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CHEMICAL CONSTITUENTS OF AQUATIC FERN *AZOLLA NILOTICA*

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Key Word Index—*Azolla nilotica*; Azzolaceae; fern; (24*R*)-6β-hydroxy-24-ethylcholest-4-en-3-one; phytyl-3,7,11,15-tetramethy-2-hexadecenoate.

Abstract—Two new components, (24*R*)-6β-hydroxy-24-ethyl-cholest-4-en-3-one and phytyl-3,7,11,15-tetramethyl-2-hexadecanenate were isolated from the whole plants of aquatic fern *Azolla nilotica*. Their structures were established by spectroscopic techniques and chemical correlation. Some typical triterpenoids of ferns were also detected.

INTRODUCTION

In the continuation of our studies on triterpenoids from fern plants, we investigated an aquatic fern, *Azolla nilotica*, collected in Egypt and isolated a new steroidal ketone and a diterpenoidal, although some typical fern triterpenoids could also be isolated. The findings may have interesting chemotaxonomical significance.

RESULTS AND DISCUSSION

The petrol extract of the whole plant of *Azolla nilotica* was collected at the Nile in Egypt and was fractionated into six fractions with silica gel chromatography (CC). All fractions contained many aliphatic compounds on TLC profile. The individual fractions were rechromatographed using Dry Si gel, the final purification was achieved by preparative HPLC yielding new compounds **1** and **2** beside the known compounds, viz squalene (**3**) [1], ethyl phytylate (**4**) [2], 6,10,14-trimethylpentadecan-2-one (**5**) [2], three cycloartanoid alcohols (**6**–**8**) [3, 4] and hydroxyhopane (**9**) [5], the last four compounds **6**–**9** being widely distributed in fern plants.

Compound **1**, colorless plates, mp 208–210° (CHC_3I–MeOH) showed a positive Liebermann–Burchard reaction. The high resolution EI-mass spectrum of **1** showed [M^+] at *m/z* 428.3674, suggesting a molecular formula of $C_{29}H_{48}O_2$. IR absorptions of **1** showed the presence of a hydroxyl (ν_{max}^{KBr} cm^{-1}: 3480, 1100) group and an α,β-unsaturated carbonyl group (ν_{max}^{KBr} cm^{-1}: 1689) and UV absorption at 237 nm (λ_{max} 13,900). The low resolution EI-mass spectrum of **1** (Scheme 1) showed major fragment ions at *m/z* 428 (M^+ the base peak), 410 (*m/z* $M^+ - H_2O$, 12), 395 (10), 343 (5), 287 (12), 245 (30) and 227 (40). These fragmentation patterns corresponded to those of sitosterol (**10**) [6] with an extra oxygen functional group at the A, B or C ring systems.

The ^{1}H NMR spectrum (Table 1) of **1** in $CDCl_3$ showed the signals for two tertiary methyls, three secondary methyls and one ethyl methyl beside a vinyl proton at δ 5.817 and a carbinyl proton at δ 4.384. The multiplicity of the carbinyl proton signal as a broad singlet indicated the hydroxyl group to be axial in nature. A comparison of the ^{1}H NMR chemical shifts of **1** with those of sitosterol [6] revealed that the tertiary methyl proton signals (δ 0.742, δ 1.37) of **1** were deshielded considerably. The skeleton of the compound as well as the location of the function groups could, however, be established by a detailed 2D spectral analyses of **1**. Initially, the chemical shifts of all the ^{1}H (Table 1) and ^{13}C (Table 2) of **1** were assignable unambiguously from its H-H COSY, HSQC, HMBC and NOESY spectra. Careful analysis of the two- and three-bond correlations of the methyl, vinyl and carbinyl proton signals with neighboring carbon observed in the HMBC spectrum clearly demonstrated the presence of three part structures shown by heavy lines in structure **1a**. The C-C connectivities shown by broken lines in **1a** could be established from H-H COSY and H-C COSY spectra. Compound **1** could, therefore, be represented by the 6β-hydroxy-

