Sterols and Fatty Acids of a Whisk Fern *Psilotum nudum*

Toshihiro Akihisa[a,*], Takeshi Kawashima[a], Shushichi Takahashi[b], Norio Sahashi[c], Takahisa Okamoto[d], Isao Niiya[d] and Toshitake Tamura[a]

[a]College of Science and Technology, Nihon University, Tokyo 101, Japan, [b]College of Pharmacy, Nihon University, Narashinodai, Funabashi, Chiba 274, Japan, [c]School of Pharmaceutical Sciences, Toho University, Miyama, Funabashi, Chiba 274, Japan and [d]Japan Institute of Oils & Fats, Other Foods Inspection, Foundation, Nihonbashi, Chuo-ku, Tokyo 103, Japan

The sterols and fatty acids of *Psilotum nudum* were investigated. The 4,4-dimethyl- and 4α-methylsterol fractions contained 24β-methyl-Δ ^{25}-unsaturated sterols, *viz.*, cycloladenol and 24β-methyl-25-dehydrolophenol, respectively, as dominant sterols among the other components common in vascular plants. 24-Methylcholesterol (mixture of C-24 epimers) and sitosterol constituted the dominant sterols in the 4-demethylsterol fraction. This is the first identification of 24-methylene-5α-lanost-8-en-3β-ol, 24β-methyl-25-dehydrolophenol, codisterol, isofucosterol, 24-methylene-25-methylcholesterol and avenasterol in a fern. The major fatty acids were 16:0, 18:1, 18:2, 18:3 and 20:3. In addition, several C_{20} fatty acids with various unsaturation were found to be present in low concentrations.

KEY WORDS: Fatty acids, 24β-methyl-25-dehydrolophenol, 24β-methyl-Δ ^{25}-sterols, *Psilotum nudum*, sterols, whisk fern.

The small family, Psilotaceae, of pteridophytes consists of about ten species in the two genera, *Psilotum* and *Tmesipteris*, and is generally considered to be closely related to the other ferns, *viz.*, club-mosses, horsetails and true ferns. Phenolic compounds of some members of Psilotaceae have been studied, and it has been suggested that psilotin, hydroxypsiotin and amentoflavone are the chemical markers peculiar to this family (1). In connection with this, we have been interested in examining the lipid constituents of psilotaceous plants. This paper describes our thorough investigations on the sterols and fatty acids of *Psilotum nudum* Griseb.

EXPERIMENTAL PROCEDURES

General methods. Preparative thin-layer chromatograph (TLC) plates (silica gel) were developed three times with hexane/EtOAc (6:1, vol/vol). Argentation preparative TLC: system 1 (for steryl acetates), silica gel/$AgNO_3$ (4:1) developed three times with CCl_4/CH_2Cl_2 (5:1, vol/vol); system 2 (for fatty acid methyl esters), silica gel/$AgNO_3$ (9:1, w/w), developed once with hexane/EtOAc (95:5, vol/vol). High-performance liquid chromatography (HPLC) was performed with a Beckman (Altex) Ultrasphere ODS 5μ column (25 cm × 10 mm i.d.) (Beckman Instruments, Inc., San Ramon, CA), a refractive index detector and methanol as the mobile phase (flow rate 4 mL/min). Gas-liquid chromatography (GLC): system 1 (for steryl acetates), DB-17 fused-silica capillary column (30 m × 0.3 mm i.d.), column temperature 275°C; system 2 (for fatty acid methyl esters), SILAR-5CP deactivated stainless-steel capillary column (50 m × 0.25 mm i.d.), column temperature 197°C. Relative retention times (RRT) of steryl acetates on HPLC and GLC are expressed relative to cholesterol (6a) acetate.

*To whom correspondence should be addressed at College of Science and Technology, Nihon University, 1—8, Kanda Surugadai, Chiyoda-ku, Tokyo 101, Japan.

