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Two new bufadienolides and one new pregnane from *Helleborus thibetanus*

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ABSTRACT

Two new bufadienolides, 3β,14β,16β-trihydroxy-5α-bufa-20,22-dienolide (**1**) and 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide (**2**), one new pregnane, 3β-hydroxypregna-5,16-diene-20-one-1β-yl sulfate (**3**), along with one known pregnane (**4**) were isolated from the dried roots and rhizomes of *Helleborus thibetanus*. Their structures were elucidated by the extensive use of 1D and 2D NMR experiments, together with IR and HRESIMS spectra and the results of enzymatic hydrolysis.

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1. Introduction

The genus of *Helleborus* is a member of the Ranunculaceae family. It comprises more than 20 species which are widely spread in Southeast Europe and West Asia. Previous phytochemical investigation on *Helleborus* illustrated that steroids including bufadienolides, phytoecdystones and steroidal saponins (Muzashvili et al., 2011; Yang et al., 2010a,b; Bassarello et al., 2008; Watanabe et al., 2003, 2005; Braca et al., 2004; Mimaki et al., 2003; Meng et al., 2001) were the main components. *H. thibetanus* Franch., an endemic plant of China, is mainly distributed in Sichuan, Gansu and Shaanxi. The roots and rhizomes of *H. thibetanus*, locally called "*XiaoTaoErQi*", have a wide use for the treatment of cystitis, urethritis, sores and traumatic injury (An et al., 2013; Yang et al., 2010a,b). One spirostanol sulfate, several bufadienolides and phytoecdystones had been isolated from *H. thibetanus* (Yang et al., 2010a,b). Herein, the isolation of two new bufadienolides (**1–2**), one new pregnane (**3**) (Fig. 1) and one known compound (**4**) from the title plant is reported. Their structures were elucidated by spectroscopic techniques including IR, MS, 1D and 2D NMR spectroscopy.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{24}H_{34}O_5$, deduced from the HRESIMS (*m/z* 425.2301 $[M+Na]^+$), as well as its ^{13}C NMR spectrum. IR absorptions at 3439 cm^{-1} and 1725 cm^{-1} supported the presence of hydroxyl and carbonyl groups. The assignments of **1** (Table 1) were established by a comprehensive analysis of ^1H and ^{13}C NMR, DEPT, COSY, HSQC, HMBC and NOESY spectra. Its ^1H and ^{13}C NMR spectroscopic data were similar to those of the known compound 14β,16β-dihydroxy-3β-[(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide (Yang et al., 2010a), which has an α-pyrone ring at C-17 position and the A/B ring junction was *trans*. Comparison of the ^1H and ^{13}C NMR spectra of compound **1** and 14β,16β-dihydroxy-3β-[(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide, allowed us to observe the absence of the signals for the β-D-glucose in **1**. The angular methyl carbon signal at δ_C 12.7 (Me-19) in **1** was very similar to the signal at δ_C 12.4 (Me-19) in both 5α-furostan and 5α-spirostan with 5α-H (Agrawal et al., 1985; Su et al., 2009), identifying an α-configuration of H-5, moreover, the correlations observed in the NOESY (Fig. 2) spectrum between Me-19 (δ_H 0.74) and Hax-2 (δ_H 1.65)/Hax-4 (δ_H 1.52)/Hax-6 (δ_H 1.14)/H-8 (δ_H 1.72)/Hax-11 (δ_H 1.17), between H-5 (δ_H 1.05) and H-3 (δ_H 3.84)/Hax-1 (δ_H 0.96)/H-9 (δ_H 0.85), and between H-3 (δ_H 3.84) and Hax-1 (δ_H 0.96) manifested the configuration of 5α-H and the A/B ring junction was *trans*. Therefore, the structure

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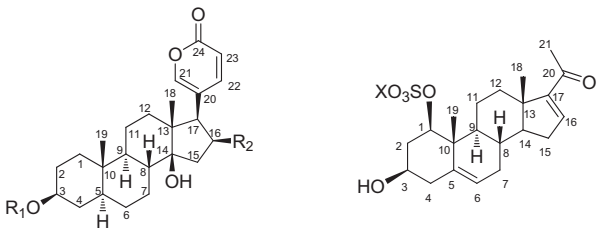

	R_1	R_2
1	H	OH
2	Glc(1→6)Glc	H

3

Fig. 1. Structures of compounds **1**–**3**, **3** given as salt (mostly K^+).

