOESY of **3** showed the H-22 to correlate with H-26, therefore, the methoxyl group oriented with the same configuration as that of **1**. On the other hand, the NOESY of **2** did not show a cross peak between H-22 and 26-OMe. Consequently, **3** was determined to be the (26*R*)-26-*O*-methyl ether of **1**, while **2** was the (26*S*)-26-*O*-methyl ether of **1**.

Cilistol q (**4**) was obtained as an amorphous powder, $[\alpha]_D$ −42.1° (MeOH). The positive FABMS showed a peak at *m*/*z* 619 due to $[M+H]^+$ corresponding to the molecular formula $C_{34}H_{50}O_{10}$, which was supported from the analysis of its ^{13}C NMR spectrum and from elemental analysis. The ^{13}C NMR spectrum (Table 1, in pyridine-d_5) showed 34 signals, in which signals due to one β-D-glucopyranosyl moiety was included and signals originated from the aglycone part was resembled to those of cilistol a (**1**). As for the location of the glucosidic linkage, HMBC disclosed a correlation between C-26 and a glucosyl anomeric proton. Therefore, **4** was represented as cilistol a 26-*O*-β-D-glucopyranoside.

Cilistol g (**5**) was obtained as colorless plates, mp 223–224°C, $[\alpha]_D$ +1.2° (MeOH). TLC analysis showed that it had two components, and when one component was isolated by preparative TLC, re-analysis immedietely revealed the pressence of two components as before. The positive FABMS displayed peaks due to $[M+Na]^+$ and $[C_8H_{16}O_4]^+$ at *m*/*z* 497 and 176, respectively, which together with the analysis of the ^{13}C NMR spectrum and the elemental analysis indicated a molecular formula of $C_{28}H_{42}O_6$. The ^1H and ^{13}C signals due to C-17, -18, -20, -22, -24, -25 and -26 appeared as splitting pairs. By comparing the ^{13}C NMR spectrum (Table 1, in pyridine-d_5) of **5** with that of **1**, a prominent difference was observed that signals due to C-24 and C-25 shifted lower field at δ 75.3; 77.3 and 74.5; 77.8, respectively. Therefore, the epoxide ring at C-24 and-25 observed in **1** was suggested to be opened as a glycol, whose relative configurations were deduced to be *R* and *R* at C-24 and-25, respectively, since they could be estimated as the same as those of cilistol j [Zhu et al., 2000]. The structure of cilistol j had been characterized as (22*R*,24*R*,25*R*,26*S*) - 22,26 - epoxy - 1α,3β,24,25,26 - pentahydroxy-ergost-5-ene β-D-glucopyranoside, in which NOESY correlations were observed between H-26 and H_3-27, and between H-22 and H_3-28, suggesting stereo configurations at C-24 and C-25 to be *R* and *R*, respectively. It indicated the epoxide ring on C-24 and-25 was opened toward to C-27 in a SN2 system to form a *trans*-glycol. Therefore, cilistol g (**5**) was characterized (22*R*,24*R*,25*R*,26Φ) - 1 - oxo - 22,26 - epoxy - 17α,24,25,26 - tetrahydroxy-ergost-2,5-diene.

Cilistol f (**6**) was obtained as colorless plates, mp 166–170°C, $[\alpha]_D$ −18.6° (MeOH) showing inseparable two spots on TLC. The positive FABMS displayed peaks due to $[M+Na]^+$ and $[C_9H_{18}O_4]^+$ at *m*/*z* 511 and 190, respectively, which together with the analysis of the ^{13}C NMR spectrum and from its elemental analysis indicated a molecular formula of $C_{29}H_{44}O_6$. The ^1H and ^{13}C signals due to C-17, -18, -20, -22, -24, -25 and -26 appeared as splitting pairs. By comparing the ^{13}C NMR spectrum (Table 1, in pyridine-d_5) of **6** with that of **5**, both resembled each other except for the presence of a methyl ether signal in **6**; therefore, **6** was suggested to be

the methyl ether derivative of **5**. The configurations could be suggested to be *R* and *R* at C-24 and 25, respectively, as for **5**.

Therefore, cilistol f (**6**) was characterized as (22*R*,24*R*, 25*R*,26Φ)-1-oxo-22,26-epoxy-24-*O*-methyl-17α,24,25,26-tetrahydroxyergost-2, 5-diene.

The occurrence of the withanolide-type steroids from the *Solanum* genera is for the first time reported and it is worthy of chemotaxonomic remark.

3. Experimental

3.1. General

Optical rotations were determined on a JASCO DIP-1000 polarimeter ($l = 0.5$). Melting points (uncorrected) were measured using Yanagimoto micro-melting point apparatus. FABMS were obtained in a glycerol matrix in the positive ion mode using a JEOL JMS-DX300 and JMS-DX 303HF, and EI-MS on a JEOL JMS-01SG and JMS-DX303HF. NMR spectra were measured in pyridine-d_5 on a JEOL α-500 spectrometer (500 MHz) and chemical shifts were referenced to TMS. Column chromatography was carried out on silica gel 60 (230–400 mesh, Merck), Sephadex LH-20 (25–100 nm, Pharmacia Fine Chemicals), MCI gel CHP-20P (75–150 μm, Mitsubishikasei), Chromatorex ODS (30–50 μm, Fuji Silysia Chemical Itd.), and TLC was performed on precoated silica gel 60F254 (0.2 mm, Merck).

3.2. Plant material

Leaves of the title plant provided from Dr. Masaharu Matsui (National Research Institute of Vegetables, Ornamental Plants and Tea, Ministry of Agriculture, Forestry and Fisheries, Ano, Mie, Japan) was identified by Dr. Monma, plant taxonomist in the above same National Institute and cultivated at the Botanical Garden of Kumamoto University.

3.3. Extraction and isolation

A methanolic extract of the leaves (560 g) of this plant was extracted with hot MeOH repeatedly and evaporated under reduced pressure to give an extract (68.0 g), which was treated with hexane. The insoluble layer was applied to a Diaion HP-20P column, which was eluted with H_2O, 30, 50, 70 and 90% aq. MeOH, successively. The respective saponin fractions eluted with 50, 70, 90% MeOH were subsequently subjected to a variety of column chromatographic procedures such as silica gel, Chromatorex ODS, and Sephadex LH-20 to afford six whithanolide-type steroids, named cilistols a (**1**, 252 mg), b (**2**, 10.2 mg), d (**3**, 36.7 mg), q (**4**, 15.2 mg), g (**5**, 337 mg), and f (**6**, 167 mg).

3.4. Cilistol a (*1*)

Colorless plates, m.p. 235–236°C; $[\alpha]_D^{25}$ −17.0°(c = 0.22, MeOH). UV λ^{MeOH} nm: 269.3; IR (KBr) ν_{max} cm^{-1} 3494 (OH), 1680, 1660 (α,β-unsaturated carbonyl group); EIMS (m/z): 439 $[M\text{-}OH]^+$, 267 $[C_{19}H_{23}O]^+$, 109 $[C_7H_{11}O_2]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.75 (3H, *s*, H_3-18), 1.21 (3H, *s*, H_3-19), 1.28 (3H, *d*, J = 6.7 Hz, H_3-21), 1.33 (3H, s, H_3-28), 1.49 (3H, *s*, H_3-27), 2.69 (1H, *dd*, J = 4.8, 21.2 Hz, H-4), 3.19 (1H, *br d*, J = 21.2 Hz, H'-4), 4.69 (1H, *br d*, J = 11.6 Hz, H-22), 5.45 (1H, *br d*, J = 6.1 Hz, H-6), 5.51 (1H, *s*, H-26), 5.95 (1H, *br d*, J = 10.4 Hz, H-2), 6.68 (1H, *ddd*, J = 2.4, 4.8, 10.4 Hz, H-3); ^{13}C-NMR: Table 1; Anal. calc. for $C_{28}H_{40}O_5$ C 73.65, H 8.83; found C 73.44, H 8.79.

3.5. X-Ray diffraction structure determination for *1*

Crystal data: $C_{28}H_{40}O_5$, M = 456.62, colorless plates; crystal size (mm) 0.25×0.25×0.25, crystal system, orthorhombic; space group $P\ 2_12_12_1$; unit cell dimensions, a = 11.090(1), b = 29.639(2), c = 7.458(2)Å, volume, 2451.2(5) $Å^3$; Z = 4; formula weight, 456.62; density, 1.237 g/cm^3; F(000), 992.00; μ(CuK_α), 6.65 cm^{-1}.

3.6. Data collection

The reflection data were collected on a Rigaku AFC7R diffractometer using graphite-monochromated CuK_α radiation ($\lambda = 1.5418\mathring{A}$) with the $\omega - 2\theta$ scan technique to a maximum 2θ of 120.1° at room temperature (23°C). Independent 1912 reflections were collected. The structure was solved by the direct method using SIR88.3. All atomic parameters, with anisotropic temperature factors for non-hydrogen atoms and isotropic ones for hydrogen atoms, were refined by block-diagonal least-squares method. Final indices $R_1 = 3.6\%$.

3.7. Cilistol b (*2*)

Colorless plates, mp 223–225°C; $[\alpha]_D^{25}$ −11.2°(c = 0.22, MeOH); Positive FABMS (m/z): 471 $[M+H]^+$, 154 $[C_9H_{14}O_2]^+$, 136 $[C_9H_{12}O]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.83 (3H, *s*, H_3-18), 1.24 (3H, *d*, J = 7.0 Hz, H_3-21), 1.27 (3H, *s*, H_3-19), 1.27 (3H, *s*, H_3-28), 1.43 (3H, *s*, H_3-27), 2.90 (1H, *dd*, J = 4.3, 21.4 Hz, H-4), 3.20 (1H, *br d*, J = 21.4 Hz, H′-4), 3.55 (3H, *s*, OMe), 4.42 (1H, *br d*, J = 11.6 Hz, H-22), 4.92 (1H, *s*, H-26), 5.46 (1H, *m*, H-6), 5.76 (1H, *dd*, J = 1.8, 9.7 Hz, H-2), 6.69 (1H, *m*, H-3); ^{13}C NMR: Table 1; Anal. calc. for $C_{29}H_{42}O_5$ C 74.01, H 9.00; found C 73.87, H 8.89.

3.8. Cilistol d (*3*)

Colorless plates, mp 236–237°C; $[\alpha]_D^{25}$ −44.0°(c = 0.13, MeOH); Positive FABMS (m/z): 471 $[M+H]^+$, 154

$[C_9H_{14}O_2]^+$, 136 $[C_9H_{12}O]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.86 (3H, *s*, H_3-18), 1.22 (3H, *s*, H_3-19), 1.26 (3H, *s*, H_3-28), 1.30 (3H, *d*, $J=7.3$ Hz, H_3-21), 1.41 (3H, *s*, H_3-27), 2.69 (1H, *dd*, $J=4.8$, 21.4 Hz, H-4), 3.19 (1H, *br d*, $J=21.4$ Hz, H′-4), 3.54 (3H, *s*, OMe), 4.05 (1H, *br d*, $J=11.6$ Hz, H-22), 4.88 (1H, *s*, H-26), 5.45 (1H, *br d*, $J=6.1$ Hz, H-6), 5.96 (1H, *dd*, $J=2.7$, 10.0 Hz, H-2), 6.70 (1H, *m*, H-3); ^{13}C NMR: Table 1; Anal. calc. for $C_{29}H_{42}O_5$ C 74.01, H 9.00; found C 74.11, H 9.10.

3.9. Cilistol q (**4**)

Amorphous powder, $[\alpha]_D^{25}$ −42.1°($c=0.63$, MeOH); Positive FABMS (*m*/*z*): 619 $[M+H]^+$, 154 $[C_9H_{14}O_2]^+$, 136 $[C_9H_{12}O]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.85 (3H, *s*, H_3-18), 1.18 (3H, *s*, H_3-19), 1.30 (3H, *d*, $J=7.3$ Hz, H_3-21), 1.43 (3H, *s*, H_3-27), 1.44 (3H, *s*, H_3-28), 3.16 (1H, *br d*, $J=21.4$, H-4), 3.94 (1H, *m*, *glc* H-5), 4.10 (1H, *t*-like, $J=8.2$ Hz, *glc* H-2), 4.25 (1H, *t*-like, $J=7.1$ Hz, *glc* H-4), 4.28 (1H, *t*-like, $J=7.1$ Hz, *glc* H-3), 4.41 (1H, $J=5.3$, 11.6 Hz, *glc* H-6), 4.54 (1H, *dd*, $J=2.4$, 11.6 Hz, *glc* H′-6), 4.84 (1H, *br d*, $J=11.6$ Hz, H-22), 5.39 (1H, *d*, $J=7.1$ Hz, *glc* H-1), 5.42 (1H, *br d*, $J=6.1$ Hz, H-6), 5.66 (1H, *s*, H-26), 5.96 (1H, *dd*, $J=2.4$, 9.8 Hz, H-2), 6.67 (1H, *m*, H-3); ^{13}C NMR : Table 1; Anal. calc. for $C_{34}H_{50}O_{10}$ C 66.00, H 8.15; found C 66.03, H 8.21.

3.10. Cilistol g (**5**)

Colorless plates, mp 223–224°C; $[\alpha]_D^{25}$ +1.2° ($c=0.44$, MeOH); Positive FABMS *m*/*z*: 497 $[M+Na]^+$, 176 $[C_8H_{16}O_4]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.82, 0.83 (each s, H_3-18), 1.23 (3H, *s*, H_3-19), 1.39 (3H, *d*, $J=6.7$ Hz, H_3-21), 1.85, 1.91 (each *s*, H_3-27), 2.00, 2.11 (each *s*, H_3-28), 2.71 (1H, *dd*, $J=4.9$, 21.4 Hz, H-4), 3.21 (1H, *br d*, $J=21.4$ Hz, H′-4), 4.31, 4.85 (each *br d*, $J=11.6$ Hz, H-22), 5.46, 5.57 (each *s*, H-26), 5.46 (1H, *br s*, H-6), 5.97 (1H, *br d*, $J=6.0$ Hz, H-2), 6.71 (1H, *m*, H-3); ^{13}C NMR: Table 1; Anal. calc. for $C_{28}H_{42}O_6$ C 70.85, H 8.92; found C 70.89, H 8.91.

3.11. Cilistol f (**5**)

Colorless needles, m.p. 166–170°C; $[\alpha]_D^{25}$ −18.6° ($c=0.40$, MeOH); Positive FABMS (*m*/*z*): 511 $[M+Na]^+$, 190 $[C_9H_{18}O_4]^+$; EIMS (*m*/*z*): 420 $[M-2\times H_2O-MeOH]^+$, 267 $[C_{19}H_{23}O]^+$, 124 $[C_7H_8O_2]^+$; ^{1}H NMR (pyridine-d_5) δ: 0.81, 0.82 (each *s*, H_3-18), 1.22, 1.23 (each *s*, H_3-19), 1.34, 1.35 (each *d*, $J=6.7$ Hz, H_3-21), 1.68, 1.69 (each *s*, H_3-27), 1.83, 1.90 (each *s*, H_3-28), 2.72 (1H, *dd*, $J=4.9$, 21.4 Hz, H-4), 3.21 (1H, *br d*, $J=21.4$ Hz, H′-4), 3.51, 3.52 (each *s*, OCH_3), 4.19, 4.85 (each *br d*, $J=12.2$ Hz, H-22), 5.30, 5.39 (each *s*, H-26), 5.47 (1H, *m*, H-6), 5.96 (1H, *dd*, $J=1.8$, 9.8 Hz, H-2), 6.70 (1H, *br d*, $J=7.3$ Hz, H-3); ^{13}C NMR: Table; Anal. calc. for $C_{29}H_{44}O_6$ C 71.28, H 9.08; found C 71.33, H 9.06.

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Pyrone and pyridone compounds in the liquid culture of *Physisporinus sanguinolentus*

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Abstract

Chromatographic separation of the liquid culture filtrate of the basidiomycete fungus *Physisporinus sanguinolentus* has yielded three new compounds viz., 2-methyl-4-pyrone, 2-methyl-5,6-dihydro-4-pyrone and the pyridone form of 4-hydroxy-2-methylpyridine, together with the known triacetic acid lactone, the sesquiterpene dialdehyde merulidial and a derivative of merulidial. Their structures were elucidated by spectroscopic analysis and by comparison to literature data and a synthetic sample. One of the compounds, merulidial, was shown to inhibit the germination of spores and the hyphal growth of the wood-rotting basidiomycete *Heterobasidion annosum* and the saprophytic mould *Cladosporium cucumerinum*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Physisporinus sanguinolentus*; Antifungal effect; 4-Pyrone; Dihydro-4-pyrone; 4-Hydroxypyridine; 4-Pyridone

1. Introduction

Micro-organisms produce a wide array of secondary metabolites with varying degrees of antibiotic activity (Russell et al., 1995). Many of these compounds diffuse readily in the growth substrate, where they play important roles in chemical signalling and in regulating the growth of other organisms in natural habitats.

Fungal pathogens are one of the main causes of crop losses all over the world. There is a long history of using chemical fungicides to control fungi. In recent years, natural products are being used increasingly as fungicides or as lead compounds for the development of new active fungicides (Demain, 1999).

One of the main forest pathogens in boreal and temperate forests is the basidiomycete fungus *Heterobasidion annosum* (Fr.) Bref., which causes root rot in conifers (Woodward et al., 1998). Airborne basidiospores of the fungus infect freshly cut stump surfaces, from where the fungus grows down into the root system, where it continues to infect nearby trees via root-to-root contact.

As part of an investigation to combat the root rot problem, many fungal culture filtrates were tested for their ability to inhibit *H. annosum* and *Cladosporium cucumerinum* (Ellis and Arth.) growth, from which *Physisporinus sanguinolentus* (Alb. & Schwein. : fr.) Pilát was selected to undergo further investigations.

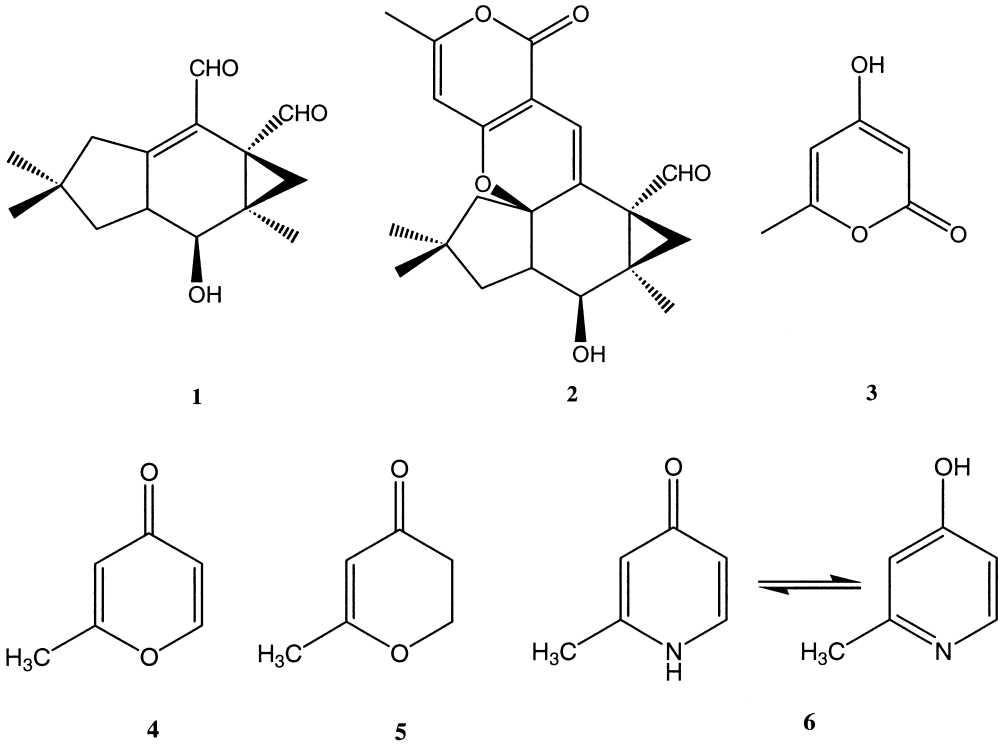
2. Results and discussion

2.1. General

The culture filtrate was fractionated using solid phase extraction (C-18), silica gel column chromatography and preparative HPLC, yielding compounds **1**–**6** (Scheme 1). During fractionation, the biological activity was followed using biotests, resulting in the isolation of compound **1**. By comparing the ^{1}H and ^{13}C NMR spectra and the mass spectrum to the literature data (Giannetti et al., 1986), the compound was identified as the sesquiterpene dialdehyde merulidial. Merulidial has been isolated previously from the culture filtrate of the fungus *Merulius tremellosus* Fr. (Quack et al., 1978). Merulidial is known to show antibiotic effects against several bacteria and fungi (Giannetti et al., 1986). In

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1 2 3

4 5 6

Scheme 1.

this study, it showed a strong antifungal effect against both *H. annosum* and *C. cucumerinum*.

Two known compounds, **2** and the triketide triacetic acid lactone **3**, produced in small quantities, were also isolated. Compound **3** has previously been analytically shown to be produced of *M. tremellosus* (Jonassohn et al., 1996) but is here isolated for the first time. Compound **2** was identified as an adduct of merulidial and **3**. The identification of **2** was based upon a comparison of the mass spectrum and the ^{1}H and ^{13}C NMR spectra to literature data (Jonassohn et al., 1994). The structure of **3** was confirmed fully by synthesis according to the literature (Evans and Staunton, 1988). Compound **2**, together with merulidial and **3**, were found previously in a culture filtrate of *Merulius tremellosus* (Jonassohn et al., 1996). The same authors noticed that **2** slowly formed from merulidial and **3** in a buffered water solution. Hence, it was concluded that **2** formed chemically during the fermentation. In our biotests, no activity was detected for compounds **2** and **3**. Compounds **1**–**3** are reported here in the genus *Physisporinus* for the first time.

Compounds **4**–**6** are low-molecular compounds and are reported here as natural compounds for the first time. Compound **4** was isolated as a colourless liquid. The molecular formula was determined to be $C_6H_6O_2$ from HR-EIMS ($[M^+]$ m/z 110.0363). The ^{13}C NMR spectrum revealed signals from a methyl group, four olefinic carbons and a carbonyl carbon. The ^{1}H NMR spectrum showed a broad singlet from a methyl group at δ 2.25, and ABX-type signals at δ 7.67 (*d*, $J=5.9$ Hz), 6.27 (*dd*, $J=5.9$ and 2.5 Hz) and 6.16 (*d*, $J=2.5$ Hz). The signal at δ 6.16 is a broad doublet because of long-range coupling to the methyl group. Furthermore, the HMBC spectrum revealed a three-bond correlation between the methyl carbon and the proton giving a signal at δ 6.16. Based on these spectroscopic data, the identity of **4** was established as 2-methyl-4-pyrone. Compound **4** has been reported as a synthetic product previously (Dorman, 1967). However, to our knowledge it is a new natural product. The ^{1}H NMR spectrum agreed well with the reported data.

High levels of compound **5**, $C_6H_8O_2$ ($[M^+]$ m/z 112.0535), were obtained (1.6 g) as a colourless liquid from the culture filtrate. The ^{1}H NMR spectrum revealed singlet signals from a methyl group at δ 1.95 and from an olefinic proton at δ 5.28, and triplet signals at δ 2.45 and 4.41 ($J=6.8$ Hz) assigned to a pair of isolated vicinal methylene groups. A signal in the ^{13}C NMR spectrum at δ 192.0 is consistent with an α,β-unsaturated keto group with oxygen linked to the β-position. Therefore, **5** is proposed to be 2-methyl-5,6-dihydro-4-pyrone. Cross peaks in the HMBC spectrum from the methyl carbon signal at δ 21.0 to the olefinic proton signal and to the methylene group signal at δ 4.41, confirmed the proposed structure. Compound **5** has been reported previously as a synthetic intermediate

(Haddad et al., 1997), and is, to our knowledge, a new natural product. The ^{1}H NMR spectral data corresponded closely with literature data.

Compound **6** was identified as the pyridone form of 4-hydroxy-2-methylpyridine. The molecular formula was determined as C_6H_7NO from HR-EIMS ([M^+] m/z 109.0527), and the ^{1}H NMR spectrum of **6** (in CD_3OD) showed resemblance to the spectrum of **4**. In addition, the ^{13}C NMR spectra of **6** and **4** were similar and differed considerably only with regard to the signals from C-2 and C-6. In **6**, these signals were shifted upfield with 15.3 and 15.6 ppm, respectively, in comparison with the corresponding signals in **4**.

In solid state, as well as in water and chloroform solutions, 4-hydroxypyridine derivatives are known to be present predominantly in the pyridone form (Albert and Spinner, 1960). Our NMR data also show that the pyridone form of **6** is predominant in both methanol and water. The chemical shift of the C-4 carbon at δ 182.0 in CD_3OD solution of **6** is consistent with a signal from a carbonyl carbon. The signals in the ^{1}H NMR spectra of **6** in H_2O/D_2O and CD_3OD corresponded closely. This indicates that the same type of heterocyclic ring system is present in both solvents.

Compound **6** has been synthesised previously and included in a study of enthalpies of combustion for different hydroxypyridines (Suradi et al., 1982) but no physical data were reported. However, **6** has not been reported as a natural product before.

2.2. Antifungal activity

2.2.1. Agar plate test

The *P. sanguinolentus* compounds were biotested with *H. annosum* and *C. cucumerinum* by placing the compounds in wells in Petri dishes containing Hagem agar, and inoculating the surface of the Hagem agar with *C. cucumerinum* or *H. annosum*. Three and five days after inoculation, respectively, hyphal growth inhibition was assessed by measuring the clear zone around the wells resulting from the prevention of hyphal growth. Compound **1** significantly prevented the growth of the isolates examined in this experiment. The clear zone from the edge of the well was 4–7 mm in Petri dishes inoculated with *C. cucumerinum* and 15–19 mm in those inoculated with *H. annosum*. The other compounds tested did not inhibit hyphal growth.

2.2.2. Microtitre plate

The germination of spores and the growth of germinating hypha were evaluated under the microscope. Only compound **1** showed a strong activity against *H. annosum* and showed a weaker effect on *C. cucumerinum*. The germination of *H. annosum* spores was inhibited in concentrations of both 12.5 and 25.0 μg ml^{-1} of compound **1**, and strongly delayed in 6.3 μg ml^{-1}. *C. cucumerinum* spore germination was delayed by at least 48 h compared with the control. The hyphae showed abnormalities in morphology, such as shorter segmented hyphae, thicker cell walls and dense pigmentation.

None of the other compounds showed conspicuous inhibition of *H. annosum* and *C. cucumerinum* spore germination.

3. Experimental

3.1. General

NMR spectra were obtained at 30°C on Bruker DRX 400 and 600 spectrometers using $CDCl_3$, CD_3OD or H_2O/D_2O (1:1) as the solvent. The signals were assigned by one- and two-dimensional experiments (^{1}H, ^{13}C, COSY, HMQC and HMBC) using standard pulse-sequences. The HMQC experiments were performed with carbon decoupling during acquisition, and the delay time for HMBC was 80 ms.

HR-EIMS and EIMS were recorded on a JEOL JMS-SX/SX-102A instrument.

TLC — silica gel 60 F 254 0.25 mm (Merck). CC — silica gel 60 230–400 mesh (Merck) and reversed phase C18, 40–70 mm particle size, 60 nm pore diameter (Isolute).

3.2. Fungal isolates

Metabolites were produced by *P. sanguinolentus* strain M 83. The isolate was grown in 500 ml E-flasks containing 200 ml of Hagem liquid medium (Stenlid, 1985) and incubated at 20°C in the dark for 5 weeks. The liquid supernatant was then separated from the fungal mycelia by filtration of the cultures.

H. annosum, S-type, strain 143-5632 VIIa, and *C. cucumerinum*, strain CW1 were used for the bioassays. Spore suspensions were obtained from 2–3-week-old sporulating cultures of *H. annosum* and *C. cucumerinum* by adding 2 ml liquid Hagem medium, agitating the surface of the culture with an inoculating needle. The spore suspensions were then filtered through several layers of autoclaved nylon mesh. The final concentration of the spore suspension was adjusted to about 1×10^5 ml^{-1} using a haematocytometer.

3.3. Bioassays

The compounds isolated from the liquid culture were dissolved in methanol to a final concentration of 25 mg ml^{-1}, and used in the following antifungal tests.

3.3.1. Agar plate test

Six evenly spaced wells, 8 mm in diameter and 4 mm in depth, were made in 9 cm diameter Petri dishes filled with approx. 15 ml of Hagem agar to a depth of 7 mm.

Four of the wells were filled with a compound under test, dissolved in 0.05 ml of methanol, a fifth well contained methanol only and the sixth well was unfilled. Spore suspensions of *H. annosum* and *C. cucumerinum* were spread over the surface of the Hagem agar. The plates were incubated in the dark at 25°C for three and five days, respectively. Each set of dishes was replicated three times. Growth inhibition was measured as the width of the clear zone around the edge of the well that lacked mycelial growth.

3.3.2. Microtitre plate

A 96-well flat-bottomed microtitre plate was sterilised by exposing it to UV light over night. The antifungal test was carried out in aseptic conditions. Each well contained 80 μl of Hagem liquid medium and 10 μl of spore suspension in sterile water. Activity was measured for the different compounds using three replications, each containing 6.3, 12.5 and 25.0 μg of the compound. In the controls, the compounds were omitted, but the same volumes of alcohol were applied. The microtitre plate was incubated at 20°C for 24 h, and then examined under the microscope (objective 25×) once a day for at least four days.

3.4. HPLC separation

Analytical HPLC separations were carried out on a Merck–Hitachi liquid chromatography system with a Nova-Pak C18 Radial-Pak cartridge (8×100 mm, Waters Associates). The separated components were monitored by UV at 254 nm. The mobile phase (mixture by volume of MeCN and aq. 0.01 M sodium phosphate buffer, pH 2.8) consisted of a linear gradient ranging from 5 to 70% MeCN for 40 min. The flow rate was 1.0 ml min^{-1}. Preparative HPLC separations were carried out on a Gilson liquid chromatography system with a Prep Nova-Pak HR C18 cartridge (25×100 mm, Waters Associates). The eluate was monitored with a UV-detector at 254 nm.

3.5. Isolation

The liquid culture filtrate (18 l) of *P. sanguinolentus* was subjected to solid phase extraction in portions of 6 l on a reversed phase column (10×15 cm). The column was eluated with 1 l of H_2O, 20% EtOH, 50% EtOH and 95% EtOH, successively.

Half of the 20% EtOH and 50% EtOH fractions were concentrated to small volumes, diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ extracts were dried with anhydrous sodium sulphate and evaporated to dryness. Silica gel column chromatography of the evaporated 20% EtOH fraction with $CHCl_3$–MeOH 50:1 as eluent, yielded **5** (1.6 g) and **4** (0.64 g). Silica gel column chromatography ($CHCl_3$–MeOH 50:1 as eluent) and preparative HPLC (60% MeCN as eluent, 5.0 min^{-1} flow rate) of the evaporated 50% EtOH fraction yielded **1** (50 mg) and **2** (8 mg).

The second half of the 20% EtOH fraction was evaporated to dryness and separated with preparative HPLC using 0.01 M sodium phosphate buffer (pH 2.8)–MeCN 9:1 as eluent and a flow rate of 5.0 ml min^{-1}. One subfraction was collected, evaporated to dryness, dissolved in H_2O and purified from inorganic salts by solid phase extraction on a Sep-Pak C18 cartridge (Waters) with H_2O and 20% CH_3OH as eluents. Evaporation of the 20% CH_3OH eluent yielded **3** (3 mg).

The combined break-through and H_2O-fraction from the initial solid phase extraction was evaporated and **6** (50 mg) was obtained from the residue by successive chromatographic separations on two silica gel columns (4×4 and 3×20 cm) with $CHCl_3$–MeOH–H_2O 10:5:1 as the mobile phase.

3.5.1. 2-Methyl-4-pyrone (*4*)

Colourless liquid, UV λ_{max} (MeOH) nm: 249, EIMS *m/z* (rel. int.): 110 (100) $[M]^+$, 82 (20), 69 (39), 54 (9), 43 (21), HR-EIMS *m/z*: 110.0363 $[M]^+$ (calcd for $C_6H_6O_2$, 110.0368), ^1H NMR ($CDCl_3$) δ 2.25 (3H, *s*, H-2′), 6.16 (1H, *d*, $J=2.5$ Hz, H-3), 6.27 (1H, *dd*, $J=5.9$ and 2.5 Hz, H-5), 7.67 (1H, *d*, $J=5.9$ Hz, H-6), ^{13}C NMR spectra (see Table 1).

3.5.2. 2-Methyl-5,6-dihydro-4-pyrone (*5*)

Colourless liquid, UV λ_{max} (MeOH) nm: 267, EIMS *m/z* (rel. int.): 112 (87) $[M]^+$, 84 (80), 69 (100), 43 (67), HR-EIMS *m/z*: 112.0535 $[M]^+$ (calcd for $C_6H_8O_2$, 112.0524), ^1H NMR ($CDCl_3$) δ 1.95 (3H, *s*, H-2′), 2.45 (2H, *t*, $J=6.8$, H-5), 4.41 (2H, *t*, $J=6.8$, H-6), 5.28 (1H, *s*, H-3), ^{13}C NMR spectra (see Table 1).

3.5.3. 2-Methyl-4-hydroxypyridine (*6*)

UV λ_{max} (MeOH) nm: 234, 249 (sh), EIMS *m/z* (rel. int.): 109 (100) $[M]^+$, 94 (8), 80 (20), 70 (25), HR-EIMS *m/z*: 109.0527 $[M]^+$ (calcd for C_6H_7NO, 109.0528), ^1H NMR (CD_3OD) δ 2.35 (3H, *s*, H-2′), 6.36 (1H, *d*, $J=2.5$ Hz, H-3), 6.41 (1H, *dd*, $J=6.8$ and 2.5 Hz, H-5), 7.72 (1H, *d*, $J=6.8$ Hz, H-6), ^1H NMR (H_2O/D_2O) δ 2.29 (3H, *s*, H-2′), 6.34 (1H, *d*, $J=2.4$ Hz, H-3), 6.38

Table 1
^{13}C NMR chemical shifts (δ) for **4**, **5** (in $CDCl_3$) and **6** (in CD_3OD)

C	4	5	6
2	166.7	174.5	151.4
2′	20.0	21.0	19.2
3	115.5	105.3	116.0
4	179.3	192.0	181.5
5	116.6	35.5	117.0
6	155.4	68.0	139.8

(1H, *dd*, $J = 7.2$ and 2.4 Hz, H-5), 7.69 (1H, *d*, $J = 7.2$ Hz, H-6), 11.7 (*br s*, NH), ^{13}C NMR spectra (see Table 1).

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Four alkaloids from *Annona cherimola*

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Abstract

Four alkaloids, annocherine A, annocherine B, cherianoine, and romucosine H, along with one known alkaloid, artabonatine B, were isolated from the MeOH extract of the stems of *Annona cherimola*. Their structures were identified on the basis of both analysis of their spectral data and from chemical evidence. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Annona cherimola*; Annonaceae; Annocherine A; Annocherine B; Cherianoine; Romucosine H

1. Introduction

Annona cherimola Mill. (Annonaceae), a subtropical fruit tree indigenous to Ecuador and Peru, is cultivated in southern Taiwan. It has been used in folk medicine for the treatment of skin disease, especially for boils (Kan, 1979). Previously, we isolated 88 compounds, including two novel compounds, cherimoline (Chen et al., 1997a) and cherinonaine (Chen et al., 1998a) and 11 kauranes (Chen et al., 1998c), three lignans (Chen et al., 1998c), eight amides (Chen et al., 1998b), two acetogenins (Chen et al., 1999a), one lactam amide (Chen et al., 1997b), two purines (Chen et al., 1997b, 1999b), nine steroids (Chen et al., 1997b, 1999b), 30 alkaloids (Chen et al., 1997b, 1999b), one *p*-quinone (Chen et al., 1999b), and 19 benzenoids (Chen et al., 1999b) from different parts of this plant. As part of our continuing investigation on the phytochemical and bioactive compounds of Formosan Annonaceous plants, four new alkaloids, annocherine A (**1**), annocherine B (**2**), cherianoine (**3**), and romucosine H (**4**), together with one known alkaloid, artabonatine B, were obtained by systematic extraction and isolation from the stems of *A. cherimola*. Artabonatine B was also isolated for the first time from this source.

2. Results and discussion

Annocherine A (**1**) was obtained as a yellow needles, $[\alpha]_D^{24}$ +135.0° (*c* 0.1, $CHCl_3$), positive to Dragendorff's test. The molecular formula, $C_{17}H_{15}O_4N$, was confirmed by high-resolution EIMS measurement (*m*/*z* 297.1014 $[M]^+$, calc. 297.1001). The presence of a 6,7,4′-oxygenated benzylisoquinoline skeleton in the molecule was deduced by its UV (absorption maxima at λ 260, 300, and 330 nm) (Botega et al., 1993). A bathochromic shift of the UV spectrum with the addition of alkali and the IR absorption at 3400 cm^{-1}, suggested the presence of a phenolic function. The ^1H NMR spectrum (Table 1) indicated the presence of signals at δ 3.94 and 4.45 corresponding to one methoxy group and one benzylic carbinol proton, along with two doublets at δ 8.29 and 7.51 (J = 6.0 Hz, each one proton), and six aromatic protons, including two singlets at δ 7.88 and 7.16 (each one proton), and two doublets at δ 7.01 and 6.65 (J = 8.8 Hz, each two protons). The above mentioned data indicated the presence of a C-6,7,4′ and C-α tetra-*O*-substituted benzylisoquinoline. The complete proton assignments of **1** were established by COSY and NOESY (Fig. 1) experiments. Proton 5 showed significant correlations to OMe-6 and H-4, and H-α displayed correlations with H-8, H-2′ and H-6′ in the NOESY spectrum. The substitution of four oxygen-bearing functional groups was determined and located. According to the literature (Bojadziev et al., 1987; Corey and Helal, 1996), a positive $[\alpha]_D$ value indicates that C-α possesses an *S* configuration. Fifteen aromatic carbon atoms were observed between δ 156.3 and 105.1, a methoxy carbon at δ 56.4, and

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Table 1
^{1}H NMR spectral data of alkaloids **1** and **2** (400 MHz, δ in ppm, J in Hz, $CDCl_3$)

Proton	1	2
3	8.29 (1H, *d*, $J=6.0$)	8.24 (1H, *d*, $J=5.6$)
4	7.51 (1H, *d*, $J=6.0$)	7.46 (1H, *d*, $J=5.6$)
5	7.16 (1H, *s*)	7.03 (1H, *s*)
8	7.88 (1H, *s*)	7.67 (1H, *s*)
2′ and 6′	7.01 (2H, *d*, $J=8.8$)*[a]	7.19 (2H, *d*, $J=8.4$)[a]
3′ and 5′	6.65 (2H, *d*, $J=8.8$)[a]	6.67 (2H, *d*, $J=8.4$)[a]
α	4.45 (1H, *s*)	5.78 (1H, *s*)
OMe-6	3.94 (3H, *s*)	3.96 (3H, *s*)
OMe-α	–	3.36 (3H, *s*)

[a] AA′BB′ system.

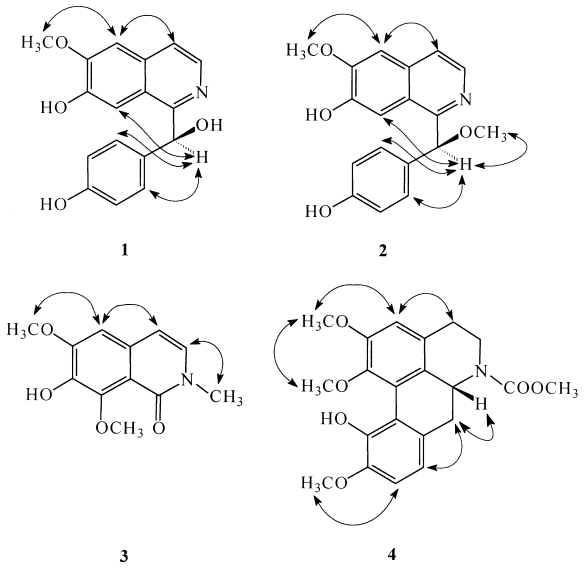


Fig. 1. 2D NOESY correlations for alkaloids **1, 2, 3**, and **4**.

a signal for a methine carbon at δ 74.1 in the ^{13}C NMR spectrum (Table 2) further confirmed the structure of **1**. The EIMS revealed significant key fragments at m/z 174 ($[C_{10}H_7O_2N+H]^+$) and 123 ($[C_7H_6O+OH]^+$), which represented an isoquinoline moiety and a benzyl moiety through cleavage between C-1 and C-α, respectively. Therefore, the structure of **1** was determined as 1(*S*)-hydroxy - *p* - hydroxybenzyl - 6 - methoxy - 7 - hydroxyisoquinoline, which we named annocherine A (**1**). Annocherine A (**1**) is the second example of a C-α hydroxy benzylisoquinoline from natural sources (Botega et al., 1993). Although Botega et al. reported that C-α hydroxy benzylisoquinolines isolated from natural sources were not stable and were oxidized in air to the corresponding ketones (Botega et al., 1993), the new alkaloid **1** is stable and is not oxidized spontaneously in air.

Annocherine B (**2**) was obtained as a yellow amorphous powder, $[\alpha]_D^{24}$ +115.0° (*c* 0.1, $CHCl_3$), positive to Dragendorff's test. The molecular formula, $C_{18}H_{17}O_4N$, was determined by high-resolution EIMS measurement (m/z 311.1174 $[M]^+$, calc. 311.1158). The UV and IR

Table 2
^{13}C NMR spectral data of alkaloids **1** and **2** (100 MHz, δ in ppm, $CDCl_3$)

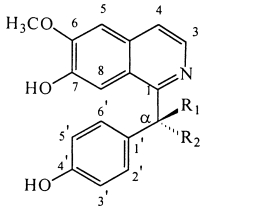
Carbon	1	2
1	156.3 (*s*)	141.5 (*s*)
3	133.3 (*d*)	133.8 (*d*)
4	120.2 (*d*)	120.0 (*d*)
4a	137.3 (*s*)	138.4 (*s*)
5	106.9 (*d*)	107.7 (*d*)
6	151.3 (*s*)	156.4 (*s*)
7	150.2 (*s*)	156.2 (*s*)
8	105.1 (*d*)	106.4 (*d*)
8a	122.3 (*s*)	122.3 (*s*)
1′	130.2 (*s*)	131.0 (*s*)
2′ and 6′	128.5 (*d*)	128.1 (*d*)
3′ and 5′	115.5 (*d*)	115.2 (*d*)
4′	145.3 (*s*)	147.0 (*s*)
α	74.1 (*d*)	85.4 (*d*)
6-OMe	56.4 (*q*)	56.0 (*q*)
α-OMe	–	57.2 (*q*)

absorptions of **2** were very similar to them of **1**. The ^{1}H and ^{13}C NMR spectra of **2** were also similar to those of **1**, except for the presence of a methoxyl signal, as revealed by the signals at δ 3.36 in the ^{1}H NMR (Table 1) and δ 57.2 in the ^{13}C NMR spectra (Table 2). The position of the methoxyl group was confirmed by ^{1}H NMR spectroscopic analysis, in which the α proton was shifted downfield to δ 5.78 as compared to **1**. This indicated that the methoxyl group was at the α carbon. The complete assignments of **2** were established by COSY and NOESY (Fig. 1) experiments. The positive $[\alpha]_D$ value suggested that C-α had an *S* configuration (Bojadziev et al., 1987; Corey and Helal, 1996). The EIMS also revealed significant fragments at m/z 174 ($[C_{10}H_7O_2N+H]^+$) and 137 ($[C_7H_6O+OCH_3]^+$), corresponding to an isoquinoline moiety and a benzylic moiety. Therefore, the structure of **2** was determined as 1(*S*)-methoxy-*p*-hydroxybenzyl-6-methoxy-7-hydroxyisoquinoline and named annocherine B (**2**).

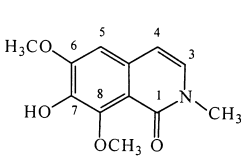
Cherianoine (**3**) was obtained as a white needles from $CHCl_3$. The molecular formula, $C_{12}H_{13}O_4N$, was established by high-resolution EIMS measurement (m/z 235.0845 $[M]^+$, calc. 235.0844). Its UV spectrum (λ_{max} 220, 260, 270 and 300 nm) indicated a 6,7,8-oxygenated isoquinolone skeleton (Castedo et al., 1981). A carbonyl group in the isoquinolone was confirmed by an IR band at 1685 cm^{-1} and a signal at δ 161.3 in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum displayed two typical doublets at δ 7.00 and 6.39 ($J=7.2$ Hz, each one proton) for H-3 and H-4, and an important amidic methyl at δ 3.55. Two methoxyl signals at δ 4.05 and 4.01 (each 3H) and a singlet at δ 6.88 (1H) needed to be assigned. These data indicated the presence of a 6,7,8-tri-*O*-substituted isoquinolone. COSY and NOESY (Fig. 1) experiments established the complete assignments of **3**. Proton 5 displayed significant correlations to OMe-6 and H-4,

and H-3 was correlated with the *N*-methyl and H-4 in the NOESY spectrum. The absence of NOE correlations between the two methoxy groups and a hydrogen bonding signal at δ 10-15 (for an 8-OH group) indicated that the hydroxyl group was positioned at C-7, and the methoxy group at C-8. Eight aromatic carbon atoms between δ 150.6 and 105.4, two methoxy carbons at δ 56.4 and 52.5, an *N*-methyl carbon at δ 37.8, and a carbonyl carbon at δ 161.3 were consistent with structure **3**. The ^{13}C NMR spectrum further supported this assignment. Alkaloid **3** was named cherianoine.

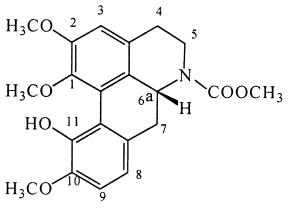
Romucosine H (**4**) was obtained as a brown amorphous powder, $[\alpha]_D^{24}$ −43.0° (*c* 0.01, $CHCl_3$), and was positive to Dragendorff's test. The molecular formula, $C_{21}H_{23}O_6N$, was deduced by high-resolution EIMS measurement (*m*/*z* 385.1532 $[M]^+$, calc. 385.1525). The presence of a 1,2,9,10-oxygenated aporphine skeleton in the molecule was deduced by its UV spectrum (λ_{max} 222, 270 and 310 nm) (Chalandre et al., 1985). IR bands at 3500 and 1630 cm^{-1} and a signal at δ 155.6 in the ^{13}C NMR spectrum indicated that a hydroxyl group and a carbamate moiety were present (Chen et al., 1996). The ^{1}H NMR spectrum indicated the presence of singlets at δ 3.92, 3.89, 3.76, and 3.70 corresponding to three methoxy groups and a *N*-(methoxycarbonyl) group. Three aromatic protons, two doublets at δ 6.88 and 6.84 (J = 8.0 Hz, each 1H), and a singlet at δ 6.73 (1H) were observed. The seven proton signals at δ 4.58 (1H, *dd*, J = 14.0, 4.5 Hz), δ 4.45 (1H, *m*), and δ 3.05-2.63 (5H, *m*) for the aliphatic protons were consistent with the features of an *N*-(methoxycarbonyl) aporphine (Chen et al., 1996; Chang et al., 2000). Two significant downfield signals at δ 4.58 (1H, *dd*) for H-6a and δ 4.45 (1H, *m*) for H-5a indicated an electron-withdrawing group bonded to the nitrogen atom. COSY and NOESY (Fig. 2) experiments were further measured to deduce the structure of **4**. Significant correlated sequences of OMe-1/OMe-2/H-3/H-4/H-5 and H-6a/H-7/H-8/H-9/OMe-10, were observed in the NOESY spectrum. The remaining hydroxyl should therefore be located at 11-position. The ^{13}C NMR spectrum showed 12 aromatic carbons between δ 151.3 and 111.0, three methoxyls at δ 62.0, 56.1, and 55.8, three methylenes at δ 42.6, 38.2, and 29.1, a carbonyl carbon at δ 155.6, a carboxylic methyl carbon at δ 52.6, and a methine at δ 54.0, which were consistent with the structure of **4**. Treatment of (−)-norisocorydine with triethylamine and methyl chlorocarbonate gave a compound that had an mp and TLC, as well as UV, IR, and ^{1}H NMR spectral data identical to **4**. Thus, the structure of **4** was determined as illustrated and named romucosine H.



	R_1	R_2
1	OH	H
2	OCH_3	H



3



4

The identity of the known alkaloid was verified by comparing UV, IR, ^{1}H NMR, ^{13}C NMR and MS spectral data with the published values of artabonatine B (Hsieh et al., 1999).

N-(Methoxycarbonyl) aporphinoid alkaloids like romucosine were shown to have significant antiplatelet aggregation activity (Wu et al., 1998). The *N*-(methoxycarbonyl) aporphinoid alkaloids cathafiline and cathaformine strongly inhibited platelet aggregation induced by AA (arachidonic acid) (Chen et al., 1999b; Wu et al., 1998). The mechanism of antiplatelet aggregation effects of these aporphine alkaloids are apparently different from aspirin, which is known to be a cyclooxygenase inhibitor (Chen et al., 1999b), and will be further investigated.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Melting points were determined using a Yanagimoto micro-melting point apparatus and were uncorrected. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra (in $CDCl_3$) were recorded with Varian NMR spectrometers, using TMS as internal standard. LRFABMS and LREIMS spectra were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC/MS spectrometer having a direct inlet system. HRFABMS spectra were measured on a JEOL JMS-HX 110 mass spectrometer. Si gel 60 (Macherey-Nagel, 230-400 mesh) was used for column chromatography, precoated Si gel plates (Macherey-Nagel, SIL G-25 UV_{254}, 0.25 mm) were used for analytical TLC, and precoated Si gel plates (Macherey-Nagel, SIL G/UV_{254}, 0.25 mm) were used for preparative TLC. The spots were detected by spraying with Dragendorff's reagent or 50% H_2SO_4 and then heating on a hot plate.

3.2. Plant material

A. cherimola was collected from Chia-Yi City, Taiwan, in September 1996, and identified by Dr. Hsin-Fu

Yen, National Museum of Natural Science, Taichung, Taiwan. A voucher specimen is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

3.3. Extraction and isolation

Fresh stems (4.0 kg) were extracted repeatedly with MeOH at rt. The combined MeOH extracts were evaporated under reduced pressure and partitioned to yield $CHCl_3$ and aq. extracts. The bases in the $CHCl_3$ solution were extracted with 3% HCl to leave the acidic portion (Part A) and $CHCl_3$ solution (Part B). The acidic portion (Part A) was basified with NH_4OH and then extracted with $CHCl_3$. The $CHCl_3$ solution was dried and evaporated to leave a brownish viscous residue (2.0 g). The residue was placed on a silica gel column and eluted with ethyl acetate gradually enriched with MeOH to afford 20 fractions. Fr. 6 (0.3 g) eluted with *n*-hexane–Me_2CO (10:1) was separated using silica gel CC and prep. TLC [*n*-hexane–Me_2CO (15:1)] and gave romucosine H (**4**) (4 mg). Fr. 9 (0.4 g) eluted with $CHCl_3$–MeOH (10:1) was repeatedly subjected to silica gel CC and prep. TLC [$CHCl_3$–MeOH (18:1)] and gave (−)-artabonatine B (6 mg). Cherianoine (**3**) (5 mg) was obtained from Fr. 11 (0.2 g) by means of silica gel CC eluting with $CHCl_3$–MeOH 9:1. Fr. 13 (0.1 g) eluted with $CHCl_3$–MeOH (8:1) was further separated using silica gel CC and prep. TLC [$CHCl_3$–MeOH (12:1)]and gave annocherine A (**1**) (3 mg) and annocherine B (**2**) (5 mg), respectively.

3.3.1. Annocherine A (*1*)

Yellow yellow needles, mp 156–158°C, $[\alpha]_D^{24}$ +135.0° (*c* 0.1, $CHCl_3$). UV λ_{max} (MeOH) nm (log ε): 260 (3.41), 300 (3.10), 330 (3.52). IR (KBr) ν_{max} cm^{-1}: 3400 (OH). EIMS *m/z* (rel. int.): 297 ($[M]^+$, 12), 296 (70), 280 (100), 263 (38), 249 (20), 174 (28), 137 (95), 123 (60), 107 (35). ^1H NMR ($CDCl_3$) δ: see Table 1. ^{13}C NMR ($CDCl_3$) δ: see Table 2. HREIMS *m/z*: 297.1014 (calcd for $C_{17}H_{15}O_4N$ 297.1001).

3.3.2. Annocherine B (*2*)

Yellow amorphous powder, mp 196–198°C, $[\alpha]_D^{24}$ +115.0° (*c* 0.1, $CHCl_3$). UV λ_{max}(MeOH) nm (log ε): 260 (3.32), 300 (3.11), 331 (3.60). IR (KBr) ν_{max} cm^{-1}: 3400 (OH). EIMS *m/z* (rel. int.): 311 ($[M]^+$, 21), 296 (35), 280 (34), 264 (5), 236 (11), 174 (15), 137 (100), 121 (41), 107 (31). ^1H NMR ($CDCl_3$) δ: see Table 1. ^{13}C NMR ($CDCl_3$) δ: see Table 2. HREIMS *m/z*: 311.1174 (calcd for $C_{18}H_{17}O_4N$: 311.1158).

3.3.3. Cherianoine (*3*)

White needles, mp 122–124°C. UV λ_{max} (MeOH) nm (log ε): 220 (4.51), 260 (4.41), 270 (3.35), 330 (3.39). IR (KBr) ν_{max} cm^{-1}: 3200 (OH), 1685 (C=O). EIMS *m/z* (rel. int.): 235 ($[M]^+$, 21), 218 (35), 204 (34), 178 (5), 164 (90), 136 (100), 69 (21), 55 (16). ^1H NMR ($CDCl_3$) δ: 7.00 (1H, *d*, *J* = 7.2 Hz, H-3), 6.88 (1H, *s*, H-5), 6.39 (1H, *d*, *J* = 7.2 Hz, H-4), 4.05 (3H, *s*, OMe-6), 4.01 (3H, *s*, OMe-8), 3.55 (*N*-Me). ^{13}C NMR ($CDCl_3$) δ: 161.3 (*s*, C-1), 150.6 (*s*, C-6), 143.5 (*s*, C-7), 143.1 (*s*, C-8), 132.8 (*s*, C-8a), 131.4 (*s*, C-4a), 131.1 (*d*, C-3), 106.1 (*d*, C-5), 105.4 (*d*, C-4), 56.4 (*q*, OMe-8), 52.5 (*q*, OMe-6), 37.8 (*q*, *N*-Me). HREIMS *m/z*: 235.0845 (calcd for $C_{12}H_{13}O_4N$: 235.0844).

3.3.4. Romucosine H (*4*)

Brown amorphous powder, mp 230–233°C, $[\alpha]_D^{24}$ −43.0° (*c* 0.01, $CHCl_3$). UV λ_{max} (EtOH) nm (log ε): 222 (4.39), 270 (4.19), 310 (3.80). IR (KBr) ν_{max} cm^{-1}: 3500 (OH), 1630 (C=O). EIMS *m/z* (rel. int.): 385 ($[M]^+$, 90), 326 (70), 312 (100), 296 (76), 284 (20), 267 (45), 252 (21), 239 (15). ^1H NMR ($CDCl_3$) δ: 6.88 (1H, *d*, *J* = 8.0 Hz, H-8), 6.84 (1H, *d*, *J* = 8.0 Hz, H-9), 6.73 (1H, *s*, H-3), 4.58 (1H, *dd*, *J* = 14.0, 4.5 Hz, H-6a), 4.45 (1H, *m*, H-5a), 3.92 (3H, *s*, OMe-2), 3.89 (3H, *s*, OMe-10), 3.76 (3H, *s*, *N*-COOMe), 3.70 (3H, *s*, OMe-1). ^{13}C NMR ($CDCl_3$) δ: 155.6 (*s*, *N-CO*OMe), 151.3 (*s*, C-1), 149.4 (*s*, C-2), 144.1 (*s*, C-10), 142.0 (*s*, C-11), 130.2 (*s*, C-7a), 130.1 (*s*, C-3a), 129.8 (*s*, C-11a), 125.6 (*s*, C-3b), 120.1 (*s*, C-11b), 118.8 (*d*, C-3), 111.7 (*d*, C-8), 111.0 (*d*, C-9), 62.0 (*q*, OMe-1), 56.1 (*q*, OMe-10), 55.8 (*q*, OMe-2), 54.0 (*d*, C-6a), 52.6 (*q*, *N*-COO*Me*), 42.6 (*t*, C-5), 38.2 (*t*, C-7), 29.1 (*t*, C-4). HREIMS *m/z*: 385.1532 (calcd for $C_{21}H_{23}O_6N$: 385.1525).

3.3.5. Preparation of N-(methoxycarbonyl) norisocorydine (Romucosine H) (*4*)

(−)-Norisocorydine (20 mg) in dry CH_2Cl_2 (10 ml) was treated with triethylamine (5 μl), with stirring at 0°C, for 10 min, and then methyl chlorocarbonate (2 ml) was slowly added. The reaction mixture was stirred for 10 min, and H_2O was added to quench excess reagent. The mixture was partitioned with $CHCl_3$ and then passed through a disposable pipette (0.6×6 cm) containing silica gel (230–400 mesh) and eluted with 10 ml of $CHCl_3$. Elution with $CHCl_3$ afforded a brown amorphous powder (5 mg) that was identified by comparison with **4** (mixed mp, co-TLC, UV, IR, ^1H and ^{13}C NMR).

3.3.6. Artabonatine B

Yellow powder, $[\alpha]_D^{25}$ −122.4° (*c* 0.80, $CHCl_3$), UV λ_{max}(EtOH) nm (log ε): 214 (4.21), 256 (4.18), 295 (3.85), 325 (3.54). MS, and ^1H and ^{13}C NMR data were identical with published data (Hsieh et al., 1999).

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Phenolic constituents from the core of Kenaf (*Hibiscus cannabinus*)

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Abstract

Four lignans, boehmenan H {2-(4-hydroxy-3-methoxyphenyl)-5-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-3-hydroxy methyl-7-methoxybenzodihydrofuran}, boehmenan K {2-(4-hydroxy-3-methoxyphenyl)-5-[3-(4-hydroxycinnamoyloxy)-1-propenyl] -3-(4-hydroxy-3-methoxycinnamoyloxymethyl)-7-methoxybenzodihydrofuran}, *threo*-carolignan H {*threo*-1-(4-hydroxy-3-methoxyphenyl)-2-{4-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-2-methoxyphenoxy}-1,3-propanodiol}, and *threo*-carolignan K {*threo*-1-(4-hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxycinnamoyloxy)-2-{4-[3-(4-hydroxycinnamoyloxy)-1-propenyl]-2-methoxy phenoxy}-1-propanol} as well as several other lignans, aldehydes and a tyramine derivative were isolated from the acetone extract of core of kenaf (*Hibiscus cannabinus*). All the structures were established by spectroscopic methods. The hitherto unreported ^{13}C NMR spectra of some compounds are also presented and discussed. 2D NMR techniques have allowed the revision of certain previously reported ^{13}C NMR assignments of some scarce naturally occurring compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Kenaf (*Hibiscus cannabinus*); Malvaceae; Lignans; Boehmenan H; Boehmenan K; *threo*-Carolignan H; *threo*-Carolignan K

1. Introduction

Kenaf (*Hibiscus cannabinus*) is an annual dicotyledonous herbaceous African plant, but also well known in other geographical areas. In Portugal, the variety Salvador has been experimentally cultivated with good results. It has already been shown that this plant has good features to be used in several applications, such as antidotes to poisoning with chemicals (acids, alkali, pesticides) and venomous mushrooms in traditional medicine (Chifundera et al., 1994) and as source of fibres to pulp and paper industries (Pande and Roy, 1996). Our previous studies on the chemical composition of the light petroleum extract of bark and core of this plant resulted in the isolation of several sterols, a triterpene, and a long chain fatty ester (Sêca et al., 2001). In the present study we report on the isolation and structure elucidation of four new lignans, designated as boehmenan H **1**, boehmenan K **2**, *threo*-carolignan K **3** and *threo*-carolignan H **4**, as well as 16 other compounds from the acetone extract of the core of *Hibiscus cannabinus*. The detailed NMR studies of these compounds **5**–**20** allowed us to correct some literature ^{13}C NMR assignments for boehmenan **5**, *erythro*- and *threo*-carolignan E, **6** and **7**, and to come forward for the first time with the ^{13}C assignments for boehmenan D **8** and *erythro*-carolignan F **9**.

2. Results and discussion

The core of *Hibiscus cannabinus* was finely ground and extracted successively with light petroleum and acetone. The acetone extract was fractionated by its solubility in chloroform and acetone, but almost all the amount of this extract was soluble in chloroform. In this way, only the latter fraction was considered; it was successively submitted to column and preparative thin-layer silica gel chromatographies to give four new lignans **1**–**4** and 16 known compounds **5**–**20** (Fig. 1).

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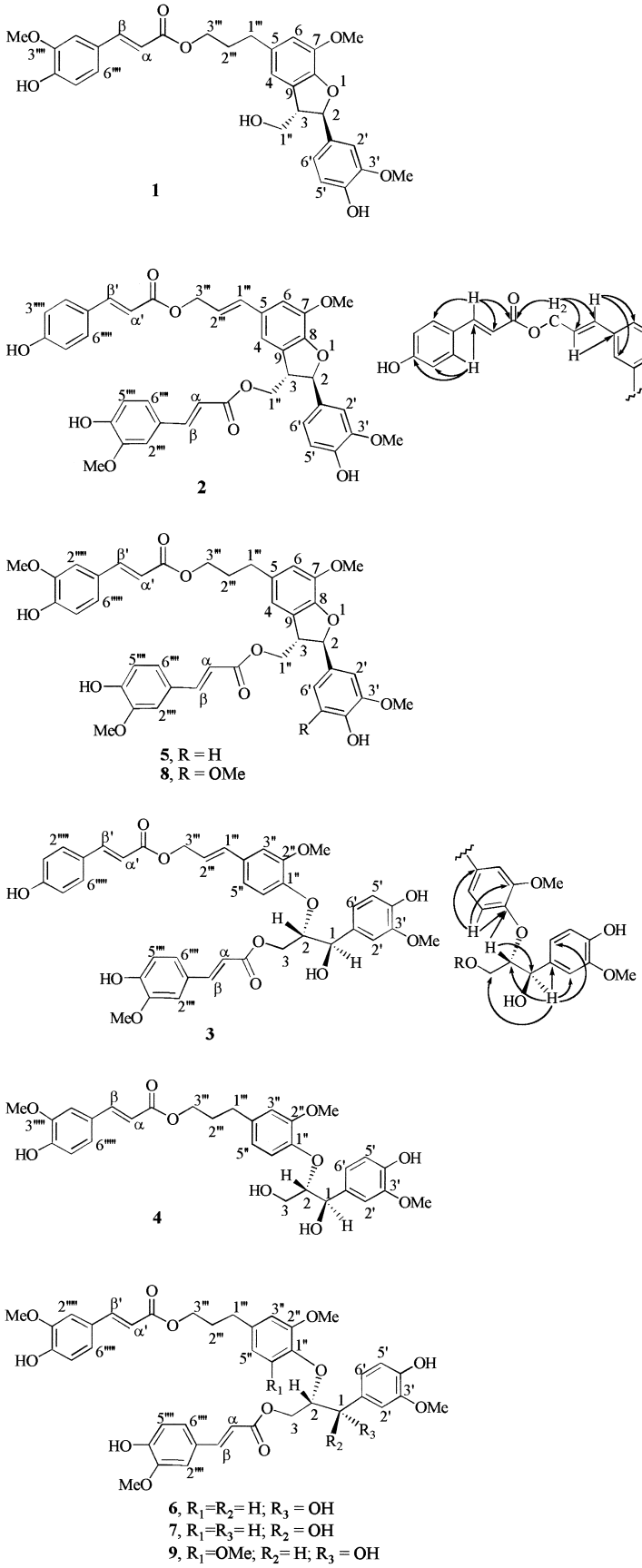


Fig. 1. Lignans **1**–**9** from *Hibiscus cannabinus* and major connectivities observed for **2** and **3**.

Due to the complexity and similarity of the ^1H and ^{13}C NMR spectra of compounds **1**, **2**, **5** and **8** and the available large amount of **5**, it was decided to start with **5** for the elucidation of these compounds. In the ^1H NMR spectrum of **5** four different spin systems can be found. One set of ABX signals corresponding to three methylene groups appear at δ_H 1.97–2.06 (2H, *m*), 2.71 (2H, *t*, $J=7.6$ Hz) and 4.23 (2H, *t*, $J=6.5$ Hz). In the HMBC spectrum of **5**, correlations between the proton resonance at δ 4.23 with that of the carbon at δ 167.3 and also between that at δ_H 2.71 with carbon resonances of an aromatic ring (δ_C 112.4, 116.1 and 134.9) were observed. From these results one can conclude that the referred three methylene groups (δ_C 30.7, 32.1 and 63.7) connect an aromatic ring to an ester group.

In the ^1H NMR spectrum of **5** an ABC set of signals can also be found. They are due to proton resonances of a methine group [δ_H 3.84–3.93 (*m*); δ_C 50.7], one benzylic methine group [δ_H 5.50 (*d*, $J=7.8$ Hz); δ_C 88.9] and also a methylene group [δ_H 4.42 (1H, *dd*, $J=7.7$ and 11.2 Hz) and 4.59 (1H, *dd*, $J=5.1$ and 11.2 Hz); δ_C 65.4]. The inequivalence of these two methylene protons and the multiplicity of their resonances indicate that this methylene group is attached to a chiral methine carbon. In the HMBC spectrum of **5** these methylene proton resonances showed connectivities with that of the carbon of another ester group (δ_C 167.0).

The aromatic region of the ^1H NMR spectrum of **5** showed two AB sets of signals at δ_H 6.23, 7.49 (2H, *2d*, $J=15.9$ Hz) and δ_H 6.30, 7.60 (2H, *2d*, $J=15.9$ Hz). The coupling constants of these two vinylic systems indicate that they have a *trans* configuration. Correlations between the proton resonances of these vinylic systems with the already referred carbon resonances of ester groups, indicate that compound **5** have two α,β-unsaturated ester moieties in their structure. The presence of these moieties was also confirmed by the presence of a band at 1700 cm^{-1} in the IR spectrum of **5**.

The ^1H NMR spectrum of **5** showed three singlets (δ_H 5.65, 5.91 and 5.92, one proton each) which disappear after shaking its $CDCl_3$ solution with D_2O, suggesting the presence of three phenolic protons involved in hydrogen bonds.

The presence of ester groups in the structure of compound **5** lead us to consider a transesterification reaction in order to aid its structure elucidation. The transesterification reaction of **5** with sodium methoxide in methanol lead to the formation of three products **21**–**23**. One of these compounds was identified as methyl ferulate **21**, suggesting the presence of esters of this acid in the structure of compound **5**. The ^1H NMR spectrum of the second compound **22** obtained in this reaction showed an aliphatic region similar to that already described for **5**. The analysis of the ^1H and ^{13}C NMR spectra of **22**, the connectivities found in their HMBC spectrum (Fig. 2) and the molecular ion (*m*/*z* 360) present in the EIMS

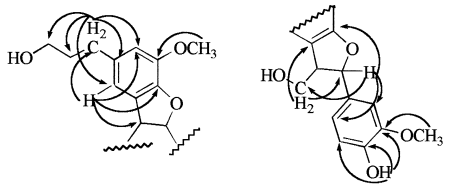
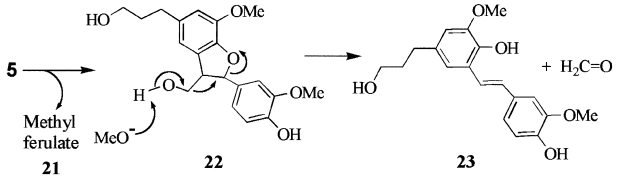


Fig. 2. Major HMBC connectivities observed for **22**.

allowed to establish its structure as 3-hydroxymethyl-5-(3-hydroxypropyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxybenzodihydrofuran **22** (Takemoto et al., 1975). The coupling constant $J_{H2\text{-}H3}=7.8$ Hz indicates that these protons have a *trans* configuration. The structure of the third compound obtained in the transesterification of **5** was identified as 2,4′-dihydroxy-3,3′-dimethoxy-5-(3-hydroxypropyl)stilbene **23**. The formation of this compound **23** in the base-catalysed transesterification reaction of **5** can be envisaged by the elimination of formaldehyde, induced by sodium methoxide, and consequent ring opening of the dihydrofuran moiety of compound **22** (Scheme 1). The formation of formaldehyde in this reaction was confirmed by GC–MS (*m*/*z* 30).

All the discussed spectroscopic data and the results obtained in the transesterification reaction of **5** support the assignment of its structure to boehmenan (Takemoto et al., 1975; Paula et al., 1995). The analysis of the ^1H, ^{13}C, COSY, HETCOR and HMBC spectra of **5** permits to correct the literature assignment of the carbon resonances of C-5, C-7, C-1′, C-4′, C-4′′′′ and C-4′′′′′.

The ^1H and ^{13}C NMR spectral data of compound **8** were very similar to those of **5** except for one additional methoxyl group signal in the case of **8**. The presence of one extra methoxyl group at the aromatic nucleus attached to the dihydrofuran ring of **8** simplified its ^1H NMR spectrum, compared with that of boehmenan **5**. In the case of **8** the resonances of H-2′ and H-6′ appear as a singlet (δ_H 6.67), since these protons are chemically equivalents. In the HMBC spectrum that proton resonance correlates with the carbon signals at δ_C 89.3 (C-2), 131.7 (C-1′), 134.7 (C-4′) and 147.0 (C-3′,5′). These results confirm the assignment of structure **8** to boehmenan D (Paula et al., 1995). The ^1H NMR spectral data of **8** have been already reported, but its ^{13}C NMR data is now being added. The assignments of all carbon



Scheme 1.

resonances of boehmenan D **8** were made by analysis of its HMBC spectrum.

In the case of compound **1**, its HR–EIMS spectrum shows a molecular ion at *m/z* 536.2053 and this is consistent with the molecular formula $C_{30}H_{32}O_9$ (calculated mass: 536.2046) (corroborated by its ^{13}C NMR spectrum). The ^1H and ^{13}C NMR spectra of **1** are similar to those of boehmenan **5** although lacking the resonances of one feruloyl moiety. The attachment site of the feruloyl residue was determinated through the connectivities found in its HMBC spectrum. The correlation between the proton resonance of H-3‴ and that of the carbonyl ester carbon indicates that the feruloyl is attached to C-3‴ of the propyl chain. The presence of the ester group in **1** was also confirmed by the presence of the band at 1700 cm^{-1} in its IR spectrum. Structure **1** was fully characterised and named as boehmenan H.

For compound **2** the molecular formula $C_{39}H_{36}O_{11}$ (calculated mass: 680.2257) was assigned by HR–FABMS (*m/z* 680.2285). The major differences between the ^1H NMR spectrum of compound **2** in relation with that of boehmenan **5** were the presence of one additional double bond and the disappearance of one methoxyl group. The signals at δ_H 1.97–2.06 (*m*, 2H) and 2.71 (*t*, 2H) due to the proton resonances of H-2″ and H-1″ in boehmenan **5** are now for **2** being replaced by others at δ_H 6.18–6.30 (*m*, 1H) and 6.66 (*d*, $J=15.9$ Hz, 1H), which are due to the vinylic protons H-2″ and H-1″. The coupling constants of these two protons indicate that this double bond has a *trans* configuration. The ^1H NMR spectrum of **2** also showed an AB set of signals at δ_H 6.84 and 7.45 (2H each), which are due to the resonances of a *p*-substituted phenyl ring. The attachment position of the coumaroyl moiety was established by the connectivities found in its HMBC spectrum (Fig. 1). All these spectroscopic data of **2** support the assignment of the structure depicted in Fig. 1; this compound is named boehmenan K. The coupling constant $J_{H2\text{-}H3}\sim 8$ Hz in the case of compounds **1** and **2** indicates that these protons have a *trans* configuration like boehmenan **5** and boehmenan D **8**.

