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Short communication

# Four new minor spirostanol glycosides from *Helleborus thibetanus*

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ABSTRACT

Four new minor spirostanol glycosides (**1–4**) were isolated from the dried roots and rhizomes of *Helleborus thibetanus*. Structures of the compounds were established by means of a combination of 1D and 2D NMR experiments, together with HRESIMS and IR measurements as well as the results of acid hydrolysis. The spirostanol glycosides with both a double bond at C-25 and glycosidation at 1-OH have seldom been reported.



## 1. Introduction

*Helleborus thibetanus* Franch., a plant endemic to China, is mainly distributed in Sichuan, Gansu and Shaanxi provinces (Guan, 1979). The roots and rhizomes of *H. thibetanus* are locally known as "Xiao-tao-er-qi". They have been used in Chinese folk medicine for the treatment of cystitis, traumatic injury, and urethritis (Guo et al., 2003). The chemical constituents of *H. thibetanus* have been studied, allowing the isolation of eight steroidal saponins, one pregnane, one spirostanol sulfate, fourteen bufadienolides and two phytoecdystones (Yang et al., 2010a, 2010b; Cheng et al., 2014; Zhang et al., 2014a, 2014b, 2016). *Helleborus* is a genus of herbaceous perennials belonging to the family Ranunculaceae. Previous phytochemical investigations on the *Helleborus* have led to the isolation of bufadienolides, phytoecdystones, steroidal saponins and flavonoids (Bassarello et al., 2008; Braca et al., 2004; Duckstein and Stintzing, 2015; Meng et al., 2001; Mimaki et al., 2003; Muzashvili et al., 2011; Watanabe et al., 2005). In continuing our investigations on the chemistry of this species, we obtained four new spirostanol glycosides **1-4** (Fig. 1). Herein, we report the isolation and structural elucidation of the new spirostanol glycosides. Compounds **1-4** were rare steroidal saponins which contained a double bond at C-25 and meanwhile were glycosylated at 1-OH and were minor steroidal saponins from *H. thibetanus*.

## 2. Results and discussion

Compound **1** was isolated as a white amorphous solid. Its molecular formula was determined as $C_{60}H_{94}O_{32}$ based on its HRESIMS ion peak at *m/z* 1349.5618 $[M+Na]^+$, as well as its $^1H$ and $^{13}C$ NMR spectroscopic data. The IR spectrum of **1** exhibited absorptions at 3423 $cm^{-1}$, suggesting the presence of hydroxyl groups. The $^1H$ NMR and $^{13}C$ NMR spectra of **1** revealed the presence of two angular Me groups at $\delta_H$ 1.00 (3H, s), 1.34 (3H, s), and $\delta_C$ 16.7, 15.0, respectively, and the $^{13}C$ NMR spectrum showed a characteristic acetal signal at $\delta_C$ 111.5, suggesting a spirostanol skeleton in **1**. The presence of two broad singlets at $\delta_H$ 5.03 and 5.15 in the $^1H$ NMR spectrum, both correlated to the carbon signal at $\delta_C$ 113.6 (C-27) in the HSQC spectrum, together with correlations with the carbon signal at $\delta_C$ 143.6 (C-25) in the HMBC spectrum, substantiated the presence of one exo double bond at C-25(27). HSQC spectrum displayed the correlation from the olefinic proton at $\delta_H$ 5.54 (1H, br d, $J$ = 5.5 Hz) to $\delta_C$ 124.5 (C-6), manifesting the other double bond at C-5(6), which was also demonstrated by the HMBC correlations from the olefinic proton at $\delta_H$ 5.54 (1H, br d, $J$ = 5.5 Hz) to the carbon resonances of $\delta_C$ 43.6 (C-4), $\delta_C$ 32.9 (C-8) and $\delta_C$ 42.8 (C-10), along with the HMBC correlation between $\delta_H$ 1.34 (3H, s, Me-19) and $\delta_C$ 139.5 (C-5). The correlations between the proton signal at $\delta_H$ 3.30 (H-20) and three different proton signals at $\delta_H$ 1.90 (1H, dd, $J$ = 8.0, 6.5 Hz, H-17), $\delta_H$ 3.97 and 4.19 ($H_2$-21) in the COSY plot (Fig. 2) were observed, and HMBC cross-peaks between C-22 ($\delta_C$ 111.5) and $H_2$-21 verified hydroxylation at C-21 ($\delta_C$ 62.2). HSQC spectrum showed the correlation of proton signal at $\delta_H$ 3.76 (1H, dd, $J$ = 12.0, 4.0 Hz) with C-1 ($\delta_C$ 83.8), providing the

