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STEROLS OF BRYOPHYTES

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Abstract—Sterol compositions of six species of bryophytes were studied. The major sterols identified were campesterol, 22-dihydrobrassicasterol, sitosterol, clionasterol, stigmasterol, cholesterol, 24-methyl-5,22-cholestadienol and 24-methyl-5,7,22-cholestatrienol. The quantitative determinations of the α- and β-epimers of 24-methyl and 24-ethylcholesterols were made based on 220 MHz ^{1}H NMR spectroscopy and capillary gas chromatography. Sterol compositions of bryophytes from other studies are reviewed, and possible sterol biosynthetic pathways in bryophytes are discussed.

INTRODUCTION

Bryophytes are plants possessing dominant leafy or thallial haploid plant bodies (gametophytes). Although most of them are of terrestrial habitats, they have no vascular system, and require water to carry out fertilization to complete the life cycle. Therefore, phylogenetically, they are between the 'higher' vascular plants (e.g. angiosperms, gymnosperms) and 'lower' thallophytes (e.g. algae, fungi). The sterol composition of this group of plants has not attracted much attention, and only a few studies concerning the identification of major sterols of bryophytes have been published [1–10]. Generally, the major desmethyl sterols were reported to be sitosterol, campesterol, and stigmasterol, commonly found in higher vascular plants.

Sitosterol, campesterol and stigmasterol are 24α-epimers of 24-ethylcholesterol, 24-methylcholesterol and 24-ethyl-5,22-cholestadienol respectively. With the exception of *Palavicinnia lyellii* in which Adler [11] found a mixture of 24α- and 24β-methylcholesterol, no 24β-epimers of the above have been found in bryophytes. Infrared spectroscopy, mass spectroscopy and conventional gas-liquid chromatography do not differentiate between sterols epimeric at C-24. Their identifications can be based on melting points, specific optical rotations at a fixed wavelength [12, 13] and specific chemical shifts in nuclear magnetic resonance. However, the differences in melting points and specific rotations are very often so small in some epimeric pairs that they do not provide reliable measurements.

Recently, by using high resolution ^{1}H NMR spectroscopy [14–20] and capillary gas-liquid chromatography [21], the 24α- and 24β-epimers of several sterols have been distinguished from one another, and separated. In the present paper, the sterol compositions of six species of bryophytes were determined and 220 MHz ^{1}H NMR spectroscopy and glass capillary gas chromatography were used to differentiate the 24α- and 24β-epimers of 24-methylcholesterol, 24-ethylcholesterol and 24-ethyl-5,22-cholestadienol in these plants. The plants studied were *Conocephalum conicum* and *Marchantia diptera* (liverworts from the Order Marchantiales); *Bazzania* sp. and *Mastigophora diclados* (leafy liverworts from the Order Jungermanniales); *Plagiomnium succulentum*, and *Sphagnum palustre* (mosses). The results obtained from these plants are compared with previously reported data for other bryophytes.

RESULTS AND DISCUSSION

The sterol content of the bryophyte species studied varied from 0.04% to 0.21% of the tissue dry weight (Table 1). Except for *Mastigophora diclados*, the plants contained more desmethylsterols than methylsterols. The desmethylsterol compositions observed from bryophytes in this work and from the recent literature are listed in Table 2. 24-Methylcholesterol, 24-ethylcholesterol and 24-ethyl-5,22-cholestadienol were the major sterols along with small amounts of 24-methyl-5,7,22-cholestatrienol. In the present work the epimeric compositions of the 24-methylcholesterol, 24-ethylcholesterol and 24-ethyl-5,22-cholestadienol were analyzed by comparing the ^{1}H NMR spectra with those of authentic standard mixtures [20] and the first two were also analyzed by glass capillary gas chromatography (Table 3). The data indicate that the 24-methylcholesterol in all the bryophyte species was actually a mixture of campesterol (24α-epimer) and 22-dihydrobrassicasterol (24β-epimer). The 24α-epimers made up 20% to 80% of the 24-methylcholesterol fraction. Epimeric mixtures were also found in the 24-ethylcholesterol fraction. Although sitosterol (24α-epimer) was shown to exist as the only epimeric form of 24-ethylcholesterol in some species, 10–40% of clionasterol (24β-epimer) was found in some other bryophyte species. On the contrary, stigmasterol (24α-epimer) was the only epimeric form of 24-ethyl-5,22-cholestadienol which existed in all the bryophytes studied here. The sterol

