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Steroidal constituents from *Helleborus thibetanus* and their cytotoxicities

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[ABSTRACT] Thibetanosides E–H (**1**–**4**), four new steroidal constituents including three rare sulfonates (**2**–**4**), were isolated from the roots and rhizomes of *Helleborus thibetanus*, together with nine known steroidal compounds (**5**–**13**). Their structures were elucidated by detailed spectroscopic analysis, including 1D and 2D NMR techniques and chemical evidence. In this study, compounds **2**–**13** were evaluated for their cytotoxic activities against HCT116, A549 and HepG2 tumor cell lines *in vitro*. Among them, compound **8** (thibetanoside C) showed cytotoxicities against A549 cells (IC_{50} 39.6 ± 1.9 μmol·L^{-1}) and HepG2 cells (IC_{50} 41.5 ± 1.1 μmol·L^{-1}), respectively. Compound **9** (23*S*, 24*S*)-24-[(*O*-*β*-D-fucopyranosyl)oxy]-3*β*, 23-dihydroxy-spirosta-5, 25(27)-diene-1*β*-yl*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside) showed cytotoxicity against HCT116 cells (IC_{50} 33.6 ± 2.1 μmol·L^{-1}).

[KEY WORDS] *Helleborus thibetanus*; Steroidal constituents; Structure identification; Cytotoxic activity

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Introduction

Helleborus thibetanus Franch., a plant endemic to China, known as "Tigencao" or "Xiao-tao-er-qi", is mainly distributed in Gansu, Sichuan and Shaanxi Provinces. The roots and rhizomes of *H. thibetanus* have been as folk medicine to treat cystitis, traumatic injury and urethritis [1]. Previous pharmacological research has exhibited that this plant produced pharmacological effects such as immune-regulation, anti-tumour and cytotoxic properties [2-3]. Previous phytochemical investigation on this plant illustrated the presence of steroidal saponins, pregnane, bufadienolides, phytoecdystones and other chemical constituents from *H. thibetanus* [4-6]. During the continual screening for bioactive compounds in medicinal herbs growing in the Qinba Mountains [7-10], four new steroidal constituents thibetanosides E–H (**1**–**4**), together with nine known steroidal compounds **5**–**13** (Fig. 1) were obtained from the roots and rhizomes of *H. thibetanus*. In the present paper, we reported the isolation and structural elucidation of steroidal compounds (**1**–**13**), as well as their antitumour cytotoxicities for three human tumor cell lines (HCT116, A549 and HepG2) *in vitro*.

Results and Discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined to be $C_{33}H_{52}O_{10}$ by its HR-ESI-MS at *m/z* 631.3439 $[M + Na]^+$ (Calcd. for $C_{33}H_{52}O_{10}Na$, 631.3453). The ^1H NMR spectrum showed four methyl protons at δ_H 0.91 (3H, s, Me-18), 1.35 (3H, s, Me-19), 1.03 (3H, d, J = 6.8 Hz, Me-21) and 1.4 (3H, s, Me-27), one olefinic proton at δ_H 5.61 (1H, d, J = 4.6 Hz, H-6), along with

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one anomeric proton at δ_H 4.98 (1H, d, J = 7.7 Hz) for the sugar moiety. The ^{13}C NMR spectrum (Table 1) showed 33 carbon signals, 27 of which belonged to the aglycone carbons, and the remaining 6 to one monosaccharide. The quaternary carbon signal at δ_C 120.7 (C-22) was exhibited in the ^{13}C NMR spectrum, characteristic of the furospirostanol skeleton [11]. In the HMQC experiment, the proton and protonated carbon NMR resonances of **1** were assigned. Comparison of the NMR data of **1** and the known compound chonglouoside SL-9 [12], indicated almost similar NMR spectroscopic features, except an increase of 1-OH, which was supported by HMBC correlations of H-19/C-1, C-5, C-9 and C-10 (Fig. 2). Therefore, the aglycone of **1** was identified as (22*S*, 25*S*)-22, 25-epoxyfurost-5-en-1, 3, 26-triol. In addition, the HMBC correlation signal of H-1' (δ_H 4.98) of Glc moiety and C-26 (δ_C 78.0) of aglycone, indicated the glucosyl group was linked to C-26. The glucosyl moiety was judged to be D-glucose by enzymatic hydrolysis of **1**, followed by TLC comparison with a reference compound and optical rotation determination [13], and identified as *β*-configuration [14] from the coupling constant of the anomeric proton (7.7 Hz). Meanwhile, in the NOESY spectrum of **1**, H-1 demonstrated NOE correlations with H-2a, H-3 and H-9, but no correlation with Me-19, verifying *α*-axial configurations of H-1 and H-3, and *β*-orientations of Me-19, 1-OH and 3-OH. The NOE correlations of H-20 (δ_H 2.17)/H-23b (δ_H 1.62) and H-23b/Me-27 (δ_H 1.4) verified that the stereochemistry C-25 was in the *S*-configuration [12]. Hence, thibetanoside E (**1**) was defined as (25*S*)-22*α*, 25-expoxy-26-[(*O*-*β*-D-glucopyranosyl)oxy]-1*β*, 3*β*-dihydroxyfurosta-5-en.

