Two New Spirostanol Glycosides from the Roots and Rhizomes of 

*Helleborus thibetanus* Franch.

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**Abstract:** Two new spirostanol glycosides, thibetanosides J and K (1 and 2), along with three known ones (3-5) were isolated from the roots and rhizomes of *Helleborus thibetanus*. Their structures were elucidated by extensive use of spectroscopic techniques and chemical evidence. In this study, compounds 1-5 were evaluated for their cytotoxic activity against HCT116, A549 and HepG2 tumor cell lines. Among them, compound 1 exhibited moderate cytotoxicity against A549 cells (IC$_{50}$ 7.69 ± 1.1 μM) and HepG2 cells (IC$_{50}$ 8.32 ± 2.63 μM). Compound 2 exhibited moderate cytotoxicity against HCT116 cells (IC$_{50}$ 20.67 ± 1.06 μM).

**Keywords:** *Helleborus thibetanus*; spirostanol glycosides; cytotoxic activity. ©2022 ACG Publications. All right reserved.

1. Introduction

*Helleborus thibetanus* Franch., a plant endemic to China, known as “Tigenceao” or “Xiao-tao-er-qi”, is mainly distributed in Gansu, Sichuan and Shaanxi Provinces [1]. Its dried rhizomes have been used as Chinese folk medicine for the treatment of cystitis, urethritis and traumatic injury [2-3]. Several bufadienolides, ecdysteroids, furostanol saponins, spirostanol saponins and flavonoids have been isolated from *H. thibetanus* [4-5]. Modern pharmacology studies revealed that the extracts and chemical components of *H. thibetanus* possess immune-regulation, anticancer, antibacterial and cytotoxic properties [6-7]. As part of an ongoing search for bioactive constituents

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from the medicinal herbs around Qinba Mountains [8-10], two new spirostanol saponins (23S,24S)-24-\([\{O-\beta-D\text{-glucopyranosyl}\}(1 \rightarrow 4)\beta-D-fucopyranosyl\text{oxy}\}3\beta,23\text{-dihydroxyspirosta-5,25(27)}\text{-dien}-1\beta\text{-yl}O-\beta-D\text{-apoifuranosyl}\}-(1 \rightarrow 3)\text{-}O-(\alpha-L\text{-rhamnopyranosyl})-(1 \rightarrow 2)\text{-}O-\alpha-L\text{-arabinopyranoside} (1), \text{and} (23S,24S)-24-\([\{O-\beta-D\text{-glucopyranosyl}\} (1 \rightarrow 4)\beta-D\text{-fucopyranosyl} \text{oxy}\}3\beta,23\text{-dihydroxyspirosta-5,25(27)}\text{-dien}-1\beta\text{-yl}O-\beta-D\text{-apoifuranosyl}-(1 \rightarrow 3)\text{-}O-(4\text{-}O\text{-acetyl}\alpha-L\text{-rhamno-pyranosyl})-(1 \rightarrow 2)\text{-}O-\alpha-L\text{-arabinopyranoside} (2), \text{and three known saponins} (23S,24S)-24-\([\{O-\beta-D\text{-glucopyranosyl}\} (1 \rightarrow 4)\beta-D\text{-fucopyranosyl} \text{oxy}\}3\beta,23\text{-dihydroxyspirosta-5,25(27)}\text{-dien}-1\beta\text{-yl}O-\beta-D\text{-apoifuranosyl}-(1 \rightarrow 3)\text{-}O-(4\text{-}O\text{-acetyl}\alpha-L\text{-rhamnopyranosyl})-(1 \rightarrow 2)\text{-}O-\alpha-L\text{-arabinopyranoside} (3) [5], (23S,24S)-24-\([\{O-\beta-D\text{-glucopyranosyl} \text{oxy}\}3\beta,23\text{-dihydroxyspirosta-5,25(27)}\text{-dien}-1\beta\text{-yl}O-\beta-D\text{-apoifuranosyl}-(1 \rightarrow 3)\text{-}O-(4\text{-}O\text{-acetyl}\alpha-L\text{-rhamnopyranosyl})-(1 \rightarrow 2)\text{-}O-\beta-D\text{-xylopyranosyl}-(1 \rightarrow 3)\text{-}\alpha-L\text{-arabinopyranoside} (4) [11], (23S,24S)-21\text{-acetoxy}3\beta,23,24\text{-trihydroxyspirosta-5,25(27)}\text{-dien}-1\beta\text{-yl}O-\beta-D\text{-apoifuranosyl}-(1 \rightarrow 3)\text{-}O-(4\text{-}O\text{-acetyl}\alpha-L\text{-rhamnopyranosyl})-(1 \rightarrow 2)\text{-}O-\beta-D\text{-xylopyranosyl}-(1 \rightarrow 3)\text{-}\alpha-L\text{-arabinopyranoside} (5) [12] (Figure 1), \text{were isolated from the roots and rhizomes of} \text{H. thibetanus. Herein, the isolation and structure elucidation of the new compounds, and their anti-tumor evaluation against A549, HepG 2 and HCT116 tumor cells were described.}
an HP-5 capillary column (30 m × 0.32 mm, 0.5 µm) and an FID detector. Standards for D-glucose (D-Glc), D-fucose (D-Fuc), L-arabopyranose (L-Ara), L-rhamnose (L-Rha) and D-apiose (D-Api) was purchased from Herbest Bio-Tech Co. (Baoji, China). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

