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APIGENIN AND AMENTOFLAVONE GLYCOSIDES IN THE PSILOTACEAE AND THEIR PHYLOGENETIC SIGNIFICANCE

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Key Word Index—*Psilotum*; *Tmesipteris*; Psilotaceae, amentoflavone-*O*-glucosides; apigenin-*O*-glycosides; apigenin *C*-glycosides; phylogeny; taxonomy.

Abstract—Documentation of amentoflavone *O*-glucosides as the predominant flavonoid glycosides in both genera of the Psilotaceae clearly distinguishes this family from all other families of vascular plants. *Psilotum* and *Tmesipteris* also possess apigenin *C*- and *O*-glycosides as common flavonoid types. Apigenin 7-*O*-rhamnoglucoside occurs in both genera and the previously undocumented apigenin 7-*O*-rhamnoglucoside-4′-*O*-glucoside, although identified only in *Tmesipteris*, may also be present in *Psilotum*. The existence of flavone *C*-glycosides in both genera may provide a phytochemical relationship between the Psilotaceae and some ferns. The phylogenetic significance of these results is discussed.

INTRODUCTION

The Psilotaceae is a small, tropical to subtropical family consisting of two genera and about a dozen species. Although the two genera are morphologically distinct, they have been shown to intergrade anatomically and developmentally [1]. The Psilotaceae is generally considered to be the most primitive living family of vascular plants and is usually interpreted as containing the extant members of the Psilophyta, the extinct members of which are likely ancestors of other groups of vascular plants. This phylogenetic position of the Psilotaceae has been challenged by Bierhorst who contends that the Psilotaceae should be included with the filicalean ferns [1, 2]. However, Bierhorst's views do not seem to be supported by phytochemical evidence [3].

Relative to the angiosperms, very little detailed information has been documented for the flavonoids of the ferns (Pteridophyta) or for the Psilotaceae [4]. The only published flavonoid data for the Psilotaceae is that *P. triquetrum* contains the biflavone amentoflavone and possibly hinokiflavone [5, 6] and that *Tmesipteris* produces 'an unknown flavonoid' [3] recently characterized as amentoflavone [5]. Additionally Voirin documents the absence of leucoanthocyanins, flavonols, and flavones in the Psilotaceae [6]. Previous work [5, 6] pointed out the lack of flavonoids other than biflavone aglycones in the Psilotaceae.

The work presented here attempts to establish the genetic potential for the synthesis of flavonoids by the Psilotaceae, through a detailed study of the flavonoids of three representative species. The data are discussed in terms of their significance to an understanding of the phylogenetic position of the Psilotaceae.

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RESULTS

Two indigenous New Zealand species of *Tmesipteris*, *T. tannensis* (Tt) and *T. elongata* (Te), and one of *Psilotum*, *P. nudum* (Pn), were studied. The predominant flavonoid in all three is amentoflavone, the level of which is so high that it masks the presence of many of the glycosidic spots detailed in Fig. 1. The 2D-PCs presented in this Figure represent the patterns of flavonoid glycosides remaining in the extracts after removal of the bulk of the amentoflavone by precipitation.

Purification of individual compounds was generally achieved via repeated paper chromatography, and physical data of each of these compounds are detailed in Table 1. Compounds common to two or more species were each identified independently and the identity then confirmed by cochromatography. Structure assignments of constituents are based as follows.

Amentoflavone

(Te-1, Tt-1 and Pn-1.) PMR and UV spectra agree with literature values [7] and PMR data demonstrate that the linkage is 3′-8″ and not the isomeric 3′-6″ which has been reported to be chromatographically similar [8]. This compound co-chromatographed with an authentic sample of amentoflavone in 5 solvents (TLC).

Amentoflavone O *glycosides*

(Pn-II, III, IV, V, VI; Tt-II, V; Te-II.) Acid hydrolysis of each of these compounds produced amentoflavone and glucose as the only products, so defining them as amentoflavone *O*-glucosides. Based on cochromatography in TBA and HOAc, the glycoside in *T. elongata* (Te-II) appears to be identical with one in *T. tannensis* (Tt-II), and it is likely also that Pn-V is identical with Tt-V. Positive identity however must await the full structure determination of each compound.

