New 23-Spirocholestane Derivatives from *Ypsilandra thibetica*

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Abstract

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Three new unusual 23-spirocholestane derivatives, ypsilanogenin (1), ypsilanogenin 3-O- β -D-glucopyranoside (2), and 4'-acetylypsilanogenin 3-O- β -D-glucopyranoside (3), were isolated from the whole plants of *Ypsilandra thibetica*. The structures of compounds 1–3 were deduced by spectroscopic and chemical methods, and the structure of 1 was further confirmed by a single-crystal diffraction analysis. All isolates were evaluated for their inhibitory activities against HIV-1.

Key words

 $Ypsilandra\ thibetica \cdot Liliaceae \cdot 23$ -spirocholestane glycosides · anti-HIV-1 activity

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Ypsilandra thibetica, a perennial plant belonging to the Liliaceae family, is mainly distributed in southwestern China [1]. The whole plant has been used for treatment of scrofula, urine negative, edema, uterine bleeding, and traumatic hemorrhage [2,3]. Our previous studies on the species have resulted in the isolation of a series of steroidal saponins with cytotoxic and antifungal activities [4–9]. Reinvestigation of the phytochemical constituents of this plant afforded three new cholestane-type steroids, ypsilanogenin (1), ypsilanogenin 3–O- β -D-glucopyranoside (2), and 4′-acetylypsilanogenin 3–O- β -D-glucopyranoside (3) (\bullet Fig. 1). Compounds 1–3 had a unique 23-spirocholestane skeleton with a ketal carbon at C-23 to link to C-16 and C-2′ of the fucosyl moiety and an oxygen bridge between C-24 and C-1′ of the fucosyl moiety. We present herein the isolation, structural elucidation, and anti-HIV activities of the three new compounds.

Compound **1**, obtained as colorless prisms, had a molecular formula of $C_{33}H_{52}O_8$ as determined by the HR-ESI-MS ion at m/z 575.3580 [M – H]⁻ (calcd. for $C_{33}H_{51}O_8$, 575.3583), thus requiring eight double-bond equivalents. The IR absorptions implied the presence of hydroxyl (3446 cm⁻¹) and carbonyl (1709 cm⁻¹) groups. In accordance with its molecular formula, all 33 carbons were well resolved in the ¹³C NMR spectrum (**© Table 1**) and were further classified by DEPT experiments as six methyls, eight methylenes, 14 methines (7 oxygenated ones), two quaternary carbons, an acetal, a ketal, and a carbonyl. In addition, two tertiary methyls at $\delta_{\rm H}$ 0.73 (s) and 0.77 (s), four secondary methyls at

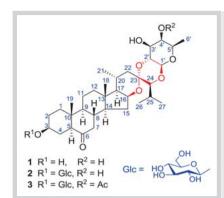


Fig. 1 Structures of compounds 1–3. (Color figure available online only.)