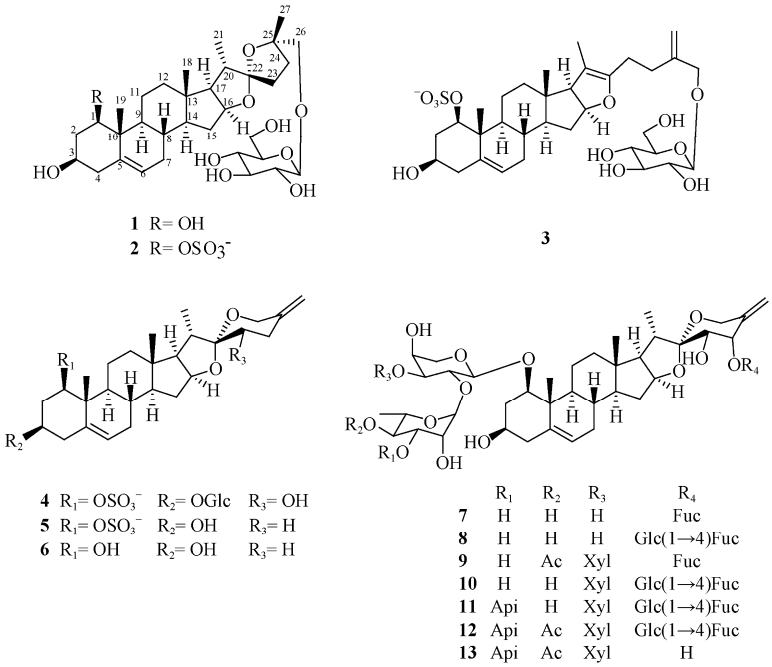

Fig. 1 Structures of compounds 1–13

Table 1 ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compounds 1–4 in pyridine-d_5

No.	1		2		3		4	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	78.6	3.83 (dd, 3.6, 11.3)	84.7	4.76 (dd, 3.9, 11.7)	85.4	4.7(dd, 3.8, 11.8)	83.8	4.7 (dd, 4.0, 11.9)
2	44.1	2.24 (*ca.*[a]) 2.63 (*ca.*)	40.3	2.32 (*ca.*) 3.57 (*ca.*)	40.2	3.46 (*ca.*) 2.28 (*ca.*)	38.0	3.65 (*ca.*) 2.26 (*ca.*)
3	68.6	3.99 (*ca.*)	68.6	3.95 (ca.)	68.3	3.92 (*ca.*)	75.6	4.04 (*ca.*)
4	44.4	2.67 (*ca.*) 2.61 (*ca.*)	44.2	2.66 (*ca.*) 2.57 (*ca.*)	44.1	2.57 (*ca.*) 2.68 (*ca.*)	40.0	2.6 (*ca.*) 2.5 (*ca.*)
5	140.7	-	139.5	-	139.1	-	138.2	-
6	124.9	5.61 (d, 4.6)	125.8	5.57 (d, 3.9)	126.0	5.61 (d, 5.3)	126.2	5.47 (d, 5.3)
7	32.7	1.96 (*ca.*) 1.52 (*ca.*)	32.4	1.8 (*ca.*) 1.48 (*ca.*)	32.5	1.88 (*ca.*) 1.55 (*ca.*)	32.6	1.81 (*ca.*) 1.53 (*ca.*)
8	33.4	1.51 (*ca.*)	33.6	1.51 (*ca.*)	33.2	1.52 (*ca.*)	32.2	1.52 (*ca.*)