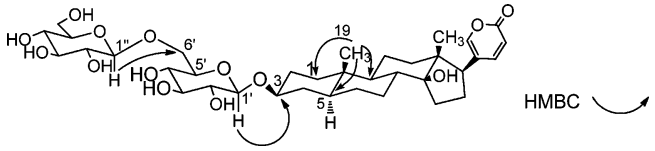

Fig. 2. Selected HMBC, NOE correlations for compound **1**.

of **1** was unambiguously identified as 3β,14β,16β-trihydroxy-5α-bufa-20,22-dienolide.

Compound **2** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{36}H_{54}O_{15}$, deduced from the HRESIMS (*m/z* 733.3399 $[M + Na]^+$), as well as its ^{13}C NMR spectrum. The assignments of **2** (Table 1) were achieved by a comprehensive analysis of ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and NOESY spectra. The ^1H and ^{13}C NMR spectroscopic data disclosed that compound **2** was similar to compound **1**, significant differences in the chemical shifts of positions 15, 16 and 17 (Table 1), indicated **2** lacking the hydroxyl group at C-16. The two anomeric proton signals at δ_H 4.95 (d, J = 7.5 Hz), 5.11 (d, J = 7.5 Hz) in the ^1H NMR spectrum and two carbon signals at δ_C 102.3, 105.3 in the ^{13}C NMR spectrum were indicative of the presence of two hexose moieties. Enzymatic hydrolysis of **2** with snailase (Hu et al., 2004) afforded glucose (Glc), which was identified by TLC analysis. The β-orientation of the glucose was supported by the J values of their anomeric H-atoms and the D configuration of the glucose was assumed from biogenetic consideration. The deshielded chemical shift observed for C-3 (δ_C 77.5) compared to C-3 (δ_C 70.9) of compound **1** provided the linkage of inner Glc to C-3, which were confirmed by HMBC (Fig. 3) correlation from H-1′ (δ_H 4.95) of inner Glc to C-3 (δ_C 77.5). The HMBC correlation between H-1″ (δ_H 5.11) of terminal Glc and C-6′ (δ_C 70.1) of inner Glc demonstrated the linkage of the two glucosyl at C-6′ (δ_C 70.1) of inner Glc, which could also be deduced by the chemical shift of C-6′ of inner Glc. Thus, the structure of **2** was characterized as 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide. Compound **3** was isolated as an amorphous solid, its molecular formula was determined as $C_{21}H_{30}O_6S$, deduced from the HRESIMS (*m/z* 409.1692 $[M - H]^-$), as well as its ^{13}C NMR spectrum. The presence of the sulfate functional group was further confirmed by a series of characteristic strong absorption bands at 1237, 1061 and 955 cm^{-1} in its IR (KBr) spectrum (Yang et al., 2010b;

Table 1
^1H (500 MHz), ^{13}C (125 MHz) NMR spectroscopic data of compounds **1** (pyridine-d_5) and **2** (pyridine-d_5)[a]

No.	1		2		No.	2	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C
1ax[b]	0.96	38.0	0.97, m	37.4	3-*O*-Glc		
1eq[c]	1.68	–	1.62	–	1′	4.95, d (7.5)	102.3
2ax	1.65	32.7	1.64	30.0	2′	3.95, dd (8.5, 8.0)	75.13
2eq	2.02, m	–	2.16, m	–	3′	4.20	78.43
3	3.84, dddd (5.0, 5.5, 10.5, 11.0)	70.9	4.02, dddd (5.5, 5.5, 10.5, 10.5)	77.5	4′	4.12	71.65
4ax	1.52	39.5	1.36	34.8	5′	4.11	77.2
4eq	1.78, m	–	1.82	–	6′	4.83, brd (11.5)	70.1
5	1.05, m	45.1	0.90	44.2		4.33	–
6ax	1.14	29.6	1.12	29.2	Glc		
6eq	1.24	–	1.22	–	1″	5.11, d (7.5)	105.3
7ax	1.16	28.6	1.08	28.0	2″	4.00	75.13
7eq	2.39, m	–	2.30, m	–	3″	4.19	78.43
8	1.72	42.3	1.63	42.0	4″	4.18	71.65
9	0.85, td (15.5, 3.5)	50.3	0.83	50.0	5″	3.89, m	78.43
10	–	36.4	–	36.0	6″	4.47, dd (12.0, 2.0)	62.7
11ax	1.17	22.0	1.11	21.7		4.32	–
11eq	1.41, m	–	1.34	–			
12ax	1.25	41.5	1.20	40.7			
12eq	1.48	–	1.35	–			
13	–	50.0	–	48.8			
14	–	84.9	–	84.3			
15	2.49, dd (14.5, 7.5)	43.5	1.91	32.9			
	2.14, brd (14.5)	–	1.81	–			
16	4.77, dd (7.5, 7.0)	73.0	2.11, m; 1.83	29.4			
17	2.76, d (7.5)	59.4	2.44	51.4			
18	0.98, s	17.6	0.84, s	17.2			
19	0.74, s	12.7	0.63, s	12.2			
20	–	119.7	–	123.3			
21	7.47, d (2.0)	150.9	7.44, brs	149.4			
22	8.48, dd (9.5, 2.5)	151.7	8.19, dd (10.0, 2.0)	147.6			
23	6.27, d (9.5)	112.9	6.33, d (10.0)	115.2			
24	–	162.6	–	162.1			