The ^1H NMR spectrum of **3** showed basically the same resonances and coupling constants of boehmenan K **2** (see Experimental), but with a few differences. The absence of the dihydrofuran ring was suggested by the following data: (a) the presence of a protonated carbon resonance at δ_C 120.0 assigned to C-6″, confirmed by ^{13}C DEPT NMR spectrum, in place of the signal at δ_C 127.7 due to the quaternary carbon C-9 of boehmenan K **2**. In the HETCOR spectrum this carbon resonance correlates with the proton resonance at δ_H 7.11 (d, $J=8.1$ Hz), which belongs to a trisubstituted aromatic ring, confirmed from their COSY spectrum; (b) some important connectivities found in the HMBC spectrum, as shown in Fig. 1. The HR-FABMS of **3** showed a $M+H^+$ ion at *m/z* 699.2445 consistent with the molecular formula of $C_{39}H_{39}O_{12}$ (calculated mass: 699.2442). All this spectral data are compatible with the structure of compound **3** shown in Fig. 1, which is called carolignan K.

Due to the presence of two chiral carbons in the 1,2,3-trioxygenated propane moiety of carolignan K **3**, two possible diastereomers, *erythro* and *threo*, can be present. The coupling constant $J_{H1\text{-}H2}=7.5$ Hz indicates the presence of the *threo* diastereomer, and so these protons have a *trans* diaxial orientation (Shimomura et al., 1987; Abraham et al., 1991).

The ^1H and ^{13}C spectra of compounds **6** and **7** are very similar to that of carolignan K **3**, except the presence of an additional methoxyl group signal and the disappearance of those due of one double bond ($C_{1''}=C_{2''}$) in both compounds and the multiplicity of the H-1 resonance in the case of **6**. Due to the presence of this methoxyl group in **6** and **7** a trisubstituted aromatic ring is in place of the *p*-substituted phenyl ring in the case of **3**. The signals at δ_H 6.24 and 7.49 (2H, 2d, $J=15.9$ Hz), due to the vinylic protons H-2″ and H-1″ in calolignan K **3** were replaced by others in the aliphatic region due to the proton resonances of H-2″ and H-1″ in the case of **6** and **7**. All these data and the connectivities found in the HMBC spectrum of these two compounds allowed us to assign their structures to carolignan E isomers. It was also possible to correct the literature assignments of some quaternary carbon resonances, C-1′, C-3′, C-4′, C-1″, C-2″, C-4″, C-4‴ and C-4⁗ in the case of **6** and those of C-1′ and C-4″ in the case of **7** (Paula et al., 1995). The proton resonance of H-1 appears in the case of **7** as a doublet and its coupling constant with H-2 ($J_{H1\text{-}H2}=8.1$ Hz) indicates these two protons have a *trans* diaxial orientation. Thus, the structure of **7** was assigned to *threo*-carolignan E. However, in the case of **6**, H-1 appears as a slightly broad singlet, suggesting a small coupling constant with H-2. The structure of **6** is then assigned to *erythro*-carolignan E.

Compound **9** has an additional methoxyl group in comparison with the carolignan E isomers **6** and **7** and in this case H-3″ and H-5″ appear as a singlet (δ_H 6.46), because they are chemically equivalent. An analysis of the spectroscopic data (^1H, ^{13}C, COSY, HETCOR and HMBC NMR spectra and MS) showed a good agreement to those assigned to carolignan F (Paula et al., 1995). In this case H-1 also appears as a singlet, indicating the presence of the *erythro* diastereomer.

The ^1H and ^{13}C NMR spectra of **4** are very similar to those of boehmenan H **1**. The absence of the dihydrofuran ring and the presence of the trioxygenated propane moiety in the structure of **4** were suggested by their spectral data as described above for carolignan K **3**. These facts and the assigned molecular formula of $C_{30}H_{34}O_{10}$ (calculated mass: 577.2049) by HR-FABMS (*m/z* 577.2041 $M+Na^+$) are only compatible with the structure of **4** (Fig. 1), which was designated as

carolignan H. The coupling constant $J_{H1\text{-}H2} = 7.5$ Hz indicates the presence of the *threo* isomer of carolignan H.

The known compounds **10**–**20**, were identified by comparisons of spectral data with the commercially available vanillin **10**, *trans*-coniferyl aldehyde **11**, syringaldehyde **12**, *trans*-sinapaldehyde **13** and *p*-hydroxy benzaldehyde **17**, and also with 3-hydroxy-3′-methoxy-4′-hydroxypropiophenone **19** (Achenbach et al., 1988), *N-trans*-feruloyl tyramine **20** (Fukuda et al., 1983; Lajide et al., 1995) and the six lignans, pinoresinol **14**, 4-ketopinoresinol **15** (Otsuka et al., 1989), medioresinol **16** (Tsukamoto et al., 1984; Deyama et al., 1987) and syringaresinol **18** (Ratnayake et al., 1992; Changzeng and Zhongjian, 1997).

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AMX 300 at 300.13 and 75.47 MHz, respectively; the chemical shifts are expressed in δ (ppm) values relative to TMS as internal reference and the coupling constants J are expressed in Hz. ^{1}H assignments were made using 2D COSY and NOESY (mixing time of 800 ms) experiments, while ^{13}C assignments were made using 2D HETCOR and HMBC experiments (long range C/H coupling constants were optimised to 4 and 7 Hz). MS were obtained at 70 eV electron impact and positive FAB ionisation (nitrobenzyl alcohol as matrix) using a VG Autospect Q mass spectrometer. IR spectra were obtained with a MATTSON 7000 FTIR spectrometer. GC–MS analysis was performed using a HP 5890 chromatograph equipped with a mass selective detector MSD series II (electron-impact ionization). Injector and interface temperatures were 220 and 250°C, respectively. Initial oven temperature was 30°C (8 min), then 10°C/min up to 200°C (5 min), carrier gas helium (flow rate 0.9 ml/min^1), split ratio, 1:100, injections 1 μl. Fused silica capillary column DB-5 (30 m×0.25 mm×0.25 μm) was used. Prep TLC was carried out on silica gel plates (Merck silica gel 60 F_{254}); spots were detected under UV (at 254 and/or 366 nm). Phenolic compounds were detected by the appearance of a brown colour when the TLC plates was sprayed with a (3:2) $CHCl_3$:py solution of $FeCl_3$ (1%) (Browning, 1967). Column chromatography was also performed on silica gel (Merck silica gel 60, 70–230 mesh).

3.2. Plant material

Hibiscus cannabinus, variety Salvador, was harvested in Figueira da Foz, Portugal, in September 1995.

3.3. Extraction and isolation

The stems were separated from foliage and air-dried at room temperature. The core of stems was separated from bark and then finely ground (1470 g) and extracted successively with light petroleum and Me_2CO. The acetone extract (18.5 g) was fractionated by its solubility in $CHCl_3$ and Me_2CO. The residue obtained from the fraction soluble in $CHCl_3$ was defatted with hexane and then separated by silica gel CC. The column was eluted with $CHCl_3$ (fr I and II) and Me_2CO (fr III). Fr I was chromatographed on prep. silica gel TLC, eluting with $CHCl_3$-light petroleum (3:2), to give **5** (58.3 mg), **10** (5.7 mg), **11** (1.5 mg), **12** (10.1 mg), **13** (17.1 mg) **14** (12.1 mg), **15** (25.6 mg), and **16** (4.3 mg). Fr II on prep silica gel TLC, eluted with $CHCl_3$–EtOAc (4:1), yielded **5** (206.5 mg), **14** (2.1 mg), **17** (5.5 mg) and **18** (17.3 mg). Fr III on prep. TLC [silica gel, iso-PrOH–$CHCl_3$ (1:25)] gave **1** (13.9 mg), **2** (12.3 mg), **3** (33.5 mg), **4** (3.3 mg), **5** (64.5 mg), **6** (40.2 mg), **7** (48.5 mg), **8** (17.1 mg), **9** (25.2 mg), **18** (5.2 mg), **19** (5.8 mg) and **20** (47.1 mg).

3.3.1. 2-(4-Hydroxy-3-methoxyphenyl)-5-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-3-hydroxymethyl-7-methoxybenzodihydrofuran (boehmenan H) **1**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3412 (O–H), 2952, 1700 (C=O), 1604, 1516 (C=C), 1430, 1271, 1032. ^{1}H NMR: δ 1.99–2.06 (2H, *m*, H-2‴), 2.71 (2H, *t*, $J = 7.5$ Hz, H-1‴), 3.53–3.67 (1H, *m*, H-3), 3.86 (3H, *s*, 3′-OCH_3), 3.89 (3H, *s*, 3⁗-OCH_3), 3.93 (3H, *s*, 7-OCH_3), 3.86–4.00 (2H, *m*, H-1″), 4.17–4.24 (2H, *m*, H-3‴), 5.54 (1H, *d*, $J = 7.8$ Hz, H-2), 5.64 (1H, *s*, 4′-OH), 5.90 (1H, *s*, 4⁗-OH), 6.31 (1H, *d*, $J = 15.9$ Hz, H-α), 6.68 (2H, *s*, H-4,6), 6.87 (1H, *d*, $J = 8.1$ Hz, H-5′), 6.88–6.93 (2H, *m*, H-6′,5⁗), 6.94 (1H, *s*, H-2′), 7.04 (1H, d, $J = 1.8$ Hz, H-2⁗), 7.09 (1H, *dd*, $J = 1.8$ and 8.4 Hz, H-6⁗), 7.62 (1H, *d*, $J = 15.9$ Hz, H-β). ^{13}C NMR: δ 30.6 (C-2‴), 32.0 (C-1‴), 53.7 (C-3), 56.0 (7,3′,3⁗-OCH_3), 63.5 (C-3‴), 63.7 (C-1″), 87.8 (C-2), 108.8 (C-2′), 109.3 (C-2⁗), 112.3 (C-6), 114.2 (C-5′), 114.7 (C-5⁗), 115.3 (C-α), 116.0 (C-4), 119.4 (C-6′), 123.0 (C-6⁗), 126.9 (C-1⁗), 127.8 (C-9), 133.0 (C-1′), 134.6 (C-5), 144.2 (C-7), 145.0 (C-β), 145.6 (C-4′), 146.6 (C-8,3′), 146.7 (C-3⁗), 148.0 (C-4⁗), 167.4 (CO_2R on C-3‴). EIMS 70eV, *m*/*z* (rel. int.): 536 [M]$^+$ (70), 519 (100), 506 (89), 342 (19), 324 (30), 177 (93). HR–EIMS *m*/*z*: 536.2053, [M]$^+$ (calculated for $C_{30}H_{32}O_9$: 536.2046).

3.3.2. 2-(4-Hydroxy-3-methoxyphenyl)-5-[3-(4-hydroxy cinnamoyloxy)-1-propenyl]-3-(4-hydroxy-3-methoxy cinnamoyloxymethyl)-7-methoxybenzodihydrofuran (boehmenan K) **2**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3400 (O–H), 2940, 1693 (C=O), 1605, 1515 (C=C), 1268, 1168, 832. ^{1}H

NMR: δ 3.84–3.93 (1H, *m*, H-3), 3.84 (3H, *s*, 3′-OCH_3), 3.92 (3H, *s*, 7-OCH_3), 3.93 (3H, *s*, 3‴-OCH_3), 4.43 (1H, *dd*, *J* = 7.5 and 11.1 Hz, H-1″), 4.59 (1H, *dd*, *J* = 5.1 and 11.1 Hz, H-1″), 4.84 (2H, *d*, *J* = 6.3 Hz, H-3‴), 5.55 (1H, *d*, *J* = 7.5 Hz, H-2), 5.65 (1H, *s*, 4′-OH), 5.91 (1H, *s*, 4⁗-OH), 6.24 (1H, *d*, *J* = 15.9 Hz, H-α), 6.18–6.30 (1H, *m*, H-2‴), 6.33 (1H, *d*, *J* = 15.9 Hz, H-α′), 6.66 (1H, *d*, *J* = 15.9 Hz, H-1‴), 6.84 (2H, *d*, *J* = 8.4 Hz, H-3⁗′,5⁗′), 6.90–6.95 (6H, *m*, H-4,6,2′,5′,6′,5‴), 6.98 (1H, *d*, *J* = 1.8 Hz, H-2⁗), 7.04 (1H, *dd*, *J* = 1.8 and 8.4 Hz, H-6⁗), 7.45 (2H, *d*, *J* = 8.7 Hz, H-2⁗′,6⁗′), 7.51 (1H, *d*, *J* = 15.9 Hz, H-β), 7.66 (1H, *d*, *J* = 15.9 Hz, H-β′). ^{13}C NMR δ: 50.4 (C-3), 55.9 (7,3′,3⁗-OCH_3), 65.2 (C-3‴), 65.3 (C-1″), 89.1 (C-2), 108.7 (C-2′), 109.4 (C-2⁗), 110.5 (C-6), 114.3 (C-5′), 114.6 (C-α), 114.7 (C-5⁗), 115.3 (C-4,α′), 115.9 (C-3⁗′,5⁗′), 119.7 (C-6′), 121.4 (C-2‴), 123.2 (C-6⁗), 126.7 (C-1⁗), 127.1 (C-1⁗′), 127.7 (C-9), 130.0 (C-2⁗′,6⁗′), 130.6 (C-5), 132.3 (C-1′), 134.3 (C-1‴), 144.4 (C-7), 144.8 (C-β′), 145.7 (C-β), 145.8 (C-4′), 146.7 (C-3′), 146.8 (C-3⁗), 148.2 (C-8,4⁗), 157.8 (C-4⁗′), 167.1 (CO_2R on C-1″), 167.2 (CO_2R on C-3‴). FABMS *m*/*z* (rel. int.): 703 $[M+Na]^+$ (8), 680 $[M]^+$ (8). HR-FABMS *m*/*z*: 680.2285 $[M]^+$ (calculated for $C_{39}H_{36}O_{11}$: 680.2257).

3.3.3. *threo-1-(4-Hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxycinnamoyloxy)-2-{4-[3-(4-hydroxycinnamoyloxy)-1-propenyl]-2-methoxyphenoxy}-1-propanol (threo-carolignan K)* **3**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3394 (O–H), 2917, 1702 (C=O), 1604, 1513 (C=C), 1428, 1166, 1031, 833. ^{1}H NMR: δ 3.83 (3H, s, 3⁗-OCH_3), 3.90 (3H, *s*, 2″-OCH_3), 3.92 (3H, *s*, 3′-OCH_3), 4.14–4.24 (1H, *m*, H-3), 4.30–4.37 (2H, *m*, H-2,3), 4.85 (1H, *d*, *J* = 5.7 Hz, H-3‴), 4.95 (1H, *d*, *J* = 7.5 Hz, H-1), 5.70 (1H, *s*, 4′-OH), 6.01 (1H, *s*, 4⁗-OH), 6.24 (1H, *d*, *J* = 15.9 Hz, H-α), 6.33 (1H, *d*, *J* = 15.9 Hz, H-α′), 6.22–6.34 (1H, *m*, H-2‴), 6.64 (1H, *d*, *J* = 15.9 Hz, H-1‴), 6.82 (2H, *d*, *J* = 8.7 Hz, H-3⁗′,5⁗′), 6.88–6.92 (3H, *m*, H-5′,6′,5‴), 6.91–6.93 (1H, *m*, H-2⁗), 6.70–6.95 (1H, *m*, H-3″), 6.94–7.00 (1H, *m*, H-5″), 7.01–7.04 (2H, *m*, H-2′,6⁗), 7.11 (1H, *d*, *J* = 8.1 Hz, H-6″), 7.41 (2H, *d*, *J* = 8.7 Hz, H-2⁗′,6⁗′), 7.49 (1H, *d*, *J* = 15.9 Hz, H-β), 7.67 (1H, *d*, *J* = 15.9 Hz, H-β′). ^{13}C NMR: δ 55.8 (2″-OCH_3), 55.9 (3′,3⁗-OCH_3), 63.1 (C-3), 65.0 (C-3‴), 74.3 (C-1), 85.8 (C-2), 109.2 (C-2⁗), 109.3 (C-2′), 109.8 (C-3″), 114.4 (C-α), 114.5 (C-5′), 114.8 (C-5⁗), 114.9 (C-α′), 115.9 (C-3⁗′,5⁗′), 120.0 (C-6″), 120.1 (C-5″), 120.3 (C-6′), 122.8 (C-2‴), 123.3 (C-6⁗), 126.6 (C-1⁗), 126.8 (C-1⁗′), 130.0 (C-2⁗′,6⁗′), 130.9 (C-1′), 132.3 (C-4″), 133.5 (C-1‴), 145.0 (C-β′), 145.7 (C-4′,β), 146.7 (C-3⁗), 146.8 (C-3′), 147.8 (C-1″), 148.2 (C-4⁗), 150.8 (C-2″), 158.2 (C-4⁗′), 166.9 (CO_2R on C-3), 167.3 (CO_2R on C-3‴). FABMS *m*/*z* (rel. int.): 721 $[M+Na]^+$ (5), 698 (3). HR–FABMS *m*/*z*: 699.2445 $[M+H]^+$ (calculated for $C_{39}H_{39}O_{12}$: 699.2442).

3.3.4. *threo-1-(4-Hydroxy-3-methoxyphenyl)-2-{4-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-2-methoxyphenoxy}-1,3-propanodiol (threo-carolignan H)* **4**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3413 (O–H), 2921, 1698 (C=O), 1600, 1515 (C=C), 1428, 1268, 1159, 1029. ^{1}H NMR: δ 1.98–2.06 (2H, *m*, H-2‴), 2.72 (2H, *t*, *J* = 7.5 Hz, H-1‴), 3.94 (1H, *dd*, *J* = 3.9 and 8.4 Hz, H-3), 3.58–3.65 (1H, *m*, H-3), 3.89 (3H, *s*, 3′-OCH_3), 3.92 (3H, *s*, 7-OCH_3), 3.94 (3H, *s*, 3‴-OCH_3), 3.86–3.99 (1H, *m*, H-2), 4.22 (2H, *t*, J = 6.5 Hz, H-3‴), 4.96 (1H, *d*, J = 7.5 Hz, H-1), 5.64 (1H, *s*, 4′-OH), 5.89 (1H, s, 4⁗-OH), 6.31 (1H, *d*, *J* = 15.9 Hz, H-α), 6.77 (1H, *dd*, *J* = 1.8 and 8.9 Hz, H-5″), 6.79 (1H, *s*, H-3″), 6.89 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.93 (1H, *d*, *J* = 8.2 Hz, H-5⁗), 6.85–6.94 (1H, *m*, H-6′), 6.97 (1H, *s*, H-2′), 7.04 (1H, *d*, *J* = 1.8 Hz, H-2⁗), 7.06 (1H, *d*, *J* = 8.9 Hz, H-6″), 7.09 (1H, *dd*, *J* = 1.8 and 8.2 Hz, H-6⁗), 7.62 (1H, *d*, *J* = 15.9 Hz, H-β). ^{13}C NMR: δ 30.4 (C-2‴), 32.0 (C-1‴), 55.9 (3′-OCH_3), 56.0 (7,3⁗-OCH_3), 61.0 (C-3), 63.6 (C-3‴), 74.0 (C-1), 89.7 (C-2), 109.3 (C-2′,2⁗), 112.3 (C-3″), 114.3 (C-5′), 114.7 (C-5⁗), 115.3 (C-α), 120.3 (C-6′), 121.1 (C-6″), 121.3 (C-5″), 123.1 (C-6⁗), 126.9 (C-1⁗), 131.4 (C-1′), 137.6 (C-4″), 144.9 (C-β), 145.5 (C-4′), 145.7 (C-1″), 146.6 (C-3⁗), 146.8 (C-3′), 148.0 (C-4⁗), 151.1 (C-2″), 167.3 (CO_2R on C-3‴). EIMS 70 eV, *m*/*z* (rel. int.): 554 $[M]^+$ (3), 536 (6), 519 (10), 177 (30), 164 (100), 149 (30), 77 (26). HR–FABMS *m*/*z*: 577.2041 $[M+Na]^+$ (calculated for $C_{30}H_{34}O_{10}$ + Na: 577.2049).

3.3.5. *2-(4-Hydroxy-3-methoxyphenyl)-5-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-3-(4-hydroxy-3-methoxycinnamoyloxymethyl)-7-methoxybenzodihydrofuran (boehmenan)* **5**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3401 (O–H), 2940, 1700 (C=O), 1602, 1515 (C=C), 1430. ^{1}H NMR: δ 1.97–2.06 (2H, *m*, H-2‴), 2.71 (2H, *t*, *J* = 7.6 Hz, H-1‴), 3.84 (3H, *s*, 3′-OCH_3), 3.90 (3H, *s*, 7-OCH_3), 3.93 (6H, *s*, 3⁗,3⁗′-OCH_3), 3.84–3.93 (1H, *m*, H-3), 4.23 (2H, *t*, *J* = 6.5 Hz, H-3‴), 4.42 (1H, *dd*, *J* = 7.7 and 11.2 Hz, H-1″), 4.59 (1H, *dd*, *J* = 5.1 and 11.2 Hz, H-1″), 5.50 (1H, *d*, *J* = 7.8 Hz, H-2), 5.65 (1H, *s*, 4′-OH), 5.91 and 5.92 (2H, 2s, 4⁗-OH and 4⁗′-OH), 6.23 (1H, *d*, *J* = 15.9 Hz, H-α), 6.30 (1H, *d*, *J* = 15.9 Hz, H-α′), 6.69 (1H, *s*, H-6), 6.71 (1H, *s*, H-4), 6.91 (2H, *d*, *J* = 8.3 Hz, H-5⁗,5⁗′), 6.92 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.90–6.94 (3H, m, H-2′,6′,2⁗′), 7.00 (1H, dd, *J* = 1.7 and 8.3 Hz, H-6⁗′), 7.05 (1H, *d*, *J* = 1.7 Hz, H-2⁗), 7.08 (1H, *dd*, *J* = 1.7 and 8.3 Hz, H-6⁗), 7.49 (1H, *d*, *J* = 15.9 Hz, H-β), 7.60 (1H, *d*, *J* = 15.9 Hz, H-β′). ^{13}C NMR: δ 30.7 (C-2‴), 32.1 (C-1‴), 50.7 (C-3), 55.9 and 56.0 (4×OCH_3), 63.7 (C-3‴), 65.4 (C-1″), 88.9 (C-2), 108.8 (C-2′), 109.3 (C-2⁗), 109.4 (C-2⁗′), 112.4 (C-6), 114.2 (C-5′), 114.7 (C-α,5⁗,5⁗′), 115.4 (C-α′), 116.1 (C-4), 119.7 (C-6′), 123.0 (C-6⁗), 123.1 (C-6⁗′), 126.7 (C-1⁗), 126.9 (C-1⁗′), 127.4 (C-9), 132.5 (C-1′), 134.9 (C-5), 144.1 (C-7), 144.9 (C-β′), 145.5 (C-β), 145.7 (C-4′), 146.2 (C-8), 146.6 (C-3′), 146.7 (C-

3‴″,3‴″″), 147.9 (C-4‴″), 148.1 (C-4′″″), 167.0 (CO_2R on C-1″), 167.3 (CO_2R on C-3‴). FABMS *m*/*z* (rel. int.): 735 $[M+Na]^+$ (4), 713 $[M+H]^+$ (4), 518 (6), 391 (8), 329 (12).

3.3.6. *erythro-1-(4-Hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxycinnamoyloxy)-2-{4-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-2-methoxyphenoxy}-1-propanol (erythro-carolignan E)* **6**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3511 (O–H), 2938, 1700 (C=O), 1631, 1592, 1513 (C=C), 1430, 1031. ^1H NMR: δ 1.97–2.07 (2H, *m*, H-2‴), 2.71 (2H, *t*, $J=7.7$ Hz, H-1‴), 3.88 (6H, *s*, 3′,2″-OCH_3), 3.92 and 3.93 (2×3H, 2s, 2×OCH_3), 4.22 (2H, *t*, $J=6.5$ Hz, H-3‴), 4.22–4.30 (1H, *m*, H-3), 4.47–4.52 (2H, *m*, H-2,3), 4.92 (1H, *s*, H-1), 5.64 (1H, *s*, 4′-OH), 5.95 (2H, s, 4‴,4′″″-OH), 6.24 (1H, *d*, $J=15.9$ Hz, H-α), 6.30 (1H, *d*, $J=15.9$ Hz, H-α′), 6.73–6.77 (2H, *m*, H-3″,5″), 6.83 (1H, *dd*, $J=1.8$ and 8.1 Hz, H-6′), 6.88 (1H, d, $J=8.1$ Hz, H-5′), 6.91* (1H, *d*, $J=8.1$ Hz, H-5′″″), 6.92* (1H, *d*, $J=8.1$ Hz, H-5‴″), 6.67 (1H, *d*, $J=8.3$ Hz, H-6″), 7.05–6.96 (4H, m, H-2′,2‴″,2′″″,6‴″), 7.08 (1H, *dd*, $J=1.8$ and 8.1 Hz, H-6′″″), 7.51 (1H, *d*, $J=15.9$ Hz, H-β), 7.62 (1H, *d*, $J=15.9$ Hz, H-β′). *They can be interchanged. ^{13}C NMR: δ 30.3 (C-2‴), 32.0 (C-1‴), 55.8 (2″-OCH_3), 55.9 (3′,3‴″,3′″″-OCH_3), 62.6 (C-3), 63.6 (C-3‴), 72.0 (C-1), 84.5 (C-2), 108.8 (C-2′), 109.3 (C-2‴″,2′″″), 112.3 (C-3″), 114.1 (C-5′), 114.7 (C-5‴″,5′″″), 114.9 (C-α), 115.3 (C-α′), 119.2 (C-6′), 120.7 (C-6″), 121.1 (C-5″), 123.0 (C-6′″″), 123.2 (C-6‴″), 126.8 (C-1′″″), 126.9 (C-1‴″), 131.0 (C-1′), 137.4 (C-4″), 144.9 (C-β′), 145.0 (C-4′,1″), 145.2 (C-β), 146.6 (C-3′), 146.7 (C-3‴″,3′″″), 148.0 (C-4‴″,4′″″), 151.3 (C-2″), 167.1 (CO_2R on C-3), 167.3 (CO_2R on C-3‴). FABMS *m*/*z* (rel. int.): 753 $[M+Na]^+$ (35).

3.3.7. *threo-1-(4-Hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxycinnamoyloxy)-2-{4-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-2-methoxyphenoxy}-1-propanol (threo-carolignan E)* **7**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3413 (O–H), 2940, 1700 (C=O), 1631, 1600, 1513 (C=C), 1430, 1172, 1031. ^1H NMR: δ 1.97–2.06 (2H, *m*, H-2‴), 2.71 (2H, *t*, $J=7.7$ Hz, H-1‴), 3.85, 3.93 and 3.94 (3×3H, 3s, 3×OCH_3), 3.90 (3H, *s*, 7-OCH_3), 4.12 (1H, *dd*, $J=4.7$ and 11.9 Hz, H-3), 4.22 (2H, *t*, $J=6.5$ Hz, H-3‴), 4.17–4.27 (1H, *m*, H-2), 4.36 (1H, *dd*, $J=3.3$ and 12.0 Hz, H-3), 4.94 (1H, *d*, $J=8.1$ Hz, H-1), 5.69 (1H, *s*, 4′-OH), 6.00 (2H, *s*, 4‴,4′″″-OH), 6.28 (1H, *d*, $J=15.9$ Hz, H-α), 6.31 (1H, *d*, $J=15.9$ Hz, H-α′), 6.74–6.78 (1H, *m*, H-5″), 6.78 (1H, *s*, H-3″), 6.85–6.94 (5H, *m*, H-2′,5′,6′,5‴″,5′″″), 7.02–7.07 (4H, *m*, H-6″,2‴″,6‴″,2′″″), 7.08 (1H, *dd*, $J=1.9$ and 8.1 Hz, H-6′″″), 7.51 (1H, *d*, $J=15.9$ Hz, H-β), 7.62 (1H, *d*, $J=15.9$ Hz, H-β′). ^{13}C NMR: δ 30.4 (C-2‴), 32.0 (C-1‴), 55.8 (7-OCH_3), 55.9 (3′,3‴″,3′″″-OCH_3), 63.0 (C-3), 63.6 (C-3‴), 74.4 (C-1), 86.3 (C-2), 109.2 (C-2′), 109.3 (C-2‴″,2′″″), 112.3 (C-3″), 114.3 (C-5′), 114.7 (C-5‴″,5′″″,α), 115.3 (C-α′), 120.4 (C-6′), 120.6 (C-6″), 121.0 (C-5″), 123.0 (C-6′″″), 123.2 (C-6‴″), 126.7 (C-1′″″), 126.9 (C-1‴″), 131.1 (C-1′), 137.4 (C-4″), 145.0 (C-β′), 145.5 (C-β), 145.6 (C-4′), 146.0 (C-1″), 146.7 (C-3′), 146.8 (C-3‴″,3′″″), 148.0 (C-4‴″), 148.1 (C-4′″″), 150.7 (C-2″), 166.8 (CO_2R on C-3), 167.3 (CO_2R on C-3‴). FABMS *m*/*z* (rel. int.): 753 $[M+Na]^+$ (12).

3.3.8. *2-(4-Hydroxy-3,5-dimethoxyphenyl)-5-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-3-(4-hydroxy-3-methoxycinnamoyloxymethyl)-7-methoxybenzodihydrofuran (boehmenan D)* **8**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3411 (O–H), 2939, 1701 (C=O), 1603, 1515 (C=C), 1429, 1269, 1159. ^1H NMR: δ 1.99–2.08 (2H, *m*, H-2‴), 2.72 (2H, *t*, $J=7.5$ Hz, H-1‴), 3.84 (6H, *s*, 3′,5′-OCH_3), 3.90 (3H, *s*, 7-OCH_3), 3.93 (6H, s, 3‴″,3′″″-OCH_3), 3.81–3.93 (1H, *m*, H-3), 4.23 (2H, *t*, $J=6.5$ Hz, H-3‴), 4.44 (1H, *dd*, $J=7.8$ and 11.1 Hz, H-1″), 4.61 (1H, *dd*, $J=5.1$ and 11.1 Hz, H-1″), 5.47 (1H, *d*, $J=8.1$ Hz, H-2), 5.52 (1H, *s*, 4′-OH), 5.90 (2H, *s*, 4‴″,4′″″-OH), 6.23 (1H, *d*, $J=15.9$ Hz, H-α), 6.30 (1H, *d*, $J=15.9$ Hz, H-α′), 6.67 (2H, *s*, H-2′,6′), 6.70 (1H, *s*, H-6), 6.71 (1H, *s*, H-4), 6.91* (1H, *d*, $J=8.1$ Hz, H-5′″″), 6.92* (1H, *d*, $J=8.1$ Hz, H-5‴″), 6.97 (1H, *d*, $J=1.8$ Hz, H-2‴″), 7.03 (1H, *d*, $J=1.8$ Hz, H-2′″″), 7.03** (1H, *dd*, $J=1.8$ and 8.1 Hz, H-6′″″), 7.08** (1H, *dd*, $J=1.8$ and 8.1 Hz, H-6‴″), 7.48 (1H, *d*, $J=15.9$ Hz, H-β), 7.60 (1H, *d*, $J=15.9$ Hz, H-β′). * And ** they can be interchanged. ^{13}C NMR: δ 30.7 (C-2‴), 32.1 (C-1‴), 50.8 (C-3), 55.9* (7-OCH_3), 56.0* (3‴″,3′″″-OCH_3), 56.3 (3′,5′-OCH_3), 63.7 (C-3‴), 65.4 (C-1″), 89.3 (C-2), 103.2 (C-2′.6′), 109.3 (C-2′″″), 109.4 (C-2‴″), 112.5 (C-6), 114.7 (C-α,5‴″,5′″″), 115.4 (C-α′), 116.1 (C-4), 123.0 (C-6‴″,6′″″), 126.6 (C-1′″″), 126.9 (C-1‴″), 127.3 (C-9), 131.7 (C-1′), 134.7 (C-4′), 135.0 (C-5), 144.2 (C-7), 144.9 (C-β′), 145.6 (C-β), 146.2 (C-8), 146.8 (C-3‴″,3′″″), 147.0 (C-3′,5′), 148.0 (C-4′″″), 148.2 (C-4‴″), 167.0 (CO_2R on C-1″), 167.3 (CO_2R on C-3‴). *They can be interchanged. FABMS *m*/*z* (rel. int.): 765 $[M+Na]^+$ (35), 743 $[M+H]^+$ (14), 742 $[M]^+$ (17), 549 (90).

3.3.9. *erythro-1-(4-Hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxycinnamoyloxy)-2-{4-[3-(4-hydroxy-3-methoxycinnamoyloxy)propyl]-2,6-dimethoxyphenoxy}-1-propanol (erythro-carolignan F)* **9**

Yellowish oil, IR ν_{max} (KBr) cm^{-1}: 3416 (O–H), 2936, 1703 (C=O), 1592, 1515 (C=C), 1428, 1271, 1173, 1125. ^1H NMR: δ 1.96–2.09 (2H, *m*, H-2‴), 2.71 (2H, *t*, $J=7.5$ Hz, H-1‴), 3.85 (6H, *s*, 2″,6″-OCH_3), 3.89, 3.92 and 3.93 (3×3H, 3*s*, 3×OCH_3), 4.24 (2H, *t*, $J=6.5$ Hz, H-3‴), 4.32 (1H, *dd*, $J=3.6$ and 11.7 Hz, H-3), 4.45 (1H, *dd*, $J=7.7$ and 11.6 Hz, H-3), 4.55 (1H, *m*, $J=3.4$ Hz, H-2), 4.90 (1H, *s*, H-1), 5.61 (1H, *s*, 4′-OH), 5.95 (1H, *s*, 4′″″-OH), 5.97 (1H, *s*, 4‴″-OH), 6.26 (1H, *d*, $J=15.9$ Hz,

H-α), 6.31 (1H, *d*, *J* = 15.9 Hz, H-α′), 6.46 (2H, *s*, H-3″,5″), 6.77 (1H. *dd*, *J* = 1,2 and 8.1 Hz, H-6′), 6.87 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.90* (1H, *d*, *J* = 8.1 Hz, H-5‴″), 6.92* (1H, *d*, *J* = 8.1 Hz, H-5‴), 7.01–7.05 (4H, m, H-2′,2‴,2‴″,6‴″), 7.08 (1H, *dd*, *J* = 1.5 and 8.1 Hz, H-6‴), 7.52 (1H, *d*, *J* = 15.9 Hz, H-β), 7.62 (1H, *d*, *J* = 15.9 Hz, H-β′). *They can be interchanged. ^{13}C NMR: δ 30.3 (C-2‴), 32.7 (C-1‴), 55.9 (2″,6″,3‴,3‴″-OCH_3), 56.1 (3′-OCH_3), 62.5 (C-3), 63.6 (C-3‴), 71.5 (C-1), 83.1 (C-2), 105.3 (C-3″,5″), 108.4 (C-2′), 109.2 (C-2‴″), 109.3 (C-2‴), 114.0 (C-5′), 114.6* (C-5‴″), 114.7* (C-5‴), 115.2 (C-α′), 115.5 (C-α), 118.8 (C-6′), 123.0 (C-6‴,6‴″), 126.8 (C-1‴,1‴″), 130.7 (C-1′), 132.6 (C-1″), 137.9 (C-4″), 144.7 (C-β,4′), 145.0 (C-β′), 146.6 (C-3‴″), 146.7 (C-3′,3‴), 147.9 (C-4‴″), 148.0 (C-4‴), 151.3 (C-2″,6″), 167.1 (CO_2R on C-3), 167.3 (CO_2R on C-3‴). *They can be interchanged. FABMS *m*/*z* (rel. int.): 783 $[M+Na]^+$ (14).

3.4. Transesterification of boehmenan **5**

Compound **5** (~ 50 mg) was added to a 1% solution of NaOMe in MeOH (7.5 ml) and the resulting mixture was refluxed for 3 h. After this period, the mixture was acidified (pH 6) with 2 M H_2SO_4, extracted with Et_2O and separated by prep. silica gel TLC, eluting with $CHCl_3$–EtOAc (9:1). The three most abundant spots were collected and identified as methyl ferulate **21**, 3-hydroxymethyl-5-(3-hydroxypropyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxybenzodihydrofuran **22** and 2,4′-dihydroxy-3,3′-dimethoxy-5-(3-hydroxypropyl) stilbene **23**.

3.4.1. 3-Hydroxymethyl-5-(3-hydroxypropyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxybenzodihydrofuran **22**

Colourless oil, ^{1}H NMR: δ 1.88–1.92 (2H, *m*, H-2‴), 2.68 (2H, *t*, *J* = 7.7 Hz, H-1‴), 3.58–3.64 (1H, *m*, H-3), 3.70 (2H, *t*, *J* = 6.3 Hz, H-3‴), 3.87 and 3.89 (2×3H, 2*s*, 2×OCH_3), 3.87–3.92 (2H, *m*, H-1″), 5.55 (1H, *d*, *J* = 7.5 Hz, H-2), 5.63 (1H, *s*, 4′-OH), 6.68 (2H, *s*, H-4,6), 6.95 (3H, *m*, H-2′,5′,6′). ^{13}C NMR: δ 32.0 (C-1‴), 34.6 (C-2‴), 53.8 (C-3), 56.0 (7,3′-OCH_3), 62.3 (C-3‴), 63.9 (C-1″), 87.9 (C-2), 108.8 (C-2′), 112.7 (C-6), 114.2 (C-5′), 115.9 (C-4), 119.4 (C-6′), 127.7 (C-9), 133.1 (C-5), 135.4 (C-1′), 144.2 (C-8), 145.6 (C-4′), 146.5 (C-3′), 146.6 (C-7). EIMS 70 eV, *m*/*z* (rel. int.): 360 $[M]^+$ (85), 342 (100), 330 (66), 167 (30), 137 (36), 77 (13).

3.4.2. 2,4′-Dihydroxy-3,3′-dimethoxy-5-(3-hydroxy propyl)stilbene **23**

Colourless oil, ^{1}H NMR: δ 1.89–1.92 (2H, *m*, H-2″), 2.67 (2H, *t*, *J* = 7.7 Hz, H-1″), 3.71 (2H, *t*, *J* = 6.5 Hz, H-3″), 3.90 and 3.96 (2×3H, 2*s*, 2×OCH_3), 5.67 (1H, *s*, 4′-OH), 5.82 (1H, *s*, 2-OH), 6.61 (1H, *d*, *J* = 1.8 Hz, H-4), 6.90 (1H, *d*, *J* = 8.3 Hz, H-5′), 7.00 (1H, *d*, *J* = 1.8 Hz, H-6), 7.04 (1H, *dd*, *J* = 1.9 and 8.3 Hz, H-6′), 7.09 (1H, *d*, *J* = 1.9 Hz, H-2′), 7.10 (1H, *d*, *J* = 16.4 Hz, H-β), 7.27 (1H, *d*, *J* = 16.4 Hz, H-α). ^{13}C NMR: δ 32.0 (C-1″), 34.5 (C-2″), 55.9 and 56.1 (2×OCH_3), 62.3 (C-3″), 108.2 (C-2′), 109.5 (C-4), 114.4 (C-5′), 118.0 (C-6), 120.6 (C-6′), 120.7 (C-α), 123.5 (C-1), 129.2 (C-β), 130.5 (C-1′), 133.0 (C-5), 141.3 (C-2), 145.4 (C-4′), 146.6 (C-3, 3′). EIMS 70 eV, *m*/*z* (rel. int.): 330 $[M]^+$ (100), 286 (13), 253 (10), 137 (13), 77 (4).

3.5. Identification of formaldehyde by GC–MS

The boehmenan **5** transesterification mixture, obtained as described above, was directly injected in the GC–MS chromatograph. The identification of the formaldehyde peak was done by comparison its mass spectrum with the equipment's mass spectral library.

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Erysubins C–F, four isoflavonoids from *Erythrina suberosa* var. *glabrescences*

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Abstract

Four isoflavonoids, erysubins C–F, together with ten known compounds were isolated from the roots of *Erythrina suberosa* var. *glabrescences*, and their structures were elucidated on the basis of spectroscopic evidence. Erysubin C is an unusual pterocarpan derivative with a formyl group. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Erythrina suberosa* var. *glabrescences*; Leguminosae; Isoflavonoids; Pterocarpans; Erysubins C–F

1. Introduction

Erythrina suberosa var. *glabrescences* has been used in Pakistan as an ornamental plant and as a folk medicine and in India as a useful medicinal plant for the treatment of various ailments (Chopra et al., 1956). The ethanol extract of the leaves was reported to have anti-tumor activity (Dhar et al., 1968). Phytochemical studies on this plant showed the presence of many alkaloids including erythrinan alkaloids (Chawla et al., 1989), sterols (Singh et al., 1970) and a flavone (Chauhan and Saxena, 1987). A recent investigation has revealed the isolation of two isoflavones (erysubins A and B) from the wood of this plant (Tanaka et al., 1998a). In continuation of our investigation on the secondary metabolites of the genus *Erythrina*, we now describe the isolation and structural elucidation of three new pterocarpans, erysubins C (**1**), D (**2**) and E (**3**), and a new isoflavone, erysubin F (**4**), along with ten known compounds from the roots of this source.

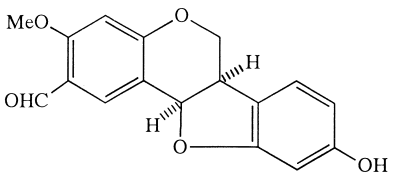
2. Results and discussion

Silica gel chromatography of the dichloromethane extract of the roots of *E. suberosa* var. *glabrescences* gave three new pterocarpans (**1–3**) and a new isoflavone (**4**) as well as ten known compounds. The known compounds were identified as orientanol D (Tanaka et al., 1998b), bidwillon B (Iinuma et al., 1992), 2-(γ, γ-dimethylallyl)-6a-hydroxyphaseollidin (Tanaka et al., 1997), phaseollin (Perrin et al., 1972), erythrabyssin II (Kamat et al., 1981), cristacarpin (Ingham and Markham, 1980), wighteone (Kinoshita et al., 1990), bidwillon A (Iinuma et al., 1992), alpinumisoflavone (Olivares et al., 1982) and erystagallin A (Tanaka et al., 1997), respectively, by comparison of spectroscopic and physical data with those of authentic samples or the reported values.

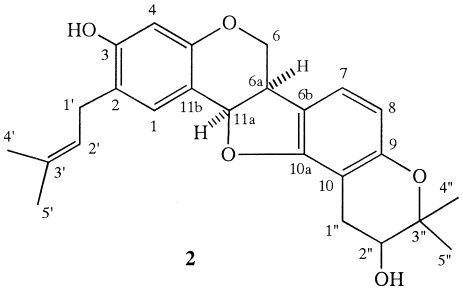
The molecular formula of erysubin C (**1**) was determined as $C_{17}H_{14}O_5$ ($[M]^+$ m/z 298.0836) from the HREI mass spectrum. The UV spectral data and a set of aliphatic proton signals (δ 3.68, 3.77, 4.41 and 5.59) in the ^{1}H NMR spectrum showed that compound (**1**) is a pterocarpan derivative. The ^{1}H NMR spectrum displayed two singlet aromatic protons (δ 6.62 and 7.93), three aromatic protons in an ABX system (δ 6.31, 6.40 and 7.17) and a methoxyl group (δ 3.96) in addition to the characteristic formyl proton (δ 10.30). The presence

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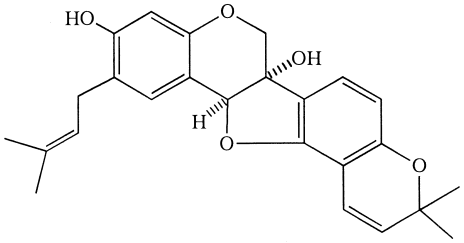
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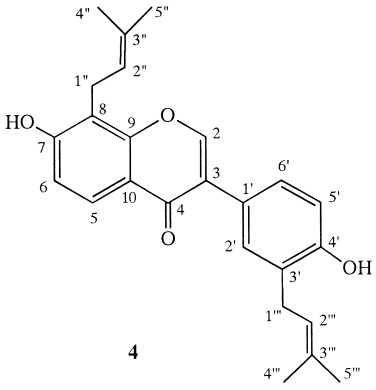
1



2



3



4

of the formyl group was also obvious from both the formyl carbon signal (δ 187.6) in the ^{13}C NMR spectrum and the absorption at 1650 cm^{-1} in the IR spectrum. The formyl group at C-2 and the methoxyl group at C-3, respectively, were located by the NOESY experiment which revealed NOE interactions between the formyl proton and the aromatic proton at C-1 (δ 7.93) and between the methoxyl group and the aromatic proton at C-4 (δ 6.62), as well as between the formyl proton and the methoxyl proton. Further support for the assignment of the formyl group at C-2 was obtained by the HMBC experiment, indicating correlations from the formyl proton to carbons at C-1 (δ 132.7) and C-2 (δ 120.8). The absolute stereochemistry at C-6a and C-11a was assigned as 6a *R*: 11a *R* from the negative optical rotation value (Ingham and Markham, 1980). Therefore, the structure of erysubin C is represented by **1** (6a *R* and 11a *R*). Erysubin C is the first example of a pterocarpan possessing a formyl group.

The molecular formula of erysubin D (**2**) was determined as $C_{25}H_{28}O_5$ ([M]$^+$ *m*/*z* 408.1929) by the HREI mass spectrum. The UV spectrum and the aliphatic proton signals (δ 3.50, 3.60, 4.21 and 5.47) in the ^{1}H NMR spectrum showed that compound (**2**) also has a pterocarpan skeleton. The ^{1}H NMR spectrum displayed two singlet aromatic protons (δ 6.41 and 7.25), a set of *ortho* coupled aromatic protons (δ 6.40 and 6.99) and a prenyl group (δ 1.79, 1.80, 3.35 and 5.34) as well as the typical protons of a hydroxydimethyldihydropyran ring (δ 1.33, 1.34, 2.68, 2.98 and 3.80). The presence of the dihydropyran moiety was also evident from the EI mass spectrum which revealed the characteristic fragment at *m*/*z* 337 [M-71]$^+$ (Nakahara et al., 1986). The dihydropyran ring fused to the C-9 and C-10 positions was assigned from the HMBC spectrum which exhibited correlations from the aliphatic protons at C-1″ (δ 2.68 and 2.98) to a carbon at C-10 (δ 103.3). The placement of the prenyl group at the C-2 position was confirmed by the NOE experiment, indicating NOE interaction between the aliphatic proton at C-1′ (δ 3.35) and the aromatic proton at C-1 (δ 7.25). The absolute stereochemistry at C-6a and C-11a was also 6a *R*: 11a *R* (negative optical rotation value), while the configuration of C-2″ was not determined. Therefore, the structure of erysubin D was represented by **2** (6a *R* and 11a *R*).

The molecular formula of erysubin E (**3**) was determined as $C_{25}H_{27}O_5$ ([M+1]$^+$ *m*/*z* 407.1874) by the HRFAB mass spectrum. The UV spectrum and the three characteristic protons (δ 3.94, 4.18 and 5.26) in the ^{1}H NMR spectrum showed it to be a 6a-hydroxypterocarpan derivative. Comparison of the ^{1}H NMR spectrum of compound (**3**) with that of **2** showed the identical positions of two singlet aromatic protons (δ 6.41 and 7.24), *ortho* coupled aromatic protons (δ 6.42 and 7.07) and a prenyl group (δ 1.79, 3.34 and 5.32); these same partial structures were also revealed by comparison of the ^{13}C NMR spectrum of **3** with that of **2**. The remaining signals (δ 1.40, 1.43, 5.58 and 6.49) were attributable to those of the 2, 2-dimethylchromene ring. The location of the dimethylchromene moiety at the C-9, 10 position was assigned via the HMBC experiment, which showed correlations from the olefinic proton at C-2″ (δ 5.58) to an sp^2 quaternary carbon at C-10 (δ 106.5) and from the olefinic proton at C-1″ (δ

6.49) to an sp^2 quaternary carbon carrying oxygen at C-9 (δ 155.2). The placement of the prenyl group at the C-2 position was confirmed from the NOESY spectrum, indicating NOE interactions between the aromatic proton at C-1 (δ 7.24) and the aliphatic protons at both C-1′ (δ 3.34) and C-2′ (δ 5.32). The absolute stereochemistry at C-6a and C-11a was 6a *S*: 11a *S* (negative optical rotation value) and, therefore, erysubin E was represented by **3** (6a *S* and 11a *S*).

The molecular formula of erysubin F (**4**) was determined as $C_{25}H_{26}O_4$ ($[M]^+$ m/z 390.1823) by the HREI mass spectrum. The IR spectrum showed the presence of a conjugated carbonyl (1625 cm^{-1}) and hydroxyl (3390 cm^{-1}) groups. The UV spectrum and the typical singlet assigned to H-2 (δ 8.21) in the ^1H NMR spectrum indicated that compound (**4**) is an isoflavone derivative. The ^1H NMR spectrum revealed ABX type aromatic protons (δ 6.88, 7.31 and 7.38), *ortho* coupled aromatic protons (δ 7.04 and 7.93) and two prenyl groups (δ 1.67, 1.71, 1.74, 1.84, 3.37, 3.58, 5.30 and 5.39). The ^{13}C NMR spectrum also indicated the presence of these partial structures and the higher shift value of C-1″ (δ 22.6) in the prenyl group suggested the prenyl group to be situated at the C-8 position (Fukai and Nomura, 1996). The assignment of the prenyl group at the C-8 position was confirmed by the HMBC experiment which indicated correlations from the aliphatic proton at C-1″ (δ 3.58) to carbons at C-7 (δ 160.1), C-8 (δ 116.3) and C-9 (δ 156.5). The other prenyl group was located at C-3′ as revealed by the HMBC experiment, indicating correlations from the aliphatic protons at C-1‴ (δ 3.37) to carbons at C-2′ (δ 131.2), C-3′ (δ 128.4) and C-4′ (δ 155.6) and the placement was confirmed from the NOESY spectrum which exhibited NOE interaction between the protons at C-1‴ and the aromatic proton at C-2′ (δ 7.38). Therefore, the structure of erysubin F is represented by **4**.

3. Experimental

3.1. General

IR spectra were recorded on a JASCO IR-810 spectrophotometer and UV spectra were recorded on a Beckman DU-530 spectrophotometer. Optical rotations

Table 1
^1H NMR spectral data for compounds **1–4**

H	1[a]	2[b]	3[b]	4[a]
1	7.93 *s*	7.25 *s*	7.24 *s*	
2				8.21 *s*
4	6.62 *s*	6.41 *s*	6.41 *s*	
5				7.93 *d* (8.8)
6	3.77 *t*-like (10.7)	3.60 *t*-like (11.0)	3.94 *d* (11.7)	7.04 *d* (8.8)
	4.41 *dd* (10.7, 4.8)	4.21 *dd* (11.0, 5.1)	4.18 *d* (11.7)	
6a	3.68 *m*	3.50 *m*		
7	7.17 *d* (7.8)	6.99 *d* (8.1)	7.07 *d* (8.1)	
8	6.40 *dd* (7.8, 2.2)	6.40 *d* (8.1)	6.42 *d* (8.1)	
10	6.31 *d* (2.2)			
11a	5.59 *d* (7.3)	5.47 *d* (6.6)	5.26 *s*	
1′		3.35 *d* (7.3)	3.34 *d* (6.6)	
2′		5.34 *t* (7.3)	5.32 *t* (6.6)	7.38 *d* (2.2)
4′		1.79 *s*[c]	1.79 *s*[d]	
5′		1.80 *s*[c]	1.79 *s*[d]	6.88 *d* (8.4)
6′				7.31 *dd* (8.4, 2.2)
1″		2.68 *dd* (17.6, 5.9)	6.49 *d* (9.5)	3.58 *d* (7.3)
		2.98 *dd* (17.6, 5.1)		
2″		3.80 *dd* (5.9, 5.1)	5.58 *d* (9.5)	5.30 *t* (7.3)
4″		1.34 *s*	1.40 *s*[c]	1.84 *s*
5″		1.33 *s*	1.43 *s*[c]	1.67 *s*
1‴				3.37 *d* (8.1)
2‴				5.39 *t* (8.1)
4‴				1.74 *s*
5‴				1.71 *s*
OMe	3.96 *s*			
CHO	10.30 *s*			
OH	8.38 *br s*	2.64 *br s*	2.37 *br s*	3.80 *s*
OH		5.27 *s*	5.29 *br s*	8.33 *br s*

[a] In acetone-d_6.
[b] In $CDCl_3$.
[c] Assignments in the same vertical column may be interchanged.
[d] Assignment is superimposed.

Table 2
^{13}C NMR spectral data for compounds **1–4**[a]

C	1[b]	2[c]	3[c]	4[b]
1	132.7	132.0	132.1	
2	120.8	121.0	121.7	153.1
3	164.0	155.8	155.8	125.0
4	101.0	104.0	104.1	176.1
4a	163.0	155.1	154.3	
5				125.3
6	67.4	66.6	69.8	114.7
6a	40.0	39.9	76.9	
6b	118.5	118.1	120.1	
7	125.9	122.7	122.8	160.1
8	108.6	109.2	109.6	116.3
9	159.7	153.8[d]	155.2	156.5
10	98.7	103.3	106.5	118.8
10a	161.6	158.5[d]	155.5	
11a	78.3	78.7	84.9	
11b	114.6	112.3	112.1	
1′		29.3	29.3	124.6
2′		121.9	121.7	131.2
3′		134.9	135.1	128.4[e]
4′		17.9	17.9	155.6
5′		25.8	25.8	115.4
6′				128.4[e]
1″		26.3	116.0	22.6
2″		69.2	129.6	122.5
3″		76.8	76.4	132.5
4″		22.1	27.9[e]	17.8
5″		24.6	27.9[e]	25.8
1‴				29.2
2‴				123.8
3‴				132.3
4‴				17.9
5‴				25.9
OMe	56.5			
CHO	187.6			

[a] Assignments are based on HSQC and HMBC spectra.
[b] In acetone-d_6.
[c] In $CDCl_3$.
[d] Assignment may be interchanged.
[e] Assignments in the same vertical column are superimposed.

were taken on a JASCO DIP-370 digital polarimeter. The ^1H NMR spectra were obtained on JEOL JNM-A 600 and 400 MHz spectrometers, while ^{13}C NMR spectra were recorded at 150.8 and 100.4 MHz on the same instruments. Mass spectra were performed on a JEOL JMS-D 300 spectrometer. Column chromatography was performed using silica gel (230–400 mesh). Thin-layer chromatography was carried out using Merck precoated silica gel (60 F_{254}). Ceric sulphate spray reagent and UV light were used for the detection of compounds.

3.2. Plant material

The roots of *E. suberosa* var. *glabrescences* were collected at Karachi, Pakistan, in August, 1998. A voucher specimen was deposited at the Department of Natural Product Chemistry in the Faculty of Pharmacy, Meijo University.

3.3. Separation and isolation

The finely powdered roots (2.9 kg) were macerated with acetone and the solvent was removed to give a dark-green residue (162 g). The residue was divided into petrol-, CH_2Cl_2- and EtOAc-soluble fractions. The CH_2Cl_2-soluble fraction (60 g) was applied to a silica gel column which was eluted with solvents of varying polarity of $CHCl_3$, $CHCl_3$–acetone (10:1.5→1:1) and $CHCl_3$–MeOH (10:1) (each volume, 400 ml) to afford 35 fractions (frs. 1–35). Fr. 21 (1 g) was purified by repeated CC on silica gel using benzene–EtOAc (3:1) to yield orientanol D (5 mg). Fr. 25 (5 g) was subjected to additional silica gel chromatography successively using benzene–EtOAc (4:1→2:1) and *n*-hexane-acetone (2:1→1:1) to furnish bidwillon B (25 mg) and 2-(γ, γ-dimethylallyl)-6a-hydroxyphaseollidin (97 mg). Fr. 19 (7.8 g) was submitted to CC on silica gel using $CHCl_3$-acetone (40:1→3:1) (each volume, 80 ml) to provide 25 fractions (frs. 36–60). Frs. 43–46 (836 mg) were separated by CC on silica gel using *n*-hexane-acetone (2:1→1:1) to give phaseollin (28 mg) and erythrabyssin II (20 mg). Frs. 49–54 (1.8 g) were repeatedly subjected to silica gel column chromatography using benzene–EtOAc (5:1→3:1) to afford cristacarpin (160 mg) and wighteone (52 mg). Frs. 55–60 (476 mg) were rechromatographed on silica gel successively using $CHCl_3$–acetone (10:1) and benzene–EtOAc (3:1→2:1) to yield **4** (6.8 mg) and bidwillon A (132 mg). Frs. 47–48 (2.61 g) were submitted to CC on silica gel using benzene–EtOAc (10:1→5:1) (each volume, 40 ml) to furnish 23 fractions (frs. 61–83). Frs. 73–80 (514 mg) were separated by CC on silica gel successively using *n*-hexane-acetone (2:1) and $CHCl_3$–acetone (20:1) to provide **2** (4.5 mg) and **3** (8 mg) and alpinumisoflavone (11 mg). Frs. 81–83 (490 mg) were applied to a silica gel column, this being successively eluted with benzene–EtOAc (5:1) and *n*-hexane–acetone (12:5) to give **1** (10 mg) and erystagallin A (6 mg).

3.4. Erysubin C (*1*)

Oil, $[\alpha]_D$ −250° (MeOH, *c* 0.1); IR (KBr) ν_{max} cm^{-1}: 3430, 1650, 1620; UV (MeOH) λ_{max} nm (log ε): 206 (4.61), 277 (4.11), 316 (3.85); EI MS *m*/*z* (rel. int.): 298 ($[M]^+$, 100), 283 (10), 269 (6), 255 (4), 189 (5); HREI MS *m*/*z*: 298.0836 ($[M]^+$, calcd for $C_{17}H_{14}O_5$: 298.0840); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

3.5. Erysubin D (*2*)

Oil, $[\alpha]_D$ −180° (MeOH, *c* 0.1); IR (KBr) ν_{max} cm^{-1}: 3400, 1620; UV (MeOH) λ_{max} nm (log ε): 209 (4.94), 284 (4.14); EI MS *m*/*z* (rel. int.): 408 ($[M]^+$, 100), 406 (6), 375 (9), 337 (11), 336 (17), 281 (13), 267 (5); HREI MS *m*/*z*: 408.1929 ($[M]^+$, calcd for $C_{25}H_{28}O_5$: 408.1935); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

3.6. Erysubin E (**3**)

Oil, $[\alpha]_D$ −274° (MeOH, *c* 0.16); IR (KBr) ν_{max} cm^{-1}: 3390, 1620; UV (MeOH) λ_{max} nm (log ε): 206 (4.70), 227 (4.49), 282 (4.05); FAB MS *m/z* (rel. int.): 407 ($[M+1]^+$, 6), 391 (9), 389 (14), 369 (30), 277 (100), 275 (4), 231 (8), 207 (6); HRFAB MS *m/z*: 407.1874 ($[M+1]^+$, calcd for $C_{25}H_{27}O_5$: 407.1857); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

3.7. Erysubin F (**4**)

Oil, IR (KBr) ν_{max} cm^{-1}: 3390, 1625; UV (MeOH) λ_{max} nm (log ε): 204 (4.66), 253 (4.56); EI MS *m/z* (rel. int.): 390 ($[M]^+$, 100), 375 (14), 373 (22), 361 (16), 347 (12), 335 (36), 305 (8), 291 (11), 279 (9); HREI MS *m/z*: 390.1823 ($[M]^+$, calcd for $C_{25}H_{26}O_4$: 390.1830); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

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A pregnane steroid from *Aglaia lawii* and structure confirmation of cabraleadiol monoacetate by X-ray crystallography

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Abstract

The pregnane steroid, (*E*)-aglawone, along with four known triterpenes, and a known sterol mixture were isolated from the bark of *Aglaia lawii* (Wight) Saldanha ex Ramamoorty (Meliaceae). The structural determination/identification was accomplished by a combination of 1D- and 2D-NMR spectroscopic techniques. The relative stereochemistry of the known triterpene, 20*S*,24*S*-epoxydammarane-3α,25-diol acetate, was also unequivocally determined for the first time by X-ray crystallography. The isolates were not active against various human cancer cell lines. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Aglaia lawii*; Meliaceae; (*E*)-Aglawone; Pregnane steroid; Triterpenes; Phytosterols; 1D- and 2D-NMR Spectroscopic techniques; X-Ray crystallography

1. Introduction

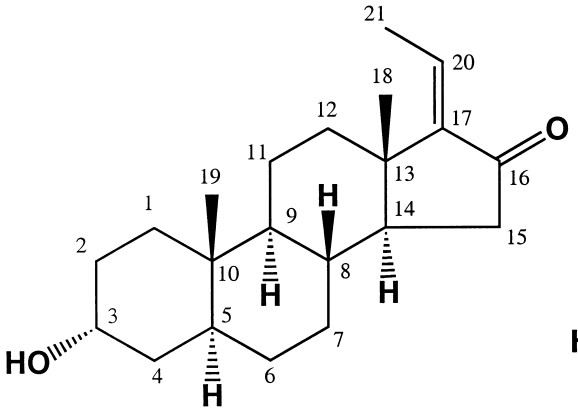
Aglaia lawii (Wight) Saldanha ex Ramamoorty is a traditional medicinal plant indigenous to Vietnam, having been used for the treatment of bacterial infection, liver and tumor diseases (Vo, 1997). In our continuing search for biologically active constituents from Vietnamese higher plants, the $CHCl_3$ fraction of a MeOH extract of the stem bark derived from *A. lawii* was found to exhibit cytotoxicity against several human cell lines (Le et al., 2000). Fractionation of $CHCl_3$-soluble components resulted in the isolation of a new pregnane steroid, namely (*E*)-aglawone (**1**), as well as the known triterpenes, β-amyrone and β-amyrin, 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate (cabraleadiol monoacetate) (**2**), cabraleadiol (**3**) and a mixture of the phytosterols, stigmasterol and poriferasterol. Structures of the isolates were characterized by means of spectroscopy, including 1D and 2D NMR spectroscopic techniques. The relative stereochemistry of 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate was unequivocally determined by X-ray crystallography.

2. Results and discussion

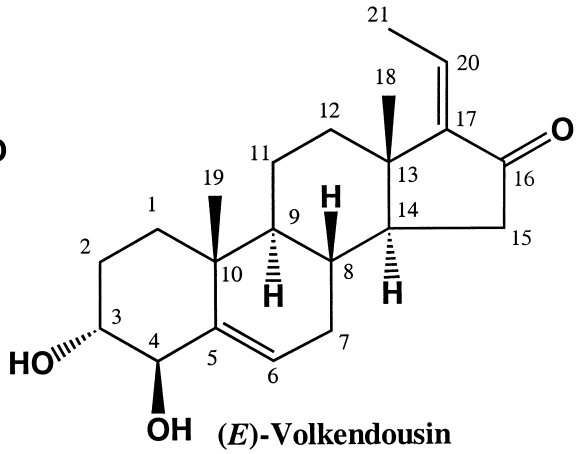
(*E*)-Aglawone (**1**), obtained as white needles, exhibited a molecular formula $C_{21}H_{32}O_2$ ($M_r = 316$) as evidenced by a quasimolecular ion $[M+H]^+$ at m/z 317, determined by electrospray mass spectroscopy, and DEPT experiments, in which three methyls, nine methylenes, six methines and four quaternary carbons were evident. Inspection of its UV, IR, and ^1H and ^{13}C NMR spectral data revealed that it was an α, β-unsaturated pregnane steroid closely related in structure to (*E*)-volkendousin (Rogers et al., 1998). The existence of the α,β-unsaturated cyclopentanone fragment was further confirmed by the strong

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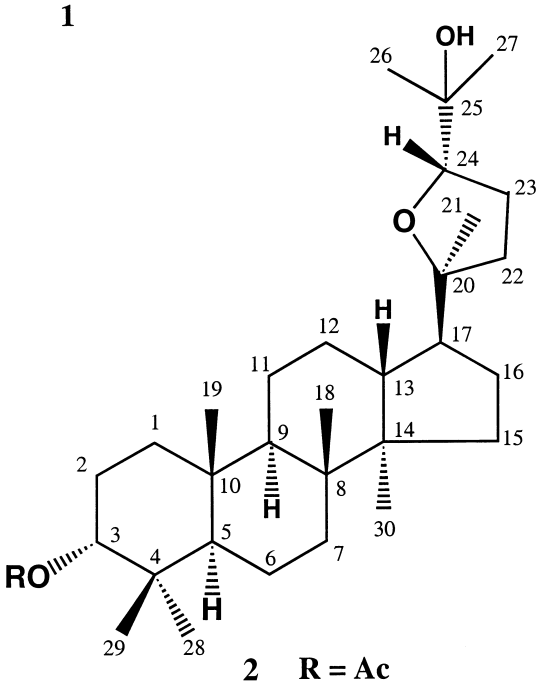
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1



(*E*)-Volkendousin



2 R = Ac

3 R = H

absorption at 1718 and 1640 cm^{-1} in the IR spectrum, and an UV absorption maximum at 242 nm (Nakano et al., 1996). In addition to confirming the tetracyclic framework of **1** with the D-ring being an α,β-unsaturated cyclopentanone, the interpretation of the NMR spectroscopic data from DQF-COSY, HOHAHA, ROESY, HMQC, and HMBC also established the placement of the hydroxyl group at the C-3 position (Fig. 1). The small coupling constants of the carbinol proton H-3 at δ 4.06 (*t*, $J = 2.6$) was indicative of the C-3 hydroxyl being axial from an examination of the Dreiding model.

It has been reported that the angular methyl groups of pregnane steroids usually show significant ^{1}H–^{13}C correlations in the long-range hetereonuclear coupling (HMBC) spectrum, and therein provided the basis for the assignments of both angular methyls and backbone skeleton carbon signals (Qiu et al., 1997). In the HMBC spectrum of **1**, the CH_3-18 signal at δ 1.01 showed strong ^{1}H–^{13}C correlations with the ^{13}C signals of C-12, C-13, C-14 and C-17 as expected. Similarly, the ^{1}H signal at δ 0.83, which showed correlations with the ^{13}C signals assignable to those of C-1, C-5, C-9 and C-10, was assigned to CH_3-19. The geometric stereochemistry of **1** was assigned to *E* by a comparison of its ^{1}H NMR spectrum with those of (*E*)- and (*Z*)-volkendousin (retrieved directly from the deposited supplemental documents of cited reference via the ACS website) (Rogers et al., 1998). It has been reported that the ^{1}H profiles of *E*- and *Z*-isomers can be regarded as being characteristic of the geometric stereochemistry of the trisubstituted exocyclic double bond, since the chemical shifts of the vinyl protons and the methyl groups of the two epimers could be differentiated by the anisotropic effect of the carbonyl group at C-16 (Rogers et al., 1998). The assignment of an *E* geometric stereochemistry to the C_{17}–C_{20} double bond was justified by

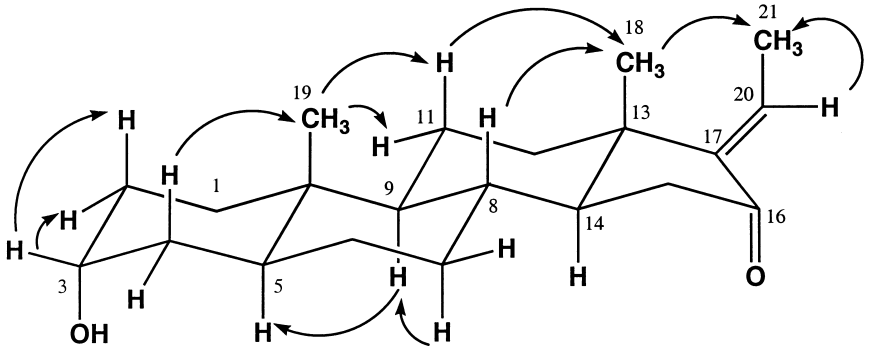


Fig. 1. Selective ROESY correlations for compound **1**.

the ^{1}H-NMR spectrum of **1**, in which the vinyl proton resonated at a relatively higher frequency at δ 6.49 (δ 6.45 and 5.88 were observed for *E*- and *Z*-volkendousin, respectively). This resonance is indicative of a *syn* configuration to the carbonyl oxygen, and would consequently experience a deshielding effect, leading the doublet methyl signal to be detected at relatively lower frequency (δ 1.85). Indeed, *Z*- and *E*-volkendousin were observed at δ 2.05 and 1.85, respectively (Rogers et al., 1998). Furthermore, the ROE cross peak observed between the signals of 18-methyl at δ 1.01 and 21-methyl at δ 1.85 in the ROESY spectrum provides solid evidence for the *E* geometric stereochemistry of the C_{17}–C_{21} double bond. Therefore, the structure of **1** was determined as (*E*)-aglawone. An unambiguous NMR assignment of **1** was achieved by means of a combination of 1D and 2D NMR experiments (Table 1). The protonated carbon signals were assigned to the corresponding proton signals by means of HMQC experiment, which also allowed the construction of spin-systems by combination with DQF-COSY experiment. Finally, the HMBC experiment enable the assignment of the quaternary carbons.

Compound **2**, which was assigned to a molecular formula of $C_{32}H_{54}O_4$ by HREIMS at *m*/*z* 502.4071 (requires 502.4022), exhibited eight tertiary methyl singlets at δ0.84, 0.87, 0.89, 0.93, 0.98, 1.11, 1.15 and 1.19, a carbinol methine proton at δ 4.47 (*br. s*), an oxymethine proton at δ3.50 (*dd*, $J = 5.7$, 9.0 Hz), an acetyl methyl at δ 2.10 (*s*) and some heavily-overlapping unresolved multiplets extending from δ 1.05 to 1.88 in its ^{1}H NMR spectrum, characteristic for dammarane-type triterpenoids (Hisham et al., 1996). The IR spectrum of **2** showed the presence of a hydroxyl absorption at 3622 (secondary OH) and an ester carbonyl absorption at 1730 and 1245 cm^{-1}. The ^{13}C NMR spectrum of **2** showed 32 ^{13}C signals which were resolved by a DEPT experiment into nine methyls, ten methylenes, six methines and six quaternary carbons, respectively. The overall spectroscopic profiles of **2** were found to be very similar to those of 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate (betulafolianediol 3-acetate; cabraleadiol monoacetate) or ocotillone-II, which are epimeric at C-24 (Nagai et al., 1973; Hisham et al., 1996), leaving the stereochemistry at positions C-20 and C-24 to be assigned.

Table 1
^{1}H and ^{13}C NMR spectral assignment for (*E*)-aglawone (**1**)[a]

No.	δ_C (mult.)	δ_H [mult., coupling (Hz)]	HMBC(H)
1	31.92 (*t*)	1.45 (*m*); 0.98 (*m*)	19
2	28.92 (*t*)	1.23 (*m*); 1.09 (*m*)	
3	66.45 (*d*)	4.06 (*t*, 2.6)	
4	36.33 (*t*)	1.25 (*m*); 1.05 (*m*)	
5	39.03 (*d*)	1.65 (*m*)	19
6	28.27 (*t*)	1.21 (*m*); 1.04 (*m*)	
7	31.87 (*t*)	1.44 (*obsc*); 1.02 (*m*)	
8	34.19 (*d*)	1.56 (*m*)	
9	54.03 (*d*)	0.91 (*m*)	19
10	36.25 (*s*)		19
11	20.52 (*t*)	1.70 (*m*); 1.45 (*m*)	
12	35.79 (*t*)	1.40 (*obsc*); 1.25 (*m*)	18
13	43.39 (*s*)		18, 20
14	50.07 (*d*)	1.42 (*m*)	18
15	37.93 (*t*)	1.96 (*dd*, 14.5; 17.0) 2.20 (*dd*, 7.4; 17.0)	
16	206.75 (*s*)		20
17	148.02 (*s*)		18, 20, 21
18	17.67 (*q*)	1.01 (*s*)	
19	11.17 (*q*)	0.83 (*s*)	
20	128.97 (*d*)	6.49 (*q*,7.5)	21
21	13.17 (*q*)	1.85 (*d*, 7.5)	20

[a] Recorded in $CDCl_3$, chemical shift values were reported as δ values (ppm) from internal TMS at 300 MHz, signal multiplicity and coupling constants (Hz) are shown in parentheses: *m* = multiplex signals; *obsc.* = obscured signals.

There has been some controversy regarding the assignment of the configuration of the C-24 chiral center of 20, 24-trisubstituted epoxides. Nagai et al. (1973) initially assigned the (*R*) configuration to C-24 for ocotillone-II, based on a series of chemical conversions and an X-ray crystallographic study, but Rao et al. (1975) claimed 24(*S*) stereochemistry and suggested 24(*R*) stereochemistry for cabraleone and its cogeners. A few years later, the C-24 stereochemistry of ocotillone and cabraleone was reassigned as *R* and *S*, respectively, by

Tanaka and Yahara (1978) and by Betancor et al. (1983). There was no controversy in regard to the C-20 configuration in both compounds.

