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Pterosterone 20,22-Acetonide, a New Ecdysteroid and Other Constituents from *Acrostichum aureum* L.

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Abstract: Chemical investigation of the EtOAc extract from the aerial parts of *Acrostichum aureum* distributed along coastlines of Vietnam lead to the isolation of a new ecdysteroid, named pterosterone 20,22-acetonide (**1**), along with twelve known compounds (**2-13**). Their structures were elucidated by spectroscopic methods including 1D, 2D NMR and HR-ESI-MS analysis as well as comparison with the results of previous studies. Five ecdysteroids (**1-5**) were evaluated for their cytotoxic activity against the LU-1, MCF7 and HepG2 human cancer cell lines using the SRB assay. Ecdysteroids **1** and **2** having a 20,22-dimethyl acetal group showed cytotoxicity against all tested cell lines with IC_{50} values in the range 51.59 to 60.14 μM. The other compounds were considered as inactive in this test.

Keywords: *Acrostichum aureum*; Pteridaceae; ecdysteroid; flavonoid; spectroscopic analyses; cytotoxicity.

1. Introduction

Ecdysteroids are steroid hormones that control the processes of insect moulting and metamorphosis. These steroids are characterized by a *cis*-fusion of the A/B rings, a 7-en-6-one chromophore, a *trans*-fusion of the C/D rings with 14α-OH group, and they usually retain the entire carbon skeleton of their original sterols. The ecdysteroids originated from plants (phytoecdysteroids) play important roles in growth regulation and in the plant protection [1]. Fifty percent of more than 500 fern species were detected containing ecdysteroids, sometimes at very high contents (4-5%). About 40 ecdysteroids have been isolated from different ferns [2,3]. The mangrove fern *Acrostichum aureum* L. (Pteridaceae) has been used as a traditional remedy in Vietnam and many Asian countries for treatment of non-healing ulcers, dermatitis, worm infections, rheumatism, gastritis and diabetes [4,5]. More than 40 compounds have previously been isolated or detected from *A. aureum*, including terpenoids, steroids, flavonoids, and other phenolic compounds [5]. However, there has been few reports on the presence of ecdysteroids from *A. aureum*. Ponasterone A, the first ecdysteroid from *A.*

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aureum, has been isolated in 1981 [6], whereas pterosterone has been found from this fern in 2006 [7]. Continuing our work on phytochemistry and cytotoxic activity of *A. aureum* [8], we carried on the isolation and structure elucidation of compounds from the ethyl acetate fraction of methanol extract of *A. aureum*. A new pterosterone 20,22-acetonide (**1**) together with twelve known compounds including ecdysteroids (**2**-**5**), flavonoids and flavonoid glycosides (**6**-**12**) and a phenolic glucoside (**13**) have been isolated. The cytotoxicity of ecdysteroids (**1**-**5**) effect against three human cancer cell lines LU-1, MCF7 and HepG2 was also evaluated.

2. Materials and Methods

2.1. General Experimental Procedures

The JASCO P-2000 polarimeter (Hachioji, Japan) was used for measuring optical rotation. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer with tetramethyl silane (TMS) as internal standard. HR-ESI-MS were obtained on X500 QTOF mass spectrometer system (MA, USA). Silica gel 60 (0.04-0.063 mm, Merck), C18 reversed-phase (RP) silica gel (150 μm, YMC), and Sephadex LH-20 (25-100 μm, Sigma-Aldrich) were used for column chromatography (CC). Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 and RP-18 F254S plates (0.25 mm, Merck). The spots were visualized under UV fluorescence at 254 nm or spraying with 1% vanillin-H_2SO_4 in MeOH, followed by heating at 100 ^oC for 1-2 min.

2.2. Plant Material

The aerial parts of *A. aureum* were collected from coastal area of Thai Binh province, Vietnam, in 2019. The identity of the plant material was confirmed by Prof. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). Voucher specimens are retained at the Herbarium of the Department of Organic Chemistry, Hanoi University of Science and Technology (HUST), Vietnam (deposit number HUST.N03).

