Fitoterapia 81 (2010) 636–639

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Two new bufadienolides from the rhizomes of *Helleborus thibetanus* Franch

Jian Yang [a], Yan-hong Zhang [b], Fang Miao [b], Le Zhou [a,*], Wei Sun [c]

[a] College of Science, Northwest A&F University, Yangling 712100, PR China
[b] College of Life, Northwest A&F University, Yangling 712100, PR China
[c] Department of Chemistry, Northwest University, Xi'an 710068, PR China

ARTICLE INFO

Article history:
Received 12 August 2009
Accepted in revised form 9 March 2010
Available online 15 March 2010

Keywords:
Helleborus thibetanus Franch
Bufadienolides
Tigencaosides A and B

ABSTRACT

Two new bufadienolides, named tigencaoside A(**1**) and tigencaoside B(**2**), were isolated from the rhizomes of *Helleborus thibetanus* Franch., along with two known bufadienolides, hellebrigenin (**3**) and 5β,14β-dihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy]bufa-20,22-dienolide (**4**). Their structures were elucidated on the basis of extensive spectroscopic analysis. Two new compounds were evaluated for their cytotoxic activities against four strains of cultured tumor cells.

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1. Introduction

Helleborus thibetanus Franch. (the family Ranunculaceae) commonly known as Tigencao or Xiaotaoqi is a perennial herb and Chinese special local plant species which is only distributed in Qinling Mountain in China. Its dried rhizomes have been used as Chinese folk medicine for the treatment of cystitis, urethritis and traumatic injury since bufadienolides19th century. Previous phytochemical investigations on the *Helleborus* genus have led to the isolation of several bufadienolides, ecdysteroids, spirostanol saponins, furostanol saponins and flavonoids [1–15]. Bufadienolides have been reported to show potent cardiotonic, blood pressure-stimulating and local anaesthetic activities, especially cytotoxic activity against cultured tumor cells [16]. However, a literature survey on *H. thibetanus* indicates that its phytochemistry has not previously been investigated. In order to find potentially bioactive secondary metabolites from *H. thibetanus* and appraise the ethnomedical properties, the present study was undertaken to investigate the chemical constituents of the rhizomes of *H. thibetanus* and led to the isolation of two new bufadienolides named tigencaoside A (**1**) and tigencaoside B (**2**), along with two known bufadienolides (**3**-**4**), hellebrigenin (**3**) and 5β,14β-dihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy]bufa-20,22- dienolide(**4**) (Fig. 1). Here, we report the isolation and structure elucidation of two new bufadienolides (**1**-**2**).

2. Experimental

2.1. General

Optical rotations were obtained on a Horiba SEPA-300 digital polarimeter. IR Spectra were taken on a Nexus FT-IR 400 spectrometer with KBr pellets. UV Spectra were performed on a SP-2100 UV/VIS spectrometer. NMR spectra were recorded with a Bruker DRX-500 instrument. ESI-MS and HR-ESI-MS were measured on Finnigan-MAT 90 and API QSTAR Pulsar i mass spectrometers respectively. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., PR China), silica gel 60 (0.015–0.04 μm, Merck, US), Sephadex LH-20 (Amersham Biosciences, Sweden) and LSA-30 macroporous resin (Xian Lanshen Chemical Inc., PR China) were used for column chromatography. Compounds were visualized on UV light. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

* Corresponding author. College of Science, Northwest A&F University, No.3 Taicheng Road, Yangling 712100, Shaanxi Province, PR China. Tel.:+86 29 87092048; fax: +86 29 87092226.
E-mail addresses: vivian54321@gmail.com (J. Yang), zhoulechem@yahoo.com.cn (L. Zhou).