In order to resolve the geometric stereochemical assignment at the chiral center at C-24 and validate the use of ^{1}H and ^{13}C NMR chemical shifts as proposed by Hisham et al. (1996) for predicting the C-20, 24 stereochemistry of these compounds, an X-ray crystallographic study was performed to conclusively determine the stereochemistry of compound **2**. In addition, the NMR spectroscopic resonances were unequivocally assigned by use of 1D- and 2D NMR spectroscopy.

A suitable crystal that formed in the monoclinic space group C2 was selected for the study. Accurate lattice constants were $a=30.2897(6)$ Å, $b=7.1071(2)$ Å, $c=13.8023(4)$ Å. Four molecules of the composition $C_{32}H_{54}O_4$ formed the asymmetric unit. All unique diffraction maxima with $2\theta \leqslant 49^{\circ}$ were collected using $2\theta{:}\theta$ scans and graphite monochromated MoK_{α} radiation. A total of 7181 unique reflections were collected. A computer-generated drawing of the final X-ray model of 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate is given in Fig. 2. All the ^{1}H and ^{13}C NMR chemical shifts were unambiguously assigned by means of 2D NMR techniques and were found to be consistent with those made by Hisham et al. (1996). Thus, the stereochemistry of 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate was conclusively determined, and the published NMR spectral assignments were confirmed.

Compound **3** was readily discerned to be 20*S*, 24*S*-epoxydammarane-3α, 25-diol, a deacetyl derivative of **2**, by a comparison of its NMR profile with that of the literature values (Hisham et al., 1996). The deacetylated derivative prepared from **2** by treatment of K_2CO_3-MeOH was found to be identical and possessing the same stereochemistry as that of **2** (20*S*, 24*S*) by a direct comparison of their mp, the ^{1}H and ^{13}C NMR spectral data. Therefore, compound **3** was unambiguously identified as 20*S*, 24*S*-epoxydammarane-3α, 25-diol (betulafolianediol 3-acetate; cabraleadiol).

β-Amyrone (Seo et al., 1975; Kikuchi et al., 1980), β-amyrin (Seo et al., 1975; Kikuchi et al., 1980), were identified by direct comparison of their physical and NMR spectral data with those from the literature.

A mixture of two phytosterols was also isolated. There are two very close but well-resolved sets of signals of equal intensity observed in both ^{1}H and ^{13}C NMR spectra taken in $CDCl_3$, which suggested that the two existed in equal population. The ^{13}C NMR spectral data were fully superimposable on those of stigmasterol and poriferasterol (Matsumoto et al., 1984; Horibe et al., 1989), resulting in the identification of this mixture as equimolecular epimeric mixture of stigmasterol and poriferasterol.

None of the isolates were shown to be cytotoxic against several human cancer cell lines (LNCaP, Col 2 or KB), using established protocols (Likhitwitayawuid et al., 1993). Further bioassay-guided of the $CHCl_3$ soluble fraction with an aim to isolate and identify the bioactives responsible for the cytotoxcity observed for the extract is in progress.

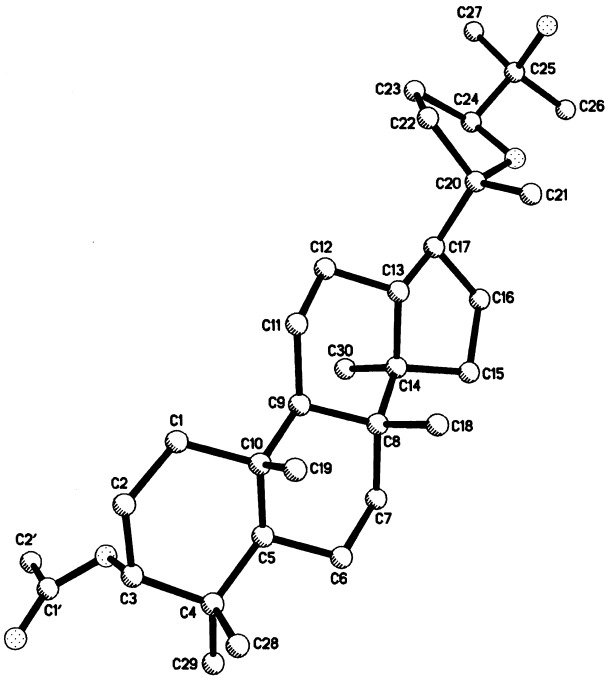


Fig. 2. Computer-generated perspective drawing of the final X-ray model of 20*S*, 24*S*-epoxydammarane-25-ol-3α-yl acetate (**2**).

3. Experimental section

3.1. General experimental procedures

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded with a MIDAC collegian FT–IR spectrometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. UV spectra were obtained with a Beckman DU-7 spectrometer. The ^{1}H and ^{13}C NMR, DEPT, DQF-COSY, HOHAHA, HMQC, HMBC and ROESY spectra were recorded with TMS as internal standard, using a Bruker Advance DPX-300 instrument equipped with a Bruker standard program in $CDCl_3$ solution. Electrospray MS/MS was performed on a Macro Mass Quattro II electrospray triple quadrupole mass spectrometer. Electron impact mass spectra (70 eV) were recorded with a Varian MAT-112S mass spectrometer. TLC was performed on precoated Kieselgel 60 F254 plates (Merck) and the visualization was achieved by spraying with either 10% H_2SO_4 followed by heating or phosphomobydbic acid spray reagent.

The X-ray diffraction study of compound **2** was conducted on a R3M/V four-circled diffractometer (Siemens)

connected to a MicroVax II computer (Digital) using CuK_α or MoK_α radiations.

3.2. Plant material

Stem bark of *A. lawii* was collected in Cuc Phuong National Park, Vietnam, and identified by one of the authors (DDS). Voucher specimens (No. MAF-1414B) have been deposited in the Herbaria of the Cuc Phuong National Park, Vietnam, and the Field Museum of Natural History, Chicago, IL, USA.

3.3. Extraction and isolation

The milled, air-dried stem bark of *A. lawii* (4 kg) was extracted with MeOH (9 l×3) by maceration. The resulting pooled extract was concentrated to dryness in vacuo and defatted with *n*-hexane to afford a *n*-hexane-soluble fraction (25 g). The defatted residue was then suspended in H_2O and partitioned with $CHCl_3$ to give, on drying under reduced pressure, 98 g of a $CHCl_3$-soluble fraction. This fraction demonstrated cytotoxic activity against the LNCaP (hormone-dependent prostate human cancer), KB (human oral epidermoid carcinoma), and Col 2 (human colon cancer) cell lines with ED_{50} values of 8.2, 11.9, and 9.0 μg/ml, respectively.

Fractionation of the $CHCl_3$-soluble fraction was initiated by column chromatography over silica gel (450 g) using a gradient mixture of petroleum and EtOAc (0–100%) as eluent. Sixteen fractions were pooled on the basis of their similar TLC patterns. Work-up of fraction 2 afforded compound β-amyrone (1.0 g) after recrystallization from a mixture of petroleum ether and $CHCl_3$ (1:1). Work-up of fractions 4 and 5, followed by recrystallization from $CHCl_3$, resulted in the isolation of β-amyrin (520 mg) and **2** (5.2 g). The epimeric mixture of stigmasterol plus poriferasterol (400 mg) was isolated from the work-up of fraction 6 and recrystallization from $CHCl_3$. Fraction 11 was further chromatographed on a silica gel column (50 g) and eluted with a mixture of petroleum/$CHCl_3$/MeOH (20:20:1, v/v/v) followed by preparative TLC (silica gel, solvent system: petroleum: EtOAc = 4:1) to afford compounds **1** (3.5 mg) and **3** (7 mg).

(*E*)-Aglawone (**1**), white powder; mp 125–126°C; $[\alpha]_D$ −0.1° (*c*, 0.2, MeOH); UV (MeOH) nm (log *E*) λ_{max}: 242 (3.82); IR (film) cm^{-1}: 3350, 1718, 1640, 1065; ^1H and ^{13}C NMR data are given in Table 1; Electrospray MS/MS *m/z*: 317 $[M+H]^+$, 281 $[M+H\text{-}H_2O\times2]^+$; HREIMS: 316.2410 for $C_{21}H_{32}O_2$ (required 316.2402).

3.4. 20S, 24S-Epoxydammarane-25-ol-3α-yl acetate (cabraleadiol monoacetate) (2)

Colorless needle, mp 148–150°C; ^1H NMR (ppm from solvent $CDCl_3$ signal at 7.27) δ: 0.84 (3H, *s*, Me-28), 0.87 (3H, *s*, Me-19), 0.88 (3H, *s*, Me-29), 0.93 (3H, *s*, Me-30), 0.98 (3H, *s*, Me-18), 1.11 (3H, *s*, Me-17), 1.15 (3H, *s*, Me-21), 1.19 (3H, *s*, Me-26), 2.09 (3H, *s*, Ac-Me), 3.65 (1H, *dd*, *J* = 9.5, 5.7 Hz, H-24), 4.62 (1H, *br.s*, H-3β). ^{13}C NMR (ppm from solvent $CDCl_3$ signal at 77.0) δ : 33.9 (*t*, C-1), 22.5 (*t*, C-2), 77.8 (*d*, C-3), 36.3 (*s*, C-4), 50.3 (*d*, C-5), 17.7 (*t*, C-6), 34.7 (*t*, C-7), 40.1 (*s*, C-8), 50.2 (*d*, C-9), 36.7 (*s*, C-10), 21.2 (*t*, C-11), 26.8 (*t*, C-12), 42.4 (*d*, C-13), 49.7 (*s*, C-14), 31.1 (*t*, C-15), 25.5 (*t*, C-16), 49.4 (*d*, C-17), 15,1 (*q*, C-18), 15.6 (*q*, C-19), 86.0 (*s*, C-20), 26.6 (*q*, C-21), 34.4 (*t*, C-22), 26.0 (*t*, C-23), 85.9 (*d*, C-24), 69.8 (*s*, C-25), 27.2 (*q*, C-26), 23.9 (*q*, C-27), 27.5 (*q*, C-28), 21.4 (*q*, C-29), 16.2 (*q*, C-30), 20.9 (*q*, Ac-Me).

Crystal data for **2**: $C_{32}H_{54}O_4$, MW = 502.75, monoclinic, C2, *a* = 30.2897(6) Å, *b* = 7.1071(2) Å, *c* = 13.8023(4) Å, α = 90.0000 (2)°, β = 95.3225(12)°, γ = 90.0000(10)°, *V* = 2958.44 (13) Å, *Z* = 4, crystal size: 0.5×0.3×0.23 mm, R1 = 0.0507, WR2 = 0.1127. All unique diffraction maxima with $2\theta \leqslant 49°$ were collected using $2\theta{:}\theta$ scans and graphite monochromated MoK_α radiation. A total of 7181 unique reflections were collected. The structure was solved by direct methods (SIR92) refined by full-matrix least-squares techniques.

Additional crystallographic details (atomic coordinates and equivalent isotropic displacement coefficients, lists of structure factors, interatomic distances, angles, anisotropic displacement coefficients for non-hydrogen atoms, hydrogen atom coordinates and their isotropic displacement coefficients) have been deposited at the Cambridge Crystallographic Data Centre. The coordinates can be obtained, upon request, from Dr. Olga Kennord, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.

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Alkyl phenols and derivatives from *Tapirira obtusa*

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Abstract

From the bark of *Tapiria obtusa,* six alkyl phenol derivatives were isolated: 1-hydroxy-3-[(*Z*)-7′-nonadecenyl]-benzene, 1-hydroxy-3-[(*Z*)-7′-heptadecenyl]-benzene, 1-hydroxy-3-[14′-phenyltetradecyl]-benzene, and 1-hydroxy-3-[16′-phenyltetradecyl]-benzene, and their possible biogenetic precursors, 1-(16′-phenyl-12′*Z*-hexadecenyl)-4-*Z*-cyclohexene-(1*S**,3*S**)-diol and (4*S**,6*S**)-dihydroxy-6-(14′*Z*-nonadecenyl)-2-cyclohexenone. The structures of these compounds were elucidated by chemical and spectroscopic analysis. (4*S**,6*S**)-Dihydroxy-6-(14′*Z*-nonadecenyl)-2-cyclohexenone showed cytotoxic activity. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Tapirira obtusa*; Anacardiaceae; Alkyl phenol derivatives; Alkenyl phenyl cyclohexenediol derivative; Alkenyl cyclohexenone derivative

1. Introduction

The genus *Tapirira*, which is composed of approximately 15 species, belongs to the Anacardiaceae family and is found primarily from Mexico throughout South America. *T. obtusa* (Benth) J.D. Mitchel is a small tree that occurs in the northeastern region of Brazil, especially in the State of Bahia. This plant is popularly known as ''pau-pombo-da-serra'' and is botanically similar to *T. guianensis* whose bark and leaves are used locally against leprosy, diarrhea and syphilis (Braga, 1976; Mello et al., 1971).

Previous bioactivity-directed fractionation of the seed extract of *T. guianensis* afforded two cytotoxic compounds (David et al., 1998). There are no other reports in the literature regarding the chemical constituents of this genus.

This paper describes studies on the chemical composition of the hexane extract from the bark of *T. obtusa* and the cytotoxic evaluation of the isolates. Eight compounds (**1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4** and **5**) were isolated, of which six are new substances. Compound **5** showed strong cytotoxic activity against several human cancer cell lines.

2. Results and discussion

The hexane extract of the bark of *T. obtusa* was subjected to column chromatography and preparative TLC on silica gel to afford compounds **1–5**, whose structures were elucidated by spectral analysis. Compounds **1a**, **1b**, **3a**, **3b**, **4** and **5** were new, and isolates **1a** and **2a**, and **1b** and **2b** were analyzed as a mixture. Their NMR spectral data were similar, and their chain lengths, as well as the location of the double bond in the alkenyl chains, were determined by MS.

The MS of biphenyl compounds **3a** and **3b** exhibited $[M]^+$ at *m*/*z* 366 ($C_{26}H_{38}O$) and 338 ($C_{24}H_{34}O$), respectively, and a base peak at *m*/*z* 108 due to benzylic cleavage of alkyl 3-hydroxybenzene. The ^1H NMR spectrum showed signals corresponding to nine hydrogens attached to aromatic rings, and a hydroxyl hydrogen exchangeable with D_2O. The two methylene carbons at δ 35.9 and 35.8, and identified by DEPT experiments, indicated that the two phenyl groups were joined by a saturated alkyl chain. Localization of the OH group at C-1 was made by comparison of the carbon resonances of the phenol moiety in **1** and **2**. Even though alkenyl phenols are common in the Anacardiaceae (Evans and Schmidt, 1980), this is the first report of alkylbenzylphenols from natural sources.

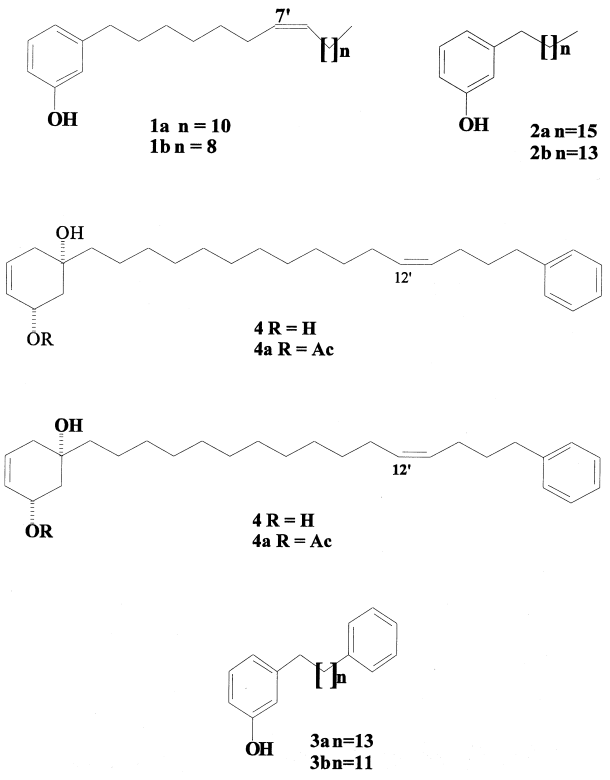
The cyclohexene moiety of **4** was identified by analysis of NMR spectroscopic data, including ^1H–^1H COSY

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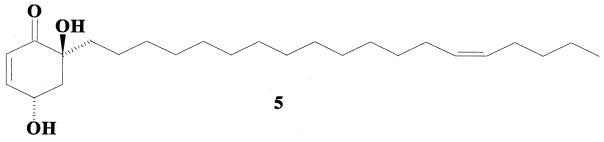
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and long-range ^{13}C–^{1}H COSY ($J=9$ Hz). The main correlations were observed for H-2 with C-4 and C-3, and of H-4 with C-1 and C-6. The ^{13}C NMR data of the 3-acetyl derivative **4a** (Table 1) confirmed the substitution pattern of **4**. The relative stereochemistry was determined by coupling constants, NOESY and NOE difference experiments.



The NMR values of 4*S**,6*S**-dihydroxy-6-(14′-nonadecenyl)-2-cyclohexenone (**5**) were quite similar to the compound previously isolated from the seeds of *T. guianensis* (David et al., 1998). However, differences were observed in the methylene ^{1}H NMR peaks and in the carbonyl data in the ^{13}C NMR spectrum. In addition, the ^{1}H–^{1}H COSY spectrum showed a correlation between H-3 and the oxymethine hydrogen (H-4). The correlations observed in the latter spectrum and the long-range ^{13}C–^{1}H COSY ($J=9$ Hz) spectra permitted establishment of a cyclohexenone ring in **5** (Table 1). The relative stereochemistry at C-6 was proposed based on NOESY experiments, which showed a correlation between H_2-1′ (δ1.8) and H-3 (δ6.87). This spatial correlation indicated that the OH group on C-6 must be *pseudo*-equatorial.

The location of the double bond in the compounds **1a**, **1b**, and **5**, was established through the MS fragments of the α,β-*bis*-(methylthio)-derivative produced by the reaction of the acetyl derivatives of **1a**, **1b**, **5**, with dimethyl disulfide (Francis and Veland, 1981). The double bond position in the alkenyl chain of **4** could not be determined by the above-mentioned methodology. However, the spatial correlation between the benzyl hydrogens (H-16′) and the olfinic hydrogen (H-13′) observed in the NOESY spectrum justifies placement of the double bond at C-12′ (Fig. 1).



The mixture of **1a**, **1b**, **2a** and **2b** was separated by GC analysis to afford four peaks which were correlated with the masses 358, 330, 332 and 304, a.m.u., respectively. Attempts to separate these compounds by reverse-phase HPLC were not successful.

The isolates were evaluated for cytotoxicity against a panel of human cancer cell lines (Table 2) using established protocols (Likhitwitayawuid et al., 1993). Only compound **5** demonstrated significant cytotoxic activity against the tested cell lines.

n-Alkylphenols are widely distributed in the Anacardiaceae family. This class of compounds are known as urushiols and are responsible for the allergic dermatitis caused by contact with some plants (e.g. *Toxicodendron* sp.) (Vogl and Mitchell, 1996). Compounds **4** and **5** would appear to be biogenetic precursors of the alkenyl phenols that are quite common in the Anacardiaceae. Walters and co-workers (Walters et al., 1990) proposed that *n*-alkyl phenols are derived from saturated and unsaturated fatty acids.

3. Experimental

3.1. General

The FTIR spectrum was recorded on a JASCO spectrophotometer Mod. Valor III. ^{1}H and ^{13}C NMR, DEPT, COSY, HETEROCOSY ($J=140$ and 9 Hz) spectra were obtained on a Varian Gemini 300 instrument employing $CDCl_3$ as solvent and reference. The MS were recorded on a Finnigan MAT 90 spectrometer. Column chromatography was carried out on Silica gel 60. Fractions were monitored by TLC on silica gel and spots were visualized with iodine fumes and UV light (254/366 nm).

3.2. Plant material

The bark of *T. obtusa* was collected in May 1997 in Chapada Diamantina, city of Lençois (BA), Brazil. The plant material was identified by Professor Maria Lenise S. Guedes and a voucher was deposited at the Herbarium Alexandre Leal Costa of the Universidade Federal da Bahia under number 032912.

Table 1
^{1}H NMR[a], ^{13}C NMR[b] spectral data and long range correlations ($J = 9$ Hz) of **4** and **5**

	4			**5**		
Position	H	C	^{13}C–^{1}H correlation	H	C	^{13}C–^{1}H correlation
1	–	72.4	2.02	–	201.0	6.87; 2.25
2	1.43 *m* 2.10 *m*	42.9		6.04 *dd* (10.2; 1.1)	126.7	6.04; 4.70
3	4.5 *m*	66.0	1.43	6.87 *dd* (10.2; 3.6)	149.4	4.70; 2.25
4	2.15 *m* 2.02 *m*	37.4	5.81	4.70 *m*	64.1	6.04; 2.25
5	5.71 *dt* (10.1; 2.2)	125.8	2.15	2.25 *m*	41.1	6.87; 2.25; 1.80
6	5.81 *d* (10.4)	130.5	2.10		74.5	6.04; 4.70; 2.25; 1.80
1′	1.50–1.48 *m*	43.3	1.43		39.1	2.25
11′, 14′	2.01–2.04 *m*	27.3/26.9		–	–	–
12′, 13′	5.36 *dt* (9.1, 4.6)	129.8 129.7		–	–	–
13′, 16′	–	–			26.9; 27.2	
14′, 15′	2.01–2.04 *m* 1.62 *q* (7.4)	26.9 31.5		5.35 *dt* (9.1; 4.6)	129.9; 129.8	
16′	2.61 *t*	36.0				
18′	–	–	–	1.27 *m*	22.3	0.90
19′	–	–		0.90 *t* (7.0)	13.9	
1″		142.6				
3″,5″	7.19 *m*	128.2	7.28			
2″,6″	7.28 *m*	128.1	7.28			
4″	7.23 *m*	125.5	7.28; 7.19			

[a] 300 MHz, $CDCl_3$, δ (ppm), *m* and *J* (Hz).
[b] 75 MHz.

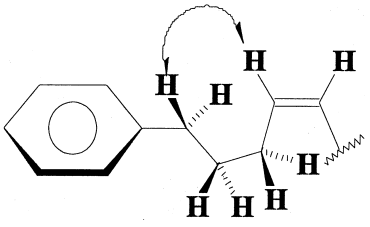


Fig. 1. Spatial correlation between the benzylic and olefinic protons.

3.3. Extraction and isolation

After drying in a ventilated oven (40°C) the trunk was separated from the bark (100 g), ground and extracted with hexane. The hexane extract (673 mg) was submitted to column chromatography over silica gel eluted with different mixtures of $CHCl_3$/EtOAc. The fractions obtained were further combined into 8 fractions on the basis of TLC. From the second fraction was isolated an oil (48 mg) composed of four compounds (**1a, 1b, 2a** and **2b**) which could only be separated by GC. These compounds were identified as 1 - hydroxy - 3 - [(*Z*) - 7′ - nonadecenyl]benzene (**1a**), 1-hydroxy-3-[(*Z*)-7′-heptadecenyl]benzene (**1b**), 1-hydroxy-3-heptadecenylbenzene (**2a**), and 1-hydroxy-3-pentadecenylbenzene (**2b**). The third fraction was submitted to PTLC eluted with hexane/EtOAc (9:1) to afford a red-brown oil (70 mg) composed of an inseparable mixture of **3a** and **3b**. From the sixth fraction **4** (11 mg) and **5** (13 mg) were isolated after PTLC using hexane/EtOAc (6:4) as eluent.

Table 2
Cytotoxic activities of isolates of *T. obtusa* (ED_{50} in μg/ml)

	Cell line[a]						
Sample	BC1	Lu1	Col2	KB	KB-V (+VLB)	KB-V (−VLB)	LNCaP
1–2	> 20	18.7	> 20	> 20	> 20	> 20	15.5
3	> 20	> 20	> 20	14.4	> 20	> 20	> 20
4	> 20	19.5	10.2	11.3	> 20	> 20	10.6
5	3.7	12.0	4.1	1.4	9.7	> 20	1.0

[a] BC1 = human breast cancer; Lu1 = human lung cancer; Col2 = human colon cancer; KB = human epidermoid carcinoma; KB-V1 (+VLB) = vinblastine (VLB)-resistant KB with 1μg/ml VLB; KB-V1 (−VLB) = VLB-resistant KB without VLB; LNCaP = human hormone-dependent prostate cancer.

3.4. 1-Hydroxy-3-[(Z)-7′-nonadecenyl]benzene (**1a**); 1-hydroxy-3-[(Z)-7′-heptadecenyl]benzene (**1b**); 1-hydroxy-3-heptadecenylbenzene (**2a**); 1-hydroxy-3-pentadecenylbenzene (**2b**)

Red oil; EIMS *m/z* 358 (M^+, 17), 332 (M^+, 20), 330 (M^+, 30), 304 (M^+, 8), 161 (5), 149 (14), 147 (14), 133 (12), 121 (35), 120 (37), 109 (30), 108 (100); IR (film) ν_{max}3378, 2923, 2854, 1653, 1593, 1487 cm^{-1}; ^1H NMR spectral data [300 MHz, $CDCl_3$, δ (ppm), *J* (Hz)]: 6.65–6.70 (*m*, H-2/H-6), 6.78 (*d*, 7.4, H-4), 7.17 (*dd*, 7.4, 7.6; H-5), 2.58 (*t*, 7.3; H-1′), 5.38 (*dt*, 9.0, 4.5; H-7′/H-8′), 0.93 (*t*, 7.0; H-19′); ^{13}C NMR spectral data [75 MHz, $CDCl_3$, δ (ppm)]: 155.3 (C-1), 115.2 (C-2), 144.6 (C-3), 120.8 (C-4), 129.2 (C-5), 112.3 (C-6), 35.7 (C-1′), 31.2 (C-2′), 27.1 and 26.9 (C-3′/C-5′, C-10′/C-16′), 129.8 (C-7′/C-8′), 31.9 (C-17′), 22.2 (C-18′) and 13.9 (C-19′).

The alkyl and alkenyl phenols **1a, 1b, 2a**, and **2b** were separated using a DB5 column (30 min, init temp 100–300°C at 10°C/min, 30 min). Four peaks were observed at 19.96 min (**2a**, *m/z* 332), 21.4 min (**1b**, *m/z* 330), 23.64 min (**2b**, *m/z* 304), and 26.94 min (**1a**, *m/z* 358).

3.5. 1-Hydroxy-3-[14′-phenyltetradecyl]benzene (**3a**); 1-hydroxy-3-[14′-phenyldodecyl]benzene (**3b**)

Red-brown oil; EIMS *m/z* 366 (M^+, 11), 338 (M^+, 69), 332 (3), 330 (17). IR (film) ν_{max} 3390, 3026, 2925, 2854, 1589, 1496, 1456 cm^{-1}; ^1H NMR spectral data [300 MHz, $CDCl_3$, δ (ppm), *J* (Hz)]: 6.74 (*m*, H-2/H-4/H-6), 7.26 (*m*, H-5), 5.12 (*brs*, OH), 2.63 (*t*, 7.3, H-′), 2.60 (*t*, 7.4, H-14′). ^{13}C NMR spectral data [75 MHz, $CDCl_3$, δ (ppm)]: 155.3 (C-1), 115.2 (C-2), 144.8 (C-3), 120.8 (C-4), 129.2 (C-5), 112.3 (C-6), 35.9 (C-1′), 31.4 (C-2′), 142.8 (C-1″), 128.0 (C-2″/C-6″), 128.3 (C-3″/C-5″), 125.4 (C-4″).

3.6. 1-(16′-Phenyl-12′Z-hexadecenyl)- 4-cyclohexene-(1S*,3S*)-diol (**4**)

Yellow oil; $[\alpha]_D^{25}$ +7.0° (*c* = 0.3, $CHCl_3$); EIMS *m/z*: 412 (M^+, 100), 398 (2), 368 (6), 341 (5), 161 (9), 131 (22), 129 (11), 128 (18), 123 (23), 121 (12), 119 (13), 109 (28), 108 (10); IR (film) ν_{max}: 3382, 2927, 2854, 1572, 1496 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data: Table 1.

3.7. 3-(16′-Phenyl-12′Z-hexadecenyl)-3S*-acetyl-4-cyclohexen-1S*-ol (**4a**)

To a solution of pyridine (0.5 ml) and acetic anhydride (0.5 ml) was added compound **4** (8.0 mg) and the mixture allowed to stand at room temperature for 36 h. Cold H_2O was added and the acetyl derivative (7.6 mg) was extracted with $CHCl_3$. ^{13}C NMR spectral data (75 MHz, $CDCl_3$, δ (ppm): 170.6 (COO), 129.9 (C-12′), 129.8 (C-13′), 128.4 (C-3″/C-5″), 128.2 (C-2″/ C-6″), 127.9 (C-4), 126.3 (C-5), 125.5 (C-4″), 71.8 (C-3), 69.3 (C-3), 43.0 (C-1′), 38.8 (C-2), 37.5 (C-6), 36.0 (C-16′), 31.5 (C-15′), 27.2 (C-11′), 26.9 (C-14′).

3.8. 4S*, 6S*-Dihydroxy-6-(14′-nonadecenyl)2-cyclohexenone (**5**)

Yellow oil; $[\alpha]_D^{25}$ +10.4° (*c* = 0.25, $CHCl_3$); EIMS *m/z*: 412 (M^+, 18), 390 (4), 374 (42), 348 (5), 346 (42); IR (film) ν_{max}: 3393, 2954, 2858, 1685, 1446 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data: Table 1.

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Book reviews

Illustrated Dictionary of Mycology

M. Ulloa and R.T. Hanlin, American Phytopathological Society Press, St Paul, Minnesota, 2000, 448 pp., $99. ISBN 0-89054-257-0

Although fungi are now recognised as a distinctive phylum unrelated either to vascular plants or to algae, the teaching of mycology is still usually included as part of plant sciences in most British universities. However, when it comes to learning the arcane vocabulary of fungal nomenclature, the students tend to rebel. They may accept the complicated terms used to describe higher plant anatomy, but they find the extra strain of learning about abhymenial, abjection, abjunction and ablastic beyond them. Here at last is a dictionary which explains in simple language the meaning of the many technical terms of fungal structures. Furthermore, this dictionary provides a wealth of illustrations, so that it is possible to visualise the shape of asymmetric ascospores, auriculiform basidiocarps and asterinoid ascostrama.

Until now, the most generally useful mycology dictionary has been Ainsworth and Bisby's *Dictionary of Fungi*, published by the Commonwealth Mycological Institute. It has been so popular that it has reached an eighth edition. This new dictionary is essentially complementary to Ainsworth and Bisby, since it lacks its taxonomic emphasis. Instead of listing the many and various fungal genera, an outline of fungal classification is present as an Appendix. Only the major fungal classes received individual entries. Interestingly, the Oomycetes are still included, in spite of the fact that some taxonomists have separated them from the fungi and have placed them in the Kingdom Protoctista.

The great strength of this new dictionary is undoubtedly the illustrations of fungi in all their varieties of shape, structure and size. My only grumble is the lack of colour. Since the fruiting bodies of many toadstools display a wealth of colours provided by anthraquinone or carotenoid pigments, it is a shame not to see some colours in these illustrations. However, one should not complain too much, since the book is priced so reasonably that it is within reach of the keen mycological student.

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PII: S0031-9422(00)00436-2

Plant–Microbe Interactions

Volume 5 — edited by G. Stacey and N.T. Keen, American Phytopathological Society, St Paul, Minnesota, 2000, 336 pp., $64. ISBN 0-89054-260-0

This volume explores a number of interesting microbial plant pathogens or symbionts and the responses they elicit in their plant hosts. The emphasis is on the biological and genetical aspects of these interactions, although biochemical pathways do get some mention. The first chapter discusses the genetics of *Pseudomonas syringae* a harmful bacterium which acts as a model system for plant–bacterial interactions. The second chapter moves onto one of the most infamous of all plant disease organisms, potato blight or *Phytophthora infestans*. Much progress has been made in our understanding of this micro-organism at the molecular level and the recent reclassification of the Oomycetes, to which *Phytophthora* belongs, is mentioned. The next chapter is devoted to quorum sensing in plant–microbe interactions, while the fourth presents a molecular overview of *Agrobacterium rhizogenes*, the causative agent of hairy root disease.

Because of its simple chromosomal complement, *Arabidopsis thaliana* has become the most popular of all plants for molecular biologists. Where else to look for the genes of plant resistance? Here, in Chapter 5, the

mapping of the many gene copies for resistance to infection is outlined in some detail. Chapter 6 moves on to consider the plant response to pathogen invasion and discusses the redox conditioning effects of salicylic acid and genistein during elicitation. Chapter 7 is devoted to the regulation and function of the pectic enzymes produced by the pathogen *Erwinia chrysanthemi* for degrading plant cell walls. Chapter 8 reviews the role of plant protein receptors in pathogen recognition, while the final Chapter 9 is devoted to *Acetobacter diazotrophicus*, a beneficial endophyte of sugar cane, which has the ability to fix agriculturally significant levels of nitrogen in this crop.

PII: S0031-9422(00)00437-4

The range and quality of the articles in this series *Plant–Microbe Interactions* compare very favourably with the older and better established annual publication *Annual Reviews in Phytopathology*. Certainly, anyone wishing to keep up with progress on plant diseases and their effects on plants will need to consult both of these review publications.

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PHYTOCHEMISTRY

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Concentricol, a taxonomically significant triterpenoid from *Daldinia concentrica*

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Dedicated to the memory of Dr. W. Kolbe, former director of the Fuhlrott-Museum, and president of the Naturwissenschaftlicher Verein, Wuppertal

Abstract

In the course of a chemotaxonomic study of xylariaceous Ascomycetes, a major metabolite was isolated and identified from the ascostromata of *Daldinia concentrica*. The compound, for which the name concentricol is proposed, constitutes a highly oxidised squalene derivative. A survey of several *Daldinia* spp. from around the world (including several type materials), employing analytical HPLC–UV/Vis (with diode array detection) and positive electrospray HPLC–MS of stromatal MeOH extracts revealed that concentricol was omnipresent in the stromata of *D. concentrica*, as well as in those of several collections of the pantropical *Daldinia eschscholzii*. All other investigated *Daldinia* spp. were found devoid of concentricol but contained binaphthalenes, benzophenones and/or azaphilones as further taxonomically relevant main metabolites. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Daldinia concentrica*; *Daldinia eschscholzii*; Xylariaceae; Ascomycetes; Chemotaxonomy; Preparative HPLC; HPLC-UV/DAD; HPLC–MS fingerprinting; Triterpenes; (6,7,12,6′,7′,12′)-Hexahydroxysqualene; Concentricol

1. Introduction

Ascomycetes of the genus *Daldinia* (Xylariaceae) are rich in secondary metabolites. European and American *Daldinia* spp. had already been studied for secondary metabolites by Allport and Bu'Lock (1958, 1960) and Stadler (1993), respectively, which had resulted in the identification of characteristic metabolites in their stromata and cultures. More recently, two Japanese *Daldinia* spp. were investigated intensively by Hashimoto and Asakawa (1998 and references therein), leading to the discovery of more than 20 new metabolites. However, no previous studies as to the chemotaxonomic significance of these metabolites had so far been conducted, even though the feasibility of chemotaxonomy had been demonstrated in other Xylariaceae genera by Whalley and Edwards (1995).

In a study on correlations between, secondary metabolism, genetic fingerprints and morphological characters in *Daldinia* and allied Xylariaceae, several main taxonomically relevant compounds produced by these fungi (Fig. 2) were identified by HPLC–UV/Vis and HPLC–MS fingerprinting (Stadler et al., 2001). These studies revealed that some *Daldinia* spp. (e.g. *Daldinia childiae*) contained benzophenones (**3**, **4**) and azaphilones (**5**), whereas the species previously shown to contain terpene alkaloids of the cytochalasin type (**6**–**9**) by Buchanan et al. (1994) was identified as *Daldinia eschscholzii*. The remaining species were found devoid of both types of compounds and preferably yielded binaphthalene derivatives such as BNT and daldinol (**2a, 2b** in Fig. 2). The occurrence of secondary metabolites was correlated with both morphology and genetic fingerprints as derived from PCR-based investigations (Stadler et al., 2001). Moreover, the metabolite profiles found in stromatal extracts were strongly correlated to the observed

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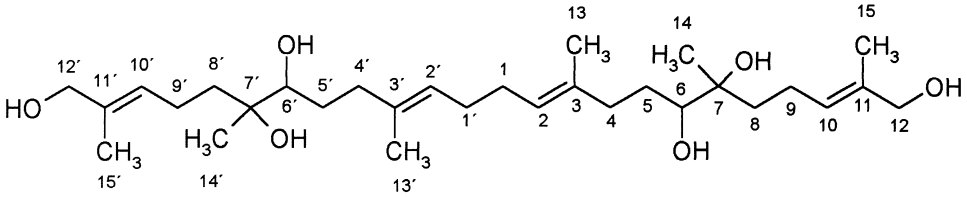


Fig. 1. Chemical structure of concentricol (**1**).

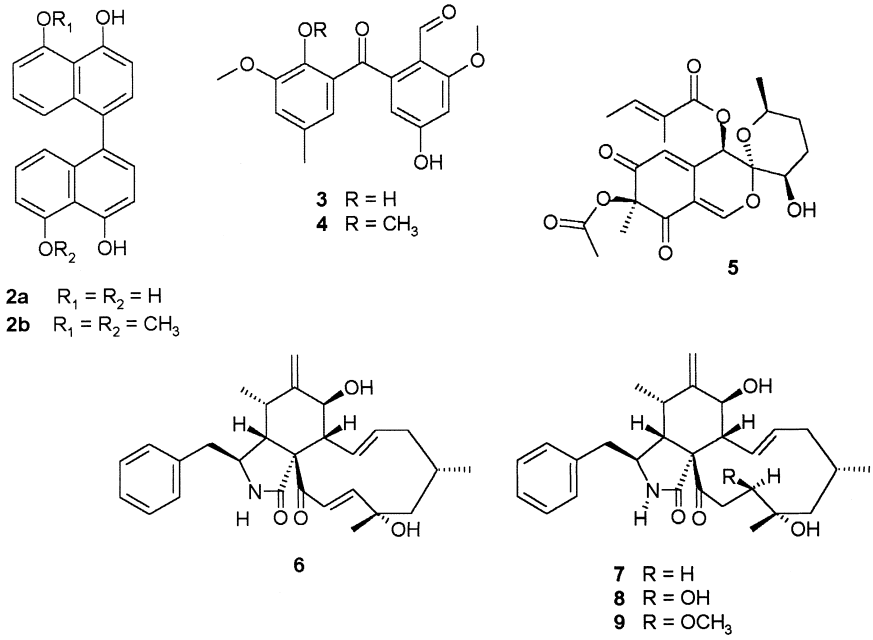


Fig. 2. Structures of secondary metabolites from stromata of *Daldinia*. **1**, concentricol, **2**, 1,1′-binaphthalene-4,4′-5,5′-tetrol (BNT); **2a**, daldinol; **3**, daldinal A; **4**, daldinal B; **5**, daldinin C; **6**, cytochalasin I; **7**, cytochalasin II; **8**, cytochalasin III; **9**, cytochalasin IV.

colours of stromatal pigments in KOH, i.e., a valuable diagnostic tool introduced by Ju et al. (1997) in their monograph to discriminate the morphologically very similar species of *Daldinia*. Besides above mentioned compounds, the HPLC-UV chromatograms of stromatal extracts of *Daldinia* spp. revealed several further characteristic peaks corresponding to taxonomically significant main metabolites, which had not yet been identified.

Recently, we collected the type species of the genus, *D. concentrica* (Bolt: Fr.) Ces. & De Not., in sufficient quantities for isolation and structure elucidation of a new natural product from stromata of this fungus. The present report deals with the identity of this compound and its distribution in *Daldinia* spp. as established by HPLC-based secondary metabolite fingerprinting methodology.

2. Results and discussion

2.1. Isolation and structure elucidation of concentricol (*1*)

The isolation of concentricol (**1**) from MeOH extracts of *D. concentrica* by preparative HPLC is described in the Experimental. The compound was fairly separated from another main metabolite, BNT (**2a**), in the employed gradient system. The physico-chemical data of concentricol are summarised further below.

The structure of concentricol (**1**) was deduced from a combination of NMR spectroscopy (^{1}H, COSY, HSQC, HMBC, DEPT-HMQC), ESI HPLC–MS and ESI high resolution mass spectrometry. In the positive ESI mode, the molecular ion was revealed at 511 (m/z + H), accounting for a molecular weight (M_r) of 510. Accordingly, the NMR signals were present in duplicate subset, pointing towards a symmetric molecule. The NMR and MS data is therefore in accordance with the structure proposal postulated as **1** (Fig. 1). No signals corresponding to the hydroxyl protons were observed during NMR measurements, and the stereochemistry of hydroxyl groups was not determined.

The biogenetic origin of concentricol (**1**) can be deduced from six isoprene moieties. Hence, concentricol (**1**) most probably represents a linear triterpenoid derived from squalene by introduction of six hydroxyl moieties (two terminal, two introduced upon opening of double bonds in the squalene chain). Squalene is the conceivable

progenitor of the higher terpenoids. Folding and cyclisation reactions and/or backbone rearrangement lead from squalene to tetracyclic and pentacyclic molecules of, e.g. the sterol, cucurbitane, and friedelane types (Torsell, 1997). However, linear triterpenoids are quite rare in fungi, as compared to cyclic triterpenes and steroids. The latter are not only abundant in fungi, but also widely occur in animals and plants.

Some examples for structurally related linear hydroxylated terpenoids from fungi are the glisoprenins and related compounds from cultures of imperfect fungi, which were described as inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT) by Tomoda et al. (1992), as neurokinin receptor inhibitors by Hedge et al. (1997), and as inhibitors of appressorium formation in phytopathogenic fungi (Sterner et al., 1998). Metabolites with remote structural similarities to concentricol were also obtained from Basidiomycetes (e.g. Findlay and He, 1991), plants (e.g. Sharma and Ali, 1998; Zdero et al., 1988) and sponges (e.g. Ovenden and Capon, 1999). Hydroxylated squalenes with strong structural similarities to concentricol were obtained from the plant, *Quassia multiflora* (Miller et al., 1995). The NMR data reported for partial structures (isoprenoid units) of these compounds were basically in accordance with those of concentricol.

Most interestingly, a group of linear polyhydroxylated squalene derivatives (berteric acid, cameronic acid, malaysic acid) have been obtained from the fruitbodies of *Xylaria* spp. (Adeboya et al., 1995). These metabolites, together with the related cubensic acid (O'Hagan et al., 1992) were proposed to constitute fatty acid derivatives. They are structurally very similar to concentricol, but possess a terminal carboxylic acid function. Further investigations are needed, however, to establish the taxonomic significance of linear squalene derivatives in *Xylariaceae* other than *Daldinia*.

2.2. Chemotaxonomy: distribution of concentricol in *Daldinia spp*.

In the course of a previous study (Stadler et al., 2001), numerous stromata and cultures of *Daldinia* spp. were compared by examination of morphology, genetic fingerprints and HPLC-based secondary metabolite profiles. The results not only pointed towards the existence of several previously undescribed species, but suggested the presence of several chemotaxonomically relevant metabolites in the stromatal extracts of known species. These compounds had not been isolated to purity and identified for lack of extractable material. As the HPLC–UV data from previous investigations, as well as the aliquots of stromatal extracts from the respective HPLC investigations (Stadler et al., 2001) had been saved for their retrospective investigations, numerous records of *Daldinia* were still available for studies on the occurrence of concentricol (**1**). Re-analysis of these HPLC data revealed that concentricol (**1**) only occurred in *D. concentrica* and the pantropical *D. eschscholzii*. Interestingly, a collection of *D. eschscholzii* from Japan (Ww 3551) which had been found by Buchanan et al. (1994) to contain large amounts of cytochalasins showed only minor quantities of concentricol, while the cytochalasins (**6**–**9** in Fig. 1) were prevailing. In other specimens of *D. eschscholzii*, (Ww 3518, Ww 3548, Ww 3591, Ww 3592) the opposite was observed: Cytochalasins did definitely not constitute their main stromatal metabolites, and the HPLC–UV chromatograms resembled those of *D. concentrica*, containing larger amounts of concentricol (**1**), as well as BNT (**2a**) as main metabolites. A morphological examination of the stromata of Ww 3551 had not revealed any significant differences between the other investigated specimens of *D. eschscholzii*. However, differences in the ascospore size range and the PCR fingerprints of *D. eschscholzii* had already pointed towards the existence of sub-populations of this taxon, which may have arisen from development of host specificity and geographic isolation (Stadler et al., 2001). Only the examination of a significant number of further materials will show whether *D. eschscholzii* can be divided into further taxa, according to secondary metabolite profiles and other means of investigation.

From a biogenetic view, both concentricol (**1**) and part of the cytochalasin molecules (**6**–**9**) probably constitute metabolites derived from the acetate–mevalonate pathway. Accordingly, the before mentioned *Daldinia* spp. contain terpenoids as main stromatal metabolites. In contrast, the main metabolites found in stromata of other *Daldinia* spp. (e.g. **2**–**5**) are probably of polyketide origin. Further main metabolites, BNT and daldinol (**2a**, **2b**) may constitute side products of the melanin biosynthesis because of their structural similarity with 1,8-dihydroxynaphthalene (DHN), the general melanin precursor in Ascomycetes (Wheeler, 1983). The involvement of DHN in secondary metabolism of *Daldinia* is also suggested by the occurrence of naphthalene derivatives in most cultures of *Daldinia* (Allport and BúLock, 1960; Anke et al., 1995; Stadler et al., 2001). The exact biogenetic origin of daldinals and daldinin (**3**–**5**), however, is not yet clear.

Daldinia spp. are being classified by Ju et al. (1997) by the colour of stromatal pigments. While the colour of stromatal pigments in KOH is yellowish in species like *D. childiae* (Rogers et al., 1999) and olivaceous in other species (e.g. *D. lloydii*), the majority of *Daldinia* spp. yields purplish stromatal pigments. Moreover, it is possible to distinguish between *Daldinia* spp. with strong or weaker purple pigments, respectively (Table 1; Stadler et al., 2001). During investigations of *D. concentrica* (collections from Wuppertal and Haan-Gruiten/Neandertal) throughout the season, it was found that the purple colour of stromatal pigments became weaker during maturation of ascospores, while young conidiogenous

Table 1
Distribution of some characteristic metabolites in stromata of *Daldinia* spp.[a]

Species	**1**	**2a, 2b**	**3–5**	**6–9**	Stromatal pigment colour (KOH)
Daldinia bambusicola	–	+	–	–	Purple
D. caldariorum	–	+	–	–	Purple
D. childiae	–	+	++–	–	Yellow
D. clavata	–	+	–	–	Purple
D. concentrica	++	+	–	(+)	Weak purple
D. eschscholzii	+/++	+	–	(+)/+/++	Weak purple
D. fissa	–	++	–	–	Dark purple
D. grandis	–	+	–	–	Dark purple
D. lloydii	–	+[b]	–	–	Olivaceous
D. loculata	–	++	–	–	Dark purple
D. petriniae	–	+[b]	–	–	Variable, mostly purple
D. singularis	–	+	–	–	Purple

[a] (+) Tentatively identified; + = present; ++ = present in large amounts (more than 70% of UV absorption at 210 nm); – = not detected.
[b] Signal overlaid by another peak corresponding to an unstable compound with similar polarity; for details see Stadler et al. (1998a).

specimens from the same collection sites had a stronger purplish pigment. HPLC investigations of these extracts showed that the amounts of BNT (**2a**) detectable in the crude extracts decreased, while the percentage of concentricol (**1**) increased upon ascospore production. Hence, the production of concentricol (**1**) is possibly related to the morphogenesis of the sexual stage in *D. concentrica*.

The importance of secondary metabolites as a means for classification of *Daldinia* spp. was only recently demonstrated by the discovery of *D. childiae* (Rogers et al., 1999). This cosmopolitan species of warmer climates, was until recently regarded as *D. concentrica* by many authors until Rogers et al. (1999) re-discovered the type material of *D. concentrica* and found it had purplish stromatal pigments as opposed to their *D. concentrica* ss. Ju et al. (1997). Later, it was established that the new taxon can be easily distinguished from *D. concentrica* by the presence of azaphilones and benzophenones as revealed by HPLC fingerprinting (Stadler et al., 2001). These compounds are held responsible for the yellow colours of their stromatal extracts in KOH (Rogers et al., 1999; Stadler et al., 2001). *D. concentrica*, however, had in the past not only been confused with *D. childiae*. Some other species of *Daldinia* occurring in Europe (e.g. *D. loculata*, *D. petriniae*) are morphologically very similar to *D. concentrica*. All these species may yield purplish stromatal pigments (Table 1), but according to our studies, the former taxa are characterised by the general absence of concentricol (**1**).

3. Conclusions

The novel triterpenoid, concentricol (**1**), constitutes a chemotaxonomically significant stromatal metabolite of *Daldinia* spp. The employed HPLC-based fingerprinting methodology will be the method of choice to extend chemotaxonomical investigations of *Daldinia* and other *Xylariaceae* on metabolites other than stromatal pigments. Such investigations can rely on minute quantities of starting material, once the taxonomically relevant metabolites have been identified.

4. Experimental

4.1. General experimental procedures

Chemicals were obtained in analytical grade from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany). NMR spectra were recorded in DMSO-d_6 using a Bruker DRX 500 spectrograph (500 MHz), whereas MS was conducted using a ThermoQuest Finnigan MAT 90 spectrometer. HPLC–MS analyses were performed using a TSP liquid chromatograph directly coupled with a MAT 900S mass spectrometer (Finnigan, Bremen, Germany) in the positive electrospray ionisation (ESI) mode, using a Waters Symmetry column as stationary phase and a previously described method (Stadler et al., 1998b). LC–MS spectra were recorded in the range of M_r between 150 and 1.200. HPLC–UV/Vis analyses were carried out on a HP 1100 Series analytical HPLC system (Hewlett-Packard, Waldbronn, Germany) comprising a G 1312A binary pump system, a G 1315A diode array detector, a G 1316A column compartment, a G 1322A degaser and a G 1313A autoinjector. As mobile phase, 0.1% H_3PO_4: acetonitrile was chosen, while a Merck (Darmstadt, Germany) Lichrospher RP 18 column (125×4 mm, particle size 7 μm) was employed as stationary phase. Aliquots of the samples (representing 2–10 μg of methanol-soluble materials, according to the conc. of main metabolites) were analysed at 40°C with a flow of 1 ml/min in the following gradient: linear from 0% acetonitrile to 100% acetonitrile in 10 min, thereafter isocratic conditions at 100% acetonitrile for 5 min; followed by regeneration of the column for 5 min. HPLC–UV chromatograms were

recorded at 210 nm with a reference wavelength of 550 nm and a bandwidth of 80 nm. Diode array detection (DAD) was employed to record HPLC–UV/Vis spectra in the range of 210–600 nm. The HP ChemStation software allowed for an automated search for calibrated standard compounds in crude extracts. Preparative HPLC was performed at room temperature on a preparative HPLC system (Gilson Abimed, Ratingen, Germany), comprising Gilson Unipoint software, 306 binary pump system, 205 fraction collector, 119 UV–Vis detector, 806 manometric module, and 811C dynamic mixer.

4.2. Secondary metabolites (standards)

Compounds **2**–**9** had been reported previously in the literature (Allport and Bu'Lock, 1958; Hashimoto and Asakawa, 1998 and references therein). While BNT (**2a**) was re-isolated from *Daldinia* spp. (Stadler et al., 2001) authentic samples of the remaining compounds were kindly provided by Y. Asakawa, Tokushima, Japan. The identity of all compounds was established based on their Rt, characteristic UV–Vis spectra and positive ESI mass spectra. Furthermore, the original starting materials used for the isolation of compounds **2b**–**9** (Hashimoto and Asakawa, 1998) were included as "standardised extracts". The taxonomy of these species [*D. childiae* Ww 3550; previously referred to as *D. concentrica*, and evaluated on its secondary metabolites by Hashimoto et al. (1994), as well as *D. eschscholzii* Ww 3551; previously referred to as *D.* cf. *vernicosa* and studied on chemical constituents by Buchanan et al. (1994)] had been revised after comparison of morphological data of the voucher specimens. All compounds were detected in the crude extracts by matching Rt, characteristic UV–Vis spectra and positive ESI mass spectra with those of external standards upon HPLC analyses.

4.3. Biological materials

The taxonomy of *Daldinia* spp. is given according to Ju et al. (1999) and Rogers et al. (1999). Reference materials were either collected by the authors or kindly provided by the individuals and institutions as listed below. All materials have been assigned a Ww number for practical purposes. They are referred to this number in the present report and concurrent publications. In total more than 250 records of *Daldinia* and allies from around the world have hitherto been studied by HPLC fingerprinting techniques and by other means of investigation. Information on the origin of the examined specimens is listed below [only data on some representative specimens are given; for further information see Stadler et al., 2001]. Besides morphological identification, the identity of most materials was also confirmed by PCR-based genetic fingerprinting (Stadler et al., 2001). If not indicated otherwise, voucher specimens were collected and identified by H.W. and M.S. and deposited at the herbarium of the Fuhlrott-Museum (H. W., Wuppertal, Germany; Ww). Abbreviations for other institutions who kindly provided us with materials: ZT = Eidgenössische Technische Hochschule, Zurich, Switzerland; K = Kew Royal Botanical Garden, U.K., M = Bot. Staatssammlung Munich, MCT = Museo Cantonale Ticino, Switzerland; WSP = herbarium of J.D. Rogers, Washington State University, Department Plant Pathology, Pullman, USA.

4.4. Selection of specimens examined

D. bambusicola Ju, Rogers and San Martín: Thailand: Ww 3707, Nakorn Nayok, X. 1994, *Bambusa* sp., WSP 69652, Holotype (Ju et al., 1997).

D. caldariorum Henn.: Spain: Ww 3292, Barra, IX. 1992, *Cytisus,* F. Candoussau 5365-595, ZT (Ju et al., 1997). UK: Ww 3692, Cornwall, Padstow, IV. 1993, *Ulex europaeus,* K (M) 47.140, M. C. Clark as *D. concentrica*.

D. childiae Rogers and Ju: Austria: Ww 3603, Rekawinkel near Vienna, VII 1940, *Carpinus,* M; immature specimens, F. Petrak. France: Ww 3587, Itxassou, VI. 1999, *Fraxinus*. Japan: Ww 3550, Tokushima, I. 1993, wood, Hashimoto et al. (1994) as *D. concentrica*; Ww 3643, Kamiyama-cho, Shikoku, X. 1999, *Quercus* sp., leg. M.S., Y. Asakawa and T. Hashimoto. Romania: Ww 3388, Mantenia Bucaresti, V. 1933, *Acer campestris,* M, leg. Savulescu. Switzerland: Ww 3714, Bremgarten-Zopfhau, Aargau, VII. 1999, *Fagus,* leg. R. de Marchi, Herbar of B. Senn-Irlet (Univ. Bern). USA: Ww 3608, St. Louis, Forest Park, VIII. 1999, wood; Ww 3609, Castlewood State Park, VII. 1999, *Quercus,* leg. D. Triebel & M.S.

D. clavata Henn.: Liberia: Ww 3708, Ganta, XI.1939, wood, WSP, Harley, G. W. 331 (Ju et al., 1997).

D. concentrica (Bolt: Fr.) Ces. & De Not.: Germany: Ww 0945, Wuppertal, XI.1979, *Fraxinus excelsior;* Ww 2992, Bielefeld, I. 1978, wood, I. & W. Sonneborn; Ww 3038, Wuppertal, XI. 1996, *Fraxinus excelsior*; Ww 3080, Bielefeld, IX. 1996, *Fagus sylvatica,* I. and W. Sonneborn; Ww 3316, Neandertal, IV. 1998, *Fraxinus excelsior*; Ww 3522, Wuppertal, XI. 1998, *Fraxinus excelsior,* immature specimens; Ww 3685, Bavaria, Salzach-Auen, VIII. 1978, wood, T.R. Lohmeyer No. 78/58. France: Ww 3588, Coulon, VI. 1999, *Fraxinus angustifolia;* Ww 3586, Angers, I. 1979, *Fraxinus,* T.R. Lohmeyer No. 79/1; Italy: Ww 3598, Pisa, IV. 1992, *Populus,* MCT 3591, leg. F. Bellu, (Lucchini, 1997); UK: Ww 3686, Renmore Common, Surrey, V. 1985, *Fraxinus,* T.R. Lohmeyer No. 85/30; Ww 3690, Halifax, Black Brook; UK, IV. 1998, *Ulmus,* K(M). SE 086208, M.W. Sykes.

D. eschscholzii (Ehrenb.: Fr.) Rehm: Cuba: Ww 3591, Cienaga de Zapata, IV. 1999, *Bucida palustris,* leg. R.F. Castañeda and C. Decock; Ww 3592, Cumanayagua, IV. 1999, *Jambosa vulgaris,* leg. R.F. Castañeda and C. Decock. Japan: Ww 3551, Tokushima, VI. 1992, wood,

leg. Y. Asakawa and T. Hashimoto; Buchanan et al. (1994); Hashimoto and Asakawa (1998). Pakistan: Ww 3548, Lahore, XI. 1961, *Morus alba,* M, Herbar Petrak, leg. S. Ahmad. Papua New Guinea: Ww 3563, VII. 1997, wood, Häffner (1999); Spain: Ww 3518, La Galga, Isla de La Palma, Canary Islands, XI. 1998, *Laurus azorica*.

D. fissa Lloyd: Canada: Ww 3590, Humber River, near Toronto, IX. 1935, *Carpinus caroliniana,* M; leg. H.D. Darkis (Herbar Petrak). France: Ww 3684, Bois de Chaumont, II. 1979, *Betula* (burnt), leg. T.R. Lohmeyer and J. Mornand. Germany: Ww 2885, Wuppertal, VIII. 1996, *Fagus* (burnt), Ju et al. (1999); Ww 3034, Gruiten, XI. 1996, *Carpinus* (burnt), leg. S. Woike; Ww 3077, Hilden, VII. 1963, *Betula,* leg. S. Woike, immature specimens; Ww 3188, Gruiten, IX. 1997, *Carpinus* (burnt), immature specimens, Ww 3191; Gruiten, IX. 1997, *Corylus* (burnt); Ww 3509, Düsseldorf, XI. 1965, *Quercus rubra,* leg. S. Woike. Switzerland: Ww 3601, Castensago, X. 1992, *Fagus sylvatica,* MCT 8110 (Lucchini, 1997). UK: Ww 2042, New Forest, Shatterfordbottom, England, VIII. 1992, *Ulex* (burnt); Ww 3693, Fairmile Common, Surrey, England, IX. 1996, *Ulex europaeus* (burnt), K (M) 39.729 leg. et det. B.M. Spooner as *D. vernicosa*.

D. grandis Child: Mexico: Ww 3700, Nuevo León state, San Roque, wood in oak forest, WSP, leg. R.F. Jorge, Ju et al. (1997).

D. lloydii Ju, Rogers and San Martín: Austria: Ww 3594, Spittal (Drau), "before 1930", *Betula,* M, leg. G. Niessl v. Mayendorf; immature specimens; Romania: Ww 3547, Bad Zaizon, VIII. 1883, *Betula alba,* M, leg. Römer; Slovak Republic: Ww 3595, Hranice, IX. 1938, *Betula pendula,* M, F. Petrak No. 1824; immature specimens; USA: Ww 3593, Chevry Mt. NH, VII. 1928, *Betula lutea,* M, leg. G. Spaulding; Ww 3610, Squaw Creek, Idaho, VII. 1967, *Betula papyrifera,* WSP 57303, leg. H. Goree, Ju et al. (1997).

D. loculata (Lév) Sacc.: Germany: Ww 0367, Wuppertal, X. 1970, *Betula*; Ww 2886, Wuppertal, IX. 1969, *Betula*; Ww 3086, Hilden, X. 1971, *Betula*, leg. S. Woike. UK: Ww 3691, Pirbright, Surrey, England, XI. 1991, *Betula pendula* (burnt), K (M) 18.694; leg. G. Butterfill, det. T. Laessøe as *D. concentrica*, Ww 3696, Esher West End Common, Surrey, England, X. 1993, *Fagus*, K(M), leg. P.M. Kirk, det. B. Spooner as *D. concentrica*. USA: Ww 3578, Trinidad, CA., VI. 1939, *Alnus*, M, leg. H.D. Darkes and C.L. Sheer as *D. occidentalis*.

D. petriniae Ju, Rogers and San Martín: Austria: Ww 3391, Zillertal, VIII. 1998, *Alnus incana,* leg. K. Helm, Ww 3392, Zillertal, VIII. 1998, *Alnus incana,* leg. J. Keller, immature specimens, Ww 3393, Jenbach, IX.1998, *Alnus incana,* leg. E. Wollweber. Czech Republic: Ww 3604, "Mährisch Weisskirchen", IX. 1928, *Carpinus,* M, leg. F. Petrak. Germany; Ww 3039, Weingarten, VIII. 1997, *Alnus glutinosa,* leg. W. Winterhof, det. L. Petrini as *D. occidentalis;* Ww 3373, Waldkraiburg, X. 1972, *Alnus,* M, leg. H. Marschner (No. 397). Switzerland: Ww 3297, Ramosch, IX. 1983, *Alnus incana,* ZT, Holotype (Ju et al., 1997). USA: Ww 3577, Newman Lake, WA. VIII. 1935, *Alnus rhombifolia,* M, leg. C.R. Stillinger as *D. occidentalis*.

D. singularis Ju, Vasilyeva and Rogers: Russia: Ww 3706, near Vladivostok, IX.1997, *Carpinus cordata*, WSP, Isotype, Ju et al. (1999).

4.5. Small-scale extraction of stromata

Aliquots of the freeze-dried stromata, always containing materials from the pigmented ectostroma, were ground in a mortar. MeOH extracts were prepared from these powdery materials (minimum amounts used for extraction were 10 mg) as described above for fermentation-derived materials.

4.5.1. Isolation of concentricol (*1*)

Stromata of *D. concentrica* were collected during a field foray to Winkelsmühle, Neandertal, Haan-Gruiten, Germany, on dead stems of *F. excelsior*. Voucher specimens, which showed the typical characteristics described by Rogers et al. (1999) are deposited in the herbaria of H. Wollweber (Wuppertal, Germany) and J.D. Rogers (Washington State University, Pullman, Washington, USA). The stromata of *D. concentrica* (fresh weight ca. 150 g) were powdered and extracted twice with each 1 l MeOH, resulting in 16.5 g of an oily crude extract. 1.2 g of this crude MeOH extract were dissolved in 5 ml of MeOH, filtered through a Bond Elut C18 500 mg solid phase extraction cartridge (Baker, Deventer, The Netherlands) and applied directly onto a MZ Analysentechnik (Mainz, Germany) Kromasil RP 8 column (particle size, 7 μm; 250×40 mm; mobile phase, 0.01% TFA: acetonitrile). The following experimental conditions were employed: flow: 10 ml/min. 25% acetonitrile at $t = 0$ min, linear gradient: 25–50% acetonitrile in 30 min; thereafter isocratic conditions at 50% acetonitrile for 15 min; thereafter linear gradient from 50 to 75% acetonitrile in 15 min; thereafter, isocratic conditions at 75% acetonitrile for 15 min, thereafter linear gradient from 75 to 100% acetonitrile in 15 min. Fractions were combined according to UV adsorption at 210 nm. Retention times (Rt) in this gradient system were observed as follows: 64–67 min for concentricol (**1**), 74–79 min for BNT (**2a**)**.** Yields were 160 mg of concentricol (**1**) and 112 mg of BNT (**2a**) per g of crude extract, which corresponded to yields of more than 1 g of each compound from the extracted 150 g fresh wt.

4.5.2. Compound characterisation

Concentricol (**1**), (6,7,12,6′,7′,12′)-hexahydroxysqualene. Colourless amorphic substance. HPLC–UV: Rt = 7.62 min. HPLC–MS: Rt = 6.05 min; ESI–MS 511 ($m/z + H$); ESI–HR–MS: 511.4048 ($m/z + H$) calc. for $C_{30}H_{54}O_6$.

^{13}C NMR spectral data (125.76 MHz, DMSO-d_6): *d* 27.9 (*t*, C-1), 123.6 (*d*, C-2), 135.4 (*s*, C-3), 36.8 (*t*, C-4), 29.4 (*t*, C-5), 76.0 (*d*, C6), 73.1 (*s*, C-7), 38.8 (*t*, C-8), 21.1 (*t*, C-9), 124.9 (*d*, C-10), 134.8 (*s*, C-11), 66.6 (*t*, C-12), 16.1 (*q*, C-13), 21.7 (*q*, C-14), 13.6 (*q*, C-15).

^{1}H spectral data (500.13 MHz, DMSO-d_6): *d* 1.98 (2H, *m*, H-1), 5.14 (1H, *m*, not resolved, H-2), 1.87 (1H, *m*, H-4), 2.18 (1H, *m*, H-4), 1.66 (1H, *m*, H-5), 1.20 (1H, *m*, H-5), 3.09 (1H, *m*, $J=9.9$ Hz, H6), 1.35 (1H, *m*, $J=12$ Hz (gem.), $J=4.9$ Hz, H8), 1.45 (1H, *m*, $J=12$ Hz (gem.), $J=4.9$, H8), 1.99 (1H, overlap, H9), 2.04 (1H, overlap, H9), 5.30 (1H, *t*, $J=6.2$ Hz, H10), 3.76 (2H, *s*, H12), 1.56 (3H, *s*, H13), 0.96 (3H, *s*, H14), 1.53 (3H, *s*, H15).

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Tetra-unsaturated sesterterpenoids (Haslenes) from *Haslea ostrearia* and related species

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Abstract

The structures and distributions of C_{25} highly branched isoprenoid (HBI) alkenes (Haslenes) have been determined following isolation from cultures of the diatoms *Haslea ostrearia, Haslea crucigera, Haslea pseudostrearia* and *Haslea saltstonica*. The distributions of the HBIs change between *Haslea* species and also between different cultures of the same species. Large scale culturing of *H. ostrearia* and *H. pseudostrearia* has enabled the structures of three new tetra-unsaturated alkenes to be determined by NMR spectroscopy. The structural relationships between different Haslenes together with the potential significance of the biosynthesis of HBIs by the *Haslea* genus is discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Haslea ostrearia*; *Haslea crucigera*; *Haslea pseudostrearia*; *Haslea saltstonica*; Microalgae; Diatoms; Isoprenoid alkenes; Highly branched isoprenoids; C_{25} alkenes; Non-saponifiable lipids

1. Introduction

In a number of recent reports, we have described the structures and distributions of a series of C_{25} highly branched isoprenoid (HBI) hydrocarbons that are present in the diatom *Haslea ostrearia* (Gaillon) Simonsen (Belt et al., 1996; Johns et al., 1999; Wraige et al., 1997, 1999). These compounds (parent structure **I**; Fig. 1) are ubiquitous in contemporary marine sediments and are often the major hydrocarbons present (for a review, see Rowland and Robson, 1990). Further, by use of axenic cultures, we have demonstrated conclusively that *H. ostrearia* is a primary producer of these lipids (Wraige et al., 1999). In culture, HBIs range in their unsaturation (e.g. **III**–**X**) between two and six double bonds, though monoenes (e.g. **II**) and the parent alkane (I) have also been observed in sediments (Robson and Rowland, 1986). Cultures of *H. ostrearia* are usually dominated by trienes and tetraenes, often as mixtures of geometric and/or diastereoisomers (Belt et al., 1996). To date, the reasons for varying distributions of HBIs in cultures of *H. ostrearia* are not fully understood, though it is apparent that temperature and not salinity is a major controlling influence over unsaturation (Wraige et al., 1998; Rowland et al., 2001). In sediments, it is likely that HBIs undergo isomerisation and cyclisation reactions depending on the degree of unsaturation (Belt et al., 2000a). More recently, two new producers of C_{25} HBI alkenes have been reported (viz. *Rhizosolenia setigera* (Sinninghe Damsté et al., 1999) and *Pleurosigma intermedium* (Belt et al., 2000b,c)). The C_{25} pentaene (**IX**) found in *R. setigera* is also produced by *H. ostrearia* (Wraige et al., 1997) but the HBIs from *P. intermedium* represent a slightly different structural type (e.g. **XI**-**XII**), since these compounds are unsaturated at the major branch point, C-7.

In the present study, we report the presence of HBI alkenes in three previously unexamined *Haslea* species (*Haslea crucigera* (Wm. Smith) Simonsen, *Haslea pseudostrearia* Massé et al. and *Haslea saltstonica* Massé et al.) together with the characterisation of three new HBI tetraenes.

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2. Results and discussion

2.1. Identification of HBIs in four species of the Haslea genus

The four *Haslea* species that have been cultured and examined for HBIs in this study are *H. ostrearia*, *H. crucigera*, *H. pseudostrearia* and *H. saltstonica*. Each of these species are pennate, benthic diatoms measuring ca. 30–100×6–20 μm. The main structural differences between them have been described elsewhere (Massé et al., 2000). After large scale culture of each *Haslea* species followed by centrifugation, freeze drying and extraction (hexane), GC and GC–MS analysis of the total hexane extracts (THEs) revealed the presence of several HBI alkenes with varying distributions. HBI production from *H. ostrearia* is well known (Volkman et al., 1994; Belt et al., 1996; Johns et al., 1999; Wraige et al., 1997, 1999), but this is the first report of HBIs from other *Haslea* species. Following saponification (KOH/MeOH/H_2O) and re-extraction (hexane), the remaining non-saponifiable lipid fractions consisted almost entirely of HBIs and phytol. Fig. 2 shows representative partial total ion current chromatograms (TICs) of these fractions for *H. ostrearia, H. crucigera, H. pseudostrearia and H. saltstonica* cultures, which clearly illustrates the presence of different HBI distributions in each *Haslea* species. The differences between these distributions are not presumed to be an indicator of each specific *Haslea* species, since analysis of a number of different cultures of *H. ostrearia* reveals a substantial variation in HBI structure(s) (Belt et al., 1996; Johns et al., 1999; Wraige et al., 1997, 1999) and the same may well be true for the other three *Haslea* species studied here. However, our analyses do verify that each species can be considered to be a primary producer of these sesterterpenoid lipids.

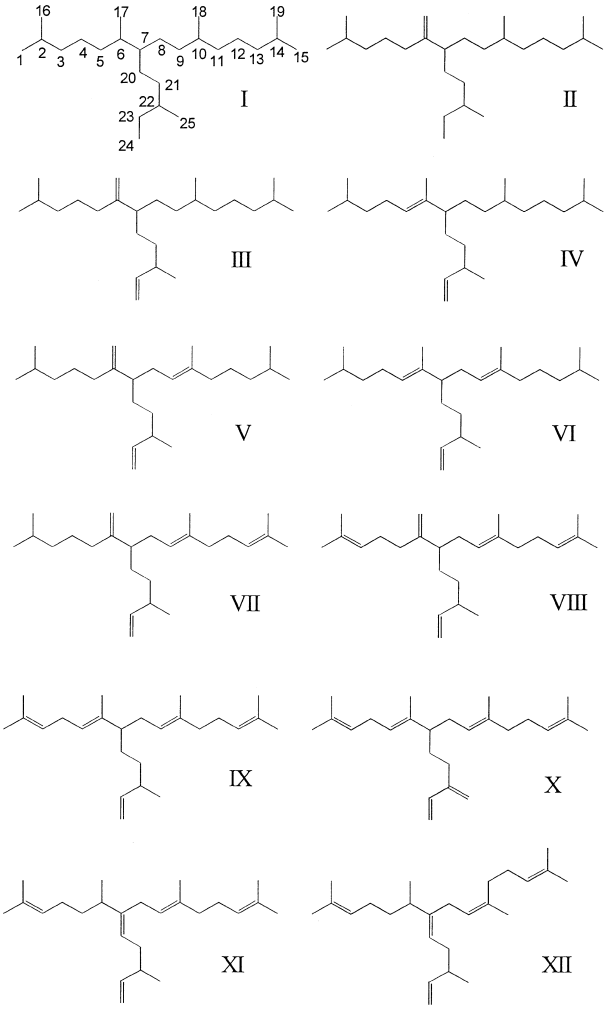


Fig. 1. Structures of C_{25} HBI alkenes previously characterised in sediments and diatoms. The numbering scheme is illustrated for the parent alkane I.