 $\delta_{\rm H}$ 0.78 (d, J = 6.3 Hz), 1.10 (d, J = 6.6 Hz), 1.16 (d, J = 6.6 Hz), and 1.58 (d, J = 6.3 Hz), and an acetal proton at $\delta_{\rm H}$ 4.58 (d, J = 6.7 Hz) were distinguished by ¹H NMR data (**Table 1**). The carbonyl group accounted for one out of the eight degrees of unsaturation, and the remaining seven degrees of unsaturation implied compound 1 to be heptacyclic (rings A-G). Interpretation of ¹H-¹H COSY spectra of 1 revealed four spin systems (a-d) as shown with bold bonds in **Fig. 2**. Further, the HMBC correlations (**Fig. 2**) from H₂-4, H-5, H₂-7, and H-8 to C-6, from Me-18 to C-12, C-13, C-14, and C-17, from Me-19 to C-1, C-5, C-9, and C-10, and from H-16, H-20, H-24, H-25, and H-2' to C-23 led to unambiguous assignment for the connectivities of the substructures $\mathbf{a} - \mathbf{c}$ to four quaternary carbons [δ_C 210.1 (C-6); δ_C 40.9 (C-10); δ_C 42.3 (C-13); and δ_C 100.3 (C-23)], and two tertiary methyls. In addition, the diagnostic HMBC correlation from H-1' to C-5' allowed connection of C-1' to C-5' by an oxygen bridge. The E-ring and G-ring were connected by extensive interpretation of the well-resolved HMBC spectrum. The HMBC correlations of H-1' ($\delta_{\rm H}$ 4.58) with C- $24 (\delta_{\rm C} 85.9)$, and H-2' ($\delta_{\rm H} 4.10$) with C-23 ($\delta_{\rm C} 100.3$) indicated the linkages in order of C-23-O-C-2' and C-24-O-C-1'. The above data revealed the planar structure of 1. The different ring junctions and the stereochemistry around rings A, F, and G were deduced from detailed analysis of the ROESY experiment (OFig. 2). The clear NOE correlations from H-8 to Me-18 and Me-19, from H-14 to H-9, H-16, and H-17, and from H-5 to H-3 and H-9 in the ROESY spectrum indicated the usual trans ring fusion for rings A/B, B/C, and C/D as well as a *cis* junction for rings D/E and β -configuration for OH-3, and that the rings A-C adopted a chair conformation. Moreover, NOEs of H-20 with Me-18 was consistent with the β -configuration for H-20. In addition, the ROESY correlations of H-17/H-22 α , H₂-22/H-2', H-1'/H-24, H-1'/H-3', H-1'/H-5', H-3'/H-4', and H-3'/H-5' indicated that H-24, H-1', H-3', H-4', and H-5' were α -oriented, while H-2' and Me-6' were β -oriented and rings F-G also adopted a chair conformation. The oxygenbridge between C-23 and C-2' was α -oriented. Finally, the structure and relative configuration of 1 were unequivocally confirmed by single-crystal X-ray diffraction (Fig. 3). Therefore, the structure of 1 was established as shown in • Fig. 1, and the compound was named ypsilanogenin.

Compound **2** was obtained as colorless prismatic crystals (MeOH). Its negative ion HR-ESI-MS exhibited a quasimolecular ion peak at m/z 737.4109, consistent with a molecular composition of $C_{39}H_{61}O_{13}$ (calcd. 737.4112). The deduced molecular formula was higher by $C_6H_{10}O_5$ than that of **1**, and the ¹H NMR spectrum (**Table 1**) showed a signal for an anomeric proton at 5.04 (d, J = 7.7 Hz), along with signals for five cholestane-type steroid methyl protons at δ_H 0.63 (s), 0.70 (s), 0.75 (d, J = 6.6 Hz), 1.08

Table 1 1 H and 13 C NMR data of compounds **1–3** in C₅D₅ N.

