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# New minor spirostanol glycosides from *Helleborus thibetanus*

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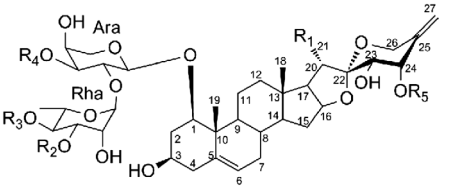
**ABSTRACT**

Phytochemical reinvestigation of the dried roots and rhizomes of *Helleborus thibetanus* afforded four new minor spirostanol glycosides (**1**–**4**) and four known spirostanol glycosides (**5**–**8**). Their structures were determined on the basis of spectroscopic analyses, including 1D and 2D NMR experiments, together with HR-ESI-MS and IR measurements and the results of acid hydrolysis.



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| **1** | $CH_3$ | Api | Ac | Xyl | Glc |
| **2** | $CH_2OAc$ | H | H | Xyl | Glc |
| **3** | $CH_3$ | H | H | H | Glc(1→4)Fuc |
| **4** | $CH_3$ | H | H | Xyl | Glc |

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**KEYWORDS**
*Helleborus*; Ranunculaceae; steroidal saponins; spirostanol

## 1. Introduction

*Helleborus thibetanus* Franch., a plant endemic to China, is mainly distributed in Sichuan, Gansu and Shaanxi (Guan 1979). The roots and rhizomes of *H. thibetanus*, commonly known as 'Xiao-Tao-Er-Qi', have been used as a folk medicine for traumatic cystitis, injury and urethritis (Guo et al. 2003). Extracts of several *Helleborus* species have immunostimulatory and anti-inflammatory properties (Čakar et al. 2014). Previously, the structure determination of several steroidal saponins, one pregnane, one spirostanol sulphate, several bufadienolides and phytoecdystones from *H. thibetanus* have been reported (Cheng et al. 2014; Zhang

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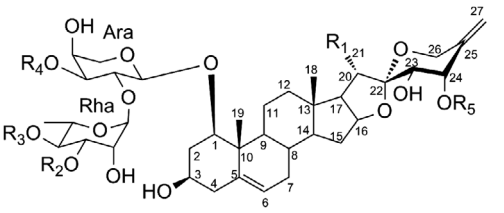

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| **1** | $CH_3$ | Api | Ac | Xyl | Glc |
| **2** | $CH_2OAc$ | H | H | Xyl | Glc |
| **3** | $CH_3$ | H | H | H | Glc(1→4)Fuc |
| **4** | $CH_3$ | H | H | Xyl | Glc |

**Figure 1.** The structures of compounds **1**–**4**.

et al. 2014a, 2014b, 2016). In continuation of our search for new steroidal glycosides from this plant, we herein report the isolation and structure elucidation of four new minor spirostanol steroidal saponins (**1**–**4**) (Figure 1) and four known spirostanol glycosides from the title plant.