Some indication of the glycoside types can be obtained

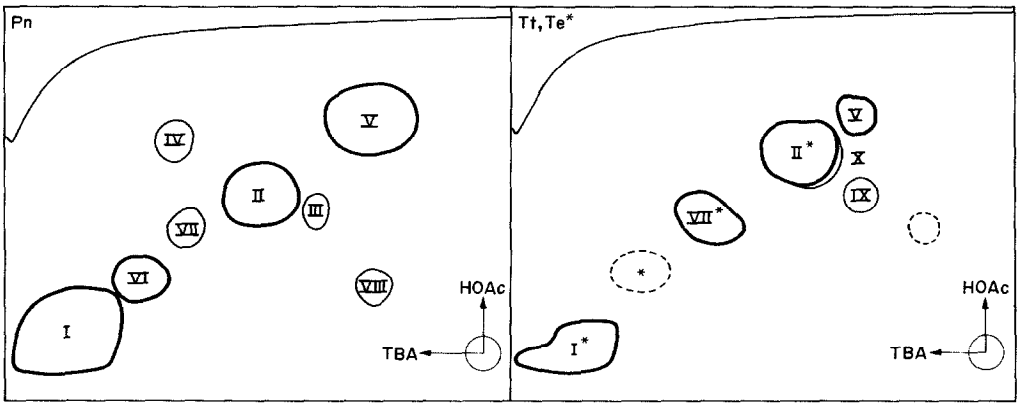

Fig. 1. Schematic diagram of the flavonoids of *Psilotum nudum* (Pn), *Tmesipteris tannensis* (Tt) and *T. elongata* (Te) as observed by 2D PC. Spot numbers for *Tmesipteris* spp. were allocated on the basis of chromatographic similarities with corresponding spots in *P. nudum*. Spots with an asterisk are present in *T. elongata*. Major components are represented by spots in bold outline and minor components by those in normal outline. Dotted spots represent trace components which were not studied.

from a comparison of the R_f values (especially in HOAc). Thus Pn-VI appears to be a monoglucoside, Pn-II, III, IV, T t-II, and Te-II appear to be di- or triglucosides while Tt-V and Pn-V are possibly tetraglycosides. The UV-visible absorption spectra (Table 1) demonstrate that a variety of different glycosylation patterns occur in this group of glycosides. Although it is not possible to assign glycosylation patterns to these compounds with certainty on the basis of chromatographic and spectrophotometric data alone, it is clear that a wide range of different amentoflavone glucosides have been isolated. A detailed chemical study of these glycosides is currently underway.

Apigenin-7-O-rhamnoglucoside

(Pn-VII, Tt-VII, Te-VII.) This is the predominant apigenin-*O*-glycoside in all three species; however it is minor, relative to amentoflavone and its glucosides. Acid hydrolysis of Pn-VII produced apigenin together with equimolar amounts of glucose and rhamnose, but it was unaffected by β-glucosidase. It is thus considered to be apigenin-7-*O*-rhamnoglucoside and its PC appearance,

Table 1. Chromatographic and spectral data of flavonoids from the Psilotaceae (according to ref. [7]).

Compound* (name)	R_f (PC) TBA	HOAc	MeOH	MeO⁻
Amentoflavone (Te–I, Tt–I, Pn–I)	0.88		333, 286 *sh*, 269	384, 275
Amentoflavone-*O*-glucoside (Pn–II)	0.48	0.53	316, 290 *sh*, 268	373‡, 282¶
Amentoflavone-*O*-glucoside (Tt–II, Te–II)	0.43	0.66	330, 270	390¶, 293 *sh*, 270 (*br*)
Amentoflavone-*O*-glucoside (Pn–III)	0.27	0.51	323, 271	391§, 282
Amentoflavone-*O*-glucoside (Pn–IV)	0.60	0.76	317, 270	366§, 287¶
Amentoflavone-*O*-glucoside (Pn–V, Tt–V)	0.2	0.81	313, 270	367‡, 286¶
Amentoflavone-*O*-glucoside (Pn–VI)	0.75	0.30	328, 287 *sh*, 269	377§, 293 *sh*, 280
Apigenin-7-*O*-rhamnoglucoside (Tt–VII, Pn–VII)	0.60	0.50	330, 267	387¶, 353 *sh*, 301 *sh*, 272
Apigenin-7-*O*-rhamnoglucoside -4′-*O*-glucoside (Tt–X)	0.31	0.64	317, 270	375‡, 286¶
Apigenin-glycosides (Tt–IX)	0.28	0.48	328 (*br*), 271	396‖, 323, 284
Apigenin-*C*-glycoside (Pn–VIII)	0.22	0.25	335, 300 *sh*, 272	393¶, 325, 282