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doi:10.1016/j.fitote.2010.03.009

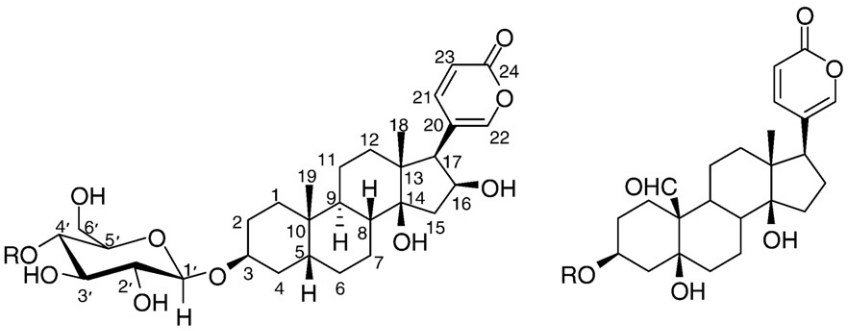

Fig. 1. The structures of **1–4**.

2.2. Plant material

The rhizomes of *H. thibetanus* Franch. were collected from Qinling Mountain, Shaanxi province, PR China in August 2006. The plants were identified by Dr. Fang Miao, a co-author of this paper. The voucher specimen (No.200602638) was deposited in botanic specimen center of Northwest A&F University, Yangling, PR China.

2.3. Extraction and isolation

The dried and powdered rhizomes of *H. thibetanus* (2.4 kg) were exhaustively extracted with 95% ethanol (2×15 L) at 40 °C assisted by ultrasonicfor 30 min for each time, and the combined extracts were concentrated in vacuo. This residue (465.2 g) was suspended in H_2O and then successively partitioned with petroleum ether (PE), EtOAc, and BuOH. The BuOH extract (248.8 g) was dissolved in 2 000 ml distilled water, and passed through a column packed with LSA-30 macroporous resin with a rate of 0.5 ml/s. The column was firstly eluted with water to remove saccharide (detected by α-naphthol test), and then subsequently eluted with 30% aqueous ethanol, 50% aqueous ethanol and 95% aqueous ethanol to afford fractions Fr.1-4 respectively. Fr.2 (50% ethanol portion, 29.8 g) was subjected to column chromatography over silica gel and eluted with $CHCl_3$–MeOH (from 9:1 to 0:1, v/v) to give subfractions Fr.2.1–2.4. Fr.2.1 was subjected to be repeated CC over silica gel 60 (stepwise, $CHCl_3$-MeOH from 15:1 to 12:1) to afford **1** (39 mg) and **2** (24 mg). Fr.3 (70% ethanol portion, 36.6 g) was further separated into subfractions Fr.3.1–3.3 by CC (SiO_2, stepwise, $CHCl_3$-MeOH-H_2O from 9:1:0.1 to 2:1:0.1). Fr.3.1 was subjected to CC (stepwise, $CHCl_3$–MeOH from 9:1 to 4:1) and Sephadex LH-20 chromatography ($CHCl_3$–MeOH, 1:1) to yield **3** (30 mg) and **4** (121 mg).

2.4. Acid hydrolysis

The compounds **1** or **2** (each 10 mg) was refluxed with 4 mol/L HCl for 2 h respectively. The reaction mixture was diluted with H_2O and extracted with EtOAc. The aqueous layer was neutralized with Na_2CO_3 and then concentrated to ca. 2 mL. D-glucose in the aqueous layer was identified by co-TLC with authentic sample using $CHCl_3$–MeOH–H_2O (16:9:1)

Table 1
^{1}H NMR and ^{13}C NMR Spectral Data of **1** and **2** (500 and 125 MHz, DMSO, *J* in Hz and δ in ppm).