The electron-impact mass spectrometry (EI-MS) was run on a Hitachi M-80B double-focusing gas chromatograph/mass spectrometer (GC/MS) (70 eV) (Hitachi Seisakusho Ltd., Tokyo, Japan) with a direct inlet system. The MS data do not include peaks at $m/z < 200$. The proton nuclear magnetic resonance (^{1}H NMR) (400 MHz) spectra were recorded on a JNM GX-400 spectrometer (Japan Electron Optics Laboratory Co., Tokyo, Japan) in $CDCl_3$ with $SiMe_4$ as internal standard. Saponification of the plant extract was carried out by reflux with 5% KOH in 90% methanol for 3 h, and the unsaponifiable lipids were extracted with diisopropyl ether. The fatty acids in the aqueous layer were extracted by hexane after acidification with dil. HCl. Acetylation of sterols was performed in Ac_2O-pyridine at room temperature overnight. Methyl ester derivatives of fatty acids were prepared by the diazomethane method. The reference compounds of sterols used were described recently (2). Fatty acid methyl esters used as the authentic standards were either purchased from Sigma Chemical Co. (St. Louis, MO) or separated from cultured yellowtails (3). The plant materials of *Psilotum nudum* were collected at the botanical garden of the College of Pharmacy, Nihon University (plant material I), and at Oahu island of Hawaii in 1990 (plant material II).

Isolation of sterols. Plant material I: Fresh aerial parts (A.P.) (537 g) were oven-dried, powdered (121 g) and Soxhlet-extracted for the lipids (5.3 g). Saponification of the lipids gave unsaponifiable lipids (954 mg) and fatty acids (2.0 g). Repeated preparative TLC of the unsaponifiable lipids yielded purified 4,4-dimethyl- (45 mg), 4α-methyl- (35 mg) and 4-demethylsterol fractions (111 mg). The same procedure was followed for the fresh rhizomes (232 g), which yielded dried and powdered rhizomes (38 g); extracted lipids (1.1 g); unsaponifiable lipids (139 mg); fatty acids (650 mg); 4,4-dimethylsterols (3 mg); 4α-methylsterols (3 mg); 4-demethylsterols (41 mg). Plant material II: The same procedure as above for the fresh A.P. (915 g) afforded the following: dried and powdered A.P. (183 g); extracted lipids (6.8 g); unsaponifiable lipids (1.2 g); fatty acids (3.0 g); 4,4-dimethylsterols (69 mg); 4α-methylsterols (45 mg); and 4-demethylsterols (123 mg). Individual sterol fractions were then acetylated, and the isolation of each component as the acetate was carried out by argentation TLC followed by HPLC. The compositions of individual sterol fractions listed in Table 1 were determined based on the argentation TLC, GLC, HPLC and ^{1}H NMR data. The melting point (m.p.), MS and ^{1}H NMR data for the acetate of 5g was given below.

4α,24β-Dimethyl-5α-cholesta-7,25-dien-3β-ol (5g; *24β-methyl-25-dehydrolophenol) acetate.* M.p. 171–173°C (uncorrected; crystallized from acetone-methanol). MS, *m/z* (assignment, rel. int.): 454.3782 ($C_{31}H_{50}O_2$, $[M]^+$, 63, calcd. 454.3808), 439.3584 ($C_{30}H_{47}O_2$, 32), 394.3544 ($C_{29}H_{46}$, 8), 379.3318 ($C_{28}H_{43}$, 12), 356.2697 ($C_{24}H_{36}O_2$, 6), 327.2295 ($C_{22}H_{31}O_2$, 100), 313.2161 ($C_{21}H_{29}O_2$, 6), 302.2239 ($C_{20}H_{30}O_2$, 9), 287.2050 ($C_{19}H_{27}O_2$, 8), 269.2236 ($C_{20}H_{29}$, 42), 241.1948 ($C_{18}H_{25}$, 18) 227.1807 ($C_{17}H_{23}$, 30), 213.1666 ($C_{16}H_{21}$, 8). ^{1}H-NMR data (multiplicity, assign-

TABLE 1

Chromatographic Data and Compositions (%) of 4,4-Dimethyl-, 4α-Methyl-, and 4-Demethylsterol Fractions from the Aerial Parts (A.P.) and Rhizomes of *Psilotum nudum*

