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Three new spirostanol glycosides from *Helleborus thibetanus*

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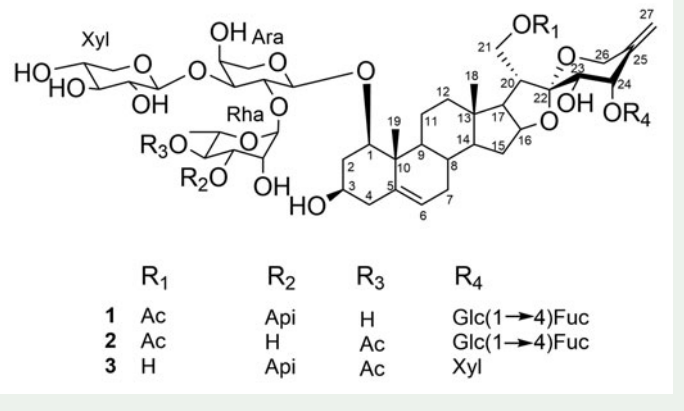
ABSTRACT

An ongoing chemical investigation on *n*-BuOH extract of roots and rhizomes of *Helleborus thibetanus* afforded three new spirostanol glycosides (**1**–**3**). Their structures were elucidated by extensive analysis of 1 D, 2 D NMR spectra, together with IR and MS methods and acid hydrolysis. This is the first report of the isolation of spirostanol glycoside with xylose at C-24 of the aglycone in *Helleborus*.



	R_1	R_2	R_3	R_4
1	Ac	Api	H	Glc(1→4)Fuc
2	Ac	H	Ac	Glc(1→4)Fuc
3	H	Api	Ac	Xyl

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Ranunculaceae; *Helleborus thibetanus*; steroidal saponins

1. Introduction

Helleborus thibetanus, which belongs to the family of Ranunculaceae, is a plant endemic to China. The dried roots and rhizomes of *H. thibetanus*, locally known as 'Xiaotaoerqi' in Chinese, are used as a traditional Chinese medicine for the treatment of cystitis, traumatic injury, and urethritis (Guo et al. 2003). In the course of our studies

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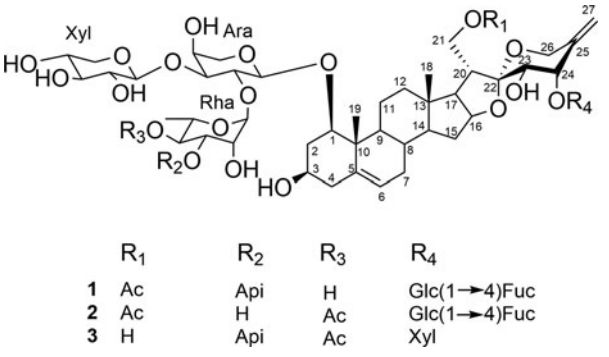

	R_1	R_2	R_3	R_4
1	Ac	Api	H	Glc(1→4)Fuc
2	Ac	H	Ac	Glc(1→4)Fuc
3	H	Api	Ac	Xyl

Figure 1. Structures of compounds **1–3**.

on this plant, we have isolated several steroidal saponins, several bufadienolides and phytoecdystones, one pregnane and one spirostanol sulfate (Zhang et al. 2014a, 2014b, 2016, 2017). Continued phytochemical research on this medicinal plant provided three new spirostanol glycosides (**1**–**3**) (Figure 1). Herein, the isolation and structure elucidation of these three new compounds are reported.