2.3. Extraction and Isolation

The air-dried aerial parts (3.2 kg) of *A. aureum* were powdered and extracted three times with MeOH (3 × 10 L) at 45 ^oC for 1 h under sonication. After removal of solvent under reduced pressure, the crude extract was suspended in H_2O and successively partitioned with *n*-hexane (3 × 1 L) and EtOAc (3 × 1 L). The EtOAc soluble fraction (61.0 g) was fractionated on a silica gel column eluted with a CH_2Cl_2/MeOH (100:1 to 1:1, v/v) gradient to afford six fractions *A-1* to *A-6*. Fraction *A-2* (2.79 g) was purified by a Sephadex LH-20 column with MeOH followed by chromatography with silica gel eluted with CH_2Cl_2/MeOH (15:1, v/v) to yield compounds **12** (2.7 mg) and **4** (75.4 mg). Fraction *A-3* (1.16 g) was subjected to Sephadex LH-20 CC eluted with MeOH to give two subfractions *A-3.1* and *A-3.2*. Subfraction *A-3.1* was further separated by RP-C_{18} silica gel CC eluted with MeOH/H_2O (4:1, v/v) to obtain compounds **1** (50.9 mg) and **2** (37.7 mg). Compound **6** (2.4 mg) was obtained from the subfraction *A-3.2* by RP-C_{18} silica gel CC eluted with MeOH/H_2O (2:1, v/v). Fraction *A-4* (11.35 g) was chromatographed repeatedly on a silica gel column eluted with CH_2Cl_2/MeOH (15:1, 10:1, 5:1, 1:1, v/v) and five subfractions *A-4.1* - *A-4.5* were obtained. Subfraction *A-4.1* was purified by Sephadex LH-20 CC with MeOH, followed by recrystallization in acetone to yield compound **3** (140.2 mg). Purification of the *A-4.3* by Sephadex LH-20 CC eluted with MeOH to give compound **10** (60.7 mg). Subfraction *A-4.4* was purified by a Sephadex LH-20 column with MeOH followed by chromatographed with RP-C_{18} silica gel eluted with MeOH-H_2O (1:2, v/v) to afford compounds **11** (13 mg) and **13** (9 mg). Compound **5** (5.6 mg) was obtained from the subfraction *A-4.5* by RP-C_{18} silica gel CC eluted with MeOH/H_2O (2:3, v/v). Fraction *A-5* (7 g) was further separated by silica gel CC eluted with CH_2Cl_2/MeOH (10:1, v/v) to give two subfractions *A-5.1* and *A-5.2*. These subfractions were purified by Sephadex LH-20 CC with MeOH elution and then recrystallized in acetone to yield compounds **7** (35.0 mg) and **8** (80 mg). Fraction *A-6* (2.5 g) was subjected to Sephadex LH-20 CC

eluted with MeOH to give two subfractions *A-6.1* and *A-6.2*. Subfraction *A-6.1* was further separated by silica gel CC eluted with CH_2Cl_2/MeOH (2:1, v/v) to afford compound **9** (9.1 mg).

*Pterosterone 20,22-acetonide (**1**)*: White powder. $[\alpha]_D^{25}$ = + 70.6 (*c* 0.1, MeOH); HR ESI-MS: *m/z* 543.3272 [*M* + Na]$^+$ (calcd. for $C_{30}H_{48}O_7Na$, 543.32922); ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data are given in Table 1.