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Table 1. A comparison of total lipids, desmethylsterols and methyl sterols in six species of bryophytes

Species	% total lipids*	% total desmethyl sterols*	% total dimethyl sterols*	% total sterols*
Conocephalum conicum	7.40	0.20	t	0.20
Marchantia diptera	3.11	0.14	t	0.14
Bazzania sp.	2.96	0.05	0.02	0.07
Mastigophora diclados	3.06	0.08	0.13	0.21
Plagiomnium succulentum	2.93	0.07	0.01	0.08
Sphagnum palustre	1.81	0.03	0.01	0.04

*All percentages are expressed on a dry weight basis; t, trace amount.

Table 2. Composition* of desmethylsterols in bryophytes

Species	Desmethylsterols A	B	C	D	E	F	Reference
Liverworts							
Conocephalum conicum	2	2	—	41	37	18	This work
Marchantia diptera	1	2	—	48	35	14	This work
Bazzania sp.	1	10	—	26	36	27	This work
Mastigophora diclados	2	9	—	31	37	21	This work
Jungermannia thermarum	3	7	—	30	41	19	[10]
J. torticalyx	3	13	—	25	46	13	[10]
Scapania undulata	1	10	—	31	41	17	[10]
Macrodiplophyllum plicatum	4	9	—	20	22	45	[10]
Chiloscyphus polyanthus	7	12	—	18	57	20	[10]
Heteroscyphus bescherellei	2	8	—	21	39	30	[10]
Plagiochila japonica	1	7	—	19	38	35	[10]
P. ovalifolia	5	8	—	22	52	33	[10]
Scapania parvitexta	1	14	—	29	39	17	[10]
Palavicinnia lyellii	—	—	—	52	15	33	[11]
Mosses							
Plagiomnium succulentum	2	3	—	27	36	32	This work
Sphagnum palustre	5	4	—	16	31	44	This work
Brachythecium rivulare	—	—	30	—	7	63	[5]
Camoylopus introflexus	—	—	36	3	10	51	[5]
Ctenidium molluscum	—	—	25	—	20	55	[5]
Racomitrium lanuginosum	—	—	—	26	61	13	[5]
Scleropodium touretti	—	—	—	5	30	65	[5]

*Percent of total desmethylsterol. A: 5-cholestenol (cholesterol); B: 24-methyl-5,22-cholestadienol; C: 24-methyl-5,7,22-cholestatrienol; D: 24-methyl-5-cholestanol; E: 24-ethyl-5,22-cholestadienol; F: 24-ethyl-5-cholestenol.

composition of more than 30 other species of bryophytes have been studied by several investigators [1–10]. The results are summarized in Table 4. The desmethyl sterols identified in these species were sitosterol (or 24-ethylcholesterol), campesterol (or 24-methylcholesterol), stigmasterol (or 24-ethyl-5,22-cholestadienol), cholesterol, ergosterol, and 24-methyl-5,22-cholestadienol. However, clionasterol identified in the present study has not been previously reported in bryophytes. Because of technical limitations, many previous studies did not attempt to distinguish between the C-24 epimeric isomers of 24-methylcholesterol and 24-ethylcholesterol. Our study suggests that the 24β-epimer of 24-methylcholesterol may exist in a significant amount in many bryophyte species. Clionasterol may also exist in many of the bryophyte species listed in Table 4. Our study indicates that at least 4% of the total desmethylsterol in *Sphagnum palustre* is clionasterol. It is probable that the sitosterol identified in the five species of *Sphagnum* shown in Table 4 was a mixture of sitosterol and clionasterol.

The major methylsterols identified in this study were cycloartenol and cyclolaudenol. In *Mastigophora diclados*, cycloartenol makes up more than 60% of the methylsterols, and significant amounts were also found in *Bazzania*. The methylsterols reported in other studies were 31-nor-cyclolaudenol, 24-methylenecycloartanol,

Table 3. C-24 epimeric composition of 24-methylcholesterol, 24-ethylcholesterol and 24-ethyl-5,22-cholestadienol in six species of bryophytes*