2. Results and discussion

Compound **1** was isolated as a white amorphous solid. Its molecular formula was determined as $C_{62}H_{96}O_{33}$ from the HR-ESI-MS data at *m/z* 1391.5725 $[M+Na]^+$ and ^{13}C NMR data. The IR spectrum displayed absorption bands relative to hydroxy groups at 3333 cm^{-1} and carbonyl group at 1730 cm^{-1}. The characteristic quaternary carbon signal at δ_C 110.9 in ^{13}C NMR spectrum suggested compound **1** had a spirostanol skeleton (Watanabe et al. 2003). The ^1H NMR spectrum showed signals for two quaternary methyl groups at δ_H 1.03 (3 H, s) and 1.40 (3 H, s) assignable to the C-18 and C-19 methyl groups, one olefinic proton at δ_H 5.56 (1 H, d, $J = 5.5$ Hz) and an exocyclic methylene group at δ_H 5.21 (1 H, s) and 5.08 (1 H, s). Comparison to the literature data for steroidal saponins containing a $\triangle^5$-spirostanol skeleton showed a good match with the aglycone of (23*S*,24*S*)-21-acetoxy-3β,23,24-trihydroxyspirosta-5,25(27)-diene-1β-*O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside (Watanabe et al. 2003). The signals at δ_H 1.93 (3 H, s) and δ_C 170.8 and 20.8 showed the presence of an acetyl group. The acetyl group at C-21 of the aglycone was evident from HMBC correlations from δ_H 4.33-4.37 (H-21a, H-21b) to δ_C 170.8. The α-configurations of H-1 and H-3 were supported by correlations between H-1 and H-3, between Me-19 and Me-18/H-2ax/H-4ax in the NOESY spectrum. In the NOESY spectrum, the correlations from H-23 to H-20 and H_a-21/H_b-21/H_a-27/H_b-27, and from H-24 to H_a-27/H_b-27, together with the small coupling constant between H-23 and H-24 ($J = 4.0$ Hz) established *S* configurations of C-23 and C-24 (Watanabe et al. 2003; Mimaki and Watanabe 2008; Hayes et al. 2009).

Six anomeric proton signals at δ_H 4.67, 6.30 (1 H, br s), 6.22 (1 H, d, $J = 2.5$ Hz), 4.97, 5.14 (1 H, d, $J = 8.0$ Hz) and 5.12 (1 H, d, $J = 8.0$ Hz) in the ^1H NMR spectrum and six anomeric carbon signals at δ_C 100.5, 101.3, 111.6, 106.4, 106.1 and 106.8 in the ^{13}C NMR spectrum indicated six sugar moieties in compound **1**. The two methyl signals at

δ_H 1.64 (3 H, d, $J = 6.0$ Hz), δ_H 1.52 (3 H, d, $J = 6.3$ Hz) in the ^{1}H NMR spectrum and two methyl signals at δ_C 18.9, 17.4 in the ^{13}C NMR spectrum suggested two of them were deoxyhexoses. The identification of six sugar moieties arabinose, xylose, rhamnose, apiose, glucose and fucose was carried out by NMR analyses and acid hydrolysis results. The HMBC spectrum allowed to establish the sequence of all sugar units by long-range correlations between the proton signal of H-1 of arabinosyl group and C-1 of the aglycone, between H-1 of xylosyl group and C-3 of arabinosyl group, between H-1 of rhamnosyl group and C-2 of arabinosyl group, between H-1 of apiosyl group and C-3 of rhamnosyl group. In the HMBC spectrum, the correlations between H-24 of the aglycone and C-1 of fucosyl group, between the proton signal of H-4 of fucosyl group and C-1 of glucosyl group indicated that fucosyl group was connected to C-24 of the aglycone, and glucosyl group was linked at C-4 of fucosyl group. The linkage of the sugar moieties was further confirmed by the correlations in NOESY spectrum between H-1 of arabinosyl group and H-1 of the aglycone, between H-1 of xylosyl group and H-3 of arabinosyl group, between H-2 of arabinosyl group and H-1 of rhamnosyl group, between H-3 of rhamnosyl group and H-1 of apiosyl group, between H-24 of the aglycone and H-1 of fucosyl group, between H-1 of glucosyl group and H-4 of fucosyl group. By a detailed analysis of ^{1}H, ^{13}C NMR, DEPT, COSY, HSQC, NOESY, and HMBC spectra, the structure of **1** was defined as (23*S*,24*S*)-21-acetyloxy-24-{[*O*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-*O*-β-D-apiofuranosyl-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside named thibetanoside E.

Compound **2** was obtained as a white amorphous solid. It was found to have a molecular formula of $C_{59}H_{90}O_{30}$ from the peak at *m/z* 1301.5410 $[M + Na]^+$ in the HR-ESI-MS and ^{13}C NMR data. IR spectrum revealed the presence of hydroxy groups at 3402 cm^{-1} and carbonyl group at 1728 cm^{-1}. A detailed comparison of the NMR data of **2** with those of **1** revealed that **2** shared the same aglycone part with **1**. The signals at δ_H 1.89, 1.98 and δ_C 170.9, 170.8 showed two acetyl groups in compound **2**. Comparing with **1**, the chemical shift of C-3 (δ_C 70.0) of rhamnosyl group in **2** moved an upfield shift of 9.6 ppm suggested there was no sugar at C-3 of rhamnosyl group in **2**. One acetyl group was at C-4 of rhamnosyl group on the basis of the correlation between H-4 (δ_H 5.73) of rhamnosyl group and C=O (δ_C 170.8) in the HMBC spectrum, which was also confirmed by the downfield shift of 4.0 ppm for C-4 (δ_C 76.5) of rhamnosyl group in **2** compared with **1**. Acid hydrolysis of **2** gave arabinose, xylose, rhamnose, glucose and fucose. Through analysis of ^{1}H, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and NOESY data of **2**, its structure was elucidated as (23*S*,24*S*)-21-acetyloxy-24-{[*o*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside named thibetanoside F.