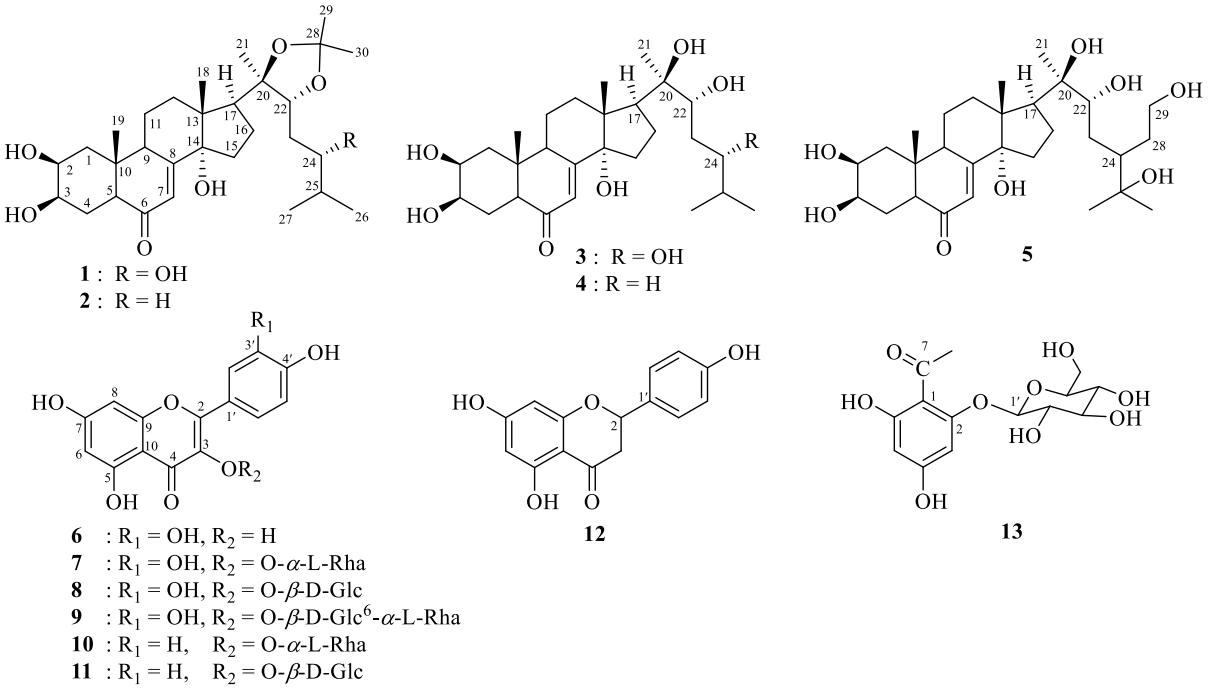

Figure 1. Chemical structures of compounds **1-13**

2.4. Cytotoxic Assay

Cytotoxic activity against three human cancer cell lines as LU-1 (lung carcinoma), MCF7 (breast carcinoma), and HepG2 (hepatocellular carcinoma) were determined using the sulforhodamine B (SRB) assay [9]. All tumor cell lines were supplied by Professor J. M. Pezzuto (Long-Island University, US) and Professor Jeanette Maier (Milan University, Italia). The detailed methodology for the cytotoxicity assay has been described in a previous paper [8]. Ellipticine was used as a positive control.

3. Results and Discussion

3.1. Structure Elucidation

The EtOAc soluble fraction of the methanol extract of *A. aureum* was separated by chromatographic techniques to afford thirteen compounds (**1-13**, Figure 1).

Table 1. ^{1}H (at 500 MHz) and ^{13}C (at 125 MHz) NMR data for compound **1** in acetone-d_6

Position (C)	δ_C	δ_H, *J* in Hz	Position (C)	δ_C	δ_H, *J* in Hz
1	37.4 (CH_2)	1.77 (1H, *m*) 1.39 (1H, *m*)	16	22.0 (CH_2)	2.04 (1H, *m*) 1.98 (1H, *m*)
2	68.1 (CH)	3.83 (1H, *brd*, *J* = 10.5)	17	49.9 (CH)	2.34 (1H, *overlapped*)
3	68.0 (CH)	3.92 (1H, *m*)	18	17.5 (CH_3)	0.81 (3H, *s*)
4	32.0 (CH_2)	1.65 (2H, *m*)	19	24.3 (CH_3)	0.91 (3H, *s*)
5	51.1(CH)	2.32 (1H, *dd*, *J* = 6.0, 11.0)	20	85.5 (C)	-
6	203.9 (C)	-	21	22.4 (CH_3)	1.17 (3H, *s*)
7	121.8 (CH)	5.73 (1H, *d*, *J* = 2.5)	22	80.4 (CH)	3.93 (1H, *dd*, *J* = 4.0, 8.5)
8	165.2 (C)	-	23	33.8 (CH_2)	1.61 (2H, *m*)
9	34.4 (CH)	3.14(1H, *ddd*, *J* = 2.5, 7.0, 11.5)	24	75.1 (CH)	3.52 (1H, *dd*, *J* = 3.0, 7.5)
10	38.6 (C)	-	25	33.7 (CH)	1.67 (1H, *m*)
11	21.0 (CH_2)	1.79 (1H, *m*) 1.65 (1H, *m*)	26	17.4 (CH_3)	0.89 (3H, d, *J* = 7.0)
12	31.8 (CH_2)	2.14 (1H, *td*, *J* = 5.0, 13.0) 1.79 (1H, *m*)	27	19.3(CH_3)	0.90 (3H, d, *J* = 7.0)
13	48.0 (C)	-	28	107.6 (C)	-
14	84.7 (C)	-	29	27.1 (CH_3)	1.31 (3H, *s*)
15	31.4 (CH_2)	1.92 (1H, *m*) 1.67 (1H, *m*)	30	29.2 (CH_3)	1.36 (3H, *s*)