Continued

No.	**1**		**2**		**3**		**4**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
9	51.8	1.37 (*ca.*)	50.4	1.78 (*ca.*)	50.3	1.69 (*ca.*)	50.1	1.69 (*ca.*)
10	44.1	-	43.7	-	43.5	-	43.5	-
11	24.7	2.91 (*ca.*) 1.78 (*ca.*)	24.2	3.09 (*ca.*) 1.71 (*ca.*)	24.3	2.92 (*ca.*) 1.59 (*ca.*)	23.8	3.06 (*ca.*) 1.68 (*ca.*)
12	41.0	1.75 (*ca.*) 1.22 (*ca.*)	41.1	1.67 (*ca.*) 1.6 (*ca.*)	40.9	1.62 (*ca.*) 1.35 (*ca.*)	41.2	1.67 (*ca.*) 1.38 (*ca.*)
13	40.8	-	40.8	-	43.6	-	41.0	-
14	57.2	1.12 (*ca.*)	57.0	1.03 (*ca.*)	55.4	0.92 (*ca.*)	56.9	1.15 (*ca.*)
15	32.9	1.87 (*ca.*) 1.36 (*ca.*)	32.9	1.92 (*ca.*) 1.48 (*ca.*)	35.1	2.10 (*ca.*) 1.48 (*ca.*)	33.1	2.01 (*ca.*) 1.51 (*ca.*)
16	81.4	4.72 (*ca.*)	81.6	4.67 (*ca.*)	85.0	4.78 (*ca.*)	82.2	4.62 (*ca.*)
17	63.2	1.76 (*ca.*)	63.1	1.68 (*ca.*)	65.2	2.38 (*ca.*)	62.9	1.84 (*ca.*)
18	17.0	0.91 (3H, s)	17.1	0.82 (3H, s)	15.2	1.3 (3H, s)	17.1	1.03 (3H, s)
19	14.4	1.35 (3H, s)	15.4	1.3 (3H, s)	14.9	0.74 (3H, s)	14.8	1.13 (3H, s)
20	39.1	2.17 (*ca.*)	39.1	2.13 (*ca.*)	104.6	-	36.0	3.01 (*ca.*)
21	15.6	1.03(d, 6.8)	15.8	1.04(d, 6.7)	12.2	1.58 (3H, s)	14.9	1.12 (d, 5.8)
22	120.7	-	120.9	-	152.1	-	112.1	-
23	33.6	2.04 (*ca.*) 1.62 (*ca.*)	33.6	2.18 (*ca.*) 1.96 (*ca.*)	25.2	2.48 (*ca.*) 2.42 (*ca.*)	68.9	3.94 (dd, 5.3, 11.5)
24	34.4	2.23 (*ca.*) 1.67 (*ca.*)	34.4	2.2 (*ca.*) 1.67 (*ca.*)	31.6	2.41 (*ca.*) 2.49 (*ca.*)	39.2	2.92 (*ca.*) 2.83 (*ca.*)
25	84.3	-	84.4	-	146.7	-	144.6	-
26	78.0	4.20(d, 9.9) 3.91(d, 10.0)	78.1	4.16 (d, 9.8) 3.87 (d, 9.9)	72.2	4.59 (d, 13.5) 4.38 (d, 13.3)	64.5	4.43 (d, 12.1) 4.02 (*ca.*)
27	24.9	1.4(3H, s)	24.9	1.39(3H, s)	112.0	5.07 (s) 5.39 (s)	109.7	4.83 (s) 4.85 (s)
3-*O*-Glc								
1							103.2	5.02 (d, 7.7)
2							75.6	4.25 (*ca.*)
3							78.7	3.97 (*ca.*)
4							72.0	4.19 (*ca.*)
5							78.7	4.28 (*ca.*)
6							63.0	4.55 (d, 11.0) 4.32 (dd, 5.8, 12.0)
26-*O*-Glc								
1	106.0	4.98 (d, 7.7)	105.8	4.94 (d, 7.7)	104.3	4.93 (d, 7.8)		
2	75.9	4.05 (*ca.*)	75.9	4.03 (*ca.*)	75.7	4.08 (*ca.*)		
3	79.0	3.97 (*ca.*)	79.0	3.94 (*ca.*)	79.0	3.98 (*ca.*)		
4	72.1	4.31 (*ca.*)	72.1	4.25 (*ca.*)	72.2	4.26 (*ca.*)		
5	78.9	4.28 (*ca.*)	78.8	4.26 (*ca.*)	79.1	4.28 (*ca.*)		
6	63.2	4.56(d,10.8) 4.45 (dd, 4.9, 11.6)	63.0	4.53 (d, 9.9) 4.41(dd, 4.9, 11.7)	63.3	4.56 (dd, 1.9, 12.5) 4.42 (dd, 5.3, 11.8)		

