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STEROL COMPOSITION OF PTERIDOPHYTES

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Key Word Index—Pteridophytes; whisk-ferns; horsetails; club-mosses; ferns; chemotaxonomy; sterols; sitosterol; stigmasterol; campesterol; 22-dihydrobrassicasterol.

Abstract—Sixteen species of pteridophytes were examined for their sterol compositions and for the configuration of the substituents at C-24. The 24-ethylsterols existed exclusively as the 24α-epimers sitosterol and stigmasterol. In all 16 species, the 24-methylsterols existed as epimeric mixtures in which campesterol (24α-) was the dominant epimer except in the genera *Lycopodium* and *Selaginella* in which 22-dihydrobrassicasterol (24β-) dominated. The quantitative determinations were made based on 220 MHz ^{1}H NMR and capillary GC. The sterol compositions of pteridophytes from other studies are reviewed and possible biosynthetic pathways in pteridophytes are discussed.

INTRODUCTION

The pteridophytes are the group of seedless, vascular plants consisting of whisk ferns (*Psilotum*), horsetails (*Equisetum*), club-mosses (*Lycopodium*, *Selaginella*) and true ferns. Phylogenetically, they are between the Spermatophyta (seed plants) and the Bryophyta (non-vascular plants). To date, few studies concerning the sterol compositions of pteridophytes have been published [1–13].

In previous studies, sitosterol (24α-ethylcholest-5-en-3β-ol) was shown to be the dominant desmethylsterol in pteridophytes [2–10] based on melting point and optical rotation. However, in studies where absolute configurations were determined by high resolution ^{1}H NMR spectroscopy, the 24-ethylsterol component consisted exclusively of sitosterol without the 24β-epimer clionasterol [11–13]. In this respect, the pteridophytes are similar to the spermatophytes and different from the bryophytes where 24-ethyl epimeric mixtures are known to occur [14]. Conversely, plants are known to contain epimeric mixtures of the 24-methylsterols [11–15]. A phylogenetic transition is exhibited in the ratio of 24α- to 24β-methylsterols from the algae [16], mosses and some liverworts [14, 15] which produce predominantly 22-dihydrobrassicasterol (24β-methylcholest-5-en-3β-ol) to the pteridophytes and spermatophytes which produce predominantly campesterol (24α-methylcholest-5-en-3β-ol) [12]. However, there are reports where campesterol is dominant in some liverworts (bryophytes) [14] and 22-dihydrobrassicasterol is dominant in *Lycopodium complenatum* (pteridophytes) [11–13]. Since the pteridophytes appear to be at the interface of this phylogenetic transition, it was of interest to examine both the sterol composition and the configuration of the methyl and ethyl substituents at C-24 of species in this group.

RESULTS

The total lipid content of the 16 pteridophyte species examined was between 1.30 and 7.44% of the tissue on a dry weight basis (Table 1). The total sterol content was between 0.03 and 0.41% on a dry weight basis (Table 1). Except for *Equisetum arvense* and *Lycopodium obscurum*, the quantity of desmethylsterols exceeded the methylsterols.

In all 16 species, sitosterol (24α-ethylcholest-5-en-3β-ol) was the dominant desmethylsterol (46–87%) (Table 2). After recrystallization from methanol, the melting point of the acetate of the isolated sitosterol was 116–119° (authentic 117–119°) as compared to authentic clionasteryl acetate (24β-epimer) 138–139°. The ^{1}H NMR spectra of the free alcohol did not exhibit multiple signals between 187.5 and 200.3 Hz for protons at C-29 indicating that clionasterol, if present, was below the limits of detection (5–10%) [17]. Additionally, capillary GC analysis of several of the sitosterol isolates did not reveal the presence of clionasterol so additional analyses were not performed.

Fourteen of the 16 species also contained stigmasterol (24α-ethylcholesta-5,22*E*-dien-3β-ol) from trace amounts (<0.5%) to 39% of the desmethylsterols (Table 2). After recrystallization from MeOH, the melting point of the free alcohol was 167–169° (authentic 168–169°) as compared to authentic poriferasterol (24β-epimer) 156–157°. The ^{1}H NMR spectra did not exhibit multiple signals between 182.1 and 185.2 Hz for protons at C-29 indicating that the 24β-epimer, if present, was below the limits of detection [17]. Capillary GC analysis of several of the stigmasterol isolates did not reveal the presence of poriferasterol so additional analysis were not performed.