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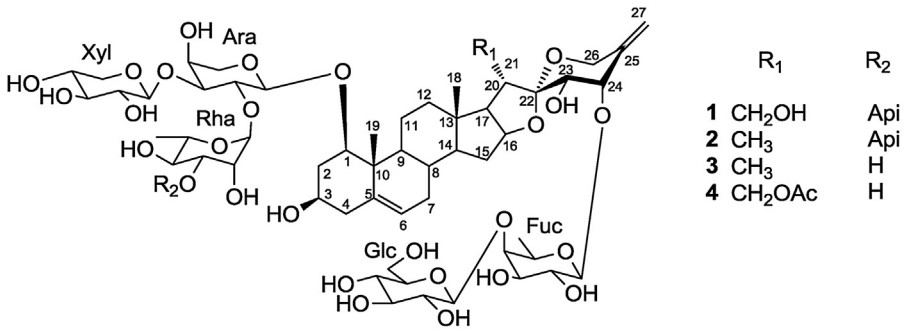


**Fig. 1.** Structures of compounds **1**–**4**.

assignment of H-1, which was also confirmed by the HMBC correlation between Me-19 (3H, s, $\delta_H$ 1.34) and C-1 ($\delta_C$ 83.8).The signal at $\delta_H$ 3.83 (1H, m) in the $^1$H NMR spectrum was ascribed to H-3 based on the COSY (Fig. 2) correlations with H-4ax/H-2ax. The β-equatorial orientations at C-1 and C-3 were revealed by NOESY cross-peaks between H-1 and H-3, between Me-19 and Me-18/H-2ax/H-4ax. Further NOESY (Fig. 3) correlations between H-23 and H-20, between H-23 and $H_2$-21/$H_2$-27, between H-24 and $H_2$-27, as well as a small coupling constant of 4.0 Hz between H-23 and H-24 supported the 23*S* and 24*S* configurations (Watanabe et al., 2003; Mimaki et al., 2003; Mimaki and Watanabe, 2008; Hayes et al., 2009). The $^1$H and $^{13}$C NMR chemical shifts arising from the aglycone moiety of **1** were in good agreement with those of bethoside A (Hayes et al., 2009), on the basis of the above analysis, therefore, the structure of the aglycone of **1** was elucidated as (23*S*,24*S*)-1β,3β,21,23,24-pentahydroxy-spirosta-5,25(27)-diene.