Species	24-methyl-5-cholestenol		24-ethyl-5-cholestenol		24-ethyl-5,22-cholestadienol	
	24α	24β	24α	24β	24α	24β
LIVERWORTS						
Marchantiales						
Conocephalum conicum	80(72)	20(28)	100(100)	0 (0)	100	0
Marchantia diptera	70(68)	30(32)	60(68)	40(32)	100	0
Jungermanniales						
Bazzania sp.	70(61)	30(39)	100(100)	0 (0)	100	0
Mastigophora diclados	40(43)	60(57)	90(88)	10(12)	100	0
MOSSES						
Bryales						
Plagiomnium succulentum	30(31)	70(69)	100(100)	0 (0)	100	0
Sphagnales						
Sphagnum palustre	20(23)	80(77)	90(84)	10(16)	100	0

*Values given were determined by 220 MHz ^{1}H NMR spectroscopy. Values in parentheses were obtained by capillary GC.

cycloeucalenol, cyclolaudenol and obtusifoliol [2–7].

The identification of methylsterols in bryophytes provides information on the possible biosynthetic pathway of sterols. Goad *et al.* [22] proposed that 24β-methyl sterols were formed through $\Delta^{25(27)}$-sterol intermediates in primitive plants based on the evidence that cyclolaudenol and 31-nor-cyclolaudenol were found in *Polypodium vulgare* [23]. Nes *et al.* [17] proposed that 24β-methylcholesterol in tracheophytes is also formed from the 24β-methyl-$\Delta^{25(27)}$-intermediates. Both cyclolaudenol and 31-nor-cyclolaudenol have been detected in bryophytes [2, 3, 5–7] suggesting that the same pathway may be applied to the formation of 24β-methylcholesterol (22-dihydrobrassicasterol). 24-Methylenesterols were suggested to be precursors for 24α-methylcholesterol and 24α-ethylcholesterol in higher plants [17, 22]. The formation of 24β-ethylcholesterol in tracheophytes was proposed by Nes *et al.* [17] to go through the 24-methylene precursor as in green algae [22]. The presence of 24-methylenecycloartenol [2–4, 6], cycloeucalenol [5], and obtusifoliol [5], supports the possibility of having 24-methylenesterols as intermediates for the formation of 24α-methyl, 24α-ethyl and 24β-ethylsterols in bryophytes. It has been demonstrated that poriferasterol can be formed from clionasterol through dehydrogenation at C-22 [24, 25]. The same mechanism was suggested for the formation of stigmasterol from sitosterol in tracheophytes [17]. However, this mechanism is not operative in *Chlorella* [26] and no evidence of its occurrence is known in bryophytes.

Several species of bryophytes studied contained over 50% of their 24-methylcholesterol as the 24β-form. In contrast, in higher plants the 24β-form is about one-third of the total while most algae and fungi contain only the 24β-epimer. Likewise higher plants have been shown to produce sitosterol 24α, but (using modern techniques) no clionasterol 24β. Algae produce clionasterol but no conclusive demonstration of the presence of sitosterol has been made. The demonstration of greater percentages of 24β-methyl sterols and the presence of 24-ethyl epimeric mixtures in bryophytes is in accord with their accepted place between the thallophytes and the tracheophytes.

EXPERIMENTAL

Sources of plant materials. Six species of bryophytes were collected for chemical analysis. They were: *Conocephalum conicum, Marchantia diptera, Bazzania* sp., *Mastigophora diclados, Plagiomnium succulentum* and *Sphagnum palustre.* (All were collected in Taiwan.)

Extraction and purification of sterols. The collected plant materials were oven dried at 65–80°, and then ground to pass a 20-mesh screen. The dry powder was extracted with $CHCl_3$–MeOH (2:1) for 24 hr with a Soxhlet extractor. The extract was evaporated under red. pres., the residue was redissolved in $CHCl_3$ and filtered through Whatman ≠1 filter paper. The solvent of the filtrate was evaporated and the remaining residue is defined as the total lipid of the plant.

The total lipid was saponified with a 20% solution of KOH in 60% EtOH by refluxing for 45 min. The soln was then acidified with 6 N HCl and the lipid partitioned with Et_2O. The Et_2O fraction was collected, dried, dissolved in a small amount of BCl_3–MeOH and heated for 5 min. This soln was then partitioned with hexane.