* Data quoted refers to first listed compound. † p = purple, ol = olive, yg = yellow-green, d = dark. ‡ Distinct decrease in intensity. § Moderate decrease in intensity. ‖ Little change in intensity. ¶ Moderate increase in intensity.

chromatographic mobility and UV-visible spectra are consistent with published data [7].

Apigenin 7-O-rhamnoglucoside-4′-O-glucoside

(Tt-X). This compound was only detected after the major amentoflavone-glucoside (Tt-II) has been partially separated from it by preparative 1D-PC (TBA; 2× overdevelopment). Its appearance and location on a PC are suggestive of a triglycoside, and spectrophotometric shift data indicate it to be an apigenin glycosylated at both the 7- and 4′-positions (Table 1). Acid hydrolysis produced apigenin together with rhamnose and glucose in the ratio 1:2. Treatment with β-glucosidase gave Tt-VII (apigenin-7-*O*-rhamnoglucoside) thereby defining the structure as apigenin-7-*O*-rhamnoglucoside-4′-*O*-glucoside.

Apigenin C-glycoside

(Tt-IXa.) Spot Tt-IX appears to be a mixture of two compounds, an apigenin *C*-glucoside (Tt-IXa) and an apigenin *O*-glucoside (Tt-IXb). Acid treatment with 2N HCl for up to 2 hr produced apigenin and glucose but left about half of Tt-IX unchanged. The flavonoid unaffected by acid treatment gave UV spectra (little different from Tt-IX, Table 1) which define it as an apigenin derivative in which all three hydroxyl functions are free [7]. Its chromatographic properties are those of a glycoside, and as it is not isomerized by acid it is thought to be an apigenin 6,8-di-*C*-glycoside. This *C*-glycoside cochromatographed with vicenin-2 from *Conocephalum conicum* [10] in TBA, HOAc and EPWM and is therefore considered to be apigenin 6,8-di-*C*-glucoside.

Apigenin O-glycoside

(Tt-IXb.) Only indirect data is available for this compound (cf. Tt-IXa). Acid hydrolysis gave apigenin and glucose and its chromatographic properties are those of an apigenin di-*O*-glucoside [11]. Its appearance on paper indicates that the 5-hydroxyl is free. The UV-visible spectrum of Tt-IX (+NaOMe) shows no sign of a band II absorption at 269 nm which would indicate the presence of an apigenin 7-*O*-diglucoside with the *C*-glycoside Tt-IXa. Instead, band II appears at 285 nm which, in view of the sizeable proportion of Tt-IXb present, suggests that Tt-IXb is apigenin 7,4′-diglucoside. The chromatographic data (TBA, HOAc) are in accord with this assignment [11].

Apigenin-C-glycoside

(Pn-VIII.) This compound occurs in trace amounts and was not detectable by PC of the total plant extract; it was isolated as a minor constituent of the 20% MeOH–80% H_2O fraction from the cellulose column. Spectral data (Table 1) suggest the compound is an apigenin derivative with both the 7- and the 4′-positions unsubstituted [7]. Acid hydrolysis (2 hr, 2N HCl) caused this compound to equilibrate with one of a higher R_f (TLC: TBA, 0.3; HOAc, 0.4) which, when isolated and treated with acid, reconverted in part to the parent compound (R_f TBA, 0.3, HOAc, 0.2). The chromatographic data for these isomers approximate those recorded for the apigenin 6,8-di-*C*-pentosides found in *Hymenophyton flabellatum* [12].

DISCUSSION

In view of the small number of species in each genus, it is considered that the present work establishes amentoflavone and its *O*-glucosides as the major flavonoids of both *Psilotum* and *Tmesipteris*. Apigenin and its *O*-glycosides are minor constituents of both genera as also are apigenin *C*-glycosides.