## 2. Results and discussion

Compound **1** was obtained as a white amorphous solid with molecular formula of $C_{56}H_{86}O_{28}$, determined by its HR-ESI-MS at *m/z* 1229.5198 ($[M + Na]^+$), as well as its $^1H$ and $^{13}C$ NMR spectroscopic data. The IR spectrum of **1** displayed the characteristic absorptions of hydroxy groups at 3400 $cm^{-1}$ and carbonyl group at 1734 $cm^{-1}$. The $^1H$ NMR spectrum of **1** showed signals for two angular methyl groups at $\delta_H$ 0.96 (3H, s), 1.37 (3H, s), and an olefinic proton signal at $\delta_H$ 5.62 (1H, s), an exomethylene group at $\delta_H$ 5.17 (1H, s) and 5.00 (1H, s). Five anomeric protons at $\delta_H$ 6.50 (1H, br s), 5.94, 5.40 (1H, d, $J$ = 8.0 Hz), 4.89 and 4.61 were observed in the $^1H$ NMR spectrum and five anomeric carbon resonances at $\delta_C$ 100.6, 112.2, 106.0, 106.8 and 100.8 were observed in the $^{13}C$ NMR spectrum, respectively. Additionally, the methyl group at $\delta_H$ 1.39 (3H, d, $J$ = 6 Hz) in the $^1H$ NMR spectrum and $\delta_C$ 18.3 in the $^{13}C$ NMR spectrum were indicative of a 6-deoxyhexose unit. The presence of an acetyl group in **1** was shown by the signals at $\delta_H$ 2.19 (3H, s) and $\delta_C$ 170.6 (C=O) and 21.0 (methyl). The above data and one characteristic acetal signal at $\delta_C$ 111.8 suggested that **1** was a spirostanol saponin with five monosaccharides and one acetyl group. Detailed comparison of NMR data of the aglycone moiety of **1** with those of Clintonioside B (Mimaki & Watanabe 2008) indicated the presence of a methyl ($\delta_H$ 1.04; $\delta_C$ 14.7) and the disappearance of a hydroxymethyl. The methyl group ($\delta_C$ 14.7) was placed at C-20 based on the COSY correlation from $\delta_H$ 1.04 (3H, d, $J$ = 7.0 Hz) to $\delta_H$ 2.88 (H-20) and the HMBC correlations of $\delta_H$ 1.04 (3H, d, $J$ = 7.0 Hz) with $\delta_C$ 37.3 (C-20), $\delta_C$ 61.4 (C-17), and $\delta_C$ 111.8 (C-22). Additionally, the NOESY correlations between H-1 and H-3, between Me-19 and Me-18/H-2ax/H-4ax, indicated the $\beta$-configurations of the oxygenated substituents at C-1 and C-3. Furthermore, the configurations of C-23 and C-24 were determined to be *S* by the NOESY correlations between H-23 and H-20, between H-23 and Me-21/$H_{2-27}$, between H-24 and $H_{2-27}$ (Watanabe et al. 2003; Mimaki et al. 2003; Mimaki & Watanabe 2008; Hayes et al. 2009). All of the data support assignment of the structure of

the aglycone of **1** as (23*S*,24*S*)-1*β*,3*β*,23,24-tetrahydroxy-spirosta-5,25(27)-diene. Acid hydrolysis of **1** with 1 M HCl in dioxane-$H_2O$ (1:1) followed by TLC analysis produced apiose (api), arabinose (ara), rhamnose (rha), xylose (xyl) and glucose (glc). The sequence and linkage positions of the sugars were verified by detailed 2D NMR spectroscopic analysis. In the HMBC spectrum, the correlations of $\delta_H$ 4.61 (H-1 of ara) with $\delta_C$ 84.2 (C-1 of the aglycone), H-1 ($\delta_H$ 6.50) of rha with C-2 ($\delta_C$ 72.5) of ara, H-1 ($\delta_H$ 4.89) of xyl with C-3 ($\delta_C$ 85.2) of ara, H-1 ($\delta_H$ 5.94) of api with C-3 ($\delta_C$ 77.7) of rha were observed, which indicated that rha and xyl attached at C-2 and C-3 of ara, respectively, api linked at C-3 of rha, and ara was attached at C-1 of the aglycone. HMBC correlations between H-24 ($\delta_H$ 4.82) of the aglycone and C-1 ($\delta_C$ 106.0) of glc established the linkage of glc and the aglycone. The linkage of the sugars was further supported by the NOESY correlations of signals at H-1 ($\delta_H$ 3.77) of aglycone with H-1 ($\delta_H$ 4.61) of ara, H-2 ($\delta_H$ 4.59) of ara with H-1 ($\delta_H$ 6.50) of rha, H-3 ($\delta_H$ 3.99) of ara with H-1 ($\delta_H$ 4.89) of xyl, H-3 ($\delta_H$ 4.73) of rha with H-1 ($\delta_H$ 5.94) of api, H-24 ($\delta_H$ 4.82) of aglycone and H-1 ($\delta_H$ 5.40) of glc. Full assignments of **1** were accomplished by a combined analysis of DEPT, COSY, HSQC, NOESY and HMBC spectra. Based on the above evidence, the structure of the new spirostanol glycoside **1** was fully elucidated as (23*S*,24*S*)-24-[(*O*-*β*-D-glucopyranosyl) oxy]-3*β*,23-dihydroxyspirosta-5,25(27)-diene-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 named thibetanoside A.

