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FLAVONOIDS AND PHYLOGENY OF THE 'PRIMITIVE' NEW ZEALAND HEPATIC, *HAPLOMITRIUM GIBBSIAE*

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Key Word Index—*Haplomitrium gibbsiae*; Calobryales; Hepaticae; acylated flavone glucosides; apigenin; isoscutellarein; phylogeny; taxonomy.

Abstract—Flavonoid glycosides have been isolated from a species of the (primitive) order of liverworts, Calobryales, for the first time. The endemic New Zealand species, *Haplomitrium gibbsiae*, was found to contain acylated derivatives of apigenin-7-glucoside, apigenin 7,4′-diglucoside and isoscutellarein-7-glucoside, all of which are unique to the Bryophytes. The possible taxonomic and phylogenetic relevance of these findings is discussed.

INTRODUCTION

The hepatics with root-like structures, *Haplomitrium* and *Takakia* are generally grouped together in the order Calobryales in modern classification systems [1, 2]. These liverworts are regarded by many morphologists as primitive or ancestral types [3, 4], *H. gibbsiae*, the endemic New Zealand representative, showing some particularly archaic features [3]. According to these workers, the general direction of evolution in the Hepaticae would be from the isophyllous radially symmetrical type (Calobryales) to the dorsiventral leafy and thallose types (such as the Jungermanniales and Marchantiales), the Calobryales perhaps even representing an intermediate position between the mosses and the hepatics [4]. However, members of the Calobryales also show some advanced features [1, 4] and their position on the evolutionary tree (and in fact the direction of evolution in the whole of the Hepaticae) still remains highly speculative in the absence of a reliable fossil record [5–7].

The present paper discusses the flavonoid chemistry of one of the few species in this order and attempts to apply these new data to the above problem. This work constitutes the first study of the flavonoids in any representative of the order Calobryales.

RESULTS

Two dimensional PC of an acetone–water extract of *Haplomitrium gibbsiae* gametophyte tissue revealed the presence of three major flavonoids. Physical data (see Table 1) indicate that two of the flavonoids (HG-1 and HG-2) are apigenin derivatives and indeed acid hydrolysis of both of these compounds yielded apigenin which was identified by MS and by cochromatography with authentic material. The major flavonoid, HG-3, had an unusual appearance on the PC and on acid hydrolysis gave an aglycone with an MS exhibiting a molecular ion at *m/e* 286 supported by an A-ring fragment of *m/e* 168. This aglycone proved to be spectrally and chromatographically (see Table 1) identical with 5,6,7,4′-tetrahydroxyflavone (scutellarein). However, the UV/visible absorption spectrum of scutellarein differs markedly from that of the original glycoside (which resembles that of 5,7,8,4′-tetrahydroxyflavone, iso-scutellarein) and it is therefore considered that the natural glycoside is an isoscutellarein glycoside which undergoes the expected Wessely–Moser rearrangement on acid treatment [8].

UV/visible spectral data (Table 1) define the glycosylation patterns [9] of HG-1 and HG-2 as 7,4′- and 7-, and the R_f values of these compounds are consistent with di- and mono-*O*-glycoside formulations respectively. Both give glucose as the only sugar on acid hydrolysis and HG-1 is partly converted to HG-2 on mild acid hydrolysis. Glucose is also produced from HG-3 on hydrolysis and the site of glucosylation is considered to be the 7-hydroxyl group rather than the 8- since the UV/visible spectrum of HG-3 is similar to that of isoscutellarein. Glycosylation at C-8 has, in the cases of isoscutellarein 8-*O*-glucuronide [8] and gossypetin 8-*O*-glucoside [9], been shown to cause a marked change in the aglycone spectrum whereas glycosylation at C-7 does not (cf. gossypetin [9]).

This data suggests that HG-2 and HG-1 are the 7- and 7,4′-*O*-glucosides of apigenin and that HG-3 is the 7-*O*-glucoside of isoscutellarein. However, none of these compounds is hydrolysed by β-glucosidase and HG-2 does not cochromatograph with authentic apigenin 7-*O*-glucoside.

The presence of acylation on each glucose unit is indicated by the total conversion in dilute alkali (and partial conversion in dilute acid) of HG-1, 2 and 3 to the chromatographically less mobile compounds, HG-1′, 2′ and 3′. These compounds possess the same phenolic substitution pattern as do their parents, but in contrast are completely hydrolysed by β-glucosidase to their

