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New cytotoxic bufadienolides from the roots and rhizomes of *Helleborus thibetanus* Franch

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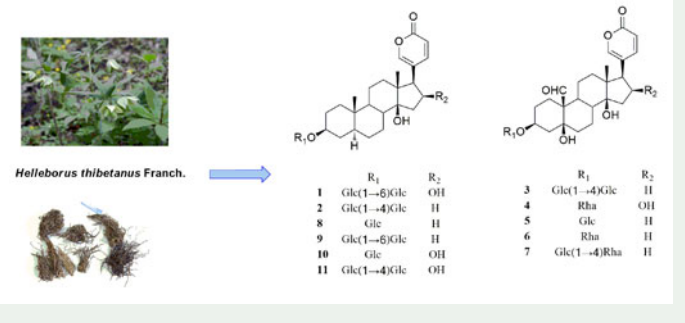
ABSTRACT

Three new bufadienolides 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**1**), 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**2**) and hellebrigenin-3-*O*-β-D-glucosyl-(1→4)-β-D-glucoside (**3**), together with eight known bufadienolides (**4**–**11**) were isolated from the roots and rhizomes of *Helleborus thibetanus*. Their structures were elucidated by extensive spectroscopic methods and acid hydrolysis. Compounds **1**–**7** were evaluated for their cytotoxic activity against HCT116, A549 and HepG2 tumor cell lines. Compound **1** exhibited moderate cytotoxicity against HepG2 cells with IC_{50} value of 15.1 ± 1.72 μM. Compounds **5** and **6** exhibited moderate cytotoxicity against HCT116 cells with IC_{50} values of 15.12 ± 0.58 μM and 13.17 ± 2.34 μM, respectively.



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bufadienolide glycosides; cytotoxic activity; *Helleborus thibetanus* Franch; structure identification

1. Introduction

Helleborus thibetanus Franch., a species in the genus *Helleborus* of the family Ranunculaceae, is used as an endemic herbal medicine, known as 'Tigencao' or 'Xiao-

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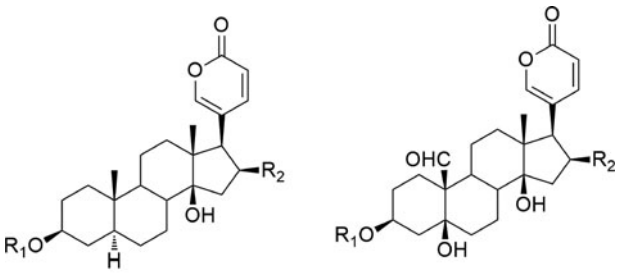

	R_1	R_2
1	Glc(1→6)Glc	OH
2	Glc(1→4)Glc	H
8	Glc	H
9	Glc(1→6)Glc	H
10	Glc	OH
11	Glc(1→4)Glc	OH

	R_1	R_2
3	Glc(1→4)Glc	H
4	Rha	OH
5	Glc	H
6	Rha	H
7	Glc(1→4)Rha	H

Figure 1. Structures of compounds **1–11**.

tao-er-qi', in the Qinba Mountains of Shaanxi Province in China (Song and Liu 2011). The roots and rhizomes of *H. thibetanus* are commonly used as folk medicine to treat cystitis, urethritis, sores and traumatic injury (An et al. 2013; Yang et al. 2010). Previous phytochemical investigation on *Helleborus* illustrated that steroids including bufadienolides, phytoecdystones and steroidal saponins (Bassarello et al. 2008; Braca et al. 2004; Meng et al. 2001; Muzashvili et al. 2011; Tsiftsoglou et al. 2018) were the main components. Pharmacological studies suggested that this plant possesses antitumour, antibacterial, immune-regulation and cytotoxic properties (Littmann et al. 2008; Rosselli et al. 2009). Previous studies disclosed the presence of bufadienolides, spirostanol glycosides, spirostanol sulfonate, pregane and phytoecdystones in the rhizomes of *H. thibetanus* (Yang et al. 2010; Zhang et al. 2016a; Zhang et al. 2016b; Zhang et al. 2017). The bufadienolides of *H. thibetanus* had both A/B *cis* and A/B *trans* ring fusion modes, and some of them were found to exhibited potent antitumour activity. (Cheng et al. 2014; Ma et al. 2018). As part of our research project to explore more diverse bioactive leading compounds from the medicinal herbs of Qinba mountains of China (Chai et al. 2014; Li et al. 2015; Song et al. 2015), the chemical constituents and pharmacological studies of *H. thibetanus* were studied, and three new bufadienolides 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20,22-dienolide (**1**), 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**2**) and hellebrigenin-3-*O*-β-D-glucosyl-(1→4)-β-D-glucoside (**3**), along with eight known bufadienolides 16β-hydroxyhellebrigenin-3-*O*-α-L-rhamnoside (**4**) (Watanabe et al. 2003), hellebrigenin-3-*O*-β-D-glucoside (**5**) (Watanabe et al. 2003), deglucohellebrin (**6**) (Watanabe et al. 2003), hellebrin (**7**) (Yang et al. 2010), 14β-hydroxy-3β-(β-D-glucopyranosyloxy)-5α-bufa-20, 22-dienolide (**8**) (Cheng et al. 2014), 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**9**) (Zhang et al. 2014), 14β, 16β-dihydroxy-3β-(β-D-glucopyranosyloxy)-5α-bufa-20, 22-dienolide (**10**) (Cheng et al. 2014), 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyloxy)]-5β-bufa-20, 22-dienolide (**11**) (Yang et al. 2010) (Figure 1) were isolated from the rhizomes of *H. thibetanus*. Their structures were