All 16 species contained 24-methylcholesterol from 9 to 35% of the total desmethylsterols (Table 2). The ^{1}H NMR spectra of the 24-methylcholesterol isolated from these species all exhibited multiple doublet signals between 167.0 and 205.3 Hz for protons at C-21, C-27 and C-28 indicating an epimeric mixture [17]. Estimations of the ratio of campesterol (24α-methylcholest-5-en-3β-ol) to 22-dihydrobrassicasterol (24β-methylcholest-5-en-3β-ol) were made by comparison of the spectra from the isolated mixtures to the spectra of known epimeric mixtures (Table 3), as previously described [17]. Capillary GC analysis confirmed the presence of both epimers and facilitated quantitation (Table 3). In all isolates except

Table 1. A comparison of total lipids, desmethylsterols and methylsterols in 16 species of pteridophytes

Species	% Total lipids*	% Total desmethylsterol*	% Total methylsterol*	% Total sterols*
Psilotum nudum	7.44	0.17	0.03	0.20
Lycopodium cernuum	7.16	0.03	t	0.03
L. clavatum	4.06	0.05	t	0.05
L. complanatum	4.44	0.04	t	0.04
L. obscurum	5.43	0.10	0.13	0.23
Selaginella delicatula	6.76	0.07	t	0.07
S. doederleinii	3.74	0.05	t	0.05
Equisetum arvense	6.39	0.17	0.24	0.41
E. ramosissimum	3.09	0.14	t	0.14
Alsophila spinulosa	3.40	0.14	0.04	0.18
Angiopteris lygodiifolia	4.42	0.14	0.03	0.17
Blechnum orientale	1.30	0.10	t	0.10
Cibotium cumingii	3.61	0.09	0.02	0.11
Diplazium dilatatum	4.19	0.18	t	0.18
Nephrolepis auriculata	4.12	0.24	0.04	0.28
Woodwardia orientalis	2.73	0.09	0.02	0.11

*All percentages are expressed on a dry weight basis; t < 0.5%.

Table 2. Composition* of desmethylsterols in pteridophytes

Species	Desmethylsterols A	B	C	D	E	F
Psilotum nudum	—	t	31	11	58	—
Lycopodium cernuum	t	1	13	39	47	—
L. clavatum	t	t	21	29	50	—
L. complanatum	—	1	18	16	65	—
L. obscurum	t	t	15	15	70	—
Selaginella delicatula	16	2	11	25	46	—
S. doederleinii	1	t	20	23	56	—
Equisetum arvense	—	—	28	t	72	—
E. ramosissimum	t	—	35	t	65	—
Alsophila spinulosa	t	t	19	t	73	8
Angiopteris lygodiifolia	t	1	21	7	71	—
Blechnum orientale	t	t	14	5	81	—
Cibotium cumingii	t	t	16	4	75	5
Diplazium dilatatum	—	t	11	3	86	—
Nephrolepis auriculata	1	t	9	3	87	—
Woodwardia orientalis	1	t	10	4	85	—

*Percent of total desmethylsterol. A: Cholest-5-enol; B: 24-methylcholesta-5,22-dienol; C: 24-methylcholest-5-enol; D: 24-ethylcholesta-5,22-dienol; E: 24-ethylcholest-5-enol; F: 24-ethyl-5α-cholestanol: (t < 0.5% of total desmethylsterol).

Angiopteris lygodiifolia, the ^{1}H NMR estimates were in close agreement with capillary GC results.

DISCUSSION

In the present study, the dominant desmethylsterols are the 24-alkylcholest-5-enols (Table 2). The 24-ethylcholest-5-enol and 24-ethylcholesta-5,22-dienol were present only as the 24α-epimers, sitosterol and stigmasterol respectively (Table 3). In previous studies, the dominant sterol in pteridophytes was shown to be sitosterol (Table 4) based on metling point and optical rotation [2–10]. However, where configurations were determined by high resolution ^{1}H NMR spectroscopy, sitosterol was the only epimer present [11–13]. In the bryophytes (non-vascular plants), although some species produce only the 24α-ethyl epimers, other species also contain clionasterol (24β-ethylcholest-5-enol) as 10–40% of the 24-ethylcholest-5-enol component [14]. In the spermatophytes (seed plants), while there are reports of the isolation of small quantities of 24β-ethylsterols from species in the Cucurbitaceae [18–24], and substantial quantities from species in the Verbenaceae [25–27] Crassulaceae [12] and Caryophyllaceae [28], this is at present atypical of spermatophytes. The pteridophytes, therefore, appear to be more closely allied to the majority of the spermatophytes with respect to their 24-ethylsterol biosynthetic capabilities.