Position	1		2		3	
	δ _H (/ in Hz)	δ_{C}	δ _H (/ in Hz)	δ_{C}	δ _H (/ in Hz)	δ_{C}
1α	1.18 m	37.0	1.03 m	36.7	1.00 m	36.9
1β	1.66 m		1.52 m		1.52 m	
2α	2.05 m	31.8	2.05 m	29.5	2.04 m	29.5
2β	1.67 m		1.57 m		1.57 m	
3	3.84 m	70.0	3.98 m	76.6	3.95 m	76.8
4α	2.31 m	31.3	2.37 m	27.0	2.36 m	27.1
4β	1.91 m		1.72 m		1.72 m	
5	2.25 m	56.9	2.08 m	56.4	2.01 m	56.5
6		210.1		209.7		209.7
7α	2.38 m	46.9	2.36 m	46.8	2.36 m	46.8
7β	1.98 m		1.99 m		1.97 m	
8	1.60 m	26.0	1.57 m	26.0	1.60 m	26.1
9	1.15 m	53.8	1.11 m	53.7	1.09 m	53.8
10		40.9		40.9		40.9
1α	1.52 m	21.6	1.45 m	21.5	1.43 m	21.5
1β	1.27 m		1.22 m		1.21 m	
2α	1.77 m	40.2	1.74 m	40.1	1.73 m	40.1
2β	1.09 m		1.05 m		1.03 m	
3		42.3		42.2		42.2
4	0.90 m	52.5	0.89 m	52.5	0.87 m	52.5
5α	1.87 m	32.8	1.87 m	32.7	1.84 m	32.7
5β	1.20 m		1.18 m		1.18 m	
6	4.47 m	71.4	4.48 m	71.4	4.43 m	71.5
7	0.80 m	58.8	0.80 m	58.8	0.80 m	58.8
8	0.73 s	14.9	0.70 s	14.9	0.71 s	14.9
9	0.77 s	13.3	0.63 s	13.1	0.64 s	13.1
20	1.82 m	37.0	1.77 m	36.9	1.76 m	36.7
21	0.78 d (6.3)	20.9	0.75 d (6.6)	20.9	0.83 d (6.1)	20.9
!2α	2.23 m	37.3	2.21 m	37.3	2.38 m	37.4
2β	1.09 m	37.3	1.10 m	57.5	1.23 m	37.4
3	1.05 111	100.3	1.10111	100.3	1.23 111	100.5
4	3.59 d (5.1)	85.9	3.58 d (6.2)	85.9	3.58 d (4.6)	86.1
5	2.08 m	28.2	2.07 m	28.2	2.09 m	28.2
.5	1.10 d (6.6)	18.1	1.08 d (6.5)	18.1	1.09 d (6.7)	18.1
7	1.16 d (6.6)	22.9	1.14 d (6.5)	22.9	1.15 d (6.7)	22.9
1'	4.58 d (6.7)	101.4	4.56 d (6.3)	101.4	4.59 d (7.9)	101.2
2'	4.10 m	72.3	4.12 m	72.3	4.29 m	70.0
3'	4.12 m	73.6	4.12 m	73.6	4.00 m	74.0
4'	4.05 brs	73.0	4.07 brs	73.0	5.60 d (3.4)	74.3
- 5'	3.86 (m)	73.5	3.87 m	73.5	3.98 m	71.8
6'	1.58 d (6.3)	17.2	1.57 d (5.9)	17.2	1.32 d (6.3)	16.6
0 1′′	1.50 (0.5)	17.2	5.04 d (7.7)	102.2	5.01 d (7.6)	102.2
2''			4.05 m	75.4	4.04 m	75.4
2 3''			4.30 m	78.7	4.28 m	78.7
3 4''			4.23 m	71.8	4.24 m	71.9
5''			4.23 III 4.00 m	78.7	4.24 III 4.00 m	71.9
6''a			4.61 d (11.2)	63.0	4.61 m	63.0
6''b					4.40 m	03.0
			4.39 dd (12.5, 5.6)		2.19 s	171.0
AC .					2.135	21.0
						21.0

(3H, d, J = 6.5 Hz), and 1.14 (d, J = 6.5 Hz). On comparison of the whole 13 C NMR spectrum of **2** with **1** (**© Table 1**), a set of six signals corresponding to a D-glucopyranosyl moiety were observed at $\delta_{\rm C}$ 102.2 (d), 75.4 (d), 78.7 (d), 71.8 (d), 78.7 (d), and 63.0 (t), and the signals due to C-3 of the aglycone and its neighboring carbon varied, while all other signals remained almost unaffected. Acid hydrolysis of **2** with 4 M TFA released D-glucose residue, as determined by GC analysis of the corresponding trimethylsilated L-cysteine adduct [10]. The β-configuration of the

anomeric center of the glucopyranosyl was supported by the relatively large J value of the anomeric proton (J = 7.7 Hz). The HMBC spectrum showed a long-range correlation peak from the anomeric proton of the glucopyranosyl group at δ_H 5.04 to C-3 of the aglycone at δ_C 76.6. Consequently, these considerations established the structure of **2** as indicated in **Fig. 1**, and the compound was named ypsilanogenin 3-O- β -D-glucopyranoside. Compound **3** was obtained as white amorphous powder and had the molecular formula $C_{41}H_{64}O_{14}$ on the basis of the negative ion