elucidated on the basis of extensive spectroscopic analysis. Compounds **1**–**7** were evaluated for their cytotoxic activity against HCT116, A549 and HepG2 tumor cell lines.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{36}H_{54}O_{15}$ from the HR-ESI-MS at *m/z* 749.3364 $[M+Na]^+$ (calcd $C_{36}H_{54}O_{15}Na$ 749.3360). Its IR spectrum revealed absorption bands for hydroxyl (3383 cm^{-1}) and carbonyl groups (1711 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** indicated the presence of a 2H-pyran-2-one unit [δ_H 6.34 (1H, d, $J = 9.7$ Hz, H-23), 7.54 (1H, d, $J = 1.6$ Hz, H-21), 8.55 (1H, dd, $J = 2.5$, 9.8 Hz, H-22); δ_C 113.1, 119.9, 151.1, 151.8, 162.8], two angular methyls [δ_H 0.68 (3H, s, H-19), 1.02 (3H, s, H-18); δ_C 12.7, 17.8], two oxygenated methines [δ_H 4.08 (1H, H-3), 4.82 (1H, t, $J = 5.5$ Hz, H-16); δ_C 78.0, 73.0], one oxygenated quaternary carbon (δ_C 85.0) and two anomeric signals [δ_H 5.01 (1H, d, $J = 7.8$ Hz, H-1′), 5.18 (1H, d, $J = 7.8$ Hz, H-1″); δ_C 102.9, 105.9]. These evidences indicated that compound **1** possessed a bufadienolide skeleton. The ^1H and ^{13}C NMR spectroscopic data of **1** were similar to those of the known compound 3β, 14β, 16β-trihydroxy-5α-bufa-20, 22-dienolide (Zhang et al. 2014), except for the obvious downfield shift of C-3 ($\delta_{C\text{-}3} = +7.1$), which resulted from glycosylation shifts. The β-configuration (Zhang et al. 2012) of the glucose was deduced from the relative large coupling constants of the anomeric protons ($J = 7.8$ Hz). The two glucosyl moieties were identified as D-glucose by acid hydrolysis of **1**, followed by TLC comparison with a reference compound and optical rotation determination (Hudson and Dale 2002). In addition, the HMBC correlation (Table S1 and Figure S1) between H-1′ (δ_H 5.01) and C-3 (δ_C 78.0), as well as between H-1" (δ_H 5.18) and C-6′ (δ_C 70.7) indicated the terminal Glc was linked at C-6 of the inner Glc, which was linked at C-3 of the aglycone. Meanwhile, in the NOESY spectrum, the NOE correlations of H-3/H-1a, H-2a, H-4a and H-5; H-19/H-1b, H-2b, H-4b and H-8 were observed and the lack of correlation between H-5 and H-19, confirming the α-configuration of H-3 and H-5 and the sugar residue to be located at the 3β-position. A key NOE correlation of H-16/H-12a showed the β-configuration of 16-OH. Thus, the structure of **1** was assigned as 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide.