Position	**1**		**2**	
	δ (C)	δ (H)	δ (C)	δ (H)
1	36.7	0.88–0.92 (1H, *m*) 1.62–1.66 (1H, *m*)	36.7	0.88–0.93 (1H, *m*) 1.65–1.68 (1H, *m*)
2	29.0	1.38–1.40 (1H, *m*) 1.72–1.74 (1H, *m*)	29.0	1.37–1.40 (1H, *m*) 1.72–1.74 (1H, *m*)
3	76.8	3.53–3.56 (1H, *m*)	76.8	3.53–3.57 (1H, *m*)
4	34.0	1.16–1.20 (1H, *m*) 1.59–1.63 (1H, *m*)	34.0	1.14–1.18 (1H, *m*) 1.64–1.66 (1H, *m*)
5	43.7	0.97–1.02 (1H, *m*, H_β)	43.6	0.98–1.02 (1H, *m*, H_β)
6	28.5	1.11–1.14 (1H, *m*) 1.27–1.30 (1H, *m*)	28.5	1.13–1.16 (1H, *m*) 1.29–1.32 (1H, *m*)
7	27.1	0.93–0.99 (1H, *m*) 2.01 (1H, *d*, *J* 10.1)	27.1	0.92–0.95 (1H, *m*) 2.01 (1H, *d*, *J* 10.7)
8	41.2	1.36–1.40 (1H, *m*, H_β)	41.2	1.37–1.41 (1H, *m*, H_β)
9	49.0	0.78–0.83 (1H, *m*)	49.0	0.82–0.85 (1H, *m*)
10	35.4		35.4	
11	20.8	1.02–1.08 (1H, *m*) 1.36–1.40 (1H, *m*)	20.8	1.07–1.09 (1H, *m*) 1.38–1.41 (1H, *m*)
12	39.7	1.18–1.23 (1H, *m*) 1.38–1.42 (1H, *m*)	39.7	1.20–1.23 (1H, *m*) 1.38–1.42 (1H, *m*)
13	48.7		48.7	
14	83.1	4.17 (1H, s, OH)	83.1	4.17 (1H, s, OH)
15	42.7	1.57 (1H, *d*, *J* 14.2, H_β) 2.48–2.49 (1H, *t*–like, H_α)	42.7	1.57 (1H, *d*, *J* 14.2, H_β) 2.48–2.49 (1H, *t*–like, H_α)
16	70.5	4.38–4.42 (1H, *m*, H_α) 4.51 (1H, *d*, *J* 3.90, OH)	70.4	4.38–4.42 (1H, *m*, H_α) 4.51 (1H, *d*, *J* 3.75, OH)
17	57.5	2.62 (1H, *d*, *J* 8.1, H_α)	57.5	2.62 (1H, *d*, *J* 8.0, H_α)
18	16.8	0.63 (3H, *s*)	16.8	0.63 (3H, *s*)
19	12.0	0.71 (3H, *s*)	12.0	0.70 (3H, *s*)
20	118.7		118.7	
21	150.3	7.46 (1H, *d*, *J* 1.5)	150.3	7.46 (1H, *d*, *J* 1.5)
22	151.5	8.08 (1H, *dd*, *J* 2.4, 9.7)	151.5	8.08 (1H, *dd*, *J* 2.1, 9.7)
23	111.0	6.11 (1H, *d*, *J* 9.7)	111.0	6.11 (1H, *d*, *J* 9.8)
24	161.7		161.7	
1′	100.6	4.20 (1H, *d*, *J* 7.8)	100.4	4.28 (1H, *d*, *J* 7.8)
2′	73.5	2.83–2.88 (1H, *m*)	73.2	2.94–3.00 (1H, *m*)
3′	76.8	3.09–3.12 (1H, *m*)	74.7	3.23–3.28 (1H, *m*)
4′	70.1	2.98–3.02 (1H, *m*)	80.8	3.24–3.28 (1H, *m*)
5′	76.3	3.02–3.05 (1H, *m*)	75.0	3.23–3.28 (1H, *m*)
6′	61.1	3.38–3.41 (1H, *m*) 3.61–3.65 (1H, *m*)	61.0	3.34–3.38 (1H, *m*) 3.67–3.72 (1H, *m*)
1″			103.2	4.23 (1H, *d*, *J* 7.8)
2″			73.3	2.96–3.01 (1H, *m*)
3″			76.6	3.13–3.18 (1H, *m*)
4″			70.0	2.99–3.04 (1H, *m*)
5″			76.5	3.10–3.14 (1H, *m*)
6″			60.5	3.55–3.58 (1H, *m*)

and *n*-BuOH–HOAc–H_2O (BAW, 4:1:5, upper layer) as developers and spraying with aniline *o*-phthalic acid solution.