Contrary to the configuration of the 24-ethylsterols, it is well established that plants produce epimeric mixtures of 24-methylsterols [11–15]. In the present study, campesterol (24α-methylcholest-5-enol) was the dominant 24-methylsterol (> 75% of the epimeric mixture) except in the genera *Lycopodium* and *Selaginella* (Table 3). In these two genera, 22-dihydrobrassicasterol (24β-methylcholest-5-enol) formed more than 57% of the 24-methylsterol component. In this respect, the whisk fern (*Psilotum*) and true ferns are similar to the spermatophytes where the 24α-methyl epimer is dominant [11] and the club mosses (*Lycopodium* and *Selaginella*) are more similar to the true mosses and some liverworts (bryophytes) where the 24β-methyl epimer is dominant [14, 15].

The major methylsterols in the present study were cycloartenol (9β,19-cyclo-4,4,14α-trimethylcholest-24-en-3β-ol) and cyclolaudenol (9β,19-cyclo-4,4,14α,24β-tetramethylcholest-25(27)-en-3β-ol). The presence of cyclolaudenol in true ferns has been reported previously [3, 5, 8, 9, 29, 30] and it was suggested to be an intermediate in the synthesis of the 24β-methylsterols [9, 12, 29, 30]. In some fern species, considerable quantities of cyclolau-

Table 3. C-24 epimeric composition of 24-methylcholest-5-enol, 24-ethylcholest-5-enol and 24-ethylcholesta-5,22-dienol in 16 species of pteridophytes*

	24-Methylcholest-5-enol		24-Ethylcholest-5-enol		24-Ethylcholesta-5,22-dienol	
Species	24α-	24β-	24α-	24β-	24α-	24β-
Psilotum nudum	90 (92)	10 (8)	100	0	100	0
Lycopodium cernuum	40 (43)	60 (57)	100	0	100	0
L. clavatum	40 (36)	60 (64)	100	0	100	0
L. complanatum	30 (33)	70 (67)	100	0	100	0
L. obscurum	20 (22)	80 (78)	100	0	100	0
Selaginella delicatula	20 (23)	80 (77)	100	0	100	0
S. doederleinii	20 (22)	80 (78)	100	0	100	0
Equisetum arvense	80 (85)	20 (15)	100	0	—	—
E. ramosissimum	80 (83)	20 (17)	100	0	—	—
Alsophila spinulosa	80 (76)	20 (24)	100	0	100	0
Angiopteris lygodiifolia	90 (82)	10 (18)	100	0	100	0
Blechnum orientale	90 (95)	10 (5)	100	0	100	0
Cibotium cumingii	90 (92)	10 (8)	100	0	100	0
Diplazium dilatatum	80 (82)	20 (18)	100	0	100	0
Nephrolepis auriculata	80 (78)	20 (22)	100	0	100	0
Woodwardia orientalis	80 (76)	20 (24)	100	0	100	0

*Values in parentheses determined by capillary GC.

denol have been isolated along with 31-norcyclolaudenol (Table 4) [3, 5, 29, 30]. In all the true ferns examined here, cyclolaudenol was the major methylsterol isolated and only *Angiopteris lygodiifolia* and *Alsophila spinulosa* also contained trace quantities of cycloartenol. However, in the club mosses *Lycopodium* and *Selaginella*, where 22-dihydrobrassicasterol (24β-methyl epimer) was the major 24-methylsterol, only trace quantities of cyclolaudenol were present.

The sterol biosynthetic capacity of pteridophytes appears to be similar to the spermatophytes. In the first C_1 transfer to cycloartenol, the enzyme systems for the production of both 24α- and 24β-methylsterols are functional. However, in the second C_1 transfer, only the 24α-ethyl epimers are produced. This indicates that the enzyme system for the production of the 24β-ethylsterols, if present, is not functional. Since some species of spermatophytes have been demonstrated to produce 24β-ethyl sterols [12, 18–28] this capacity may have been conserved through the phylogenetic line. This suggests that the capacity to produce 24β-ethylsterols was either lost in most pteridophytes or the ability to express these enzymes was genetically suppressed in the pteridophytes.

EXPERIMENTAL

Plant material. Lycopodium cernuum L., *L. clavatum* L., *L. complanatum* L., *Selaginella delicatula* (Desv.) Alston, *S. doederleinii* Hieron., *Equisetum ramosissium* Desf., *Angiopteris lygodiifolia* Rosenst., *Alsophila spinulosa* (Hook.) Tyron, *Blechnum orientale* L., *Cibotium cumingii* Kunze, *Diplazium dilatatum* Blume, *Nephrolepis auriculata* (L.) Trimen and *Woodwardia orientalis* Sw. were collected in Taiwan. All species identification were independantly confirmed by Ming-Jou Lai of the National Taiwan University. *Lycopodium obscurum* L. and *Equisetum arvense* L. were collected in Maryland. *Psilotum nudum* (L.) Pal. Beauv. was grown in the greenhouse at the University of Maryland.