Compound **2** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{36}H_{54}O_{14}$ from the HR-ESI-MS at *m/z* 711.3588 $[M+H]^+$ (calcd $C_{36}H_{55}O_{14}$ 711.3592). The ^1H and ^{13}C NMR spectra of **2** indicated the presence of a 2H-pyran-2-one unit [δ_H 6.38 (1H, d, $J = 9.7$ Hz, H-23), 7.49 (1H, brs, H-21), 8.26 (1H, dd, $J = 1.3$, 9.7 Hz, H-22); δ_C 115.5, 123.7, 147.9, 149.6, 162.4], two angular methyls [δ_H 0.66 (3H, s, H-19), 0.89 (3H, s, H-18); δ_C 12.4, 17.4], one oxygenated methines [δ_H 4.01 (1H, H-3), δ_C 77.6], one oxygenated quaternary carbon (δ_C 84.6) and two anomeric signals [δ_H 5.03 (1H, d, $J = 7.8$ Hz, H-1′), 5.23 (1H, d, $J = 7.8$ Hz, H-1″); δ_C 102.1, 105.2]. These evidences indicated that compound **2** possessed a bufadienolide skeleton. Comparison of the NMR, HR-ESI-MS data of **2** and **1**, compound **2** exihibited spectroscopic features similar to those of **1**, except sugar chain difference and an absence of 16-OH. The proton and carbon NMR signals of [δ_H 2.49 (1H, ca., H-15a), 2.16 (1H, ca., H-15b); δ_C 43.6], [δ_H 4.82 (1H, t, $J = 5.5$ Hz, H-16); δ_C 73.0] and [δ_H 2.82 (1H, d, $J = 7.7$ Hz, H-17); δ_C 59.5]

in **1**, were replaced by [δ_H 1.87 (1H, ca., H-15a), 1.99 (1H, ca., H-15b); δ_C 33.1], [δ_H 2.15 (1H, ca., H-16a), 1.86 (1H, ca., H-16b); δ_C 29.7] and [δ_H 2.47 (1H, dd, $J = 6.1$, 9.3 Hz, H-17); δ_C 51.7] in **2**, which was supported by ^{1}H - ^{1}H COSY, HMQC, HMBC and NOESY spectra. In addition, the HMBC correlation between H-1′ (δ_H 5.03) and C-3 (δ_C 77.6), as well as between H-1" (δ_H 5.23) and C-4′ (δ_C 81.8) indicated the terminal Glc was linked at C-4 of the inner Glc, which was linked at C-3 of the aglycone. Similarly as compound **1**, the results of the acid hydrolysis procedure and analysis of detail NOESY spectra data showed (Figure S2) the structure of compound **2** was deduced as 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide.

Compound **3** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{36}H_{52}O_{16}$ from the HR-ESI-MS at *m/z* 763.3155 $[M + Na]^+$ (calcd $C_{36}H_{52}O_{16}Na$ 763.3153). The ^{1}H and ^{13}C NMR spectra of **3** indicated the presence of a 2H-pyran-2-one unit [δ_H 6.36 (1H, d, $J = 9.6$ Hz, H-23), 7.48 (1H, d, $J = 1.6$ Hz, H-21), 8.25 (1H, dd, $J = 2.5$, 9.7 Hz, H-22); δ_C 115.8, 123.5, 147.9, 149.9, 162.6], one aldehyde hydrogen [δ_H 10.45 (1H, s, H-19); δ_C 209.1], one angular methyl [δ_H 0.89 (3H, s, H-18); δ_C 17.5], one oxygenated methine [δ_H 4.08 (1H, H-3); δ_C 73.6], two oxygenated quaternary carbons (δ_C 73.9, 84.7) and two anomeric signals [δ_H 5.04 (1H, d, $J = 7.9$ Hz, H-1′), 5.22 (1H, d, $J = 7.9$ Hz, H-1′′); δ_C 101.4, 105.6]. These evidences indicated that compound **3** possessed a bufadienolide skeleton. The ^{1}H and ^{13}C NMR spectroscopic data of **3** were similar to those of **5**, except for the sugar chain difference. And the HMBC correlation between H-1′ (δ_H 5.04) and C-3 (δ_C 73.6), as well as between H-1" (δ_H 5.22) and C-4′ (δ_C 82.1) indicated the terminal Glc was linked at C-4 of the inner Glc, which was linked at C-3 of the aglycone. The β-configuration (Zhang et al. 2012) of the glucose was deduced from the relative large coupling constants of the anomeric protons ($J = 7.9$ Hz). The two glucosyl moieties were identified as D-glucose by acid hydrolysis of **3**, followed by TLC comparison with a reference compound and optical rotation determination (Hudson and Dale 2002). Meanwhile, in the NOESY spectrum (Figure S3), the NOE correlations of H-3/H-4, H-4a/H-7a and H-9, and H-19/H-8, indicated α-axial configurations of H-3 and β-orientation of H-19, 3-OH and 5-OH, which supported the A/B cis ring junction pattern. Thus, the structure of **3** was formulated as hellebrigenin-3-*O*-β-D-glucosyl-(1→4)-β-D-glucoside.