Table 1. Physical data for *H. gibbsiae* flavonoids

Compound	R_f values, PC (TLC)*			Spot colour	UV/Visible spectra†		
	TBA	HOAc	BzAW	UV–UV (NH_3)	MeOH	NaOMe	$AlCl_3$ (HCl)
HG-1	0.35	0.67		d-d	267, 314, 335 *sh*	286,† 374	—
HG-1′	0.26	0.44					
HG-2	0.60	0.37		d-yg	266, 331	270, 301, 388	273, 297, 338, 380 *sh*
HG-2′	0.53	0.16					
HG-1,2 aglycone	0.9	0.03		d-green	267, 332	275, 323, 391	276, 299, 340, 381
HG-3	0.53	0.22		d-olive	274, 307, 325 *sh*	decomposed	282, 320, 348
HG-3′	0.43	0.11					
HG-3 aglycone	0.59	0.03					
	(0.71)		(0.15)	d-olive	280, 305, 322 *sh*	decomposed	284, 320, 350
Scutellarein	0.47	0.03					
	(0.56)		(0.16)	d-olive	284, 335	decomposed	302, 360
Isoscutellarein	0.58	0.04					
	(0.70)		(0.15)	d-olive	280, 305, 322 *sh*	decomposed	284, 320, 350

* Cellulose TLC. † Shift reagents as described in ref. [9]. ‡ The 286 nm peak is very intense.

respective aglycones. Further. HG-2′ is chromatographically identical with apigenin 7-*O*-glucoside and HG-1′ possesses chromatographic properties similar to those of the previously reported [10] apigenin 7,4′-diglucuronide. The 7,4′-diglucoside has been reported only once before and it does not appear to have been well characterized [11].

The nature of the acyl group has not been positively determined due to the minute amounts of material available. However, TLC analysis of the saponification product using the solvents of Carles *et al.* [16] revealed the presence of two acids, one phenolic (detected with diazotized sulphanilic acid) and the other non-phenolic (detected with bromocresol green). The phenolic acid was identified as *p*-hydroxybenzoic acid by MS (*m/e* 138, M^+; 121, M^+-OH; 93, M^+-(CO_2H) and by further chromatography, but the more dominant non-phenolic acid remains unidentified in spite of extensive TLC comparisons with a wide range of naturally occurring organic acids (see Experimental).

DISCUSSION

The current work is of particular interest because it represents the first study of the flavonoid chemistry of a member of the order Calobryales. The flavonoids isolated are distinct in a number of ways from those previously isolated from other liverworts. Although flavones predominate as they seem to in most of the Bryophytes, the flavone *C*-glycosides, which are commonly found in the orders Jungermanniales, Marchantiales and Metzgeriales, are absent. The occurrence of apigenin glycosides in *H. gibbsiae* is of no particular significance since they occur widely in the bryophytes. However, the presence of isoscutellarein is of interest in that to date it has been isolated from only one other liverwort, *Marchantia berteroana* [8], in which it occurs as the 8-*O*-glucuronide.

The presence of *O*-glucosides is also distinctive since few have been isolated previously from liverworts. For example apigenin and naringenin 7-*O*-glucosides occur in *Riccia crystallina* and isovitexin 7-*O*-glucoside in *Porella platyphylla*, but in neither case does *O*-glucosylation represent the plant's sole form of flavonoid glycosylation as in *H. gibbsiae*. Yet another unusual feature of *H. gibbsiae* is its ability to acylate the sugar of a flavonoid glycoside. Such acylation has not previously been reported in bryophytes, although a caffeic acid diglucoside ester is thought to occur in the moss *Pohlia wahlenbergii* [12]. Also, recent unpublished work in our laboratory has revealed the existence in *Riccia fluitans* of luteolin 7,3′-di-*O*-glucuronide acylated on both sugar units with ferulic acid.

The question of whether the flavonoid biochemistry of *H. gibbsiae* contributes to the problem of the phylogenetic position of the order Calobryales, must wait until some of the remaining nine species in the group have been investigated.

EXPERIMENTAL

A voucher specimen of *Haplomitrium gibbsiae* has been deposited with Massey University, Palmerston North (MPN 17044). 2-D PC was routinely carried out on Whatman 3 mm paper using *t*-BuOH–HOAc–H_2O, 3:1:1 (TBA) and 15% HOAc (HOAc), and TLC on Schleicher and Schüll plastic-backed cellulose and polyamide plates. Spot detection: UV light, UV/NH_3; or spray, Naturstoffreagenz A (Carl Roth) in MeOH. MS were determined by direct insertion on an AEI MS-30 spectrometer and UV/visible spectra were measured as previously described [9].

Isolation procedure. Fresh, clean, *H. gibbsiae* was oven dried at 80° for 1.5 hr to give 0.8 g dry plant material. This was ground finely and extracted several times at 100 M temp. with Me_2CO–H_2O (1:1). The extract was applied to 16 PCs and 2-D PC resolved the major constituents HG-1, 2 and 3, which were subsequently extracted from the paper with MeOH–H_2O. HG-3 was further purified by 1-D PC in H_2O (triple over-run).