[a] Full assignments of the protons and carbons were accomplished by analysis of COSY, HSQC and HMBC spectra, and coupling pattern and coupling constants (J in Hz) are in parentheses. Overlapped signals were given without designating multiplicity.
[b] ax = axial.
[c] eq = equatorial.

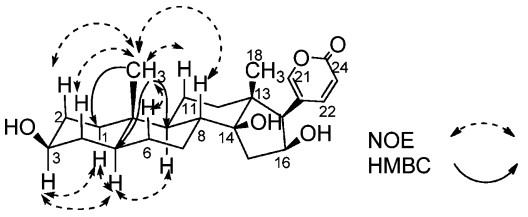

Fig. 3. Selected HMBC correlations for compound **2**.

Asano et al., 1993). Its ^{1}H NMR and ^{13}C NMR spectra revealed the presence of two angular methyl groups at δ_H 0.94 (s), 1.27 (s) and δ_C 16.1, 14.6. Evidence for the presence of a methyl ketone and two double bonds at C-5 and C-16 came from a three-proton singlet at δ_H 2.20 and two vinylic proton signals at δ_H 5.60 and δ_H 6.56 in the ^{1}H NMR of **3**, which showed a close similarity to that of lβ,3β-dihydroxy-5,16-pregnadien-20-one (Gamboa-Angulo et al., 1996), suggesting that **3** had a pregnane skeleton. Full assignments of the protons and carbons (Table 2) of **3** were accomplished by analysis of ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC and NOESY spectra. The obvious HMBC correlation between H-19 and C-1 confirmed that the carbon signal at δ_C 84.9 was designated to C-1 in the ring A, and the proton signal at δ_H 4.75 was assigned to H-1 by its HSQC correlation with C-1. The signal at δ_C 84.9 (C-1) of **3** was 7.3 ppm higher than δ_C 77.6 (C-1) of lβ,3β-dihydroxy-5,16-pregnadien-20-one (Gamboa-Angulo et al., 1996), but similar to δ_C 85.5 (C-1) of ruscogenin 1-sulfate (Asano et al., 1993), δ_C 84.1 (C-1) of spirost-5-en-1β,3β-diol 1-sulfate (Oulad-Ali et al., 1996), and δ_C 85.1 (C-1) of spirost-5,25(27)-dien-1β,3β-diol 1-sulfate from the title plant (Yang et al., 2010b), confirming the sulfate group at C-1. In the NOESY plot, the intense correlations between H-1 (δ_H 4.75) and H-3 (δ_H 3.90) verified the β-configurations of the oxygenated substituents at C-1 and C-3. The ^{1}H and ^{13}C NMR signals of A/B ring and Me-19 were identical to those of spirost-5-en-1β,3β-diol 1-sulfate (Oulad-Ali et al., 1996) and spirost-5,25(27)-dien-1β,3β-diol 1-sulfate from the title plant (Yang et al., 2010b), further verifying one sulfate connected to C-1, one hydroxy at C-3 and their β-configurations. Based on the above evidence, compound **3** was indicated to be 3β-hydroxyl-pregna-5,16-diene-20-one-1β-yl sulfate.

In addition to the three new steroids (compounds **1–3**), one previously known compound (**4**) was also isolated and identified by the spectroscopic data and physical data as pregna-5,16-diene-20-one-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside, which was identical to the structure reported in the patent (Li and Shen, 2012).