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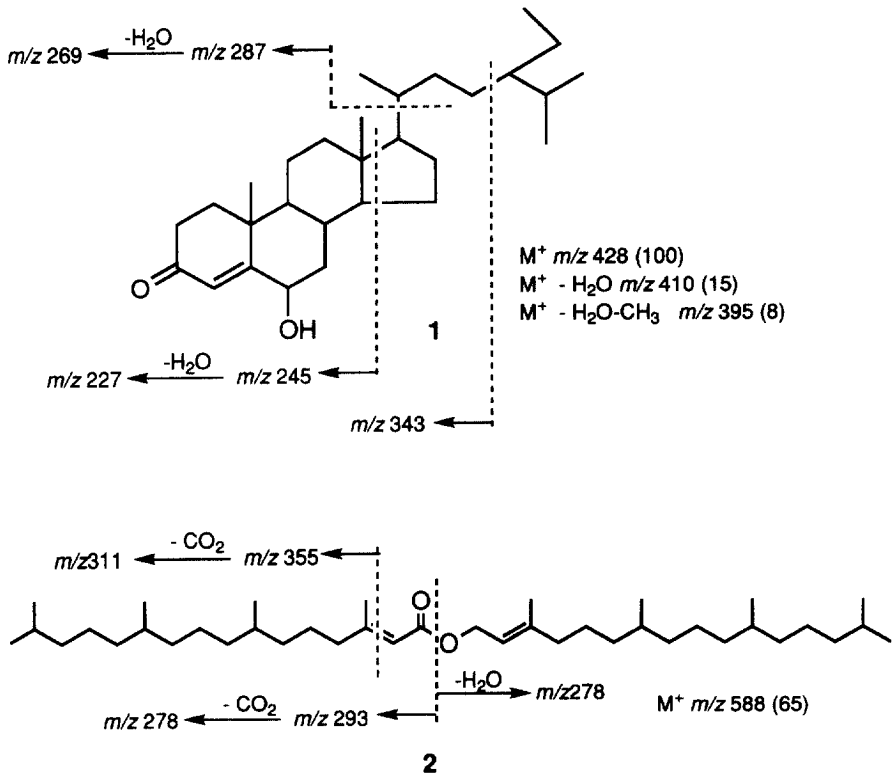

Scheme 1. Mass fragmentations of compounds **1** and **2**.

Table 1. ^{1}H NMR spectral data (δ ppm) for compounds **1** and **11**

^{1}H	**1**	**11**
18	0.742	0.711
19	1.375	0.181
21	0.923	0.918
	(*d*, 6.4)	(*d*, 6.4)
26	0.814	0.813
	(*d*, 7.0)	(*d*, 6.7)
27	0.837	0.837
	(*d*, 7.0)	(*d*, 6.7)
24^1	1.25	1.25
24^2	0.847	0.848
	(*dd*, 7.0, 7.0)	(*dd*, 6.9, 6.9)
3		4.332
		(*dddd*, 10.8, 10.8, 4.3, 4.3)
4	5.817	
6	4.348	
	(*brs*)	

Coupling constants are shown in parentheses.

24-ethylcholest-4-en-3-one structure. The configuration of the C-24 ethyl group in **1** was deduced by comparison of the ^{13}C chemical shift of the side chain carbons (C-21, C-24, C-26, C-27 and C-29) with those of a series of sterols having *R*- and *S*-configurations at C-24, particularly (24*S*)-24-ethylcholest-5-en-3β-ol and (24*R*)-24-ethylcholest-5-en-3β-ol [7–10] showing a close proximity of the chemical shifts to the 24*R*-series. Therefore, the structure of **1** was established as (24*R*)-6β-hydroxy-24-ethylcholest-4-en-3-one.

From this same fraction (24*R*)-3β-hydroxy-ethylcholest-5-en-6-one (**11**) [7, 8] was also isolated and its structure was elucidated by the same methods as discussed above.

Oily compound **2** showed the molecular formula $C_{40}H_{76}O_2$ from its high resolution EI-mass spectrum (M^+ *m/z* 588.5282). Its IR spectrum displayed a strong absorption band for an ester function (1644 cm^{-1}). The simple fragmentation pattern of compound **2** in the EI-mass spectrum indicated that **2** was an ester of two linear diterpenoid moieties (Scheme 1). The ^{1}H NMR spectrum of **2** exhibited signals for protons of eight secondary and two olefinic methyl groups, two vinyl protons and two protons of a -CH_2-O- group. Furthermore, characteristic aliphatic methylene signals at δ 1.254 were observed. The ^{13}C NMR spectrum (Table 3) of **2** showed 40 carbon signals of which 10 were methyl, 19 methylene, eight methine and three quartenary carbons, suggesting that **2** is a derivative of the linear diterpenoid like phytol (**12**) [2]. Hydrolysis of **2** with alcoholic sodium hydroxide gave an alcohol, identified as phytol, and an acid **13**. Compound **13** showed a molecular weight *m/z* 310 in the EI-mass spectrum, and five methyl proton signals consisting of four secondary and the olefinic methyl in its ^{1}H NMR spectrum. The above data indicated that **13** was