Pterosterone 20,22-acetonide (**1**) was obtained as a white powder. Its molecular formula was determined as $C_{30}H_{48}O_7$ by the HR-ESI-MS at *m/z* 543.3272 $[M + Na]^+$ (calcd. for $C_{30}H_{48}O_7Na$, 543.3292) and NMR spectroscopic data (Table 1), indicating seven degrees of unsaturation. The NMR spectroscopic data shows that **1** possesses an ecdysteroid skeleton with a 7-ene-6-one unsaturated ketone group [1]. The ^{13}C NMR and DEPT spectra of **1** displayed 30 carbon resonances including seven methyl, seven methylene, nine methine (comprising four oxygenated carbons at δ_C 68.0, 68.1, 75.1, 80.4 and an olefinic carbon at δ_C 121.8) groups, seven non-protonated carbons (comprising a ketone carbon at δ_C 203.9, an olefinic carbon at δ_C 165.2, an acetal carbon at δ_C 107.6, and two oxygenated carbons at δ_C 84.7 and 85.5). It also exhibited the presence of an *α,β*-unsaturated ketone system [δ_C 203.9, 165.2 and 121.8]. In addition, the ^{1}H NMR spectrum of **1** revealed signals of an olefinic proton as a doublet at δ_H 5.73 (1H, *d*, *J* = 2.5 Hz, H-7), four oxygenated methine protons at δ_H 3.93 (1H, *dd*, *J* = 4.0, 8.5 Hz, H-22), 3.92 (1H, *m*, H-3), 3.83 (1H, *brd*, *J* = 10.5 Hz, H-2), and 3.52 (1H, *dd*, *J* = 3.0, 7.5 Hz, H-24), two geminal methyl groups at δ_H 0.89 (3H, d, *J* = 7.0 Hz, H_3-26) and 0.90 (3H, d, *J* = 7.0 Hz, H_3-27), as well as five methyl singlets at δ_H 0.81 (3H, *s*, H_3-18), 0.91 (3H, *s*, H_3-19), 1.17 (3H, *s*, H_3-21), 1.31 (3H, *s*, H_3-29), and 1.36 (3H, *s*, H_3-31). Comparison of the NMR spectroscopic data of **1** and pterosterone (**3**) indicates their structural similarity except an additional acetonide group [δ_C/δ_H 107.6, 29.2/1.36 (3H, *s*), 27.1/1.31 (3H, *s*)] and the downfield shifts of C-20 (7.8 ppm) and C-22 (2.8 ppm) signals relative to those for **3**, thus suggesting the 20,22-acetonide structure in **1** [10]. The proposed structure of **1** was confirmed by detailed analysis of its 2D NMR (^{1}H-^{1}H COSY and HMBC) spectra. The spin system of H-22/H_2-23/H-24/H-25/H_3-26/H_3-27 in the ^{1}H-^{1}H COSY spectrum and the HMBC correlations of H-22 to C-17, C-20, C-21, C-23, C-24; H_3-21 to C-17, C-20, C-22; and H_3-26, H_3-27 to C-24, C-25 was confirmed a 20,22-acetonide-24-hydroxylated steroid side-chain at C-17 of **1**. Noted that the HMBC spectrum also showed the correlations from two methyl groups H_3-29 (δ_H 1.31) and H_3-30 (δ_H 1.36) to acetal carbon C-28 (δ_C107.6). In addition, the NOESY spectrum of **1** exhibited correlations H_3-21/H_3-30 and H-22/H_3-29, indicating the dimethyl acetal moiety. The relative configuration of **1** resembles that of pterosterone (**3**) [12], which was substantiated by NOESY spectroscopic analysis. The existence of correlation between H(ax)-5 (δ_H 2.32) and H_3-19 (δ_H 0.91) proved the *cis*-fusion of A/B rings, which was consistent with the coupling constant between H-5 and H_2-4 (*J* = 6.0, 11.0 Hz). The NOE correlations between H(ax)-2 (δ_H 3.83) and H(ax)-9 (δ_H 3.14), H(ax)-2 and H(eq)-3 (δ_H 3.92) indicated that H-2, H-3 and H-9 were on the same side and judged to be *β*-oriented of the hydroxy groups at C-2 and C-3. The NOE correlations