3. Experimental

3.1. General experimental procedure

Optical rotation was determined on a Rudolph Research Analytical Autopol II automatic polarimeter. IR spectra were recorded on a Bruker Tensor 27 spectrometer using KBr disks. NMR spectra were recorded on a Bruker AVANCE DRX-500 spectrometer and a Varian INOVA 500 MHz spectrometer. HRMS spectra were made on a Varian IonSpec FT 7.0 T mass spectrometer. All solvents used were of analytical grade (Tianjin Jiangtian Chemical Technology Co., Ltd.). Silica gel (100–200 and 200–300 mesh, Qingdao Haiyang Chemical Co., Ltd.), D101 macroporous resin (Tianjin Haiguang Chemical Co., Ltd.), ODS Silica gel (40–63 μm, LiChroprep RP-18, Merck KGaA) and Sephadex LH-20 (Amersham Pharmacia Biotech AB) were used for open-column chromatography. TLC was carried out using precoated plates with GF_{254} silica gel (Qingdao Haiyang Chemical Co., Ltd.). Spots on TLC were visually observed under UV light and by spraying with 5% sulfuric acid in alcohol reagent followed by heating. Preparative HPLC was performed using ODS columns (Agilent ZORBAX SB-C18, 21.2 mm × 250 mm, 7 μm).

3.2. Plant material

The roots and rhizomes of *H. thibetanus* were collected from Mei County, Shaanxi Province in the People's Republic of China in September 2007 and were authenticated by Prof. Zhen Hai Wu. A voucher specimen (S200609002) is deposited in School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, People's Republic of China.

3.3. Extraction and isolation

The dried roots and rhizomes of *H. thibetanus* (8.0 kg) were pulverized and refluxed with 95% (v/v) EtOH twice (each time 6 l) and then 60% (v/v) EtOH once (6 l). The combined extracts were concentrated to give a residue (1.5 kg) which was suspended in water to a final volume of 5 l and then partitioned sequentially with petroleum ether (PE) (60–90 °C), $CHCl_3$, EtOAc, and *n*-BuOH.

The $CHCl_3$ extract (34.7 g) was applied to silica gel column chromatography with a step gradient of PE–Me_2CO (9:1 to 6:4, v/v), producing fractions 75–76 repeatedly followed by silica gel column chromatography ($CHCl_3$–Me_2CO, 7:3 to 5:5, v/v and $CHCl_3$–MeOH, 98:2 to 9:1, v/v) and purified by Sephadex LH-20 (MeOH), compound **1** (30 mg) was obtained by further recrystallization with Me_2CO.

Table 2
^{1}H (500 MHz), ^{13}C (125 MHz) NMR spectroscopic data of compound **3** (pyridine-d_5)[a]

No.	δ_H	δ_C	No.	δ_H	δ_C
1	4.75, dd (12.0, 4.5)	84.9	11ax	1.74	23.6
2ax[b]	2.24, ddd (12.0, 11.5, 11.5)	39.6	11eq	3.02, m	–
2eq[c]	3.43, m	–	12ax	1.60	35.8
3	3.99, dddd (5.0,5.0,10.5,12.5)	67.8	12eq	2.50, m	–
4ax	2.56, m	43.6	13	–	45.9
4eq	2.65, m	–	14	1.37, m	56.5
5	–	139.0	15	2.10, m; 1.89	32.4
6	5.60, brd (5.5)	125.3	16	6.56, brs	144.3
7	1.85,1.56	31.4	17	–	155.7
8	1.53	31.6	18	0.94, s	16.1
9	1.73	50.4	19	1.27, s	14.6
10	–	43.2	20	–	196.2
			21	2.20, s	27.1

[a] Full assignments of the protons and carbons were accomplished by analysis of COSY, HSQC and HMBC spectra, and coupling pattern and coupling constants (*J* in Hz) are in parentheses. Overlapped signals were given without designating multiplicity.
[b] ax = axial.
[c] eq = equatorial.

The *n*-BuOH extract (934 g) was submitted to D101 macroporous resin column chromatography eluting with EtOH–H_2O (0:100, 30:70, 50:50, 70:30 and 95:5, v/v) to get five fractions. The fraction A (378 g), eluted with 30% EtOH, was partitioned into 126 fractions by silica gel column chromatography (EtOAc–MeOH, 9:1 to 6:4, v/v). Fractions A37–A46 (20 g) were purified by silica gel column chromatography successively ($CHCl_3$–MeOH, 84:16 to 7:3, v/v and EtOAc–MeOH, 94:6 to 9:1, v/v), followed by LiChroprep RP-18 column chromatography (MeOH–H_2O, 3:7 to 100:0, v/v), finally afforded compound **3** (9 mg).