Table 2. ^{13}C NMR spectral data (δ ppm) for compounds **1** and **11**

	1	11
1	37.09	36.28
2	34.27	33.81
3	200.47	68.72
4	126.32	41.52
5	168.52	171.48
6	73.28	119.65
7	38.56	199.46
8	29.79	34.15
9	53.62	53.75
10	37.99	39.04
11	20.98	21.04
12	39.60	39.44
13	42.51	42.45
14	56.05	55.96
15	24.15	24.18
16	28.18	28.14
17	55.89	55.56
18	12.01	11.94
19	19.51	18.28
20	36.12	36.09
21	18.73	18.70
22	33.89	33.86
23	26.00	26.05
24	45.83	45.83
25	29.15	29.14
26	19.83	19.82
27	19.03	19.02
24^1	23.07	23.08
24^2	11.98	11.99

Table 3. ^1H NMR spectral data (δ ppm) for compounds **2** and **3**

^1H	2	3
1		4.155
		(*d*, 7.0)
2	5.676	5.410
	(*d*, 1.3)	(*dd*, 7.0, 7.0)
16	0.865	0.867
	(*d*, 6.7)	(*d*, 6.7)
17	0.865	0.867
	(*d*, 6.7)	(*d*, 6.7)
18	0.848	0.845
	(*d*, 6.7)	(*d*, 6.7)
19	0.843	0.853
	(*d*, 6.7)	(*d*, 6.7)
20	0.154	1.671
	(*d*, 1.2)	(*d*, 1.0)
1′	4.163	
	(*d*, 7.0)	
2′	5.363	
	(*d*, 6.4)	
16′	0.865	
	(*d*, 6.4)	
17′	0.865	
	(*d*, 6.7)	
18′	0.848	
	(*d*, 6.7)	
19′	0.843	
	(*d*, 6.7)	
20′	1.701	
	(*d*, 1.1)	

Coupling constants are shown in parentheses.

3,7,11,15-tetramethyl-2-hexadecanoic acid. Thus the structure of **2** was determined to be phytyl-3,7,11,15-tetramethyl-2-hexadecanoate.

Although the isolation of **9**, a typical fern constituent, suggested resemblance of the triterpenoid biogenetic pathway in both aerial and aquatic fern plants, the occurrence of compounds **2** and **12** seem to have significance in the chemotaxonomic identification of an aquatic fern plant.

EXPERIMENTAL

General

Mps were measured on a Yanagimoto micro apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. The ^1H (500 MHz) and ^{13}C NMR (125.65 MHz) spectra were recorded on a JEOL α-500 spectrometer and the chemical shifts were expressed on the δ (ppm) scale with TMS as int. standard. The conditions for 2D NMR were cited in Ref. [5]. MS were recorded on a JEOL JMS D-300 spectrometer. TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm) using *n*-hexane–EtOAc (2:1) as the developing phase and the detection was carried out by spraying with conc. H_2SO_4 followed by heating.

Plant material

Azolla nilotica was collected at the Nile in August 1992. The voucher specimen has been deposited in the herbarium of Showa College of Pharmaceutical Sciences, Tokyo (No. 930402).

Extraction and separation

Whole plants (10 kg) were extracted with petrol to give a green extract (12 g). The extract was fractionated with Si gel CC to six fractions as follows. Fr. 1 oily (*n*-C_6H_{14}) 4.8 g; Fr. 2 oily [*n*-C_6H_{14}–C_6H_6 (7:3)], 0.27 g; Fr. 3 waxy [*n*-C_6H_{14}–C_6H_6 (1:1)], 3.0 g; Fr. 4 waxy (C_6H_6), 0.7 g; Fr. 5 waxy (C_6H_6), 2.7 g; Fr. 6 waxy (C_6H_6), 0.5 g.

Squalene (**3**). Fr. 1 was rechromatographed over dry Si Gel to give a trace of **3**, identified by comparison of its ^1H NMR spectrum with that of an authentic sample.