[a] *Ca.* means multiplet.

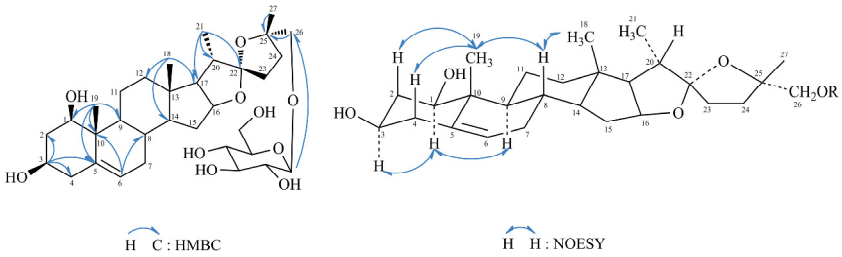

Fig. 2 Key HMBC and NOESY correlations of the compound 1

Compound **2** was obtained as a white amorphous power. Its molecular formula was deduced as $C_{33}H_{51}O_{13}S^-$, from the quasi-molecular ion peak at *m/z* 687.3000 $[M]^-$ (Calcd. for $C_{33}H_{51}O_{13}S^-$, 687.3056) in the HR-ESI-MS, which indicated the presence of a sulfonate group. The sulfonate functional group was further confirmed by a series of characteristic strong absorption bands at 1211, 1053 and 925 cm^{-1} in its IR spectrum [15]. Careful comparison of NMR spectroscopic data of **2** with those of **1**, compound **2** exihibited spectroscopic features similar to those of **1**, except for one oxygenated carbon signal at C-1 ($\delta_{C\text{-}1}$= + 6.1) and one carbon signal at C-2 ($\delta_{C\text{-}2}$ = – 3.8), which resulted from one sulfonate group. And the HMBC correlation of H-1 (δ_H 4.76, dd, *J* = 3.9, 11.7 Hz) and C-19 (δ_C 15.4), further illustrated the presence of the sulfonate functional group at C-1. The above inference were certified by HMQC, HMBC and NOESY spectrum. Similarly as compound **1**, the results of NOESY spectra data analysis and enzymatic hydrolysis procedure revealed the structure of thibetanoside F (**2**) was elucidated as (25*S*)-22*α*, 25-expoxy-26-[(*O*-*β*-D-glucopyranosyl)oxy]-3*β*-hydroxyfurosta-5-ene-1*β*-yl sulfonate.

