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Phytosteroids from Roots of *Psammosilene tunicoides* W.C.Wu et C.Y.Wu

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Abstract: To explore structurally and pharmacologically interesting secondary metabolites from *Psammosilene tunicoides*, a crude extract of this plant was investigated, leading to the isolation of a new phytoecdysteroid, furoecdysterone (**1**) and four known phytosteroid compounds, ecdysterone (**2**), 22-deoxyecdysterone (**3**), *β*-sitosteryl D-glucoside (**4**), and *β*-sitosterol (**5**). Their structures were identified based on a detailed analysis of NMR spectra and MS data. Those compounds, except for **2**, showed moderate NO inhibition activities on lipopolysaccharide (LPS)-induced release in RAW 264.7 cells.

Keywords: *Psammosilene tunicoides*; phytoecdysteroid; nitric oxide inhibition activity; RAW 264.7 cells.

1. Plant Source

The roots of *Psammosilene tunicoides* W. C. Wu et C. Y. Wu (Caryophyllacea) were purchased from Kunming Luosiwan medicinal material market of Yunnan Province in China and authenticated by A/Prof. Yong Xiong of the School of Ethnic Medicine, Yunnan Minzu University. A voucher specimen (JTS202201) was deposited in the herbarium of the above department.

2. Previous Studies

Psammosilene tunicoides is a unique monotype genus plant in southwestern China, and widely used to benefit the stomach and treat traumatic injuries and rheumatism [1]. *P. tunicoides* (so-called “Jin-Tie-Suo”) is one of the important ingredients in the famous Chinese patent medicines “Yunnan Baiyao” and “Baibaodan Capsule” in Yunnan province. A growing number of the modern studies are focusing on

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the chemical composition and biological activity of *P. tunicoides*. More than 100 compounds have been obtained from roots of the plant [2,3]. Phytochemical studies on this plant have afforded triterpenoid saponins [4-7], cyclic peptides [8-10], alkaloids [11-13], maltol glycosides [14,15], and nitro-substituted compounds [16]. In our previous study, we have isolated oleanan-type triterpene saponins and cyclic peptides from *P. tunicoides*, and elucidated their structures and analgesic effects [17,18]. However, so far there were few reports on phytosteroid compounds from *P. tunicoides*.

3. Present Study

As part of the effort aimed at exploring structurally and pharmacologically interesting secondary metabolites from *P. tunicoides*, a crude extract of this plant was investigated, leading to the isolation of a new phytoecdysteroid, furoecdysterone (**1**) and four known phytosteroid compounds, ecdysterone (**2**) [19], 22-deoxyecdysterone (**3**) [19], *β*-sitosteryl D-glucoside (**4**) [20], and *β*-sitosterol (**5**) [21] (Figure 1).