Compound **3** was obtained as a white amorphous solid. It was assigned the molecular formula $C_{55}H_{84}O_{28}$ on the basis of its positive HR-ESI-MS at *m/z* 1215.5040 $[M + Na]^+$ and ^{13}C NMR data. Its IR spectrum revealed absorptions at 3391 cm^{-1} and 1730 cm^{-1} due to hydroxy and carbonyl groups, respectively. Comparison of the ^{1}H and ^{13}C NMR signals of **3** with those of **1**, revealed that the aglycone structure of **3** was similar to that of **1**, except for one hydroxy group at C-21 in **3** instead of an

acetoxy group in **1**. The hydroxy group at C-21 was further confirmed by the correlations between H-17/H-20 and C-21 in the HMBC spectrum. Acid hydrolysis of **3** gave arabinose, xylose, rhamnose and apiose. The position of the acetyl group at C-4 of rhamnosyl group was assigned by the HMBC cross-peaks between C=O (δ_C 170.4) and H-4 (δ_H 5.89) of rhamnosyl group. The signals for fucosyl group and glucosyl group in **3** disappeared and two sets of NMR signals for xylosyl group were observed in **3**. Xylosyl group-1 was located at C-3 of arabinosyl group by the HMBC correlation between H-1 of xylosyl group-1 and C-3 of arabinosyl group. The correlation between H-24 and C-1 of xylosyl group-2 indicated xylosyl group-2 was linked at C-24 of the aglycone. Thus, the structure of **3** was established as (23*S*,24*S*)-24-[(*O*-β-D-xylopyranosyl) oxy]-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-*O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside named thibetanoside G.

3. Experimental

3.1. General experimental procedures

Optical rotations were acquired on a Rudolph Research Analytical Autopol II automatic polarimeter (Rudolph Research Analytical, USA). IR spectra with KBr pellets were measured on a Bruker Tensor 27 spectrometer (Bruker Co., Ettlingen, Germany). NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker Daltonics Inc., Faellanden, Switzerland) at room temperature (25 °C), and the chemical shifts (δ) were expressed in ppm and were referenced to the residual solvent peaks. The HR-ESI-MS data were obtained on Bruker microTOF-Q II mass spectrometer (Bruker Biospin Inc., Germany) and Agilent Technologies 6230 TOF LC/MS (Agilent, Karlsruhe, Germany). Column chromatography (CC) was performed with D101 macroporous resin (Tianjin Haiguang Chemical Co., Ltd, Tianjin, China), silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), and ODS (40-63 μm, LiChroprep RP-18, Merck KGaA, Darmastadt, Germany). ODS was also used as a stationary phase for the medium-pressure liquid chromatography (MPLC) system (Shanghai Tauto Biotech Co., Ltd, Shanghai, China). Analytical HPLC was performed on a Wufeng LC-100 chromatography system equipped with a LC-UV100 tunable UV absorbance detector using ODS columns (Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 μm, CA, USA). Preparative HPLC was carried out using an ODS column (Agilent ZORBAX SB-C18, 21.2 × 250 mm, 7 μm, CA, USA). Thin layer chromatography (TLC) was performed using precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd, Qingdao, China). All solvents were of analytical grade (Tianjin Concord Technology Co. Ltd, Tianjin, China).

3.2. Plant material

The roots and rhizomes of *Helleborus thibetanus* were collected from Mei County, Shaanxi Province in China in September 2007. The plant material was authenticated by Prof. Zhen-Hai Wu of College of Life Sciences in Northwest A&F University, China. The voucher specimen (No. S200609002) has been deposited in School of Pharmaceutical Science and Technology, Tianjin University, Tianjin.