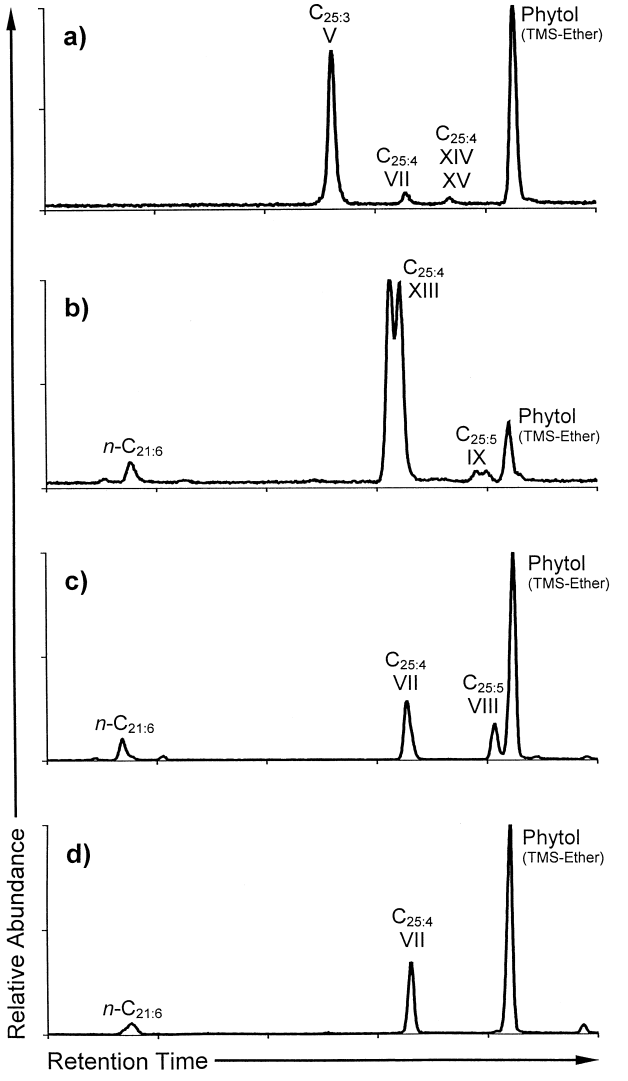


Fig. 2. Representative partial GC (HP-1) total ion current chromatograms (TICs) of non-saponifiable hexane extracts from (a) *Haslea ostrearia,* (b) *Haslea pseudostrearia,* (c) *Haslea crucigera,* and (d) *Haslea saltstonica.*

Closer examination of the TICs reveals that they consist of mixtures of HBIs reported previously in cultures of *H. ostrearia* and new, uncharacterised HBI compounds. Thus, of the three large scale cultures of *H. ostrearia* analysed in this present study, one culture (HO-1; Fig. 2a) was found to contain mainly triene **V** (ca. 90% of total HBIs) and a trace amount of the tetraene **VII** characterised from previous cultures (Wraige et al., 1997), together with two previously unreported tetraenes (**XIV**, **XV;** see Table 1 for GC RIs). The other two cultures of *H. ostrearia* consisted mainly of HBI triene **V** with trace quantities of the pseudo-homologues; tetraene (**VII**) and pentaene (**VIII**). The new tetraenes found in HO-1 (vide infra) were also present, though their concentrations represented less than 10% of the total HBIs for all three cultures. The single large scale culture of *H. crucigera* was found to yield C_{25} tetraene **VII** (45%) and pentaene **VIII** (22%) but no new HBI alkenes were detected (Fig. 2c). Following further fractionation of tetraene **VII** and pentaene **VIII** (SiO_2/hexane), NMR analysis of these individual components demonstrated that they were present as single stereoisomers rather than mixtures of diastereoisomers even though diastereoisomeric mixtures of HBIs have been observed previously in cultures of *H. ostrearia* (Belt et al., 1996; Johns et al., 1999). Similarly, the bulk culture of *H. saltstonica* yielded only one stereoisomeric C_{25} HBI (Fig. 2d), which corresponded to tetraene **VII** (Belt et al., 1996). Finally, large scale culture of the second *Haslea* species isolated from the Kingsbridge estuary (UK), *H. pseudostrearia*, contained predominantly a C_{25} HBI alkene (**XIII**; resolves as 2 GC peaks) that has not been previously characterised, together with a trace of pentaene **IX** (Fig. 2b).

2.2. Characterisation of 2,6,10,14-tetramethyl-9-(3-methylpent-4-enyl)pentadec-2,6,10-triene (*XIII*)

81 mg of a C_{25} HBI tetraene (94% purity, GC) was isolated from a single large scale culture of *H. pseudostrearia* (Massé et al., 2000) by extraction (hexane), saponification (KOH/MeOH/H_2O), re-extraction (hexane) and column chromatography (SiO_2/hexane). GC analysis of this compound reveals that it resolves into two peaks on all three phases used (e.g. Fig. 2b) and that the GC RIs for both peaks are different to those obtained for the previously characterised HBI tetraene **VII** (n.b. diastereomeric HBI **VII** only resolves into two peaks on DB-WAX; Table 1). In addition, while the mass spectra for the two tetraenes in this fraction are identical to each other, they show differences when compared to ms data obtained for tetraene **VII** (Fig. 3). In particular, the mass spectrum of tetraene **VII** exhibits a relatively large number of fragment ions at $m/z > 200$ with the most abundant ion at m/z 259, whereas the same spectral region for **XIII** is dominated by two ions at m/z 207 and 275. The intensity of ion m/z 95 is also significantly different between the two isomers. Examination of the ^{1}H NMR spectrum obtained for **XIII** demonstrates the presence of a vinyl moiety ($CH{=}CH_2$), three trisubstituted double bonds, and a 1:1 ratio of isopropyl ($(CH_3)_2CH$) and isoprenyl ($(CH_3)_2C{=}$) groups. The ^{13}C NMR spectrum reveals six CH_3, eight CH_2, seven CH and three quaternary ^{13}C resonances which confirms the substitution multiplicity of the double bonds. The appearance of a resonance at ca. 49 ppm confirms that the major branch point of the carbon

Table 1
GC retention indices of four isomeric C_{25} HBI tetraenes obtained from *Haslea ostrearia* and related species

	Retention index		
Tetraene structure	HP-1	HP-5	DB-WAX
VII	2140	2145	2232, 2238
XIII	2134, 2138	2143, 2146	2221, 2227
XIV	2157	2159	2267
XV	2157	2159	2277

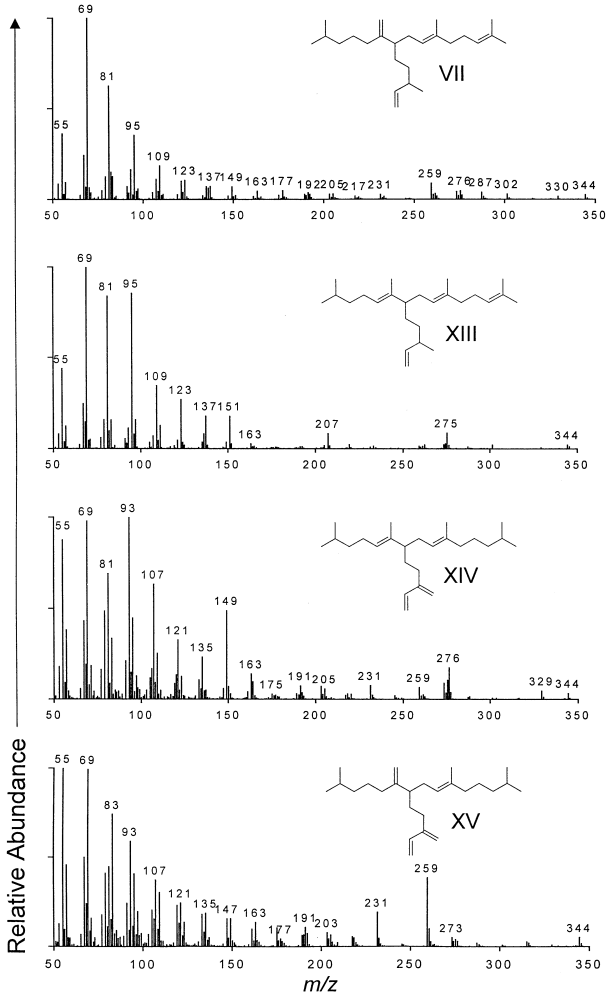


Fig. 3. Mass spectra for 4 isomeric C_{25} HBI tetraenes isolated from cultures of *Haslea ostrearia* and related species.

chain (C-7) is saturated, consistent with other HBI structures from *H. ostrearia* (Belt et al., 1996; Wraige et al., 1997; Johns et al., 1999). Since the presence of a vinyl group and three trisubstituted double bonds are readily identified from the ^{1}H and ^{13}C NMR data, the four double bonds must be positioned at C23–C24 (vinyl), C5–C6, C9–C10 and either of C2–C3 or C13–C14. Double bonds in both of these latter two positions can be ruled out from the observation of an equal number of isopropyl and isoprenyl groups. In order to determine which end of the carbon chain is unsaturated, the allylic region of the ^{1}H NMR spectrum was examined in more detail. Methylene (CH_2) protons that are allylic to two double bonds resonate (δ 2.6–2.8 ppm) to higher frequency compared to those which are monoallylic (δ 1.8–2.1 ppm). Thus, the chemical shifts for H-4 in pentaene **IX** (Sinninghe Damsté et al., 1999) and hexaene **X** (Wraige et al., 1997) appear at 2.8 and 2.7 ppm, respectively. The ^{1}H NMR spectrum of the tetraenes isolated from *H. pseudostrearia* does not have any resonances in this region and integration of the monoallylic region (10H) is consistent with the terminal double bond being in the C13–C14 position. The low frequency chemical shifts for C-17 (11.8 ppm) and C-18 (16.1 ppm) confirm that the stereochemistries for the C5–C6 and C9–C10 double bonds are *E* in both cases. The structure for the C_{25} HBI tetraene can thus be confirmed as **XIII** (Fig. 4).

Finally, since most of the individual resonances in the ^{13}C spectrum are split into two peaks, we conclude that the tetraenes exist as a mixture of diastereoisomers, a feature that has been observed for other HBIs from *H. ostrearia* (Belt et al., 1996; Johns et al., 1999). In these cases, C-7 has been shown to have a fixed (but unknown) configuration, with variable stereochemistry at C-22 (Johns et al., 2000). The appearance of two compounds (GC) possessing identical mass spectra (vide infra) can also be explained by the presence of a mixture of diastereoisomers.

*2.3. Characterisation of 2,6,10,14-tetramethyl-7-(3-methylenepent-4-enyl)pentadec-5,9-diene (**XIV**) and 2,6,14-trimethyl-10-methylene-9-(3-methylenepent-4-enyl) pentadec-6-diene (**XV**)*

For virtually all cultures of *H. ostrearia* studied, two additional HBI tetraenes could be detected as minor components (< 10% of total HBIs) that had different GC and mass spectral properties to either **VII** or **XIII** (Table 1, Fig. 3). Following repeated fractionation (SiO_2/hexane), these two new tetraenes were obtained from several cultures of *H. ostrearia* in sufficient quantities and purity for structural analysis by GC–MS and NMR spectroscopy.

The ^{1}H NMR spectra obtained for both compounds contains a resonance due to the vinylic proton H-23 which has a significantly higher frequency chemical shift

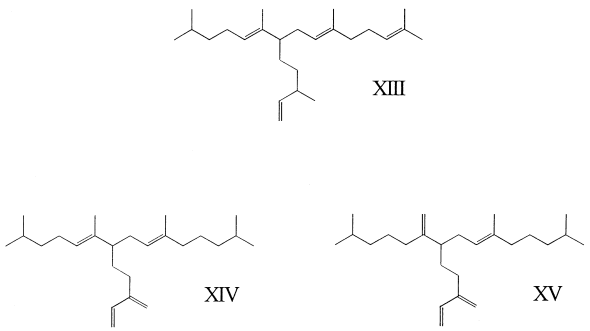


Fig. 4. Structures of new C_{25} HBI tetraenes described in this study.

(δ 6.33 ppm) compared to the analogous resonance observed for **VII** and **XIII**, and appears as a dd due to coupling to H-24$_{cis}$ and H-24$_{trans}$ only. Further methylene (alkene) proton resonances are observed at 4.95 ppm. These observations are consistent with double bonds located at C23–C24 and C22–C25. This arrangement of conjugated double bonds is unusual for HBI alkenes and has only been reported for hexaene **X** which has also been isolated from *H. ostrearia* (Wraige et al., 1997). Additional ^{1}H and ^{13}C NMR spectral analysis of these two tetraenes confirmed their structures to be **XIV** and **XV** (Fig. 4). HBI **XIV** contains two trisubstituted double bonds while XV has one methylene and one trisubstituted double bond. Both compounds are saturated (isopropyl) at each end of the main carbon chain and the stereochemistries of the trisubstituted double bonds (C5–C6 and C9–C10 (**XIV**); C9–C10 (**XV**)) were found to be *E* by analysis of ^{13}C data for C-17 and C-18.

The results presented here extend our understanding of the different structural isomers of HBIs biosynthesised by *H. ostrearia*. The generality of HBIs possessing a vinyl moiety together with a double bond in either of the C5–C6 or C6–C17 positions now appears to be fully established. The presence of additional double bonds account for further degrees of unsaturation in the most common tetraenes and pentaenes, though new tetraene structures, with methylenic double bonds in the C22–C25 position have now been determined. Although the reasons for the co-occurrence of individual HBIs are not clear at present, it appears that there are structural relationships between them. Thus, for cultures containing HBIs with different degrees of unsaturation, there is normally a double bond in either the C5–C6 or C6–C17 position (e.g. **V**, **VII** and **VIII**). Similarly, co-occurring isomers also exhibit structural similarities (e.g. **VII** and **XV**).

Our observations are of further significance in that we have shown the biosynthesis of HBIs to be common to four *Haslea* species and this may be the case for the entire genus, though planktonic species such as *Haslea gigantea* and *Haslea wawrikae* (Simonsen, 1974; Von Stosch, 1985) have yet to be analysed. Apart from these taxonomic relationships, the results of these rigorous structural identifications have possible wider implications.

For example, in a geochemical context, by careful comparison with the authenticated algal compounds, the haslenes found in sediments worldwide have recently been shown to be principally isomers with C7–C20, rather than C5–C6 or C6–C17 unsaturation (Belt et al., 2000b,c). The C7–C20 unsaturation is therefore consistent with the positions of sulphur incorporation in the analagous isoprenoid thiophenes found in more ancient sediments and crude oils and which are useful 'biological markers' of ancient algal communities (e.g. Sinninghe Damsté et al., 1989; Kohnen et al., 1990). Finally, the identification of new haslenes is also important in terms of their apparently variable pharmacological activities (Rowland et al., 2001). Some isomers appear to slow the growth of a human lung cancer cell line in vitro, whilst others do not. Examination of the bioactivities of newly discovered isomers is therefore a worthwhile goal of such pharmacological investigations.

3. Experimental

3.1. Algal cultures

H. ostrearia and *H. crucigera* were isolated from the Bay of Bourgneuf (France) while *H. pseudostrearia* and *H. saltstonica* were isolated from the Kingsbridge estuary (Devon, UK). *H. ostrearia* was grown in bulk between April 1991 and June 1998. Large scale cultures (440 l) of each of the other three diatoms were grown in an outdoor facility during September and October 1999 as described previously (Belt et al., 1996).

3.2. Isolation and purification of HBIs

Each of the large scale cultures of the four diatoms were centrifuged, freeze dried and extracted (Soxhlet) with hexane to yield total hexane extracts (THEs). The THEs were saponified ($KOH/MeOH/H_2O$) and the non-saponifiable lipids re-extracted into hexane and purified by column chromatography (SiO_2/hexane). Fractions containing >90% (GC) of individual HBIs were combined and analysed by ^1H and ^{13}C NMR spectroscopy. In all cases, the yield of purified HBIs from each culture corresponded to ca. 1 mg per 1 g wet weight (centrifuged algal paste). Small aliquots of each HBI were hydrogenated ($PtO_2.2H_2O$/hexane) to the parent alkane **I** in order to confirm the carbon skeleton.

3.3. NMR spectroscopy

NMR spectra were recorded in $CDCl_3$ using a JEOL EX 270 spectrometer. Chemical shifts (δ) are referenced to residual $CHCl_3$ (7.24 ppm) and $CDCl_3$ (77.0 ppm) for ^1H and ^{13}C respectively. Resonances marked * indicate the presence of diastereoisomers.

*3.3.1. 2,6,10,14-Tetramethyl-9-(3-methylpent-4-enyl) pentadec-2,6,10-triene (**XIII**)*

3.3.1.1. ^1H NMR (270 MHz). δ 5.66 (ddd, 1H, J=7, 10.5, 17.5 Hz, H-23), 5.07 (m, 3H, H-5, H-9, H-13), 4.91 (m, 2H, H-24), 2.01 (m, 10H, H-4, H-7, H-8, H-11, H-12, H-22), 1.67 (s, 3H, H-15), 1.59 (s, 3H, H-19), 1.57 (s, 3H, H-18), 1.53 (m, 1H, H-2), 1.45 (s, 3H, H-17), 1.24 (m, 6H, H-3, H-20, H-21), 0.96 (d, 3H, J=6.9 Hz, H-25), 0.88 (d, 6H, J=6.6 Hz, H-1, H-16).

3.3.1.2. ^{13}C NMR (67.8 MHz). δ 145.1, 144.8* (C-23), 136.3 (C-6), 134.8 (C-10), 131.1 (C-14), 126.2, 126.1* (C-5), 124.5 (C-13), 123.5 (C-9), 112.4, 112. 1* (C-24), 49.4, 49.3* (C-7), 39.8 (C-11), 39.1 (C-3), 37.8, 37.7* (C-22), 34.6, 34.5* (C-21), 32.4, 32.3* (C-8), 30.4, 30.3* (C-20), 27.5 (C-2), 26.8 (C-12), 25.7 (C-15), 25.5 (C-4), 22.6 (C-1, C-16), 20.5, 19.9* (C-25), 17.7 (C-19), 16.1 (C-18), 11.8, 11.7* (C-17).

*3.3.2. 2,6,10,14-Tetramethyl-7-(3-methylenepent-4-enyl) pentadec-5,9-diene (**XIV**)*

3.3.2.1. ^1H NMR (270 MHz). δ 6.33 (dd, J=10.7, 17.7 Hz, H-23), 5.20–4.99 (m, H-5, H-9, H-24), 4.95 (br, s, H-25), 2.2–1.9 (m, H-4, H-7, H-8, H-11, H-21), 1.6–1.1 (m, H-2, H-3, H-12, H-13, H-14, H-20), 1.54 (s, H-18), 1.47 (s, H-17), 0.86, 0.84 (2×d, J=6.6 Hz, H-1, H-15, H-16, H-19).

3.3.2.2. ^{13}C NMR (67.8 MHz). δ 146.9 (C-22), 139.1 (C-23), 135.9 (C-10), 135.4 (C-6), 126.6 (C-5), 123.1 (C-9), 115.4 (C-25), 113.1 (C-24), 49.4 (C-7), 39.9 (C-11), 39.0 (C-3), 38.5 (C-13), 32.2 (C-8), 31.3 (C-21), 29.4 (C-20), 27.9 and 27.5 (C-2, C-14), 25.7 (C-12), 25.5 (C-4), 22.7 and 22.6 (C-1, C-15, C-16, C-19), 16.0 (C-18), 11.9 (C-17).

*3.3.3. 2,6,14-Trimethyl-10-methylene-9-(3-methylenepent -4-enyl)pentadec-6-diene (**XV**)*

3.3.3.1. ^1H NMR (270 MHz). δ 6.33 (dd, J=10.7, 17.7 Hz, H-23), 5.20–4.99 (m, H-9, H-24), 4.95 (br, s, H-25), 4.78 (br, d, H-17a), 4.74 (br, s, H-17b), 2.2–1.9 (m, H-5, H-7, H-8, H-11, H-21), 1.6- 1.1 (m, H-2, H-3, H-4, H-12, H-13, H-14, H-20), 1.54 (s, H-18), 0.86, 0.84 (2×d, J=6.6 Hz, H-1, H-15, H-16, H-19).

3.3.3.2. ^{13}C NMR (67.8 MHz). δ 152.2 (C-6), 146.8 (C-22), 139.0 (C-23), 135.8 (C-10), 122.9 (C-9), 115.3 (C-25), 113.0 (C-24), 108.9 (C-17), 46.6 (C-7), 39.9 (C-11), 38.9 (C-3), 38.5 (C-13), 33.8 (C-5), 32.8 (C-8), 31.8 (C-21), 29.2 (C-20), 28.0 (C-14), 27.9 (C-2), 25.7 (C-12), 25.5 (C-4), 22.7 and 22.6 (C-1, C-15, C-16, C-19), 16.0 (C-18).

3.4. Chromatography

GC–MS was performed using a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector fitted with fused

silica capillary columns (12 m (0.2 mm i.d.) HP-1 and 25 m (0.2 mm i.d.) HP-5 Ultra stationary phases; 15 m (0.32 mm i.d.) DB-WAX stationary phase). Auto-splitless injection and helium carrier gas were used. The gas chromatograph oven temperature was programmed from 40 to 300°C at 5°C min^{-1} (HP-1, HP-5) and 40 to 200° at 5°C min^{-1} (DB-WAX) and held at the final temperature for 5–10 min. Mass spectrometer operating conditions were: ion source temperature 250° and 70 eV ionisation energy. Spectra (35–500 Daltons) were collected using Hewlett Packard Chemstation™ software.

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Sterols from the fungus *Lactarium volemus*

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Abstract

Seven ergostane-type sterols and two mono-glucosides were isolated from the ethyl acetate soluble fraction of *Lactarium volemus*. Three are previously unknown, i.e. 3-*O*-β-D-glucopyranosyl-22*E*,24*R*-5α,8α-epidioxyergosta-6,22-diene, 3-*O*-β-D-glucopyranosyl-22*E*, 24*R*-5β,8β-epidioxyergosta-6,22-diene and 22*E*,24*R*-ergosta-7,22-diene-3β,5α,6β,9α-tetraol. The structural elucidation of these compounds was mainly achieved by spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Lactarium volemus*; Russullaceae; 3-*O*-β-D-Glucopyranosyl-22*E*,24*R*-5α,8α-epidioxyergosta-622-diene; 3-*O*-β-D-Glucopyranosyl-22*E*24*R*-5β,8β-epidioxyergosta-6,22-diene; 22*E*,24*R*-Ergosta-7,22-diene-3β,5α,6β9α-tetraol

1. Introduction

Lactarius volemus Fr. (Russullaceae) is a fungus widely distributed in China, especially in the southwest of China. The fungal sample was collected from Lijiang in Yunnan province. The whole body of this fungus, containing a large amount of milky juice called "milk mushroom" by local residents, is edible. Its ethanol extract was reported to inhibit the growth of several tumor cell lines in vitro (Zang and Ying, 1994). An initial chemical investigation of the whole body of this fungus led to the identification of nine ergostane-type sterols and their mono-glucosides (**1**–**9**). Although ergosterol peroxides are very common metabolites of fungal species, their mono-glucosides have not been identified. A series of polyhydroxy ergostane-type sterols was isolated from marine soft coral and sponge (Wright et al., 1978; Piccialli and Sica, 1987; Migliuolo et al., 1990; Kobayashi and Kanda, 1991; Kobayashi et al., 1993), but identification of this type of highly oxygenated polar compound (**6**–**9**) from fungi is very rare. We report herein the isolation and structure identification of nine sterols, three of which are new (**3**, **5** and **9**).

2. Results and discussion

Analysis of the ^{1}H NMR and ^{13}C NMR spectra of the compounds **1**–**9** and comparison with the literature (Wright et al., 1978; Piccialli and Sica, 1987; Kobayashi and Kanda, 1991; Kobayashi et al. 1993) indicated that compounds **1**–**9** have an ergostane-type side-chain with a 22*E*,24*R*-configuration. Although both the 24α- and 24β-configurations are found naturally, fungi, in general, only produce sterols with the 24β-configuration (24α-methyl group) (24*S* if a saturated side-chain, 24*R* if a Δ^{22} unsaturated side-chain) indicating a phylogenetic significance of the configuration at C-24 (Wright et al., 1978). This is consistent with the assignment of the side-chain configuration at C-24 for compounds **1**–**9** by ^{1}H NMR and ^{13}C NMR spectroscopy. The assignments of the proton signals (H-26 and H-27) and the carbon signals (C-26 and C-27) of the side-chain were made according to literature values (Zhou et al., 1996).

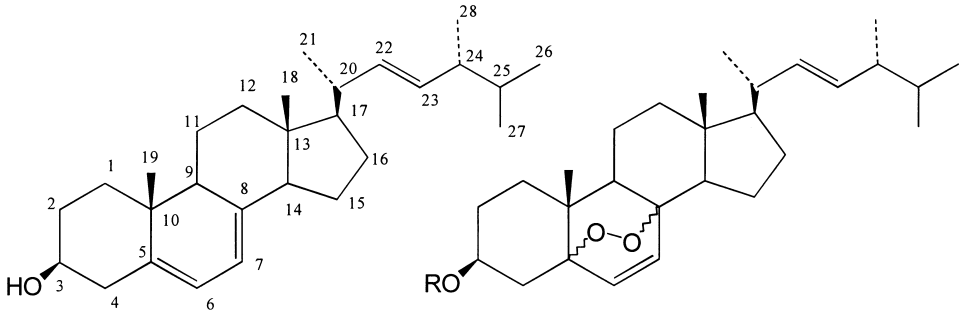
*2.1. 3-O-β-D-Glucopyranosyl-22E,24R-5α,8α-epidioxyergosta-6,22-diene (**3**)*

3-*O*-β-D-Glucopyranosyl-22*E*,24*R*-5α,8α-epidioxyergosta-6,22-diene (**3**). The molecular formula of **3** was determined to be $C_{34}H_{54}O_8$ on the basis of the HREIMS (*m*/*z* 428 [M-glc]$^+$) and ^{13}C NMR data. A weak molecular ion at *m*/*z* 590 [M]$^+$(1%) in the EIMS was confirmed by quasimolecular ions at *m*/*z* 591

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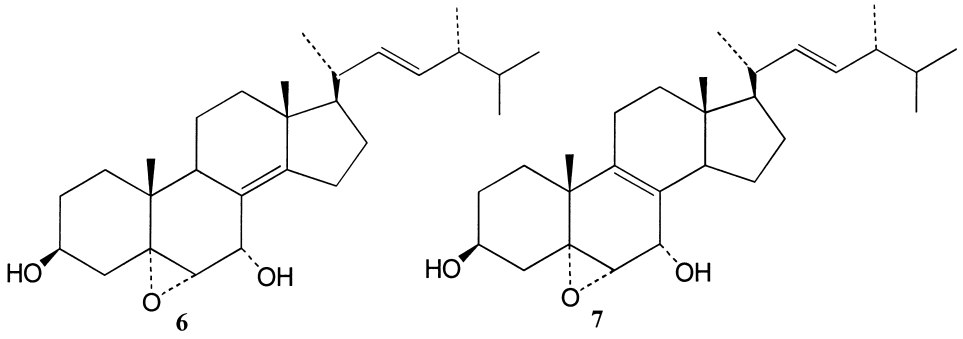
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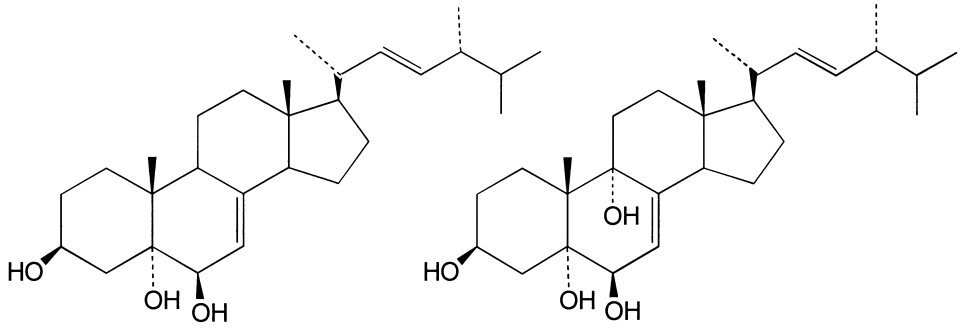


1

2. R=H, 5α,8α–Form
3. R=Glc, 5α,8α–Form
4. R=H, 5β,8β–Form
5. R=Glc, 5β,8β-Form



6 **7**



8 **9**

$[M+H]^+$ and 613 $[M+Na]^+$ in the positive FABMS. Its ^{13}C NMR spectra, with the DEPT technique, showed 33 carbon signals corresponding to 34 carbon atoms. The EIMS ion at m/z 428[M-162]$^+$ and the positive FABMS ion at m/z 429 $[M+H\text{-}162]^+$ inferred the presence of a six carbon sugar moiety in compound **3**. The carbon resonances of the sugar moiety in the ^{13}C NMR spectrum (see Table 1) and the proton signals of the sugar moiety in the ^1H NMR spectrum (see Experimental) indicated that the sugar moiety was glucose. Furthermore, in the ^1H and ^{13}C NMR spectra, the signals at δ 4.81 (1H, *d*, $J=8.0$ Hz) and δ 103.02 were, respectively, assignable to the proton and the carbon of the anomeric center of glucose with a β-configuration. Observation of the ^1H NMR and ^{13}C NMR spectra of **3** indicated that the aglycone of **3** is an ergostane-type sterol. Two proton signals at δ 6.53 and 6.23 (each 1H, *d*, $J=8.6$ Hz) and two tertiary carbon signals at δ 135.91 and 131.07 were attributable to a Δ^6 double bond. Two quaternary carbon signals at δ 82.15 and 79.47 were, respectively, assignable to C-5 and C-8 bearing a 5α,8α-peroxide bond. The proton signal at δ 4.10 (1H, *m*) was assigned to H-3α, and the tertiary carbon signal at δ 74.07 was assigned to C-3 bearing an oxygenated function. The ^1H NMR and ^{13}C NMR spectral data mentioned above suggested that the aglycone of **3** was 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**2**). This was supported by the ion at m/z 428 [M-162]$^+$ in the EIMS. Comparison of the C-3 carbon signal at δ 74.04 (in pyridine-d_5) of **3** with the C-3 carbon signal at δ 66.47 (in $CDCl_3$) of ergosterol peroxide indicated that the glycosylation shift ($\Delta\delta$) of C-3 was +7.6 ppm, suggesting that the glucose is definitely attached to the C-3 position. The structure of **3** was thus elucidated to be 3-

Table 1
^{13}CNMR spectral data of compounds **2**, **3**, **6**, **7** and **9**

Carbons	**2**[a]	**3**[b]	**6**[b]	**7**[b]	**9**[b]
C-1	30.20	30.12	32.35	32.07	29.14
C-2	34.77	34.63	27.76	31.08	32.46
C-3	66.47	74.07	68.29	68.21	67.38
C-4	39.44	39.79	40.88	40.45	42.09
C-5	82.15	82.15	66.95	64.77	75.06
C-6	135.22	135.91	62.53	63.71	73.87
C-7	130.77	131.07	65.24	67.08	121.31
C-8	79.42	79.47	127.36	133.86	143.04
C-9	51.25	51.96	39.57	128.33	78.72
C-10	37.03	37.52	36.50	38.81	41.35
C-11	20.66	21.20	19.62	24.16	28.53
C-12	37.03	37.52	37.11	36.34	36.00
C-13	44.61	44.89	43.22	42.48	44.23
C-14	51.76	52.21	150.50	50.70	51.30
C-15	23.44	23.80	25.18	24.24	23.50
C-16	28.59	29.05	33.05	29.61	28.27
C-17	56.33	56.64	57.31	54.11	56.33
C-18	12.90	13.21	18.43	11.66	12.13
C-19	18.18	18.31	16.71	22.66	22.44
C-20	39.64	40.02	40.13	40.80	40.77
C-21	20.90	21.29	21.51	21.35	21.40
C-22	135.45	135.91	136.01	136.28	136.23
C-23	132.39	132.60	132.32	132.20	132.20
C-24	42.82	43.25	43.12	43.10	43.13
C-25	33.11	33.51	33.38	33.38	33.36
C-26	19.64	20.05	19.86	19.86	19.85
C-27	19.93	20.35	20.14	20.14	20.14
C-28	17.56	18.02	17.81	17.84	17.66
C-1′		103.02			
C-1′		75.05			
C-3′		78.82			
C-4′		71.83			
C-5′		78.30			
C-6′		62.96			

[a] Recorded in $CDCl_3$ (100.61 MHz).
[b] Recorded in pyridine-d_5 (100.61 MHz).

O-β-D-glucopyranosyl-22*E*,24*R*-5α,8α-epidioxyergosta-6,22-diene (**3**).

The occurrence of a small amount of another new compound, 3-*O*-β-D-glucopyranosyl-22*E*,24*R*-5β,8β-epi dioxyergosta-6,22-diene (**5**) in **3** was observed in the ^{1}H NMR spectrum. The ratio of 3 and 5 in the mixture was estimated to be about 20:1 from the measurement of ^{1}H NMR integration for the proton signals of H-6, H-7 and H-1′ in both compounds. The separation of **5** from the mixture was unsuccessful due to the structural similarity of the two compounds and the limited amount of sample.

2.2. 22E,24R-5α,6α-Epoxyergosta-8(14),22-diene-3β,7α-diol (6)

Compound **6** was isolated as a white powder. The EIMS revealed a molecular ion at *m*/*z* 428 and its ^{13}C NMR spectra showed 28 carbons in the molecule, suggesting the molecular formula $C_{28}H_{44}O_3$. This molecule exhibited an UV maximum at 239 nm. Its IR absorption band at 3436 cm^{-1} indicated the presence of hydroxyl groups. The molecular formula of **6** and its oxygenated features in the ^{13}C NMR spectrum implied seven degrees of unsaturation. A signal at δ 4.30 (1H, *br m*) for H-3α and the oxygenated carbon signal at δ 68.29 for C-3 indicated the presence of a 3β-hydroxyl group. The ^{1}H NMR spectrum at δ 3.37 (1H, *d*, $J = 3.2$ Hz) and the ^{13}C NMR spectrum δ 66.95 and 62.53 inferred the presence of a trisubstituted 5α,6α-epoxide ring (Kobayashi and Kanda, 1991; Greca et al., 1993). The downfield shift of H-3α in **6** results from the deshielding effect of the 5α-oxygen (Aeillo et al., 1990). The proton at δ 4.67 (1H, *d*, $J = 3.2$ Hz) for H-7β and the resonance at δ 65.24 for C-7 suggested the presence a 7α hydroxyl group. The two quaternary carbon signals at δ 127.36 and 150.50 for a fully substituted double bond suggested possible assignments at $\Delta^{8,9}$ or $\Delta^{8(14)}$. The assignment of a $\Delta^{8(14)}$ double bond seemed to be favorable from the comparison of its ^{1}H NMR and ^{13}C NMR spectra with those of melithasterol B and the diacetate of 5α,6α-epoxy-22*E*,24*R*-methyl-cholesta-8,22-diene-3β,7α-diol (Kobayashi and Kanda, 1991; Greca et al., 1993). In the cases of a $\Delta^{8,9}$ and $\Delta^{8(14)}$ double bond, the carbon signals for C-8 appeared at about δ 127 to 129 ppm, while for C-9 and C-14 they appeared at about δ 134 and 151 ppm, respectively (Kobayashi and Kanda, 1991; Greca et al., 1993). The above functionalities account for six of seven degrees of unsaturation present in the sterol. The remaining unsaturation is attributable to the Δ^{22} double bond corresponding to the carbon signals at δ 136.01 (C-22) and 132.32 (C-23). Compound **6** was thus identified as 22*E*,24*R*-5α,6α-epoxyergosta-8(14),22-diene-3β,7α-diol. Compound **6** was reported previously as a minor constituent of *Arum italicum*, but it was characterized as the diacetate (Greca et al. 1993). We report herein the structural identification and spectral data of **6** in its native form.

2.3. 22E,24R-5α,6α-Epoxyergosta-8,22-diene-3β,7α-diol (7)

Compound **7** was isolated as white crystalline prisms along with **6**. A weak molecular ion at *m*/*z* 428 in its EIMS and 28 carbon signals in its ^{13}C NMR spectrum suggested a molecular formula of $C_{28}H_{44}O_3$. Analysis of the ^{1}H NMR and ^{13}C NMR spectra revealed that compound **7** also possesses a 3β-hydroxyl group, a trisubstituted 5α,6α-epoxide ring and a 22*E*,24*R*-methylergostane-type side-chain. Comparison of the spectral features of **7** with those of **6** revealed the close relationship of two structures. The only difference was the assignment of the tetrasubstituted double bond (δ 128.33 (C-8) and 133.86 (C-9)) to $\Delta^{8,9}$ in **7**. Compound **7** was, therefore, identified as 22*E*,24*R*-5α,6α-epoxy-

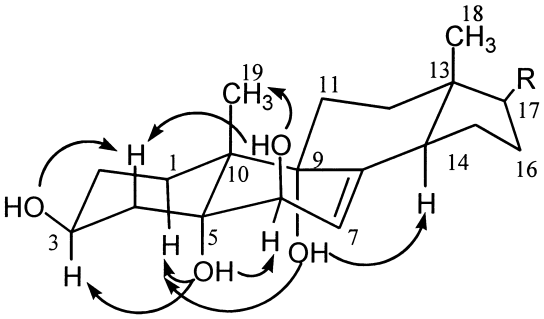


Fig. 1. Pyridine-induced deshielding of compound **9**.

ergosta-8,22-diene-3β,7α-diol. Compound **7** was also reported to occur as a minor constituent in *Arum italicum*, but it was characterized as the diacetate. (Greca et al. 1993). This paper deals with the spectral data of **7** in its native form.

2.4. 22E,24R-Ergosta-7,22-diene-3β,5α,6β,9α-tetraol (9)

Compound **9** had the molecular formula $C_{28}H_{46}O_4$ on the basis of its HREIMS (*m*/*z* 428[M-H_2O]$^+$) and ^{13}C NMR spectral data. Its ^{13}C NMR spectrum showed 28 carbon signals corresponding to 28 carbon atoms, comprising six methyls, eight methylenes, nine methines, and five non-protonated carbons as indicated in DEPT experiments. The signals for six methyl groups at δ 0.69 (3H, *s*, H-18), 1.60 (3H, *s*, H-19), 1.06 (3H, *d*, J=6.7 Hz, H-21), 0.84 (3H, *d*, J=6.8 Hz, H-26), 0.85 (3H, *d*, J=6.8 Hz, H-26) and 0.94 (3H, *d*, J=6.8 Hz, H-28) in the ^{1}H NMR spectrum indicated a sterol nucleus (Migliuolo et al.,1990). Four of the carbon signals at δ 67.38, 75.06, 73.87 and 78.72 in the ^{13}C NMR spectrum were assigned to C-3, C-5, C-6 and C-9, respectively, bearing hydroxyl groups. The presence of four hydroxyl groups in **9** was also observed from its EIMS and ^{1}H NMR spectrum. Its EIMS exhibited fragment ions at *m*/*z* 428 [M-H_2O]$^+$, 410 [M-2H_2O]$^+$, 392 [M-3H_2O]$^+$ and a weak ion at *m*/*z* 374 [M-4H_2O]$^+$. Two less-than-one-proton singlets (partially exchangeable) at δ 6.20 and 7.56, and two less-than-one-proton doublets (partially exchangeable) at δ 5.79 and 7.49 in the ^{1}H NMR spectrum (in pyridine-d_5), all of which disappeared in the D_2O exchange experiment, were attributable to four hydroxyls. One proton signal at δ 4.81 (1H, *br m*) for the H-3α indicated the presence of a 3β-hydroxyl group. The proton signal at δ 4.43 (1H, *d*, J=4.0 Hz) assigned to H-6α inferred the presence of a 6β hydroxyl. One tertiary and one quaternary carbon signals at δ 121.31 and 143.04, respectively, were attributable to C-7 and C-8. The olefinic proton at δ 5.82 (1H, *d*, J=4.0 Hz) was, therefore, assigned to H-7 based on chemical shift and coupling constant with H-6α. Two tertiary carbon signals at δ 136.23 and 132.20 assigned to C-22 and C-23, respectively, suggested a Δ^{22} double bond. This was supported by the proton signals at δ 5.21 (2H, *m*) for H-22 and H-23. The ^{1}H NMR spectrum of **9** measured in pyridine-d_5 showed significant pyridine-induced deshielding effects on the signals of H_{ax}-1, H_{ax}-3, H_{ax}-4, H_{ax}-14, and Me-19. This can be easily observed by comparison with the ^{1}H NMR spectrum (recorded in CD_3OD) of the 24*S*-epimer of **9** (Migliuolo et al., 1990). The pyridine-induced downfield shifts inferred that the four hydroxyls had 3β, 5α, 6β, and 9α configurations, and fully rationalized the stereochemistry of **9** (see Fig. 1) (Migliuolo et al., 1990; Kobayashi et al., 1993). The structure of **9** was thus assigned as 22*E*,24*R*-ergosta-7,22-diene-3*β*,5*α*,6*β*,9*α*-tetraol. A 24*S*-epimer of **9**, (22*E*,24*S*)-24-methyl-5α-cholesta-7,22-diene-3β,5,6β,9-tetraol, was isolated from *Spongia officinalis* (Migliuolo et al., 1990). The structural elucidation of **9** was confirmed by comparison of its ^{13}C NMR spectrum with that of the 24*S*-epimer. The ^{13}C NMR spectrum patterns were very similar except for some distinguishable changes in the side-chain.

2.5. Ergosterol (1), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (2), 5β,8β-epidioxyergosta-6,22-dien-3β-ol (4) and 22E,24R-Ergosta-7,22-diene-3β,5α,6β-triol (8)

The least polar compounds **1**, **2** and **4** were identified as ergosterol (**1**) and its peroxides (**2**, **4**) on the basis of ^{1}H NMR and ^{13}C NMR spectral analysis. The ergosterol peroxides were characterized as a mixture of 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**2**) and 5β,8β-epidioxyergosta-6,22-dien-3β-ol (**4**). The ratio of **2** and **4** in the mixture was estimated to be 15:1 by measurement of the ^{1}H NMR integration for the proton signals of H-6 and H-7 in both compounds. This is in agreement with earlier results (Greca et al., 1990). Compound **8** was identified as 22*E*,24*R*-ergosta-7,22-diene-3*β*,5*α*,6*β*-triol on the basis of spectral data, in comparison with the literature (Cafeiri and Fattoruso, 1985; Piccialli and Sica, 1987; Kobayashi et al., 1993).

3. Experimental

3.1. General

Melting points (uncorrected) were determined on a Kofler apparatus. UV spectra were recorded in chloroform on a Shimadizu UV-210A spectrometer. IR spectra were recorded on a Perkin-Elmer 577 spectrometer with KBr disc. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM-400 spectrometer with TMS as internal standard. ^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.61 MHz, broad band and DEPT) were measured in solvents $CDCl_3$ and pyridine-d_5. EIMS (70

eV) and FAB$^+$MS were carried out on a VG Auto Spec 3000 instrument. The matrix for FAB$^+$MS was *meta*-nitrobenzyl alcohol (MNBA). All solvents used were of analytical grade (Shanghai Chemical Plant). Silica gel (200–300 mesh) was used for column chromatography, and pre-coated silica gel GF_{254} plate (Qingdao Haiyang Chemical Plant) was used for TLC. C18 silica gel (Merck) and MCI GEL CHP20P (75–150 μ) (Mitsubishi Chemical Industries LTD) were also used for column chromatography.

3.2. Plant material

Lactarius volemus Fr. was collected from the Lijiang district of Yunnan province of China and authenticated by Prof. Zang Mu of the Kunming Institute of Botany, where a voucher specimen is deposited.

3.3. Extraction and isolation

Fresh mushroom bodies (20 kg) were collected from the mountains (over 3000 m above sea level) near the town of Qiao-Tou in the Lijiang district. The fresh bodies of *Lactarius volemus* were crushed with a blender and immediately immersed in 95% ethanol. The ethanol-immersed sample was then extracted four times with 95% ethanol at room temperature (10 h each) and the liquid was obtained by suction filtration. The residue (276 g) yielded by removal of the solvent was dissolved in water (2 l) to form a suspension. The aqueous suspension was successively partitioned with petrol (to defat) and ethyl acetate to obtain a petrol soluble fraction (89 g) and an ethyl acetate soluble fraction (52 g), respectively. The aqueous part was then condensed to give a water soluble fraction (135 g). The ethyl acetate soluble fraction (52 g) was subjected to column containing GEL CHP20P eluting with 70% methanol in water, methanol and acetone, respectfully to give fr. 1 (18 g), fr. 2 (27 g) and fr. 3 (5.5 g). Fraction 3 was subjected to silica gel column chromatography eluted with petrol containing increasing amounts of acetone to afford five major frs. 3a–3e. Fr. 3b was crystallized from petroleum–acetone (10:1) to yield ergosterol (**1**, 1.5 g). Fr. 3c was applied to a silica gel column eluted with petrol–ethyl acetate (5:1) to give a mixture of ergosterol peroxides (**2** and **4**, 2.1 g). Fr. 2 was separated by column chromatography (packed with 300 g silica gel H) eluted with petrol–acetone (5:1–1:1 and acetone) to collect eleven major frs. 2a–2k. Frs. 2a, 2b and 2c were mainly composed of ergosterol and its peroxides. Frs. 2d, 2e and 2f were combined and separated by silica gel (H) column chromatography eluted with petrol–ethyl acetate (4–1) to give two major fractions. Each of the two fractions was further purified by column chromatography (C18 silica gel) eluted with 70% MeOH in H_2O to yield compound **6** (104 mg) and compound **7** (63 mg). Fr. 2g was applied to a C18 silica gel column eluted with 70% MeOH in H_2O to afford compound **8** (247 mg). Fr. 2i was repeatedly subjected to a C18 silica gel column, eluted with 70% MeOH in H_2O to afford compound **9** (31 mg). Fr. 2j and fr. 2k were combined and applied to a C18 silica gel column eluted with 60% MeOH–H_2O to give a mixture of compounds **3** and **5** (22 mg).

3.4. 5α,8α-Epidioxyergosta-6,22-dien-3β-ol (*2*)

Compound **2** was identified by comparing its physical constants and spectral data with those reported in the literature (Greca et al., 1990). White crystalline needles, mp 172–174°. UV ($CHCl_3$): 237 nm. IR (KBr): 3521, 3389, 2957, 2872, 1459, and 1377 cm^{-1}. ^{1}HNMR ($CHCl_3$): 6.47 (1H, *d*, $J=8.5$ Hz), 6.21 (1H, *d*, $J=8.5$ Hz), 5.17 (1H, *d*, $J=9.0$ Hz), 5.12 (1H, *d*, $J=9.0$ Hz), 3.94 (1H, m), 0.97 (3H, *d*, $J=6.7$ Hz), 0.89 (3H, *d*, $J=6.5$ Hz), 0.87 (3H, *s*), 0.84 (3H, *d*, $J=6.6$ Hz), 0.81 (3H, s) and 0.80 (3H, *d*, $J=6.7$ Hz). EIMS *m*/*z* (%): 428 [M]$^+$ (10), 410 (12), 396 (100), 376 (13), 363 (50), 337 (25) and 251 (30).

3.5. 3-O-β-D-Glucopyranosyl-22E,24R-5α,8α-epidioxyergosta-6,22-diene (*3*)

Amorphous powder, ^{1}H NMR (pyridine-d_5): 6.53 (1H, *d*, $J=8.6$ Hz, H-6), 6.23 (1H, *d*, $J=8.6$ Hz, H-7), 5.35 (1H, *dd*, $J=15.4$, 8.1 Hz, H-22), 5.24 (1H, *d*, $J=15.4$, 8.1 Hz, H-23), 4.81 (1H, *d*, $J=8.0$ Hz, H-1′), 4.40 (1H, *dd*, $J=11.8$, 2.7 Hz, H-6a), 4.28 (1H, *dd*, $J=11.8$, 4.9 Hz, H-6b), 1.09 (3H, *d*, $J=6.7$ Hz, H-21), 1,04 (3H, *d*, $J=6.7$ Hz, H-24), 0.94 (6H, $J=6.8$ Hz, H-26, H-27), 0.94 (3H, *s*, H-19), and 0.84 (3H, *s*, H-18). EIMS *m*/*z* (%): 590 [M]$^+$ (1), 428 [M-*glc*]$^+$ (3), 410 [M-*glc*-H_2O]$^+$ (10), 392 [M-*glc*-2H_2O]$^+$ (10) and 378 [M-*glc*-H_2O-O_2]$^+$ (25). FABMS: 613 [M + Na]$^+$(10), 558 [M-O_2]$^+$ (20), and 377 [M-*glc*-H_2O-O_2]$^+$ (100). HREIMS *m*/*z*: 428.3288 ($C_{28}H_{44}O_3$[M-*glc*]$^+$, $C_{28}H_{44}O_3$ requires 428.3290).

3.6. 22E,24R-5α,6α-Epoxyergosta-8(14),22-diene-3β,7α-diol (*6*)

Compound **6** was identified on the basis of spectral data, in comparison with the data of its diacetate in the literature (Greca et al., 1993). White powder, mp 124–125°. UV ($CHCl_3$): 239 nm, IR (KBr): 3436, 2960, 2937, 2872, 1466, and 1377 cm^{-1}. ^{1}H NMR (pyridine-d_5): 5.19–5.31 (2H, *m*, H-22 and H-23), 4.67 (1H, *d*, $J=3.2$ Hz, H-7β), 3.37 (1H, *d*, $J=3.2$ Hz, H-6β), 4.30 (1H, *m*, H-3α), 0.84 (3H, *d*, $J=7.0$ Hz, H-26), 0.86 (3H, *d*, $J=7.0$ Hz, H-27), 0.90 (3H, *s*, H-18), 0.91(3H, *s*, H-19), 0.96 (3H, *d*, $J=6.8$ Hz, H-28), and 1.03 (3H, *d*, $J=6.7$ Hz, H-21). EIMS *m*/*z* (%): 428 [M]$^+$ (8), 410 (80), 392 (30), 377 (50), 285 (20), and 267 (45).

3.7. 22E,24R-5α,6α-Epoxyergosta-8,22-diene-3β,7α-diol (7)

Compound **7** was identified on the basis of spectral data, in comparison with the data of its diacetate in the literature (Greca et al., 1993). White powder, mp 178–179°. UV ($CHCl_3$): 239 nm, IR (KBr): 3412, 2959, 2931, 2871, 1460, and 1372 cm^{-1}. ^1H NMR (pyridine-d_5): 5.14–5.28 (2H, *m*, H-22 and H-23), 4.53 (1H, *d*, $J=1.8$ Hz, H-7β), 3.52 (1H, *d*, $J=1.8$ Hz, H-6β), 4.34 (1H, *m*, H-3α), 0.82 (3H, *d*, $J=7.0$ Hz, H-26), 0.84 (3H, *d*, $J=7.0$ Hz, H-27), 0.71 (3H, *s*, H-18), 1.17(3H, *s*, H-19), 0.93 (3H, *d*, $J=6.7$ Hz, H-28), and 1.06 (3H, *d*, $J=6.6$ Hz, H-21). EIMS *m/z* (%): 428 $[M]^+$ (12), 410 (75), 392 (35), 377 (60), 285 (50), and 267 (55).

3.8. 22E,24R-Ergosta-7,22-diene-3β,5α,6β,9α-tetraol (9)

White powder. ^1H NMR (pyridine-d_5): 5.82 (1H, *d*, $J=4.0$ Hz, H-7), 5.21 (2H, *m*, H-22, 23), 4.81 (1H, *m*, H-3), 4.43 (1H, *d*, $J=4.0$ Hz, H-6), 3.05 (1H, *dd*, $J=12.8$, 12.8 Hz, H-4ax), 2.90 (1H, *ddd*, $J=13.6$, 13.6, 3.7 Hz, H-1ax), 2.94 (1H, *m*, H-14), 2.48 (1H, *dd*, $J=13.6$, 4.9 Hz, H-4eq), 2.35 (1H, *br.d*, $J=12.8$ Hz, H-2eq), 2.08 (1H, *m*, H-2ax), 1.60 (3H, *s*, H-19), 1.06 (3H, *d*, $J=6.7$ Hz, H-21), 0.94 (3H, *d*, $J=6.8$ Hz, H-28), 0.85 (3H, *d*, $J=6.8$ Hz, H-27), 0.84 (3H, *d*, $J=6.8$ Hz, H-26), 0.69 (3H, *s*, H-18), 5.80 (*d*, $J=4.9$ Hz, 3-OH), 6.18 (*s*, 9-OH), 7.49 (*d*, $J=6.0$ Hz, 6-OH), and 7.56 (*s*, 5-OH). EIMS *m/z* (%): 446 $[M]^+$ (2), 428 $[M\text{-}H_2O]^+$ (100), 410 $[M\text{-}2H_2O]^+$ (80), 382 $[M\text{-}3H_2O]^+$ (75), 318 (45), 300 (30), 285 (40), and 257 (30). HREIMS *m/z*: 428.3296 ($C_{28}H_{44}O_3[M\text{-}H_2O]^+$, $C_{28}H_{44}O_3$ requires 428.3290).

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seco-Cuparane-type sesquiterpenoid from the Japanese liverwort *Jungermannia infusca*

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Abstract

A 2,3-*seco*-cuparane-type sesquiterpenoid and the previously known barbatane-type sesqui- and *ent*-kaurane-type diterpenoids were isolated from the Japanese liverwort *Jungermannia infusca* (Mitt.) Steph. The structure of the 2,3-*seco*-cuparane-type sesquiterpenoid was determined by NMR spectroscopic analyses. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Jungermannia infusca*; Jungermanniaceae; Hepaticae; Secocuparenal; Rostronal F; 16,17-Dihydrorostronol F; *seco*-Cuparane-type; Barbatane-type; *ent*-Kaurane-type; Sesquiterpenoid; Diterpenoid

1. Introduction

As part of a search for the novel compounds and biologically active substances of the Hepaticae, we are continuing to study the chemical constituents of liverworts (Asakawa, 1982, 1995). The liverwort *J. infusca* (Mitt.) Steph. belonging to Jungermanniaceae in particular contains a number of clerodane-, *ent*-kaurane- and labdane-type diterpenoids (Asakawa, 1982, 1995; Nagashima and Asakawa, 1998). It is known that there are occasionally geographical differences in the main components in the same species. With this in mind we investigated the chemical constituents of *J. infusca* collected in Awajishima, Japan. In this paper, we report the structure elucidation of a new 2,3-*seco*-cuparane-type sesquiterpenoid and chemosystematics of the present *J. infusca*.

2. Results and discussion

A combination of CC on silica gel, Sephadex LH-20 and preparative HPLC of the ether extract of *J. infusca* gave a new 2,3-*seco*-cuparane-type sesquiterpenoid, secocuparenal (**1**). The eight known sesqui- and diterpenoids, 3-gymnomitrien-15-ol (**2**) (Buchanan et al., 1996), rostronol F (**3**) (Nomoto et al., 1976), 16,17-dihydrorostronol F (**4**) (Nomoto et al., 1976), *ent*-kauren-15α-ol (**5**) (Matsuo et al., 1977), *ent*-kauren-15-one (**6**) (Matsuo et al., 1977), *ent*-11α-hydroxykauren-15-one (**7**) (Connolly and Thornton, 1973; Benés et al., 1977), *ent*-11α-hydroxy-16*R*-kauran-15-one (**8**) (Connolly and Thornton, 1973; Benés et al., 1977) and *ent*-14α-hydroxy-kauren-15-one (**9**) (Liu et al., 1997), were also isolated from this species.

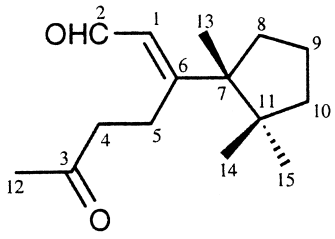
The ^{1}H MMR spectra of compounds **2** and **3** were in good agreement with those of rostronol F, isolated from *Englerastrum scandens*, and its chemical derivative, 16,17-dihydrorostronol F, respectively (Nomoto et al., 1976). But, the complete assignments of the ^{13}C NMR spectra of these compounds has as yet not been reported. Therefore, the complete ^{13}C spectroscopic assignments (Table 1) of **2** and **3** were carried out by the detailed analysis of ^{1}H–H COSY, HMQC, HMBC and NOESY spectra.

The high resolution-EIMS of **1** (*m*/*z* 236.1769 $[M]^+$) showed the molecular formula $C_{15}H_{24}O_2$, indicating four degrees of unsaturation. The ^{1}H NMR spectrum showed the presence of three tertiary methyls (δ 0.57, 0.74 and 0.80 *s*), an acetyl methyl (δ 1.56 *s*), an olefinic proton (δ 5.95 *d*) and a formyl proton (δ 9.88, *d*). The ^{13}C NMR (Table 1) and the DEPT spectra of **1** also displayed trisubstituted olefinic carbons (δ 127.6 *d*, 170.4 *s*), a formyl carbon (δ 190.4 *d*) and a carbonyl

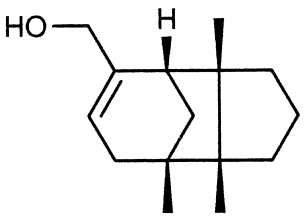
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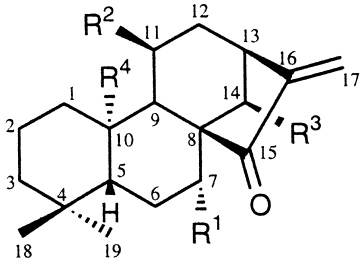
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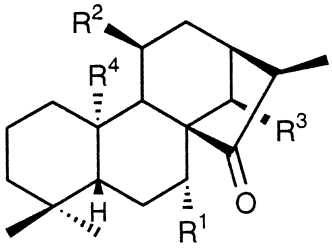
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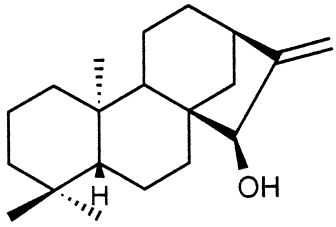
2



3 R^1=R^3=OH, R^2=OAc, R^4=CH_2OH
6 R^1=R^2=R^3=H, R^4=CH_3
7 R^1=R^3=H, R^2=OH, R^4=CH_3
9 R^1=R^2=H, R^3=OH, R^4=CH_3



4 R^1=R^3=OH, R^2=OAc, R^4=CH_2OH
8 R^1=R^3=H, R^2=OH, R^4=CH_3



5

Table 1
^{13}C NMR spectral data for **1**, **3** and **4** (150 MHz, $CDCl_3$)

C	1[a]	3[b]	4
1	127.6	33.8	33.7
2	190.4	18.2	18.1
3	204.2	41.2	41.1
4	45.4	33.1	33.0
5	24.0	53.2	53.1
6	170.4	27.7	28.0
7	53.5	75.3	75.6
8	35.8	59.6	58.8
9	19.5	61.6	60.8
10	40.1	43.8	43.4
11	44.9	68.5	67.6
12	29.2	35.8	29.1
13	22.2	45.1	42.2
14	24.2	75.8	75.9
15	26.5	207.4	219.9
16		148.7	44.2
17		116.2	10.0
18		22.5	33.9
19		34.1	22.4
20		60.5	60.5
$OCO\underline{CH}_3$		21.5	21.5
$O\underline{C}OCH_3$		169.5	169.3

[a] Measured in C_6D_6.
[b] Measured by 100 MHz.

carbon (δ 204.2 *s*), along with four methyls, five methylenes and two quaternary carbons. The above spectral data suggested that compound **1** was a monocyclic sesquiterpenoid.

^1H–^1H COSY, HMQC and HMBC experiments were carried out for **1**. The HMBC experiment displayed long-range ^1H–^{13}C correlations as shown in Fig. 1. Accordingly, the structure of **1** must be a 2,3-*seco*-cuparane-type sesquiterpenoid. The phase sensitive NOESY experiment showed the NOEs between (i) H-2 and H-5, (ii) H-1 and H-8α, H-13, (iii) H-13 and H-4, H-8β, H-14, (iv) H-14 and H-13, H-15, and (v) H-15 and H-8α, H-14, respectively. Therefore, the C_1–C_2 double bond has an *E* configuration.

Thus, the structure of secocuparenal (**1**) is a 2,3-*seco*-cuparene-type sesquiterpenoid with an aldehyde at C-2 and a ketone at C-3. However, the absolute configuration remained to be clarified.

The isolation of 16,17-dihydrorostronol F (**4**) is the first report of this as a natural product. There are at least four chemo-types of *J. infusca*, [I] *ent*-kaurane-, [II] clerodane- and labdane-type diterpenoids, [III] bis(bibenzyl)-type aromatic compounds, and [IV] cuparane-type sesqui- and labdane-type diterpenoids as main components, respectively (Nagashima and Asakawa,

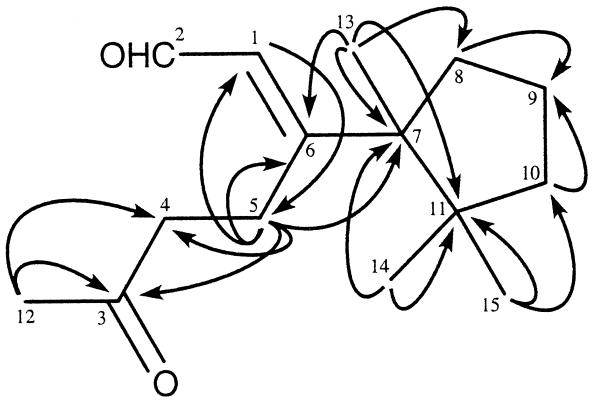


Fig. 1. Long-range ^{1}H–^{13}C correlations of **1** by HMBC spectral analysis.

1998; Nagashima et al., 1999). The present *J. infusca*, which contained *ent*-kaurane-type diterpenoids as major components, is classified into chemo-type [I].

3. Experimental

3.1. General

^{1}H and ^{13}C NMR: 400 and 600 MHz (^{1}H NMR), and 100 and 150 MHZ (^{13}C NMR). Chemical shift values were expressed in δ (ppm) downfield from tetramethylsilane as an internal standard (^{1}H NMR), and δ 77.03 (ppm) from $CHCl_3$ and δ 128.0 (ppm) from C_6D_6 as a standard (^{13}C NMR). TLC: visulaized under UV (254 nm) light and by spraying with 10% H_2SO_4 or Godin reagent (Godin, 1954) followed by heating. MeOH–CH_2Cl_2 (1:1) was used for Sephadex LH-20. $[\alpha]_D$: $CHCl_3$.

3.2. Plant material

Jungermannia infusca (Mitt.) Steph. (F8021) was collected in Awajishima, Hyogo pref., Japan, 1997. This species was identified by Dr. M. Mizutani (The Hattori Botanical Laboratory, Miyazaki, Japan). The voucher specimen was deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

3.3. Extraction and isolation from J. infusca

The ether extract (822 mg) of *J. infusca* (23 g) was divided into nine fractions by CC on silica gel using *n*-hexane–EtOAc gradient solvent system. Fr. 3 was applied to a Sephadex LH-20 and silica gel (*n*-hex.-Et_2O 19:1) column to give *ent*-kauren-15α-ol (**5**) (22.7 mg) and *ent*-kauren-15-one (**6**) (16.6 mg). 3-Gymnomitrien-15-ol (**2**) (4.4 mg) was isolated from Fr. 5 by the preparative HPLC (NUCLEOSIL, *n*-hex.–EtOAc 9:1). Fr. 6 was applied to both Sephadex LH-20 and silica gel (CH_2Cl_2–Et_2O 19:1 columns) and divided into two fractions. Preparative TLC of Fr. 6-1 gave secocuparenal (**1**) (4.6 mg). Fr. 6-2 was subjected to C_{18} silica gel chromatography (CH_3CN) to afford *ent*-11α-hydroxy-16*R*-kauran-15-one (**8**) (12.3 mg). The CC on silica gel (*n*-hex.–EtOAc 7:3) of Fr. 7 gave *ent*-14α-hydroxy-kauren-15-one (**9**) (2.2 mg). Fr. 8 was subjected to Sephadex LH-20 and silica gel chromatography (CH_2Cl_2–Et_2O 19:1) to yield *ent*-11α-hydroxykauren-15-one (**7**) (37.7 mg). Fr. 9 was applied to Sephadex LH-20, and silica gel ($CHCl_3$–MeOH 49:1) chromatography and finally purified on prep. HPLC (NUCLEOSIL, *n*-hex.–EtOAc 1:1) to afford rostronol F (**3**) (6.1 mg) ($[\alpha]_D$ -107.8° *c* 3.1) and 16,17-dihydrorostronol F (**4**) (2.5 mg) ($[\alpha]_D-111.9^\circ$ *c* 1.1).

3.4. Secocuparenal (*1*)

Oil; $[\alpha]_D-59.1^\circ$ (*c* 0.44); HR-EIMS: found 236.1769 $C_{15}H_{24}O_2$ requires 236.1776; FTIR ν_{max} cm^{-1}: 1715, 1665; ^{1}H NMR (600 MHz): δ 5.95 (1H, *d*, $J=7.4$ Hz, H-1), 9.88 (1H, *d*, $J=7.4$ Hz, H-2), 2.02 (2H, *m*, H-4), 2.62 (1H, *ddd*, $J=14.0$, 10.7, 6.3 Hz, H-5), 2.67 (1H, *ddd*, $J=14.0$, 10.2, 6.3 Hz, H-5), 1.11 (1H, *m*, H-8), 1.85 (1H, *m*, H-8), 1.35–1.43 (3H, *m*, H-9, H-10), 1.26 (1H, *m*, H-10), 1.56 (3H, *s*, H-12), 0.74 (3H, *s*, H-13), 0.80 (3H, *s*, H-14), 0.57 (3H, *s*, H-15); ^{13}C NMR: Table 1: EIMS *m*/*z* (rel. int.): 236$[M]^+$(8), 218(12), 193(10), 175(13), 149(36), 121(17), 109(37), 93(23), 81(22), 69(29), 55(29), 43(100).

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We thank Dr. M. Mizutani (The Hattori Botanical Laboratory, Miyazaki, Japan) for identification of the species. Thanks are also due to Dr. M. Tanaka (TBU) and Miss. Y. Okamoto (TBU) for measurements of 600 MHz NMR and mass spectra.

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Labdane and pimarane diterpenes from *Croton joufra*☆

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Abstract

From the chloroform extract of the leaves of *Croton joufra,* the diterpenes 2α,3α-dihydroxy-labda-8(17),12(13),14(15)-triene and 3β-hydroxy-19-*O*-acetyl-pimara-8(9),15-dien-7-one, were isolated. Their structures were establised by spectroscopic methods. One of the compounds showed weak lethality in the brine shrimp assay. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Croton joufra*; Euphorbiaceae; Labdane; 2,3-Dihydroxy-labda-8(17),12(13),14(15)-triene; Pimarane; 3-Hydroxy-19-*O*-acetyl-pimara-8(9),15-dien-7-one; Brine shrimp lethality test

1. Introduction

Croton joufra Roxb. (Euphorbiaceae) is a medium size shrub commonly named in Thai as ''Plau noi'', the same name as that used for *Croton sublyratus*.

A decoction of the leaves and bark have been used as an antidysentery and peptic promotor and a decoction of the flowers as an anthelmintic (Phupattanapong and Wongprasart, 1987). The heartwood and stems have been used as a blood tonic and antipyretic (Mokkhasmit et al., 1971). In previous studies, two furanoditerpenes, plaunol A and plaunol C, were reported from the methanolic extract of the stems by TLC (Ogiso et al., 1981). Subsequently, Roengsumran et al. (1982) isolated another furanoditerpene swassin from the stems of this plant. We report here on the isolation and structural characterization of a labdane and a pimarane diterpene from the leaves of this plant.

2. Results and discussion

In our search for bioactive compounds from plants of the Euphorbiaceae, we used the *Artemia salina* (brine shrimp) toxicity test as an in-house bioassay method (Meyer et al., 1982). Among the hexane, chloroform and methanol extracts, the chloroform extract, which showed $LC_{50} = 62.8$ μg/ml, was chosen for further purification. Two novel compounds were obtained from the chloroform extract after several chromatographic separations. Identification of these compounds was based on spectral data.

Compound **1** was obtained as a pale yellow powder, mp 72–74°C. The ElMS gave a molecular ion at m/z 304, corresponding to the molecular formula $C_{20}H_{32}O_2$. The FT–IR spectrum indicated secondary alcohol absorptions (ν_{max} 3405 and 1054 cm^{-1}) and an olefinic double bond (ν_{max} 1645 and 891 cm^{-1}). The ^1H NMR spectrum showed three methyl group singlets at δ 0.78, 0.80 and 1.02, in addition to a downfield methyl group signal at δ 1.76 H(*s*) assignable to CH_3–C=C. The spectrum also exhibited olefinic protons at δ 5.08 (1H, *d*, $J = 10.8$ Hz), 5.17 (1H, *d*, $J = 17.2$ Hz), 5.26 (1H, *t*, $J = 6.4$ Hz) and 6.73 (1H, *dd*, $J = 10.8, 17.3$ Hz), together with additional exocyclic methylene group signals as two broad one-proton singlets at δ 4.50 and 4.86. These signals, particularly the olefinic proton signals, resemble those reported in 12,13*E*-biformen (Bohlmann and

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Czerson, 1979). Two sets of additional one-proton signals at δ 3.02 (*d*, $J=9.6$ Hz) and 3.70 (*ddd*, $J=4.1$, 9.8 and 11.2 Hz) were characterized as two vicinal oxymethine protons of which the former carbinolic carbon was bonded to a tertiary carbon; these two protons were assigned to H-3 and H-2, respectively. The ^{1}H–^{1}H COSY spectrum further indicated that the one-proton signal at δ 3.70 (H-2) was coupled to the one-proton doublet signal at δ 3.02 (H-3) and also to two other signals at δ 1.21 (1H, *dd*, $J=4.3$ and 12.4 Hz, H-1e) and 2.11 (1H, *dd*, $J=3.0$ and 3.6 Hz, H-1a). The ^{13}C NMR spectrum showed 20 carbons comprising four quaternary (of which two were olefinic), and six methine [including two oxymethine carbons at δ 69.1(*d*) and 83.4 (*d*)]. The compound was proposed as a 2,3-dihydroxylabda-8 (17),12(13),14(15)-triene (**1**). Extensive use of NMR spectroscopic techniques, including ^{1}H–^{1}H COSY, HETCOR, COLOC, led to the complete assignments of the ^{1}H and ^{13}C shift values, as shown in Table 1. The important long-range- ^{1}H–^{13}C (COLOC) correlations are shown in Fig. 1.

The relative stereochemistry of **1** was deduced from the NOESY spectrum. The key NOE effects observed between H-2/H-3, H-2/C-19-Me, H-2/C-20-Me, H-3/C-18-Me and H-5/C-18-Me suggested that the two hydroxyl groups at C-2 and C-3 are both α oriented (Fig. 1).

Compound **2** was obtained as a colorless gum. The HR-FABMS (glycerol matrix) gave an $[M+H]^+$ ion at m/z 361.23804 corresponding to the molecular formula $C_{22}H_{32}O_4+H$. The FT-IR spectrum indicated a hydroxyl group (ν_{max} 3460 cm^{-1}), an ester group (ν_{max} 1730 cm^{-1}), an α/β-unsaturated ketone (ν_{max} 1650 cm^{-1}), and a vinylidene group (ν_{max} 3090 and 908 cm^{-1}). The ^{1}H NMR spectrum showed three methyl group singlets at δ 1.02, 1.11 and 1.15, together with a low field acetate methyl group signal at δ 2.08 (*s*). Three

Table 1
^{1}H and ^{13}C NMR spectral data for **1** and **2** ($CDCl_3$)[a]

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
1	1.21 (*m*)	45.0 (*t*)	1.92 (*m*)	34.0 (*t*)
1′	2.11 (*dd*, 4.3, 12.4)			
2	3.70 (*ddd*, 4.2, 9.8, 11.3)	69.1 (*d*)	1.36 (*m*)	27.2 (*t*)
2′			1.85 (*m*)	
3	3.02 (*d*, 9.6)	83.4 (*d*)	3.35 (*dd*, 4.8, 11.5)	78.0 (*d*)
4		39.4 (*s*)		41.8 (*s*)
5	1.19 (*br d*, 12.3)	54.5 (*d*)	1.73 (*dd*, 3.9, 14.0)	49.4 (*d*)
6	1.38 (*dddd*, 4.1, 12.8, 12.8 12.9)	23.6 (*t*)	2.53 (*dd*, 14.0, 17.6)	35.6 (*t*)
6′	1.69 (*m*)		2.63 (*dd*, 3.9, 17.6)	
7	1.99 (*ddd*, 4.7, 12.8, 12.9)	37.7 (*t*)		199.5 (*s*)
7′	2.39 (*m*)			
8		147.2 (*s*)		129.0 (*s*)
9	1.73 (*m*)	56.9 (*d*)		164.3 (*s*)
10		40.2 (*s*)		39.3 (*s*)
11	2.19 (*dd*, 7.0, 10.9)	22.3 (*t*)	2.00 (*m*)[b]	23.1 (*t*)
11′	2.37 (*m*)		2.18 (*m*)	
12	5.26 (*t*, 6.4)	130.9 (*d*)	1.28 (*m*)	33.5 (*t*)
12′			1.62 (*m*)	
13		131.9 (*s*)		34.4 (*s*)
14	6.73 (*dd*, 10.8, 17.3)	133.7 (*d*)	2.00 (*m*)[b]	33.2 (*t*)
14′			2.38 (*dd*, 1.5, 17.8)	
15	5.08 (*d*, 10.8)	113.5 (*t*)	5.66 (*dd*, 10.8, 17.5)	144.9 (*d*)
15′	5.17 (*d*, 17.3)			
16	1.76 (*s*)	19.7 (*q*)	4.83 (*dd*, 1.2, 17.5)	111.7 (*t*)
16′			4.93 (*dd*, 1.2, 10.8)	
17	4.50 (*br s*)	108.7 (*t*)	1.02 (*s*)	28.2 (*q*)
17′	4.86 (*br s*)			
18	1.02 (*s*)	28.8 (*q*)	1.15 (*s*)	21.9 (*q*)
19	0.80 (*s*)	16.6 (*q*)	4.23 (*d*, 11.8)	65.0 (*t*)
19′			4.41 (*d*, 11.8)	
20	0.78 (*s*)	15.5 (*q*)	1.11 (*s*)	17.5 (*q*)
CO				171.1 (*s*)
CH_3			2.08 (*s*)	21.0 (*q*)

[a] δ in ppm and J (in parentheses) in Hz.
[b] These signals overlapped.