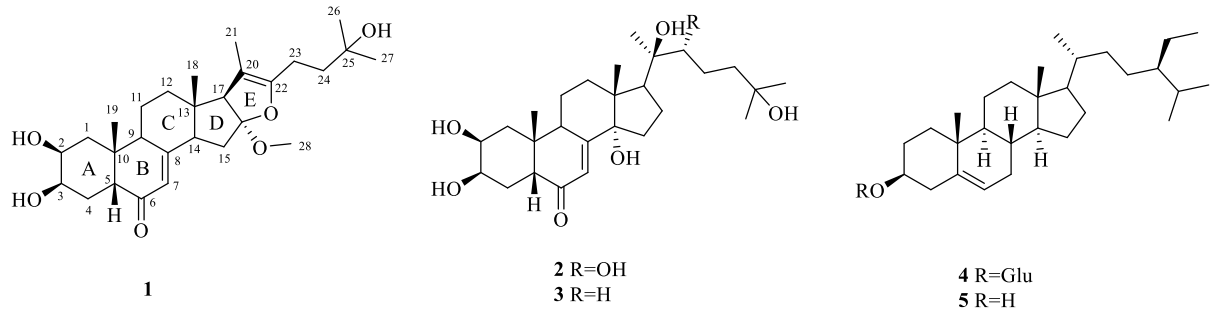

Figure 1. Chemical structure of compounds **1**-**5**

Extraction and Separation: The roots of *P. tunicoides* were air-dried and powdered to afford a material of 4.5 kg, which was infiltrated and extracted with 95% EtOH at room temperature (48 h × 4). A sticky residue (204.8 g) was obtained after the organic solvent was removed on a rotary evaporator at 50 °C under reduced pressure. The residue was directly subjected to silica gel CC and eluted with $CHCl_2$/MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1; v/v) and pure MeOH to obtain eight fractions. Fr.1 (2.3 g) with crystal precipitation was prepared by recrystallization to yield compound **5** (98.3 mg). Fr.3 (4.1 g) was separated by Sephadex LH-20 CC and eluted with 100% MeOH to yield four subfractions, Frs.3-1~3-4. Fr.3-4 (0.6 g) was purified by semi-preparative HPLC (MeOH/H_2O, 60:40, v/v) to give compound **1** (8.0 mg, t_R=30 min). Fr.4 (3.0 g) was subjected to a Sephadex LH-20 column (100% MeOH) to yield three subfractions, Frs.4-1~4-3. Fr.4-2 (1.1 g) was separated by Sephadex LH-20 column eluting with $CHCl_2$/MeOH (1:1, v/v) and semi-preparative HPLC (MeOH/H_2O, 46:54, v/v) to give compounds **2** (48.0 mg, t_R=13 min) and **3** (10.0 mg, t_R=43 min). Fr.5 (22.8 g) was subjected to silica gel CC eluting with $CHCl_2$/MeOH (50:1, 30:1, 10:1, 5:1; v/v) to give five fractions, Frs.5-1~5-5. Fr.5-5 (0.8 g) was chromatographed by Sephadex LH-20 column eluting with $CHCl_2$/MeOH (1:1, v/v) to yield two subfractions, Frs.5-5-1 and 5-5-2. Fr.5-5-2 was purified by recrystallization (100% MeOH) to obtain compound **4** (11.3 mg).

*Furoecdysterone (**1**)*: White powder, $[\alpha]_D^{20.2}$ + 15 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.16), 248 (3.98) nm; IR (KBr) ν_{max}: 3444, 2965, 2360, 2025, 1632, 1597, 1385, 1353, 1066, 1016, and 559 cm^{-1}; ^{1}H- and ^{13}C-NMR spectral data, see Table 1; HRESIMS: *m/z* 497.2884 $[M+Na]^+$ (calcd for $C_{28}H_{42}O_6Na^+$, 497.2874).

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1** in CD_3OD ([a]400 MHz, [b]100 MHz)

Position	δ_H (*J* in Hz)[a]	δ_C, mult.[b]	Position	δ_H (*J* in Hz)[a]	δ_C, mult.[b]
1	1.48, m	36.9, CH_2	15	1.52, m	37.2, CH_2
	1.82, m			1.85, m	
2	3.65, dt (11.6, 3.5)	69.1, CH	16		103.9, C
3	3.94, brs	68.6, CH	17	2.41, d (9.5)	54.2, CH
4α	1.74, m	33.3, CH_2	18	1.41, s	32.1, CH_3
4β	1.53, m				
5	2.35, dd (13.4, 4.0)	50.8, CH	19	0.90, s	23.9, CH_3
6		205.9, C	20		135.0, C
7	6.04, d (2.2)	122.8, CH	21	1.61, s	9.9, CH_3
8		175.3, C	22		144.4, C
9	2.46, m	35.0, CH	23	2.48, m	22.3, CH_2
				1.94, m	
10		40.2, C	24	1.76, m	42.3, CH_2
				1.46, m	
11α	2.01, m	22.7, CH_2	25		71.4, C
11β	1.65, m				
12α	1.93, m	43.9, CH_2	26	1.29, s	29.1, CH_3
12β	1.79, m				
13		49.3, C	27	1.29, s	29.3, CH_3
14	3.25, m	42.1, CH	28	3.03, s	50.5, CH_3

Nitric Oxide (NO) Production Inhibition Assay: We used RAW264.7 cells for the NO production inhibition assay, which were incubated in 96-well plates with test samples at concentrations of 6.25, 12.5, 25, 50, 100 μM, and LPS (1 g/mL) for 24 hours. RAW264.7 cells were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences (Yunnan, China). A microplate reader was used to measure the absorbance at 540 nm after incubating Griess reagent with culture medium for 15 minutes at room temperature.

Compound **1**, a white amorphous powder, was assigned a molecular weight of *m*/*z* 497.2884 $[M+Na]^+$ (calcd for 497.2874) by HRESIMS, indicating that its molecular formula was $C_{28}H_{42}O_6$. The IR absorption spectrum indicated the presence of hydroxyl (3444 cm^{-1}) and α,β-unsaturated cyclic ketone (1632 cm^{-1}) [22]. The ^{1}H NMR spectrum (Table 1) displayed characteristic signals for a methoxyl (δ_H 3.03) and five methyls (δ_H 0.90, 1.29, 1.29, 1.41, and 1.61), one broad singlet signal (δ_H 3.94), and two doublet signals (δ_H 6.04, *J* = 2.2 Hz and δ_H 2.41, *J* = 9.5 Hz). Its ^{13}C and DEPT NMR spectrum (Table 1) showed 28 carbon resonances, including a carbonyl (δ_C 205.9), a methoxyl (δ_C 50.5), five methyls (δ_H 9.9, 23.9, 32.1, 29.1, and 29.3), seven methylenes, seven methines, and seven quaternary carbons. Among them, the downfield signals at δ_C 205.9, 175.3, and 122.8, and two oxygen-substituted methine signals at δ_C 68.6 and 69.1, and one oxygen-substituted quaternary carbon at δ_C 71.4, suggesting that compound **1** was a phytoecdysteroid, as well as by comparison with 22-deoxyecdysterone (**3**), which was also isolated in this work [23]. Since the molecular formula of **1** had eight degrees of unsaturation, with the nucleus accounting for the six ones, one double bond (δ_C 135.0 and 144.4) accounting for the one, and no other unsaturated atoms found, it was further convinced of the fifth ring.