Extraction and purification of sterols. The plants were washed, cleaned of all necrotic tissue, oven-dried at 80° and then ground to pass a 20-mesh screen. The dry powder was extracted with $CHCl_3$–MeOH (2:1) in a Soxhlet for 24 hr. The extract was evapd under red. pres. and the residue was dissolved in $CHCl_3$ and filtered through Whatman No. 1 filter paper. The solvent of the filtrate was evaporated and the remaining residue was defined as the total lipid.

The total lipid was saponified for 45 min at reflux in 20% KOH (w/v) in 60% EtOH. The soln was acidified with 6N HCl and partitioned against Et_2O. The Et_2O fraction was reduced to dryness and the residue was dissolved in BCl_3–MeOH and heated for 5 min. This solution was then partitioned against hexane.

The sterols were isolated from the saponification and methylation products by Al_2O_3 CC by eluting with hexane, hexane–C_6H_6 (1:1), C_6H_6 and Et_2O as previously reported [14]. The Et_2O fraction was rechromatographed by Al_2O_3 CC by eluting with increasing gradients of Et_2O in hexane. The desmethylsterols were eluted in the 60–100% Et_2O in hexane fraction.

The individual sterol components were separated and purified by repetitive chromatography on lipophilic Sephadex (eluted with 5% hexane in MeOH) and Anasil B chromatography (eluted with 20% Et_2O in hexane).

Sterol identification. The major sterols were identified and quantitated on a 3% SE-30 column (2 m × 2 mm) at 245° in a Varian Model 3700 gas chromatograph equipped with a CDS data system. ^{1}H NMR was performed at 220 MHz at 20° in $CDCl_3$ with TMS as int. standard. The 24α- and 24β-epimers were estimated by comparison of the chemical shifts of the ^{1}H NMR spectra with those of authentic standard mixes [17]. The TMS ethers of the C-24 epimeric mixtures were analysed

Table 4. Sterols of pteridophytes

Species	Sterols	Reference
Whisk ferns		
Psilotum nudum	B, C*, D, E, J, K	†
Club mosses		
Lycopodium cernuum	A, B, C*, D, E, J	†
L. clavatum	A, B, C*, D, E, J	†
L. complanatum	B, C*, D, E, J	†
L. complanatum	C*, D, E, G*	11, 12
L. obscurum	A, B, C*, D, E, J, K	†
Selaginella delicatula	A, B, C*, D, E	†
S. doederleinii	A, B, C*, D, E	†
Horsetails		
Equisetum arvense	C*, D, E, J, K	†
E. ramosissimum	A, C*, D, E	†
True ferns		
Acrostichum aureum	E	2
Alsophila spinulosa	A, B, C*, D, E, F, J, K	†
Angiopteris lygodiifolia	A, B, C*, D, E, F, J, K	†
Asplenium trichomanes	J	9, 10
Blechnum orientale	A, B, C*, D, E	†
Ceterach officinarum	J	3
Cheilanthes longissima	E	4
C. marantae	E	7
Cibotium cumingii	A, B, C*, D, E, F, J	†
Cyathea spinulosa	E	12
Dennstaedtia puctilobula	A, C*, E	3, 11, 12
Diplazium cumingii	B, C*, D, E	†
Dryopteris filix-mas	A, C, E, J	8
D. noveboracensis	A, C*, E, H, I	11, 12
Nephrolepis auriculata	A, B, C*, D, E, J	†
Osmunda cinnamonea	A, C*, E	12
Phyllitis scolopendrium	J	3
Polystichum acrostichoides	A, C*, E	12
P. filix	A, C, E	8
Polypodium vulgare	A, E, J, L, M, N, O	3, 5, 30, 31
Pyrrosia lingua	E	10
Woodwardia orientalis	A, B, C*, D, E, J	†

Sterols: A = cholest-5-enol; B = 24-methycholesta-5,22-dienol; C = 24-methylcholest-5-enol; D = 24,-ethylcholesta-5,22-dienol; E = 24-ethylcholest-5-enol; F = 24-ethyl-5α-cholestanol; G = 24-methycholesta-5,7,22-trienol; H = cholest-7-enol; I = 24-ethylcholest-7-enol; J = cyclolaudenol; K = cycloartenol; L = cycloartanol; M = 31-norcyclolaudenol; N = 31-norcycloartanol; O = pollinasterol.

*Contains epimeric mixture.

†This work.

separately by capillary GC on a 100 m × 0.25 mm glass capillary column coated with SP-2340 (Quadrex Corp., New Haven, CT) in a Varian Model 3400 gas chromatograph as previously described [31]. Mps: uncorr.

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