3.3 Extraction and isolation

The extraction and partition of the dried roots and rhizomes of *H. thibetanus* (8.0 kg) were conducted as reported in our previous study (Zhang et al. 2017).

The *n*-BuOH extract (934 g) was subjected to a D101 macroporous resin column chromatography (CC), eluting with EtOH–H_2O (0:100, 30:70, 50:50, 70:30 and 95:5 gradient system) to afford five fractions (fractions A-E). The fraction A (378 g), eluted with 30% EtOH aq., was separated on a silica gel column using EtOAc–MeOH (9:1–6:4) to give 126 fractions (fractions A1-A126).

Fractions A77–A92 (50 g) were subjected to a silica gel column with a gradient of CH_2Cl_2–MeOH (8:2–6:4), followed by an open ODS CC eluting with a step gradient of MeOH–H_2O (3:7–100:0) to give subfractions 17–19 (9.1 g) and subfractions 20–22 (6 g). Subfractions 17–19 were further separated by silica gel CC with $CHCl_3$–MeOH (83:17–7:3), ODS CC with MeCN-H_2O (19:81–100:0) and repeated preparative HPLC with MeCN–H_2O (21:79) to afford compound **2** (8 mg). Subfractions 20–22 were subjected to MPLC using MeOH–H_2O (34:66-100:0) as the eluent and further purified by preparative HPLC repeatedly to yield compound **3** (8 mg).

Fractions A93–A107 (68 g) were applied to silica gel CC using EtOAc–MeOH (8:2–5:5) to get subfractions 63–78 (24 g), which were separated by MPLC with a step gradient of MeOH–H_2O (3:7–100:0) to give subfractions 42–46 (2 g). Subfractions 42–46 were subjected to preparative RP-HPLC repeatedly with MeCN–H_2O (17:83) to afford compound **1** (10 mg).

3.3.1. Thibetanoside E (1)

White amorphous solid; $[\alpha]^{25}_D$ −50 (*c* 0.1, MeOH); IR (KBr) ν_{max}: 3333, 2923, 1730, 1046 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data, see Tables S1 and S2. HR-ESI-MS: *m/z* 1391.5725 $[M + Na]^+$ (calcd for $C_{62}H_{96}O_{33}Na$, 1391.5727).

3.3.2. Thibetanoside F (2)

White amorphous solid; $[\alpha]^{25}_D$ −85 (*c* 0.28, MeOH); IR (KBr) ν_{max}: 3402, 2917, 1728, 1050 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data, see Tables S1 and S2; HR-ESI-MS: *m/z* 1301.5410 $[M + Na]^+$ (calcd for $C_{59}H_{90}O_{30}Na$, 1301.5409).

3.3.3. Thibetanoside G (3)

White amorphous solid; $[\alpha]^{25}_D$ −95 (*c* 0.2, MeOH); IR (KBr) ν_{max}: 3391, 2921, 1730, 1043 cm^{-1}; ^1H NMR and ^{13}C NMR spectroscopic data are presented in Tables S1 and S2; HR-ESI-MS: *m/z* 1215.5040 $[M + Na]^+$ (calcd for $C_{55}H_{84}O_{28}Na$, 1215.5041).

3.4. Acid hydrolysis of compounds 1–3

Compounds (4 mg for **1**, 3 mg for **2**, 3 mg for **3**) in 1 M HCl (dioxane-H_2O, 1:1, 6 ml) were hydrolyzed by heating at 80 °C for 2 h. After cooling down, each of the reaction mixture was dried under reduced pressure to remove dioxane and diluted to 8 ml with H_2O, and then the mixture was extracted with EtOAc (4 × 6 ml). The aqueous layer was neutralized by Ag_2CO_3 to pH = 6–7 and filtered with the microporous membrane and further concentrated to an appropriate volume. D-glucose, D-xylose, L-

arabinose, D-fucose, D-apiose and L-rhamnose in the sugar fraction were identified by comparison with authentic samples by TLC (silica gel) with the solvent systems of $CHCl_3$: MeOH: HAc: H_2O (16:10:3:3) and EtOAc: MeOH: HAc: H_2O (20:5:3:3).

4. Conclusion

The present phytochemical investigation of roots and rhizomes of *Helleborus thibetanus* revealed the presence of three new spirostanol glycosides. Isolation of spirostanol glycoside with xylose at C-24 of the aglycone is reported from *Helleborus* for the first time.

Disclosure statement

No potential conflict of interest was reported by the authors.

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