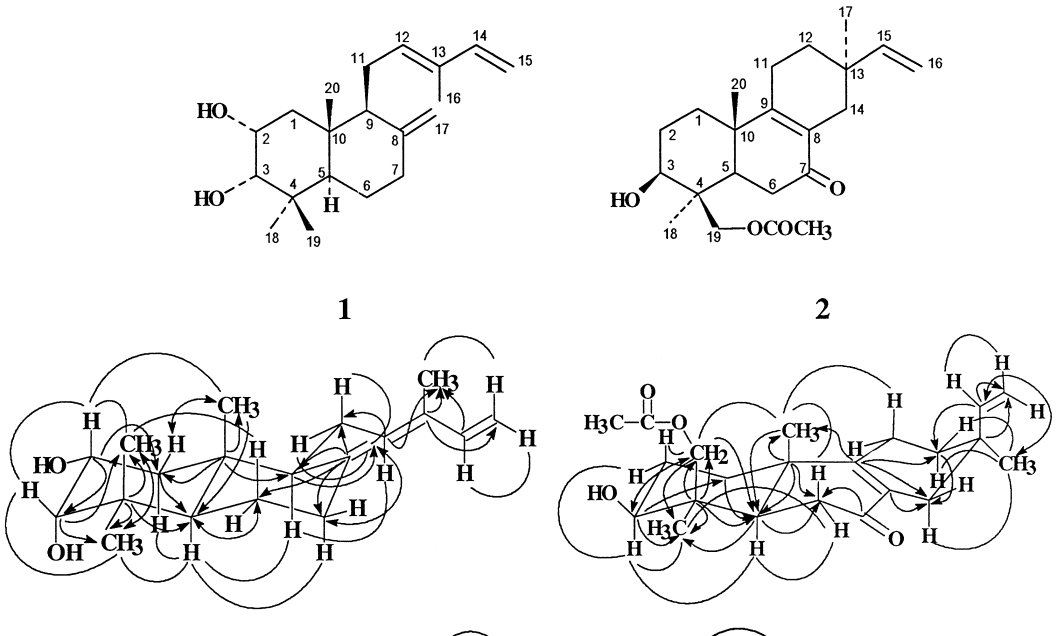


Fig. 1. Structures: key long-range ^{1}H–^{13}C (C H) and NOESY (H H) correlations of **1** and **2**.

sets of one proton signals δ 4.83 (1H, *dd*, $J=1.2$ and 17.5 Hz), 4.93 (1H, *dd*, $J=1.2$ and 10.8 Hz) and 5.66 (1H, *dd*, $J=10.8$ and 17.5 Hz), in addition to the ^{13}C NMR spectroscopic signals at δ 111.7 (*t*) and 144.9 (*d*), indicated a vinylidene group. The key ^{1}H–^{13}C long range correlation (HMBC) between a methyl proton signal at δ 1.02 and the ^{13}C signal at δ 144.9 (*d*), in addition to correlations of the two vinylidene proton signals at either δ 4.83 or 4.93 to the ^{13}C signal at δ 34.4 (*s*), indicated a pimarane diterpene with a vinyl group bonded to C-13 (Rao et al., 1968). The one-proton signals at δ 4.23 (*d*, $J=11.8$ Hz) and 4.41 (*d*, $J=11.8$ Hz) and the ^{13}C signal at δ 65.0 (*t*) implied an oxymethylene moiety bonded to a $COCH_3$ group. The signals of two non-equivalent protons at δ 2.53 (*dd*, $J=14.0$ and 17.6 Hz) and 2.63 (*dd*, $J=3.9$ and 17.6 Hz), which were coupled to a methine proton at δ 1.73 (*dd*, $J=3.9$ and 14.0 Hz) indicated a CH–CH_2–CO moiety. The absence of either an α- or β-proton signal of an α,β-unsaturated carbonyl group, in addition to the ^{13}C signals at δ 129.0 (*s*), 164.3 (*s*), and 199.0 (*s*), suggested a fully substituted olefinic double bond at C-8(9) and a keto group at C-7. The ^{3}J coupling between a one-proton signal at δ 3.35 (*dd*, $J=4.8$ and 11.5 Hz) and the ^{13}C signals at δ 21.9 (*q*), 65.0 (*t*) and 34.0 (*t*) indicated the location of a hydroxyl group at C-3. Another ^{3}J correlation between the oxymethylene proton signals at δ 4.23 and 4.41 and the ^{13}C signal at δ 49.4 (*d*, assigned to C-5) implied the link of an $OCOCH_3$ group at either C-18 or C-19. The NOEs obtainable from the NOESY spectrum indicated the bonding between the $OCOCH_3$ group and C-19 from the interactions between C-20-Me/C-19-CH_2O-$COCH_3$ and H-2a/C-19-CH_2OCOCH_3. Furthermore, the NOE correlations between H-3/H-5, H-3/C-18-Me and H-3/H-2e also suggested the stereochemistry of C-3-OH as β (equatorial). Compound **2** was therefore proposed to be 3β-hydroxy-19-*O*-acetyl-pimara-8(9),15-dien-7-one. Complete ^{1}H and ^{13}C chemical shifts (Table 1) were obtained from ^{1}H–^{1}H COSY, HMQC and HMBC spectra. The key HMBC and NOESY correlations are illustrated in Fig. 1.

The brine shrimp assay of **1** and **2** indicated weak toxicity in comparison to colchicine. The percent deaths at 204.2, 204.2 and 1.0 μg/ml of **1**, **2** and colchicine after 24 h were 53.3, 20.0 and 50, respectively. The LC_{50} were found to be 197.1, >204.0 and 1.0 μg/ml, respectively.

3. Experimental

3.1. General

Mps uncorrected; ^{1}H and ^{13}C NMR spectra were acquired at 400 and 100 MHz, respectively. The solvent signals were employed for reference purposes.

3.2. Plant material

The leaves of *Croton joufra* were collected from Kalasin Province, in the Northeast of Thailand, during December, 1995. The plant was kindly identified by Nijsiri Ruangrangsi. Voucher specimens (SSCJ/1995) are deposited at the Department of Chemistry, Faculty of Science, Ramkhamhaeng University and at the

Herbarium, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.3. Extraction and isolation

The dried leaves of *Croton joufra* were milled to obtain a fine powder (624 g) which were extracted successively with *n*-hexane, chloroform and methanol in a Soxhlet extraction apparatus. After evaporation of the solvents under reduced pressure, dark green gums of *n*-hexane (21.52 g), chloroform (31.70 g) and methanol (91.80 g) extracts were obtained.

The chloroform extract was fractionated by silica gel column chromatography using a gradient of *n*-hexane–chloroform (1:1 to 0:1) followed by chloroform–MeOH (10:0 to 1:1) to yield eight major frs. after combination of similar frs. as judged by TLC. Fr. 6 was subjected to additional silica gel cc ($CHCl_3$–MeOH (1:0 to 1:1) to give eight subfrs. (subfrs. 6.1–6.8). Subfr. 6.4 was subjected to chromatography (2×, silica gel cc, CH_2C1_2–MeOH 19:1 to 1:1 and *n*-hexane–EtOAc 17:3) to yield **2** (5.5 mg). Subfr. 6.5 was subjected to chromatography (2×, silica gel cc, $CHC1_3$–MeOH 10:0 to 4:1 and *n*-hexane ($CHC1_3$ 3:7 to 1:4) to yield **1** (103.6 mg).

3.4. Compound **1** (2α, 3α-dihydroxy-labda-8(17),12(13),14(15)-triene)

Pale yellow powder, mp 72–74°C; $[\alpha]_D^{25}$ −18.24° ($CHCl_3$, *c* 0.34); HR–EIMS *m/z*: 304.24150, $[M]^+$, $C_{20}H_{32}O_2$ requires 304.24023; EI–MS 70 eV *m/z* (rel. int.): 304 ($[M]^+$, 14.5), 248 (50.5), 187 (25.0), 135 (100), 93 (98.5), 81 (36.5), 55 (99.5), 43 (67); IR (film, cm^{-1}): 3405, 2941, 1715, 1645, 1456, 1385, 1054, 954, 891; ^1H and ^{13}C NMR spectral data are shown in Table 1.

3.5. Compound **2** (3β-hydroxy-19-O-acetyl-pimara-8(9),15-dien-7-one)

Colorless gum; $[\alpha]_D^{25}$ −127.62° ($CHCl_3$, *c* 0.105); HR–FABMS (glycerol) *m/z*: 361.23804, $[M+H]^+$, $C_{22}H_{32}O_4+H$ requires 361.23788; IR (film, cm^{-1}): 3460, 3090, 2931, 2863, 1731, 1648, 1373, 1241, 1094, 1036, 908, 756; ^1H and ^{13}C NMR spectral data are shown in Table 1.

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Dammarane derivatives from the dried fruits of *Forsythia suspensa*

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Abstract

Anti-inflammatory activity guided fractionation of the *n*-hexane soluble fraction of a 70% aqueous methanolic extract of the dried fruits of *Forsythia suspensa* afforded two new triterpenes. The structures of these compounds were elucidated as 3β-acetyl-20,25-epoxydammarane-24α-ol (**1**) and 3β-acetyl-20,25-epoxydammarane-24β-ol (**2**) on the basis of spectral data interpretation as well as by comparison with those of structurally similar compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Forsythia suspense*; Oleaceae; Damaranes; Triterpenoids

1. Introduction

Forsythia suspensa Vahl (Oleaceae) is a climbing plant, which is widely distributed in China, North and South Korea and Japan (Namba, 1993). This plant is also cultivated in Japan for its beautiful yellow flowers. The extract of the dried fruits has long been used in the Chinese and Japanese folk medicines to treat gonorrhea, erysipelas, inflammation, pharyngitis, pyrexia, tonsillitis, and ulcer (Jinasu, 1977; Tagaki et al., 1982). Moreover, the extract of the dried fruits showed potential antibacterial, antiviral, choleretic and antipyretic effects (Hikino, 1982; Ishizuka et al., 1992; Nishibe et al., 1992; Miura et al., 1987). Previous studies with the fruits have shown them to contain lignans, including a few with platelet activating factor (PAF) inhibitory effects (Iwakami et al., 1992; Nishibe et al., 1989). The 70% methanolic crude extract of *F. suspensa* fruits and its *n*-hexane soluble fraction showed anti-inflammatory activity in Wistar rats (Ozaki et al., 1997). In this paper, we report the isolation and characterization of two triterpenes (**1**,**2**) from the *n*-hexane soluble fraction of a 70% methanolic extract of fruits of same species. Although triterpenoids **1** and **2** have been synthesized from a hydroxydammaren-one-II (Biellmann, 1966), and 20,25-epoxydammarane-3β,24β-diol, and the deacetyl derivative of **2** has been prepared by reduction of an isolate (20,25-epoxy-24β-hydroxy-3-dammaranone) of *Citrus libanotis* (Teresa et al., 1982), this is the first report of their occurrence from a natural source. The complete ^{1}H and ^{13}C NMR spectral data for these compounds are also reported here for the first time.

2. Results and discussion

Compound **1** was the major isolate obtained in this investigation. HRFABMS showed a protonated ion peak at 503.4076 which corresponded to the molecular formula $C_{32}H_{54}O_4$, suggesting six double-bond equivalents in the molecule. The mass spectral fragments at *m*/*z* 443 due to loss of acetic acid from the pseudo molecular ion suggested the presence of an acetyl moiety in **1**. Additionally, it showed strong fragment ion peaks at *m*/*z* 485 ($C_{32}H_{52}O_3$) and 425 ($C_{30}H_{49}O$) due to the loss of water and water plus acetic acid, respectively from the molecular ion, $[M+H]^+$. The ^{13}C NMR spectrum of **1** displayed 32 carbon resonances, while the HSQC experiment confirmed that 25 out of the 32 carbons were attached to protons. The multiplicities of the carbon signals were

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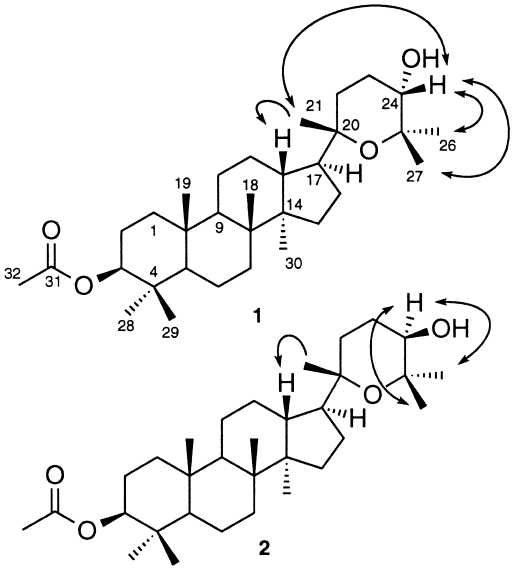
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determined by performing DEPT experiments using last pulse angles (θ) 45, 90 and 135°, which revealed the presence of nine methyls, ten methylenes, six methines, and seven quaternary carbons. The ^{13}C NMR spectral data of **1** showed close correspondence to that of cabraleadiol monoacetate (3α-acetyl-20*S*,24*S*-epoxydammarane-25-ol) (Hisham et al., 1996), ocotillol-II (20*S*, 24*R*-epoxydammarane-3β,24-diol) (Ohmoto et al., 1978), 20*S*,25-epoxydammarane-3β,6α,12β,24α-tetrol (Duc et al., 1994), 20*S*,25-epoxy-3β,12β,23ξ,24ξ-tetrahydroxy dammarane (Fujita et al., 1995), and (20*R*)- and (20*S*)-panaxadiols (Duc et al., 1994; Fujita et al., 1995). The ^1H NMR spectrum of **1** (Table 1) showed eight tertiary methyl singlets, an acetyl group at δ 2.01, two oxymethine protons at δ 3.69 (1H, *t*, = 7.0 Hz) and 4.44 (1H, *dd*, = 10.5, 5.5 Hz) and a series of resolved and unresolved multiplets extending from δ 0.79 to 1.83. These structural features revealed that the compound **1** was a triterpenoid. Therefore, the unsaturation index exhibited by the molecular formula ($C_{32}H_{54}O_4$) of **1** was satisfied by the four rings of a dammarane skeleton, the cyclized side chain at C-17 and an ester carbonyl group (δ_C 170.9).

Table 1
NMR assignments for compounds **1** and **2** in $CDCl_3$

Position	**1**				**2**			
	^{13}C	^1H	mult	*J* (Hz)	^{13}C	^1H	mult	*J* (Hz)
1	38.7	1.00	*m*		38.7	1.03	*m*	
		1.65	*m*			1.67	*m*	
2	23.7	1.60	*m*		23.7	1.60	*m*	
		1.62	*m*			1.62	*m*	
3	80.9	4.44	*dd*	10.5, 5.5	80.9	4.46	*dd*	10.3, 5.5
4	37.9	–	–		37.9	–		
5	55.9	0.79	*m*		55.9	0.80	*m*	
6	18.1	1.38	*m*		18.2	1.42	*m*	
		1.47	*m*			1.47	*m*	
7	35.2	1.23	*m*		35.2	1.24	*m*	
		1.48	*m*			1.50	*m*	
8	40.4	–	–		40.4	–	–	
9	50.7	1.32	*m*		50.7	1.31	*m*	
10	37.1	–	–		37.1	–	–	
11	21.5	1.15	*m*		21.8	1.18	*m*	
		1.46	*m*			1.48	*m*	
12	27.3	1.18	*m*		26.9	1.18	*m*	
		1.73	*m*			1.75	*m*	
13	42.9	1.52	*m*		42.8	1.60	*m*	
14	50.0	–	–		50.0	–	–	
15	31.4	1.03	*m*		31.4	1.03	*m*	
		1.42	*m*			1.42	*m*	
16	25.7	1.43	*m*		25.8	1.28	*m*	
		1.75	*m*			1.70	*m*	
17	49.5	1.76	*m*		49.8	1.83	*m*	
18	15.4	0.92 (3H)	*s*		15.5	0.93 (3H)	*s*	
19	16.4	0.81 (3H)	*s*		16.4	0.81 (3H)	*s*	
20	86.3	–	–		86.5	–	–	
21	23.5	1.09 (3H)	*m*		24.1	1.09 (3H)	*s*	
22	35.7	1.58	*m*		34.9	1.63	*m*	
		1.68	*m*			1.81	*m*	
23	26.1	1.74	*m*		26.3	1.73	*m*	
		1.83	*m*			1.82	*m*	
24	83.3	3.69	*t*	7.0	86.3	3.62	*dd*	10.5, 5.3
25	71.4	–	–		70.2	–	–	
26	24.3	1.08 (3H)	*s*		27.1	1.12 (3H)	*s*	
27	27.4	1.17 (3H)	*s*		27.8	1.17 (3H)	*s*	
28	16.2	0.83 (3H)	*s*		16.3	0.84 (3H)	*s*	
29	27.9	0.81 (3H)	*s*		27.9	0.82 (3H)	*s*	
30	16.4	0.83 (3H)	*s*		16.5	0.85 (3H)	*s*	
$\underline{CH_3}CO$	21.3	2.01 (3H)	*s*		21.3	2.02 (3H)		
$CH_3\underline{C}O$	170.9	–	–		171.0	2.00 (3H)	*s*	



Arrows indicate key nOe interactions in **1** and **2**

Table 2
Key HMBC correlations observed for **1**

Proton	^2J	^3J
H-3	C-2	C-1, C-5, C-28, C-29, C-31
H-5	C-6	C-3, C-7, C-9, C-19, C-29
Ha-7	C-6, C-8	C-5, C-9, C-18
H-9	C-8, C-10	C-5, C-11, C-12, C-14, C-19
H-13	C-14	C-8, C-20, C-30
Ha-15	C-14, C-16	C-13, C-17, C-30
H-17	C-13, C-16, C-20	C12, C-14, C-15, C-21
H-18	C-8	C-7, C-9, C-14
H-19	C-10	C-1, C-5, C-9
H-21	C-20	C-17, C-22
Hb-23	C-22	C-20, C-25
H-24	C-23, C-25	C-22, C-26, C-27
H-26	C-25	C-24, C-27
H-27	C-25	C-24, C-26
H-28	C-4	C-3, C-5, C-29
H-29	C-4	C-3, C-5, C-28
H-30	C-14	C-13, C-15
C-32	C-31	–

The downfield resonance (δ_H 4.44) of one of the oxymethine protons in **1**, which was attached to a carbon at 80.9 ppm suggested it to be esterified. The large couplings (10.5 and 5.5 Hz) of this proton demonstrated the equatorial (β-) orientation of the acetyl moiety. In the HMBC spectrum (Table 2), the proton at δ 4.44 showed two bond correlations to δ_C 23.7 (C-2) and 37.9 (C-4), and connectivities over to ^3J 38.7 (C-1), 55.9 (C-5) and the C-4 methyl groups at δ_C 16.2 and 27.9. These HMBC correlations defined the site of esterification at C-3. The chemical shift and splitting pattern of the upfield oxymethine proton (identical to those observed for the synthetic 3β-acetyl-20,25-epoxydammarane-24α-ol) (Biellmann, 1966) in the side chain implied that the secondary hydroxyl group must be situated next to a quaternary carbon, namely C-22 or C-24. The location of this functionality at C-24 was confirmed by HMBC correlations from this proton to δ_C 71.4 (C-25), 24.3 (C-26) and 27.4 (C-27). The axial (α-) orientation of the hydroxyl function at C-24 was determined from the coupling constant (7.0 Hz), between H-24β (equatorial) and H_2-23. The assignments of the remaining carbon signals in **1** were based on HMBC experiments using ^2J and ^3J correlations from the methine and methyl group protons to carbons, while the ^1J C–H interactions observed in the HSQC spectrum allowed unambiguous assignments of the methylene protons in **1**.

The relative stereochemistry of the C-17 side chain and the hydroxyl substituent at C-24 in **1** was finally determined by 1D NOESY experiments as shown on the structure. Irradiation at the resonance frequency of H-24 produced strong enhancements of the methyl signals, H_3-21 and H_3-27 and weak enhancement of H_3-26. On the other hand, similar irradiations of the H_3-21 and H_3-27 signals showed significant enhancement of H-24, while the former one also showed enhancement of H-13, suggesting their close proximity. On the basis of above spectral data, the structure of compound **1** was elucidated as 3β-acetyl-20,25-epoxydammarane-24α-ol.

HRFABMS of **2** showed the pseudomolecular ion peak at m/z 635.3104 $[M+Cs]^+$, compatible with the molecular formula, $C_{32}H_{54}O_4$, identical to that for **1**. The mass spectrum showed a major fragment ion and the base peak at m/z 485 and 425 attributable to the loss of water and water plus acetic acid from the molecular ion, $[M+H]^+$ at m/z 503. The ^1H and ^{13}C NMR spectra of compound **2** (Table 1) were almost identical to those recorded for 3β-acetyl-20,25-epoxydammarane-24α-ol (**1**). However, the upfield oxymethine proton appeared as a double doublets ($J=10.2$, 5.3 Hz) at δ 3.62, which was attached to δ_C 86.3 as evident from the HSQC experiment. The splitting pattern of this proton as well as the downfield shift of the carbon signal suggested the β-orientation of the C-24 hydroxyl group, as opposed to its α-orientation in **1**. A similar 3.3 ppm difference in the ^{13}C signal was observed between the C-24 epimeric triterpenoids, ocotillone and cabraleone (Tanaka and Yahara, 1970). The assignments of the ^1H and ^{13}C resonances in **2** were made by comparison with those for compound **1** as well as on the basis of HSQC data.

The α-configuration of H-24 was substantiated by 1D NOESY experiments as depicted on the structure **2**. Irradiation of the H-24 signal produced strong enhancements of the C-24 methyl signals, suggesting their close proximity. Similar irradiation at the resonance frequency of H_3-21 caused a significant enhancement of H-13, indicative of a *cis* relationship between these. Thus compound **2** was identified as 3β-acetyl-20,25-epoxydammarane-24β-ol.

Although triterpenoids **1** and **2** were isolated from the fraction exhibiting anti inflammatory activity, the purified compounds have not been tested yet.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on a Beckman DU-640 and Perkin-Elmer 1600 FTIR spectrometer, respectively. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian VXR-300 or Varian 500S spectrometer and the chemical shifts are reported in ppm relative to the residual undeuterated solvent. The number of attached protons for ^{13}C signals was determined using the DEPT pulse sequence. Inverse detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{CH}=140$ Hz) and HMBC (optimized for $^nJ_{CH}=8.3$ Hz) pulse sequences. Mass spectra and accurate mass measurements were performed on a VG Micromass ZAB mass spectrometer.

3.2. Plant materials

The dried fruits of *F. suspensa* Vahl used in this study were purchased from the Tianjin market, China in 1996 and identified by Jing Rui, Tianjin Municipal Institute for Drug Control, Tianjin 300070, China. Voucher specimen are on deposit at the Tianjin Municipal Institute for Drug Control, China.

3.3. Extraction and isolation

The dried and powdered fruits (500 g) were refluxed twice with 70% methanol in H_2O. The combined extract was concentrated under reduced temperature and pressure, and freeze dried to give a powdered mass (84.5 g). The crude extract was then re-dissolved in water and extracted twice with *n*-hexane to yield 4.53 g of *n*-hexane soluble fraction. An aliquot (2.0 g) of the hexane soluble

material was subjected to column chromatography over silica gel 60 eluted with $CHCl_3$. Based on TLC characteristics, four major fractions (FS-I to FS-IV) were made. Assay for in vivo anti-inflammatory activity (RE) on Wister male rats revealed FS-IV as the active fraction. The active fraction FS-IV (200 mg), predominantly consisting of triterpenoids and fat, was further purified by silica gel 60 column chromatography using *n*-hexane-ethyl acetate (9:1) as the developing solvents to afford triterpenes **1** (40 mg) and **2** (15 mg).

3.4. 3β-Acetyl-20,25-epoxydammarane-24α-ol (*1*)

White amorphous; $[\alpha]_D$ +22.7° (*c* 0.022, $CHCl_3$) [lit. (Biellmann, 1966) +52°]. IR (film) ν_{max} cm^{-1}: 3492, 2949, 1714, 1451, 1378, 1250, 1143, 1042, 980. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data — see Table 1. HRFABMS *m/z* 503.4076 $(MH)^+$, calc. for $[C_{32}H_{55}O_4]^+$ 503.4100. FABMS: *m/z* 503 $[M+H]^+$, 485 $[503\text{-}H_2O]^+$, 443 $[503\text{-}CH_3COOH]^+$, 425 $[503\text{-}(H_2O+CH_3COOH)]^+$.

3.5. 3β-Acetyl-20,25-epoxydammarane-24β-ol (*2*)

White gum; $[\alpha]_D$ +81° (*c* 0.05, $CHCl_3$) [lit. (Biellmann, 1966) +50°]. IR (film) ν_{max} cm^{-1}: 3446, 2948, 2875, 1730, 1451, 1368, 1250, 1o89, 1025, 980. HRFABMS *m/z* 635.3104 $(M+Cs)^+$, calc. for $[C_{32}H_{54}O_4Cs]^+$ 635.3076. FABMS *m/z*: 503 $[M+H]^+$, 485, 543, 425. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data — see Table 1.

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The (3*R*,9*R*)-3-hydroxy-7,8-dihydro-β-ionol disaccharide glycoside is an aroma precursor in tea leaves

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Abstract

The disaccharide glycoside, (3*R*,9*R*)-3-hydroxy-7,8-dihydro-β-ionyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside was isolated as an aroma precursor from the leaves of *Camellia sinensis* var. *sinensis* cv. Yabukita. Its stereochemistry was elucidated on the basis of spectral data and chemical synthesis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Camellia sinensis*; Theaceae; Glycosidic aroma precursor; Disaccharide glycoside; (3*R*,9*R*)-3-Hydroxy-7,8-dihydro-β-ionyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside; C_{13}-Norisoprenoid

1. Introduction

Thirteen-carbon (C_{13}-) norisoprenoids are well known as important aroma constituents of grape juices and wines (Strauss et al., 1987; Winterhalter et al., 1990) and recent studies have revealed that the volatile norisoprenoids are derived from glycosidic precursor forms (Skouroumounis and Winterhalter, 1994). A number of these compounds have been detected in grapes, wine (Strauss et al., 1987; Winterhalter et al., 1990), quince fruit (Güldner and Winterhalter, 1991; Winterhalter et al., 1991), tobacco (Baumes et al., 1994) and *Osmanthus absolute* (Kaiser and Lamparsky, 1978). However, the absolute configuration of a limited number have been determined with both isomers having 9*R* (Andersson and Lundgren, 1988; Winterhalter et al., 1991; Pabst et al., 1992; Matsuda et al., 1997) and 9S (Miyase et al., 1989; Pabst et al., 1992; Baumes et al., 1994) configurations being reported. Most ionone-type glycosides on the other hand, have a 9*R*-configuration.

In tea leaves, many kinds of alcohols such as geraniol, linalool, etc., which contribute to the characteristic floral tea aroma of oolong tea and black tea, have been reported to be present as glycosides and liberated from their glycosidic aroma precursors by the action of endogenous enzymes during the tea manufacturing process (Takeo, 1981; Fischer et al., 1987; Morita et al., 1994, Moon et al., 1994). In the course of our study on the molecular basis of aroma formation during tea processing, we have isolated and identified glycosidic aroma precursors such as β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides) of linalool, geraniol, 2-phenylethanol, benzyl alcohol, etc., 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides of methyl salicylate and of linalool oxides II and III and a β-D-glucopyranoside of (*Z*)-3-hexenol from fresh leaves of oolong tea cultivars (*C. sinensis* var. *sinensis* cv. Shuixian and Maoxie) (Guo et al., 1993, 1994; Moon et al., 1994; Ogawa et al., 1995). We also purified a β-primeverosidase which is considered to be a specific glycosidase to hydrolyze the disaccharide glycosidic aroma precursors (Guo et al., 1996; Ogawa et al., 1997; Ijima et al., 1998).

In the course of these studies, 3-hydroxy-7,8-dihydro-β-ionol, a C_{13}-norisoprenoid, was found to be generated from some glycosidic fractions from tea leaves (*C. sinensis* var. *sinensis* cv. Yabukita) when treated with a crude enzyme prepared from the fresh tea leaves. Because 3-hydroxy-7,8-dihydro-β-ionol has never been reported as a tea aroma, it was searched among volatile flavor compounds from green, oolong and black tea by the Simultaneous Distillation and Extraction (SDE) method (Schultz et al., 1977; Berderich et al., 1992) and found only from a black tea made from *C. sinensis* var. *assamica*.

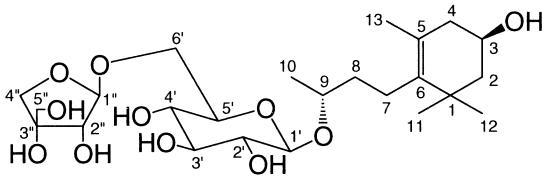
3-Hydroxy-7,8-dihydro-β-ionol has been isolated only as the 3-glycosylated form from quince fruits and

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Prunus spinosa (Hump and Schreier, 1992; Winterhalter and Schreier, 1994), but its absolute configuration has not been reported. This compound is the first example with a hydroxyl at C-9 from tea leaves (*C. sinensis* var. *sinensis*), although several kinds of C_{13}-norisoprenoid compounds have been identified in tea volatiles (Yamanishi, 1995). So we decided to determine the absolute configuration at C-9 of this compound, even though the isolated amount was minute.

This paper addresses with the isolation of the new aroma precursor (**1**) of 3-hydroxy-7,8-dihydro-β-ionol and the elucidation of its absolute stereochemistry.



2. Results and discussion

The glycosidic fractions obtained from hot water extracts of tea leaves (cv. Yabukita) were subjected to HPLC on an ODS column (gradient elution of 20–60% MeCN in H_2O). The 25.5–29% MeCN eluate containing a glycoside (**1**) of 3-hydroxy-7,8-dihydro-β-ionol was further purified by repeated HPLC on an ODS column (gradient elution of 10–50% MeCN in H_2O) and then on Fluofix column (eluted with 21% MeOH in H_2O) to give 1.1 mg of **1**.

The molecular formula $C_{24}H_{42}O_{11}$ of **1** was determined by high resolution FAB-MS analysis [*m*/*z*; found: 507.2798, calcd. for $C_{24}H_{43}O_{11}$ ($[M+H]^+$): 507.2806]. The ^{13}C NMR spectrum (Table 1) showed 11 carbon signals ascribed to a sugar moiety including two anomeric carbons (δ 102.4 and 110.9) besides those of an aglycone, 3-hydroxy-7,8-dihydro-β-ionol. The large coupling constant of an anomeric proton (δ 4.35, 1H, d, $J=7.9$ Hz, H-1′) and the sequential *trans*-1,2-diaxial relationship of H-1′ to H-5′ ($J=7.9$–9.2 Hz) in the ^1H NMR spectrum suggested the presence of a glucopyranosyl moiety. The identification of the remaining sugar moiety was established by comprehensive ^1H and ^{13}C NMR measurements including HSQC and HMBC. Another anomeric proton (δ 4.92, 1H, d, $J=3.1$ Hz, H-1″) having a small coupling constant and a signal corresponding to a quaternary carbon (δ 80.6, C-3″) indicated a furanose moiety. This was determined as apiofuranose based on the presence of AB quartet methylene signals (δ 3.71 and 3.85, 1H each, d, $J=10.4$ and 10.1 Hz, H-4″a and -4″b, respectively) and a singlet (δ 3.50, 2H, s, H-5″) as well as a cross peak between methylene (δ 3.50, H-5″) and methine (δ 3.82, 1H, d, $J=3.1$ Hz, H-2″) protons in the NOESY experiment. The cross peaks between H-1″ (δ 4.92, 1H, d, $J=3.1$ Hz) of the apiofuranose moiety and C-6′ (δ 68.5) of the glucopyranosyl moiety were clearly detected in the HMBC spectrum. The apiofuranose was confirmed to have a β-configuration by comparing the ^{13}C and ^1H NMR data of the apiofuranose moiety of **1** with those of previously reported papers (Moon et al., 1996; Oka et al., 1997). Therefore, the disaccharide moiety of **1** was concluded to be 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside.[1]

Thirteen carbon signals in the ^{13}C NMR spectrum accounted for the aglycone part of **1**. The ^1H NMR spectrum showed the presence of two quaternary methyl protons (δ 0.88 and 0.90, 3H each, s, H-11 and 12), one doublet methyl protons (δ 1.07, 3H, d, $J=6.1$ Hz, H-10) and one vinyl methyl protons (δ 1.49, 3H, s, H-13), indicating an ionone derivative. Two oxygen bearing carbons (δ 65.8, C-3 and δ 76.4, C-9) and two sets of downfield-shifted methylene protons (δ 1.89–1.95, 2H, m, H-7 and δ 1.37–1.43, 1.54–1.59, 1H each, m, H-8a,

Table 1
^{13}C NMR spectral data for compounds **1**, **2**, **5a** and **5b** in CD_3OD

C	1	5a	5b
1	40.0	33.5	33.5
2	49.8	32.7	32.6
3	65.8	23.9	23.9
4	40.5	121.0	121.1
5	125.4	137.9	137.8
6	138.6	50.7	50.8
7	25.4	27.8 (27.9[a])	27.7
8	38.9	38.8 (38.9[a])	38.0 (38.1[a])
9	76.4	76.1 (76.2[a])	78.0 (78.1[a])
10	19.9	19.8 (19.9[a])	21.8 (21.9[a])
11	30.4	28.1	28.1
12	29.0	28.0	28.1
13	20.2	24.0	24.0
1′	102.4	102.0 (102.1[a])	103.7 (103.8[a])
2′	75.2	75.2	75.3
3′	78.0	78.2	78.2
4′	71.8	71.8	71.8
5′	76.9	77.8	77.8
6′	68.5	62.9	62.9
1″	110.9	–	–
2″	78.2	–	–
3″	80.6	–	–
4″	75.3	–	–
5″	65.8	–	–

[a] Chemical shifts due to the epimer at C-6.

[1] The absolute configurations of the sugar moieties were not determined, but we had determined the sugar moiety (D-glucose) of geranyl β-primeveroside in a previous paper (Guo et al., 1993). Since only D-apiose has been found in plant kingdom so far, both sugars were deduced to be D-series.

-8b) show that this aglycone is 3-hydroxy-7,8-dihydro-β-ionol. The glycosidic linkage of the glucopyranosyl moiety to C-9 position of the aglycone was confirmed by the cross-peak between an anomeric carbon signal (δ 102.4, C-1′) of the glucopyranose and H-9 (δ 3.74–3.78, 1H, m) of the aglycone in HMBC spectrum.

The relative stereochemistry at C-3 in the aglycone moiety of **1** was confirmed as follows. In NOE experiments, strong interactions were observed between H-11 and H-7 and between H-12 and H-3, which indicates the hydroxyl group at C-3 is equatorial. This is the same configuration as those of previously reported 3-oxygenated ionone derivatives, all of which have been reported having 3*R*-configurations (Miyase et al., 1988, 1989; Matsushita et al., 1991; Güldner and Winterhalter, 1991; Baumes et al., 1994). Based on these results, the absolute configuration of C-3 was deduced to be *R*.

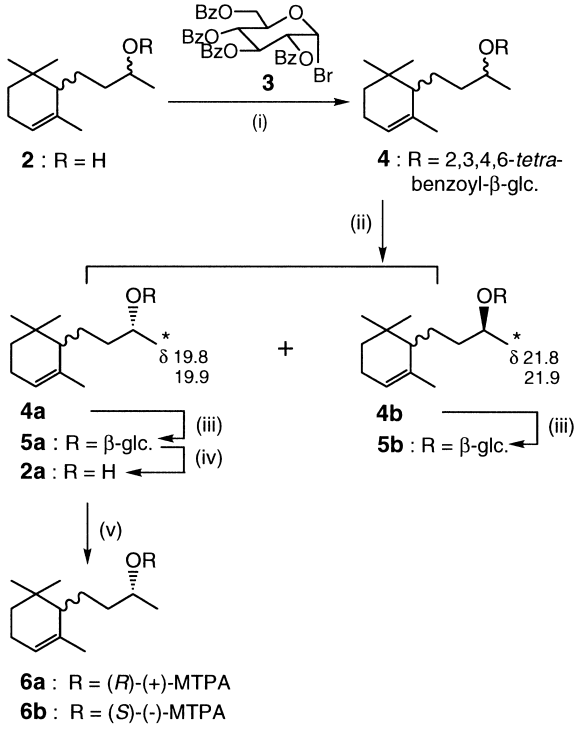


Fig. 1. Synthesis of model compounds (**6a** and **6b**) to determine the absolute configuration of C-9 of aglycone of **1**. *Indicates C-10 chemical shift of **5a** and **5b**. (i) $Hg(CN)_2$, dry MeCN, 36 h, rt; (ii) Silica gel column chromatography (CH_2Cl_2); (iii) NH_3 in MeOH, 24 h; (iv) β-glucosidase, citrate buffer (pH 4.4), 16 h, 38°; (v) (+) or (−)-MTPA chloride, dry pyridine, dry CH_2Cl_2, 12 h, rt.

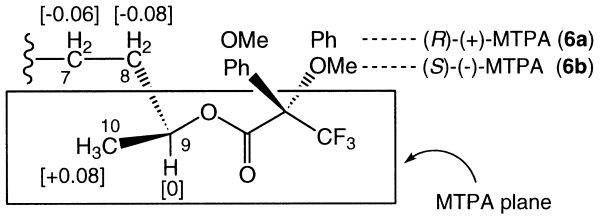


Fig. 2. The conformation of MTPA esters (**6a** and **6b**) with Δδ values (shown in bracket, $\Delta\delta = \delta_S - \delta_R$).

The absolute configuration at the C-9 position with a glycosyl substituent was determined using the model compound, 7,8-dihydro-α-ionol (**2**), having a similar structure to the aglycone moiety of **1** (Fig. 1), since the isolated quantity of **1** was too small for further analysis.

A racemic mixture (6*RS*, 9*RS*) of **2** was reacted with 2,3,4,6-*tetra*-*O*-benzoyl-α-D-glucopyranosyl bromide (**3**) to give the diastereoisomeric mixture of **4**. The mixture was separated on a silica gel column to afford two sets [(6*RS*, 9*R*) and (6*RS*, 9 *S*)] of diastereomers (**4a** and **4b**), which gave diastereomixtures of α-ionyl glycosides (**5a** and **5b**), respectively, after deacylation with methanolic ammonia. Seo et al. and Tagawa et al. reported the absolute configuration at C-9 could be determined based on the glucosidation shift rule i.e. based on the C-10 chemical shift difference between 9*R*- and 9 *S*-glycosylated ionol epimers (Seo et al., 1978; Tagawa et al., 1996). Actually, the C-10 carbon signals of **5a** and **5b** were observed at δ 19.8 (19.9*), 21.8 (21.9*) respectively (Asterisks indicate chemical shifts of an epimer at C-6). The C-10 carbon signal of the isolated glycoside (**1**) was observed at δ 19.9 (Table 1), indicating the configuration at C-9 is identical with that of **5a**.

The absolute configuration of C-9 of **5a** was confirmed by use of Mosher's Method (Dale et al., 1969; Dale and Mosher, 1973). Chiral alcohol (**2a**) was prepared by enzymatic hydrolysis of **5a**, this being converted into the *R*- and *S*-MTPA esters (**6a** and **6b**). From the Δδ values ($\Delta\delta = \delta_S\delta_R$), where protons on the right-hand side of the MTPA plane must have positive values, and those on the left-hand side must be negative (Ohtani et al., 1991, 1995), were calculated. For the MTPA derivatives (**6a** and **6b**), the Δδ value for methyl protons on C-10 was positive ($\Delta\delta_{H10} = +0.08$) and for the methylene protons at C-8 and C-7 were negative ($\Delta\delta_{H8} = -0.08$, $\Delta\delta_{H7} = -0.06$). The Δδ value for H-9, however, was zero ($\Delta\delta_{H9} = 0$), since it is located on the MTPA plane (Fig. 2). These observations clearly show that the absolute configuration at C-9 of **2a** is *R* and consequently the C-9 stereochemistry of **1** is unambiguously assigned as *R*.

On the basis of the foregoing, the structure of **1** has been concluded to be (3*R*,9*R*)-3-hydroxy-7,8-dihydro-β-ionyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside.

3. Experimental

3.1. General

NMR spectra were recorded on JEOL JNM-AL 400 and JEOL LAMBDA 500 spectrometers, using either CD_3OD or D_2O as solvent. Chemical shifts were given in δ values with TMS or TSP being employed as internal

standards. Mass spectra (EI, at 70 eV; FAB, using glycerol or NBA as a matrix) were obtained with a JEOL JMS 700 spectrometer. GC–MS analysis was carried out with a JEOL JMS-DX 302 mass spectrometer linked to a Hewlett–Packard 5890A gas chromatograph (column, PEG-20 M, 0.25 mm×50 m; carrier gas, He at 1 ml min^{-1}; temp. program, 60° to 230°C at 3°C min^{-1}). TLC was performed on silica gel plates (Kieselgel 60 F_{254}, Merck) using CH_2Cl_2 for **2** and **4**, $CHCl_3$-MeOH (5:1) for **5** and hexane-$CHCl_3$ (1:3) for **6**. They were detected under UV 254 nm and by spraying with 0.2% naphthoresorcinol in H_2SO_4-EtOH (1:19) (Touchstone, 1978).

3.2. Materials

3.2.1. Plant

Fresh tea leaves (*Camellia sinensis* var. *sinensis* cv. Yabukita) were collected at the farm of the National Research Institute of Vegetables, Ornamental Plants and Tea at Kanaya, Shizuoka, Japan in May, 1997. The leaves were steamed just after plucking and stored at −70°C before use.

3.2.2. Chemical

The racemic mixture of 7,8-dihydro-α-ionol (**2**) was kindly provided by Prof. Hideo Etoh (Faculty of Agriculture, Shizuoka University).

3.3. Simultaneous distillation and extraction (SDE) method

SDE was performed for 2 h using pentane-CH_2Cl_2 (2:1) as previously reported (Schultz et al., 1977). The extracts were dried over anhydrous Na_2SO_4 and concentrated under N_2 stream for GC–MS analysis.

3.4. Detection of aroma precursor

To detect aroma precursors, enzymatic hydrolysis was carried out as previously reported (Ogawa et al., 1995). The characteristic signals in the ^1H NMR spectrum, corresponding to four methyl signals of the aglycone moiety of **1**, were used as a guide for the isolation.

3.5. Extraction and isolation of aroma precursor

Frozen tea leaves (3.8 kg) were extracted with hot water (20 l, 10 l×twice) for 20 min and subjected to active charcoal CC (85×350 mm) eluted with H_2O–MeOH (0, 10, 20, 40, 60, 80 and 100% MeOH; 2 l each). The 60–100% MeOH fractions were treated with 20 g of Polyclar SB-100 (Wako Chemical Co. Ltd, eluted with H_2O) to remove catechins, and then subjected to Amberlite XAD-2 CC (40×310 mm) eluted with H_2O–MeOH (0, 10, 20, 40, 60 and 100% MeOH; 450 ml each). The 10–100% MeOH fractions were combined and loaded on an ODS column (YMC-GEL, ODS-A 60–200/60, 30×480 mm, YMC Co. Ltd), which was eluted with H_2O–MeOH (10, 20, 30, 40, 60 and 80% MeOH, 800 ml each). The 40% MeOH fraction from which 3-hydroxy-7,8-dihydro-β-ionol was liberated after enzymatic hydrolysis, was subjected to HPLC on an ODS (YMC-Pack S-343 I-15 ODS column, 20×250 mm, YMC Co. Ltd) with gradient elution of 20–60% MeCN in H_2O.

The 25.5–29% MeCN fractions containing a glycoside (**1**) were successfully purified by repeated HPLC on an ODS (YMC-PACKED S-5 120A ODS column, 20×250 mm, YMC Co.; gradient elution of 10–50% MeCN in H_2O) and Fluofix (Fluofix 120N column, 10×150 mm, NEOS Co. Ltd; eluted with 21% MeOH in H_2O) column to give **1** (1.1 mg).

3.6. (3R,9R)-3-Hydroxy-7,8-dihydro-β-ionyl 6-O-β-D-apiofuranosyl-β-D-glucopyranoside (*1*)

HR-FABMS *m/z*; found: 507.2798, calcd. for $C_{24}H_{43}O_{11}$ ($[M+H]^+$): 507.2806; $[\alpha]_D^{27}$ −7.2° (MeOH, *c* 1.0); ^1H NMR (500 MHz, D_2O, TSP) δ 0.88 (3H, s, H-12), 0.91 (3H, s, H-11), 1.07 (3H, d, $J=6.1$ Hz, H-10), 1.24 (1H, t, $J=12.1$ Hz, H-2a), 1.40 (1H, m, H-8a), 1.49 (3H, s, H-13), 1.54–1.59 (1H, m, overlapped with H-2b, H-8b), 1.55 (1H, overlapped with H-8b, H-2b), 1.80 (1H, dd, $J=4.7$ and 11.6 Hz, H-4a), 1.89–1.95 (2H, m, H-7), 2.10 (1H, dd, $J=4.7$ and 11.6 Hz, H-4b), 3.08 (1H, dd, $J=7.9$ and 9.2 Hz, H-2′), 3.29 (1H, dd, $J=8.2$ and 9.2 Hz, H-4′), 3.32 (1H, dd, $J=8.2$ and 9.2 Hz, H-3′), 3.37 (1H, m, H-5′), 3.50 (2H, s, H-5″), 3.54 (1H, dd, $J=6.6$ and 11.6 Hz, H-6′a), 3.71 (1H, d, $J=10.4$ Hz, H-4″a), 3.75 (1H, m, overlapped with H-3, H-9), 3.76–3.80 (1H, m, overlapped with H-9, H-3), 3.82 (1H, d, $J=3.1$ Hz, H-2″), 3.84 (1H, overlapped with H-2″ and 4″b, H-6′b), 3.85 (1H, d, $J=10.4$ Hz, H-4″b), 4.35 (1H, d, $J=7.9$ Hz, H-1′), 4.92 (1H, d, $J=3.1$ Hz, H-1″); ^{13}C-NMR (125.7 MHz, CD_3OD, TMS) Table 1.

3.7. Determination of the absolute configuration in the C-9 position of *1*

3.7.1. Preparation of *4a* and *4b* by Koenigs–Knorr Reaction (Sone and Misaki, 1986)

To a mixture of 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**3**, 0.96 g, 1.45 mmol), which was prepared from D-(+)-glucose by a general method (Fletcher and Hudson, 1947), $Hg(CN)_2$ (0.3 g, 1.2 mmol) and activated 4 Å molecular sieves (4 g) in dry MeCN (15 ml) was added to the racemic mixture (6*RS*, 9*RS*) of **2** (0.96 g, 1.45 mmol); The reaction mixture was then allowed to stir at room temp. under argon for 36 h, after which it was extracted with CH_2Cl_2 and washed with

1 M $NaHCO_3$ soln. The organic layer was applied to flash chromatography (eluted with CH_2Cl_2) to afford two sets [(6*RS*, 9*R*) and (6*RS*, 9 *S*)] of diastereomers, **4a** (*Rf* 0.47, CH_2Cl_2) and **4b** (*Rf* 0.29, CH_2Cl_2) (100 mg each, 42%).

3.7.2. Preparation of *5a* and *5b*

Each diastereomer (**4a** and **4b**, 100 mg each, 129 μmol) was treated with 30 ml of the methanolic ammonia for 24 h at room temp. and purified by flash chromatography ($CHCl_3$–MeOH, 9:1) to give **5a** and **5b** (30 mg each, 90%), respectively. **5a**: *Rf* 0.35, ($CHCl_3$–MeOH, 5:1); HR-FABMS *m/z*; found: 359.2437, calcd. for $C_{19}H_{35}O_6$ ($[M+H]^+$): 359.2434; ^{1}H NMR (500 MHz, CD_3OD, TMS) δ 0.87 (3H, s, H-12), 0.93 (3H, s, H-11), 1.13 (1H, m, H-2a), 1.17 (3H, d, $J=6.1$ Hz, H-10), 1.44 (1H, overlapped with H-2b, H-6), 1.45 (1H, overlapped with H-6 and H-7, H-2b), 1.48 (2H, overlapped with H-2b, H-7), 1.53 (1H, m, H-8a), 1.62 (1H, m, H-8b), 1.68 (3H, s, H-13), 1.96 (2H, m, H-3), 3.14 (1H, dd, $J=7.8$ and 8.5 Hz, H-2′), 3.24 (1H, m, H-5′), 3.30 (1H, dd, $J=7.8$ and 8.6 Hz, H-4′), 3.34 (1H, dd, $J=7.8$ and 8.9 Hz, H-3′), 3.67 (1H, dd, $J=5.5$ and 11.9 Hz, H-6′a), 3.83 (1H, m, overlapped with H-6′b, H-9), 3.86 (1H, overlapped with H-9, H-6′b), 4.33 (1H, d, $J=7.8$ Hz, H-1′), 5.28 (1H, m, H-4); ^{13}C NMR (125.7 MHz, CD_3OD, TMS) Table 1. **5b**: *Rf* 0.35, ($CHCl_3$–MeOH, 5:1); HR-FABMS *m/z*; found: 359.2428, calcd. for $C_{19}H_{35}O_6$ ($[M+H]^+$): 359.2434; ^{1}H NMR (500 MHz, CD_3OD, TMS) δ 0.88 (3H, s, H-12), 0.94 (3H, s, H-11), 1.13 (1H, m, H-2a), 1.23 (3H, d, $J=6.1$ Hz, H-10), 1.44 (1H, overlapped with H-2b, H-6), 1.46 (1H, overlapped with H-6 and H-8, H-2b), 1.48 (2H, overlapped with H-2b, H-7), 1.53 (1H, m, H-8a), 1.63 (1H, m, H-8b), 1.69 (3H, s, H-13), 1.96 (2H, m, H-3), 3.15 (1H, dd, $J=7.7$ and 8.5 Hz, H-2′), 3.23 (1H, m, H-5′), 3.28 (1H, dd, $J=7.7$ and 8.6 Hz, H-4′), 3.34 (1H, dd, $J=7.7$ and 8.6 Hz, H-3′), 3.66 (1H, dd, $J=5.5$ and 11.9 Hz, H-6′a), 3.76 (1H, m, H-9), 3.86 (1H, dd, $J=1.8$ and 11.9 Hz H-6′b), 4.32 (1H, d, $J=7.7$ Hz, H-1′), 5.29 (1H, m, H-4); ^{13}C-NMR (125.7 MHz, CD_3OD, TMS) Table 1.

3.7.3. Preparation of *2a* from *5a*

An epimeric mixture (**5a**; 30 mg, 84 μmol) was enzymatically hydrolyzed with β-glucosidase (30 mg, 120 units) from sweet almond (Oriental Yeast Co.) in 100 mM acetate buffer (2 ml, pH 4.4) at 38° for 16 h (Yoshikawa et al., 1998). The reaction mixture was extracted with EtOAc, washed with satd aq. NaCl, respectively, and purified by flash chromatography ($CHCl_3$–MeOH, 9:1) to give the aglycone, 7,8-dihydro-α-ionol (**2a**, 9 mg, 55%). **2a**: *Rf* 0.22 (CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 0.87 (3H, s, H-12), 0.92 (3H, s, H-11), 1.13 (1H, m, H-2a), 1.19 (3H, d, $J=6.1$ Hz, H-10), 1.30 (1H, m, H-2b), 1.43 (1H, overlapped with H-7 and H-8, H-6), 1.44 (2H, overlapped with H-6, H-7), 1.54 (2H, m, H-8), 1.68 (3H, s, H-13), 1.96 (2H, m, H-3), 3.74 (1H, m, H-9), 5.30 (1H, m, H-4); ^{13}C NMR (125.7 MHz, $CDCl_3$, TMS) δ 23.0 (C-10), 23.4 (C-3), 23.5 (C-13), 27.0 (C-7), 27.5 (C-12), 27.6 (C-11), 31.6 (31.7*) (C-2), 32.6 (C-1), 39.8 (C-8), 49.3 (C-6), 68.7 (68.9*) (C-9), 120.3 (C-4) 136.4 (C-5) (Asterisks indicate chemical shifts of epimer at C-6).

3.7.4. Preparation of (R)-MTPA ester of 7,8-dihydro-α-ionol (*6a*)

To a solution of 7,8-dihydro-α-ionol (**2a**, 4.5 mg, 23 μmol) in pyridine (1.5 ml) and CH_2Cl_2 (1.5 ml) was added (+)-MTPA chloride (10 μl, 52 μmol, Aldrich Chemical Co.), and stirred at room temp. for 12 h (Dale and Mosher, 1973). The reaction mixture was added with ether, washed with cold diluted HCl, satd aq. Na_2CO_3 and NaCl, respectively, and purified by flash chromatography (hexane–$CHCl_3$, 1:1) to afford the desired (*R*)-(+)-MTPA ester (**6a**, 3 mg, 30%). **6a**: *Rf* 0.60, (hexane–$CHCl_3$, 1:3); HR-FABMS *m/z*; found: 412.2218, calcd. for $C_{23}H_{31}O_3F_3$ (M^+): 412.2225; ^{1}H-NMR (500 MHz, $CDCl_3$, TMS) δ 0.85 (3H, s, H-12), 0.87 (3H, s, H-11), 1.11 (1H, m, H-2a), 1.26 (3H, d, $J=6.1$ Hz, H-10), 1.33 (1H, m, H-2b), 1.41 (1H, overlapped with H-7, H-6), 1.42 (2H, overlapped with H-6, H-7), 1.59 (1H, m, H-8a), 1.63 (3H, s, H-13), 1.72 (1H, m, H-8b), 1.94 (2H, m, H-3), 3.54 (3H, s, H-2′-OC$\underline{H}_3$), 5.09 (1H, m, H-9), 5.30 (1H, m, H-4), 7.38–7.40 (3H, m, H-Ph-3″, -5″ and 4″), 7.52–7.54 (2H, H-Ph-2″ and 6″); ^{13}C NMR (125.7 MHz, $CDCl_3$, TMS) δ 19.5 (C-10), 23.0 (C-3), 23.4 (C-13), 26.5 (26.6*) (C-7), 27.4 (C-12), 27.5 (C-11), 31.4 (31.6*) (C-2), 32.5 (C-1), 35.9 (36.2*) (C-8), 49.0 (49.1*) (C-6), 55.3 (C-2′-OC$\underline{H}_3$), 74.6 (74.7*) (C-9), 84.4 (C-2′, $^2J_{CF}=28.3$ Hz), 120.5 (120.6*) (C-4), 123.4 [C-3′ (C$\underline{F}$3), $^1J_{CF}=289.2$ Hz], 127.2 (C-Ph-2″ and 6″), 128.4 (C-Ph-3″ and 5″), 129.5 (C-Ph-4″), 132.4 (C-Ph-1″), 135.8 (136.0*) (C-5), 166.2 (C-1′) (asterisks indicate chemical shifts of an epimer at C-6).

3.7.5. Preparation of a (S)-MTPA ester of 7,8-dihydro-α-ionol (*6b*)

2a (4.5 mg, 23 μmol) was treated with (−)-MTPA chloride (10 μl, 52 μmol, Aldrich Chemical Co.) in the same way as preparation for **6a** to give an (*S*)-(−)-MTPA ester (**6b**, 3 mg, 30%). **6b**: *Rf* 0.60, (hexane–$CHCl_3$, 1:3); HR-FABMS *m/z*; found: 412.2231, calcd. for $C_{23}H_{31}O_3F_3$ (M^+): 412.2225; ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 0.80 (0.81*) (3H, s, H-12), 0.82(0.83*) (3H, s, H-11), 1.08 (1H, m, H-2a), 1.29 (1H, m, H-2b) 1.34 (3H, d, $J=6.1$ Hz, H-10), 1.36 (2H, overlapped with H-6, H-7), 1.38 (1H, overlapped with H-7, H-6), 1.51 (1H, m, H-8a), 1.57 (3H, s, H-13), 1.64 (1H, m, H-8b), 1.92 (2H, m, H-3), 3.57 (3H, s, H-2′-OC$\underline{H}_3$), 5.09 (1H, m, H-9), 5.28 (1H, m, H-4), 7.37-7.39 (3H, m, H-Ph-3″, -5″ and 4″), 7.52–7.54 (2H, H-Ph-2″ and 6″); ^{13}C-

NMR (125.7 MHz, $CDCl_3$, TMS) δ 19.7 (C-10), 23.0 (C-3), 23.3 (C-13), 26.3 (26.4*) (C-7), 27.3 (C-12), 27.5 (C-11), 31.4 (31.6*) (C-2), 32.5 (C-1), 35.9 (36.2*) (C-8), 49.0 (49.1*) (C-6), 55.4 (C-2′-O$\underline{C}H_3$), 74.6 (74.7*) (C-9), 84.4 (C-2′, $^2J_{CF}=28.3$ Hz), 120.5 (120.6*) (C-4), 123.4 [C-3′ ($\underline{C}F_3$), $^1J_{CF}=289.2$ Hz], 127.4 (C-Ph-2″ and 6″), 128.3 (C-Ph-3″ and 5″), 129.5 (C-Ph-4″), 132.6 (C-Ph-1″), 135.8 (136.0*) (C-5), 166.2 (C-1′) (asterisks indicate chemical shifts of an epimer at C-6).

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Constituents of *Afzelia bella* stem bark

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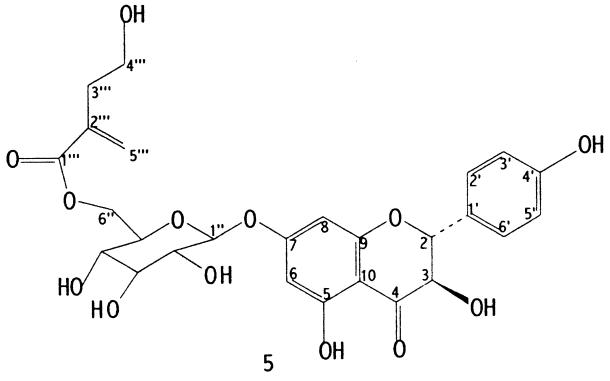
Abstract

The stembark of *Afzelia bella* yielded an acylated dihydroflavonol glycoside identified as 2*R*,3*R*-*trans*-aromadendrin-7-*O*-β-D-glucopyranoside-6″-(4‴-hydroxy-2‴-methylene butanoate), along with five known flavonoids and the lignan glycoside (+)-isolariciresinol 9-*O*-xyloside. Their structures were determined by detailed 1D and 2D NMR analyses. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Afzelia bella*; Leguminosae; Flavonoids; Lignan; 2*R*,3*R*-*trans*-Aromadendrin-7-*O*-β-D-glucopyranose-6″-(4‴-hydroxy-2‴-methylene-butanoate)

1. Introduction

Afzelia bella Harms var. Glacicolor (Leguminosae) is a handsome shade tree found on the West Coast of Africa. The aril is edible, and the heartwood is used as timber. The powdered bark is used in the Ivory Coast as a decoction for supurative skin diseases (Burkill, 1985). No previous phytochemical investigations were reported on this species. As part of our continuing search for plant-derived antimicrobial agents, and especially those with antitubercular properties, the butanol extract of the stem bark of this plant, which was found to exhibit significant antimicrobial properties against the Gram-positive and Gram-negative organisms (Binutu, 1998), was fractionated. We now report the isolation of five known flavonoids; dihydrokaempferol (aromadendrin) (**1**), 7-*O*-methyl-dihydrokaempferol (**2**), dihydrokaempferol-7-*O*-β-D-glucoside (**4**), kaempferol (**6**), kaempferol-7-*O*-β-D-glucopyranoside (**7**), and the lignan glycoside (+)-isolariciresinol 9-*O*-β-D-xylopyranoside (**3**). A new acylated dihydroflavonol glycoside identified as 2*R*,3*R*-*trans*-aromadendrin-7-*O*-β-D-glucopyranose-6″-(4‴-hydroxy-2‴-methylenebutanoate) (**5**), was also isolated. The present communication deals with the structure elucidation of this new flavonoid. Dihydrokaempferol (**1**) and kaempferol (**6**) were previously reported from an incompletely identified species of *Afzelia* (King and Acheson, 1950).



5

2. Results and discussion

Compound **5** was isolated from the methanolic stem bark extract of *A. bella* as a light yellow powder. The positive FABMS showed the molecular peak at *m*/*z* 549 $[M+H]^+$ and the negative FABMS at *m*/*z* 547$[M–H]^+$; the former ion analyzed for $C_{26}H_{29}O_{13}$. The ^{13}C NMR spectrum, particularly the APT and DEPT experiments, revealed the presence of 26 carbons, consisting of 4 methylene, 13 methine, and 9 quaternary carbons. These data allowed the establishment of the molecular formula as $C_{26}H_{28}O_{13}$. Unambiguous assignments were determined

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by heteronuclear shift correlation spectroscopy (^{13}C–^{1}H COSY), ^{1}H detected heteronuclear multiple-bond connectivity (HMBC) and nuclear Overhauser enhancement and exchange spectroscopy (NOESY) experiments. The UV–vis absorption spectra of **5** indicated a dihydroflavonol derivative with free hydroxyl groups at C-5 and C-4′, and the observed shift in alkali suggested the 7-hydroxyl group to be substituted (Mabry et al., 1970). The ^{1}H NMR spectrum showed two doublets at δ 4.70 and 5.10 ($J=11.6$ Hz) characteristic of *trans* H-2/H-3 protons in a dihydroflavonol. The two proton singlet at δ 6.15 correlated with the carbons at δ 97.6 and 96.1 in the HMQC spectrum and were assigned to the H-6 and H-8 protons of ring A. From the HMBC experiment, ring B was assigned as a 1,4-disubstituted benzene ring (δ 7.34, *d*, 2H, $J=8.5$ Hz; 6.81, *d*, 2H, $J=8.5$ Hz). Collectively, these data indicated that the aglycone moiety was dihydrokaempferol. The sugar functionality was identified as a β-glucopyranose by the ^{1}H and ^{13}C spectral data. The HMBC experiment displayed a long-range correlation between C-7 (δ 166.0) and the anomeric proton (δ 5.05, *d*, $J=7.2$ Hz), revealing the site of glycosidation as the 7-OH of dihydrokaempferol. Of the 26 resonances present in the ^{13}C NMR spectra of **5**, 20 were similar to those of aromadendrin-7-*O*-β-D-glucoside (Agrawal, 1989). The remaining acyl portion showed the presence of five carbons as an ester carbonyl (δ 167.1), an unsaturated quaternary carbon (δ 137.8), a geminal methylene (δ 128.0, δ_H 6.07, 1H, *s*; 5.44, 1H, *s*), an oxygenated methylene (δ 60.3, δ_H 3.45, 2H, *q*, $J=6.5$ Hz); and an allylic methylene (δ 35.8, δ_H 2.34, 2H, *t*, $J=6.5$ Hz). Consequently, this fragment was identified to be a 4-hydroxy-2-methylenebutanoyl ester. The linkage of this fragment to the 6″-OH was established by the significant downfield shift of glucose H_2-6″ (δ 4.46, 1H, *d*, $J=11.0$ Hz; δ 4.03, 1H, *dd*, $J=7.7$, 11.7 Hz). The HMBC spectrum, in which the ester carbonyl carbon at δ 167.1 was correlated to H-6″, further substantiated these findings. Dihydroflavonols have chiral centers at C-2 and C-3, which translates into four possible orientations. There are cases in where the all four isomers of a given compound are known (Gaffield, 1996), although one of them usually predominates. Compound **5** showed a CD curve with a negative Cotton effect at $[\theta]_{290}$ and positive Cotton effects at $[\theta]_{336}$ and $[\theta]_{289}$. Comparison with dihydroflavonols of established absolute configuration suggested the dominance of the 2*R*,3*R* isomer (Gaffield, 1970). Thus the structure of compound **5** was determined as 2*R*,3*R*-*trans*-aromadendrin-7-*O*-β-D-glucopyranose-6″-(4‴-hydroxy-2‴-methylenebutanoate). β-D-Glucopyranose-6-(4-hydroxy-2-methylenebutanoate) (6-Tuliposide A) and β-D-glucopyranose-1-(4-hydroxy-2-methylenebutanoate) (1-Tuliposide A) were identified previously (Tschesche et al., 1968, 1969; Hutchinson, 1974). The assignments for this fragment in **5** are in agreement with the ^{13}C NMR (Hutchinson, 1974) and ^{1}H NMR spectral data for 6-tuliposide A (Tschesche et al., 1968, 1969). These findings were further confirmed by the negative FABMS, which gave fragment ions at m/z 449 $[M–H\text{-}98]^+$, formed by elimination of the 4-hydroxy-2-methylenebutanoyl moiety, and at m/z 287 $[M–H\text{-}261]^+$, due to loss of the acylated glucose fragment. Tuliposides have been found among several plant genera belonging to the Liliiflorae, where they demonstrate both fungitoxic and allergenic properties (Slob, 1973; Slob et al., 1975). The observed activities are linked to the ability of the glycosides to hydrolyse easily into glucose and a α-methylene-γ-butyrolactone. The lactone is responsible for the skin irritation caused by excessive handling of tulip bulbs.

The five-carbon ester fragment at C-6″ is biosynthetically significant, and may have been formed from a 3-methyl-buten-2-yl group. Although the prenyl function is frequently seen as a C-alkylating function (Smalberger et al., 1974; McCormick et al., 1986), some prenyl ethers have also been reported (Bolmann et al., 1981). Many acylated glycosides have been reported in the literature. Several different organic acids have been found esterified to one or more hydroxyl groups of flavonoid glycosides (Aguinagalde and Del Pero Martinez, 1982; Nawwar et al., 1989; Veit et al., 1990) or directly to a flavonoid hydroxyl group (Urzua and Cuadra, 1989). This, to the best of our knowledge, is the first report of an acylated dihydroflavonol glycoside.

Compounds **1**–**4**, **6** and **7** were identified by comparison of their spectral data (UV, MS, ^{1}H NMR and ^{13}C NMR) with literature values as dihydrokaempferol (aromadendrin) (**1**) (Herz et al., 1972), 7-*O*-methyl-dihydrokaempferol (**2**) (Herz et al., 1972), dihydrokaempferol-7-*O*-β-D-glucoside (**4**) (Batterham and Highet, 1964; Agrawal, 1989), kaempferol (**6**) (Matthes et al., 1980), kaempferol-7-*O*-β-D-glucopyranoside (**7**) (Markham et al., 1978), and the lignan glycoside (+)-isolariciresinol 9-*O*-D-xylopyranoside (**3**) (Fonseca et al., 1978; Takani and Ohya, 1979).

3. Experimental

3.1. General

All melting points were measured on a Koffler hot plate and are uncorrected. ^{1}H and ^{13}C NMR spectra were run at 300 and 75 MHz, respectively (Brüker Avance 300 MHz spectrometer) in DMSO-d_6 or MeOD using tetramethylsilane (TMS) as internal standard. Coupling constants (J) are given in Hz. Standard Brüker sequences were used for APT, COSY, HMQC, HMBC and NOESY experiments. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Column chromatography (CC) was carried out using silica gel, Sephadex LH-20 or MCI gel CHP-20 and thin layer chromatography (TLC)

were conducted on silica gel or RP-18. UV spectra were recorded in MeOH.

3.2. Plant material

The stem bark of *Afzelia bella* was collected in Ibadan, Nigeria in August 1997. The plant was identified by Dr. Z. Gbile, and a herbarium specimen is deposited at the Forestry Research Institute of Nigeria, Ibadan (FRIN No. 105335).

3.3. Extraction and isolation

The air-dried powdered stem bark of *Afzelia bella* (3 kg) was percolated to exhaustion with MeOH at rt. The solution was evapd in vacuo to yield a brown gummy extract (319 g). An aq suspension of this extract (150 g) was extracted successively three times each with $CHCl_3$ and BuOH. The respective extracts were combined, evapd under reduced pressure to give $CHCl_3$ (93 g) and BuOH (37 g) fractions. The BuOH extract (35 g) was cc on Si gel (70–230 mesh, Merck) eluting with $CHCl_3$ followed by gradient mixtures of $CHCl_3$–EtOAc, EtOAc–MeOH, and MeOH. Fractions were collected and pooled according to their similarity by TLC eluting with EtOAc–MeOH–H_2O (9:1:1), to give four fractions (A1–A4). Further repeated purification of subfractions A1 and A2 on Si gel afforded compounds **1** (320 mg) and **2** (29 mg), respectively. Fraction A3 (8.75 g) was fractionated on Sephadex LH-20, eluting with methanol to give three subfractions. Subfraction A3.1 contained compound **3** (55 mg). Subfraction A3.2 was further fractionated on MCI gel with H_2O–MeOH (7:3) to give compounds **4** (3.72 g) and **5** (2.58 g). Repeated fractionation of A3.3 on Sephadex LH-20 eluting with H_2O–MeOH (5:5) furnished compounds **6** (35 mg) and **7** (92 mg). Purification of subfraction A3 was monitored by TLC on RP-18 eluted with MeOH–H_2O (1:1).

3.4. 2R,3R-trans-Aromadendrin-7-O-β-D-glucopyranose-6″-(4‴-hydroxy-2‴-methylene butanoate) (5)

Amorphous light yellow powder from MeOH–H_2O, mp 134–135°C, $[\alpha]_D$ −67° (MeOH, *c* 0.1). UV_{max} (MeOH) nm (log 284 (4.23) and 326.5 *sh* (3.52)), + NaOMe: 243.5, 287, 362.5; + NaOAc: 284.5, 329; + NaOAc + H_3BO_3: 284.5, 325.5; + $AlCl_3$: 280 *sh*, 312, 389; + $AlCl_3$ + HCl: 282, 309, 384.5. CD ($c = 9.1\times10^{-6}$ mol, MeOH): $[\theta]_{337}$ +2,012; $[\theta]_{290}$ −10,560; $[\theta]_{255}$ +1,548; $[\theta]_{216}$ +17,850. EI-MS *m/z* (rel. int.%): $[M]^+$, 287 (21), 286 (74), 285 (23), 270 (31), 166 (14), 153 (35), 134 (11), 121 (19), 108 (21), 107 (100). Negative FABMS $[M-H]^+$ 547 (7), 449 (3), 367 (3), 329 (5) 287 (15), 165 (14), 163 (10), 151 (21). positive FABMS $[M+H]^+$ 549 (6), 386 (6), 371 (8), 330 (16), 315 (27), 289 (24), 253 (35), 215 (20), 153 (29), 119 (96), 117 (100). HR–FAB–MS: calc. for $C_{26}H_{29}O_{13}$ $(M+H)^+$, 549.1608; found: 549.1628. ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) data (DMSO-d_6) are reported in Table 1.