Compound **3** was isolated as a white amorphous powder, which showed positive reactions in the Ehrlich, Liebermann-Burchard and Molisch reactions, indicating that **3** was a furostanol glycoside. Its molecular formula was determined as $C_{34}H_{51}O_{11}S^-$ from the HR-ESI-MS peak at *m/z* 669.2967 $[M]^-$ (Calcd. for $C_{34}H_{51}O_{11}S^-$, 669.2950). The ^1H NMR spectrum showed three methyl protons at δ_H 1.3 (3H, s, Me-18), 0.74 (3H, s, Me-19) and 1.58 (3H, s, Me-21), two exo-methylene protons (δ_H 5.39 (1H, s, H-27a) and 5.07 (1H, s, H-27b)), one olefinic proton at δ_H 5.61 (1H, d, *J* = 5.3 Hz, H-6), as well as one anomeric proton at δ_H 4.93 (1H, d, *J* = 7.8 Hz, H-Glc-1). The NMR spectroscopic data of **3** were similar to those of the reference compound (23*S*)-26-*O*-*β*-D-glucopyranosyl-3*β*, 23-dihydroxyfurosta-5, 20(22), 25(27)- trien-1*β*-yl sulfonate [16], except an absence of 23-OH. The proton and carbon NMR signals of [δ_H 4.18 (1H, dd, *J* = 3.6, 3.6 Hz, H-23), δ_C 68.1 (C-23)] and [δ_H 1.69 (1H, *ca.*, H-24a), 1.84 (1H, *ca.*, H-24b) and δ_C 28.4 (C-24)] in known compound, were replaced by [δ_H 2.42 (1H, *ca*., H-23a), 2.48 (1H, *ca.*, H-23b) and δ_C 25.2 (C-23)] and [δ_H 2.41 (1H, *ca.*, H-24a), 2.49 (1H, *ca.*, H-24b) and δ_C 31.6 (C-24)] in **3**, which was supported by HMQC, HMBC and NOESY spectrum. In the HMBC spectrum of **3**, HMBC correlations of H-24/C-22, C-23, C-25, C-26 and C-27, H-27/C-24, C-25 and C-26 were observed. Moreover, in the NOESY spectrum, H-1 demonstrated NOE correlations with H-2a, H-3 and H-9, but no correlation with Me-19, verifying *α*-axial configurations of H-1 and H-3, and *β*-orientations of Me-19, 1-OSO_3^- and 3-OH. Similarly as compound **2**, the results of NOESY spectra data analysis and enzymatic hydrolysis procedure illustrated the structure of thibetanoside G (**3**) was identified as 26-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyfurosta-5, 20(22), 25(27)-trien-1*β*-yl sulfonate.

Compound **4** was obtained as a white amorphous powder, and its molecular formula was deduced as $C_{33}H_{49}O_{13}S^-$ by HR-ESI-MS at *m/z* 685.2727 $[M]^-$ (Calcd. for $C_{33}H_{49}O_{13}S^-$, 685.2819). The ^1H NMR spectrum showed three methyl protons at δ_H 1.03 (3H, s, Me-18), 1.13 (3H, s, Me-19) and 1.12 (1H, d, *J* = 5.8 Hz, Me-21), two exo-methylene protons (δ_H 4.83 (1H, s, H-27a) and 4.85 (1H, s, H-27b)), one olefinic proton at δ_H 5.47 (1H, d, *J* = 5.3 Hz, H-6), as well as one anomeric proton at δ_H 5.02 (1H, d, *J* = 7.7 Hz, H-Glc-1). Comparison of the NMR, HR-ESI-MS data of **4** and the know compound **5**, compound **4** exihibited spectroscopic features similar to those of **5**, except for an increase of 3-*O*-*β*-D-glucopyranoside and 23-OH, which was confirmed by the HMBC correlations of H-2/C-1, C-3, C-4 and C-10, and H-23/C-22 and C-24. Instead of a methylene carbon δ_C 33.5 (C-23) in **5**, an oxygen-bearing methine signal at δ_C 68.9 (C-23) which correlated with a proton signal at δ_H 3.94 (dd, *J* = 5.3, 11.5 Hz) in the HMQC spectrum. The chemical shift values of C/H-23, proton coupling constants of H-23, and detailed 2D NMR analysis of ^1H-^1H COSY and HMBC correlations (Fig. 3) further revealed the hydroxyl group was linked at C-23 position. The big coupling constant (11.5 Hz) assigned the axial orientation of H-23, corresponding to *α*-configuration of 23-OH, which was in close agreement with those reported for similar compounds [17], and was confirmed by the NOESY correlation of H-23/H-20. In addition, the HMBC correlation signal of H-1' (δ_H 5.02) of Glc moiety and C-3 (δ_C 75.6) of aglycone, indicated that sugar group was connected as (Glc-1-*O*-C-3). Therefore, thibetanoside H (**4**) was elucidated as (23*S*)-3-*O*-*β*-D-glucopyranosyl-23-hydro- -xyspirost-5, 25(27)-dien-1*β*-yl sulfonate.