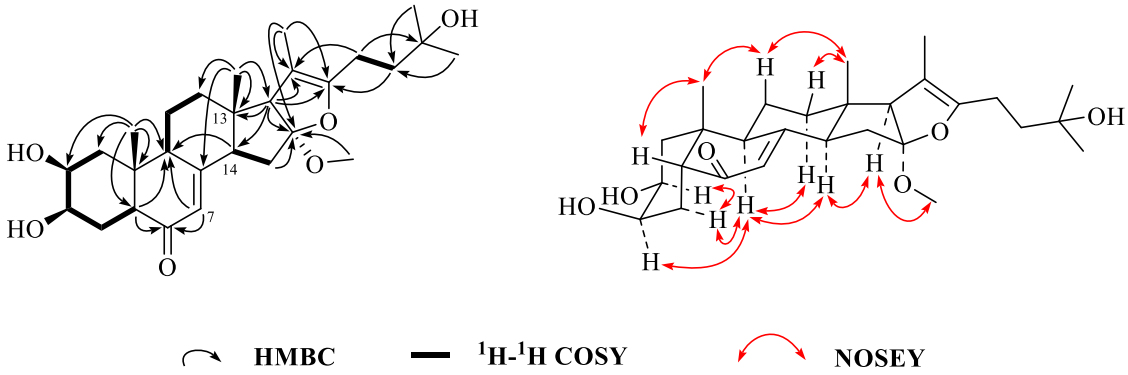

Figure 2. Key 2D NMR correlations of compound **1**

Further analysis of HSQC and HMBC experiments (Figure 2) indicated that compound **1** possessed another ring (ring E), which was proven by HMBC correlations as follows: from H-17 (δ_H 2.41, d, J = 9.5 Hz) to C-13 (δ_C 49.3), C-14 (δ_C 42.1), C-16 (δ_C 103.9), C-20 (δ_C 135.0), and C-22 (δ_C 144.4); from H_3-21 (δ_H 1.61, s) to C-16, C-20, and C-22; from H_2-15 (δ_H 1.52, 1.85) to C-16; from H_2-23 (δ_H 2.48, 1.94) to C-20; from H_2-24 (δ_H 1.76, 1.46) to C-22. The HMBC correlation from a methoxyl (δ_C 50.5) to C-16 indicated that the C-16 position was substituted by a methoxyl. The COSY and residual HMBC correlations, as shown in Figure 2, helped to elucidate the planar structure of **1**. In terms of stereochemistry, the NOESY correlation (Figure 2) between H_3-19 (δ_H 0.90) and H-5 (δ_H 2.35 dd, J = 13.4, 4.0 Hz) was observed, revealing a *cis* junction of ring A/B. The correlations were also detected between H_3-18 (δ_H 1.41) and H-11β (δ_H 1.65) [24], between H-14 (δ_H 3.25) and H-9, and between H-9 and 12α (δ_H 1.93), indicating a *trans*-linked C/D ring. The *cis* junction of D/E ring was determined based on the correlations between H-17 and both MeO-28 and H-14, which coincides with the D/E ring junction of naturally occurring spirostanes [25], thus the MeO-28 group was further deduced as α-oriented. Moreover, the NOESY correlations observed between H-9α and both H-3 and H-4, and between H-2 and H-4 indicated the β-orientation of the hydroxyl groups at C-2 and C-3. According to the afore mentioned findings and other detailed NOESY correlations (Figure 2), compound **1** was elucidated as 16,22-epoxy-16α-methoxyl-2β,3β,25-trihydroxyergost-7,20-dien-6-one, and named furoecdysterone.

Table 2. Inhibitory effects of compounds **1**–**5** on LPS-stimulated NO release from RAW264.7 cells

Compound	CC_{50} (μM)	IC_{50} (μM)
1	> 400.00	44.76 ± 2.60
2	> 400.00	> 100.00
3	> 400.00	69.59 ± 3.28
4	> 400.00	78.31 ± 4.12
5	> 400.00	47.83 ± 2.67
L-NMMA	> 400.00	10.71 ± 2.11

Those compounds were evaluated for their NO inhibitory effects by the lipopolysaccharide (LPS)-induced RAW 264.7 cells assay. L-NMMA was used as a positive control with an IC_{50} value of 10.71 ± 2.11 μM. Except for **2**, the other four compounds (**1** and **3**-**5**) showed moderate NO inhibitory activities (Table 2). When their concentration was more 400.00 μM, none of the compounds showed cytotoxicity against RAW264.7 cells.

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Supporting Information

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