Hydrolysis experiments. (a) *Normal acid hydrolysis.* All 3 compounds were converted completely to aglycone material with 1.5 N HCl at 100° for 1 hr. Sugars produced were identified by PC in EtOAc–Pyr–H_2O (12:5:4); spray, aniline HCl. (b) *Mild acid hydrolysis.* Treatment of HG-1, 2 and 3 with 1.5 N HCl at 100° for 10 min gave, in part, glycosidic products with R_f values slightly lower than starting material (see table 1). HG-1 gave HG-2 and HG-2′, HG-2 gave HG-2′ and HG-3 gave HG-3′. (c) *Enzyme hydrolyses.* HG-1, 2 and 3 were unaffected by β-glucosidase or β-glucuronidase (room temp., H_2O, 24 hr). HG-1′, 2′ and 3′ were completely hydrolysed by β-glucosidase to apigenin, apigenin and isoscutellarein respectively. (d) *Alkaline hydrolysis.* Conc aq. solns of the acylated glycoside (2 μl) were thoroughly mixed with 2 N NaOH (5 μl) in a 10 μl microsyringe (using the plunger) and left in the syringe at room temp. with all air expelled. After 2 hr the product was ejected into

3 N HCl (5 μl) and the mixture ether-extracted to give the acid fraction. The H_2O-soluble glycosides, HG-1′, 2′ and 3′ were isolated by PC for further study.

Attempted identification of acid. The acid fraction was co-chromatographed with a wide range of organic acids on cellulose, polyamide and Si gel TLC. Solvents used were (A) *n*-BuOH–formic A–H_2O, 10:1:5 (cellulose); (B) EtOH–0.88 NH_4OH, 95:5 (cellulose); (C) MeCN–EtOAc–HCO_2H, 81:8:9.1:9.1 (polyamide) and (D) EtOAc–toluene, 1:1 (Si gel). Detecting methods: UV, 2% bromocresol green spray (Me_2CO–H_2O) and diazotized sulphanilic acid–Na_2CO_3 spray. By a combination of these procedures (and MS), *p*-hydroxybenzoic acid was identified. The major acid produced, co-chromatographed only with succinic acid out of all the 60 naturally occurring acids for which R_f values have been listed by Carles *et al.* [16] for solvents A and B. Confirmatory evidence however was not obtained.

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NEW ISOFLAVONE GLYCOSIDES FROM THE WOODS OF *SOPHORA JAPONICA*

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The flavonoids of the rhizomes and the fruits of *Sophora japonica* [1–3] have been well studied but less is known of those in the wood. We have isolated biochanin A, irisolidone, maackiain, pratensein, sissotrin, irisolidone 7*4O*-glucoside and rutin [4–6] and now report two new isoflavone glycosides. These were purified by the droplet counter-current chromatography (DCC) [7, 8].

The first glycoside, mp 224–226° afforded on acid hydrolysis biochanin A and glucose. From the UV spectrum it was assumed to be a 7-glucoside [9]. Exhaustive methylation by Kuhn's method gave the methylated product, which showed nine *O*-methyls and the molecular ion peak at *m/e* 720, the typical permethylated diglucose fragment ion *m/e* 423 in the ms. Methanolysis products of the permethyl ether were methyl 2,3,4,6-tetra-*O*-methyl-glucopyranoside, and methyl 2,3,4-tri-*O*-methylgluco-pyranoside. The PMR spectrum of the glycoside shows the presence of two anomeric protons on the β-linkage as judged from the *J* values (δ, 4.62, *d*, *J* = 10Hz, 4.84, *d*, *J* = 6Hz). Thus, it is 7-*O*-[β-D-glucopyranosyl(1-6)-β-D-glucopyranosyl]-biochanin A.

The second glycoside, mp 228–230°, afforded on acid hydrolysis biochanin-A and glucose and xylose and had similar spectral properties to the first compound. Methanolysis of its permethylether gave methyl 2,3,4-tri-*O*-methylglucopyranoside and methyl 2,3,4-tri-*O*-methylxylopyranoside, thus showing that xylose is attached to the 6-position of glucose. The glycosidic bonds are β-configuration by PMR (δ 4.90, *d*, *J* = 10Hz, 5.60, *d*, *J* = 8Hz, respectively). Thus, it is biochanin A 7-(xylosyl(β1 → 6)glucoside).

EXPERIMENTAL

All mps were uncorrected. PMR spectra were taken at 100 MHz in $CDCl_3$ and/or d_5-Pyridine solution and chemical shifts are given in δ (ppm) scale. GLC was run with flame ionization detector using a glass column. PPC was conducted on Toyo Roshi No. 50 using the upper layer of *n*-BuOH–(C_5H_5N–H_2O (6:2:3) and aniline hydrogen phthalate as spray reagent. TLC was performed on Kieselgel G (Merck) using (a) $CHCl_3$–MeOH–H_2O (35:65:40) (lower layer), (b) C_6H_6–Me_2CO (4:1), (c) EtOAc–MeCOEt–HCO_2H–H_2O–C_6H_6 (4:3:1:1:2) (upper layer).

Isolation. Powdered woods of *Sophora japonica* (880g) collected at the Botanic Garden of Nagoya City University were extracted with MeOH for 3 hr on reflux. The MeOH extract was concentrated and partitioned between BuOH and H_2O. The BuOH layer was concentrated and precipitated with Et_2O.