Sterol[a]	Acetate RRT[b]		Compositions (%)[c]		
			I		II
	HPLC	GLC	A.P.	Rhizomes	A.P.
4,4-Dimethylsterol (triterpene alcohol) fraction					
1a 9β,19-Cyclolanostanol (cycloartanol)	1.28	1.50	1.8[d]	1.6	1.2[d]
1b 24-Dehydrocycloartanol (cycloartenol)	1.00	1.81	63.4[d]	27.3	30.0[d]
1f 24-Methylenecycloartanol	1.09	1.99	17.6[d]	33.7	42.4[d]
1g 24β-Methyl-25-dehydrocycloartanol (cyclolaudenol)	1.07	1.95	11.1[d]	15.5	22.5[d]
2f 24-Methylenelanost-8-enol	1.00	1.72	1.5[d]	3.8	1.5
8 Urs-12-enol (α-amyrin)	1.08	1.91	1.3	8.1	1.3[d]
9 Olean-12-enol (β-amyrin)	0.99	1.69	1.1	6.0	0.4[d]
Others, unidentified			2.2	4.0	0.7
4α-Methylsterol fraction					
3f 24-Methylene-31-norcycloartanol (cycloeucalenol)	0.97	1.72	3.1[d]	4.8	3.0[d]
4f 24-Methylene-31-norlanost-8-enol (obtusifoliol)	0.89	1.47	4.4[d]	12.3	11.7[d]
4g 24β-Methyl-31-norlanosta-8,25-dienol	0.87	1.45	2.0[d]	7.2	4.2
5f 4α-Methyl-24-methylenecholest-7-enol (24-methylenelophenol)	0.93	1.72	25.1[d]	19.2	22.0[d]
5g 24β-Methyl-25-dehydrolophenol	0.92	1.68	20.9[d]	21.9	18.8[d]
5j 24Z-Ethylidenelophenol (citrostadienol)	1.21	2.22	43.7[d]	25.2	33.5[d]
Others, unidentified			0.8	9.4	6.8
4-Demethylsterol fraction					
6a Cholest-5-enol (cholesterol)	1.00	1.00	0.6	0.6	0.5
6c 24α-Methylcholesterol (campesterol)[e]	1.14	1.28	20.6[d]	24.0[d]	24.6[d]
6d 24β-Methylcholesterol (22-dihydrobrassicasterol)[e]	1.14	1.28	5.5[d]	4.9[d]	4.3[d]
6e 24ξ-Methyl-22-dehydrocholesterol	0.90	1.13	trace	trace	trace
6f 24-Methylenecholesterol	0.84	1.33	0.7[d]	trace	0.5
6g 24β-Methyl-25-dehydrocholeserol (codisterol)	0.79	1.30	trace[d]	trace	trace
6h 24α-Ethylcholesterol (sitosterol)	1.27	1.54	60.8[d]	51.5[d]	55.0[d]
6i 24α-Ethyl-22-dehydrocholesterol (stigmasterol)	1.10	1.37	6.6[d]	13.3[d]	8.3[d]
6j 24Z-Ethylidenecholesterol (isofucosterol)	1.02	1.72	1.6[d]	1.2	1.2
6k 24β-Ethyl-25-dehydrocholesterol (clerosterol)	0.93	1.56	trace	0.3[d]	0.5[d]
6l 24-Methylene-25-methylcholesterol	0.96	1.62	0.2	trace	0.5[d]
7j 24Z-Ethylidenecholest-7-enol (avenasterol)	1.02	2.02	2.1[d]	0.5	1.6[d]
Others, unidentified			1.3	3.7	2.9

[a]All sterols and triterpene alcohols possess the C-3β hydroxyl group. All compounds, with the exception of Δ^5-sterols, are 5α-compounds. All C-22–C-23 double bonds are *trans* (*E*)-oriented.

[b]Standard cholesterol (**6a**) acetate. RRT, relative retention time; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography.