Compound **2** was isolated as a white amorphous solid. HR-ESI-MS analysis revealed the molecular formula $C_{51}H_{78}O_{25}$ (*m/z* 1113.4729 $[M + Na]^+$). The IR absorptions at 3427 $cm^{-1}$ and 1725 $cm^{-1}$ indicated the presence of hydroxy groups and a carbonyl group. Comparing the $^1H$, $^{13}C$ NMR spectra of **1** and **2**, the chemical shift of methyl signal of rha of **1** at $\delta_H$ 1.39 (3H, d, *J* = 6.0 Hz) moved downfield to $\delta_H$ 1.66 (3H, d, *J* = 6.0 Hz) in **2**, indicating the acetyl linked at C-4 of rha was absent in compound **2** (Takaashi et al. 1995). The $^{13}C$ NMR of **2** showed an upfield shift of C-3 ($\delta_C$ 72.4) of rha, suggesting threre was no sugar attached to the C-3 of rha. The carbonyl group was placed at C-21 based on the HMBC correlation between C=O ($\delta_C$ 170.7) and $H_{2-21}$ ($\delta_H$ 4.31–4.34) of the aglycone. Acid hydrolysis of **2** with 1 M HCl in dioxane-$H_2O$ (1:1) followed by TLC analysis produced ara, rha, xyl and glc. Consequently, **2** was established to be (23*S*,24*S*)-21-acetyloxy-24-[(*O*-*β*-D-glucopyranosyl)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 named thibetanoside B.

Compound **3** was isolated as a white amorphous solid and its molecular formula was determined as $C_{50}H_{78}O_{23}$ by HR-ESI-MS at *m/z* 1069.4826 $[M + Na]^+$. Comparison of the $^1H$ and $^{13}C$ NMR signal assignments of **3** with **2**, the signals of xyl were absent, which was also deduced by an upfield shift of 8.4 ppm of C-3 of ara ($\delta_C$ 75.8) compared with **2**. Furthermore, resonances of the acetyl group in **2** were replaced by a methyl signal at $\delta_C$ 14.7 in **3**. A set of six additional signals corresponding to a fuc unit in compound **3** could be observed at $\delta_C$ 105.9, 73.6, 76.1, 83.2, 70.6, 17.4. Correspondingly, the anomeric proton signal at $\delta_H$ 5.13 (1H, d, *J* = 8.0 Hz) was observed in the $^1H$ NMR spectrum of **3**. The sequence of the sugar chain at C-1 was deduced from the following HMBC correlations: H-1 ($\delta_H$ 4.68) of ara with C-1 ($\delta_C$ 83.3) of the aglycone, H-1 ($\delta_H$ 6.33) of rha with C-2 ($\delta_C$ 75.0) of ara. An HMBC correlation from H-1 ($\delta_H$ 5.13) of fuc and C-24 ($\delta_C$ 82.2) of the aglycone, H-1 ($\delta_H$ 5.12) of glc and C-4 ($\delta_C$ 83.2) of fuc, gave evidence for sugar linkage to C-24 of the aglycone. Acid hydrolysis of **3** with 1 M HCl in dioxane-$H_2O$ (1:1) followed by TLC analysis produced ara, rha, fuc and glc. From the above evidence, the structure of **3** was defined as (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)-*α*-L-arabinopyranoside named thibetanoside C.

Compound **4** was isolated as a white amorphous solid with a molecular formula of $C_{49}H_{76}O_{23}$, as determined by HR-ESI-MS with the molecular ion peak at *m/z* 1055.4669 $[M + Na]^+$, suggesting the loss of $C_2H_2O_2$ from **2**. Its IR spectrum revealed absorptions at 3427 $cm^{-1}$ due to hydroxy groups. The NMR spectra of **4** were superimposable with those of **2**, except for loss of the acetyl group. In the HMBC spectrum, a correlation between the resonances of H-1 of glc ($\delta_H$ 5.39, d, $J$ = 8 Hz) and C-24 ($\delta_C$ 82.4) of the aglycone established that glc unit was attached at C-24 of the aglycone. Acid hydrolysis of **4** with 1 M HCl in dioxane-$H_2O$ (1:1) produced ara, xyl, rha and glc. Thus, the structure of **4** was assigned as (23*S*,24*S*)-24-[(*O*-*β*-D-glucopyranosyl)oxy]-3*β*,23-dihydroxyspirosta-525(27)-diene-1*β*-yl *O*-(*α*-L-rhamnopyranosyl)-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*α*-L-arabinopyranoside named thibetanoside D.