Tigencaoside A (**1**), white amorphous powder; $[\alpha]^{21.0}{}_D$: −11.0° (*c* 0.052, MeOH); UVmax (MeOH): 293 (logε 3.62), 217 (3.53) nm; IR bands (KBr): 3425, 2923, 2854, 1693, 1118, 1081, 1027 m^{-1}; ^1H and ^{13}C NMR data: see Table 1; ESI-MS (negative) *m/z* 563 $[M–H]^-$, 401$[M–H–162]^-$; HR-ESI-MS (positive) *m/z* 564.2931 $[M]^+$ (Calcd. for $C_{30}H_{44}O_{10}$, 564.2934).

Tigencaoside B (**2**), white amorphous powder; $[\alpha]^{21.0}{}_D$: −11.0° (*c* 0.026, MeOH); UVmax (MeOH): 290 (logε 3.56), 206 (3.43) nm; IR bands (KBr): 3423, 2926, 2853, 1715, 1158, 1079, 1043; ^1H and ^{13}C NMR data: see Table 1; ESI-MS (positive) *m/z* 726 $[M]^+$; HR-ESI-MS (negative) *m/z* 725.3426 $[M–H]^-$ (Calcd. for $C_{36}H_{53}O_{15}$, 725.3384).

3. Results and discussion

Compound **1**, obtained as a white amorphous powder, has a molecular formula of $C_{30}H_{44}O_{10}$ based on HR–ESI–MS, showing a molecular ion peak at *m/z* 564.2931 ($C_{30}H_{44}O_{10}$, calc. 564.2934). Its UV spectrum exhibited absorption bands at λ_{max} (log ε): 293 (3.62) and 217 (3.53)nm due to the presence of a conjugated system. The IR analysis of **1** showed the presence of OH (3425 cm^{-1}), C=C (1629 cm^{-1}) and C=O (1693 cm^{-1}) functional groups. The ^1H NMR spectrum (Table 1) showed the presence of three olefinic protons at δ 6.11 (*d*, *J*=9.7 Hz), 7.46 (*d*, *J*=1.5 Hz), 8.08 (*dd*, *J*=2.4, 9.7 Hz) and two methyl resonances at δ 0.63, 0.71, supporting the fact that **1** had a bufadienolide skeleton. The ^{13}C NMR spectrum (Table 1) revealed a total of 30 carbon atoms and their multiplicity assignments using DEPT established the presence of two methyl (CH_3), nine methylene (CH_2), fourteen methenyl (CH) groups, and five quaternary C-atoms, of which five signals from δ 77 to 61, and a methine signal at δ 105.0 (C-1′) as well as in conjunction with the fragment peak at *m/z* 401 $[M–H–162]^-$ in ESI-MS (negative) suggested the presence of one hexose moiety in the molecule of **1**. The sugar moiety was determined as D-glucose by co-TLC of acid hydrolyzate with an authentic sample. Large coupling constant of the anomeric proton at δ 4.20 (*d*, *J*=7.8 Hz) pointed out to the β-configuration of D-glucose unit.

The aglycone of **1** was determined by comparison of its ^1H and ^{13}C NMR data with that of desacetylbufotalin isolated from *Vietnamese toad* venom [17]. The NMR data of **1** were in good agreement with the literature values except for the signals of an additional glucose moiety, leading to the aglycone of **1** as desacetylbufotalin. Based on the correlation between H-1′ (δ 4.20, *d*, *J*=7.8 Hz) and C-3 (δ 76.8) in HMBC and the downfield shift of C-3 (δ 67.9) of desacetylbufotalin [18], the glucose moiety was assigned to be attached to 3-OH group.