Compounds **1**–**7** were evaluated for their cytotoxic activity against HCT116, A549 and HepG2 tumor cell lines, 5-fluorouracil was used as positive control. As shown in Table S2, compound **1** exhibited moderate cytotoxicity against HepG2 cells with IC_{50} value of 15.1 ± 1.72 μM, compounds **5** and **6** exhibited moderate cytotoxicity against HCT116 cells with IC_{50} values of 15.12 ± 0.58 μM and 13.17 ± 2.34 μM, respectively. Compounds **3**, **5**–**7** shared the same aglycone, but exhibited different activities. This suggested that the structural differences such as the category and the number of the oligosaccharide at C-3 played a role in terms of antitumor effect.

3. Experimental

3.1. General experimental procedures

Optical rotation indices were determined in methanol on a Rudolph Autopol II digital polarimeter (Rudolph, Hackettstown, NJ, USA). The IR spectra were recorded on a

TENSOR-27 instrument (Bruker, Rheinstetten, Germany). ESI-MS was performed on a Quattro Premier instrument (Waters, Milford, MA, USA). The HR-ESI-MS spectra were recorded on an Agilent Technologies 6550 Q-TOF (Santa Clara, CA, USA). 1D and 2D NMR spectra were recorded on Bruker-AVANCE 400 instrument (Bruker, Rheinstetten, Germany) with TMS as an internal standard. The analytical HPLC was performed on a Waters 2695 Separations Module coupled with a 2996 Photodiode Array Detector and a Accurasil C18 column (4.6 mm ×250 mm, 5 mm particles, Ameritech, Chicago, IL, USA). Semipreparative HPLC was performed on a system comprising an LC-6AD pump (Shimadzu, Kyoto, Japan) equipped with a SPD-20A UV detector and a Ultimate XB-C18 (10 mm ×250 mm, 5 mm particles) or YMC-Pack-ODS-A (10 mm ×250 mm, 5 mm particles). D101 was from Sunresin New Materials Co. Ltd. (Xi'an, China). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

3.2. Plant material

The roots and rhizomes of *H. thibetanus* were collected from the Taibai region of Qinba Mountains in Shaanxi Province, China, in June 2016, and identified by senior experimentalist Jitao Wang. A voucher specimen (herbarium No. 20160915) has been deposited in the Medicinal Plants Herbarium (MPH), Shaanxi University of Chinese Medicine, Xianyang, China.

3.3. Extraction and isolation

The air-dried and powdered underground parts of *H. thibetanus* (15 kg) were extracted with 60% EtOH (15 L) three times at 80 °C. After removing the solvent, the concentrated residue was successively partitioned with petroleum ether and n-BuOH. The n-BuOH extract (500 g) was subjected to column chromatography (CC) on silica gel with gradient elution ($CHCl_3$-MeOH-H_2O, 100:0:0-65:35:10), which yielded ten fractions (Fr.1-10). Fr.3 (80 g) was subjected to column chromatography (CC) on silica gel, eluting with gradient solvent system ($CHCl_3$-MeOH, 100:0-50:50) to yield five fractions (Fr.3-1–Fr.3-5). Fr.3-3 (220 mg) were purified by HPLC with CH_3CN-H_2O (35:65) as mobile phase to afford **4** (50 mg), **5** (62 mg), **6** (14 mg). Fr.4 (60 g) was subjected to column chromatography (CC) on silica gel, eluting with gradient solvent system ($CHCl_3$-MeOH, 100:0-50:50) to yield six fractions (Fr.4-1–Fr.4-6). Fr.4-2 (220 mg) were purified by HPLC (YMC-Pack-ODS-A, 10 mm ×250 mm, 5 μm particles, flow rate: 1.0 mL·min^{-1}) with CH_3CN-H_2O (30:70) as mobile phase to afford **8** (13 mg), **10** (11 mg), **9** (15 mg) and **11** (21 mg). Fr.6 (65 g) were loaded on a silica gel column and eluted with a gradient solvent system ($CHCl_3$-MeOH, 100:0-50:50) to yield four fractions (Fr.6-1–Fr.6-4). Fr.6-2 was purified by HPLC with CH_3CN-H_2O (25:75) as the mobile phase to obtain **2** (100 mg) and **7** (12 mg). Fr.6-4 was purified by HPLC with CH_3CN-H_2O (27:73) as the mobile phase to obtain **1** (10 mg) and **3** (8 mg).