Additionally, the known compounds were identified by comparison of their spectroscopic data with those reported in the literature as spirost-5, 25(27)-dien-1*β*, 3*β*-diol 1-sulfonate (**5**) [15], spirost-5, 25(27)-dien-1*β*, 3*β*-diol (**6**) [15], (23*S*, 24*S*)-

spirosta-5, 25(27)-dien-1*β*, 3*β*, 23, 24-tetrol 1-*O*-{[*α*-L-rhamnopyranosyl-(1→2)]-*α*-L-arabinopyranoside}24-*O*-*β*-D-fucopyranoside (**7**) [18], thibetanoside C (**8**) [19], (23*S*, 24*S*)-24-[(*O*-*β*-D-fucopyranosyl)oxy]-3*β*, 23-dihydroxy-spirosta-5, 25(27)-diene-1*β*-yl*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**9**) [20], (23*S*, 24*S*)-24-{[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-fucopyranosyl]oxy}-3*β*, 23-dihydroxyspirosta-5, 25(27)-diene-1*β*-yl*O*-(*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**10**) [6], (23*S*, 24*S*)-24-{[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-fucopyranosyl]oxy}-3*β*, 23-dihydroxyspirosta-5, 25(27)-diene-1*β*-yl*O*-*β*-D-apiofuranosyl-(1→3)-*O*-(*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**11**) [6], (23*S*, 24*S*)-24-{[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-fucopyranosyl]oxy}-3*β*, 23-dihydroxyspirosta-5, 25(27)-dien-1*β*-yl*O*-*β*-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**12**) [16], (23*S*, 24*S*)-3*β*, 23, 24-trihydroxyspirosta-5, 25(27)-dien-1*β*-yl*O*-*β*-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside (**13**) [21].

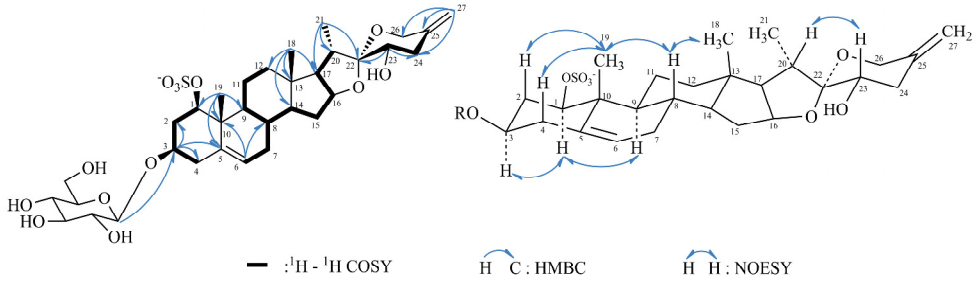

Fig. 3 Key ^{1}H-^{1}H COSY, HMBC and NOESY correlations of the compound 4

Compounds **1**–**13** were examined for their cytotoxic activities against three human tumor cell lines (HCT116, A549 and HepG2) by MTT method. Among them, compound **8** showed cytotoxicities against A549 cells (IC_{50} 39.6 ± 1.9 μmol·L^{-1}) and HepG2 cells (IC_{50} 41.5 ± 1.1 μmol·L^{-1}), respectively. Compound **9** showed cytotoxicity against HCT116 cells (IC_{50} 33.6 ± 2.1 μmol·L^{-1}). Other compounds **4**–**6**, **11** and **13** showed no cytotoxicity with the cells. Compounds **8** and **10** exhibited different cytotoxicities against A549 and HepG 2 cells, it seemed that the introduction of D-Xylose to the inner L-Arabinose could decrease the cytotoxicity. Compounds **7**–**13** shared the same aglycone, but exhibited different activities. This suggested that the structural differences such as the category, the number and the sequence of the oligosaccharide chain at C-1 and C-24 played a role in terms of antitumor effect.