With a gradient of EtOAc–MeOH (90:10 to 60:40, v/v), the fraction B (110 g), eluted with 50% EtOH, was chromatographed over a silica gel column to give 78 fractions. Fractions B23–B31 (9 g) were submitted to silica gel column chromatography ($CHCl_3$–MeOH, 8:2 to 6:4, v/v), LiChroprep RP-18 column chromatography (MeOH–H_2O, 3:7 to 100:0, v/v). Subfractions 13–14 were further applied to Sephadex LH-20 (MeOH) to yield compound **2** (203 mg) and subfractions 27–28 were washed with MeOH to get compound **4** (75 mg).

3.4. 3β,14β,16β-trihdroxy-5α-bufa-20,22-dienolide (**1**)

White amorphous solid, $[\alpha]_D^{25} = 0.7$ (*c* 1.50, C_5H_5N); IR (KBr) ν_{max}: 3439, 2932, 1725, 1088 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS: *m/z* 425.2301 $[M + Na]^+$ (calcd. for $C_{24}H_{34}O_4Na$, 425.2298).

3.5. 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide (**2**)

White amorphous solid, $[\alpha]_D^{25} = -56.0$ (*c* 1.00, C_5H_5N), IR (KBr) ν_{max}: 3420, 2934, 1710, 1071 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS: *m/z* 733.3399 $[M + Na]^+$ (calcd. for $[C_{36}H_{54}O_{14}Na]^+$, 733.3406).

3.6. 3β-hydroxypregna-5,16-diene-20-one-1β-yl sulfate (**3**)

White amorphous solid, $[\alpha]_D^{29.7} = -6.67$ (*c* 0.60, C_5H_5N); IR (KBr) ν_{max}: 3445, 2973, 1663, 1237, 1061, 955 cm^{-1}; ^1H and ^{13}C NMR data see Table 2; HRESIMS: *m/z* 409.1692 $[M - H]^-$ (calcd. for $C_{21}H_{30}O_6S$, 409.1690).

3.7. Pregna-5,16-diene-20-one-3β-yl O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**4**)

Amorphous white solid, $[\alpha]_D^{25} = -51.3$ (*c* 1.50, C_5H_5N); IR (KBr) ν_{max}: 3435, 2933, 1669, 1064 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ_H: 0.88 (s, H-18), 1.00 (s, H-19), 5.27 (brd, *J* = 5.0 Hz, H-6), 6.56 (dd, *J* = 3.0 Hz, H-16), 2.19 (s, H-21), 4.88 (d, *J* = 7.0 Hz, H-1 of inner glucose), 5.05 (d, *J* = 8.0 Hz, H-1 of terminal glucose), 6.17 (d, *J* = 1.5 Hz, H-1 of rhamnose); ^{13}C NMR (C_5D_5N, 125 MHz) δ_C: 37.7 (C-1), 30.5 (C-2), 78.5 (C-3), 39.3 (C-4), 141.7 (C-5), 121.9 (C-6), 32.2 (C-7), 30.7 (C-8), 51.2 (C-9), 37.5 (C-10), 21.3 (C-11), 35.5 (C-12), 46.7 (C-13), 56.9 (C-14), 32.7 (C-15), 145.0 (C-16), 155.6 (C-17), 16.3 (C-18), 19.6 (C-19),196.6 (C-20), 27.5 (C-21), inner glucose: 100.4 (C-1), 77.7 (C-2), 78.1 (C-3), 82.4 (C-4), 76.6 (C-5), 62.5 (C-6), terminal glucose: 105.6 (C-1), 75.3 (C-2), 78.6 (C-3), 71.6 (C-4), 78.8 (C-5), 62.3 (C-6), rhamnose: 102.2 (C-1), 72.8 (C-2), 73.1 (C-3), 74.5 (C-4), 69.8 (C-5), 19.0 (C-6); HRESIMS *m/z* 807.3776 $[M + Na]^+$ (calcd. for $C_{39}H_{60}O_{16}Na$, 807.3774).

3.8. Enzymatic hydrolysis of **2**

Compound **2** (55 mg) was treated with snailase (Sangon, SB0870, 27.2 mg) in HCl buffer (pH 5.0) at 37 °C for 48 h. The crude hydrolysate was extracted with $CHCl_3$ to give an aglycone (16 mg) and glucose. Glucose was identified by direct TLC comparison with an authentic sample.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.08.024.

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