The linkage positions of two hydroxyl groups in the aglycone were substantiated by HMBC spectrum. In the HMBC spectrum, the correlations between H-17 (δ 2.62) and C-16 (δ 70.5) , and between H-16 (δ 4.38–4.42) and C-17 (δ 57.5) showed that one hydroxyl group was attached to C-16, and the correlations between C-14 (δ 83.1)/14-OH (δ 4.17), H-18 (δ 0.63), H-8 (δ 1.36–1.40) and H-15β (δ 1.57) showed that the other hydroxyl group was located at C-14. Based on the long range correlations between H-18 (δ 0.63)/C-12 (δ 39.7), C-13 (δ 48.7), C-14 (δ 83.1) and C-17 (δ 57.5), as well as between H-19 (δ 0.71) and C-1 (δ 36.7), C-10 (δ 35.4), C-5 (δ 43.7) and C-9 (δ 49.0), two methyl groups at δ 0.63 and 0.71 were assigned to be at C-13 and C-10, respectively. The assignments (Table 1) of all C-atoms and H-atoms of **1** were carried out by ^1H,^1H-COSY and HSQC as well as in conjunction with comparison with literature values [17,18].

The stereochemistry of **1** was confirmed by the ROESY analysis. The results were showed in Fig. 2. The cross relationships between 14-OH (δ 4.17)/H-18 (δ 0.63) and H-8 (δ 1.36-1.40) established the β-configuration of 14-OH. The correlation between 14-OH (δ 4.17)/16-OH (δ 4.51) proved the β-configuration of 16-OH and the α-configuration of H-16. Based on the cross relationships between H-18 (δ 0.63)/H-22 (δ 8.08) and H-16α (δ 4.38–4.42)/H-17 (δ 2.62), H-17 was determined as α-configuration. Moreover, the correlations of the protons of β-orientated Me-19 with H-5 (δ 0.97–1.02) and H-8 (δ 1.36–1.40) established H-5 and H-8 as β-configuration. Based on the evidences above, **1** was identified as 14β,16β-dihydroxy-3β-[(β-D-glucopyranosyl) oxy]bufa-20,22-dienolide, named tigencaoside A.

Compound **2**, obtained as a white amorphous powder, possessed a molecular formula $C_{36}H_{54}O_{15}$, as deduced by HR-ESI-MS (negative) showing a quasi-molecular ion peak ($[M-H]^-$) at *m/z* 725.3426 ($C_{36}H_{53}O_{15}$, calc.725.3384). Comparison of the ^1H and ^{13}C NMR spectra for **2** with those of **1** and the co-TLC of the acid hydrolyzate of **2** with authentic sample of

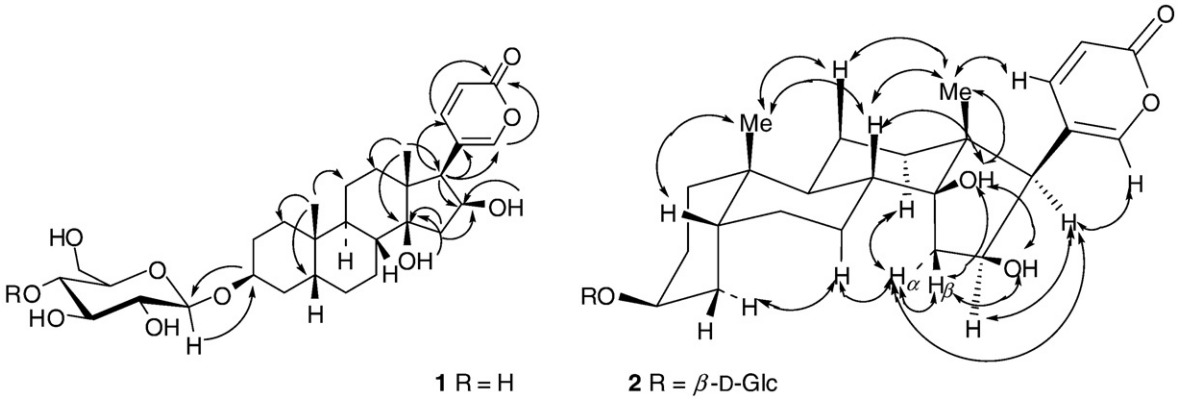

Fig. 2. Key HMBC (left) and ROESY (right) correlations of **1** and **2**.

Table 2
Cytotoxic activities of two new bufadienolides against 3LL, MCF-7, QGY-7701 and BGC-823.