[c]Composition in each fraction. 4,4-Dimethyl- and 4α-methylsterol fractions contained large amounts of the compounds that eluted faster than **6a**-acetate on GLC. These were considered to be aliphatic alcohols and were excluded from the calculation of sterol compositions. See Experimental Procedures section for the origin of the plant materials I and II.

[d]Identification was confirmed by proton nuclear magnetic resonance spectroscopy.

[e]Isolated as the C-24 epimeric mixture.

ment): δ 5.172 (*br. d*, J = 5.8 Hz, H-7), 4.663 (*br. s*, H_2-27), 4.402 (*dt*, J = 3.8, 11.3 Hz, H-3α), 2.055 (*s*, OAc-3β), 1.638 (*t*, J = 1.1 Hz, H_3-26), 0.995 (*d*, J = 6.9 Hz, H_3-28), 0.916 (*d*, J = 6.6 Hz, H_3-30), 0.851 (*d*, J = 6.3 Hz, H_3-21), 0.836 (*s*, H_3-19), 0.525 (*s*, H_3-18). Comparison of the MS and ^{1}H-NMR data with those of the same (4) and related compounds (2,5) confirmed the structure of **5g**.

Identification of fatty acids. A portion of the fatty acid methyl esters was fractionated by argentation TLC based on the degree of unsaturation, and the fractionated methyl esters were then subjected to GLC analysis. Identification and quantitation of individual fatty acids were performed by GLC comparison with authentic standards (Table 2).

RESULTS AND DISCUSSION

Sterols. Sterols identified in the aerial parts and rhizomes of plant material I and in the aerial parts of plant material II of *Psilotum nudum* are listed in Table 1 (see Fig. 1 for the structures of the sterols). Table 1 also gives the abundance and chromatographic data of each compound. Individual sterols were identified by comparison of their chromatographic and MS data with those of the reference compounds with the exception of **5g**, for which the reference compound was unavailable. The 400MHz ^{1}H-NMR was used to confirm the identification and to determine the stereochemical purity at C-24 of 24-alkylsterols (when enough material was available). The sterols for which identification was confirmed by ^{1}H-NMR are indicated in Table 1. There was no significant difference in the compositions of sterols among the plant materials of *P. nudum* investigated, as shown in Table 1.

Among the seven 4,4-dimethylsterols identified in this study, cycloartenol (**1b**), 24-methylenecycloartanol (**1f**) and cyclolaudenol (**1g**) constituted the dominant components. Cyclolaudenol, a sterol possessing a 24β-methyl-Δ^{25}-side chain, has previously been detected as one of the domi-

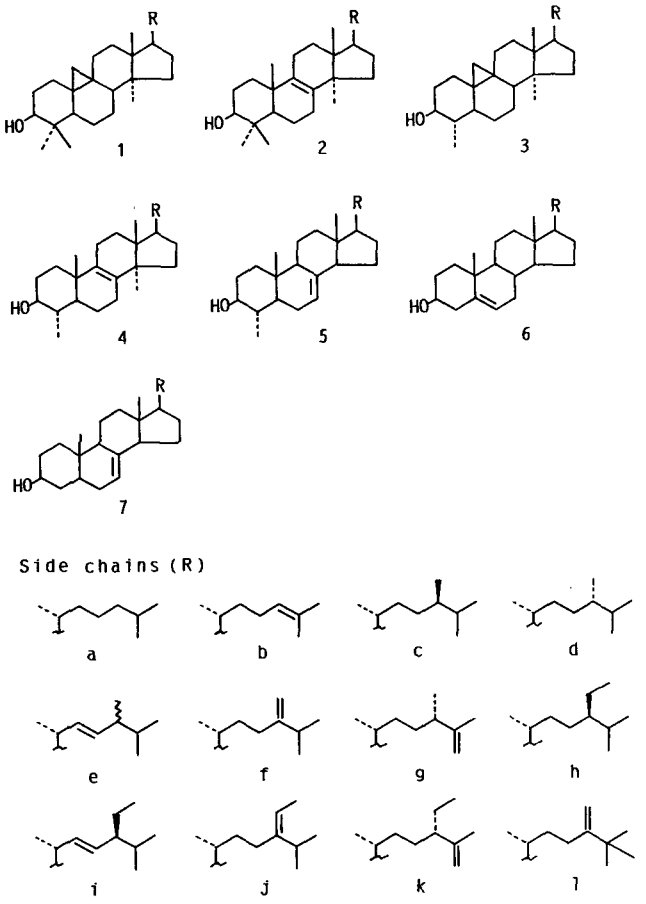

FIG. 1. Structures of sterols found in *Psilotum nudum*.