As to the sugar moiety, the $^1$H NMR spectrum of **1** displayed six anomeric proton signals due to monosaccharide units at $\delta_H$ 6.34 (br s), 6.22 (d, $J$ = 2.5 Hz), 5.14 (d, $J$ = 8.0 Hz), 5.13 (d, $J$ = 8.0 Hz), 4.96 (d, $J$ = 7.5 Hz) and 4.65 (d, $J$ = 7.5 Hz), which were associated with six anomeric carbon resonances at $\delta_C$ 101.3, 111.5, 105.9, 106.8, 106.4 and 100.4 in the HSQC spectrum, respectively, suggesting six sugar units in compound **1**. Two three-proton doublet signals at $\delta_H$ 1.61 (3H, d, $J$ = 6.0 Hz) and 1.50 (3H, d, $J$ = 6.3 Hz) in the $^1$H NMR spectrum, and the methyl carbon signals at $\delta_C$ 18.8, 17.3 in the $^{13}$C NMR spectrum, indicating that two of the six sugars were 6-deoxyhexose units. Acid hydrolysis of **1** with 1 M HCl in dioxane-$H_2O$ (1:1) followed by TLC analysis showed the presence of apiose (Api), arabinose (Ara), rhamnose (Rha), xylose (Xyl), fucose (Fuc) and glucose (Glc). The HMBC (Fig. 2) cross-peak between $\delta_H$ 4.65 (H-1 of Ara) and the carbon resonance at $\delta_C$ 83.8 (C-1 of the aglycone) unambiguously located one sugar chain on C-1 position of the aglycone. The sequence of sugars were determined by HMBC correlations of H-1 ($\delta_H$ 6.34) of Rha with C-2 ($\delta_C$ 73.4) of Ara, H-1 ($\delta_H$ 4.96) of Xyl with C-3 ($\delta_C$ 84.5) of Ara, H-1 ($\delta_H$ 6.22) of Api with C-3 ($\delta_C$ 79.4) of Rha, which was also supported by the NOESY correlations of signals at H-1 ($\delta_H$ 3.76) of aglycone with H-1 ($\delta_H$ 4.65) of Ara, H-2 ($\delta_H$ 4.59) of Ara with H-1 ($\delta_H$ 6.34) of Rha, H-3 ($\delta_H$ 4.05) of Ara with H-1 ($\delta_H$ 4.96) of Xyl, H-3 ($\delta_H$ 4.66) of Rha with H-1 ($\delta_H$ 6.22) of Api. The 9.2 ppm downfield shift observed for C-24 ($\delta_C$ 82.3) relative to the carbon ($\delta_C$ 73.1) with a free hydroxyl group (Ono et al., 2007) indicated glycosylation at this position, which was corroborated by a cross-peak in the HMBC spectrum between H-24 ($\delta_H$ 4.73) of the aglycone and C-1 ($\delta_C$ 105.9) of Fuc. And the HMBC correlations between C-4 ($\delta_C$ 83.2) of Fuc to H-1 ($\delta_H$ 5.13) of Glc established the linkage of the sugars, which was further supported by the NOESY cross-peaks between H-24 ($\delta_H$ 4.73) of aglycone and H-1 ($\delta_H$ 5.14) of Fuc, between H-4 ($\delta_H$ 4.03) of Fuc and H-1 ($\delta_H$ 5.13) of Glc. The β-anomeric orientations for the D-glucose, D-fucose, and D-xylose moieties were determined by the relatively large $^3J_{H\text{-}1,H\text{-}2}$ values of the anomeric protons of these glycose moieties in the $^1$H NMR spectra. The relatively large $J$ value of the anomeric proton of the arabinosyl (7.5 Hz) indicated an α anomeric orientation for the L-arabinose (Watanabe et al., 2003). The broad singlet of the anomeric proton of L-rhamnose combined with the carbon signals of C-3 ($\delta_C$ ca. 72.5) and C-5 ($\delta_C$ ca. 69.5) (Berrue et al., 2012) illustrated the α-configuration. The $^{13}$C NMR shift of the anomeric carbon of the D-apiose at $\delta_C$ 111.5 was indicative of a β-orientation of the anomeric center (Kitagawa et al., 1989). Full assignments of **1** were achieved by a careful examination of DEPT, COSY, HSQC, NOESY and HMBC spectra. On the basis of the above evidence, the structure of the new spirostanol glycoside **1** was fully determined to be (23*S*,24*S*)-21-hydroxymethyl-24-{[*O*-β-D-gluco-pyranosyl-(1 → 4)-β-D-fucopyranosyl]oxy}-3β,23-

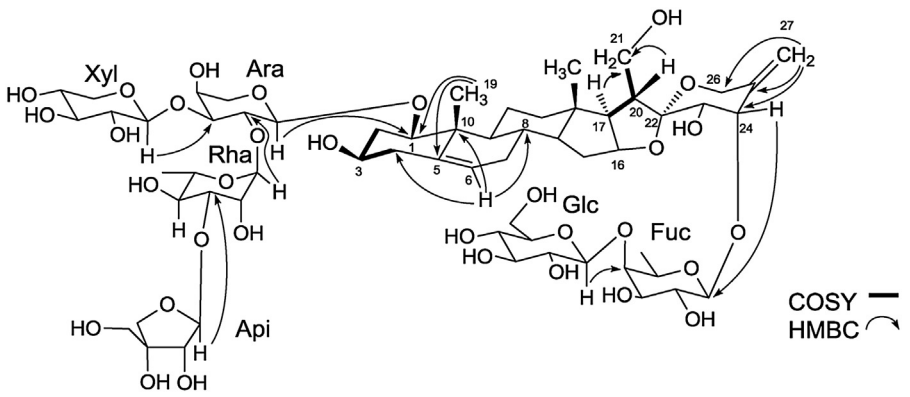


**Fig. 2.** Selected COSY and HMBC correlations of compound **1**.