Phytyl-3,7,11,15-tetramethyl-2-hexadecanoate (**2**). Compound **2** (150 mg) was obtained from Fr. 2

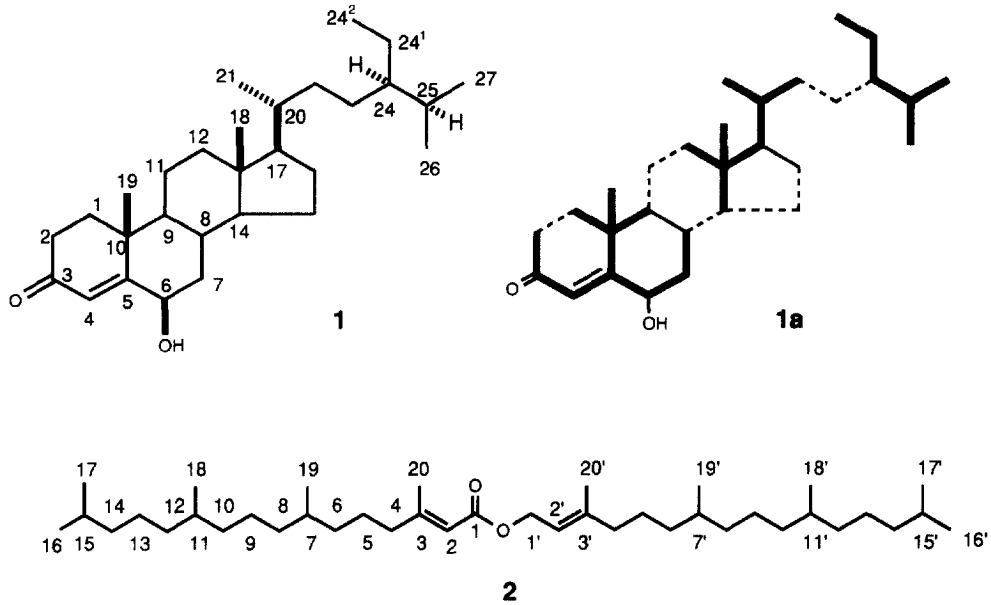

Fig. 1. Compounds **1** and **2**, and partial structures of **1** deduced from HMBC spectrum Scheme 1. Mass fragmentations of **1** and **2**.

through dry Si gel CC followed by HPLC using CH_3CN–MeOH (7:3) as eluting solvent.

6,10,14-*trimethylpentadecane-2-one* (**5**) *and Hydroxyhopane* (**9**). Fr. 3 was chromatographed over dry Si gel to give **5** (20 mg) ^{1}H NMR (δ, $CDCl_3$): 2.163 (3H, *s*, H-1), 0.841 (3H, *d*, H-6), 0.860 (3H, *d*, H-10), 0.867 (6H, *d*, H-14, 15). EI-MS (rel. int.) *m/z*: 268 (100, M^+), 250 (15), 235 (8), 210 (30). 194 (15), 179 (20), 163 (23) and **9** (4 mg). ^{1}H NMR (δ, $CDCl_3$): 0.848 (3H, *s*, H-23), 0.790 (3H, *s*, H-24), 0.812 (3H, *s*, H-25), 0.954 (3H, *s*, H-26), 0.954 (3H, *s*, H-27), 0.759 (3H, *s*, H-28), 1.179 (3H, *s*, H-29), 1.207 (1H, *s*, H-30).

Cycloartanoids

The cycloartanoid mixture (60 mg) obtained from Fr. 4 through dry Si gel CC was purified by prep. HPLC [CHCl–MeOH (7:3)] to give cycloartenol (**6**) (40 mg), 24-methylenecycloartanol (**7**) (trace) and cycloartenol (**8**) (trace).

Sitosterols

The sitosterol mixture (80 mg) was purified through Si-gel CC was by prep. (first fraction from C_6H_6 of Fr. 5) followed by Dry Si-gel CC [*n*-C_6H_{12}–EtOAc (8:3)] of Fr. 5, and were estimated to be phytosterols composed of sitosterol (80%), RR_f 2.73, LR-MS *m/z*: 414 (M^+), campesterol (8%), RR_f 2.41, LR-MS *m/z*: 400 (M^+), stigmasterol (3%), RR_f 2.50, LR-MS *m/z*: 412 (M^+) and cholesterol (8%), RR_f 1.72 LR-MS *m/z*: 386 (M^+).

6β-Hydroxy-24-ethylcholesta-4-en-3-one (**1**) *and 3β-hydroxy-24-ethylcholesta-5-en-7-one* (**2**). The second fraction from rechromatography of Fr. 5 yielded **1** and **2** by HPLC. Compound **1** (40 mg, second peak in HPLC), mp 208–209° and **2** (3 mg, first peak in HPLC), mp 208–209° were separated from them by HPLC [$CHCl_3$–MeOH (3:7)].

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