between H(eq)-12 (δ_H 1.79) and H_3-18 (δ_H 0.81), H_3-18 and H_3-21 (δ_H 1.17), and between H(ax)-12 (δ_H 2.14) and H(ax)-17 (δ_H 2.34) confirmed not only the *trans*-fusion of C/D rings, but also the *β*-orientation of the side chain at C-17. Furthermore, the NOE cross-peaks between H_3-18 and H_3-21, H_3-21 and H_3-30 (δ_H 1.36), as well as between H-22 (δ_H 3.93) and H_3-29 (δ_H 1.31) allowed the spatial differentiation between CH_3-29 and CH_3-30. These NOESY cross-peaks also proved the relative configurations of C-20 and C-22. The presence of 20,22-acetonide group in **1** caused the smaller upfield shift in the C-24 resonance (2.4 ppm) relative to that for **3**, hence the configuration at C-24 in **1** was deduced 24*S*, the same as 24*S*-configuration of **3** [11,12]. Based on the above discussion, the compound **1** was determined to be pterosterone-20,22-acetonide, a new compound. Compound **1** was not a degradation product from pterosterone (**3**), because acetone solvent was not used through out of isolation process. Moreover, compounds **1** and **3** showed as different spots on the TLC of EtOAc extract.

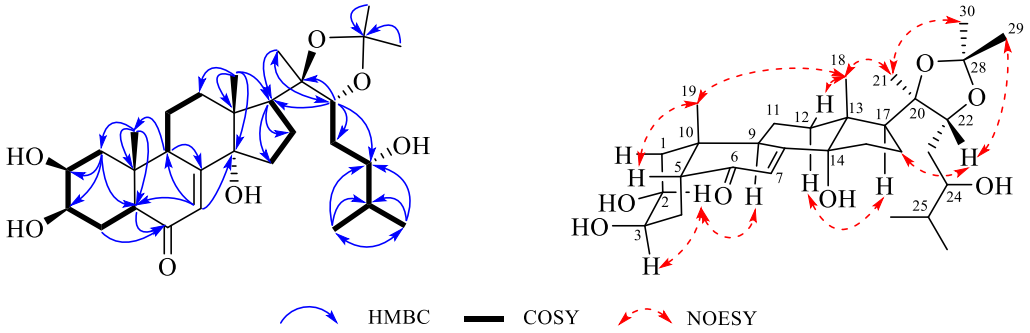

Figure 2. ^{1}H-^{1}H COSY and the selected HMBC, NOESY correlations of compound **1**

By comparison of the isolates NMR data with those reported in the literature, twelve known compounds were identified as ponasterone A 20,22-acetonide (**2**) [10], pterosterone (**3**) [11, 12], ponasterone A (**4**) [13], 24-(2-hydroxyethyl)-20-hydroxyecdysone (**5**) [14], quercetin (**6**) [15], quercitrin (**7**) [16], isoquercitrin (**8**) [17], rutin (**9**) [15], afzelin (**10**) [18], astragalin (**11**) [19], naringenin (**12**) [20], and myrciaphenone A (**13**) [21]. To our best knowledge, six compounds **2**, **5**, **10**-**13** have not been reported previously from *A. aureum*.

3.2. Cytotoxicity Activity

Table 2. Cytotoxicity of ecdysteroids **1**-**5**

Compound	IC_{50} (μM)		
	LU-1	MCF7	HepG2
1	60.14	54.65	59.67
2	55.30	56.43	51.59
3	>100	>100	>100
4	>100	>100	>100
5	>100	>100	>100
Ellipticine	1.65	1.51	1.72

Ecdysteroids **1**-**5** were evaluated for their cytotoxicity against a human lung carcinoma cell line (LU-1), a human breast carcinoma cell line (MCF7), and a human hepatocellular carcinoma cell line (HepG2) using SRB method with ellipticine as the positive control (Table 2). Interestingly, new compound **1** and known ponasterone A 20,22-acetonide (**2**) having a 20,22-dimethyl acetal group showed cytotoxic activity against all the tested cell lines with IC_{50} ranging from 51.59 to 60.14 μM, whereas the other ecdysteroids (**3**, **4** and **5**) were inactive in this test (IC_{50} > 100 μM).

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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