Experimental

General procedures

Optical rotations measured on a Rudolph Autopol II digital polarimeter (Rudolph, Hackettstown, NJ, USA). UV spectra were recorded on a Shimadzu-2201 (Kyoto, Japan). The IR spectra were recorded on a IRAFFINITY-1S instrument (Shimadzu, Kyoto, Japan). The HR-ESI-MS spectra were obtained with 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 tetramethylsilane (TMS) as an internal standard. High-performance liquid chromatography (HPLC) was performed on a 2695 Separations Module (Waters, Milford, MA, USA) equipped with octadecylsilyl (ODS)-3 column and a 2996 Photodiode Array Detector (4.6 mm × 250 mm, 5 mm particles; Ameritech, Chicago, IL, USA). Semi-preparative HPLC was conducted using a LC-6AD pump equipped with an Ultimate XB-C18 column (10 mm × 250 mm, 5 mm particles, Welch, Shanghai, China) and a SPD-20A ultraviolet detector (Shimadzu, Kyoto, Japan). Silica gel (100–200 and 200–300 mesh) was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

Plant materials

The roots and rhizomes of *H. thibetanus* Franch. were collected in June of 2016 from the Taibai region of Qinba Mountains in Shaanxi Province of China and was authenticated by senior experimentalist WANG Ji-Tao, the college of Pharmacy, Shaanxi University of Chinese Medicine. A voucher specimen (herbarium No. 20160915) has been deposited in the Medicinal Plants Herbarium (MPH), Shaanxi University of Chinese Medicine, Xianyang, China.

Extraction and isolation

The dried roots and rhizomes of *H. thibetanus*. (15 kg) were extracted three times with 60% EtOH (15 L) under reflux at 80 °C. The concentrated residue was successively partitioned with petroleum ether and *n*-BuOH. The *n*-BuOH extract (500 g) was applied to silica gel column chromatography with a step gradient of $CHCl_3$–MeOH–H_2O (from 100 : 0 : 0 to 65 : 35 : 1, *V/V/V*), and ten fractions (Frs. 1–10) were obtained based on the TLC analysis. Fr. 2 (40 g) was chromatographed on silica gel eluting with $CHCl_3$–MeOH gradients (from 100 : 0 to 80 : 10, *V/V*) to yield three fractions (Frs. 2-1–2-3). Fr. 2-2 (10 g) eluted with $CHCl_3$–MeOH (20 : 1, *V/V*), was further purified by silica gel column chromato- - graphy using the same solvent system to afford compounds **1**

(9 mg) and **2** (10 mg) and **7** (15 mg). Fr. 2-3 (1.5 g) recry- - stallized with $CHCl_3$–MeOH (1 : 1, *V/V*) to obtain compound **8** (13 mg). Fr. 7 (58 g) was chromatographed on silica gel eluting with $CHCl_3$–MeOH gradients (from 100 : 0 to 0 : 100, *V/V*) to yield six fractions (Frs. 7-1–7-6). Fr. 7-2 (0.6 g) was purified by HPLC (flow rate: 1.0 mL·min^{-1}) with CH_3CN–H_2O (27 : 73, *V/V*) as mobile phase to afford compounds **3** (8 mg, t_R = 32.8 min), **4** (13 mg, t_R = 25.6 min) and **5** (16 mg, t_R = 48.5 min). Fr. 7-3 (3.5 g) was purified by HPLC with CH_3CN–H_2O (22 : 78, *V/V*) as the mobile phase to obtain compounds **6** (16 mg, t_R = 35.5 min), **9** (13 mg, t_R = 47.3 min), **10** (12 mg, t_R = 55.6 min) and **11** (13 mg, t_R = 65.5 min). Fr. 7-5 (1.2 g) was purified by HPLC with CH_3CN–H_2O (18 : 82, *V/V*) as mobile phase to afford compounds **12** (12 mg, t_R = 33.8 min) and **13** (8 mg, t_R = 45.1 min).

Identification of compounds

Thibetanoside E (**1**): A white amorphous powder, $[\alpha]_D^{16.4}$ –96.0 (*c* 0.6, MeOH), UV (MeOH) λ_{max}: 204 nm; IR (KBr) ν_{max}: 3361, 2939, 1454, 1377, 1166, 1026, 916, 790 cm^{-1}. ^{1}H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table **1**; *m/z* 631.3439 $[M + Na]^+$ (Calcd. for $C_{33}H_{52}O_{10}Na$, 631.3453).