TABLE 2

Fatty Acid Compositions (%) of the Total Lipids from the Aerial Parts (A.P.) and Rhizomes of *Psilotum nudum* Determined by Gas-Liquid Chromatography

	Composition in each fraction (%)[a]		
	I		II
Fatty acid	A.P.	Rhizomes	A.P.
C12:0	0.7	0.3	0.4
C14:0	0.5	0.3	0.4
C14:1 (*n*-5)	0.1	0.1	—
C15:0	0.2	0.3	0.2
C16:0	21.6	17.9	30.4
C16:1 (*n*-9)	0.2	0.2	0.1
C16:1 (*n*-7)	0.8	0.6	0.7
C16:1 (*n*-5)	0.1	4.7	0.1
C17:0	0.6	0.7	0.7
C17:1 (*n*-8)	0.2	0.1	0.1
C18:0	4.8	3.1	5.6
C18:1 (*n*-9)	12.3	4.7	11.7
C18:1 (*n*-7)	0.9	3.7	0.6
C18:1 (*n*-5)	—	0.1	—
C18:2 (*n*-6)	18.9	35.4	22.4
C18:3 (*n*-6)	—	0.1	—
C18:3 (*n*-3)	22.4	10.4	14.5
C20:0	1.1	0.5	1.3
C20:1 (*n*-9)	0.3	0.3	0.2
C20:1 (*n*-7)	—	0.1	—
C20:2 (*n*-6)	1.3	1.7	1.0
C20:3 (undetermined)	5.3	7.7	5.2
C20:3 (*n*-3)	1.5	0.2	0.5
C20:4 (*n*-6)	—	0.7	—
C20:5 (*n*-3)	0.6	0.5	0.7
Others, unidentified	5.6	5.6	3.2

[a]See Experimental Procedures section for the origin of the plant materials I and II.

nant 4,4-dimethylsterols in *P. nudum* (6) and in true ferns (5–8), whereas only some species of spermatophyte plants are known to contain this sterol as major component (2). The predominance of **1g**, in addition to **1b** and **1f**, which are common in vascular plants (2), in the 4,4-dimethylsterol fraction may be a characteristic feature of pteridophyte plants. This study is the first identification of 24-methylene-5α-lanost-8-en-3β-ol (**2f**) in a fern.

Three Δ^7-unsaturated sterols, 24-methylenelophenol (**5f**), 24β-methyl-25-dehydrolophenol (**5g**) and citrostadienol (**5j**), constituted the dominant components in the 4α-methylsterol fractions. Presence of a 24β-methyl-Δ^{25}-sterol (**5g**) as one of the major sterols was consistent with the 4,4-dimethylsterol fractions. There is only one previous report for the natural occurrence of **5g** in *Myriophyllum verticillatum* (Haloragenaceae) (4). Although the presence of 24α-methyl-25-dehydrolophenol, the 24α-eipmer of **5g**, has been reported in a true fern *Polypodium niponicum* (9), our recent study has suggested that rather it is the 24β-epimer (**5g**), based on the ^{1}H-NMR spectral evidence (2,5). The occurrence of 24β-methyl-31-nor-5α-lanosta-8,25-dien-3β-ol (**4g**) has been reported so far only in *Polypodium formosanum* (5).