Of particular phytochemical interest is the finding of a wide range of amentoflavone *O*-glucosides in these plants. Amentoflavone has never before been found to

UV spectral data			Spot Appearance†	
$AlCl_3$	$AlCl_3$/HCl	NaOAc	UV	$+NH_3$
380, 346, 297 *sh*, 273	377, 342, 297 *sh*, 273	372, 274	p	d.ol
	382, 335, 295 *sh*, 279	360–330 *sh*, 312 *sh*, 285, 267	p	p
383 *sh*, 345, 300 *sh*, 269	383, *sh*, 342, 300 *sh*, 268	393, 266 (*br*)	p	ol
377 *sh*, 339, 296 *sh*, 276	376 *sh*, 336, 295 *sh*, 275	381 (*br*), 280 (*br*)	p	p
382, 337, 297 *sh*, 280	382, 334, 297 *sh*, 280	370–350 *sh*, 274	p	p
375*sh*, 334, 295 *sh*, 282	375 *sh*, 333, 294 *sh*, 281	334 (*br*), 276 (*br*)	p	p
382 *sh*, 347, 297 *sh*, 274	377 *sh*, 343, 296 *sh*, 274	358, 271	p	p
372 *sh*, 343, 296 *sh*, 272	370 *sh*, 342, 295 *sh*, 271	386‖, 265	p	yg
			p	p
			p	ol
			p	yg

exist as a glycoside and the occurrence of biflavonyl glycosides in general is exceedingly rare [13]. Thus the presence of amentoflavone glucosides clearly distinguishes this family from all other plants in which flavonoids have been documented [4] and reinforces the close genetic relationship believed to exist between the two morphologically distinct genera of the Psilotaceae [1]. We could not detect hinokiflavone in *P. nudum* and believe that its reported occurrence in *P. triquetrum* [6] should be verified. A number of minor compounds, of appearance and mobility on paper similar to that of amentoflavone, were isolated from *P. nudum* and *T. tannensis* but all differed from authentic hinokiflavone (UV, MS data).

In addition to containing an amentoflavone-*O*-glucoside (Pn-V, Tt-V) in common, the species of both psilotaceous genera also contain apigenin-7-*O*-rhamnoglucoside. *T. tannensis* produces apigenin 7-*O*-rhamnoglucoside-4′-*O*-glucoside (Tt-X) which although not identified in *Psilotum*, nevertheless may be present since the work-up of Pn-II (the major amentoflavone *O*-glucoside) produced traces of apigenin. The same range of flavonoid *O*-glycosides could only partly be recognized in the small sample of *T. elongata* available.

The classical phylogenetic position of the Psilotaceae as the extant family of the Psilophyta, an ancient group of plants dating back to the Devonian, has recently been challenged on morphological and anatomical grounds [1, 2]. No-one has questioned the antiquity of the group, only its taxonomic position. According to Bierhorst it should be considered as the most primitive family of the Filicales (ferns).

The accepted antiquity of the group is mirrored in the structures of the flavonoids it contains. Thus amentoflavone is considered to be the most primitive of the biflavonyls [14] and flavone *C*-glycosides have been recognized as indicators of primitive evolutionary status [15]. The predominance of glucose as the glycosylating sugar may also be a primitive character [11].

There appear to be three possible taxonomic positions for the Psilotaceae relative to the other vascular plants: (i) it may be classified in a distinct division (Psilophyta), (ii) it may be included in the Filicales, or (iii) it may be positioned as an equal subtaxon with the ferns and the lycopods when they are interpreted as belonging to the same division. The many morphological and anatomical features of both sporophyte and gametophyte, shared by the two genera of the Psilotaceae and the primitive fern *Stromatopteris moniliformis* (Stromatopteridaceae) [2] definitely suggest a relationship. Several of the same features are also found in species of the primitive fern families, Schizaeaceae and the Gleicheniaceae [1]. In contrast to this no flavonoid types have been found common to both the Psilotaceae and these primitive ferns [3, 5, 6]. Neither biflavones (amentoflavone or its *O*-glycosides) nor flavones (apigenin or its *O*-glycosides) have been found in *Stromatopteris moniliformis* [3, 5, 19], *Schizeae bifida* (Schizaeaceae), or *Gleichenia cunninghamii* (Gleicheniaceae) [19]. The presence of flavonols and the absence of biflavones in the ferns generally, contrasts with the presence of biflavones and flavones but the absence of flavonols in the Psilotaceae. The present work fully supports earlier observations [3, 5, 6] as even trace amounts of flavonols were not found in either *Psilotum* or *Timesipteris*.