In addition to the new steroidal saponins, **1**–**4**, four known compounds, **5**–**8**, were also isolated and identified by comparison with published spectroscopic and physical data as Hellebosaponin B (**5**) (Mimaki et al. 2003), Recurvoside D (**6**) (Takaashi et al. 1995), (23*S*,24*S*)-21-acetoxy-24-[(*O*-*β*-D-glucopyranosyl)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 (**7**) (Watanabe et al. 2003) and (23*S*,24*S*)-21-hydroxymethyl-24-[(*O*-*β*-D-fucopyranosyl)oxy]-3*β*,23-dihydroxyspirosta-5,25(27)-diene-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 (**8**) (Duckstein & Stintzing 2015).

## 3. Experimental

### 3.1. General experimental procedures

IR spectra were measured on a Bruker Tensor 27 spectrometer (Bruker Co., Ettlingen, Germany) using KBr pellets. Optical rotations were determined on a Rudolph Research Analytical Autopol II automatic polarimeter (Rudolph Research Analytical, USA). UV spectra were obtained using an Agilent Cary 60 UV–vis spectrophotometer (Agilent, CA, USA). NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker Daltonics Inc., Switzerland). High-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) was measured on a Bruker microTOF-Q II mass spectrometer (Bruker Biospin Inc., Germany). All solvents used were of analytical grade (Tianjin Jiangtian Chemical Technology Co., Ltd, Tianjin, China). Column chromatographic isolations were performed using silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), D101 macroporous resin (Tianjin Haiguang Chemical Co., Ltd, Tianjin, 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. Thin-layer chromatography was performed on precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd, Qingdao, China). Spots were visualised with UV light and by spraying with 5% $H_2SO_4$ in EtOH, followed by heating. Analytical HPLC was performed using an ODS column (Agilent ZORBAX SB-C18, 4.6 × 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).

### 3.2. Plant material

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

### 3.3. Extraction and isolation

The air-dried roots and rhizomes of *H. thibetanus* (8.0 kg) were extracted twice with 6 L of 95% ethanol under reflux for 2 h and then with 6 L of 60% ethanol for 2 h. After removal of the solvent *in vacuo*, the residue (1.5 kg) was suspended in water to a final volume of 5 L and then successively extracted at room temperature with petroleum ether (PE), $CHCl_3$, EtOAc, and *n*-BuOH to give four extracts. The *n*-BuOH extract (934 g) was subjected to adsorption on a D101 macroporous resin column, successively eluted with EtOH-$H_2O$ (0:100, 30:70, 50:50, 70:30 and 95:5) to afford five fractions. Fraction A (378 g), eluted with 30% EtOH, was chromatographed on silica gel eluting with EtOAc-MeOH (9:1→6:4) affording 126 fractions. Fractions A77–A92 (50 g) were further chromatographed on a silica gel column eluted with $CH_2Cl_2$-MeOH (80:20→0:100), yielding fractions 20–22 (6 g), which were then rechromatographed by MPLC on an ODS with a step gradient of MeOH-$H_2O$ (34:66→100:0). Sub-fractions 46–47 (180 mg) were pooled and purified by preparative HPLC on ODS with $CH_3CN$-$H_2O$ (18:82) to yield compound **1** (10 mg) and compound **4** (8 mg). Repeated separation of MPLC sub-fractions 48–49 (90 mg) by preparative HPLC on ODS with $CH_3CN$-$H_2O$ (20:80) yielded compound **7** (7 mg) and compound **8** (15 mg). MPLC sub-fractions 50–52 (70 mg) were subjected to preparative HPLC on ODS with $CH_3CN$-$H_2O$ (20:80) as the isocratic eluent system to give fractions 8–21, which were further rechromatographed by preparative HPLC with $CH_3CN$-$H_2O$ (19:81) to yield compound **2** (8 mg) and compound **3** (12 mg). MPLC sub-fractions 57–58 (100 mg) were purified by repetitive preparative HPLC with $CH_3CN$-$H_2O$ (21:79) to afford compound **5** (10 mg). Fraction B (110 g), which eluted from the D101 resin column with 50% EtOH, was chromatographed over silica gel with EtOAc-MeOH (9:1→6:4) to give 78 fractions. Fractions B56-B72 (20 g) were fractionated by MPLC on ODS with MeOH–$H_2O$ (35:65→100:0) and by preparative HPLC on ODS using $CH_3CN$–$H_2O$ (19:81) to afford compound **6** (6 mg).