Thibetanoside F (**2**): A white amorphous powder, $[\alpha]_D^{14.1}$ –77.3 (*c* 0.8, MeOH), UV (MeOH) λ_{max}: 203 nm; IR (KBr) ν_{max}: 3350, 2947, 1452, 1379, 1211, 1053, 925, 741 cm^{-1}. ^{1}H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table **1**; *m/z* 687.3000 $[M]^-$ (Calcd. for $C_{33}H_{51}O_{13}S^-$, 687.3056).

Thibetanoside G (**3**): A white amorphous powder, $[\alpha]_D^{14.8}$ –40.0 (*c* 0.5, MeOH), UV (MeOH) λ_{max}: 205 nm; IR (KBr) ν_{max}: 3356, 2947, 1452, 1375, 1215, 1059, 920, 741 cm^{-1}. ^{1}H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table **1**; *m/z* 669.2967 $[M]^-$ (Calcd. for $C_{34}H_{51}O_{11}S^-$, 669.2950).

Thibetanoside H (**4**): A white amorphous powder, $[\alpha]_D^{13.4}$ –77.0 (*c* 0.7, MeOH), UV (MeOH) λ_{max}: 204 nm; IR (KBr) ν_{max}: 3348, 2949, 1450, 1362, 1197, 1029, 921, 740 cm^{-1}. ^{1}H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table **1**; *m/z* 685.2727 $[M]^-$ (Calcd. for $C_{33}H_{49}O_{13}S^-$, 685.2819).

*Enzymatic hydrolysis of compounds **1–4** and absolute sugar configuration determination*

The solutions of compounds **1** (5 mg), **2** (6 mg), **3** (5 mg) and **4** (4 mg) in H_2O (3 mL) were individually hydrolyzed with *β*-glucosidase (10 mg, Almonds Lot 1264252, Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 24 or 36 h. Each reaction mixture was partitioned with EtOAc for three times (3 × 3 mL) to yield the H_2O phase and EtOAc extract. The aqueous extracts were chromatographed on silica gel eluting with MeCN/H_2O (8 : 1) to yield glucose with positive optical rotation, and the value of $[\alpha]_D^{20.0}$: +46.9 – +49.2 (*c* 0.16, H_2O). TLC analysis of glucose and authentic sugar samples were by using the solvent system MeCN/H_2O (6 : 1).

Cytotoxicity assay

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 in 96-well plates and allowed to adhere for 24 h. Compounds **2**–**13** were dissolved in dimethylsulfoxide (DMSO) and 6-fold dilutions were prepared in complete medium (from 0.001 mmol·L^{-1} to 0.3 mmol·L^{-1}) for determination of inhibition rate. After incubation at 37 °C for 24 h, the supernatant was removed and DMSO (100 μL) was added to each well. The inhibition rate (IR) and half-maximal inhibitory concentration (IC_{50}) were calculated (see Table 2). Compound **8** showed cytotoxicities

Table 2 Activities (IC_{50}, μmol·L^{-1}) of some compounds from *H. thibetanus* against human cancer cell lines (Mean ± SD, *n* = 3)

Compounds	Cell lines		
	HCT116	A549	HepG2
2	70.7 ± 1.1	85.3 ± 2.6	76.2 ± 1.6
3	68.4 ± 2.5	81.3 ± 1.9	74.9 ± 2.1
7	>100	>100	68.3 ± 3.2
8	>100	39.6 ± 1.9	41.5 ± 1.1
9	33.6 ± 2.1	88.9 ± 1.3	74.8 ± 1.4
10	>100	88.2 ± 1.2	78.6 ± 2.5
12	84.3 ± 1.3	>100	63.1 ± 2.2
[a]5-FU	4.1 ± 1.2	6.5 ± 1.6	9.8 ± 1.3

[a] 5-fluorouracil (5-Fu) as positive control

against A549 and HepG2 cells with IC_{50} values of 39.6 ± 1.9 μmol·L^{-1} and 41.5 ± 1.1 μmol·L^{-1}, respectively. Compound **9** showed cytotoxicity against HCT116 cells with IC_{50} values of 33.6 ± 2.1 μmol·L^{-1}, while compounds **4**–**6**, **11** and **13** showed no cytotoxicity with the cells.

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