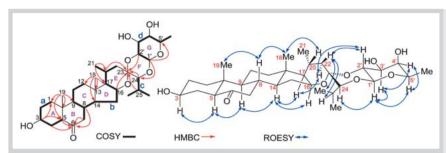


Fig. 2 Selected 2D NMR correlations of compound **1**. (Color figure available online only.)

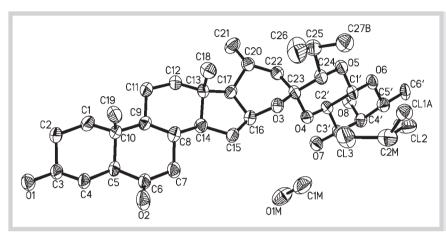


Fig. 3 Single-crystal X-ray structure of compound **1**.

HR-ESI-MS (m/z 779.4201 [M – H]⁻; calcd. 779.4217). Comparison of the 1 H- and 13 C NMR spectra of **3** with those of **2** showed their considerable structural similarity. However, **3** had an additional acetyl group, which was supported by the NMR signals at $\delta_{\rm H}$ 2.19 (3H, s), $\delta_{\rm C}$ 21.0 (q), and $\delta_{\rm C}$ 171.0 (s), and by the mass difference of m/z = 42 between **3** and **2**. The above evidence suggested that compound **3** is a monoacetate of **2**. The acetoxyl group was positioned at C-4′ on the basis of HMBC correlations of H-4′ ($\delta_{\rm H}$ 5.60) with the acetyloxy group ($\delta_{\rm C}$ 171.0), C-2′ ($\delta_{\rm C}$ 70.0), C-3′ ($\delta_{\rm C}$ 74.0), and C-5′ ($\delta_{\rm C}$ 71.8). Accordingly, the structure of **3** was assigned as depicted in **©** Fig. 1, and the compound was named 4′-acetylypsilanogenin 3-*O*- β -D-glucopyranoside.

To the best of our knowledge, helojaposide [11] obtained from *Heloniopsis japonica* (Liliaceae) was the only example having the same skeleton as compounds **1–3**, but it possessed a hydroxyl group at C-22 and a β -orientation for the oxygen-bridge between C-23 and C-2′. Compounds **1–3** were tested for cytotoxicities against C8166 cells (CC₅₀), and anti-HIV-1 activities were evaluated by the inhibition assay for the cytopathic effects of HIV-1 (EC₅₀), using AZT as a positive control. Each experiment was repeated three times. The results (**© Table 2**) indicated that only compound **1** showed weak anti-HIV-1 activity with an EC₅₀ value of 19.34 µg/mL and a therapy index (TI) of 6.57.

Materials and Methods

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The whole plants of *Y. thibetica* were collected in Zhaotong, Yunnan Province, China, in May 2011, and were identified by one of the authors, Prof. Chang-Xiang Chen. A voucher specimen (No. 0303771) was deposited at the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Chinese Academy of Sciences.