Campesterol (**6c**) and sitosterol (**6h**) constituted the dominant components among the twelve 4-demethylsterols identified. Five of them, *viz.*, **6c**, 22-dihydrobrassicasterol (**6d**), 24ξ-methyl-22-dehydrocholesterol (**6e**), **6h** and stigmasterol (**6l**), have previously been identified in *P. nudum* (6). As has been shown previously (6), the 24-ethylsterols (lacking a Δ^{25}-bond) existed exclusively as the 24α-epimers, *viz.*, **6h** and **6i**, whereas the 24-methylsterols existed as epimeric mixtures, *viz.*, **6c** and **6d**, in which the 24α-epimer (**6c**) predominated in *P. nudum*. This feature of 4-demethylsterols in *P. nudum* is closely allied with those of the other pteridophytes (2,5,6,9) and the majority of spermatophytes (2,10). We believe that this study is the first report of codisterol (**6g**), isofucosterol (**6j**), 24-methylene-25-methylcholesterol (**6l**) and avenasterol (**7j**) in a fern.

The 4,4-dimethyl- and 4α-methylsterols of *P. nudum* are thus closely allied with those of the other pteridophytes, whereas its 4-demethylsterols are similar to the other pteriodphytes as well as to the majority of spermatophytes. Thus, the sterol biosynthetic capacity of *P. nudum* appears to be similar to the other pteridophytes and to the spermatophyte plants (11).

Fatty acids. Twenty-five fatty acids identified in the plant materials of *P. nudum* are shown in Table 2. No significant difference was observed in the fatty acid composition among the aerial parts of the plant materials I and II and the rhizomes of I. Palmitic (16:0), oleic (18:1, *n*-9), linoleic (18:2, *n*-6), linolenic (18:3, *n*-3) and eicosatrienoic acids (20:3, double bond system undetermined) constituted the dominant components, and the composition was found to be similar to that reported in the pinnae of *P. triquetum* (12). In addition to the eicosatrienoic acid, *P. nudum* contains several other C_{20} acids of differing unsaturations (20:0–20:5) in low concentrations. This was

observed also in the pinnae of *P. triquetum* (12) and other pteridophytes (13). The presence of various C_{20} polyunsaturated acids is believed to be a unique feature of the pteridophyte fatty acids by which this group of plants can be distinguished from angiosperms (13).

REFERENCES

1. Takahashi, S., F. Nakamura, N. Sahashi, T. Ohmoto, U. Mizushima, U. Sankawa and G.H.N. Towers, *Biochem. Syst. Ecol. 18*:11 (1990).
2. Akihisa, T., W.C.M.C. Kokke and T. Tamura, in *Physiology and Biochemistry of Sterols*, edited by G.W. Patterson and W.D. Nes, American Oil Chemists' Society, Champaign, 1991, p. 172.
3. Okamoto, T., T. Maruyama, I. Niiya and T. Matsumoto, *J. Jpn. Oil Chem. Soc. 35*:44 (1986).
4. Greca, M.D., P. Monaco and L. Previtera, *Phytochemistry 28*:629 (1989).
5. Akihisa, T., S. Takahashi, N. Sahashi, W.C.M.C. Kokke and T. Tamura, *Ibid. 31*, 1353 (1992).
6. Chiu, P.-L., G.W. Patterson and T.A. Salt, *Ibid. 27*:819 (1988).
7. Laonigro, G., M. Adinolfi, G. Barone, R. Lanzetta and L. Mangoni, *Gazz. Chim. Ital. 112*:273 (1982).
8. Tanaka, Y., R. Hiyama and H. Ageta, *Chem. Pharm. Bull. 38*:2130 (1990).
9. Ageta, H., and Y. Arai, *Phytochemistry 23*:2875 (1984).
10. Nes, W.R., K. Krevitz, J. Joseph, W.D. Nes, B. Harris, G.F. Gibbons and G.W. Patterson, *Lipids 12*:511 (1977).
11. Benveniste, P., *Ann. Rev. Plant Physiol. 37*:275 (1986).
12. Radunz, A., *Phytochemistry 6*:399 (1967).
13. Jamieson, G.R., and E.H. Reid, *Ibid. 14*:2229 (1975).

[Received June 8, 1992; accepted September 20, 1992]