	IC_{50}(μg/ml)			
	3LL	MCF-7	QGY-7701	BGC-823
Tigencaoside A	153.45	105.23	128.76	253.12
Tigencaoside B	68.56	86.45	56.54	78.75

D-glucose showed that **2** differed from **1** only in the presence of an additional D-glucose. Furthermore, the key correlations of HMBC and ROESY spectra of **2** being identical to those of **1** showed that **2** had the same aglycone with **1**. In the HMBC spectrum, the correlations of H-1′ (δ 4.28, *d*, J = 7.8 Hz) with C-3 (δ 76.8) and H-1″ (δ 4.23, *d*, J = 7.8 Hz) with C-4′ (δ 80.8) disclosed that the second D-glucose moiety was attached to C-4' of **1**. Moreover, the large coupling constants J = 7.8 Hz of two anomeric protons and the downfield chemical shifts C-1′ (δ 100.4) and C-1″ (δ 103.2) of two anomeric carbons indicated the β-configuration for the glucopyranose moiety. Thus, **2** was assigned as 14β,16β-dihydroxy-3β-[(β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl)oxy] bufa-20,22-dienolide, named tigencaoside B.

The structures of two known compounds were identified as 3,5,14-trihydroxy-19-oxo-3-[(α-L-rhamnopyranosyl)oxy] bufa-20,22-dienolide (**4**) and hellebrigenin (**3**) by comparison of their spectroscopic data with corresponding literature values [2,19]. Both of them were reported for the first time from this plant.

The cytotoxic activities of the compounds **1** and **2** against four human cancer cell lines: 3LL, MCF-7, QGY-7701 and BGC-823 were determined using the MTT assay method [9,20]. Their IC_{50} values are given in Table 2.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (*NNSF*; No. 30571402, 30771454).

References

[1] Tschesche R, Wagner R, Jha HC. Phytochemistry 1984;23:695.
[2] Kissmer B, Wichtl M. Planta Med 1986;52:152.
[3] Kissmer B, Wichtl M. Arch Pharm 1987;320:541.
[4] Vladimirov SM, Zivanov-Stakic DS, Ribar BJ. Phytochemistry 1991;30:1724.
[5] Dirsch V, Lacaille-Dubois MA, Wagner H. Nat Prod Lett 1994;4:29.
[6] Meng YH, Whiting P, Sik V, Rees HH, Dinan L. Phytochemistry 2001;57:401.
[7] Males Z, Medic-Saric M. J Pharmaceut Biomed 2001;24:353.
[8] Watanabe K, Mimaki Y, Sakuma C, Sashida Y. Chem Lett 2002;31:772.
[9] Watanabe K, Mimak Y, Sakagami H, Sashida Y. J Nat Prod 2003;66:236.
[10] Mimaki Y, Watanabe K, Sakuma C, Sakagami H, Sashida Y. Helv Chim Acta 2003;86:398.
[11] Braca A, Prieto JM, Tommasi ND, Tome F, Morelli I. Phytochemistry 2004;65:2921.
[12] Watanabe K, Sakagami H, Mimak Y. Heterocycles 2005;65:775.
[13] Prieto JM, Siciliano T, Braca A. Fitoterapia 2006;77:203.
[14] Muzashvili T, Benidze M, Skhirtladze V, Sulakvelidze P. Chem Nat Compd 2006;42:613.
[15] Bassarello C, Muzashvili T, Skhirtladze A, Kemertelidze E, Pizza C, Piacente S. Phytochemistry 2008;69:1227.
[16] Kamano Y, Kotake A, Hashima H, Inoue M, Morita H, Takeya K, et al. Bioorg Med Chem 1998;6:1103.
[17] Verpoorte R. Phan-quoc-Kinh, Svendsen AB. J Nat Prod 1980;43:347.
[18] Li WX, Sun H, Li Q, Zhang XQ, Ye WC, Yao XS. Chin Trad Herb Drugs 2007;38:183.
[19] Heerden FR, Vleggaar R. Mag Reson Chem 1988;26:464.
[20] Vercammen D, Beyaert R, Denecker G, Goossens V, Loo GV, Declercq W, et al. J Exp Med 1998;187:1477.