Table 1
^{13}C and ^1H NMR spectral data for **5** in DMSO-d_6 (δ values)[a]

Position C	^{13}C	^1H	HMBC (H–C)
C-2	84.0	5.1, 1H, *d*, (11.6)	3, 1′, 2′ 6′
C-3	72.5	4.70, 1H, *dd*, (11.6, 6.25)	2, 5, 4′
4	199.7		
C-5	163.5		
C-6	97.6	6.15, 1H, *s*	5, 8
C-7	166.0		
C-8	96.1	6.15, 1H, *s*	6, 10
C-9	163.4		
C-10	103.0		
3-OH		5.83, 1H, *d*, (6.25)	2
5-OH		11.80, 1H, *s*	5, 10
4′-OH		9.60, 1H, *s*	3′, 4′, 5′
C-1′	128.3		
C-2′, 6′	130.5	7.34, 2H, *d*, (8.5)	2, 4′
C-3′, 5′	115.8	6.81, 2H, d, (8.5)	1′
C-4′	158.7		
C-1″	100.1	5.05, 1H, *d*, (7.2)	7
C-2″	73.7	3.31, 1H, *m*	3″
C-3″	76.8	3.36, 1H, *m*	
C-4″	70.7	3.16, 1H, *m*	
C-5″	74.6	3.76, 1H, *t*	
C-6″	64.9	4.46, 1H, *d*, (11.7)	1‴
		4.03, 1H,	*dd*, (7.7, 11.7) 1‴
C-1‴	167.1		
C-2‴	137.8		
C-3‴	35.8	2.34, 2H, *t*, (6.5)	1‴, 2‴, 4‴, 5‴
C-4‴	60.3	3.45, 2H, *q*, (6.5)	2‴, 3‴
C-5‴	128	6.06, 1H, *s*	3‴
		5.44, 1H, *s*	3‴
4‴-OH		4.4, 1H, *t*, (5.4)	4‴

[a] Coupling constants (*J* in Hz) in parentheses. Assignments were made on the basis of ^1H–^1H COSY, HMQC and HMBC pulse sequences. ^{13}C NMR multiplicities were determined by APT and DEPT sequences.

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3-Desoxyanthocyanidins from *Arrabidaea chica*

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Abstract

The new 3-desoxyanthocyanidins 6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium and 6,7,3′,4′-tetrahydroxy-5-methoxy-flavylium and the known 6,7-dihydroxy-5,4′-dimethoxy-flavylium (Carajurin) were isolated by bioguided fractionation from the leaves of *Arrabidaea chica*, with transcription factor NF-κB as target. The structure of Carajurone was revised to be 6,7,4′-trihydroxy-5-methoxy-flavylium. Additionally, the flavone acacetin was found. All structures were mainly established on the basis of MS- and NMR data (^{1}H, ^{1}H–^{1}H COSY and partly ^{13}C, GHMQCR and GHSQCR). Carajurin, which failed to give a positive result in the DPPH TLC assay completely inhibited NF-κB, but not NF-AT at a 500 μM concentration. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Arrabidaea chica*; Bignoniaceae; 3-Desoxyanthocyanidins; Flavone; Transcription factors; NF-κB; NF-AT

1. Introduction

The Bignoniaceae *Arrabidaea chica* (Bonpl.) B. Verl. (syn. *Bignonia chica* (Bonpl.)) with the common names Carajuru, Puca Panga, Chica or Pariri is a liana growing in tropical America (Borroso, 1986). Leaves of this plant are used as an anti-inflammatory and astringent agent and as a remedy for intestinal colic, sanguine diarrhoea, leucorrhoea, anaemia and leukaemia in traditional medicine (Castro da Costa and Araújo Lima, 1989). In former times Indians of the Rio Meta and the Orinoco prepared a red pigment from the leaves for tattoos (Chapman et al., 1927). It was found that the red colour was related to the 3-desoxyanthocyanidin named Carajurin (6,7-dihydroxy-5,4′-dimethoxy-flavylium) which readily formed the corresponding carajuridin chloride and stains the skin with a brilliant red shade (Ponniah and Seshadri, 1953). Up to now a further 3-desoxyanthocyanidin, not yet identified, and three flavones have been reported from the extract prepared from leaves (Chapman et al., 1927; Takemura et al., 1995). In the flowers two anthocyanin glycosides have been found in addition to Carajurin (Scogin, 1980). However, no studies for their anti-inflammatory effective compounds have been undertaken.

We here present a bioguided phytochemical study of *A. chica* leaves to gain more information about the secondary metabolites which may contribute to its anti-inflammatory activity. The transcription factor NF-κB was mainly used as a molecular target. NF-κB is a central mediator of the human immune response and regulates the transcription of genes encoding various inflammatory cytokines, chemokines, adhesion molecules and inflammatory enzymes like iNOS, COX-2, 5-LOX and cytosolic phospholipase A_2 (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). These mediators are involved in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel diseases or sepsis syndrome (Blackwell and Christman, 1997). Pharmacological inhibition of NF-κB in vivo may thus substantially modulate inflammatory processes.

2. Results and discussion

A first screening of the lipophilic and hydrophilic extract in the NF-κB EMSA showed that only the lipophilic extract completely inhibited NF-κB at a concentration of

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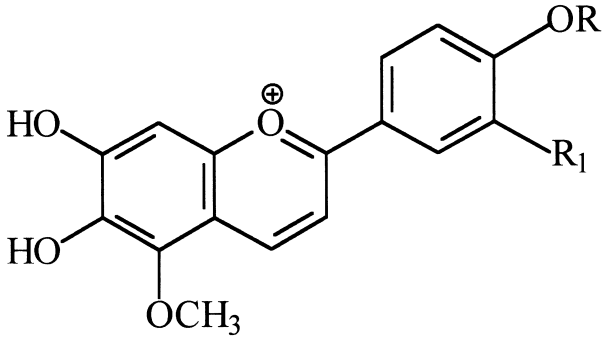
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200 μg/ml. The hydrophilic extract showed no effect up to a concentration of 500 μg/ml (data not shown). Therefore, only the lipophilic extract was investigated.

Bioguided fractionation of the methanol soluble part of the lipophilic extract using NF-κB as a molecular target led to the isolation of four 3-desoxyanthocyanidins. After TLC analysis using an acidic solvent compounds **1** and **2** gave a yellow orange colour, **3** and **4** a red one on cellulose plates without any reagent (for structures see Fig. 1).

Compound **1**, which was isolated as a red amorphous solid, is already known from *A. chica* as Carajurin (Chapman et al., 1927), but up to now only UV spectral data have been available (Scogin, 1980). To complete its spectral data MS and NMR spectra were recorded. The mass spectra in the EI, CI and ESI mode proposed the occurrence of anthocyanidin **1** as a neutral base during the measurement. ESI-MS data showed ions at *m/z* 299 and 321 of the pseudomolecular ion $[M+H]^+$ and the sodium adduct $[M+Na]^+$ respectively and corresponded to the molecular formula of $C_{17}H_{14}O_5$. CI-MS (NH_3) also yielded an ion peak at 299 $[M+H]^+$. Accordingly, the EI mass spectrum exhibited a base peak at *m/z* 298 and further ions at 283 and 255 which resulted from the loss of $[CH_3]^+$ and $[CH_3OC]^+$, respectively.

The ^{1}H NMR spectra displayed signals characteristic for 3-desoxyanthocyanidins: two 1H doublets at δ 7.37 and 8.19 ($J=8$ Hz) represented the aromatic protons H-3 and H-4 in *ortho* position; two doublets at δ 7.10 and 8.02 ($J=9$ Hz), each from two protons, indicated an AA′BB′ system and were assigned to the B-ring protons H-3′/5′ and H-2′/6′. A one proton singlet appeared at δ 6.53 and was due to H-8. Two three protons singlets at δ 3.90 and 4.05 resulted from the presence of two methoxy groups which were assigned from the GHMQCR spectrum to C-4′ and C-5, respectively. The number and characteristic shifts of the ^{13}C signals which were assigned by the GHSQCR (=gradient heteronuclear single quantum correlation reverse configuration) and GHMQCR (=gradient heteronuclear multiple quantum correlation reverse configuration) spectra confirmed the structure of Carajurin to be 6,7-dihydroxy-5,4′-dimethoxy-flavylium. However, according to mass spectral data compound **1** may be in equilibrium with the neutral quinonoidal base (Brouillard, 1988).



1: R = CH_3, R_1 = H

2: R, R_1 = H

3: R = H, R_1 = OH

4: R = CH_3, R_1 = OH

Fig. 1. Structures of the isolated anthocyanidins **1–4**.

Compound **2** showed a similar ^{1}H NMR spectrum as **1**, only the B-ring protons were slightly shifted upfield indicating a free C-4′ hydroxy group. Additionally, only one signal for a methoxy group appeared. The ESI-MS exhibited a pseudomolecular ion at *m/z* 285 as well as an ion of the sodium adduct at *m/z* 307, which was in agreement with the mass calculated for $C_{16}H_{12}O_5$. Thus, compound **2** is 6,7,4′-trihydroxy-5-methoxy-flavylium in equilibrium with the neutral base. This 3-desoxy-anthocyanidin was already isolated in microcrystalline condition and named as Carajurone. It was suggested that Carajurone differs from **1** by the occurrence of only one methoxy group (Chapman et al., 1927). Later Harborne (1967) proposed that the compound should be 5,6,7-trihydroxy-4′-methoxy-flavylium. According to our data this proposal has to be revised.

Compound **3** was identified as 6,7,3′,4′-tetrahydroxy-5-methoxy-flavylium. The mass spectra in the ESI and EI mode agreed with a molecular formula of $C_{16}H_{12}O_6$. This is in accordance with an additional hydroxy group compared to compound **2**. The chemical shifts in the ^{1}H NMR spectrum were similar to those of **2**. Only the chemical shifts for the B-ring protons differed from each other. A doublet at δ 6.91 and a two proton multiplet at δ 7.46–7.52 caused by overlapped signals indicated the presence of an *ortho* disubstituted B-ring with two hydroxyl groups.

NMR data of compound **4**, which only showed differences in the chemical shifts for the B-ring protons compared to anthocyanidin **1**, revealed a 3′-OH, 4′-OCH_3 substitution of the B-ring. In case of a 3′-OCH_3, 4′-OH-substitution the chemical shift of H-5′ should be about δ 6.91 and the signals for H-2′ and H-6′ should be found closer together (Merfort, 1985). The ESI mass spectrum was in agreement with the identity of compound **4** as 6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium.

Additionally, the known flavone acacetin (**5**) and the triterpene oleanolic acid (**6**) were isolated and identified by MS and ^{1}H and ^{13}C NMR data, which agreed well with those reported in literature (Wagner and Chari, 1976; Adesina and Reisch, 1985).

Whereas anthocyanidin **1** is known from *A. chica* leaves and flowers, compounds **2–4** are here reported for the first time. Interestingly, 3-desoxyanthocyanidins are

rarely found in plants. They occur as regular constituents in leaf and petal of members of the Gesneriaceae, subtribe Gesnerioideae (Harborne, 1967) as well as in some Bryophyta (Bendz and Martensson, 1961), Pteridophyta (Harborne, 1966) and Poaceae (Misra and Seshadri, 1967). In the Bignoniaceae they have only been found in *A. chica* (Scogin, 1980). Moreover, anthocyanidins are most often found in the 3-O-glycosylated form (Harborne, 1967). Interestingly, all isolated anthocyanidins possess a 6-hydroxylation. Previously, a 6-hydroxylated flavone was also found in *A. chica* (Takemura et al., 1995). These findings support the view of Harborne (1967) that 6-hydroxylation is a common feature of the Bignoniaceae flavonoids and contradict that of Blatt et al. (1998). They proposed that 6-hydroxylation is only characteristic for the tribe Tecomeae, but not for the Bignonieae to which the genus Arrabidaea belongs.

Anthocyanidins **2–4** were isolated with small amounts of impurities, therefore only compound **1** was studied in the NF-κB and NF-AT assay. In order to investigate whether **1** interferes with the activation of transcription factor NF-κB, Jurkat T cells were incubated with 50, 100, 200 and 500 μM concentrations of **1** for 1 h and subsequently stimulated with TNF-α for 1 h. Total protein extracts were prepared and analysed for NF-κB DNA binding activity in an electrophoretic mobility shift assay (EMSA) (see Fig. 2). Stimulation with TNF-α induced one novel DNA binding activity in Jurkat T cells (Fig. 2, lane 2). Antibody reactivity and competition assays identified this complex as an NF-κB p50/p65 heterodimer (data not shown; Brown et al., 1993; see Pahl and Baeuerle, 1995). Significant inhibition of **1** was observed at a 200 μM and complete inhibition at a 500 μM concentration.

Compound **1** shows a similar behaviour as quercetin, for which we previously obtained an inhibitory concentration of 500 μM in the NF-κB assay (Lyβ et al., 1999). It can be assumed that quercetin inhibits partly NF-κB because of its antioxidative properties (Musonda and Chipman, 1998) and because of its ability to inhibit IKK-activity (Peet and Li, 1999). Recently, it was also shown for some anthocyanins (aglycons and glycosides) that they are powerful antioxidants in vitro (Pool-Zobel et al., 1999). To elucidate, whether **1** may also inhibit the transcription factor NF-κB by its antioxidative activity compounds **1–4** were studied for their antioxidative properties by TLC analysis with DPPH (1,1-diphenyl-2-picryl-hydrazyl) as a spray reagent (Yamasaki et al., 1994). The results were not unambiguous as it cannot be excluded that these anthocyanidins are oxidized during TLC analysis and therefore fail to react with DPPH.

Preliminary data provided evidence that the anthocyanidin **1** inhibits DNA binding of NF-κB in the same way as quercetin for which an interference with IκB-α degradation could be proven by SDS page and Western-blotting with anti-IκB-α polyclonal antibody (data not shown). This effect should also be due to the inhibition of IKK-activity as already shown in the case of quercetin (Peet and Li, 1999).

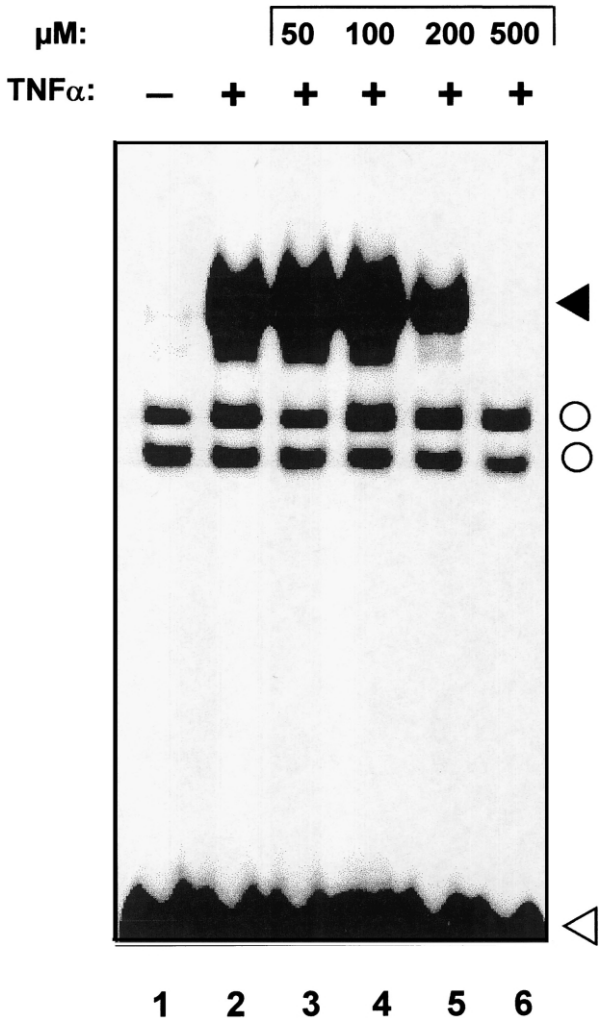


Fig. 2. The effect of anthocyanidin **1** on NF-κB DNA binding. Lane 1 shows unstimulated control cells, in lane 2 cells were treated with 200 U/ml TNF-α alone. In lanes 3–4 cells were pre-treated for 1 h with various concentrations of anthocyanidin **1** and subsequently stimulated with TNF-α for 1 h. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a non-specific activity binding to the probe. The open arrowhead shows unbound oligonucleotide.

Furthermore, it was studied whether the transcription factor NF-AT which is also involved in the regulation of various cytokines (Rao, 1994) is influenced by this anthocyanidin. The flavonol quercetin was included in this study. Jurkat T cells were incubated with **1** or with quercetin at 50, 100, 200 and 500 μM concentrations for 1 h prior to stimulation with ionomycin and PMA for 1 h. Nuclear protein extracts were prepared (Schreiber et al., 1989) and analysed for NF-AT DNA binding activity in an EMSA using an oligonucleotide comprising the distal NF-AT sequence from the IL-2 promoter (Jain et al., 1993). Stimulation with ionomycin and PMA

induced one novel DNA binding activity in Jurkat T cells, which was identified by competition assay as NF-AT (Jain et al., 1993). The NF-AT activity was influenced neither by **1** nor by quercetin at a concentration up to 500 μM (data not shown).

After comparing the inhibitory concentrations of the lipophilic extract and the pure anthocyanidin **1** (200 μg/ml and 500 μM, respectively) it is evident that these phenolic natural compounds are not solely responsible for the NF-κB inhibition by which the anti-inflammatory activity of *A. chica* preparations may be explained. Thus, other yet unknown compounds should inhibit the transcription factor NF-κB, too. Together with the 3-desoxyanthocyanidins they may contribute to the anti-inflammatory activity of leaves from *A. chica*.

3. Experimental

3.1. General experimental procedures

TLC analysis: silicagel 60 F_{254}, solvent: $CHCl_3$:MeOH (75:5, v:v) for the flavone; cellulose, solvent: HCl (conc.):HCOOH:H_2O (24.9: 23.7: 51.4, v:v:v) for the anthocyanidins (Andersen and Francis, 1985); detection: UV-light (366 nm) after spraying with 2-aminoethyl diphenyl borate and polyethylenglycol (2% in EtOH); solid phase extraction: MCI-Gel CHP 20P (Mitsubishi Kasei Corporation, Japan) or Varian Mega Bond Elut C 18 with aq. MeOH as solvent.

3.2. Plant material

Leaves of *A. chica* were collected in Siquirres, Limón region in Costa Rica in February 1998 and identified by L. Poveda, Professor of Botany, Universidad Nacional, Costa Rica. Voucher specimens (No. USJ 75132) are deposited at the Herbarium de la Universidad de Costa Rica, Escuela de Biologia.

3.3. Cell culture

Jurkat T cells were maintained in RPMI 1640 medium supplement with 10% fetal calf serum and 100 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco-BRL).

3.4. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as previously described (Lyβ et al., 1999).

3.5. Extraction and isolation

Dried leaves of the plant (102 g) were crushed and extracted with Et_2O. The solvent was removed and 2.56 g of a lipophilic extract were obtained. Additionally, extraction of the plant residue with MeOH gave 0.76 g of a hydrophilic extract. Both extracts were analysed for NF-κB inhibitory activity. The methanol soluble part of the lipophilic extract (2.07 g), which inhibited NF-κB, was separated into eleven fractions by Sephadex LH 20 using MeOH as solvent. Fractions 7, 10 and 11 showed inhibitory activity against NF-κB, therefore, further separation was performed with these fractions. Fraction 7 was chromatographed at first on Sephadex LH-20 with a mixture of cyclohexane:CH_2Cl_2:MeOH (7:4:1 v:v:v) followed by CC on silicagel with a mixture of *n*-pentane:Et_2O (1:3 v:v). Thus, 18.0 mg of compound **6** were obtained.

Fraction 10 afforded compound **2** (3.5 mg) among other subfractions by CC on Sephadex LH-20 with a mixture of C_6H_{12}:CH_2Cl_2:MeOH (7:4:1). Compound **1** (3.6 mg) was obtained by purification of subfraction 10.3 and **4** (2.1 mg) by that of 10.6 using SPE with Varian Mega Bond Elut and MeOH (70%). Fraction 11 was separated by CC on Sephadex LH-20 with a mixture of C_6H_{12}:CH_2Cl_2:MeOH (7:4:1) into 9 subfractions. CC of subfraction 11.6 on Sephadex LH-20 with MeOH followed by MPLC on RP18 with aq. MeOH (80%) gave compound **5** (5.2 mg). Subfraction 11.9 afforded compound **3** (2.5 mg) after CC on Sephadex LH-20 with MeOH and CC on MCI-Gel with aq. MeOH (70%). Compounds **2–4** were isolated with slight impurities.

3.6. 6,7-Dihydroxy-5,4′-dimethoxy-flavylium (Carajurin, *1*)

R_f: 0.65; ESI-MS *m/z* (rel. int.): 299 $[M+H]^+$ (48), 321 $[M+Na]^+$ (79), EIMS *m/z* (rel. int.): 298 $[M]^+$ (100), 283 $[M–CH_3]^+$ (22), 255 $[M\text{-}CH_3OC]^+$ (70); CI (NH_3) *m/z* (rel. int.): 299 $[M+H]^+$ (100); UV^{λ}_{max} (nm) MeOH + HCl: 488; 315; 203; ^1H NMR data see Table 1; ^{13}C NMR (75 MHz, CD_3OD): δ 161.5 (*s,* C-2), 104.5 (*d,* C-3), 136.9 (*d*, C-4), 138.0 (*s,* C-5), 151.0 (*s*, C-7), 99.6 (*d,* C-8), 158.0 (*s,* C-9), 118.6 (*s,* C-10), 124.5 (*s,* C-1′), 129.2 (*d,* C-2′ and C-6′), 115.9 (*d,* C-3′ and C-5′), 164 (*s,*

Table 1
NMR data of the isolated anthocyanidins **1–4**[a]

H	1	2	3	4
3	7.37 *d* (8)	7.33 *d* (8)	7.36 *d* (8)	7.32 *d* (9)
4	8.19 *d* (8)	8.18 *d* (8)	8.20 *d* (8)	8.19 *d* (9)
8	6.53 *s*	6.53 *s* (br)	6.55 *s* (br)	6.57 *s* (*br*)
2′	8.02 *d* (9)	7.93 *d* (8)	7.48 *d* (2)	7.46–
6′	8.02 *d* (9)	7.93 *d* (8)	7.60 *dd* (3, 9)	7.52 *m*
3′	7.10 *d*	6.94 *d* (8)		
5′	7.10 *d*	6.94 *d* (8)	7.10 *d* (9)	6.91 *d* (9)
OCH_3 at C-4′	3.90 *s*		3.20 *s*	
OCH_3 at C-5	4.05 *s*	4.05 *s*	4.05 *s*	4.05 *s*

[a] *J* (Hz) in parentheses; CD_3OD, 300 MHz; δ-values in ppm relative to TMS.

C-4′), 55.7 (*q*, OCH_3 at C-4′), 60.6 (*q*, OCH_3 at C-5); C-6 not detectable.

3.7. 6,7,4′-Trihydroxy-5-methoxy-flavylium (**2**)

R_f: 0.47; ESI-MS *m*/*z* (rel. int.): 285 $[M+H]^+$ (35), 307 $[M+Na]^+$ (65); ^1H NMR data see Table 1.

3.8. 6,7,3′,4′-Tetrahydroxy-5-methoxy-flavylium (**3**)

R_f: 0.32; ESI-MS (re. int.): 301 $[M+H]^+$ (34), 323 $[M+Na]^+$ (100), EIMS *m*/*z* (rel. int.): 300 $[M]^+$(100), 284 (60), 257 (65); ^1H NMR data see Table 1.

3.9. 6,7,3′-Trihydroxy-5,4′-dimethoxy-flavylium (**4**)

R_f: 0.35; ESI-MS *m*/*z* (rel. int.): 315 $[M+H]^+$ (100); ^1H NMR data see Table 1.

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Flavonoids from the stem bark of *Bolusanthus speciosus*

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Abstract

Three flavonoids (bolusanthols A–C), viz an isoflavan and two prenylated isoflavanones, were isolated from the stem bark of *Bolusanthus speciosus* in addition to four known flavonoids, 5,7,3′-trihydroxy-4′-methoxy-5′-γ,γ-dimethylallylisoflavanone, 5,7,2′-trihydroxy -4′-methoxy-6,5′-di(γ,γ-dimethylallyl)isoflavanone, 3,5,7,3′,4′-pentahydroxy-6-γ,γ-dimethylallylflavone and 5,7,2′,4′-tetrahydroxy-8,3′-di(γ,γ-dimethylallyl)-isoflavanone. The structure of the new compounds were determined to be 4,2′,3′,4′-tetrahydroxy-6,7-methylenedioxyisoflavan (bolusanthol A), 5,7,3′,4′-tetrahydroxy-5′-γ,γ-dimethylallylisoflavanone (bolusanthol B), and 5,7,4′-trihydroxy-6,3′-di(γ,γ-dimethylallyl)isoflavanone (bolusanthol C) by spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Bolusanthus speciosus*; Fabaceae; Isoflavan; Isoflavanones; Bolusanthols A–C

1. Introduction

Bolusanthus speciosus Harms (Fabaceae), commonly called Tree Wisteria, is a tree that grows up to 7 m tall (Van-Wyk and Van-Wyk,1997). It occurs in bushveld, often in heavy alkaline soils, it flowers in loose hanging racemes which are pale blue to violet and has flat pods (Van-Wyk, 1998). The tree extends from South Africa to Botswana, Zimbabwe, Mozambique and Zambia. It is used as an ornamental tree and the dried inner bark is used to relieve abdominal pains, emetism and tuberculosis (Palgrave, 1984). Previous work on the seeds of *B. speciosus* revealed the presence of isoflavonoids (Ascres et al., 1985). From methanolic extracts of the stem bark of *B. speciosus*, three new isoflavonoids were isolated and characterization of these compounds is presented below.

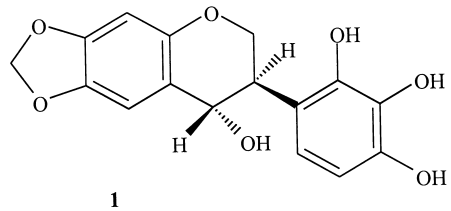
2. Results and discussion

The methanolic extract from the stem bark of *B. speciosus* was worked up as described in the experimental section to give four known compounds and three new compounds, which were identified using spectroscopic methods.

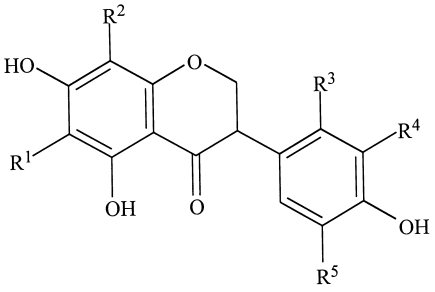
Compound **1** was isolated from a methanolic stem extract of *B. speciosus* as a white solid, mp 112–114°C, gave a HR EIMS ion at *m/z* 300.0653 corresponding to formula $C_{16}H_{12}O_6$ [M-H_2O]. In the low resolution EIMS the highest ion was also observed at *m/z* 300 which formed the base peak. This loss of 18 mass units from the molecular ion $(C_{16}H_{14}O_7)^+$ is due to easy dehydration of the molecule under EIMS conditions (Porter and Baldas, 1985). The ^{1}H NMR spectrum (Table 1) showed a pair of one-proton doublet of doublets at δ 3.74 (J = 11.0, 10.8 Hz), 4.32 (5.0, 10.8), a doublet of double doublets at δ 3.52 (5.0, 7.0, 11.0) and a doublet at δ 5.52 (7.0). These signals were assignable to two H-2 protons, H-3 and H-4 protons respectively of a 4-hydroxyisoflavan skeleton. The corresponding carbons were identified by HMQC as, a methylene carbon at δ 67.4 and two methine carbon atoms at δ 40.7 and 78.7 respectively. In the ^{1}H NMR further signals were observed which showed the presence of *ortho*-coupled aromatic doublets (δ 6.70 and 7.00, J = 8.5 Hz each), a methylenedioxy group (δ 5.93, 2H, *d*, J = 7.8), and a set of one-proton aromatic singlets (δ 6.74 and 6.45). The presence of a methylenedioxy unit in A-ring was confirmed by a significant mass fragment at *m/z* 167 consistent with a *retro*-Diels–Alder fragmentation (Mabry and Markham, 1975). The methylenedioxy protons (δ 5.93) showed HMBC correlations with C-6 (δ 142.2) and C-7 (δ 148.6) while H-5 (δ 6.45) showed correlations with C-6, C-7, C-4 (δ 78.7), C-9 (δ 154.7) and C-10 (δ 117.9). The H-8 proton (δ 6.74)

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1



2 R^1 = H, R^2 = H, R^3 = H, R^4 = OH, R^5 = Prenyl

3 R^1 = Prenyl, R^2 = H, R^3 = H, R^4 = Prenyl, R^5 = H

4 R^1 = H, R^2 = Prenyl, R^3 = OH, R^4 = Prenyl, R^5 = H

showed HMBC correlations with C-6, C-7, C-9 and C-10. Consequently the A-ring was shown to have a 6,7 methylenedioxy substitution. The H-4 proton (δ 5.52) displayed key HMBC correlations which showed connectivity with C-5 (δ 105.0) and C-9 [correlations with A-ring] and C-1′(δ 112.1) [correlation with B-ring]. The H-2 protons (δ 3.74 and 4.32) showed correlations with C-4, C-9 and C-1′ further confirming linkage of the three rings. The H-6′ (δ 7.00) showed HMBC correlations with C-2′ (δ 143.5), C-4′ (δ 144.8) and C-3, while H-5′ (δ 6.70) showed correlations with C-1′, C-3′ (δ 131.9) and C-4′. Thus the substitution in the B-ring was determined to be 2′,3′,4′-trihydroxy and consequently structure **1** was determined to be 4,2′,3′,4′-tetrahydroxy-6,7-methylenedioxyisoflavan, named bolusanthol **A**. The problem that remains unresolved is the absolute stereochemistry at C-3 and C-4. What was determined from our data is that the two H's at C-3 and C-4 show *trans* relationship and is only on that basis that the structure is drawn as shown.

Compound **2** was obtained as a brown paste and showed the molecular ion peak at m/z 356.1259 consistent with the molecular formula $C_{20}H_{20}O_6$. In the ^{1}H NMR spectrum two one-proton double doublets were observed at δ 4.52 (J = 6.3, 11.4 Hz) and 4.53 (J = 4.7, 11.4 Hz) and a one-proton triplet at 3.76 (J = 6.3 Hz) and these were assignable to protons at C-2 and C-3 in a 3′,4′ oxygenated isoflavanone. The spectrum also showed the presence of a γ,γ-dimethylallyl group [δ 1. 68 (6H, *s*, Me x2), 3.30 (2H *d*, J = 7.2 Hz, CH_2), 5.31(1H, *t*, J = 5.7)], a set of *meta*-substituted aromatic protons at δ 5.93 and 5.94 (1H each, *d*, J = 2.1), 6.62 and 6.67 (1H each, *d*, J = 2.0) and a chelated hydroxy at δ 12.31 (1H, *s*). The EIMS exhibited mass fragments at m/z 153 and 204 resulting from a *retro*-Diels–Alder fragmentation (Mabry and Markham, 1975), indicating that the γ,γ-dimethylallyl substituent was not in ring A but in ring B. The position

Table 1
^{1}H NMR data for compounds **1**, **2**, **3** and **4** in acetone-d_6[a]

Position	1	2	3	4
2a (1H)	3.74, *dd*, (11.0, 10.8)	4.52, *dd*, (6.3,11.4)	4.53, *dd*, (8.0, 11.4)	4.62, *dd*, (11.8, 9.0)
b (1H)	4.32, *dd*, (5.0, 10.8)	4.53, *dd*, (4.7, 11.4)	4.55, *dd*, (5.4, 11.4)	4.79, *dd*, (11.8, 4.4)
3 (1H)	3.52, *ddd*, (5.0,7.0, 11.0)	3.76, *t*, (6.3)	3.88, *dd*, (8.0, 5.4)	3.89, *dd*, (9.0, 4.5)
4 (1H)	5.52, *d*, (7.0)			
5 (1H)	6.74, *s*			
6 (1H)		5.93, *d*, (2.1)		5.89, *s*
8 (1H)	6.45, *s*	5.94, *d*, (2.1)	5.97, *s*	
2′ (1H)		6.67, *d*, (2.0)	7.01, *d*, (2.2)	
5′ (1H)	6.70, *d*, (8.5)		6.77, *d*, (8.9)	6.32, *d*, (8.5)
6′ (1H)	7.00, *d*, (8.5)	6.62, *d*, (2.0)	7.02, *dd*, (2.2, 8.9)	7.10, *d*, (8.5)
-OCH_2O-	5.93, *d*, (7.8)			
5-OH		12.31, *s*	12.50, *s*	11.3, *s*
2′-OH				7.71, *s*
4′-OH				5.30, *s*
1″ (2H)		3.30, *d*, (7.2)	3.50, *d*, (7.1)	3.23, *d*, (7.1)
2″ (1H)		5.31, *t*, (7.2)	5.29, *t*, (7.1)	5.14, *t*, (7.1)
4″ (3H)		1.68, *s*	1.83, *s*	1.74, *s*
5″ (3H)		1.68, *s*	1.77, *s*	1.67, *s*
1‴ (2H)			3.35, *d*, (7.1)	3.38, *d*, (7.1)
2‴ (1H)			5.29, *t*, (7.1)	5.14, *t*, (7.1)
4‴ (3H)			1.83, *s*	1.74, *s*
5‴ (3H)			1.77, *s*	1.67, *s*

[a] Assignment were confirmed by COSY, HMQC, HMBC and DEPT. J values given in parentheses in Hz.

of γ,γ-dimethylallyl group was confirmed by HMBC which showed correlations between a vinylic proton H-2″ (δ 5.31) of the γ,γ-dimethylallyl substituent with C-5′ (δ 128.6). Furthermore the H-1″ methylene protons (δ 3.30) of the γ,γ-dimethylallyl substituent showed correlations with C-5′, C-4′ (δ 142.9) and C-6′ (δ 121.1). The appearance of *meta*-coupled aromatic doublets at δ 6.62 and 6.67 indicated that ring B had a 3′,4′,5′-substitution and the chemical shifts of C-3′, C-4′ and C-5′ confirmed a 3′,4′-dihydroxy-5′-γ,γ-dimethylallyl substitution. Other salient HMBC correlations involved those between the H-2 protons (δ 4.52 and 4.53) and C-3 (δ 50.6), C-4 (δ 196.9), C-9 (δ 163.6), and C-1′ (δ 127.2); between H-3 (δ 3.76) and C-1′, C-2′ (δ 113.3), C-6 and C-10 (δ 102.3); between H-2′ (δ 6.67) and C-1′, C-6′, C-3′ (δ 144.9),and C-4′; and between H-6′ (δ 6.62) and C-3, C-4′ and C-5′. Compound **2** was thus identified as 5,7,3′,4′-tetrahydroxy-5′-γ, γ-dimethylallylisoflavanone, named bolusanthol B. The 4′-methoxy derivative of **2,** viz 5,7,3′-trihydroxy-4′-methoxy-5′–γ,γ-dimethylallyl isoflavanone, **5**, was earlier reported by Maillard et al. (1989).

Compound **3**, mp 130–132°C was isolated as a white solid and gave HREIMS molecular ion at *m*/*z* 408.1926 consistent with molecular formula $C_{25}H_{28}O_5$. In the ^{1}H NMR spectrum a set of one-proton double doublets were observed at δ 4.53 and 4.55 and another one-proton double doublet was observed at δ 3.88 and these signals were assignable to protons attached to C-2 and C-3 respectively in a 4′-oxygenated isoflavanone. The ^{1}H NMR spectrum further showed the presence of two γ,γ-dimethylallyl units [δ 1.77 (6H *s*, Me×2),1.83 (6H *s*, Me×2), 3.35, 3.50 (2H each, *d*, *J*=7.1 Hz, CH_2×2), 5.29 (2H, *t*, *J*=7.1 Hz, CH= ×2)], a single aromatic proton at δ 5.97, an ABM system [δ 7.01 (1H *d*, *J*=2.2 Hz), 7.02 (1H *dd*, *J*=2.2, 8.9), 6.77 (1H *d*, *J*=8.9)] and a chelated hydroxy at δ 12.50. A one-proton singlet at δ 5.97 and a mass fragment at *m*/*z* 221$[A_{1+}H]^+$ confirmed that ring A had two hydroxy groups and a γ,γ-dimethylallyl unit. The γ,γ-dimethylallyl unit could either be at C-6 or C-8. From our experience and that of others, O'neill et al. (1986) and Shirataki et al (1999), the presence of a prenyl unit at either positions C-6 and C-8 of an isoflavanone unit influences the chemical shift of the chelated hydroxy (C-5 hydroxy) in a predictable manner. So far we have noted that when the prenyl unit is at C-8, the chemical shift of the chelated hydroxy is less than δ 12.40, while a value of more than δ 12.40 indicates that the prenyl unit is at position C-6. The chemical shift of the chelated hydroxy (δ 12.50), in **3**, was slightly downfield suggesting the location of the γ,γ-dimethylallyl group at C-6 position. This was confirmed by HMBC correlations between the chelated hydroxy and C-10 (δ 103.3), C-6 (δ 107.4), C-5 (δ 162.1), C-4 (δ 197.4). The carbon signal at δ 107.4 was further correlated to the methylene signal at δ 3.35 assignable to the H-1 of the γ,γ-dimethylallyl group, and also to a methine signal at δ 5.29 assignable to the H-2 of the γ,γ-dimethylallyl group. Hence the A-ring substitution is 5,7-dihydroxy- 6-γ,γ-dimethylallyl. The observed ABM pattern in the ^{1}H NMR spectrum and a mass fragment at *m*/*z* 188 showed that the other γ,γ-dimethylallyl group was in ring B, which showed a 3′, 4′- substitution pattern. This substitution pattern was confirmed by HMBC correlations, the key interactions being those between the methylene protons at δ 3.50 (H-1 of the B-ring γ,γ-dimethylallyl group) and C-2′, (δ 130.6), C-3′ (δ 127.8), and C-4′ (δ 154.5). The two H-2 protons of the two prenyl units both resonate at δ 5.29. However, only one of these showed HMBC correlation with C-3′. This further confirms the presence of one of the prenyl units in the B-ring. Consequently the substitution in B-ring was 4′-hydroxy-3′-γ,γ-dimethylallyl and thus the structure of **3** was established as 5,7,4′-trihydroxy-6,3′-di(γ,γ-dimethylallyl)isoflavanone, named bolusanthol C.

Compound **4** was isolated as a yellow paste and gave a HREIMS molecular ion at *m*/z 424.1875, consistent with molecular formula $C_{25}H_{28}O_6$. The ^{1}H NMR of **4** showed three one-proton double doublets at δ 3.89 (*J*=4.5, 9.0 Hz), 4.62 (9.0, 11.8 Hz) and 4.79 (4.5, 11.8). The latter two signals were correlated with a carbon at δ 70.1 tentatively identified as C-2 in the HMQC spectrum. The proton signals were assigned to hydrogens at C-2, which suggested **4** was also an isoflavanone. The ^{1}H NMR spectrum also showed, like **3**, the presence of two γ,γ-dimethylallyl groups [δ 1.67 (6H *s*, Me×2), 1.72 (6H, *s*, Me×2), 3.23, 3.28 (2H each, *d*, *J*=7.1 Hz, CH_2×2), 5.14 (2H, *t*, *J*=7.1 Hz CH= ×2)], a single shielded aromatic proton at δ 5.89, a set of *ortho*-coupled aromatic doublets [δ 6.32, 7.10 (1H each, *d*, *J*=8.5 Hz) and a chelated hydroxy at δ 11.3. A one-proton singlet at δ 5.89 together with a mass fragment at *m*/*z* 221$[A_{1+}H]^+$ indicated the presence of a γ,γ-dimethylallyl and two hydroxy groups as substituents in ring A. The A-ring thus had a 5,7-dihydroxy-8 (or 6)γ,γ-dimethylallyl substitution. The appearance of the chelated hydroxy group at C-5 at higher field (δ 11.3) suggested the position of the γ,γ-dimethylallyl group on the A-ring was at C-8, which was supported by HMBC correlations. That is, unlike in **3**, the chelated hydroxy did not show any HMBC correlations with the quaternary carbon at δ 106.9. However the one-proton singlet at δ 5.89 showed HMBC correlations to C-5 (δ 163.4), C-7 (δ 165.0), C-8 (δ 106.9) and C-10 (δ 102.2). The methylene proton assignable to H-1 (δ 3.23) of the A-ring γ,γ-dimethylallyl substituent showed HMBC correlation with C-7, C-8, and C-9 (160.1), while the methine protons (δ 4.53) assignable to H-2 of the same unit showed a correlation with C-8. Thus A-ring has 5,7-dihydroxy-8-γ,γ-dimethylallyl substitution. The appearance of aromatic *ortho*-coupled protons at δ 6.32 and 7.10 indicated that B- ring moiety had substituents at 2′,3′ and 4′ positions. The substitution could not be 2′, 3′-dihydroxy or 3′, 4′-dihydroxy because in *ortho*-dihydroxy

substitution this would lead to mutual shielding of those positions in the ^{13}C NMR spectrum, an effect which brings both hydroxylated aromatic carbons to resonate below δ 150 (Agrawal, 1989). In compound **4** however, these two oxygen bearing aromatic carbons signals were observed at δ 154.5 and 155.6. The substitution should therefore be 2′,4′-dihydroxy thus placing the γ,γ-dimethylallyl group at 3′ position. This is further confirmed by the methylene protons (δ 3.38) of the H-1 of the B-ring γ,γ-dimethylallyl substituent which exhibited an HMBC correlation three quaternary carbons at δ 154.5 (C-2′), 116.1 (C-3′) and 155.6 (C-4′). Furthermore the methine proton (δ 5.14) of the H-2 of the B-ring γ,γ-dimethylallyl group showed HMBC correlation with C-3′. The above information enable the identification of structure **4** as 5,7,2′,4′-tetrahydroxy-8,3′-di(γ,γ-dimethylallyl)isoflavanone named 3′-(γ,γ-dimethylallyl)-kievitone which was isolated by O'Neill et al (1986). The 2′-methoxy derivative of **4,** viz 5,7,4′-trihydroxy-2′-methoxy-8,3′-di (γ,γ-dimethylallyl)isoflavanone (phyllanone) was reported by Fukai et al. (1994).

Compound **6** was identified as 5,7,2′-trihydroxy-4′-methoxy-6, 5′-di(γ,γ-dimethylallyl)isoflavanone (sophraisoflavanone B), and had earlier been isolated from *Sophora franchetiana*, by Komatsu et al.(1981) and **7** was identified as 3,5,7,3′,4′-pentahydroxy-6-γ,γ-dimethylallylflavone which had earlier been isolated from *Glycyrrhiza uralensis* by Fukai et al. (1990).

3. Experimental

3.1. General experimental procedures

Melting point: Stuart Scientific (SMP1) melting point apparatus; Specific rotation $[\alpha]_D$: Polatronic-D (Schimdt + Haensch) polarimeter; UV: Shimadzu UV-2101PC spectrophotometer; IR: Perkin Elmer 2000 FT-IR spectrometer. The 1D {^1H (300 MHz), ^{13}C (75.5 MHz), DEPT} and 2D (COSY, HMQC, HMBC) spectra acquired on Bruker Avance DPX 300 spectrometer and referenced to residual solvent signal. HMBC experiments were run using the standard pulse sequence. MS: HREIMS was done on autospec TOF spectrometer. The Autospec was run using an EI ion source. EI on Finnigan MAT SSQ 700 single quadrupole instrument. HPLC: Shimadzu LC-6AD, waters RI 401 detector, Techopak10C18 normal phase. Column chromatography-silica gel 60 particle size 0.040–0.063 mm for column chromatography (Merck); vacuum liquid chromatography- silica gel HF_{254} 5–15 μm mesh (Merck); Sephadex LH-20 (Sigma); preparative TLC-silica gel 60 $PF_{254+366}$ for preparative layer chromatography (Merck); analytical TLC-TLC silica gel 60-F_{254} precoated alumina sheets (Merck), and visualized using UV (254 and 366 nm) and vanillin-sulphuric acid spray.

3.2. Plant material

The stem bark of *B. speciosus* was collected from Mapoka, North East District, Botswana in August 1997, identified by Dr. L.M. Turton and a voucher specimen (B 0897) was deposited at the University of Botswana Herbarium.

3.3. Extraction and isolation

Air-dried and pulverized stem bark (1.5 kg) was extracted sequentially in MeOH–$CHCl_3$ and aqueous MeOH. Removal of solvent from the combined methanolic extracts gave a brown residue (150 g), which was subjected to vacuum liquid chromatography by elution with hexane, CH_2Cl_2, and MeOH mixture of increasing polarity. The flavonoid constituents were eluted in CH_2Cl_2–MeOH fractions. The combined fractions were applied to a Sephadex LH-20 column (eluted with 1:1 $CHCl_3$–MeOH) to give three sets of fractions A, B and C. The concentrated eluent A was resolved by multiple development on preparative TLC with toluene–EtOAc–HOAc (35:14:1) to afford 4,2′,3′,4′-tetrahydroxy-6,7-methylenedioxyisoflavan **1** (30 mg) and 5,7,2′,4′-tetrahydroxy-8,3′-di(γ,γ-dimethylallyl)isoflavanone **4** (15 mg). Multiple development of concentrated eluent B on preparative TLC with $CHCl_3$-EtOAc (6:1) gave 5,7,3′,4′-tetrahydroxy-5′-γ,γ-dimethylallylisoflavanone **2** (400 mg), 5,7,4′-trihydroxy-6,3′-di(γ,γ-dimethylallyl)lisoflavanone **3** (100 mg) and 5,7,2′-trihydroxy-4′-methoxy-6,5′-di(γ,γ-dimethylallyl)isoflavanone **6** (60 mg). Concentrated fraction C was separated on HPLC (running conditions for HPLC: mobile phase, Hexane–EtOAc 80:20, injection volume, 200 μl, flow rate 2 ml/min) to yield 5,7,3′-trihydroxy-4′-methoxy-5′-γ,γ-dimethylallylisoflavanone **5** (10 mg), and 3,5,7,3′,4′-pentahydroxy-6-γ,γ-dimethylallylflavone **7** (15 mg).

3.4. 2′,3′-Tetrahydroxy-6,7-methylenedioxyisoflavan (*1*)

White solid; mp 112–114°C; $[\alpha]_D^{25}$ +26.7° (MeOH, *c* 0.01); UV λ_{max} MeOH nm (logε) 308 (4.43), 210 (5.08), +NaOMe 344, 309, 211, +$AlCl_3$ 308, 231, +$AlCl_3$/HCl 308, 210, +NaOAc 342, 309, 212, +NaOAc/H_3BO_3 308, 220; IR (KBr) $\nu_{max}cm^{-1}$, 3408, 2920, 1641, 1600; ^1H and ^{13}C NMR in aceton-d_6 (see Tables 1 and 2 respectively); EIMS *m/z* 300 $[M\text{-}H_2O]^+$ (100), 175(10), 167 (50), 162 (25), HRTOFMS *m/z* 300.0617 (calc. for $C_{16}H_{12}O_6$, 300.0634).

3.5. 5,7,3′,4′-Tetrahydroxy-5′-γ,γ-dimethylallylisoflavanone (*2*)

Brown paste; $[\alpha]_D^{25}$ +10.0° (MeOH, *c* 0.015); UV λ_{max} MeOH nm (log ε) 288 (4.06), 204 (4.51), +NaOMe 323, 206, +$AlCl_3$ 294, 208, +$AlCl_3$/HCl 307, 203, +NaOAc

Table 2
^{13}C NMR data for compounds **1**, **2**, **3** and **4** in acetone-d_6[a]

Position	1	2	3	4
2	67.4	71.7	71.8	70.1
3	40.7	50.6	51.0	45.4
4	78.7	196.9	197.4	197.7
5	105.0	165.2	162.1	163.3
6	142.2	95.4	107.4	97.1
7	148.6	168.1	164.2	165.0
8	94.3	96.7	95.6	106.9
9	154.7	163.6	161.5	160.1
10	117.9	102.3	103.3	102.2
1′	112.1	127.2	127.4	115.6
2′	143.5	113.3	130.6	154.5
3′	131.9	144.9	127.8	116.1
4′	144.8	142.9	154.5	155.6
5′	110.0	128.6	116.6	108.9
6′	122.1	121.1	127.9	125.8
-OCH_2O-	101.7			
1″		28.5	21.6	22.0
2″		123.1	121.8	121.9[b]
3″		131.9	135.9	135.2
4″		17.3	18.3	18.3
5″		25.4	26.2	26.2
1‴			30.2	23.2
2‴			121.8	122.0[b]
3‴			135.5	135.6
4‴			18.3	18.3
5‴			26.2	26.2

[a] Assignment were confirmed by HMQC, HMBC and DEPT.
[b] Can be interchanged within same column.

325, 209, NaOAc/H_3BO_3 289, 211; IR (KBr) $\nu_{max}cm^{-1}$, 3448, 2920, 1640, 1600 cm^{-1}; ^1H and ^{13}C NMR in aceton-d_6 (see Tables 1 and 2 respectively); EIMS *m/z* 356 $[M]^+$ (80), 301(10) 204 (90), 153 (100), 149 (70), 148 (85); HRTOFMS *m/z* 356.1259 (calc. for $C_{20}H_{20}O_6$, 356.1260).

3.6. 5,7,4′-Trihydroxy-6,3′,-di(γ, γ-dimethylallyl) isoflavanone (*3*)

White solid; mp 130-132°C; $[\alpha]_D^{25}$ −10.0° (MeOH, *c* 0.020); UV λ_{max} MeOH (log ε) 294 (4.29), 201 (4.65), +NaOMe 330, 205, +$AlCl_3$ 294, 203, +$AlCl_3$/HCl 294, 203, +NaOAc 331, 215, +NaOAc/H_3BO_3 296, 221; IR (KBr) ν_{max} cm^{-1}, 3417, 2922, 1638, 1600; ^1H and ^{13}C NMR in aceton-d_6 (see Tables 1 and 2 respectively); EIMS *m/z* 408 $[M]^+$ (80), 353 (15), 339 (90), 221 (100), 188 (30); 165 (80), 133 (25), 69 (40) HRTOFMS *m/z* 408.1926 (calc. for $C_{25}H_{28}O_5$, 408.1937).

3.7. 5,7,2′,4′-Tetrahydroxy-8,3′-di(γ, γ-dimethylallyl) isoflavanone (*4*)

Yellow paste; $[\alpha]_D^{25}$ −20.3° (MeOH, *c* 0.010); UV λ_{max} MeOH nm, (log ε) 293 (4.15), 213 (5.30), +NaOMe 333, 214, +$AlCl_3$ 312, 218, +$AlCl_3$/HCl 312, 213, +NaOAc 335, 301, 216, +NaOAc/H_3BO_3 294, 219; IR (KBr) ν_{max} cm^{-1}, 3415, 2917, 1638, 1600; ^1H and ^{13}C NMR in aceton-d_6 (see Tables 1 and 2 respectively); EIMS *m/z* 424 $[M]^+$ (71),369 (40), 221 (89), 204 (30), 165 (100), 149 (370, 135 (14) 69 (40). HRTOFMS *m/z* 424.1875 (calc. $C_{25}H_{28}O_6$, 424.1886).

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Identification of a sucrose diester of a substituted β-truxinic acid in oats

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Abstract

A novel compound, 4,4′-dihydroxy-3,3′-dimethoxy-β-truxinic acid esterified to sucrose through the fructosyl 3-and 6-carbons (**1**), was isolated from oat grains (*Avena sativa* L.). Its structure was determined by a combination of mass spectrometry and 1-D and 2-D NMR. The amounts of **1** in groats of six different oat cultivars ranged from 101 to 150 μg g^{-1} (dry wt). None was detected in the hulls. The free diacid, 4,4′-dihydroxy-3,3′-dimethoxy-β-truxinic acid (**2**), could not be detected in groats nor in hulls. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Avena sativa*; Cyclobutane; Diferulic acid; Lignan; MS; NMR

1. Introduction

Phenolic compounds are the largest and the most widely distributed category of phytochemicals in the plant kingdom. Hydroxycinnamates play a very important role in the life of plants in that they are principal components governing cell wall structure, integrity and shape, and are involved in defense against invading pathogens (for a review, see Faulds and Williamson, 1999).

The types of hydroxycinnamic acid derivatives in plants most frequently reported are those having ester or amide linkages. Among cereals, for instance, rice and maize bran oils contain several steryl ferulates (Norton, 1994; Xu and Godber, 1999). Oat groats contain a series of hydroxy-*n*-alkanes acylated with caffeic and/or ferulic acids (Daniels and Martin, 1967; 1968) and several *N*-hydroxycinnamoyl anthranilates, collectively known as the avenanthramides (Collins, 1989; Collins and Mullin, 1988; Dimberg et al., 1993; 1996).

There are few occurrences of glycosides of hydroxycinnamic acids. Glycosides containing monosaccharides are most frequently encountered. Esters with the disaccharide sucrose have been found in a few plant species, especially in the Liliáceæ family (Bäumker et al., 1988; Harrison et al., 1995; Sashida et al., 1991; Strack et al., 1981).

In the grasses, ferulic and *p*-coumaric acids are ubiquitous components of the cell walls, and are believed to cause cross-linking of the polysaccharide chains (for a review, see Ishii, 1997). Part of the hydroxycinnamic acids are present as dimers, both dehydrodimers and cyclodimers. This has been revealed by isolation of dimers of *p*-coumaric and ferulic acids in extracts of saponified or mild acid hydrolysed cell walls (Hartley et al., 1990; Ralph et al., 1994; Saulnier et al., 1999). The major dehydrodimers identified are 8-5′, 8-8′, 8-*O*-4′, and 5-5′ di-cinnamic acids (Ralph et al., 1994; Saulnier et al., 1999). The cyclodimers identified are 4,4′-dihydroxy-α-truxillic acid, 4,4′-dihydroxy-3-methoxy-α-truxillic acid and 4,4′-dihydroxy-3,3′-dimethoxy-α-truxillic acid (Hartley et al., 1990). The cyclodimers that are reported in literature have all been found *after* subjecting the cell walls to hydrolysis. To the best of our knowledge no hydroxycinnamic cyclodimers conjugated to sucrose have been reported previously.

This paper reports the isolation and identification of a sucrose diester of a substituted truxinic acid from oat grains, without prior hydrolysis of cell walls.

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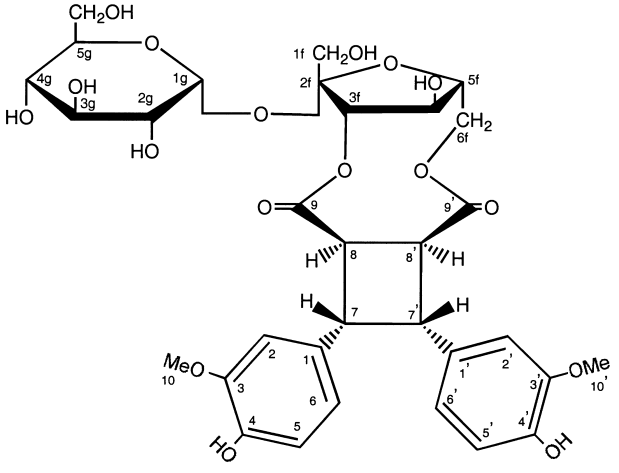
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2. Results and discussion

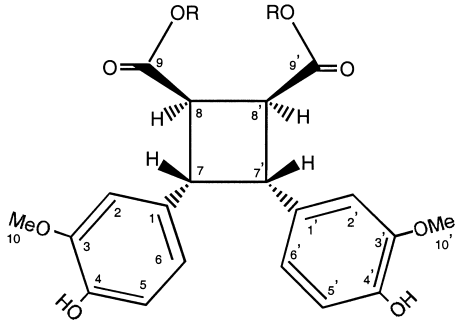
Solid phase extraction and preparative HPLC of a methanol extract from oat bran oil yielded compound **1** as an off-white solid having a UV absorption characteristic of lignans (λ_{max} (MeOH) nm: 208 and 283). The compound was freely soluble in water and methanol.

The high resolution FABMS (positive) mass spectrum gave an $M+H^+$ at m/z 695.2244 corresponding to a possible molecular formula of $C_{32}H_{38}O_{17}$.

In the ^1H NMR (in CD_3OD) spectrum, two methoxyl groups at δ_H 3.62 and 3.64 ppm were observed along with six aromatic protons (δ_H ca 6.5 ppm) that had a pattern characteristic of a dual 3,4-disubstituted phenyl ring system. There was a group of carbohydrate protons in the region δ_H 3.0–5.5 ppm and one anomeric doublet at δ_H 5.46 ppm with $^3J = 3.6$ Hz, a possible indication of an α-glucosyl unit.



Compound **1**



Compound **2** : R=H
Compound **3** : R=Me

The ^{13}C NMR revealed all the 32 carbons and the multiplicity of carbons was obtained using DEPT and HMBC. The four signals on the downfield side of the aromatic region δ_C 146–149 ppm suggested that four carbons bearing oxygen were present in the di-substituted phenyl rings. The carbohydrate signals at δ_C 63–110 ppm included an anomeric for an α-glucosyl unit (δ_C 94.5 ppm) and a quaternary carbon signal at δ_C 109.8 ppm that showed a three-bond ^{13}C–^1H correlation across to the anomeric proton (H-1g) in the HMBC spectrum, suggesting fructose as part of a disaccharide. The two downfield C=O signals at δ_C 173.1 and 175.5 ppm had correlations with H-3f and H-6f, respectively, of the fructose unit and hence a diester was indicated. HMBC data together with COSY data (not shown) for the four remaining methine carbons (signals at δ_C ca 45 ppm) proved the presence of a cyclobutane ring, and further, distinguished the corresponding shifts of the aromatic protons and carbons as summerized in Table 1.

High resolution FAB (negative) mass spectrum of the ether extract of a hydrolysis mixture, containing compound **2** (see Experimental), gave an $M-H^-$ ion at m/z 387.1118 corresponding to a molecular formula $C_{20}H_{20}O_8$.

Table 1
^1H, ^{13}C NMR and HMBC correlation data of **1** in CD_3OD

Position[a]	^{13}C Shift δ_C	^1H Shift δ_H (no. of H, multi.)	HMBC correlations via $^{2,3}J_{CH}$
1	131.7	–	H-5; H-8
2	113.4	6.45 (1H, *d*)	H-6; H-7
3	148.6	–	H-2; H-5; H-10
4	146.1	–	H-2; H-6
5	115.7	6.60 (1H, *d*)	
6	121.9	6.55 (1H, *d*)	H-2; H-7
7	44.7	4.09 (1H, *dd*)	H-2; H-6; H-8; H-7′ and/or H8′.
8	46.4	3.80 (1H, *m*)	H-7; H-7′ and/or H-8′
9	173.1	–	H-7; H-8; H-8′; H-3f
10	56.5	3.64 (3H, *s*)	
1′	132.0	–	H-5′.
2′	113.2	6.42 (1H, *d*)	H-6′; H7′
3′	148.5	–	H-10′; H-5′
4′	145.9	–	H-2′; H-6′
5′	115.5	6.59 (1H, *d*)	
6′	121.7	6.54 (1H, *dd*)	H-2′; H-7′
7′	45.4	4.37 (1H, *m*)	H-2′; H-6′; H-8′
8′	45.0	4.37 (1H, *m*)	H-7′
9′	175.5	–	H-7′ and/or H-8′; H-8; H-6f
10′	56.4	3.62 (3H, *s*)	
1g	94.5	5.46 (1H, *d*)	
2g	73.7	3.50 (1H, *m*)	H-3g
3g	75.4	3.77 (1H, *m*)	H-1g; H-2g
4g	71.4	3.48 (1H, *m*)	H-3g; H-6g
5g	74.1	4.20 (1H, *m*)	H-1g; H-4g
6g	62.8	3.90 (1H, *m*)	H-4g
		3.82 (1H, *m*)	
1f	63.7	3.81 (2H, *s*)	
2f	109.8	–	H-1g; H-1f; H-3f; H-4f; H-5f
3f	79.6	4.90 (1H, *s*)	H-1f; H-4f
4f	75.6	4.41 (1H, *s*)	H-3f; H-6f
5f	89.3	4.18 (1H, *m*)	H-3f; H-4f; H-6f
6f	65.4	4.15 (1H, *dd*)	H-4f; H-5f
		5.01 (1H, *d*)	

[a] Numbering of carbons as in the figure.

The EI mass spectrum exhibited a fragmentation pattern that confirmed the diester nature of **1**. The ions at *m*/*z* 272 and 194, formed by asymmetrical splittings of the cyclobutane ring (Hartley et al., 1988), indicated that the two 4-hydroxy-3-methoxyphenyl groups were vicinal. Methanolysis yielded a dimethyl ester (compound **3**) that behaved in a similar way in EIMS giving the corresponding asymmetrical cleavage ions (*m*/*z* 272 and 208) providing a further verification of vicinal aromatics. Hence it was indicated that a head-to head dimer of ferulic acid was present, i.e. a truxinic rather than a truxillic type of cyclobutane diacid. The aqueous layer (see Experimental) was dried and derivatized into TMSi ethers and analyzed by GC–MS. A mass spectrum and retention time comparable with that of an authentic sucrose TMSi derivative was obtained.

The stereochemistry of the truxinic acid could not be deduced from the NMR experiments in CD_3OD since the proton signals of the cyclobutane ring (especially H-7′ and H-8′) overlapped. Compound **1** was reexamined in dioxane-d_8 whereby the shifts and the coupling constants of the methine protons of the cyclobutane ring (see Experimental) could be revealed. The observed large coupling constants ($^3J_{7,7'} = 9.5$ and $^3J_{8,8'} = 11.3$ Hz) and the contacts observed in a NOE difference spectrum, established a *cis* configuration for the pairs H-7/H-7′ and H-8/H-8′. The protons H-2 and H-6 (also H-2′ and H-6′) had contacts with H-8 (H-8′) which inferred a *trans* configuration of the H-7/H-8 and H-7′/H-8′ pairs.

The free diacid (**2**) and its dimethyl ester (**3**) were subjected to ^{1}H NMR. Both gave symmetrical patterns for the methine protons of the cyclobutane ring. The shift values for **3** compared reasonably well with a related compound, the dimethyl ester of a dimer of 3,4-dimethoxycinnamic acid (Monache et al., 1996). However, in the present study, HSQC and HMBC data for **3** clearly assigned the downfield proton signals ($\delta_H = 4.17$ ppm) as those belonging to the protons nearest the aromatic ring, i.e. H-7 and H-7′. From the above spectroscopic results the structure of compound **1** was deduced as 4,4′-dihydroxy-3,3′-dimethoxy-β-truxinic acid, esterified to sucrose through the 3f and 6f carbons.

When the groats of six oat cultivars were analysed **1** ranged from 101 to 150 ug g^{-1} (dry wt). No **1** was detected in the hulls. Compound **2** could not be detected in either groats or hulls.

Two mechanisms for the dimerization of cinnamic acids have been proposed in literature. One is an oxidative coupling reaction leading to the formation of dehydrodimers (Ralph et al., 1994). The other mechanism is the cycloaddition between the ethylenic carbons of two cinnamic acids which is photo-induced and leads to the formation of truxillic or truxinic acids (Hanley et al., 1993; Mustafa, 1951; Turner et al., 1993). As the isolation of **1** was performed in day-light, one might expect that this compound spontaneously was formed through photo-induced cyclo-dimerization during the work up. However, since similar amounts of **1** was obtained in a separate experiment done in darkness (not shown) this possibility could be ruled out. One might also assume that oat kernels contain truxillic or truxinic acid derivatives comprising other hydroxycinnamic acids as well, but, so far, no such compounds have been detected.

3. Experimental

3.1. General experimental procedures

Solvents for extractions and column chromatography were reagent grade and used without further purification. Reversed phase HPLC (HP ODS Hypersil, 5 μm, 125×4 mm^2, Germany) was performed on a Hewlett-Packard 1100 instrument, equipped with a diode-array detector. Mobile phases consisted of solvent A: 0.01 M phosphate buffer, pH 2.8, acetonitrile (95:5, v/v) and solvent B: acetonitrile. Samples were run with a linear gradient over 60 min from 0 to 40% B in A with a flow rate of 1 ml min^{-1}. The components were detected at 280±2 nm. The identification of peaks to isolate was based on their retention times and UV absorption profiles. All solvents for HPLC were HPLC grade.

^{1}H, ^{13}C and 2-D NMR experiments (COSY, TOCSY, HSQC, HMBC and NOESY) were performed on a Bruker DRX-400 (400 MHz) and/or Bruker DRX-600 (600 MHz) instruments. Chemical shifts are reported in ppm using the solvent peak as reference (δ_H 3.31 and δ_C 49.0 ppm for CD_3OD; δ_H3.53 and δ_C 66.66 ppm for 1,4-dioxane-d_8). UV measurements were made on a Shimadzu UV–visible recording (UV-160) spectrophotometer. Mass spectra were recorded using a JEOL JMS-SX/SX102A (JEOL, Japan) mass spectrometer. EIMS (70 eV) spectra were obtained using a direct probe with evaporation of the sample into the ion source kept at ca 200°C. FABMS were obtained by bombardment of samples (dissolved in glycerol as matrix) with Xe atoms with 5 keV average energy. GC–MS were carried out with a Hewlett-Packard HP5970B system coupled to an HP5890A gas chromatograph using He (flow rate of 1 ml min^{-1}) as carrier gas.

3.2. Oat extract

Oat oil, a viscous liquid, obtained after evaporation of an ethanol extract of a variety blend of oat bran, was donated by Swedish Oat Fibre.

3.3. Extraction and isolation

The oat oil (10 g), suspended in 10 ml hexane, was consecutively extracted four times with 20 ml methanol at room temperature. The lower clear methanol phases

were collected, pooled and evaporated under vacuum at 40°C. The residue was suspended in distilled water and subjected to solid phase extraction (C18 SEP-PAK® Cartridge, Waters, Millipore Corporation, USA) with increasing concentration of aqueous methanol (stepwise 0, 10, 20, 40, 60, 80 and 100%) as eluents, whereby **1** was eluted with 40% methanol. This fraction was further purified by preparative RP–HPLC using aqueous acetonitrile (20%) as an eluent. This yielded ca 10 mg of **1** which was used for the identification.

Six Swedish oat cultivars were analysed for the contents of **1** and **2**. Triplicates of five grams of groats or hulls (dehulled by hand and stored at −20°C until extraction) ground in a Retch mill (0.5 mm sieve) were extracted with 2×35 ml of methanol. The pooled extracts from each sample were evaporated, the residues dissolved in 1 ml methanol and then subjected to RP–HPLC analysis (see above). Duplicate samples of one of the cultivars were also extracted and analysed in tentative darkness (down drawn curtains, the light switched off and all flasks and tubes wrapped in aluminium foil). Amounts of **1** and **2** were determined by using the purified compounds as external standards.

3.4. Compound *1* (sucrose diester of 4,4′-dihydroxy-3,3′-dimethoxy-β-truxinic acid)

Compound **1** was obtained as an off-white solid. UV λ_{max} (MeOH) nm: 208 and 283. HR–FABMS (positive) found: $M+H^+$, 695.2244, $C_{32}H_{39}O_{17}$ requires 695.2187. ^{1}H NMR spectral data (400 MHz, 1,4-dioxane-d_8), δ_H3.83 (1H, dd, H-8′), 4.10 (1H, *dd*, $^3J_{7',8'}=5.1$, H-7′), 4.15 (1H, *dd*, $^3J_{8,8'}=11.3$, H-8), 4.34 (1H, *m*, $^3J_{7,7'}=9.5$, $^3J_{7,8}=8.8$, H-7), 6.39 (2H, *d*, H-2 and H-2′), 6.50 (1H, *dd*, H-6), 6.53 (1H, *dd*, H-6′). ^{1}H, ^{13}C and HMBC NMR data in CD_3OD are shown in Table 1.

3.5. Compound *2* (4,4′-dihydroxy-3,3′-dimethoxy-β-truxinic acid)

Hydrolysis was performed by incubating compound **1** (ca 100 μg) with 1 M NaOH at room temp. for 16 h. After acidification using 1M HCl the mixture was dried with a stream of N_2 gas. The dried residue was extracted with Et_2O (2×1 ml). The combined Et_2O contained the free acid that, after removing the ether, was further purified by HPLC.

UV spectrum (MeOH) was similar with that of **1**. ^{1}H NMR spectral data (400 MHz, CD_3OD), δ_H3.61 (6H, *s*, CH_3-10, CH_3-10′), 3,78 (2H, *bs*, H-8, H-8′), 4.17 (2H, *d*, $^3J_{7,8}=4.7$, H-7, H-7′), 6.39 (2H, *d*, $^4J_{2,6}=1.7$, H-2, H-2′), 6.55 (2H, *dd*, $^3J_{5,6}=8.1$, $^4J_{2,6}=1.7$, H-6, H-6′), 6.60 (2H, *d*, $^3J_{5,6}=8.1$, H-5, H-5′). HR–FABMS (negative) found: MH^-, 387.1118, $C_{20}H_{19}O_8$ requires 387.1080; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 388 M^+ (<1%), 370 M-H_2O^+ (19), 342 M-H_2O-CO^+ (19), 272 Ar-CH=CH-Ar $^+$ (24), 194 Ar–CH=CH–$COOH^+$ (43), 177 Ar–CH=CH=C≡O^+ (100), 145 (14), 117 (5), 89 (5). Ar: 4-hydroxy-3-methoxyphenyl.

3.6. Compound *3* (dimethyl 4,4′-dihydroxy-3,3′-dimethoxy-β-truxinate)

A dried sample of compound 1 (ca 100 μg) was dissolved in a solution of sodium methoxide (ca 0.2 M in MeOH). After incubation at room temp. for 16 h it was acidified with 1M HCl. The volume was gently reduced by a stream of nitrogen gas and 300 μl H_2O was added. The aqueous solution was extracted with 2×1 ml $CHCl_3$, washed with water and dried with anhydrous Na_2SO_4. The $CHCl_3$ phase contained **3**. The remaining aqueous phase, containing sucrose, was lyophilized overnight for further analysis (see Section 3.7).

^{1}H NMR spectral data (400 MHz, CD_3OD), δ_H3.60 (6H, *s*, CH_3-10, CH_3-10′), 3.72 (6H, *s*, $COOCH_3$-9, $COOCH_3$-9′), 3.85 (2H, *d*, $^3J_{7,8}=6.1$, H-8, H-8′), 4.17 (2H, *d*, $^3J_{7,8}=6.1$, H-7, H-7′), 6.37 (2H, *d*, $^4J_{2,6}=2.0$, H-2, H-2′), 6.54 (2H, *dd*, $^3J_{5,6}=8.2$, $^4J_{2,6}=2.0$, H-6, H-6′), 6.60 (2H, *d*, $^3J_{5,6}=8.2$, H-5, H-5′). HR-EIMS found: M^+, 416.1484, $C_{22}H_{24}O_8$ requires 416.1471; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 416 M^+ (2%), 385 M-OMe^+ (2), 272 Ar–CH=CH–Ar^+ (5), 208 Ar–CH=CH–$COOMe^+$ (100), 193 (7), 177 Ar–CH=CH–C≡O^+ (83), 149 (14), 145 (27), 133 (16), 117 (13), 89 (12), 77 (10).

3.7. Sucrose

The lyophilized residue from hydrolysis (Section 3.5), methanolysis (Section 3.6) and authentic sucrose were silylated by incubating with trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) in dry pyridine (TMCS–HMDS–pyridine 1:2:10 v/v) for 10 min at 60° C. After evaporating the reagents, TMSi ethers were dissolved in $CHCl_3$ and subjected to GC–MS analysis using an HP-5 (0.2 mm×25 m) fused silica WCOT column (Hewlett-Packard). The oven was programmed at an initial temperature of 130°C (for 1 min) with a ramp of 6 to 290°C and final time of 5 min. The injector port (split 1:80) and interface temperature were 210 and 310°C. The mass spectra and the GC retention times obtained from the products compared well with those of the TMSi derivative of authentic sucrose.

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Bianthraquinones from *Cassia siamea*

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Abstract

The isolation of two bianthraquinones, 1,1′,3,8,8′-pentahydroxy-3′,6-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone and 7-chloro-1,1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone, from the root bark of *Cassia siamea* is reported. The structures were established by analysis of spectroscopic data and 7-chloro-1,1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone was determined on the direct comparison with synthetic compound. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Cassia siamea*; Leguminosae; Bianthraquinone

1. Introduction

Anthraquinones are important naturally occurring pigments that are widely distributed in nature. They are particularly prominent in fungi (Turner and Aldridge, 1983; Gill and Steglich, 1987; Gill, 1994), higher plants (Thomson, 1987), and lichens (Huneck, 1973, 1984, 1991; Huneck, et al., 1991, 1994; Elix et al., 1984).