The powdered air-dried whole plants of Y. thibetica (30 kg) were extracted with 70% EtOH (3 × 180 L, total amount 540 L) under reflux for a total of 6 h. After removal of the solvent in vacuo, the residue was suspended in H₂O (20 L) and successively extracted with petroleum ether (3 × 15 L), EtOAc (3 × 15 L), and n-BuOH (3 × 15 L). TLC of the EtOAc extract showed different spots from the reported steroidal saponins isolated from the species. The solvent was removed to give the EtOAc extract (820 g), of which 800 g was further defatted over a column (10 × 140 cm) of MCI gel CHP20P (4 L) eluted with 90% methanol (25 L) and then applied to silica gel column chromatography (20 × 120 cm, 200-300 mesh, 6.4 kg; gradient CHCl₃-MeOH, $20:1 \rightarrow 0:1$, 300 L) to afford fractions 1-8. Fr. 4 (8.0 g) was further separated by RP-18 column chromatography (3 × 48 cm, 150 g; 70–90% aqueous MeOH, 10 L) to afford 1 (30 mg). Fr. 5 (15 g) was repeatedly purified by silica gel $(5 \times 60 \text{ cm}, 300 \text{ g}; \text{ CHCl}_3\text{-MeOH} = 12:1, 15 \text{ L}), \text{ Sephadex LH-}$ 20 (3×180 cm, MeOH, 1.5 L), and then semiprep. HPLC $(MeOH-H_2O = 75:25, flow rate 3 mL/min)$ to yield **3** (10 mg). Fr. 6 (3.3 g) was subjected to silica gel CC (2.5 \times 60 cm, 100 g), eluted with CHCl3-MeOH (10:1, 5.5 L) and then Sephadex LH-20 $(1.5 \times 180 \text{ cm}, \text{MeOH}, 0.5 \text{ L}) \text{ to give } 2 (100 \text{ mg}).$

Isolates

Ypsilanogenin (1): colorless prisms (CHCl₃-MeOH 4:1); mp 208–209 °C; [α]_D²⁵ +5.3 (c 0.76, pyridine); IR (KBr) ν_{max} 3446, 2957, 2870, 1709, 1454, 1383, 1061 cm⁻¹; ¹H NMR (pyridine-d₅, 400 MHz) and ¹³C NMR (pyridine-d₅, 100 MHz), see **© Table 1**; FAB-MS (negative): m/z 575 [M − H]⁻; HR-ESI-MS m/z 575.3580 [M − H]⁻ (calcd. for C₃₃H₅₁O₈, 575.3583).

Ypsilanogenin 3-β-D-glucopyranoside (**2**): colorless prisms (MeOH); mp 228–229 °C; $[\alpha]_D^{22}$ – 25.7 (*c* 0.70, pyridine); IR (KBr) ν_{max} 3456, 2951, 2872, 1703, 1456, 1389, 1370, 1062, 892 cm⁻¹; ¹H NMR (pyridine- d_5 , 400 MHz) and ¹³C NMR (pyridine- d_5 ,

Compound	EC ₅₀ ^a (μg/mL)	CC ₅₀ ^b (µg/mL)	TI ^c
1	19.34 ± 2.16	127.05 ± 12.11	6.57
2	96.33 ± 7.83	> 200	> 2.07
3	69.24 ± 4.46	> 200	>2.89
AZT ^d	0.00351 ± 0.00034	1210.00 ± 74.68	344729.34

Table 2 Anti-HIV-1 activities of compounds **1–3**.

100 MHz), see • **Table 1**; FAB-MS (negative): m/z 737 [M - H]⁻; HR-ESI-MS m/z 737.4109 [M - H]⁻ (calcd. for $C_{39}H_{61}O_{13}$, 737.4112).

4'-Acetylypsilanogenin 3-β-D-glucopyranoside (3): colorless amorphous powder; $[\alpha]_0^{26}$ – 13.3 (c 0.95, pyridine); IR (KBr) $v_{\rm max}$ 3443, 2957, 2872, 1743, 1711, 1454, 1382, 1371, 1061, 892 cm⁻¹; ¹H NMR (pyridine- d_5 , 400 MHz) and ¹³C NMR (pyridine- d_5 , 100 MHz), see **© Table 1**; FAB-MS (negative): m/z 779 [M – H]⁻, 737 [M – H – 42]⁻, 617 [M – H – 162]⁻, 575 [M – H – 42–162]⁻; HR-ESI-MS m/z 779.4201 [M – H]⁻ (calcd. for C₄₁H₆₃O₁₄, 779.4217).