3.3.1. 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (1)

A white amorphous powder, $[\alpha]_D^{27.2}$-16.9 (*c* 1.1, MeOH), IR (KBr) ν_{max}: 3382, 2941, 1711, 1055. ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) spectral data, see Table S1; *m/z* 749.3364 $[M + Na]^+$ (calcd. for $C_{36}H_{54}O_{15}Na$ 749.3360).

3.3.2. 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (2)

A white amorphous powder, $[\alpha]_D^{26.8}$-36.0 (*c* 1.3, MeOH), IR (KBr) ν_{max}: 3382, 2943, 1709, 1056. ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) spectral data, see Table S1; *m/z* 711.3588 $[M + H]^+$ (calcd. for $C_{36}H_{55}O_{14}$ 711.3592).

3.3.3. Hellebrigenin-3-O-β-D-glucosyl-(1→4)-β-D-glucoside (3)

A white amorphous powder, $[\alpha]_D^{27.0}$-19.5 (*c* 0.7, MeOH), IR (KBr) ν_{max}: 3407, 2944, 1715, 1630, 1056. ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) spectral data, see Table S1; *m/z* 763.3155 $[M + Na]^+$ (calcd. for $C_{36}H_{52}O_{16}Na$ 763.3153).

3.4. Acid hydrolysis of compounds 1-3 and absolute sugar configuration determination

The solutions of compounds **1** (4 mg), **2** (3 mg) and **3** (5 mg) were hydrolyzed with 2*N* HCl (5 mL) for 5 h at 80 °C, respectively. The reaction mixtures were concentrated and dried by N_2, and then water (5 mL) was added and the mixtures were extracted with EtOAc (3 × 5 mL). The aqueous layers of **1**–**3** were subjected to CC over silica gel eluted with MeCN-H_2O (8:1) to yield D-glucose, which was determined by TLC comparison (MeCN-H_2O, 6:1) with the authentic sugar and the optical rotation determination $[\alpha]_D^{20.0}$ +49.2 (*c* 0.16, H_2O).

3.5. Cytotoxicity experiments

The cytotoxic activity assays towards the HCT116, A549 and HepG2 tumor cell lines were measured by the MTT method *in vitro*, using 5-fluorouracil as positive control. Briefly, 1×10^4 mL^{-1} cells were seeded into 96-well plates and allowed to adhere for 24 h. Compounds **1**–**7** were dissolved in DMSO and diluted with complete medium to six concentration levels (from 0.001 mmol·L^{-1} to 0.3 mmol·L^{-1}) for inhibition rate determination. After incubation at 37 °C for 4 h, the supernatant was removed before adding DMSO (100 μL) to each well. 5-Fluorouracil (5-Fu) was used as positive control. The inhibition rate (IR) and IC_{50} were calculated. Values are mean ± SD, $n = 3$, ** $p < 0.01$ *vs.* DMEM control. Compounds **1**–**7** showed cytotoxicity against human HCT116, A549 and HepG2 cell lines; the IC_{50} values are shown in Table S2.

4. Conclusion

Three new bufadienolides 14β, 16β-dihydroxy-3β-[β-D-glucopyranosyl-(1→6)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**1**), 14β-hydroxy-3β-[β-D-glucopyranosyl-(1→4)-(β-D-glucopyranosyl)oxy]-5α-bufa-20, 22-dienolide (**2**) and hellebrigenin-3-*O*-β-D-glucosyl-(1→4)-β-D-glucoside (**3**), together with eight known bufadienolides (**4**–**11**) were isolated from the roots and rhizomes of *H. thibetanus*. Compounds **1**, **5** and **6** showed moderate cytotoxicity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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