In continuation of our work on the constituents of plants, we have isolated from *Cassia siamea* the minor new bianthraquinones, 1,1′,3,8,8′-pentahydroxy-3′,6-dimethyl [2,2′-bianthracene]-9,9′,10,10′-tetrone (**1**) and 7-chloro-1, 1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone (**2**) together with three known anthraquinones and four known bianthraquinones. Among lower plants, lichens and fungi are known to produce a variety of anthraquinones, including several chlorinated metabolites (Elix et al., 1984). Chlorinated bianthra-quinone, (**2**) however, is extremely rare and unique in higher plants.

2. Results

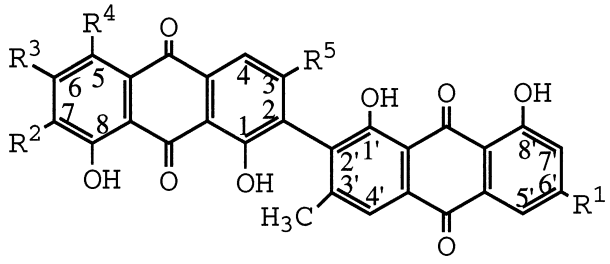
The methanol extract of *C. siamea* was dissolved in water and sequentially partitioned with diethyl ether, chloroform, and ethyl acetate. Silica gel column chromatography of the ether and chloroform soluble layers were used to isolate crysophanol, physcion, emodine, cassiamin A, cassiamim B, cassiamin C (Patil et al., 1970), madagascarin (Buckley et al., 1972; Sreeramulu et al., 1999), and two new bianthraquinones. The bianthraquinones **1** and **2** are minor constituents of *C. siamea*, and were isolated from fractions containing the main constituent, cassiamin A, by using TLC on silica gel.

Compound **1**, $C_{30}H_{18}O_9$, showed IR absorptions due to the carbonyl groups (1672, 1618 cm^{-1}). Its ^1H NMR spectrum showed signals for two methyl groups [δ 2.36 (3H, *s*), 2.47 (3H, *s*)], three 1,2,3-correlated aromatic protons [δ 7.32 (1H, *dd*), 7.70 (1H, *t*), 7.80 (1H, *dd*)], four singlet aromatic protons [δ 7.10, 7.54, 7.65, 7.77], and two phenolic hydroxy groups [δ 12.05, 12.68]. The ^{13}C NMR spectrum showed 29 carbon signals consisting of two methyl carbons (δ 20.92, 22.20), seven methine carbons, 16 quaternary carbons, and four carbonyl carbons (δ 181.67, 181.84, 190.89,192.22). One quaternary carbon is overlapped. The structure of **1** was determined by analysis of ^1H, ^{13}C NMR, HMQC, HMBC (Fig. 1), and IR spectroscopy as 1,1′,3,8,8′-pentahydroxy-3′,6-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone.

Compound **2**, $C_{30}H_{17}O_9Cl$, showed IR absorptions due to the carbonyl groups (1674, 1623 cm^{-1}). The ^1H NMR spectrum indicated the presence of three 1,2,3-correlated aromatic protons [δ 7.32 (1H, *dd*), 7.71 (1H, *t*), 7.88 (1H, *dd*)], two methyl groups [δ 2.23(3H, *s*), 2.24 (3H, *s*)], three singlet aromatic protons [δ 7.54, 7.85, 7.86], and

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(**1**) $R^1=R^2=R^4=H$, $R^3=CH_3$, $R^5=OH$

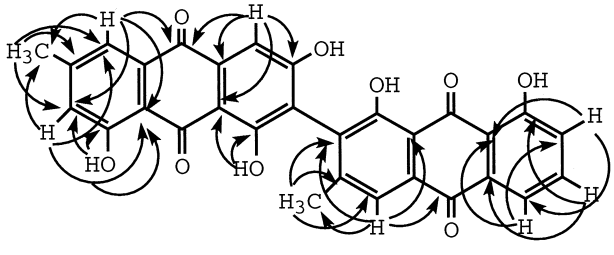
(**2**) $R^1=R^4=H$, $R^2=Cl$, $R^3=OH$, $R^5=CH_3$

(**3**) $R^1=R^2=R^4=H$, $R^3=OH$, $R^5=CH_3$

(**4**) $R^1=R^2=H$, $R^3=OH$, $R^4=Cl$, $R^5=CH_3$

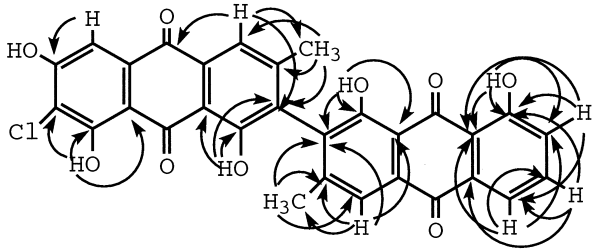
Chart 1.

four phenolic hydroxy groups [δ 12.06, 12.27, 12.38, 12.87]. The ^{13}C NMR spectrum showed 29 carbon signals consisting of two methyl carbons (δ 20.61, 20.65), six methine carbons, 17 quaternary carbons, and four carbonyl carbons (δ 180.99, 181.80, 190.83,192.70). This quinone exhibited a molecular ion base peak at $m/z=556$ with a satellite peak at $m/z=558$. The relative intensities of M^{+2} to M as well as those of the $M^+ - 15$ peak ($m/z=541$) to its satellite peak ($m/z=543$) were 38% which suggested the presence of a chlorinated quinone. Thus, the structure **2** was determined by means of analysis of the ^1H, ^{13}C NMR, HMQC and HMBC (Fig. 2), IR, and MS spectra as 7-chloro-1,1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone. Finally, this structure was confirmed by a direct comparison of the physical data and TLC behaviour with a synthetic product, 7-chloro-1,1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]- 9,9′,10,10′-tetrone, synthesized from cassiamin A (**3**) by chlorination.



(**1**)

Fig. 1. ^{13}C–^1H long range correlation in the HMBC spectrum.



(**2**)

Fig. 2. ^{13}C–^1H long range correlation in the HMBC spectrum.

Models of bianthraquinones, **1** and **2** show that rotation about the 2-2′ bond is hindered, which should give rise to the biphenyl type chirality. The strong CD-values show clearly that they are derived from the CD-coupling (Harada and Nakanishi, 1983) between the two practically identical achiral chromophores. The CD-coupling at the longest wavelength was found with negative first (**1**: 455 nm, $\Delta\varepsilon_{max}=-1.67$; **2**: 439 nm, $\Delta\varepsilon_{max}=-6.48$) and positive second (**1**: 397 nm, $\Delta\varepsilon_{max}=+0.53$; **2**: 388 nm, $\Delta\varepsilon_{max}=+1.72$). Cotton effects due to the long axis of the two anthraquinone moieties showed the two long axes of the anthraquinone nuclei in **1** and **2** are twisted in an anticlockwise manner. Compounds **1** and **2** thus exist as stable atropisomers (Alemayehu et al., 1988; Kitanaka and Takido, 1995).

3. Experimental

NMR: 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR, $CDCl_3$, TMS as int. standard; CC: silica gel (Mallinckrodt, AR) at amounts equivalent to 50 times of the extracts; PTLC: silica gel (Merck, $60F_{254}$; thickness, 0.5 mm).

3.1. Plant material

The dried root bark of *C. siamea* was procured from the University of Maiduguri Campus and Ahmadu Bello University Campus Nigeria during November 1998. The plant was identified by Associate Professor Azmatullah Khan, Department of Botany, A.B.U., Zaria. A voucher specimen was deposited in that department.

3.2. Isolation

The dried powdered root bark of *C. siamea* (0.8 kg) was extracted with hot MeOH. The MeOH extract was concd under red. pres., the residue (77 g) was dissolved in water and sequentially partitioned with diethyl ether (residue 25.9 g), $CHCl_3$ (7.7 g), and EtOAc (30.0 g). The ether and $CHCl_3$ layers were subject to Si gel chromatography using solvent systems of $CHCl_3$ and $CHCl_3$–

MeOH, respectively. Following chromatography, cassiamin A was the main component and fractions containing same were subjected to prep. TLC on Si gel using benzene–acetone (4:1) as eluent to give **1** (3 mg) (R_f = 0.74) (cassiamin A, R_f = 0.61), and using benzene–diethyl ether–EtOAc (60:60:4.5) to give **2** (5 mg) (R_f = 0.24) (cassiamin A, R_f = 0.45).

3.3. 1,1′,3,8,8′-Pentahydroxy-3′,6-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone (*1*)

Mp: > 300°C, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1672, 1618; ^{1}H NMR ($CDCl_3$) δ: 2.36 (3H, *s*, CH_3-3′), 2.47 (3H, *s*, CH_3-6), 7.10 (1H, *s*, H-7), 7.32 (1H, *dd*, *J* = 8.0 and 1.0 Hz, H-7′), 7.54 (1H, *s*, H-4), 7.65 (1H, *s*, H-5), 7.70 (1H, *t*, *J* = 8.0 Hz, H-6′), 7.77 (1H, *s*, H-4′), 7.80 (1H, *dd*, *J* = 8.0 and 1.0 Hz, H-5′), 12.05 (1H, s, OH), 12.68 (1H, *s*, OH); ^{13}C NMR ($CDCl_3$) ppm: 20.92, 22.20, 109.17, 110.33, 113.64, 114.07, 115.25, 115.59, 120.16, 121.45, 121.95, 124.71, 125.07, 125.85, 133.07, 133.14, 133.20, 135.37, 137.35, 148.71, 150.27, 160.77, 162.28, 162.55, 162.60, 181.67, 181.84, 190.89, 192.22; MS *m*/*z*: 522.0950 (M^+, calcd. for $C_{30}H_{18}O_9$, 522.0949); $[\alpha]_D$ −60° (*c* 0.2, $CHCl_3$); CD (*c* 1.5×10^{-4}, MeOH) λ_{max} ($\Delta\varepsilon$): 455 (−1.67), 397 (+0.53), 296 (+2.47), 276 (−5.26).

3.4. 7-Chloro-1,1′,6,8,8′-pentahydroxy-3,3′-dimethyl[2,2′-bianthracene]-9,9′,10,10′-tetrone (*2*)

Mp: 183–188°C, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1674, 1623; ^{1}H NMR ($CDCl_3$) δ: 2.23 (3H, *s*, CH_3-3 or 3′), 2.24 (3H, *s*, CH_3-3′ or 3), 7.32 (1H, *dd*, *J* = 8.5 and 1.0 Hz, H-7′), 7.54 (1H, *s*, H-5), 7.71 (1H, *t*, *J* = 8.5 Hz, H-6′), 7.85 (1H, *s*, H-4), 7.86 (1H, *s*, H-4′), 7.88 (1H, *dd*, *J* = 8.5 and 1.0 Hz, H-5′), 12.06 (1H, *s*, OH), 12.27 (1H, *s*, OH), 12.38 (1H, *s*, OH), 12.87 (1H, s, OH); ^{13}C NMR ($CDCl_3$) ppm: 20.61, 20.65, 108.48, 110.57, 113.65, 113.72, 114.05, 115.89, 120.09, 121.78, 122.02, 124.75, 130.18, 130.64, 132.59, 132.99, 133.01, 133.60, 137.22, 148.04, 148.18, 158.76, 159.85, 159.89, 162.54, 180.99, 181.80, 190.83, 192.70; MS *m*/*z*: 556.0560 (M^+, calc. for $C_{30}H_{17}O_9Cl$, 556.0541), 558.0539 (M^+, calc. for $C_{30}H_{17}O_9Cl$*, 558.0548, 38% intensity); $[\alpha]_D$ −220° (*c* 0.16, $CHCl_3$); CD (*c* 2.2×10^{-4}, MeOH) λ_{max} ($\Delta\varepsilon$): 439 (−6.48), 388 (+ 1.72), 311 (−2.14), 292 (+8.96), 274 (−15.50).

Cassiamin A (**3**) (20 mg, 0.04 mM) was added to 50 ml dry DMF. The red solution was stirred at room temperature until all **3** had dissolved. *N*-Chlorosuccinimide (40 mg, 0.3 mM) was added in one portion to the stirred solution, and stirred for 24 h. The solvent was removed under reduced pressure to give an orange solid. The crude product was separated by PTLC to give **2** (5 mg, 23%), **3** (7 mg), and 5-chlorocassiamin A (**4**) (2 mg, 9%).

(**4**)IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1670, 1625; ^{1}H NMR ($CDCl_3$) δ: 2.26 (6H, *s*, CH_3-3, 3′), 6.89 (1H, *s*, H-7), 7.30 (1H, *d*, *J* = 8 Hz, H-7′), 7.31 (1H, *s*, H-4), 7.74 (1H, *t*, *J* = 8 Hz, H-6′), 7.86 (1H, *d*, *J* = 8 Hz, H-5′),7.89 (1H, *s*, H-4′), 12.09 (1H, *s*, OH), 12.28 (1H, *s*, OH), 12.38 (1H, *s*, OH); MS *m*/*z*: 556.0549 (M^+, calc. for $C_{30}H_{17}O_9Cl$, 556.0560), 558.0559 (M^+, calc. for $C_{30}H_{17}O_9Cl$*, 558.0531, 41% intensity).

Acknowledgements

We are thankful to Associate Professor Azmatullah Khan (Department of Botany, A.B.U., Zaria, Nigeria) for the identification and supply of plant material.

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Xanthones and flavonoids from *Leiothrix curvifolia* and *Leiothrix flavescens*

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Abstract

8-Carboxymethyl-1,6-dihydroxy-3,5-dimethoxyxanthone, 8-carboxymethyl-1,5,6-trihydroxy-3-methoxyxanthone and 8-carboxymethyl-1,3,5,6-tetrahydroxyxanthone were isolated from the capitula of *Leiothrix curvifolia* and *Leiothrix flavescens* and characterized by spectroscopic methods, mainly 1D and 2D NMR experiments, as well as by electrospray mass spectrometry. Eight known flavonoids were also isolated and they were identified by 1D and 2D NMR experiments and comparison with literature data.
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Keywords: Xanthone; Flavonoids; *Leiothrix* sp.; Eriocaulaceae

1. Introduction

The Eriocaulaceae, comprising around 1200 species in 10 genera (Giulietti et al., 2000), are a natural group of herbaceous monocotyledons, characterized by small flowers densely arranged in capitula. The Brazilian ornamental plant popularly known as ''sempre-viva'' (everlasting flowers), native to the Cipó Mountains, Minas Gerais, are exported to the United States, Europe and Japan (Giulietti et al., 1988). Systematic investigations have been carried out with the aim to identify biologically active substances from these plants, as mainly pyrethrins were found previously (Salatino et al., 1990). *Leiothrix* Ruhland is an exclusively South American genus of about 37 species mainly restricted to Brazil, with only two species occurring elsewhere: *L. flavescens* (Bong.) Ruhland that occurs in Brazil, Venezuela, Guyana and Peru, and *L. celiae* Moldenke which is found only in Venezuela (Giulietti et al., 1994). The other species are mostly endemic to small areas in Minas Gerais and in Bahia (Giulietti et al., 1988). While studies on *Leiothrix* are scarce, Salatino et al., (1990) studied the contents of soluble phenolic compounds of the capitula of 21 species of *Leiothrix*. Dokkedal and Salatino (1992) determined the presence of nepetin-7-*O*-glucopyranoside, nepetin-7-*O*-arabinopyranoside and luteolin *O*- and *C*-glucopyranoside in *Leiothrix* species. The distribution of flavonoids also provided useful data for the infrageneric taxonomy of *Leiothrix* (Ricci et al., 1996). The present paper reports the isolation and structural determination of xanthones (Fig. 1) and flavonoids from *L. curvifolia* Ruhland and *L. flavescens*.

2. Results and discussion

Compound **1** was obtained as yellow needles. The EI MS (70 eV, positive ion) spectrum gave a molecular ion $[M]^{+\cdot}$ at m/z 346 and a protonated molecular ion $[M+H]^{+\cdot}$ at m/z 347, corresponding to the molecular formula $C_{17}H_{14}O_8$. Other fragments were observed: 315 $[M\text{-}OCH_3]^{+\cdot}$, 285 $[M\text{-}2OCH_3+H]^{+\cdot}$ The complete structure of **1** could be elucidated by 1D and 2D NMR spectroscopic experiments at 600 MHz. The ^1H NMR spectrum (Table 1) displayed two signals for *meta*-coupled protons ($J=1.5$ Hz) at δ 6.28 and 6.54 and one

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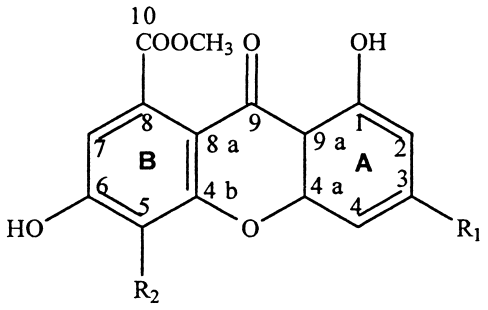
Table 1
^1H and ^{13}C NMR spectral data (CD_3OD, 600 MHz) for compounds **1–3**[a]

Position	**1**		**2**		**3**	
	δ ^{13}C	δ ^1H	δ ^{13}C	δ^1H	δ ^{13}C	δ ^1H
1	164.2	–	164.3	–	164.1	–
2	98.3	6.28 *d* (1.5)	98.1	6.30 *d* (1.5)	98.9	6.20 *d* (1.5)
3	168.2	–	168.1	–	166.7	–
4	93.2	6.54 *d* (1.5)	93.6	6.57 *d* (1.5)	94.8	6.47 (1.5)
4 a	158.5	–	158.6	–	158.5	–
4 b	155.6	–	147.2	–	146.7	–
5	136.9	–	135.1	–	135.1	–
6	157.1	–	151.9	–	152.5	–
7	113.6	6.86 *s*	112.8	6.83 *s*	112.5	6.82 *s*
8	130.3	–	125.6	–	124.9	–
8 a	112.7	–	111.9	–	111.1	–
9	180.3	–	180.5	–	180.0	–
9 a	103.9	–	103.9	–	102.7	–
10	171.3	–	171.9	–	171.9	–
3-OCH_3	56.5	3.89	56.4	3.90	–	–
5-OCH_3	61.9	4.02	–	–	–	–
10-OCH_3	53.4	3.95	53.3	3.94	52.9	3.94

[a] *J* in Hz; assignments confirmed by HSQC and HMBC experiments.

singlet at δ 6.86. Further signals at δ 3.89, 3.95 and 4.02 (3H each) are typical of methoxyl groups. Assignments of all ^1H and ^{13}C NMR spectroscopic (Table 1) signals were based on HSQC and HMBC experiments. Diagnostic long-range correlations in the HMBC spectrum of compound **1**, were observed between the proton signal at δ 6.28 (H-2) and carbon resonances at δ 93.2 (C-4), 103.9 (C-9 a), 164.2 (C-1) and 168.2 (C-3); the proton signal at δ 6.54 (H-4) and the carbon resonances at δ 98.3 (C-2), 103.9 (C-9 a), 158.5 (C-4 a) and 168.2 (C-3), the proton signal at δ 6.86 (H-7) and the carbon resonances at δ 112.7 (C-8a), 136.9 (C-5), 157.1 (C-6) and 171.3 (C-10). These data allowed us to deduce the skeleton of a xanthone and to establish the substitution pattern of rings A and B. Furthermore, the HMBC spectrum permitted definition of the position of the methoxy groups, showing correlations between the ^1H singlet at δ 3.89 and C-3 (δ 168.2), the ^1H singlet at δ 3.95 and C-10 (δ 171.3) and the ^1H signal at δ 4.02 and C-5 (δ 136.9). Thus, the structure of **1** was 8-carboxymethyl-1,6-dihydroxy-3,5-dimethoxyxanthone.

The EI-MS (70 eV, positive ion) spectrum of compound **2** showed a molecular ion at *m/z* 332 $[M]^{+\cdot}$ and of the protonated ion $[M+H]^{+\cdot}$ at *m/z* 333, indicating a molecular formula of $C_{16}H_{12}O_8$. The ^1H NMR spectral features of **2** were similar to those of **1**, the only significant difference being the absence of one of the three signals corresponding to a methoxyl group. A comparison of the ^{13}C NMR spectral data of compound **2** to those of **1** suggested that the two compounds had the same substitution pattern of the A ring, but showed differences in the B ring values. The HMBC spectrum



1 - $R_1 = OCH_3$ $R_2 = OCH_3$
2- $R_1 = OCH_3$ $R_2 = OH$
3 - $R_1 = OH$ $R_2 = OH$

Fig. 1. Xanthones from *Leiothrix curvifolia* and *Leiothrix flavescens*.

clearly showed correlations between the methoxy signal at δ 3.90 and C-3 (δ 168.1) and between the methoxy signal at δ 3.94 and C-10 (δ 171.9), suggesting that **2** differed from **1** except for substitution of the methoxyl group at C-5 with a hydroxyl group. Thus compound **2** is 8-carboxymethyl-1,5,6-trihydroxy-3-methoxyxanthone (**2**).

The EI-MS spectrum of compound **3** (70 eV, positive ion) showed a protonated ion $[M+H]^{+\cdot}$ at *m/z* 319, indicating a molecular formula of $C_{15}H_{10}O_8$. The ^1H and ^{13}C NMR data of **3** when compared to those of **2** suggested the occurrence in **1** of a single methoxyl group located at C-10 on the basis of the HMBC correlation between the proton signal at δ 3.94 and the carbon signal at δ 171.9 (C-10). Thus the structure 8-carboxymethyl-1,3,5,6-tetrahydroxyxanthone was assigned to compound **3**.

It should be noted that the occurrence of a $COOCH_3$ group at C-8 of a xanthone molecule is unusual. The large majority of xanthones, isolated from higher plants commonly have the methyl group at C-3 whereas those from lichens, possess a methyl group at C-8 (Elix and Bennett, 1990). The xanthones isolated from lichens are proposed to be biosynthesized by cyclization of a single, linear polyketide chain (Tanahashi et al., 1999). The xanthones isolated from *Leiothrix* seems to be biosynthetically related to the naphthopyranones previously isolated from species belonging to the *Paepalanthus* genus (Eriocaulaceae) (Vilegas et al., 1990; 1998, 1999a,b).

The flavonoids 3′,4′,5,6-tetrahydroxy-7-methoxyflavone (**4**), 3′,4′,5-trihydroxy-7-methoxy-6-C-glucopyranosylflavone (**5**), 4′,5-dihydroxy-3′,6-dimethoxy-7-*O*-β-D-glucopyranosylflavone (**6**), 3′,4′,5,7-tetrahydroxy-6-C-glucopyranosylflavone (**7**), 3′,4′,5,6,7,8-hexahydroxyflavone (**8**), luteolin (**9**) and apigenin (**10**) were isolated and identified based on their ^1H and ^{13}C NMR spectral data compared to those reported in the literature

(Bate-Smith and Harborne, 1969; Markham, 1982; Agrawal, 1989). 5,3′-dihydroxy-7,4′,5′-trimethoxyisoflavone (**11**), previously reported only in *Ceiba pentandra* (Bombacaceae) (Noreen et al., 1998), was identified in the capitula of *Leiothrix curvifolia*.

Table 2 shows the compounds present in each *Leiothrix* species. The presence of flavones in *Leiothrix flavescens* and *Leiothrix curvifolia* corroborates the data previously reported (Dokkedal and Salatino, 1992). However, this is the first report of xanthones and isoflavones in the *Leiothrix* genus. The presence of xanthones and absence of naphthopyranones allows chemical differentiation between *Leiothrix* and *Paepalanthus*, as naphthopyranones are the major compounds of many *Paepalanthus* species (Vilegas et al., 1998, 1999a,b). The presence of flavonoids (**4–10**) in the capitula of *Leiothrix* species (Table 2) also contributes to the chemical differentiation, since *Paepalanthus* species produces 6- and/or 7-methoxyflavonols derivatives, whereas *Leiothrix* seems to produce mainly flavone derivatives. On the other hand, it has already been postulated that *Leiothrix* is a genus closer to *Syngonanthus* than to *Paepalanthus* (Giulietti et al., 2000). The presence of flavones both in *Leiothrix* and *Syngonanthus* (Dokkedal and Salatino, 1992; Ricci et al., 1996) provides further support to this hypothesis.

3. Experimental

3.1. General experimental procedures

NMR spectra in CD_3OD were obtained using a Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^1H and 150.85 MHz for ^{13}C. ^1H–^1H DQF-COSY (Bodenhausen et al., 1977), inverse detected ^1H–^{13}C HSQC (Bodenhausen et al., 1977; Bodenhausen and Ruben, 1980), HMBC (Martin and Crouch, 1991) were obtained using UXNMR software. EI-MS spectra were performed by using a TRIO 2000 apparatus (70 eV); the mass range (50–400 μm) was analysed using a scan time of 0.6. UV spectra were obtained on a Beckman DU 670 spectrophotometer. HPLC separations were achieved on a Waters 590 system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak reversed phase C-18 columns and U6K injector. Elemental analyses were made with a CE Instrument. TLC were performed on silica gel SiF254 (Merck) eluted with n-BuOH–AcOH–H_2O (12:3:5) the plates were visualized using UV light (254 and 365 nm).

3.2. Plant material

Capitula of *Leiothrix curvifolia* (voucher number WV 11) and *Leiothrix flavescens* (voucher number CFCR 4680) were collected in May of 1997, at the Serra do Cipó, Minas Gerais State, Brazil. Both species were authenticated by Professor Paulo Takeo Sano from Instituto de Biociências, USP, São Paulo. Voucher specimens are deposited at the Herbarium of the IB-USP.

3.3. Extraction and isolation

The separated capitula and powdered scapes (300 g each) of *L. flavescens* and *L. curvifolia* were extracted successively with hexane, CH_2Cl_2, EtOH (1 week for each solvent). The solvents were evaporated in vacuum yielding black syrups. A portion (2.0 g) of the EtOH extract of *L. flavescens* was submitted to CC on Sephadex LH-20 (100×5 cm), with MeOH as eluent. Fractions (8 ml) were collected and checked by TLC [Si gel plates, *n*-BuOH–AcOH–H_2O (20:5:8)]. Fractions 65–73 (69.8 mg) were further purified by HPLC on a Waters (μ-Bondapak RP-18) column (30 cm×4.6 mm i.d) using MeOH–H_2O (1:1) as eluent to afford **3** (4.2 mg, R_t = 8 min) and **4** (10.8 mg, R_t = 11 min). Fractions 47–51 using MeOH–H_2O (2:3) as eluent gave compound **5** (6.5 mg, R_t = 18 min). Fractions 40–46 using MeOH: H_2O (2:3) as eluent afforded compounds **7** (8.5 mg, R_t = 9 min) and **6** (5.8 mg, R_t = 16 min). Fractions 82–83 contained pure **8** (13.3 mg). The EtOH extract (2.0 g) of *L. curvifolia* was submitted to the same procedure on Sephadex LH-20 eluted with MeOH. Fractions 25–28 (101.0 mg), were further purified by repeated CC on polyvinylpolypyrrolidone (Sigma) eluted with MeOH, yielding compound **1** (10.0 mg) and compound **11** (15.0 mg). Fractions 32–36 (69.0 mg), purified under the same conditions reported for fractions 25–28, yielded compound **2** (12.0 mg) and finally fractions 43–47 (43.0 mg) under the same conditions reported for fractions 25–28 yielded compound **9** (7.0 mg) and **10** (9.0 mg).

3.4. Compound *1*

UV λ_{max} nm: (MeOH) (log ε):205 (3.57), 244 (3.69), 288 (3.22), 315(3.31), 362.1 (3.16). EI-MS (70 eV) *m*/*z*

Table 2
Compounds isolated and identified in *Leiothrix curvifolia* and *Leiothrix flavescens*

Specie	1	2	3	4	5	6	7	8	9	10	11
L. curvifolia	+	+	–	–	–	-	–	–	+	+	+
L. flavescens	–	–	+	+	+	+	+	+	–	–	–

346 (66.4%) $[M]^{+\cdot}$, 315 (32.2%) $[M\text{-}OCH_3]^{+\cdot}$, 285 (29.03%) $[M\text{-}2OCH_3+H]$. Elemental analyses: C (58.96%), H (4.07%) calculated molecular formula to $C_{17}H_{14}O_8$ C (58.95%), H (4.04%). For ^1H and ^{13}C NMR spectral analyses, see Table 1.

3.5. Compound 2

UV λ_{max} nm: (MeOH) (log ε): 204 (4.10), 252 (4.13), 292 (3.75), 325 (3.77). The EI-MS (70 eV) m/z 332 (33.1%) $[M]^{+\cdot}$, 333(7.9%) $[M+H]^{+\cdot}$ 301 (13.9%) $[M\text{-}OCH_3]^{+\cdot}$, 273 (0.66%) $[M\text{-}COOCH_3]$. Elemental analyses: C (57.84%), H (3.64%) calculated molecular formula to $C_{16}H_{12}O_8$ C (57.83%), H (3.61%). For ^1H and ^{13}C NMR spectral analyses, see Table 1.

3.6. Compound 3

UV λ_{max} nm: (MeOH) (log ε): 204 (3.77), 250 (3.98), 290.9 (3.42), 328 (3.60). EI-MS (70 eV) m/z 319 (48.2%). Elemental analyses: C (56.61%), H (3.17%) calculated molecular formula to $C_{15}H_{10}O_8$ C (56.60%), H (3.14%). For ^1H and ^{13}C NMR spectral analyses see Table 1.

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Antimicrobial alkaloids from *Zanthoxylum tetraspermum* and *caudatum*

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Abstract

Two benzophenanthrene alkaloids, 8-acetonyldihydronitidine and 8-acetonyldihydroavicine were isolated from *Zanthoxylum tetraspermum* stem bark along with liriodenine, sesamin, lichexanthone and (+)-piperitol-γ,γ-dimethylallylether. The species endemic to Sri Lanka, *Z. caudatum*, contained sesamin, savinin, liriodenine, decarine and 8-*O*-desmethyl-*N*-nornitidine. 8-Acetonyldihydronitidine and 8-acetonyldihydroavicine showed significant antibacterial activity while the former along with liriodenine was strongly antifungal. Savinin exhibited potent spermicidal activity. Both savinin and sesamin exhibited significant insecticidal activity. © 2001 Published by Elsevier Science Ltd.

Keywords: *Zanthoxylum tetraspermum*; *Z. caudatum*; Rutaceae; Benzophenanthrene alkaloids; 8-Acetonyldihydronitidine; 8-Acetonyldihydroavicine; Antimicrobial; Spermicidal; Insecticidal

1. Introduction

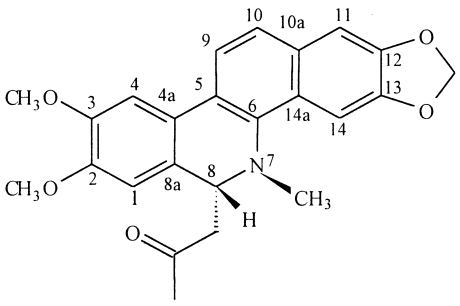
Plants of the pantropical genus *Zanthoxylum* of the family Rutaceae are known to elaborate a variety of biologically active secondary metabolites including alkaloids, lignans and coumarins (Gray, 1983). Many *Zanthoxylum* species find applications in traditional medicines especially in Asia and Africa. In Sri Lanka, the genus *Zanthoxylum* is represented by three species, viz. *Z. caudatum, Z. rhetza* and *Z. tetraspermum* (Stone, 1985). Of these, *Z. tetraspermum* (*Sinhala*-Katukeena) is employed in the indigenous system of medicine in Sri Lanka for the treatment of dyspepsia, rheumatism, and some forms of diarrhea (Jayaweera, 1982). In our continuing search for biologically active constituents of medicinal and related plants of Sri Lanka, we have investigated the stem bark extracts of *Z. caudatum* Alston, which is rare and endemic to Sri Lanka, and *Z. tetraspermum* Wight and Arn. found in Sri Lanka and South India. In this paper we report the isolation and structure elucidation of several metabolites, including two new benzophenanthridine alkaloids, 8-acetonyl dihydronitidine (**1**) and 8-acetonyldihydroavicine (**2**). A number of these constituents have been evaluated for their antimicrobial, spermicidal and insecticidal activities. A literature search revealed that there are no reported chemical or biological studies on these two species.

2. Results and discussion

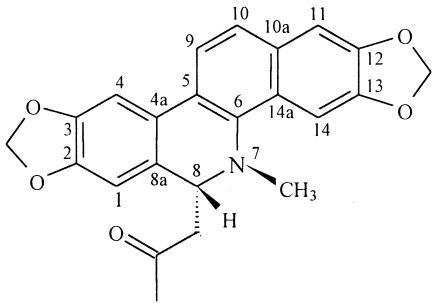
The dichloromethane extract of *Z. tetraspermum* yielded two new alkaloids, namely 8-acetonyldihydronitidine (**1**) and 8-acetonyldihydroavicine (**2**). Compound (**1**) showed in HRMS a peak at m/z 405.1582 corresponding to an elemental composition of $C_{24}H_{23}NO_5$. The ^{13}C NMR spectrum contained sixteen

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olefinic carbons (eight double bonds). Thus, the compound possessed five ring systems. The presence of an acetonyl group at C-8 was indicated by a methyl singlet at δ 1.97 and the AMX system with $J=4.5$, 7 and 12.5 Hz at δ 2.33, 2.70, and 4.56 due to 8-H and the acetonyl methylene protons in its ^{1}H NMR spectrum and signals at δ 207.8 (C=O), 31.5 ($CO\underline{C}H_3$), 48.5 ($-\underline{C}H_2CO$) in the ^{13}C NMR. The pair of three-proton singlets at δ 3.94 and 3.98 (^{13}C δ 56.1, 56.2) were assigned to two OCH_3 groups and the signal at δ 6.03 (*dd*, 2H, $J=1,3$ Hz; ^{13}C δ 101.1) to a methylenedioxy group. The NOESY spectrum of compound (**1**) showed a strong interaction between the two OCH_3 groups, indicating that they occupied adjacent positions on an aromatic ring. The three-proton singlet at δ 2.62 (NCH_3) showed cross peaks with the methyl singlet at δ 1.97 and the doublet of a doublet at δ 4.56 (H_c). Furthermore, the doublet of doublets at δ 2.33 and 2.70 (acetonide methylene protons) showed correlation with each other and with H-8. The foregoing data firmly established the position and the relative stereochemistry of the acetonyl group in ring B. Furthermore, the NMR spectral data indicated that the five ring systems constituted a benzophenanthridine skeleton (Ng et al., 1987).

The aromatic region of the ^{1}H NMR spectrum of compound (**1**) exhibited a pair of one-proton doublets (δ 7.70 and 7.51) and four one-proton singlets (δ 7.32, 6.88, 7.11, 7.57) indicating the presence of two aromatic protons *ortho* to each other and four isolated protons. In the NOESY spectrum the two OCH_3 groups showed cross peaks with H-4 (δ 7.32) and H-1 (δ 6.88) which in turn interacted with H-8 and the acetonide methylene protons indicating their proximity to C-8. Furthermore, the signal at δ 7.57 (H-14) showed correlation with the peak at δ 7.11 and the protons signals of $O-CH_2-O$ and NCH_3. The latter signal showed cross peaks with the $O-CH_2-O$ signals as well as the pair of doublets at δ 7.70 and 7.51. These data established that the protons at δ 7.57 and 7.71 were *para* to each other. Based on the above evidence, it was possible to assign the structure of compound (**1**) as 8-acetonyldihydronitidine.

The second alkaloid (**2**) ($C_{23}H_{19}NO_5$), based on its ^{1}H and ^{13}C NMR, also showed characteristics of a benzophenanthridine. An acetonyl group at C-8 was indicated by the close similarity of signals with those of compound (**1**). Although, the signals corresponding to the OCH_3 groups were absent, there was an additional signal at δ 5.97 (*dd*, 2H, $J=1,3$ Hz) which was attributed to a second methylenedioxy group. By comparison of the ^{1}H and ^{13}C NMR spectra of this compound along with COSY and NOESY spectra with those of 8-acetonyldihydronitidine (**1**), the identity of compound (**2**) was established as 8-acetonyldihydroavicine.

While the parent alkaloids, nitidine (Gopinath et al., 1963) and avicine (Arthur and Hui, 1959) have been reported previously, they are known to be unstable. These compounds were isolated in the present work as their acetone adducts (**1**) and (**2**). Acetone adducts of two other benzophenanthridine alkaloids, chelerythrine (Desai et al., 1967) and corynoline (Ng et al., 1987) are known in nature. It is therefore possible that the acetone adducts are natural products although the presence of nitidine and avicine or their acetonides in the original extract could not be conclusively shown. The absence of nitidine and avicine could be due to the known instability of these compounds. It is possible that the acetonides (**1**) and (**2**) are present in the extract or isolated as artefacts because of the relative stability of the acetone adducts in comparison with the alkaloids.

8-Acetonyldihydronitidine (**1**) and 8-acetonyldihydroavicine (**2**) showed significant growth inhibition of *S. aureus* (Minimum Inhibitory Concentration (MIC): 1.56 and 3.12 μg/ml, respectively; Minimum Bactericidal Concentration (MBC): 25 and 12.5 μg/ml, respectively) while savinin (**3**) and liriodenine (**4**) showed moderate activity (MBC: 200 and 100 μg/ml, respectively). None of the compounds had any inhibitory effect on *E. coli*. 8-Acetonyldihydronitidine (**1**) exhibited strong antifungal activity against *C. cladosporioides* (area of inhibition 100 mm^2 at 2 mg) while liriodenine (**4**) showed comparable activity against both *C. cladosporioides* and *C. gloeosporioides* (area of inhibition 150 and 100 mm^2, respectively at 2 mg). Both compounds inhibited the germination of over 90% conidia of *C. gloeosporioides* at a concentration of 200 μg/ml in 6 h. Savinine (**3**) showed total immobilisation of human spermatozoa at concentrations of 10 mg/ml in 20 s, and at 5 mg/ml in

2 min. Both savinine (**3**) and sesamin (**5**) showed activity (86.6 and 90% mortality, respectively in 24 h at 4000 ppm) against the groundnut aphid *Aphis craccivora*. None of the compounds showed significant activity against the diamond back moth *P. xylostella*. Savinin (**3**), an α-arylidine-γ-lactone lignan has been previously isolated as a cytotoxic constituent of *Aristolochia indica* (Che et al., 1984).

The stem bark of *Z. caudatum* contains bioactive compounds such as sesamin (**5**) (Furuya et al., 1972), liriodenine (**4**) and savinin (**3**). There are no reports of its ethnomedical uses presumably because of the extreme rarity of the plant. The strong bactericidal action of 8-acetonyldihydronitidine (**1**) and 8-acetonyl dihydroavicine (**2**) along with the antifungal activity of the former, and the biological importance of sesamin (**5**) and liriodenine (**4**), found in *Z. tetraspermum* corroborates the medicinal significance of the plant.

3. Experimental

3.1. General

Mps: uncorr; optical rotations: $CHCl_3$; IR: KBr discs; UV: EtOH; ^1H NMR (60 and 500 MHz) and ^{13}C NMR (100.5 MHz): $CDCl_3$ with TMS int. standard, unless otherwise noted; EIMS (probe): 70 eV; cc: silica gel 60 (230–400 mesh, Merck); TLC: silica gel GF 254 (Merck), visualisation, UV (254 nm) and by spraying with acidified anisaldehyde followed by charring with heat.

3.2. Plant materials

Stem barks of *Z. caudatum* and *Z. tetraspermum* were collected at Sinharaja forest and Hakgala, respectively of Sri Lanka and the plants were identified by Dr. D.S.A. Wijesundara. Voucher specimens have been deposited at the Royal Botanic Gardens, Peradeniya, Sri Lanka.

3.3. Bioassays

The following assays were carried out on sesamin (**5**), savinin (**3**), liriodenine (**4**), 8-acetonyldihydronitidine (**1**), 8-acetonyldihydroavicine (**2**): Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the serial dilution method; Antifungal activity against *Cladosporium cladosporioides* and *Cladosporium gloeosporioides* using the TLC-bioassay (Klarman and Sanford, 1968) and in a spore germination assay against *C. cladosporioides*; Insecticidal activity against *Aphis craccivora* (ground nut aphid) and *Plutella xylostella* at a concentration of 4000 ppm (Heinrichs et al., 1981). All experiments which were replicated three times and arranged in a completely randomised design were carried out at ambient temperature (25–34°C), humidity (77–84 RH) and a 12 h photoperiod; Spermicidal activity was evaluated in vitro using ejaculated human spermatozoa (Belsey et al., 1980).

3.4. Extraction and isolation

The air-dried and powdered stem barks of *Z. tetraspermum* (2 kg.) and *Z. caudatum* (3.2 kg.) were successively and exhaustively extracted with light petroleum and $CHCl_3$ or CH_2Cl_2. Evaporation of the solvents gave the respective extracts.

3.4.1. Z. tetraspermum

The light petroleum extract (69 g) was crystallised from hot light petroleum to give an off-white solid (21 g) and a pale green supernatant which was filtered off and concentrated to give a pale green oil (47 g). The solid on repeated recrystallisation from light petroleum/$CHCl_3$ yielded sesamin (**5**) (1.0 g). CC of the green oil (47 g) on silica gel (5×100 cm) and elution with mixtures of light petroleum to light petroleum:EtOAc as indicated afforded the following compounds; 19:1, β-sitosterol (198 mg); 9:1, β-amyrin (600 mg); 17:3, iso-arborenol, further separation by PLC (light petroleum/EtOAc 3:2, 23 mg); 5:1, betulinic acid (35 mg); 4:1, lichexanthone (**6**) (15 mg); 3:1, α-amyrin (20 mg); 7:3, spathulenol (**7**) (83 mg), (+)-piperitol-γ,γ-dimethylallylether (**8**) (40 mg), liriodenine (**4**) (400 mg). CC of the CH_2Cl_2 extract (38 g), on silica gel (5×100 cm) and elution with hexane:acetone 3:2 afforded 8-acetonyldihydronitidine (**1**) (200 mg) and 8-acetonyldihydroavicine (**2**) (24 mg).

3.4.2. Z. caudatum

CC of the light petroleum extract (13 g) on silica gel (2×50 cm) and elution with solvent gradients ranging from light petroleum to mixtures of light petroleum and EtOAc as indicated afforded the following: 19:1, β-sitosterol (760 mg); 17:3, stigmasterol (76 mg); 4:1, sesamin (**5**) (1.23 g); 3:2, savinin (**3**) (210 mg). CC of the $CHCl_3$ extract (21.5 g) on silica gel and elution with solvent gradients ranging from hexane to mixtures of hexane and acetone as indicted yielded the following: 7:3, liriodenine (**4**), (2.47 g); 1:1, decarine (**9**) (40 mg), 8-*O*-desmethyl-*N*-nornitidine (**10**) (15 mg). The fractions were combined based on their TLC patterns. Known compounds were identified by comparison of their physical and spectral properties with those of authentic samples or with those reported in the literature.

3.4.3. 8-Acetonyldihydronitidine (*1*)

Colourless crystals, m.p. 165–167°C; $[\alpha]_D^{25}$ 0°; UV λ_{max} nm (log ε) 316 (3.87); IR ν_{max} 1670, 1636, 1557, 1527,

1482 cm^{-1}; ^1H NMR: δ 1.97 (3H, *s*, $COCH_3$), 2.33 (1H, *dd, J* = 4.5, 12.5 Hz, $C\underline{H}_2$), 2.62 (3H, *s*, NCH_3), 2.70 (1H, *dd, J* = 7, 12.5 Hz, $C\underline{H}_2$), 3.98 and 3.94 (3H each, *s*, OCH_3), 4.56 (1H, *dd, J* = 4.5, 7 Hz, H-8), 6.03 (2H each, *dd, J* = 1, 3 Hz, $O-CH_2-O$), 6.88 (1H, *s*, H-1), 7.11 (1H, *s*, H-14), 7.32 (1H, *d, J* = 7 Hz, H-10), 7.51 (1H, *s*, H-4), 7.57 (1H, *s*, H-11), 7.70 (1H, *d, J* = 7 Hz, H-9); ^{13}C NMR: δ 31.5, ($CO\underline{C}H_3$), 42.5 ($N\text{-}CH_3$), 48.5 ($CO\underline{C}H_2-$), 56.1 and 56.2 ($2\times OCH_3$), 60.1 (C-8), 100.5 (C-1), 101.1 ($-OCH_2O-$), 104.4 (C-14), 106.6 (C-4), 110.5 (C-10), 119.6 (C-9), 123.4 (C-10), 123.5 (C-8a), 123.9 (14a), 127.1 (C-5),127.4 (C-10a), 130.0 (C-6), 147.6 and 148.2 (C-2 and C-3), 148.8, 149.1 (C-12 and 13), 207.8 ($\underline{C}OCH_3$); HRMS: for ($C_{24}H_{23}NO_5$) requires 405.1576; found 405.1582.

*3.4.4. 8-Acetonyldihydroavicine (**2**)*

Colourless crystals, m.p. 184–185°C; $[\alpha]_D^{25}$ −6.6°; UV λ_{max} nm (log ε) 321 (4.20); IR ν_{max} 1676, 1586, 1436 cm^{-1}; ^1H NMR: δ 1.96 (3H, *s*, $COCH_3$), 2.30 (1H, *dd, J* = 4.5, 12.5 Hz, CH_2), 2.61 (3H, *s*, NCH_3), 2.62 (1H, *dd, J* = 7, 12.5 Hz, CH_2), 4.53 (1H, *dd, J* = 4.5, 7 Hz, H-8), 5.97 and 6.04 (2H each, *dd, J* = 1, 3 Hz, $O-CH_2-O$), 6.82 (1H, *s*, H-1), 7.11 (1H, *s*, H-14), 7.29 (1H, *d, J* = 7 Hz, H-10), 7.49 (1H, *s*, H-4), 7.56 (1H, *s*, H-11), 7.62 (1H, *d, J* = 7 Hz, H-9); ^{13}C NMR: δ 31.4 ($CO\underline{C}H_3$), 42.3 ($N\text{-}CH_3$), 48.4 ($CO\underline{C}H_2-$), 60.3 (C-8), 100.6 (C-1), 101.3 (C-9, 10 $O-\underline{C}H_2O-$), 101.4 ($O\text{-}CH_2O\text{-}$), 104.4 (C-14), 103.7 (C-4), 107.8 (C-10), 119.8 (C-9), 123.9 (C-10), 123.6 (C-8a), 124.9 (C-14a), 127.2 (C-5),128.4 (C-10a), 131.1 (C-6), 147.3 and 147.6 (C-2 and C-3), 148.3, 148.7 (C-12 and 13), 207.6 ($\underline{C}OCH_3$); HRMS for $C_{23}H_{19}NO_5$ requires 389.1263; found 389.1262.

*3.4.5. Savinin (**3**)*

Colourless needles m.p. 145–147°C (lit. 147–149°C, Che et al., 1984); $[\alpha]_D^{25}$ −18° (lit. −24°, Che et al., 1984); UV, IR, ^1H NMR, ^{13}C NMR, HRMS for ($C_{20}H_{16}O_6$) requires 352.0947; found 352.0951.

*3.4.6. Liriodenine (**4**)*

Yellow crystals, m.p. 280–282°C (lit. 282–284°C, Buchanan and Dicky, 1960); UV, IR, ^1H NMR, ^{13}C NMR, HRMS for ($C_{17}H_9NO_3$) requires 275.0583; found 275.0576.

*3.4.7. Sesamin (**5**)*

Off-white crystals, m.p. 124°C (lit.; Ludwig et al., 1964; 123–124°C); $[\alpha]_D^{25}$ + 66° (lit.; Ludwig et al., 1964; + 68°); UV, IR, ^1H NMR, ^{13}C NMR, EIMS: *m/z* 354 (M^+).

*3.4.8. Lichexanthone (**6**)*

Light yellow needles, m.p. 182–184°C (lit.; Jain et al., 1969; 189–191°C); UV, IR, ^1H NMR, ^{13}C NMR, HRMS for ($C_{16}H_{14}O_5$) requires 286.0841; found 286.0837.

*3.4.9. Spathulenol (**7**)*

Pale green oil, $[\alpha]_D^{25}$ + 40.2° (lit.; Terhune et al., 1974; + 56°); UV, ^1H NMR, ^{13}C NMR, HRMS for ($C_{15}H_{24}O$) requires 220.1828; found 220.1832.

*3.4.10. (+)-Piperitol-γ,γ-dimethylallylether (**8**)*

White sticky solid, m.p. 58–60°C, $[\alpha]_D^{25}$ + 25° (lit. 60°C, $[\alpha]_D^{25}$ + 21°, Vaquette et al., 1979) UV, IR, ^1H NMR, ^{13}C NMR, HRMS for ($C_{25}H_{28}O_6$) requires 424.1886; found 424.1898.

*3.4.11. Decarine (**9**)*

Pale orange yellow crystals m.p. 245–247°C (lit. 245–246°C, Ishii et al., 1978); UV, IR, ^1H NMR, ^{13}C NMR, HRMS for $C_{19}H_{13}NO_4$) requires 319.0845; found 319.0848.

*3.4.12. 8-O-Desmethyl-N-nornitidine (**10**)*

Pale yellow semi-solid, m.p. 235–237°C; UV λ_{max} nm (log ε) 280(6.29); IR ν_{max} 3317, 1617, 1592 cm^{-1}; ^1H NMR: δ 4.03 (3H, *s*, OCH_3), 6.20 (2H, *s*, $O-CH_2-O$), 7.50 (1H, *s*, H-1), 7.58 (1H, *s*, H-7), 7.94 (1H, *d, J* = 9 Hz, H-12), 8.16 (1H, *s*, H-10), 8.54 (1H, *s*, H-4), 8.60 (1H, *d, J* = 9 Hz, H-11), 9.21 (1H, *s*, H-6), 9.95 (1H, br *s*, exchangeable with D_2O, OH); HRMS for ($C_{19}H_{13}NO_4$) requires 319.0845; found 319.0844.

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The co-occurrence of C(3) epimer N_b,C(21)-secocuran alkaloids in *Strychnos diplotricha* and *Strychnos myrtoides*

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Abstract

From the stem bark of *Strychnos diplotricha*, three N_b,C(21)-secocuran alkaloids, viz., 3-*epi*-myrtoidine, 11-demethoxy-3-*epi*-myrtoidine and 11-demethoxy-12-hydroxy-3-*epi*-myrtoidine, were isolated together with the known myrtoidine and 11-demethoxymyrtoidine. They also occur in different parts of *S. myrtoides*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Strychnos diplotricha*; *Strychnos myrtoides*; N_b,C(21)-secocuran alkaloids; 3-*epi*-Myrtoidine; 11-Demethoxy-3-*epi*-myrtoidine; 11-Demethoxy-12-hydroxy-3-*epi*-myrtoidine

1. Introduction

Genus *Strychnos* (Loganiaceae) is represented in Madagascar by 14 species, five of which are endemic to the island (Leeuwenberg, 1984). The investigation of these plants, suggested by their use as chloroquine-adjuvant in the treatment of chronic malaria, led to the identification of two major *Plasmodium*-resistance modulating alkaloids, strychnobrasiline and malagashanine, from *Strychnos myrtoides* (Rasoanaivo et al., 1994) and *S. mostueoides* (Rasoanaivo et al., 1996). Other alkaloids of the N_b,C(21)-secocuran series, as malagashanine, were later isolated from the former species (Martin et al., 1999).

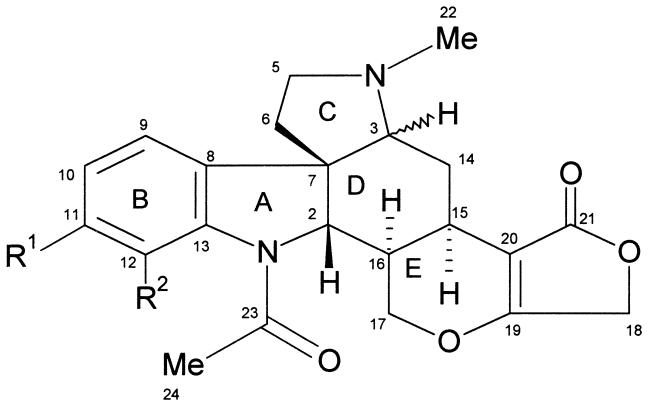
Strychnos diplotricha Leeuwenberg is an endemic species used as an antimalarial in the north-eastern part of Madagascar. The separation by counter-current distribution (CCD) of the basic extract of stem bark of *S. diplotricha* led to the isolation of three new alkaloids (obtained also from the leaves of *S. myrtoides*), viz., 3-*epi*-myrtoidine, **1**, 11-demethoxy-3-*epi*-myrtoidine, **2**, and 11-demethoxy-12-hydroxy-3-*epi*-myrtoidine, **3**, together with myrtoidine, **4**, 11-demethoxymyrtoidine, **5**, strychnofendlerine, strychnobrasiline and malagashanine, previously isolated from the stem bark of *S. myrtoides* (Martin et al., 1999). The MS fragmentation, besides the differential NOE, can discriminate between the C(3) epimer myrtoidines, **1** and **4**, and **2** and **5**.

2. Results and discussion

Alkaloid **1** corresponds to molecular formula $C_{23}H_{26}N_2O_5$ (in HREIMS m/z 410.2031). The preliminary analysis of the ^1H and ^{13}C NMR spectra of this N-acetylindoline indicated the large predominance of a conformer as for myrtoidine, **4**, and suggested that these substances were diastereoisomers. However, the strong positive Cotton effect of the CD curve of **1** at 254 nm ($[\Theta]$ +77900) pointed out the same A-D *cis* junction (2*S*,7*R*) on the basis of Klyne's rule for the *N*-acetylindolinic chromophore (Klyne et al., 1968). The complete assignment of the ^1H chemical shifts was made by the interpretation of the ^1H–NMR spectrum and the ^1H–^1H 2D COSY data. The full assignment of the ^{13}C chemical shifts of **1** was based on the spectral data comparison with the reference alkaloid myrtoidine, **4**, assisted by the interpretation of the direct and long range ^1H–^{13}C heteronuclear correlation experiments (Table 1). The relative configuration of **1** was established by differential NOE experiments. The irradiation

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	R^1	R^2	H-3	
1	OMe	H	α	3-*epi*-myrtoidine
2	H	H	α	11-demethoxy-3-*epi*-myrtoidine
3	H	OH	α	11-demethoxy-12-hydroxy-3-*epi*-myrtoidine
4	OMe	H	β	myrtoidine
5	H	H	β	11-demethoxymyrtoidine

Table 1
NMR data of 3-*epi*-myrtoidine, **1**, *E* and *Z* conformers

Atom	δ_C of E	δ_C of *Z*	δ_H of *E* (*J* in Hz)	INEPT-LR	NOE
2	65.8	65.8	4.60, *d* (8.8)		5b, 6b, 14
3	65.5	64.6	2.83, *t* (5.0)		9, 15
5a	53.5	53.5	3.00, *m* (12.5;8.2;4.0)		
5b			2.33, *m* (12.5;8.4;3.6)		
6a	40.1	38.0	1.65, *m* (12.3;8.2;3.6)	2	
6b			2.02, *m* (12.3;8.4;4.0)		
7	52.2	52.2			
8	133.0	133.0			
9	123.4	122.4	7.10, *d* (8.5)	11	3, 10
10	108.2	110.8	6.62, *dd* (8.5;1.5)	8	
11	159.1	159.1			
12	105.1	106.1	6.71, *d* (1.5)	8, 10, 11	24
13	141.1	141.1			
14a	26.0	24.2	2.00, *m* (12.4;5.0)		
14b			2.02, *m* (12.4;5.0)		
15	26.4	26.4	2.80, *m*		
16	36.6	35.5	1.84, *m* (8.8;5.7;2.1)	2, 20	3, 15, 17a, 17b
17a	70.2	70.2	4.36, *dd* (10.1;5.7)		
17b			4.42, *dd* (10.1;2.1)		
18a	66.1	66.1	4.58, *d* (16.0)		
18b			4.68, *d* (16.0)		
19	172.6	172.6			
20	102.8	103.3			
21	173.0	173.0			
22	41.1	41.1	2.43, *s*	3, 5	
23	169.1	169.1			
24	23.3	23.2	2.43, *s*	23	
OMe	55.7	55.7	3.81, *s*	11	

of H-9 (δ 7.10, *d*, *J* = 8.5 Hz) resulted in the enhancement of the signal of H-3 (δ 2.83) in addition to H-10 (δ 6.62), thus accounting for the α position of H-3. Likewise, signal enhancement was observed for H-3, H-15 (δ 2.80), H-17a and H-17b (δ 4.36 and 4.42, respectively) upon irradiation of H-16 (δ 1.84), thus giving evidence for its α orientation in agreement with the *trans*-relationship with H-2 (δ 4.60, *d*, *J* = 8.8 Hz). On the other side, the irradiation of the latter hydrogen enhanced the signals, inter alia, of H-5b (δ 2.33) and H-6b (δ 2.02). Furthermore, the irradiation of H-12 (δ 6.71, *d*, *J* = 1.5 Hz) enhanced the signal of 3H-24 (δ 2.43), thus indicating that **1** predominantly adopted the *E*-conformer in deuterochloroform solution at room temperature. Compound **1** was therefore unambiguously established as 3-*epi*-myrtoidine.

Alkaloid **2**, corresponding to molecular formula $C_{22}H_{24}N_2O_4$ (in HREIMS *m*/*z* 380.1652), was found likewise to be a diastereoisomer of 11-demethoxymyrtoidine, **5**, on the basis of its NMR data. The full assignment of its ^1H and ^{13}C NMR chemical shifts was assisted by ^1H-^1H and direct and long range ^1H–^{13}C correlation experiments. The data concerning the predominant *E* conformer are summarised in Table 2. The relative configuration of compound **2** was determined by differential NOE experiments. Thus, the irradiation of H-3 (δ 2.88) resulted in the signal enhancement for H-9 (δ 7.25, *dd*, *J* = 7.8 and 1.1 Hz) and H-15 (δ 2.51), indicating the α position of H-3 and H-15, and, vice versa, the irradiation of H-15 intensified the signals of H-3, H-5a (δ 3.01) and H-16 (δ 1.87). The irradiation of

Table 2
NMR data of 11-demethoxy-3-*epi*-myrtoidine, **2**, *E* conformer

Atom	δ_C	δ_H of *E* (*J* in Hz)	INEPT-LR	NOE
2	65.2	4.60, *d* (8.9)		
3	65.4	2.88, *dd* (5.9;5.6)	2, 22	6b, 9, 15
5a	53.5	3.01, *m* (12.5;8.6;4.4)		5b, 9
5b		2.34, *m* (12.5;8.5;4.2)		2, 5a, 6b
6a	40.0	2.01, *m* (12.6;8.6;4.2)		
6b		1.68, *m* (12.6;8.5;4.4)		3, 5b, 6a, 14b
7	52.8			
8	138.0			
9	127.7	7.25, *dd* (7.8;1.1)		
10	123.1	7.12, *dt* (7.8;1.1)		
11	124.7	7.21, *dt* (7.8;1.1)		
12	116.8	7.16, *dd* (7.3;1.1)		
13	140.8			
14a	25.9	1.99, *m* (12.8;5.9)		
14b		2.01, *m* (12.8;5.6)		
15	26.4	2.51, *m*		3, 5a, 16
16	36.4	1.87, *m* (8.9;5.8;3.6)		
17a	70.3	4.42, *dd* (12.0;5.8)		15, 16
17b		4.39, *dd* (12.0;3.6)		15, 16
18a	66.1	4.64, *d* (15.3)		
18b		4.55, *d* (15.3)		
19	172.5			
20	102.8			
21	172.9			
22	41.1	2.44, *s*		
23	169.2			
24	23.2	2.43, *s*		

Table 3
NMR data of 11-demethoxy-12-hydroxy-3-*epi*-myrtoidine, **3**

Atom	δ_C	δ_H (*J* in Hz)	INEPT-LR	NOE
2	66.5	4.13, *d* (10.4)		
3	64.5	2.83, *t* (4.5)		9, 14b, 16
5a	53.7	3.09, *m* (11.1;9.5;4.0)	3, 7	2, 5b, 6b
5b		2.35, *m* (11.1;9.3;4.4)		
6a	37.0	2.00, *m* (13.0;9.3;4.0)		
6b		1.73, *m* (13.0;9.5;4.4)	2, 8	
7	54.7			
8	140.0			
9	113.8	6.70, *dd* (7.4;1.0)	13, 11	3
10	128.5	7.14, *dd* (8.2;7.4)	12, 13	
11	118.5	6.88, *dd* (8.2;1.0)	9, 13	
12	147.9			
13	127.9			
14a	23.2	2.49, *dt* (14.9;4.5)	3, 7, 15, 16	2, 5a, 14b
14b		1.89, *m* (14.9;4.5)		
15	26.4	2.80, *m*		14b, 16
16	34.9	1.91, *m* (10.4;2.3;1.9)		
17a	70.2	4.50, *dd* (12.0;1.9)	15, 19	
17b		4.15, *dd* (12.0;2.3)		
18a	65.8	4.70, *d* (15.9)		
18b		4.59, *d* (15.9)		
19	171.5			
20	104.0			
21	171.8			
22	41.1	2.40, *s*		
23	171.1			
24	22.7	2.41, *s*		
OH		10.08, *s*		11

H-5b (δ 2.34) enhanced the signal of H-2 (δ 4.60), thus accounting for the identical orientation, which is β with A–D *cis* junction (2*S*,7*R*) in agreement with the strong positive Cotton effect at 252 nm. Compound **2** was therefore established as 11-demethoxy-3-*epi*-myrtoidine.

The third alkaloid, **3**, corresponding to molecular formula $C_{22}H_{24}N_2O_5$ (in HREIMS *m*/*z* 396.1675) was found to be structurally related to 11-demethoxy-3-*epi*-myrtoidine, **2**, on the basis of the spectral data comparison. The missing proton on C(12) suggested that this compound possessed one hydroxyl group in that position with respect to the reference alkaloid. The low-field chemical shift of this hydroxyl group (δ 10.08) indicated that it is engaged in a hydrogen bond with the N_b-carbonyl group and, as the result of the restricted rotation of the acetyl group, only the *Z* conformer is present in **3** in contrast to what usually encountered in the *N*-acetylindolinic alkaloids. Table 3 reports its ^1H and ^{13}C assignments based on ^1H–^1H homonuclear and ^1H-^{13}C heteronuclear direct and long-range correlation experiments. The relative configuration in **3** was likewise established by differential NOE experiments. The irradiation of H-9 (δ 6.70) produced signal enhancement for H-3 (δ 2.83), accounting for the α-orientation of both hydrogens. The signals of H-9, H-16 (δ 1.91) and H-14b (δ 1.89) were intensified by irradiation of H-3 and this proved the α-position of H-16 and H-14b. The irradiation of H-14a (δ 2.49) increased the signals of H-2 (δ 4.13, not deshielded as in **1** and **2** by the N_b-carbonyl group) and H-5a (δ 3.09), while the irradiation of the latter hydrogen resulted in the enhancement of H-2, thus giving evidence for the β-position of H-2 in agreement with the strong positive Cotton effect at 256 nm (see Experimental). Compound **3** was therefore 11-demethoxy-12-hydroxy-3-*epi*-myrtoidine.

N_b,C(21)-secocuran alkaloids, as malagashanine, malagashanol, myrtoidine, **4**, and 11-demethoxymyrtoidine, **5**, have been so far isolated only from Malagasy *Strychnos* species, *S. mostueoides* (Rasoanaivo et al., 1996) and *S. myrtoides* (Martin et al., 1999). The last two alkaloids, **4** and **5**, possess a α-β unsaturated γ-lactonic ring between C(18) and C(21), resulting in a furo [3,4-e]pyran system, which has never been found among the indolic alkaloids. This investigation on the stem bark of *Strychnos diplotricha* showed another structural peculiarity which is the co-occurrence of alkaloids epimeric at C(3), viz., **1** and **4**, and **2** and **5**. The comparative examination of their MS data allowed to identify another diagnostic clue to characterise the α configuration for H-3, besides the differential NOE which evidences the interaction between H-9 and H-3 α. The fragmentation peaks at *m*/*z* 273 (78%) and 243 (75) for

1 and **2**, respectively [and at *m/z* 259 (55) in **3**], are missing in the MS spectra of the corresponding alkaloids with configuration H-3 β, i.e., **4** and **5**. A double fragmentation on the D ring in the H-3 α alkaloids, i.e. 3-*epi*-myrtoidine series, with the loss of the furo[3,4-e]pyran moiety, accounts for these peaks.

Only alkaloids of the myrtoidine series, viz., **4** and **5**, were found in the stem bark of *Strychnos myrtoides* (Martin et al., 1999), whereas, remarkably, only alkaloids of the 3-*epi*-myrtoidine series, viz., **1**, **2** and **3**, together with strychnobrasiline and malagashanine, have been isolated from the leaves of the same plant. In the leaves of *S. diplotricha*, the alkaloids were practically undetectable.

Investigation is on the way to evaluate the in vitro and in vivo chloroquine enhancing activity of the new N_b-C(21)-secocuran alkaloids of the *epi*-myrtoidine series.

3. Experimental

A Craig-Post apparatus (200 stages, 10:10 ml, upper and lower phase) was used for the separations performed by CCD. NMR spectra were recorded in $CDCl_3$ on a Varian Gemini 300, observing ^1H and ^{13}C at 300 and 75 MHz, respectively. The ^1H and ^{13}C chemical shifts are given in ppm relative to TMS (internal standard). EI and HREI MS spectra were obtained with HP 5989 A and VG 7070 EQ-HF instruments, respectively. CD curves were recorded on a Jasco 710.

3.1. Plant material

Stem bark and leaves of *Strychnos diplotricha* were collected in the Antalaha region (Madagascar) and identified by Mr. A. Rakotozafy, Département de Botanique, Parc Botanique et Zoologique de Tsimbazaza (Antananarivo). A voucher specimen is kept at the Institut Malgache de Recherches Appliquées (IMRA). Leaves of *S. myrtoides* were collected in Reserve Nationelle d'Ankarafantsika (Martin et al., 1999).

3.2. Extraction and separation

The air-dried, milled stem bark (0.5 kg) of *Strychnos diplotricha* was exhaustively extracted with 2% aqueous AcOH. The combined extracts were made alkaline by $NaHCO_3$ and extracted twice with CH_2Cl_2 to afford crude alkaloids (12.4 g). This extract was fractionated by portions of 4 g by CCD between CH_2Cl_2 and phosphate/citric acid buffer (mobile phase) at discontinuously decreasing pH. At pH 7.0 was eluted strychnofendlerine ($K_r \times K_b = 10^{-7}$) (mg 30), at pH 5.0 strychnobrasiline (6×10^{-10}) (mg 450), at pH 4.0 by recycling 11-demethoxy-3-*epi*-myrtoidine (9×10^{-11}) (mg 68), **2**, and 11-demethoxymyrtoidine (7×10^{-11}) (mg 365), **5**, at pH 3.6 by recycling 3-*epi*-myrtoidine (6×10^{-11}) (mg 56), **1**, myrtoidine (4×10^{-11}) (mg 105), **4**, and malagashanine (1.5×10^{-11}) (mg 12) and at pH 3.2 11-demethoxy-12-hydroxy-3-*epi*-myrtoidine (8×10^{-12}) (mg 43), **3**. Strychnofendlerine, strychnobrasiline, 11-demethoxymyrtoidine, myrtoidine and malagashanine were identified by direct comparison.

1, **2** and **3** (0.05% for each respect to the vegetable material) have been isolated from the basic extract of leaves of *S. myrtoides*, as well, together with strychnobrasiline (0.12%) and malagashanine (0.15%).

3.3. 3-epi-Myrtoidine (*1*)

Crystals from EtOAc and cyclohexane, mp 105–107°C. $[\alpha]_D^{20} = +24.7$ ($CHCl_3$; *c* 0.2). HREIMS, *m/z* : found 410.2031, calc. for $C_{23}H_{26}N_2O_5$ 410.1842. EIMS, *m/z* (rel. int.): 410 (83, M^+), 273 (78), 215 (35), 70 (100). UV (MeOH), λ_{max} (log ε): 220 (4.48), 241 (4.10), 294 (3.67). CD (MeOH): $[\Theta]_{254}$ +77900, $[\Theta]_{230}$-49000.

3.4. 11-Demethoxy-3-epi-myrtoidine (*2*)

Crystals from EtOAc and cyclohexane, mp 103–105°C. $[\alpha]_D^{20} = +37.6$ ($CHCl_3$; *c* 0.2). HREIMS, *m/z*: found 380.1652, calc. for $C_{22}H_{24}N_2O_4$ 380.1736. EIMS, *m/z* (rel. int.): 380 (64, M^+), 243 (75). UV (MeOH), λ_{max} (log ε): 210 (4.45), 244 (4.18), 276 (3.50). CD (MeOH): $[\Theta]_{252}$ +112000, $[\Theta]_{216}$-133000.

3.5. 11-Demethoxy-12-hydroxy-3-epi-myrtoidine (*3*)

Crystals from EtOAc and cyclohexane, mp 230–232°C. $[\alpha]_D^{20} = -184.5$ ($CHCl_3$; *c* 0.2). HREIMS, *m/z*: found 396.1675, calc. for $C_{22}H_{24}N_2O_5$ 396.1685. EIMS, *m/z* (rel. int.): 396 (67), 259 (55), 170 (40), 141 (35). UV (MeOH), λ_{max} (log ε): 217(4.43), 247(3.96), 288 (3.44). CD (MeOH): $[\Theta]_{256}$ +39000, $[\Theta]_{228}$-47000.

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Bromoallenic lipid compounds from lichens of central Asia

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Abstract

The composition of two bromoallenic aliphatic fatty acids obtained from lichens collected around Lake Issyk-Kul (central Asia) is described. These compounds were identified by means of ^{1}H and ^{13}C NMR, MS and IR spectra. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bromoallenic acid; Lichens; *Acarospora gobiensis*

1. Introduction

Although the lipid composition of lichen species is well documented (Dembitsky, 1992), there is only a limited amount of information on the lipids and fatty acids present in lichens growing in the Tian Shan Mountains.

The presence of various chemical components in lichens, in particular aromatic compounds, carotenoids (Huneck and Yoshimura, 1996) and fatty acids (Dembitsky, 1992) has long been used for chemotaxonomic purposes. The fatty acid composition of lichens suggests a great biochemical diversity of these organisms (Dembitsky et al., 1991, 1992; Řezanka and Dembitsky, 1998, 1999a). The present work reports on the ^{1}H and ^{13}C NMR, MS and IR spectroscopic analysis of Tian Shan lichen fatty acids which revealed unusual bromoallenic fatty acids.

2. Results and discussion

In connection with our phytochemical interest in lichens, we examined specimens of *Acarospora gobiensis* H. Magn.; *Cladonia furcata* (Huds.) Schrad., *Lecanora fructulosa* (Dicks.) Ach.; *Leptogium saturninum* (Dicks.) Nyl.; *Peltigera canina* (L.) Willd.; *Rhizoplaca peltata* Ram.; *Xanthoparmelia camtschadalis* (Ach.) Hale, *Xanthoparmelia tinctina* (Maheu & Gill.) Hale and *Xanthoria elegans* (Link.) Th. Fr. collected in August 1995 around the Lake Issyk-Kul in the Central Asian Tian Shan Mountains, and which proved to be a rich source of halogenated metabolites (Řezanka and Dembitsky, 1998, 1999a; Řezanka and Guschina, 1999).

The new bromoallenes (**1** and **2**) as well as some known compounds (Řezanka and Dembitsky, 1999b) and bromine-acetylenic fatty acids (Řezanka and Dembitsky, 1998, 1999a), were isolated from the chloroform-soluble part of the extract of these lichens by a combination of column chromatography on Sephadex LH-20 and preparative HPLC.

Compound **1**, mp 129°, $[\alpha]_D$ +142 showed the presence of a hydroxyl group (3400 cm^{-1}), an allene (1958 cm^{-1}) and a carboxyl group (1710 cm^{-1}) in its IR spectrum. The acid **1** had a molecular formula $C_{18}H_{29}BrO_3$, which was established by HR–MS–PCI (high-resolution mass spectrometry with positive chemical ionization). The mass spectrum from low-resolution PCI-MS contained peaks at m/z 358, 360 $[M+NH_4]^+$ and peak at m/z 234 derived from $[M+NH_4]^+$ by successive losses of C_3H_2Br. The ^{1}H and ^{13}C-NMR spectra indicated the existence of a bromoallene side chain [δ 5.51 (1H, *dd*, $J=5.5$, 6.0 Hz) and 6.09 (1H, *dd*, $J=5.5$, 1.5 Hz); δ 201.9, 102.4 and 74.1] and a double bond [δ 5.39 (1H, *ddd*, $J=9.7$, 7.1 and 6.2 Hz) and 5.46 (1H, *ddd*, $J=9.7$, 5.7 and 0.6 Hz); δ 127.0 and 132.1]. Furthermore, the *Z*-configuration of the double bond at C-12 was indicated by the values ($J=9.7$ Hz) of the coupling constants between the pertinent olefinic protons. Further a downfield methane carbon, i.e. attributed to C_{15} which bears a hydroxy group and eleven methylenes belonging to a long chain were indicated in the ^{13}C NMR spectrum (Table 2). The

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configuration of the hydroxyl group was established by two independent methods. One of them is the modified method of Mosher (Ohtani et al., 1991; Rieser et al., 1992), in which the reaction of the hydroxyl group with *R*- and *S*-MTPA acid chlorides gives *S* and *R* esters. The configuration of these esters was determined by calculating the differences in the chemical shifts of the appropriate hydrogens caused by anisotropic effects. The results given in Fig. 1 show that C_{15} has an S configuration.