X-ray crystal structure analysis

Colorless crystals of **1** were obtained from the mixture of chloroform and methanol. Diffraction intensity data were collected with a MAC DIP-2030K diffractometer employing graphite monochromated Mo $K\alpha$ radiation and operating in the ω scan mode. The crystal structure was solved by the direct method SHELXS-97 [12], expanded by using difference Fourier techniques and refined by the program and method NOMCSDP and full-matrix least-squares calculations. The hydrogen atoms were fixed at their calculated positions.

Crystal data: C₃₃H₅₂O₈·CH₃OH·CHCl₃, MW = 576.77 (no solvent of crystallization); monoclinic system, space group $P2_1$; crystal cell parameters a = 15.161 (2) Å, b = 8.649 (1) Å, c = 15.652 (2) Å, β = 108.44 (2)°; V = 1947.0 (3) Å³, Z = 2, D_{calc} = 1.122 g/cm³. A crystal of dimensions $0.20 \times 0.50 \times 1.50$ mm was used for measurements ($2\theta_{\text{max}}$ = 50.0°). The total number of independent reflections measured was 3319, of which 3306 were observed ($|F|^2 \ge 3\sigma |F|^2$). The final indices were R_1 = 0.0684, wR_2 = 0.1904, and S = 1.001. Crystallographic data for 1 has been deposited in the Cambridge Crystallographic Data Centre with the deposition number CCDC 885109. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif.

Acid hydrolysis

Compounds **2** and **3** (3 mg) were refluxed with 4 M TFA-dioxane (1:1 v/v, 2 mL) in a water bath for 2 h. After cooling, the reaction mixture was extracted with EtOAc (3×5 mL). The aqueous layer was evaporated to dryness with MeOH until neutral. The dried residue was dissolved in 0.5 mL anhydrous pyridine and treated with L-cysteine methyl ester hydrochloride (1.0 mg) stirred at 60 °C for 1 h. Trimethylsilylimidazole (0.5 mL) was added to the reaction mixture which was then kept at 60 °C for 30 min. The supernatants ($2 \mu L$) were analyzed by GC, respectively, under the following conditions: H₂ flame ionization detector; column: 30QC2/AC-5 quartz capillary column ($30 \text{ m} \times 0.32 \text{ mm}$); column temperature: $180-280 \, ^{\circ}\text{C}$ with the rate of $3 \, ^{\circ}\text{C/min}$, and carrier gas N₂ ($1 \, \text{mL/min}$); injector temperature: $250 \, ^{\circ}\text{C}$; split ratio: 1/50. The configuration of D-glucose for compounds **2** and **3** was

determined by comparison of the retentions times of the corresponding derivatives with those of standard D-glucose giving a single peak at 19.01 min.

Anti-HIV-1 assay

The cytotoxicity assay against C8166 cells (CC_{50}) was performed using the MTT method, and anti-HIV-1 activity was evaluated by the inhibition assay for cytopathic effects of HIV-1 (EC_{50}) [13]. Three tested compounds gave a single peak upon HPLC analysis, and their purity was therefore at least 98%. AZT (Sigma, 98%) was used as the positive control.

Supporting information

1D and 2D NMR spectra of compounds **1–3** and crystal data of **1** are available as Supporting Information.

Acknowledgements



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Conflict of Interest

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The authors declare no conflict of interest in this work.

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 $^{^{}a}$ EC₅₀: effective concentration required to protect C8166 cells against the cytopathogenicity of HIV-1 by 50%. b CC₅₀: cytotoxic concentration required to reduce C8166 cell proliferation by 50% tested by the MTT method. c TI: therapeutic index, ratio of the CC₅₀ value/EC₅₀ value. d AZT was used as a positive control

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