However, some similarities between the flavonoid chemistry of the Psilotaceae and the ferns (and lycopods) are now becoming evident. Our present finding of *C*-glycosylflavones in the Psilotaceae does provide a feature in common with ferns such as the somewhat more advanced, *Sphenomeris* [16], *Cyathea* [26, 27], *Dryopteris* [18] and *Mecodium* (*Hymenophyllum*) [19]. Although flavone *C*-glycosides have not been found in the lycopods, this group of plants is known to possess both flavones and biflavones as flavonoid types in common with the Psilotaceae [20].

Assuming that the retention of chemical characters is as conservative as the retention of morphological features, the Psilotaceae, or their ancestors, could well have given rise to both the ferns (primarily morphological similarity) and the lycopods (primarily chemical similarity). Thus the Psilotaceae appear to be more closely related to both the ferns and lycopods than the divisional classification (Psilophyta, Pteridophyta, Lycopodophyta) implies.

EXPERIMENTAL

Plant material. Psilotum nudum (L) Beauv. (100 g dry wt) was collected 21 December 1976, at The Waimangu Thermal Area, Rotorua; *Tmesipteris tannensis* (Spreng.) Bernh. (18.5 g dry wt) was collected at Howden's Scenic Reserve, Endeavour Inlet, Queen Charlotte Sound on 10 December, 1976; and *T. elongata* Dang. (2 g dry wt) was collected in The Mangakotukutuku Valley on 8 November 1976. Voucher specimens are on file in The Herbarium of Western Carolina University.

Work-up procedure. All plant material was oven dried at 100° and pulverized in a blender. The dried material was extracted ca 18 hr with Me_2CO–H_2O (1:1) and filtered. The solvents were removed *in vacuo*, and the residue taken up in MeOH for chromatography. Unless stated otherwise PCs were developed in *t*-BuOH–HOAc–H_2O (3:1:1) (TBA) and 15% HOAc (HOAc) using Whatman 3 MM paper [7]. Cellulose TLC's (Schleicher and Schull F1440) were developed with TBA, HOAc, or C_6H_6–HOAc–H_2O (125:72:3) (BAH) [21] and Si gel TLCs (Schleicher and Schull F1500) were developed with C_6H_6–Py–HCO_2H (36:9:5) (BPF), C_6H_6–Py–HCO_2Et–dioxan (5:1:2:2) (BPEFD) and C_6H_6–EtOAc–HOAc, (10:3:2) (BEAA) [9]. EtOAc–Py–H_2O–MeOH (16:4:2:1) (EPWM) was used in the comparison of *C*-glycosides. The *T. elongata* extract was processed by heavily loading PCs and developing them in TBA and HOAc; two major and one minor compound were detected. The *T. tannensis* extract was separated into 5 bands with 1D-PC (TBA); subsequently the components of each area were purified using 2D-PC (TBA, HOAc). 4 major and 4 minor compounds were characterized. The *P. nudum* extract was separated into 15 fractions on a cellulose column (Whatman CF-11; 6.5 × 45 cm; development initially with 100% H_2O followed by H_2O–MeOH combinations and finally 100% MeOH). Subsequent PC purification indicated the presence of 2 major and 6 minor flavonoids. (See Table 1 and Fig. 1). For each species the naturally occurring amentoflavone aglycone was further purified from PCs or columns by 1D-TLC (BPF; 1 mm kieselgel HF_{254} nach stahl; E. Merck AG, Darmstadt). Structures were determined using the procedures of ref. [7] (UV, PMR) and by cochromatography of the aglycones of each glycoside in TBA, BAH, BPF, BPEFP and BEAA with authentic apigenin and amentoflavone. Following acid hydrolysis (2 N HCl, 2 hr) the aglycones were recovered by EtOAc extraction. The remaining acid and H_2O were removed *in vacuo* and the sugars identified as their TMSi ethers by GLC on 3% OV-1 (Chromsorb W, acid washed; 60–80 mesh). Enzyme hydrolyses (Koch Light: pectinase from *A. niger* and sweet almond β-glucosidase) were carried out in distilled H_2O (20°).

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