#### 3.3.1. (23S,24S)-24-[(O-β-D-glucopyranosyl)oxy]-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-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 (1)

White amorphous solid; $[\alpha]_D^{25}$ − 65 (*c* = 0.4, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 255 (3.63) nm; IR (KBr) $\nu_{max}$: 3400, 2935, 1734, 1640, 1036 $cm^{-1}$; HR-ESI-MS: *m/z* 1229.5198 $[M + Na]^+$ (calcd. for $[C_{56}H_{86}O_{28}Na]^+$, 1229.5198). $^1H$ NMR and $^{13}C$ NMR spectroscopic data are presented in Table S1and Table S2.

#### 3.3.2. (23S,24S)-21-acetyloxy-24-[(O-β-D-glucopyranosyl)oxy]-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl O-(α-L-rhamnopyranosyl)-(1→2)-O-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside (2)

White amorphous solid; $[\alpha]_D^{25}$ − 34.3 (*c* = 0.12, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 255 (3.17) nm; IR (KBr) $\nu_{max}$: 3427, 2926, 1725, 1641, 1043 $cm^{-1}$; HR-ESI-MS: *m/z* 1113.4729 $[M + Na]^+$ (Calcd

for $[C_{51}H_{78}O_{25}Na]^+$, 1113.4724). $^1$H NMR and $^{13}$C NMR spectroscopic data are presented in Table S1 and Table S2.

#### *3.3.3. (23S,24S)-24-{[O-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl O-(α-L-rhamnopyranosyl)-(1→2)-α-L-arabinopyranoside* (3)

White amorphous solid; $[\alpha]_D^{25}$ − 69.6 (*c* = 0.29, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 255 (2.71) nm; IR (KBr) $\nu_{max}$: 3426, 2923, 1638, 1052 cm$^{-1}$; HR-ESI-MS: *m/z* 1069.4826 $[M + Na]^+$ (Calcd for $[C_{50}H_{78}O_{23}Na]^+$, 1069.4826). $^1$H NMR and $^{13}$C NMR spectroscopic data are presented in Table S1 and Table S2.

#### *3.3.4. (23S,24S)-24-[(O-β-D-glucopyranosyl)oxy]-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl O-(α-L-rhamnopyranosyl)-(1→2)-O-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside* (4)

White amorphous solid; $[\alpha]_D^{25}$ − 66.7 (*c* = 0.15, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 255 (3.54) nm; IR (KBr) $\nu_{max}$: 3427, 2927, 1647, 1062 cm$^{-1}$; HR-ESI-MS: *m/z* 1055.4669 $[M + Na]^+$ (Calcd for $[C_{49}H_{76}O_{23}Na]^+$, 1055.4669). $^1$H NMR and $^{13}$C NMR spectroscopic data are presented in Table S1 and Table S2.

### *3.4. Acid hydrolysis of* 1–4

A solution of compound **1** (3 mg) was heated in 1 M HCl (dioxane-$H_2O$, 1:1, 6 mL) at 80 °C for 2 h. The dioxane was removed under reduced pressure, the residue allowed to cool to ambient temperature then diluted to 8 mL with $H_2O$, and extracted with EtOAc (4 × 6 mL). The aqueous layer was neutralised with $Ag_2CO_3$ to pH 6–7, filtered through a microporous membrane and concentrated to a minimal volume. Five sugars were identified as glc, xyl, ara, api and rha by comparison with authentic samples (glucose, xylose, arabinose, apiose and rhamnose) by TLC (silica gel) with the solvent system EtOAc: MeOH: HOAc: $H_2O$ (20:5:3:3) and $CHCl_3$: MeOH: HOAc: $H_2O$ (16:10:3:3). The same procedures were carried out for compound **2** (3 mg). TLC analysis of the sugar fraction under the same condition as in the case of **1** indicated the presence of glc, xyl, ara and rha. Compound **3** (4 mg) was subjected to acid hydrolysis as described for **1** to produce a sugar fraction. TLC analysis of which showed the presence of glc, fuc, ara and rha. Compound **4** (3 mg) was subjected to acid hydrolysis as described for **1** to produce a sugar fraction. TLC analysis of which showed the presence of glc, xyl, ara and rha.

## 4. Conclusion

Four new minor spirostanol steroidal saponins (**1**–**4**) and four known compounds (**5**–**8**) were isolated from the roots and rhizomes of *H. thibetanus*. Compound **6** was not reported from *Helleborus* before. Compounds **5**, **7**, **8** were isolated from the title plant for the first time. This is the first report of NMR data for compound **8** (Table S3).

## Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S38 and Tables S1–S3.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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