The hexane extract was applied to a 5.5 cm i.d. glass column packed with 200 g of alumina (Act II-III, pH 10 from Brockmann). Hydrocarbons, fatty acid methyl esters, sterols and fatty alcohols were eluted, respectively with 400 ml fractions of hexane, hexane–C_6H_6 (1:1), C_6H_6, and Et_2O. The Et_2O fraction containing sterols and long chain fatty alcohols were applied to a 2.6 cm i.d. glass column packed with 100 g of alumina, and eluted with increasing gradients of Et_2O in hexane. Long chain fatty alcohols eluted first in 50–60% Et_2O in hexane. Desmethylsterols eluted next in 60–100% Et_2O in hexane. The 24-methylcholesterol, 24-ethylcholesterol and 24-ethyl-5,22-cholestadienol in the desmethylsterol fraction were purified by repetitive chromatography on lipophilic Sephadex (eluted with 5% hexane in MeOH), and Anasil B column chromatography (eluted with 20% Et_2O in hexane). Data concerning fatty acids and fatty alcohols will be reported elsewhere.

Table 4. Sterols of bryophytes

	Sterols	Reference
LIVERWORTS		
Order Jungermanniales		
Scapania undulata	A, B, D, E, F	[10]
Scapania parvitexta	A, B, D, E, F	[10]
Chiloscyphus polyanthus	A, B, D, E, F	[10]
Heteroscyphus bescherellei	A, B, D, E, F	[10]
Jungermannia thermarum	A, B, D, E, F	[10]
Jungermannia torticalyx	A, B, D, E, F	[10]
Macrodiplophyllum plicatum	A, B, D, E, F	[10]
Plagiochila ovalifolia	A, B, D, E, F	[10]
Plagiochila japonica	A, B, D, E, F	[10]
Bazzania sp.	A, B, D, E, F	*
Mastigophora diclados	A, B, D, E, F	*
Order Metzgeriales		
Pallavicinnia lyellii	D, E, F	[11]
Order Marchantiales		
Conocephalum conicum	A, B, D, E, F	*
Marchantia diptera	A, B, D, E, F	*
MOSSES		
Order Hypnobryales		
Abietinella abietina (=*Thuidium*)	E	[9]
Abietinella abietina (=*Thuidium*)	D, E, F	[6]
Brachythecium rivulare	C, E, F	[5]
Climacium dendroides	D, E, F	[6]
Ctenidium molluscum	C, E, F	[5]
Hypnum cupressiforme	D, E, F	[6]
Platyhypnidium riparioides	C, E	[2]
Pseudoscleropodium purum	D, E, F	[7]
Rhytidiadelphus squarrosus	D, E, F	[6]
Rhytidiadelphus triquetrus	E	[2]
Thuidium tamariscifolium	D, E, F	[4]
Order Andreaeales		
Andreaea rupestris	E, F	[2]
Order Dicranales		
Campylopus introflexus	C, D, E, F	[5]
Dicranum elongatum	A D, E, F	[3]
Leucobryum glaucum	E	[2]
Order Isobryales		
Neckera crispa	D, E, F	[6]
Thamnium alopecurum	C E, F	[8]
Order Cottiales		
Tortella inclinata	E	[9]
Order Polytrichales		
Polytrichum formosum	D, E, F	[6]
Order Grimmiales		
Racomitrium lanuginosum	D, E, F	[5]
Racomitrium lanuginosum	C, E, F	[2]
Order Hookeriales		
Hookeria lucens	E	[2]
Order Bryales		
Plagiomnium succulentum	D, E, F	*
Order Sphagnales		
Sphagnum recurvum	C E, F	[2]
Sphagnum teres	D, E, F	[6]
Sphagnum sp.	E, F	[1]
Sphagnum palustre	D, E, F	*

*This work.

Sterols: A = 5-cholestenol; B = 24-methyl-5,22-cholestadienol; C = 24-methyl-5,7,22-cholestatrienol; D = 24-methyl-5-cholestenol; E = 24-ethyl-5-cholestenol; F = 24-ethyl-5,22-cholestadienol.

Identification of sterols. The major sterols were identified and quantitated by comparison with authentic standards on a Varian Model 3700 gas chromatograph with a CDS data system, using a 3% SE-30 column at 245°.

Sterol peaks seen on GC were isolated by Sephadex chromatography, recrystallized in MeOH, and dissolved in 0.5–0.7 ml Silanor-C $CDCl_3$ with 1% tetramethylsilane for ^1H NMR analysis. The ^1H NMR spectroscopy was performed on a Varian HR-220 NMR spectrometer (220 MHz) under the conditions previously described [20]. The amount of 24α- and 24β-epimers of each sterol were estimated by comparing the chemical shifts of the ^1H NMR spectra with those of authentic standards [20]. Capillary GC was performed as previously described [21].

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