Detailed ^1H NMR spin decoupling experiments on **1** combined with a 2D COSY (^1H–^1H) spectrum of **1** led to the following structure:

$$HOOC-(CH_2)_{10}-CH=CH-CH_2-CHX-CH=C=CHX \text{ where } X=O \text{ or } Br$$

The full confirmation of structure **1** (and also **2**) was obtained by the second method, which involved hydrogenation of **1** over platinum oxide in ethanol to give the fully saturated compound **1a**, $C_{18}H_{36}O_3$. The mass spectrum of **1a**, like that of 15-hydroxyoctadecanoic acid, revealed a prominent peak at *m*/*z* 257, ascribable to a fragment caused by elimination of a C_3 unit, and a base peak at *m*/*z* 225 (loss of MeOH from peak at *m*/*z* 257).

Further, the position of a hydroxyl group was established by fragmentation of the TMS ether of the hydroxy pyrrolidide as shown in Fig. 2; intensities of the appropriate ions are listed in the Experimental. The TMS ether has two nearly equivalent centers where ionization and fragmentation can occur. Fragmentation gives the ion **D**, which contains the terminal methyl part of the chain and is formed by α-cleavage on the amide side chain of the oxygenated carbon with the charge remaining on the silyl oxygen. The ion **D** is intense in the spectrum of 15-trimethylsilyloxy pyrrolidide, indicating that fragmentation occurs more readily when the oxygenated carbon is well separated from the amide group. The second ion **C** results from the transfer of the TMS group to the amide carbonyl followed by a second α-cleavage on the amide side chain of the oxygenated carbon. Ion **C** is rather weak (3%) in the spectra listed in Experimental. Ion **B** was expected in the spectrum of the TMS derivative because hydrogen migration is likely. Ion **A**, the other expected ion, results from α-cleavage on the methyl terminal side of the oxygenated carbon. This ion is also strong. Another important ion is the $[M\text{-}15]^+$ ion, usually prominent in the spectra of silyl ethers (Tulloch, 1985), formed by loss of a methyl group. The intensity is about 20%. The $[M\text{-}90]^+$ ion (loss of TMSiOH) and M^+ are weak ions in the spectrum.

Chiral HPLC was used to confirm the chirality of the carbon bearing the hydroxyl group. The enantiomers of both hydroxy fatty acids **1a** and **2a** (i.e. fully hydrogenated **1** and **2**) were separated by HPLC using a chiral stationary phase. In this paper, we demonstrate that chiral HPLC on a (*R*)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine (DNBPG) column provides a convenient methods for analyzing the steric configurations of 15-hydroxystearic acid.

The methyl esters of **1a** and/or **2a** were reacted with naphthoyl chloride in pyridine (the *R* or *S* configuration of the chiral center is not changed by derivatization) and the resulting aromatic esters were separated by chiral HPLC. The enantiomers of the hydroxy fatty acid methyl naphthoyl esters separated with good resolution (the α value is 1.08). For methyl esters derived by naphthoylation of a secondary alcohol group, the *R* enantiomer elutes more rapidly than the *S* enantiomer. This is in accordance with the literature reports (Hawkins et al., 1988).

In view of the strong positive rotation of **1**, the bromoallene moiety of **1** was assigned the *S*-configuration

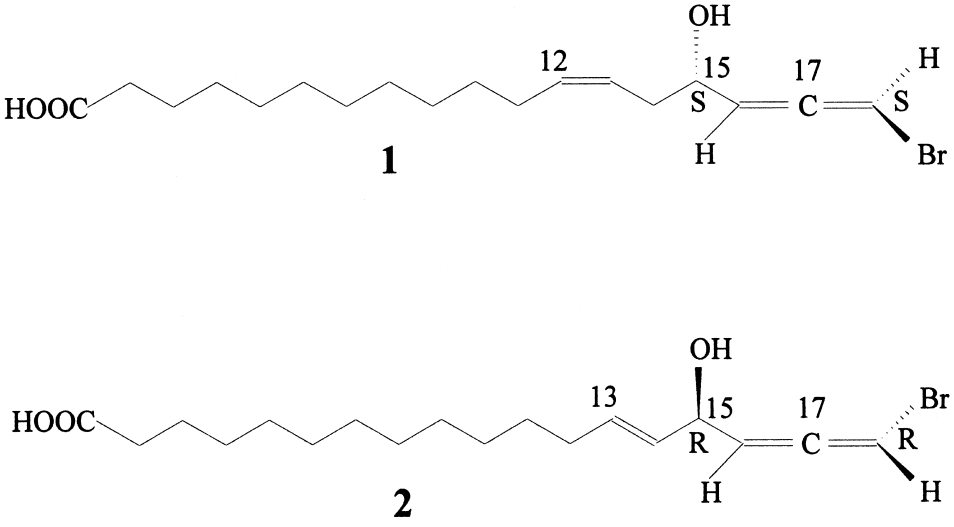


Fig. 1. The structures of the (12*E*, 15*S*, 18*S*)-15-hydroxy-18-bromo-12,16,17-octadecatrienoic acid (**1**) and (13*Z*, 15*R*, 18*R*)-15-hydroxy-18-bromo-13,16,17-octadecatrienoic acid (**2**).

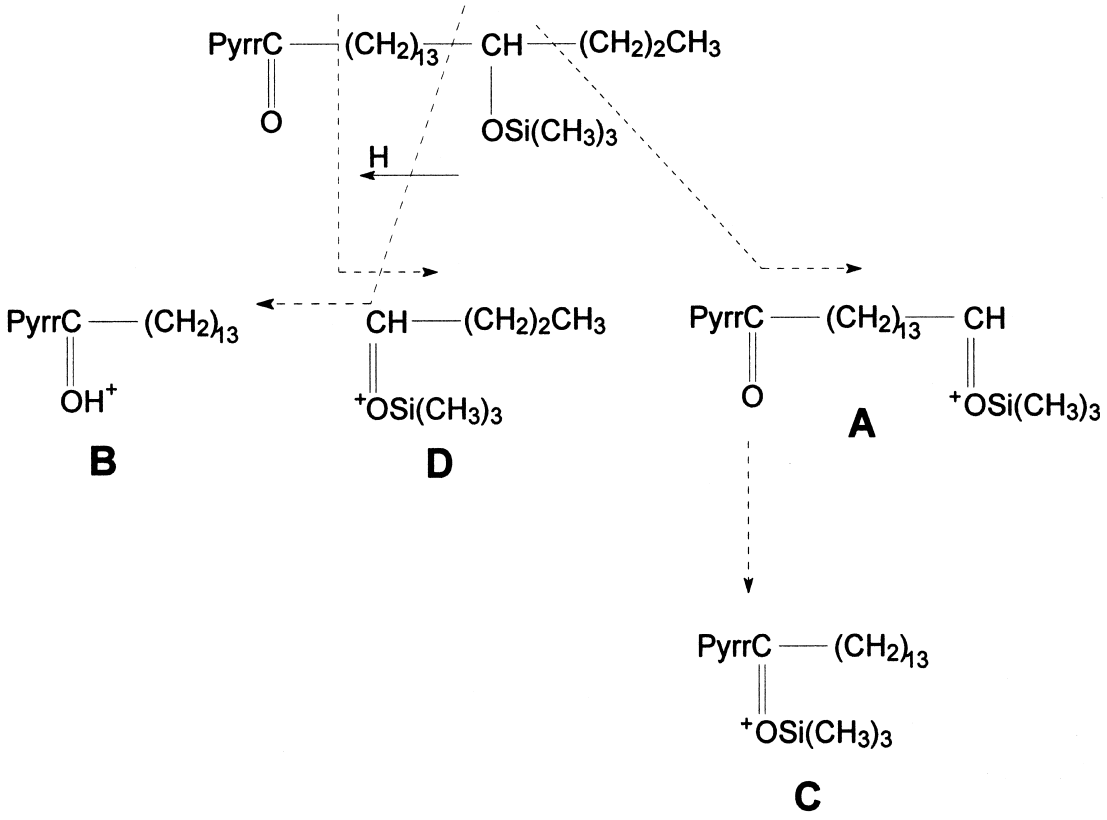


Fig. 2. Fragmentation of trimethylsilyl ether of 15-hydroxyoctadecanoic pyrrolidide.

by application of Lowe's rule (Lowe, 1965). Consequently, the configuration of the new C-18 bromoallenic acid with the two chiral centres at C-15 and C-18 was assigned as illustrated in formula **1**.

Compound **2**, $C_{18}H_{29}BrO_3$, mp 147°, $[\alpha]_D$ −211 exhibited those ^1H NMR spectral resonances expected for a disubstituted allene (δ 5.55 (1H, *dd*, $J=5.8$, 5.7 Hz) and 6.01 (1H, *dd*, $J=5.7$, 1.6 Hz)), (see Table 3), and in addition, the expected ^{13}C NMR chemical shifts (200.6, 102.8 and 76.3) as well as the ^{13}C–H coupling of the two remaining allenic carbon atoms ($J^{13}C\text{-}H_{16}=169$, $J^{13}C\text{-}H_{18}=211$ Hz). Observation of a weak allenic stretching band at 1945 cm^{-1} in the infrared spectrum supported this assignment. In addition, the mass spectral values *m/z* 358, 360 $[M+NH_4]^+$, 234 $[M+NH_4\text{-}C_3H_2Br]^+$ established the presence of a bromoallene group. Compound **2** was also established to have an additional *E* double bond [δ5.62 (1H, *ddd*, $J=15.7$, 6.4, 3.7 Hz) and 5.75 (1H, *dd*, $J=15.7$, 8.6 Hz); δ 134.9 and 127.8]. It thus has a total of three double bond equivalents (two in the bromoallene moiety and the *E* double bond) and is therefore obviously a long-chain triunsaturated fatty acid.

As indicated by the downfield carbon at δ 76.3 and by chemical shifts of H-15, i.e. δ 4.42, and also by the neighboring hydrogens (H-14 and H-16), compound **2** contains a hydroxyl group. The configuration of the hydroxyl group was determined by the modified method of Mosher (Ohtani et al., 1991; Rieser et al., 1992) and by the fact that full hydrogenation of **2** yielded compound **2a**. This compound was methylated and silylated and the appropriate ester-ether presented the same ions as compound **1a**.

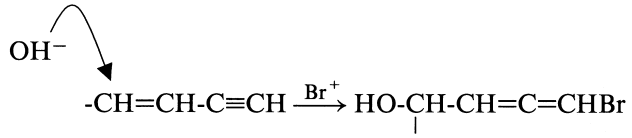
The following partial structure was established by detailed proton spin decoupling experiments:

$$HOOC-(CH_2)_{10}-CH_2-CH=CH-CHX$$
$$-CH=C=CHX \text{ where } X=O \text{ or } Br$$

The application of Lowe's rule (Lowe, 1965) to the bromoallenic acid (**2**) allows a tentative assignment of the absolute configuration of the allene moiety. According to this rule, unsymmetrically substituted allenes with the absolute configuration given in Fig. 1 show negative rotations at the sodium D line. The strong negative rotation observed for **2** $[\alpha]_D^{21}$ −211 corresponds to the configuration shown in Fig. 1. On the basis of the above-mentioned data, the structure of **2** depicted in Fig. 1 was established.

While the bromoallenic acids **1** and **2** have considerable novelty as natural halo-allenes, their partial structure indicating that they are closely related to the large group of halogenated acetylenic natural products of marine origin, all of which are based on sesquiterpenes and acetogenins with C15 skeletons. Representatives of this group have been found chiefly in red algae, but they also occur in sea hares (Grible, 1996). It has been suggested that

brominated marine terpenoids owe their biogenesis to an electrophilic bromination. It seems reasonable to postulate that bromoallenic acid is biosynthesized from a precursor bearing the typical enyne terminus via an analogous process, as shown below.



The acid F (6-bromo-(5*E*,15*Z*)-octadeca-5,15-diene-11,13,17-triynoic acid) from our previous paper (Řezanka and Dembitsky, 1999a) has a terminal enyne system and can serve as precursor of the above-mentioned bromoallenic acids (**1** and/or **2**).

Even though fatty acids with an allenic moiety have been known in the nature for a long time (Aitzetmüller et al., 1997; Spitzer et al., 1997) as indeed are halogenated fatty acids (Grible, 1996), this paper describes the first bromoallenic fatty acids from natural sources.

3. Experimental

The $CHCl_3$ extracts were obtained by the method of Blight and Dyer (1959) from the following lichens: *Acarospora gobiensis* H. Magn.; *Cladonia furcata* (Huds.) Schrad.; *Lecanora fructulosa* (Dicks.) Ach.; *Leptogium saturninum* (Dicks.) Nyl.; *Peltigera canina* (L.) Willd.; *Rhizoplaca peltata* Ram.; *Xanthoparmelia camtschadalis* (Ach.) Hale; *Xanthoparmelia tinctina* (Maheu & Gill.) Hale; and *Xanthoria elegans* (Link.) Th. Fr. All nine lichen species were extracted independently and the extracts were also chromatographed separately by means of the Sephadex LH-20 column with chloroform-hexane 65:35 and then separated by RP-HPLC (Supelco, RP-18, 85% MeOH-H_2O). This afforded compounds **1** and **2** (0.7–5.7 mg from 100 g of lichen, see Table 1).

A Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT, USA) infrared spectrophotometer was used for scanning infrared spectroscopy of methyl esters as neat film. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H), 125.7 MHz (^{13}C). High- and also low-resolution mass spectra were recorded using a VG 7070E- HF spectrometer (Micromass, Manchester, U.K), (positive chemical ionization mode with NH_3, source temperature 130°C). The ionizing condition for CI was 70 eV. GC–MS was performed using a Finnigan 1020 B instrument in EI mode. A fused silica capillary column (60 m×0.32 mm, ID/SPB-1 Supelco), using splitless injection and hydrogen as the carrier gas (a linear velocity of 600 mm/s) was used. The temperature program was 100–320°C, at a gradient of 4°C/min, ionization energy was 70 eV. All spectra were scanned within the range of m/z 50–600.

3.1. Catalytic hydrogenation

About 1 mg of the compounds (**1** and/or **2**) was dissolved in 1 ml of dry EtOH and a catalytic amount of PtO_2 was added. The mixture was stirred under H_2 for 2 h, the solution filtered and the EtOH evaporated to give, after chromatography on TLC (hexane-diethyl ether-acetic acid, 70:30:3), the pure compounds (**1a** and/or **2a**).

3.2. HPLC of enantiomers of 15-hydroxyoctadecanoic acid

The methyl esters were prepared by treating the fatty acid with an excess of ethereal diazomethane for 5 min at room temperature. The methyl esters were then further derivatized by reaction with 25 μl of 1-naphthoyl chloride in 30 μl of anhydrous pyridine and the reaction mixtures were incubated for 40 min at 60°C. The reaction mixtures were evaporated to dryness under a stream of nitrogen and the aromatic ester derivates extracted into hexane. Naphthoylated methyl esters were chromatographed on a DNBPG chiral stationary phase HPLC

Table 1
Occurrence of bromoallenic acids from lichens of Tian Shan mountains

Name of lichen	Name of compounds (mg/100 g of dry weight)	
	Compound **1**	Compound **2**
Acarospora gobiensis	2.8	0.7
Cladonia furcata	–[a]	1.9
Lecanora fructulosa	3.2	–
Leptogium saturninum	2.5	–
Peltigera canina	–	1.8
Rhizoplaca peltata	5.7	–
Xanthoparmelia camtschadalis	–	3.6
X. tinctina	–	0.7
Xanthoria elegans	1.5	–

[a] Less than 0.1 mg/100 g of dry weight.

Table 2
^{13}C NMR of compounds **1** and **2**

Carbon no.	Compound **1**	Compound **2**
1	174.2 *s*	176.1 *s*
2	34.4 *t*	34.3 *t*
3	25.9 *t*	25.3 *t*
4–10	28.7–29.4	28.8–29.4
11	26.4 *t*	29.3 *t*
12	127.0 *d*	31.9 *t*
13	132.1 *d*	127.8 *d*
14	40.0 *t*	134.9 *d*
15	74.4 *d*	76.3 *d*
16	102.4 *d*	102.8 *d*
17	201.9 *s*	200.6 *s*
18	74.1 *d*	73.6 *d*

Table 3
^{1}H NMR of compounds **1** and **2**

Hydrogen	Compound **1**	Compound **2**
2	2.24 (2H, *m*)	2.19 (2H, *m*)
3	1.55 (2H, *m*)	1.57 (2H, *m*)
4–10	1.28-1.35 (16H, *br s*)	1.28 -137 (16H, *br* s)
11	1.96 (2H, *m*)	1.30 (2H, *m*)
12	5.39 (1H, *ddd*, $J_{12,13}=9.7$, $J_{12,11a}=6.2$, $J_{12,11b}=7.1$)	2.02 (2H, *m*)
13	5.46 (1H, *ddd*, $J_{13,14a}=0.6$, $J_{13,14b}=5.7$, $J_{13,12}=9.7$)	5.62 (1H, *ddd*, $J_{13,12a}=3.7$, $J_{13,12b}=6.4$, $J_{13,14}=15.7$)
14a	2.48 (1H, *ddd*, $J_{14a,14b}=13.2$, $J_{14a,15}=10.0$, $J_{14a,13}=0.6$)	5.75 (1H, *dd*, $J_{14,13}=15.7$, $J_{14,15}=8.6$)
14b	2.01 (1H, *ddd*, $J_{14b,14a}=13.2$, $J_{14b,15}=5.0$, $J_{14b,13}=5.7$)	
15	4.86 (1H, *dddd*, $J_{15,16}=6.0$, $J_{15,18}=1.5$ $J_{15,14a}=10.0$, $J_{15,14b}=5.0$)	4.42 (1H, *ddd*, $J_{15,14}=8.6$, $J_{15,16}=5.8$,$J_{15,18}=1.6$)
16	5.51 (1H, *dd*, $J_{16,15}=6.0$, $J_{16,18}=5.5$)	5.55 (1H, *dd*, $J_{16,15}=5.8$, $J_{16,18}=5.7$)
18	6.09 (1H, *dd*, $J_{18,15}=1.5$, $J_{18,16}=5.5$)	6.01 (1H, *dd*, $J_{18,15}=1.6$, $J_{18,16}=5.7$)

column, (5 μm modified Chirex, 25×4.6 mm i.d. obtained from Phenomenex, CA, USA). The derivatives were eluted with a mobile phase solvent system of *n*-hexane containing 0.5% 2-propanol (v/v); the products were eluted isocratically at a flow rate of 0.5 ml per minute. The UV absorbance of the effluent was monitored at 235 nm.

The resolution α for both *R* and *S* isomers of 15-hydroxyoctadecanoic acids was calculated according to the following formula: $\alpha = k'/k$, where k is the capacity factor of the first eluting peak and k' the capacity factor of the second eluting peak.

3.3. GC–MS of TMS ethers of pyrrolidides of 15-hydroxy octadecanoic acid

Glacial acetic acid (0.1 ml) was added to pyrrolidine (1 ml). The mixture was cooled to 0°C, methyl 15-hydroxyoctadecanoate (~1 mg) was added and the solution was kept at 25°C for 24 h. The solution was poured into water, the product extracted with $CHCl_3$ and the extract washed with 1 M HCl and with water. TMS ethers were prepared by treating the hydroxy pyrrolidides in CH_2Cl_2 with bis(trimethyl)silyl acetamide.

Mass spectrum of trimethylsilyl ether of 15-hydroxyoctadecanoic pyrrolidide: MS *m*/*z* (rel. int.) 425 $[M]^+$ (4), 410 $[M-15]^+$ (21), 382 $[D]^+$ (74), 353 $[C]^+$ (3), 335 $[M-90]^+$ (5), 281 $[B]^+$ (9), 145 $[A]^+$ (68), 113 (100).

(12*Z*,15*S*,18*S*)-15-Hydroxy-18-bromo-12,16,17-octadecatrienoic acid (1), *m*p 129°C; $[\alpha]_D^{21}$ +142°; IR (as neat film) 3400, 1958 and 1710 cm^{-1} ; MS-CI (NH_3, *m*/*z*) $(M+NH_4)^+$ 358 and 360 (intensity ratio 1:0.97)., $(M+NH_4\text{-}C_3H_2Br)^+$ 234; HR-MS-PCI (NH_3, *m*/*z*) calcd for $(C_{18}H_{29}O_3{}^{79}Br+NH_4)^+$ 373.3304, found 373.3308.

(13*E*,15*S*,18*S*)-15-Hydroxy-18-bromo-13,16,17-octadecatrienoic acid (2), *m*p 147°C; $[\alpha]_D^{21}$ −211°; IR (as neat film) 3400, 1945 and 1715 cm^{-1} ; MS-CI (NH_3, *m*/*z*) $(M+NH_4)^+$ 358 and 360 (intensity ratio 1:0.98)., $(M+NH_4\text{-}C_3H_2Br)^+$ 234; HR-MS-PCI (NH_3, *m*/*z*) calcd for $(C_{18}H_{29}O_3{}^{79}Br+NH_4)^+$ 373.3304, found 373.3306.

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Triterpene and flavanone glycoside from *Rhododendron simsii*

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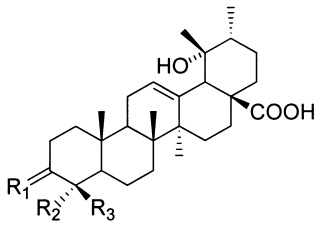
Abstract

Antioxidative substances were isolated from the leaves of *Rhododendron simsii*. These were a triterpene and flavanone glycoside, together with the known matteucinol and two known benzoic acid derivatives. Their structures were characterized as 19,24-dihydroxyurs-12-en-3-one-28-oic acid and 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranosylmatteucinol by spectroscopic analysis. © 2001 Published by Elsevier Science Ltd.

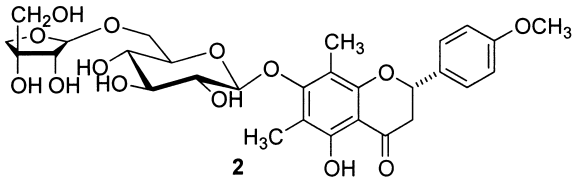
Keywords: *Rhododendron simsii*; Ericaceae; Flavanone glycoside; Apiose; Ursane-type triterpene; Antioxidant

1. Introduction

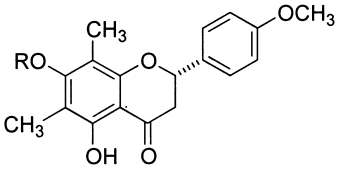
As a part of our ongoing studies on antioxidant natural products, we have already reported a variety of antioxidant compounds isolated from *Garcinia subelliptica* (Fukuyama et al., 1991; Minami et al., 1994) and *Casuarina equisetifolia* (Takahashi et al., 1999). Our screening program for antioxidants in natural products uses three in vitro assay systems, namely anti-lipid peroxidation activity (ALPO) (Stocks et al., 1974), free radical scavenging activity of the α,α-diphenyl-β-picrylhydrazyl radical (DPPH) (Blois, 1958) and superoxidation anion scavenging activity (O_2^-) (McCord and Fridovich, 1969). This indicated that the methanol extract of *Rhododendron simsii* contained potent antioxidative compounds. *Rhododendron simsii* belongs to the Ericaceae and is an evergreen short tree distributed in China, Taiwan, the Ogasawara islands and Japan, and no chemical studies on this plant have been documented. In this report, we describe chemical components of the methanol extract of *R. simsii*, the purpose being, aimed at the isolation of its antioxidative compounds. Antioxidant activity was concentrated in the resulting methylene chloride–ethyl acetate (1:1) and ethyl acetate soluble fractions, from which were isolated a new triterpene **1**, flavanone glycoside **2** and compounds **7** and **8** as antioxidative principles. This paper describes the characterization of two new compounds and the identification of antioxidative substances.



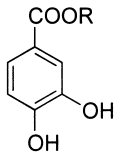
1 R_1 = O, R_2 = CH_3, R_3 = CH_2OH
3 R_1 = O, R_2 = CH_2OH, R_3 = CH_3
4 R_1 = α-H, β-OH, R_2 = CH_3, R_3 = CH_3



2



5 R = H
6 R = β-D-glc



7 R = H
8 $R = CH_3$

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2. Results and discussion

The leaves of *R. simsii* were extracted with methanol and the methanol extract was absorbed on celite and eluted through a glass column with *n*-hexane, methylene chloride, methylene chloride–ethyl acetate (1:1), ethyl acetate and methanol. Each fraction was tested for anti-lipidperoxidation activity, free radical scavenging activity and superoxide anion scavenging activity (Table 1).

The active fractions that eluted with methylene chloride–ethyl acetate (1:1) and ethyl acetate were combined and further subjected to silica gel and Sephadex LH-20 chromatography to afford a new triterpene **1** and flavanone glycoside **2** in addition to 3,4-dihydroxybenzoic acid (**7**) and methyl 3,4-dihydroxybenzoate (**8**).

Compound **1** had a molecular formula of $C_{30}H_{46}O_5$ (HR-EIMS, *m*/*z* 486.3366 $[M]^+$). Its IR spectrum revealed the presence of hydroxyl groups at 3572, 3457 cm^{-1} and a carbonyl group at 1688 cm^{-1}. The ^1H NMR spectrum of **1** had signals due to five tertiary methyl groups [δ_H 1.11 (6 H), 1.45 (3 H), 1.49 (3 H), 1.69 (3 H)], one secondary methyl group [δ_H 1.13 (*d*, $J=6.9$ Hz)], an oxymethylene group [δ_H 3.83, 4.34 (each *d*, $J=11.0$ Hz)] and an olefin proton (δ_H 5.59). The ^{13}C NMR and DEPT spectra of **1** displayed 30 carbon signals which consisted of one carbonyl group (δ_C 214.6), one trisubstituted double bond (δ_C 127.6, 140.1), one carboxylic group (δ_C 180.6), an oxygen-bearing quaternary carbon (δ_C 72.6), one oxymethylene (δ_C 65.1), six methyl groups, nine methylenes, three methines and five quaternary carbons. These spectral data indicated that compound **1** was an ursane-type triterpene. In fact, the ^{13}C NMR spectral data of **1** were very similar to those of pomolic acid (**4**) (Mahato and Kundu, 1994) except for signals due to the A-ring (Table 2). We presumed that the C-3 hydroxyl group in **4** was oxidized to a ketone (δ_C 214.6) and either the C-23 or the C-24 methyl group was hydroxylated to an oxymethylene group [δ_H 3.83, 4.34; δ_C 65.1] in **1**, respectively. In order to confirm this structure, HMBC experiments (Fig. 1) were carried out. The C-24 oxymethylene signals showed cross-peaks to the C-3 ketone and C-23, and the H_3-23 (δ_H 1.49) signal showed cross-peaks to the C-3 ketone and the C-24 methylene. These results suggested that **1** might be rotundanoic acid (**3**) (Wen and Chen, 1996), but its spectral data (^1H NMR, $[\alpha]_D$ and mp) were different from those of **3**. In the 2D-NOESY spectrum (Fig. 2), the oxymethylene signals at C-24 correlated to the H_3-25, and the H_3-23 proton signal correlated to the H-5 signal. Thus, compound **1** was established as 19,24-dihydroxyurs-12-en-3-one-28-oic acid.

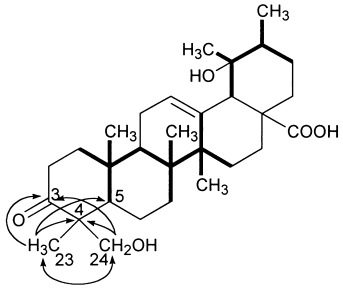


Fig. 1. Partial structures (bold lines) obtained by HMBC correlations from methyl proton signals and representatives HMBC correlations (arrows) from particular proton signals of **1**.

Compound **2** showed IR (3383, 1628 cm^{-1}) and UV absorptions (212, 247, 253, 259 and 279 nm) characteristic of a flavanone. The FAB-MS of **2** showed a quasi-molecular ion peak at *m*/*z* 631 $[M+Na]$ and the molecular formula of **2** was determined as $C_{29}H_{36}O_{14}$ by high-resolution FAB–MS. The ^1H NMR spectrum of **2** showed signals due to two methyl groups at δ 2.57 (3H, *s*) and 2.65 (3H, *s*) which should be bonded to a benzene ring, a 4′-methoxyphenyl moiety at δ 3.66 (3H, *s*), 7.02 (2H, *d*, $J=7.0$ Hz) and 7.58 (2H, *d*, $J=7.0$ Hz), a hydrogen-bonded hydroxyl group at δ 12.6 (1H, s), an ABX-system [δ 2.89 (1H, *dd*, $J=3.0$, 17.0 Hz), 3.25 (1H, *dd*, $J=13.0$, 17.0 Hz) and 5.42 (1H, *dd*, $J=3.0$, 13.0 Hz)] corresponding to a C-ring of a flavanone. These data indicated that the flavanone moiety was matteucinol (**5**) (Tanaka et al., 1985; Basnet et al., 1993). Further, the ^{13}C NMR spectra data (Table 3) showed 27 carbon signals, of which 22 were similar to those of 7-*O*-β-D-glucopyranosyl matteucinol (**6**) (Tanaka et al., 1985) and the remaining five carbon signals could be assigned to an apiose unit (δ_C 111.1, 80.4, 77.8, 75.0 and 65.7). The β configuration on C-1 anomeric orientation of apiose was confirmed by comparing the ^{13}C NMR spectra data of **2** with those of α-D- (δ_C 104.5) and β-D-apiofuranosides (δ_C 111.5), respectively (Kitagawa et al., 1993). The HMBC experiment of **2** showed long-range correlations (Fig. 3) between the H-1 (δ_H 5.66) of apiose and the C-6 (δ_C

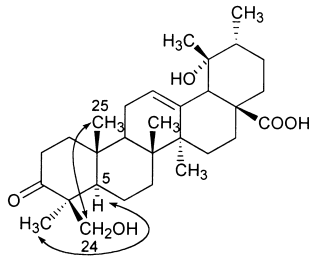


Fig. 2. NOESY correlations of **1**.

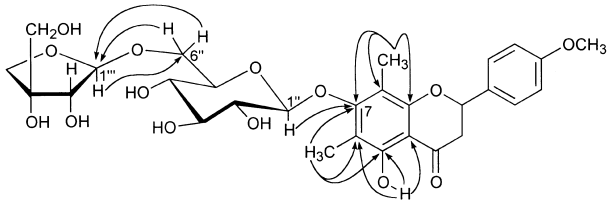


Fig. 3. HMBC correlations of **2**.

68.6) of glucose as well as between the H-6 (δ_H 4.58) of glucose and the C-1 (δ_C 111.1) of apiose, thus suggesting the linkage of apiose-(1→6)-glucose. Additionally, the HMBC correlation between the H-1 (δ_H 5.41) of glucose and the C-7 (δ_C 162.6) suggested that the glucose was located at the C-7 position. Finally, the CD spectrum of **2** observed a positive Cotton effect ($[\theta]_{285}$ −37,600), thereby indicating that the absolute configuration on C-2 is *S* in the light of the literature (Gaffield, 1970). Thus, **2** was established as 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranosyl matteucinol.

Compounds **7** and **8** were isolated as antioxidant principles in the title plants, and tested by using three in vitro assay systems such as anti-lipid peroxidation activity (ALPO), free radical scavenging activity of the α,α-diphenyl-β-picrylhydrazyl radical (DPPH) and superoxidation anion scavenging activity (O_2^-). As shown in Table 1, protocatechuic acid (**7**) and protocatechuic acid methyl ester (**8**) showed inhibitory activities in the three assays whose degree are higher than those of methanol extracts and catechin. However, compounds **1** and **2** had no antioxidant properties.

Table 1
Antioxidant activity of compounds **7** and **8**

	ALPO [a] (%)			DPPH [b]			O_2^- [c] (%)		
Concentration (μg/ml)	10	5	1	100	10	1	150	15	1
MeOH extract	93.3	46.5		94.8	54.2	7.2	100	47.8	
7	100	100	49.2	96.3	95.7	42.3	93.1	48.3	
8	71.8	44.6		95.8	86.0	19.8	96.6	27.6	
Catechin	40.3			88.5	54.5	16.4	57.1	14.3	

[a] Anti-lipid peroxidation activity (per cent inhibition in rat homogenates).
[b] Chemically stable radical scavenging activity (per cent inhibition of α,α-diphenyl-β-picrylhydrazyl (DPPH) radical).
[c] Superoxidation anion scavenging activity (per cent inhibition in xanthine and xanthine oxidase system).

Table 2
^{13}C NMR spectral data of compounds **1** and **4** in py-d_5[a]

C	**1**	**4**[a]
1	40.3	38.7
2	35.6	28.0
3	214.6	78.2
4	55.2	39.3
5	58.1	55.8
6	20.1	18.9
7	33.7	33.6
8	40.4	40.3
9	47.3	47.7
10	37.2	37.3
11	24.3	24.0
12	127.6	128.1
13	140.1	139.9
14	42.1	42.1
15	29.3	29.2
16	26.3	26.6
17	48.3	48.2
18	54.6	54.5
19	72.6	72.7
20	42.4	42.3
21	26.9	27.0
22	38.5	37.4
23	20.8	15.5
24	65.1	28.7
25	15.6	16.7
26	17.1	17.1
27	24.6	24.6
28	180.6	180.6
29	27.1	26.8
30	16.8	16.4

[a] Values taken from Mahato and Kundu 1994.

Table 3
^{13}C NMR spectral data of compounds **2**, **5** and **6** in py-d_5

	2	**5**[a]	**6**[b]
C-2	78.8	78.5	78.7
3	43.6	43.3	43.3
4	198.4	196.6	198.3
5	159.5	159.3	159.4
6	112.3	102.9	112.3
7	162.6	160.8	162.7
8	111.3	102.0	111.1
9	158.3	157.8	158.1
10	105.8	102.9	105.7
1′	131.7	131.0	131.5
2′	128.3	127.5	128.2
3′	114.5	114.1	114.5
4′	160.3	159.8	160.2
6-Me	9.4	6.9	9.4
8-Me	10.0	7.6	10.0
O-Me	55.2	55.4	55.2
Glc-1	105.8		105.8
2	75.7		75.7
3	78.3		78.4
4	71.5		71.5
5	77.4		78.7
6	68.6		62.6
Api-1	111.1		
2	77.8		
3	80.4		
4	75.0		
5	65.7		

[a] In $CDCl_3$, 100 MHz.
[b] Values taken from Tanaka et al., 1985.

3. Experimental

3.1. General

Mps: uncorr. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a Varian Unity 600 instrument. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. IR spectra were measured on a Jasco FT-IR 5300 infrared spectrophotometer. The MS were recorded on a JEOL AX-500 instrument. CC: Silica gel (Merck, 230~400 mesh and Wakogel C-300) and Sephadex LH-20 (25~100 μm, Pharmacia). TLC: precoated silica gel 60 F_{254} (Merck, 0.25 mm) and RP-8 F_{254} (Merck, 0.25 mm). The spots were visualized by UV (254 nm) and $CeSO_4$–H_2SO_4.

3.2. Plant material

Rhododendron simsii was collected in Ishigaki islands, Japan and identified by Dr. Hiroyuki Murata (Kagoshima, Japan). A voucher specimen has been deposited in this institute.

3.3. Extraction and isolation

The dried and powdered leaves were immersed in MeOH at room temperature for 1 month. The MeOH extract was evaporated in vacuo to give a gummy extract (250 g). The MeOH extract was mixed with celite (250 g) and evapd in vacuo to give the solids. The resultant powder was packed in a glass column and then eluted with *n*-hexane (1L), CH_2Cl_2 (1L), CH_2Cl_2–EtOAc (1:1) (1L), EtOAc (1L), EtOAc–MeOH (9:1) (1L) and MeOH (1L) to give 6 frs (1-6). Frs. 3 and 4 (4.6 g) were subjected to CC on Sephadex LH-20 with $CHCl_3$–MeOH (1:4) to give 5 frs (11–15). Fr.12 (1.97 g) was purified by CC on silica gel (*n*-hexane–EtOAc, 5:1) to give **1** (15.5 mg) and **2** (11.8 mg). Fr. 13 (687 mg) was again subjected to repeated CC on silica gel (CH_2Cl_2–EtOAc) to give protocatechuic acid (**7**) (20.2 mg) and protocatechuic acid methyl ester (**8**) (15.1 mg). Fr 14 (424 mg) was purified by CC on silica gel ($CHCl_3$–MeOH) to give matteucinol (**5**) (7.9 mg), **7** (5.2 mg) and **8** (200 mg).

3.4. 19,24-Dihydroxyurs-12-en-3-one-28-oic acid (*1*)

Colorless prisms, mp 189.5–191.0°C, $[\alpha]_D^{17.6}$ +27.4° (*c* 1.55, acetone). EI-MS *m*/*z* (rel. int.): 486.3366 $[M]^+$ (calc. 486.3346 for $C_{30}H_{46}O_5$), 440 (27), 410 (71), 338 (42), 146 (199); IR ν_{film}^{FT} cm^{-1}: 3572, 3457 (OH), 1688 (C=O); ^{1}H NMR (600 MHz, Py-d_5) δ: 1.11 (6H, *s*, CH_3-25, 26), 1.13 (3H, *d*, *J* = 6.9 Hz, CH_3-30), 1.39 (1H, *m*, H-5), 1.45 (3H, *s*, CH_3-29), 1.49 (3H, *s*, CH_3-23), 1.51 (1H, *m*, H-19), 1.69 (3H, *s*, CH_3-27), 1.76 (1H, *ddd*, *J* = 2.7, 6.0, 13.2 Hz, H-1), 1.86 (1H, *dd*, *J* = 8.8, 9.1 Hz, H-9), 2.79 (1H, *ddd*, *J* = 2.8, 6.0, 14.3 Hz, H-2), 3.06 (1H, *s*, H-18), 3.13 (1H, *ddd*, *J* = 8.5, 12.9, 13.2 Hz, H-16), 3.83 (1H, *d*, *J* = 11.0 Hz, H-24), 4.34 (1H, *d*, *J* = 11.0 Hz, H-24), 5.59 (1H, *dd*, *J* = 3.4, 3.4 Hz, H-12); ^{13}C NMR (150 MHz, Py-d_5) : Table 1.

3.5. 7-O-β-D-Apiofuranosyl-(1→6)-β-D-glucopyranosyl matteucinol (*2*)

$[\alpha]_D^{22.6}$ −16.8° (*c* 0.37, acetone). HR-FABMS *m*/z: Found 631.2003 $[M+Na]^+$, calc. 631.2003 for $C_{29}H_{36}O_{14}Na$; IR ν_{film}^{FT}cm^{-1}: 3383 (OH), 1628; UV λ_{max}^{EtOH} nm (ε):212 (36600), 247 (15800), 253 (17900), 259 (16500), 279 (19400); CD (MeOH, *c* 0.017): $[\theta]_{249}$ +18700, $[\theta]_{285}$ −37600, $[\theta]_{354}$ +13000; ^{1}H NMR (600 MHz, Py-d_5) δ: 2.57 (3H, *s*, C_8-CH_3), 2.65 (3H, *s*, C_6-CH_3), 2.89 (1H, *dd*, *J* = 17.0, 3.0 Hz, H-3eq), 3.25 (1H, *dd*, *J* = 17.0, 13.0 Hz, H-3ax), 3.66 (3H, *s*), 4.06 (1H, *ddd*, *J* = 9.9, 6.0, 1.6 Hz, *glc*-5), 4.08 (2H, *s*, *api*-5), 4.16 (1H, *dd*, *J* = 9.9, 8.5 Hz, *glc*-4), 4.18 (1H, *dd*, *J* = 11.3, 6.0 Hz, *glc*-6), 4.32 (1H, *dd*, *J* = 9.1, 8.5 Hz, *glc*-3), 4.29 (1H, *d*, *J* = 9.3 Hz, *api*-4), 4.34 (1H, *dd*, *J* = 9.1, 7.4 Hz, *glc*-2), 4.45 (1H, *d*, *J* = 9.3 Hz, *api*-4), 4.58 (1H, *d*, *J* = 11.3, 1.6 Hz, *glc*-6), 4.61 (1H, *d*, *J* = 2.2 Hz, *api*-2), 5.41 (1H, *d*, *J* = 7.4 Hz, *glc*-1), 5.42 (1H, *dd*, *J* = 3.0, 13.0 Hz, H-2), 5.66 (1H, *d*, *J* = 2.2 Hz, *api*-1), 7.02 (2H, *d*, *J* = 7.0 Hz, H-3′ and 5′), 7.58 (2H, *d*, *J* = 7.0 Hz, H-2′ and 6′) 12.6 (1H, *s*, 5-OH); ^{13}C NMR (150 MHz, Py-d_5): Table 2.

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Passifloricins, polyketides α-pyrones from *Passiflora foetida* resin

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Abstract

Three polyketides α-pyrones, named passifloricins, were isolated from *Passiflora foetida* resin; their structures and relative configurations were assigned through 2D NMR spectroscopic analyses. These types of compounds were not detected in other passion flowers. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Passiflora foetida*; Passifloraceae; Resin; Leaves; Polyketides; 2D NMR; Passifloricins

1. Introduction

The family of passion flowers includes edible species such as *Passiflora quadrangularis*, *P. ligularis*, *P. mollisima* and *P. edulis*. Several years ago we found in the resin of the leaves of *Passiflora foetida* (Echeverri et al., 1991) a flavonoid which exhibited antifeedant activity in in vitro assays against the phytophagous larvae *Dione juno*.

In the course of this investigation, we obtained several waxy compounds, one being related structurally to another lactone isolated from *Eupatorium pilosum* (Herz and Ramakrishnan, 1978; Nakata et al., 1989). In this paper we describe the isolation and structure elucidation of these new polyketides.

2. Results and discussion

The extraction of the leaf resin compounds with ethanol and subsequent repeated column chromatography of this fraction yielded several new polyketides **1**–**3**. Passifloricin A (**1**), was isolated as a white amorphous solid and its positive HRFABMS gave a quasimolecular peak at m/z 441.346642 $[M+H]^+$, corresponding to an empirical molecular formula of $C_{26}H_{48}O_5$. The IR absorption bands at 3256 and 1723 cm^{-1} suggested the presence of hydroxyl and carbonyl groups. The ^1H NMR spectrum (Table 1) had signals at δ 7.00 (*dddd*, 1H, $J=2.0$, 2.9, 5.9, 9.6 Hz), δ 5.90 (*dd*, 1H, $J=1.7$, 9.6 Hz), δ 2.46 (*dt*, 1H, $J=4.9$, 18.1 Hz) and δ 2.26 (*dd*, 1H, $J=11.5$, 18.5 Hz) indicating an AMX_2-spin system. Additionally, three doublets which disappeared with D_2O addition were observed at δ 4.63, δ 4.48 and δ 4.20, indicating the presence of three secondary hydroxyl groups. This was in good agreement with signals as doublets in the ^{13}C NMR spectrum at δ 70.33, δ 69.09 and δ 65.47 (Table 2). Other signals in ^1H NMR spectrum were: a double triplet at δ 4.51 (1H, $J=7.2$, 11.5 Hz) and three broad singlets at δ 3.73 (1H), δ 3.52 (1H) and δ 3.29 (1H), assignable to oxygenated-methine groups. According to an HMQC experiment, signals at δ 1.73 (*dt*, 2H, $J=7.5$, 15.5 Hz), δ 1.47 (4H, *m*) and a strong peak at δ 1.26 were attributable to methylene groups The triplet signal at δ 0.81 (3H, $J=7.1$ Hz) indicated a methyl group

The ^{13}C NMR spectrum of **1** (Table 2, DEPT) exhibited resonances for an α,β-unsaturated lactone (δ 164.24, δ 147.28 and δ 120.02), four oxymethine carbons (δ 75.91, δ 70.33, δ 69.09 and δ 65.47), one methyl (δ 14.28), and several methylenes at δ 44.74, δ 42.18, δ 37.58, δ 34.17, δ 33.63 and δ 31.31, among others. Peaks between 29.60 and 28.96 ppm corresponding to methylene groups were also detected.

On the basis of this preliminary NMR spectroscopic analysis, passifloricin A has a long hydroxylated chain

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Table 1
^{1}H NMR spectral data for passifloricins A (**1**), B (**2**) and C (**3**); multiplicities *J* (Hz) values are given in parentheses

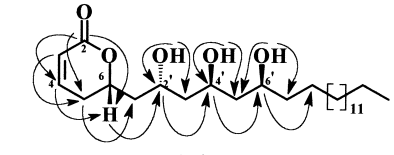
		1	Lactone from *E. pilosum*[a]	**2**	**3**
Proton	DMSO-d_6	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$
3eq	5.90 *d* (1.7, 9.6)	6.05 *d* (9.7)	6.02 *dt* (10.0, 3.0)	2.91 *d* (19.3)	2.91 *d*, 19.2
3ax				2.84 *dd* (19.3, 5.2)	2.77 *dd*, 5.30, 19.2
4	7.00 *dddd* (2.0, 2.9, 5.9, 9.6)	6.92 *ddd* (9.7, 5.0, 3.5)	6.91 *ddd* (10.0, 5.0, 4.0)	4.43 *sbr*	4.33 *s*
5eq	2.46 *dt* (4.9, 18.1)	2.44 *m* (15.2, 9.8, 5.3)	244 *m*	2.04 *brd* (13.9)	2.0 *sbr*
5ax	2.26 *dd* (11.5, 18.5)			1.99 *d* (2.0)	1.91 *m*
6	4.51 *dt* (7.2,11.5)	4.71 *dt* (15.5, 13.0, 5.8)	4.68 *m*	4.90 *sbr*	4.88 *s*
1′eq	1.73 *dt* (7.5, 15.5)	2.06 *m*	2.04 *ddd* (14.0, 8.0, 7.0)	2.02 *m*	1.76 *m*
1′ax		1.69 *m*	1.77 *ddd* (14.0, 6.0, 4.0)	1.69 *m*	
2′	3.73 *d* (5.8)	4.14 *t* (8.6)		4.06 *dd* (9.3, 2.2)	5.05 *m*
3′	1.47 *m*	1.69 *m*			
4′	3.52 *d* (4.5)	3.98 *sbr*		3.90 *m*	3.91 *m*
6′	3.29 (*sbr*)	3.68 *t* (6.0)		3.63 *d* (4.5)	3.57 *m*
Me-21′	0.81 *t* (7.1)	0.88 *t* (6.5)		0.90 *t*	0.89 *t*, 7.0
$[CH_2]_n$					1.27 *sbr*
Other signals	4.63, 4.48, 4.20, *s*		CH(OH): 4.14, *m*; 3.86, *m*		
OAc					2.03 *s*, 3H

[a] From Herz and Ramakrishnan (1978).

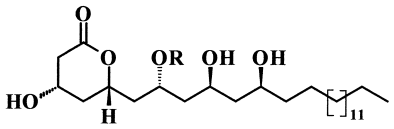
attached to its δ-lactone (Fig. 1). The placement of the hydroxyl groups of **1** along the aliphatic side chain was deduced on the basis of COSY ^{1}H–^{1}H, DEPT, HMQC and HMBC experiments. The signal at *δ* 4.51 was assigned to the proton at C-6 which was flanked by two methylene groups, since COSY cross peaks with signals at *δ* 2.26 and *δ* 1.73 were observed. In the HMQC spectra, these resonances had correlations to carbon signals at *δ* 31.31 and *δ* 42.18, respectively, which correspond to methylene carbons according to DEPT analyses. The proton signal at *δ* 1.73 also displayed a COSY interaction with a methine at *δ* 3.73 which displayed correlations with two other signals; one was a geminal hydroxyl group (*δ* 4.63) and the second one was the multiplet at *δ* 1.47, which in turn gave a cross peak with proton at *δ* 3.52. However, the multiplet at *δ* 1.47 in the ^{1}H NMR, spectrum integrates for four protons and was assignable to overlapping methylene groups at *δ* 44.74 and *δ* 34.17, respectively, according to DEPT and HMQC analyses. Other COSY interactions were detected among methine protons at *δ* 3.52 and *δ* 3.29 and their geminal hydroxyl signals at *δ* 4.48 and *δ* 4.20, respectively. The attachment of the last hydroxyl group was easily deduced by HMBC analysis since the carbon atom of the methylene group at *δ* 34.17 showed a long-range correlation (^2J) to the protons at *δ* 3.29 and *δ* 3.52 (Fig. 1).

Finally, the structure of passifloricin A was assigned by FAB mass spectra. The fragmention at *m*/*z* 97.0287 ($C_5H_5O_2$) is produced by fission of the complete side-chain. Furthermore, another important fragment *m*/*z* 225.1048 ($C_{12}H_{17}O_4$), originated after loss of water and aliphatic $C_{14}H_{29}$ from the molecular ion. The fragmentation pattern is shown in Fig. 2.

Although passifloricin A seems to be similar to the *δ*-lactone of (5*S*,7*S*,9*R*,11*R*) β-hydroxy-tetrahydroxy-hexacos-2-enoic acid, isolated from *Eupatorium pilosum* (Herz and Ramakrishnan, 1978; Nakata et al., 1989) we have observed several differences. Thus, the lactone from *Eupatorium* exhibited an $[\alpha]_{D=}$ −35.7° while that of passifloricin A was +123.45°; in addition the ^{13}C NMR spectroscopic signals for C-5 and C-5′ differed. Unfortunately, it was not possible to crystallise passifloricin A



Passifloricin A, 1



Passifloricin B, 2, R= H
Passifloricin C, 3, R= Ac

Fig. 1. Structure of passifloricins and HMBC of passifloricin A.

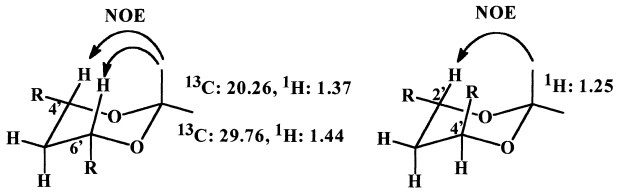


Fig. 2. Relative configuration of passifloricin A.

Table 2
^{13}C NMR spectral data for compounds **1**–**3** and passifloricin A acetonide (100 MHz)

C	**1**		1-Acetonide	Lactone from *E. pilosum*[a]	**2**	**3**
	$CDCl_3$	DMSO	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$
2	164.91	164.24	164.81	164.10	169.56	170.06
3	121.57	120.02	121.71	121.17	36.84	36.56
4	145.96	147.28	145.69	145.39	66.45	66.24
5	32.29	31.31	31.31	29.69	32.31	29.74
6	76.64	75.91	74.98	76.13	72.90	73.25
1′	42.68	42.18	41.21	43.42	37.95	40.13
2′	72.60	65.47	65.40	73.62	68.66	71.73
3′	42.95	44.74	36.74	43.42	42.65	40.31
4′	69.71	69.09	69.64	69.29	71.96	63.72
5′	34.35	34.17	33.53	42.19	33.59	33.30
6′	72.10	70.33	71.79	73.21	72.08	72.09
7′	37.73	37.58	37.81	38.40	37.72	37.30
8′	26.17	25.62	26.20	25.30	26.18	26.02
9′	29.73	29.64	29.74	29.39	30.06	30.07
10′	–	29.42	30.07	–	30.03	–
11′	–	29.24	30.50	–	–	–
12′	–	29.59	30.04	–	–	–
13′	–	29.50	30.05	–	–	–
14′	–	29.51	–	–	–	–
15′	30.06	29.25	–	–	–	–
16′	–	29.15	–	–	30.01	–
17′	–	29.08	29.68	–	29.91	29.72
18′	–	28.96	26.20	–	29.73	30.05
19′	32.92	33.63	32.69	–	33.95	–
20′	23.06	22.45	22.45	–	23.07	23.10
21′	14.31	14.28	14.52	14.14	14.48	14.56
OAc						171.25
						21.69
CH_3 eq.			20.26			
CH_3 ax.			29.76			

[a] Herz and Ramakrishnan (1978).

or its benzoate, *p*-bromobenzoate, and acetate derivatives to establish its absolute stereochemistry. The relative configurations of the 2′,4′,6′-hydroxy groups in the side chain of passifloricin A and the lactone were assigned on the basis of NMR spectroscopic analysis of the acetonide derivatives. This stereochemical approach was previously applied to polyene macrolide antibiotics (Richnovsky et al., 1997) and was carried out through ^{13}C and ^{1}H NMR experiments with detection of the NOE effects between the methyl groups of the acetonide and the protons geminal to the oxygen atoms. Passifloricin A yielded two acetonide derivatives one of which was located between C-4′ and C-6′, according to HMQC-HMBC analysis. The ^{13}C methyl signals of this acetonide derivative appeared at δ 20.26 ppm and δ 29.76 ppm (δ 1.44 and δ 1.37 ppm in ^{1}H NMR); the axial methyl group exhibited a NOE with H-4′ and H-6′, corresponding to a *syn* configuration (Fig. 3). The other acetonide was placed at C-2′ and C-4′ because both methyl groups appeared as a singlet in the ^{1}H NMR (δ 1.27) and the NOE interaction observed was only to H-2′; an *anti* configuration was assigned accordingly.

The relative configuration in C-6, was established through modeling using PC Model 6.0 (1996) and determining both the dihedral angles and the ^{1}H NMR coupling constants. The large value of $J_{5ax,6} = 11.5$ Hz, was in agreement with a *trans* diaxial orientation.

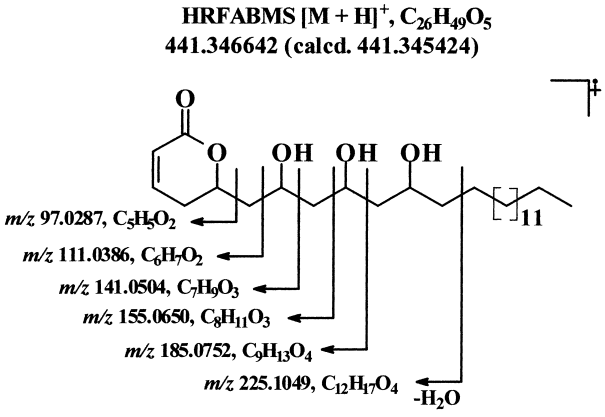


Fig. 3. Fragmentation of passifloricin A in MS.

Besides, a ROESY experiment showed a NOE effect between H-6 and H_{4ax}; therefore, the 6β configuration was assigned for passifloricin A. Thus, passifloricin A possesses the relative configuration 6β,2′α, 4′β,6′β.

Compound **2**, $C_{26}H_{50}O_6$, $[\alpha]_D = -23.92$, was obtained as a waxy substance. The ^1H and ^{13}C NMR spectra were almost identical to those of passifloricin A (**1**), except that the resonance signals of the AMX_2-spin system were absent. However, the ^{13}C NMR spectrum exhibited new signals for methylene and oxygenated-methine groups indicating the presence of a 4-hydroxy-3,4-dihydropassifloricin A. The relative configuration C-$4_{\alpha OH}$ can be deduced because of the small coupling constant to H_{3ax} and H_{5ax} ($J = 3.4$, and 3.0 Hz, respectively). Additionally, these coupling constants were similar to the ones reported for similar pyrones (Evans et al., 1988).

The third compound, passifloricin C (**3**), $[\alpha]_D = -79.13$ was similar to passifloricin B (**2**), with a new signal corresponding to an acetoxy group at δ 2.00 (3H,*s*) in ^1H NMR spectrum(δ 171.0 (*s*), δ 21.7 (*q*) in ^{13}C NMR). The position of this acetoxyl was easily assigned: position of the protons in the ^1H NMR spectrum for H-6, H-4, H-4′ and H-6′ in **3** were identical to those of **2**, but the signal attributable to H-2′ appeared at δ 5.05

That passifloricin B and C might be artifacts was excluded since both were obtained when the plant was extracted with acetone and chromatographed by TLC. Besides, extracts of *Passiflora manicata*, *P. edulis*, *P. coerulea*, *P. sanguinolenta*, *P. maliformis*, *P. mollissima*, and *P. quadrangularis* were analyzed by HPLC and TLC. Passifloricin type compounds were absent and flavonoids were the main compounds; prunasin was a common substance. In addition to *Eupatorium pilosum*, α-pyrones with a side-long chain have been reported in *Ravensara* and *Cryptocarya* species (Lauraceae) (Andrianaivoravelona et al., 1999; Cavalheiro and Yoshida, 2000). Passifloricin A exhibited a $LC_{50} = 0.014$ μg/ml in *Artemia salina* test, while passifloricins B and C were inactive.

3. Experimental

3.1. General

UV spectra were measured using a Spectronic GENESYS 2PC; whereas IR spectra was obtained with a Bruker IFS 28/55 (FTIR). NMR spectroscopic analysis utilized a Bruker Avance DRX 400 instrument. Chemical shifts are expressed in ppm and are reported relative to TMS, with coupling constants being reported in Hz. FAB MS were recorded in the positive mode using the magic bullet and nitrobenzyl alcohol. $[\alpha]_D^{20}$ were taken in a Jasco equipment in MeOH.

3.2. Plant material

Aerial parts of *Passiflora foetid*a var. *hispida* (Killip, E.P) were collected in March 1998 and 1999 in Santa Fe de Antioquia (Colombia). Voucher specimens are deposited in the Herbario Universidad de Antioquia (HUA).

3.3. Extraction and isolation

Fresh aerial parts of the plant (2 kg) were extracted at room temperature with 95% EtOH, to yield after evaporation of the solvent in vacuo 15 g of crude extract. This extract was dissolved in MeOH-H_2O (3.1) and sequentially extracted with hexane, CH_2Cl_2 and EtOAc, respectively. The EtOAc solubles afforded 1.2 g of a dark gum which was applied to a silica gel column using a gradient of $CHCl_3$–Me_2CO (9:1, v/v) The fractions eluted with $CHCl_3$–Me_2CO (6:1) yielded, after evaporation, 15 mg of compound **3** and fractions eluted with $CHCl_3$–Me_2CO (5:1) were combined and purified by repeated prep. TLC ($CHCl_3$–Me_2CO, 4:1) to give **1** (30 mg) and **2** (20 mg)

3.3.1. Passifloricin A

Amorphous powder Mp 97°C; UV λ_{max} (EtOH, nm) 232, 283, log $\varepsilon = 1.028$; IR (KBr) ν_{max} 3256, 1723, 1247, 1039 cm^{-1}. ^1H NMR, ^{13}C NMR (see Tables 1 and 2) FAB-MS: *m*/*z* (rel. int.) 463 $M + Na^+$ (94); 441.346642 $[M + H]^+$ (77); 423 $(M + H)\text{-}H_2O^+$ (27); 153 $(M + H)\text{-}C_{19}H_{28}O_2^+$ (100); 135 $(M + H)\text{-}C_{19}H_{31}O_3^+$ (92); 106 $(M + H)\text{-}C_{21}H_{35}O_3^+$ (22). Calcd for FAB-MS: *m*/*z* (rel. int.) 481 \left $[M + Na]^+$ (100), $[\alpha]_D^{20} = +123.45°$ (MeOH; *c* 0.1134).

3.3.2. Passifloricin B

Amorphous powder Mp 86°C; UV λ_{max} (EtOH, nm) 232, 283, log $\varepsilon = 1.024$; IR (KBr) ν_{max} 3374, 1717, 1101 cm^{-1}. FAB-MS: *m*/*z* (rel. int.) 481 $M + Na^+$ (100); 453 $(M + Na)\text{-}CO^+$ (90); 153 $(M + H)\text{-}C_{18}H_{41}O_3^+$ (87); 135 $(M + Na)\text{-}C_{18}H_{43}O_3^+$ (81); $[\alpha]_D^{20} = -23.92°$ (MeOH; *c* 0.2926).

3.3.3. Passifloricin C

Amorphous powder Mp 64°C; UV λ_{max} (EtOH, nm) 241, 286, log $\varepsilon = 1.027$; IR (KBr) ν_{max} 3430, 1726, 1065, 1039 cm^{-1}. FAB-MS: *m*/*z* (rel. int.) 523.3613, $M + Na^+$ (100), $C_{28}H_{52}O_7$.; calc. for $C_{28}H_{52}O_7 = ?$; $[\alpha]_D^{20} = -79.13°$ (MeOH; *c* 0.2926).

3.4. Passifloricin A acetonides

Passifloricin A **1** (15 mg) was dissolved in acetone (1 ml) and then PPTS (106 mg) were added. Solution was stirred for 2 h under N_2, concentrated and compounds purified by TLC. The main product isolated was 4′, 6′-

isopropiliden-passifloricin A: ^{1}H NMR 6.81 (1H, *ddd*, $J = 3.2,5.9, 9.7$), 5.92H (1H, *ddd*, $J = 1.0, 2.5, 9.8$), 4.52 (1H, *ddd*, $J = 5.2, 11, 16.9$), 4.08 (1H, *m*) 3.79 (1H, *m*), 3.51 (1H, *m*), 2.32 *m*, 1.98 *m*, 1.68 *m*, 1.44 (3H, *s*), 1.37 (3H, *s*) 0.81 (3H, *t*, $J = 7.0$). Minor product was the 2′, 4′-isopropiliden-passifloricin A: ^{1}H NMR 6.92 (1H, *ddd*, $J = 3.4,6.4,9.5$), 6.03 (1H, *d*, $J = 9.5$), 4.63 (1H, *dd*, $J = 5.3, 11.8$), 3.94 (1H, *m*) 3.62 (2H, *m*), 2.44 *m*, 2.14 *m*, 1.86 *m*, 156 (3H, *s*), 0.90 (3H, *t*, $J = 6.9$).

3.5. Assay of cytotoxicity

The lethality towards *Artemia salina* (brine shrimp) was carried out using the procedure of Meyer et al., 1982.

Acknowledgements

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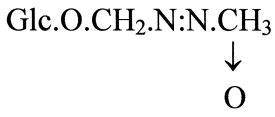
Phytochemistry, 2001, **56**, 223

Ecological biochemistry and its development

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Selected examples of chemical and biochemical interactions between plants and animals are used to illustrate the complexity and fascination of this rapidly developing subject.



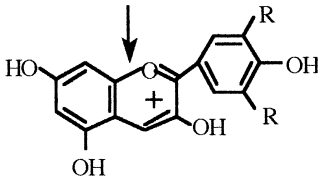
Phytochemistry, 2001, **56**, 229

Contributions of Jeffrey Harborne and co-workers to the study of anthocyanins

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A tribute is made to the many contributions that Jeffrey Harborne and his co-workers have made to the field of anthocyanins.



Phytochemistry, 2001, **56**, 237

Phytochemistry and medicinal plants

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A short history of the contribution of plants to Medicine is presented. The clinical use of drugs such as artemisinin, etoposide and taxol has once more focussed on plants as sources of novel drug entities. Examples of research into natural products with activity on the central nervous system, on pain receptors, the malaria parasite *Plasmodium falciparum*, the wound healing properties of the sap of species of *Croton* (Dragon's blood), and a traditional Chinese medicine used to treat eczema are presented.

Phytochemistry, 2001, **56**, 245

Importance of flavonoids in insect–plant interactions: feeding and oviposition

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Jeffrey Harborne and colleagues have been responsible for collating the majority of data on the role of flavonoids in insect–plant interactions. This article examines some of this information and assesses our knowledge about the role flavonoids play in insect feeding and oviposition behaviour. It is clear that insects can discriminate among flavonoids and that these compounds can modulate the feeding and oviposition behaviour of insects, but further work is required to understand the neural mechanisms associated with these behavioural responses. Despite the wealth of data about the diversity of flavonoids in plants, very few of these compounds have been tested against insects and their role in the evolution of host range in insect–plant interactions has yet to be determined.

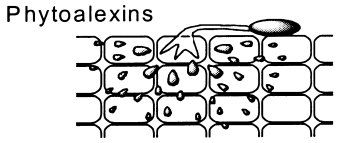
Phytochemistry, 2001, **56**, 253

Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants

Renée J. Grayer, Tetsuo Kokubun

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A brief review is given of some biological, chemical and chemotaxonomic aspects of phytoalexin research. Emphasis is placed on the search for antifungal compounds in the plant families Leguminosae and Rosaceae, and in rice, *Oryza sativa*.



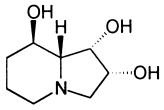
Phytochemistry, 2001, **56**, 265

Polyhydroxylated alkaloids — natural occurrence and therapeutic applications

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Over 100 polyhydroxylated alkaloids have been isolated from plants and micro-organisms. These alkaloids, such as swainsonine, can be potent and highly selective glycosidase inhibitors and are arousing great interest as tools to study cellular recognition and as potential therapeutic agents. Their distribution and activity is reviewed.



Phytochemistry, 2001, **56**, 297

Recent advances in phytochemistry of bryophytes-acetogenins, terpenoids and bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts

Yoshinori Asakawa

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Bryophytes contain a large number of terpenoids and phenolic compounds. Recent topics relating to the chemical constituents found in 36 Japanese, 3 New Zealand, 2 European, 1 Argentinean and 1 Taiwanese liverworts and 2 Japanese mosses and their biological activity are discussed. The chemosystematics of some liverworts as well as the chemical relationship between liverworts and mosses, and bryophytes and ferns are also discussed.

PERGAMON

Phytochemistry 56 (2001) xiii

PHYTOCHEMISTRY

Announcement

The Pergamon Phytochemistry Prize

This international prize of $5500 will be awarded annually by Elsevier Science in recognition of sustained, outstanding contribution to the subject of phytochemistry. It is open to candidates of any nationality, subject to nomination. It is expected that those winning the award will normally have published a significant part of their research findings in *Phytochemistry*.

Nominations for the 2001 award should be sent for onward transmission *either* to, the secretary of one of the established Phytochemical Societies (i.e. PSE, PSNA, PSJ, PSSA, PSLA) or to one of the Regional Editors of *Phytochemistry* (for addresses see inside front cover). They must be received in the editorial office by 31 May 2001, accompanied by appropriate documentation. This should consist of a one or two page letter making out the case for the candidate, a list of appointments (one page) and a list of major publications (no more than three pages). The award will be decided by the Editorial Board, which reserves the right not to make a recommendation in any given year. The result will be announced in the October issue.

Previous prize winners:

1990 E. Leete
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1992 O.R. Gottlieb
1993 J.B. Harborne
1994 E.E. Conn
1995 J. Macmillan
1996 J.D. Phillipson
1997 Y. Asakawa
1998 T.J. Mabry
1999 K.R. Markham
2000 G.H.N. Towers

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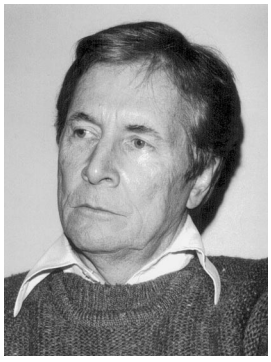
PHYTOCHEMISTRY

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Announcement

The Pergamon Phytochemistry Prize for Creativity in Plant Biochemistry 2000

has been awarded to
Professor G.H. Neil Towers



Neil Towers' contributions to the subject of phytochemistry span a distinguished career of some 42 years. After gaining a PhD at Cornell, under F.C. Steward, he was appointed to McGill University where he initiated a research program in phytochemistry. He was subsequently invited by A.C. Neish to the NRC laboratories in Halifax, Nova Scotia, to continue his programme in natural products biochemistry. In 1962 he was appointed Head of the Botany Department, University of British Columbia, Vancouver where he has worked ever since. His first real contributions to plant science concerned the chemotaxonomy of lignins which expanded to numerous studies on the biosynthesis of cinnamate derivatives such as phloridzin, hydrangenol and hispidin. His name is associated with the cinnamate pathway in plants and fungi. He has established the role of light in the *E*/*Z* isomerism of hydroxycinnamates in plants and in the biosynthesis of styrylpyrones in fungi. More recently, he and Norman Lewis demonstrated for the first time the important role of phenylalanine ammonia lyase (PAL) in the recycling of ammonium nitrogen in the cinnamate pathway in plants. He has also helped to establish the role of sesquiterpene lactones in allergic contact dermatitis from plants, particularly *Parthenium hysterophorus*. The potent light-mediated bioactivities of natural products, particularly the polyynes and their sulfur derivatives, was discovered in his laboratory as was the photogenotoxicity of harmane and furanoquinoline alkaloids and many other natural products. This subject continues to be vigorously pursued in his laboratory and elsewhere. He has also developed extensive programmes on insecticidal phenolics and liminoids of the Meliaceae and the ethnopharmacology of antibiotics and antivirals from plants and fungi.

Professor Towers has trained many phytochemists who now hold important positions in the biological sciences and continues to collaborate in research with many distinguished phytochemists and biologists in various fields. He has served on the Editorial Board of *Phytochemistry* for many years, and was both founding member and President (twice!) of the Phytochemical Society of North America. Other distinctions include recipient of a Gold Medal from the Canadian Society of Plant Physiologists, and as a long-standing Fellow and Gold Medal recipient of the Royal Society of Canada.

With sincere congratulations and best wishes from the Editors and Editorial Board of *Phytochemistry*, and from the Directors of Elsevier Science, Oxford.

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Honours and Awards:

President, Can. Soc. Plant Physiologists, 1965; Fellow of Royal Society, Canada, 1967; Commonwealth Visiting Professor, U. East Anglia, 1970–1971; Killam Fellow, Canada, 1970–1971; Gold Medallist, Can. Soc. Plant Physiologists, 1973; Special Killam Research Scholarship, 1975–1976; Erskine Fellow, U. of Canterbury, New Zealand, 1986 and 1989; President Phytochemical Society of N. America, 1974 and 1986; Flavelle Medal, Royal Society of Canada, 1986; NATO Panel on Cell–Cell Signalling in Plants and Animals.

Selected Publications and Patents:

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Announcement

The Phytochemical Society of Europe Research Award



2000 PSE Research Award Lecturer

Dr. Teris A. van Beek

The Phytochemical Society of Europe Research Award for 2000 (formerly known as the Rhône-Poulenc Rorer Award) has been awarded to Dr. Teris A. van Beek for his outstanding work on chemical ecology and the phytochemical analysis of medicinal plants. Dr van Beek (1956, Haarlem, The Netherlands) studied Pharmacy at Leiden University obtaining his MSc in 1980 and his Pharmacist's degree in 1985. In between he worked as a PhD student at the Department of Pharmacognosy of Leiden University, under the supervision of Professor R. Verpoorte. In 1984 he obtained his PhD degree on a thesis called "Pharmacognostical studies of *Tabernaemontana* species". In 1985 he was appointed as assistant professor in phytochemistry at the Department of Organic Chemistry of Wageningen Agricultural University and in 1992 he was appointed as associate professor. In 1994/1995 he spent his sabbatical leave at the Department of Chemistry of the Virginia Polytechnic Institute (Professor L. Taylor) on supercritical fluid extraction and chromatography.

Dr van Beek's outstanding contribution to phytochemistry resides in the isolation, analysis and quality control of economically relevant compounds from plants, e.g. terpenes from *Ginkgo biloba* and *Taxus baccata*, anthraquinones from *Rubia tinctorum* and antioxidants from various herbs. He is renowned for his research on chemical ecology, in particular for the analysis of compounds involved in plant–insect and insect–insect relationships (pheromones). In his research he has shown a great interest in developing new phytochemical techniques, including the on-line collection and GC analysis of pheromones from intact insects and the on-line HPLC techniques for antioxidant activity. The work of Dr. van Beek and his co-workers is described in more than 110 publications. Recently he edited "*Ginkgo biloba*", Vol. 12 in the series Medicinal and Aromatic Plants — Industrial Profiles. He has presented his work orally at more than 25 international symposia. He is a member of the Editorial Boards of *Phytochemical Analysis* and *Journal of Chromatography A*. From 1993 to 1998 he was the General Secretary of the PSE and was involved in organising the 1992 PSE-meeting "Phytochemistry and Agriculture". Additionally he has helped organise three "PSE-Young Scientists Symposia" (1996, 1998, 2000) and the joint meetings "Communication of Plants with the Environment" (Noordwijkerhout, 1997) and "2000 Years of Natural Products Research — Past, Present and Future" (Amsterdam, 1999). It is hoped that winning the Phytochemical Society of Europe Research Award will encourage him to continue his successful line of research.

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