2.2. Plant Material

The roots and rhizomes of H. thibetanus Franch were collected in June 2016 from the Taibai region (height: 2276.6 m, longitude: 107°47′/28.4581°), of Qinba Mountains in Shaanxi Province, China, and were authenticated 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.

2.3. Extraction and Isolation

The air-dried underground parts (1.5 kg) of H. thibetanus Franch were powdered and extracted three times with 60% EtOH under reflux at 80 °C. After removing the solvent, the concentrated residue was successively partitioned with petroleum ether (PE) and n-BuOH. The n-BuOH extract (200 g) was chromatographed on silica gel column, eluted with gradient solvent system (CHCl3-MeOH-H2O, 100:0:65:35:1) to yield ten fractions (Fr.1 - 10). Fr.5 (40 g) was separated on silica gel column, eluting with gradient solvent system (CHCl3-MeOH, 100:0:50:50) to yield six fractions (Fr.5.1-Fr.5.6). Fr.5-2 (150 mg) was further purified by HPLC (Ultimate XB-C18, 10 mm × 250 mm, 5 µm particles, flow rate: 1.0 mL/min) with CH3CN-H2O (32:68) to afford compounds 1 (13 mg; τr = 27.6 min) and 2 (20 mg; τr = 35.2 min). Fr.8 (150 g) was subjected to a silica column chromatography, eluting with gradient solvent system (CHCl3-MeOH, 100:0:80:10) to yield five fractions (Fr.8-1-Fr.8-5). Fr. 8-3 (0.7 g) was purified by HPLC (Ultimate XB-C18, 10 mm × 250 mm, 5 µm particles, flow rate: 1.0 mL/min) with CH3CN-H2O (20:80) to get compounds 3 (9 mg; τr = 24.7 min), 4 (7 mg; τr = 32.1 min) and 5 (14 mg; τr = 49.3 min).

2.4. Spectroscopic Data

Thibetanoside J (1): A white amorphous powder, [α]D -56.8 (c 1.4, MeOH); IR (KBr) νmax: 3383, 2932, 1450, 1377, 1250, 1050, 837 and 782 cm⁻¹; 1H-NMR (400 MHz, pyridine-d5) and 13C-NMR (100 MHz, pyridine-d5) spectral data, see Table 1; HR-ESI-MS: m/z 1177.5253 [M - H]⁻ (calcd. for C₅₅H₆₅O₃₇ 1177.5278).

Thibetanoside K (2): A white amorphous powder, [α]D +69.5 (c 1.1, MeOH); IR (KBr) νmax: 3384, 2935, 1732, 1452, 1374, 1243, 1040, 835 and 783 cm⁻¹; 1H-NMR (400 MHz, pyridine-d5) and 13C-NMR (100 MHz, pyridine-d5) spectral data, see Table 1; HR-ESI-MS: m/z 1087.4911 [M - H]⁻ (calcd. for C₅₂H₇₉O₃₄ 1087.4961).

3. Results and Discussion

3.1. Structure Elucidation

Thibetanoside J (1) was isolated as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard and Molisch tests. Its molecular formula was determined as C₅₅H₆₅O₃₇ from the HR-ESI-MS at m/z 1177.5253 [M - H]⁻ (calcd. C₅₅H₆₅O₃₇ 1177.5278). In the 1H-
NMR and HSQC spectra, five anomic protons at $\delta_H$ 5.16 (1H, d, $J = 7.8$ Hz, H-Glc-1), 5.16 (1H, d, $J = 7.8$ Hz, H-Fuc-1), 4.68 (1H, d, $J = 7.9$ Hz, H-Ara-1), 6.33 (1H, br s, H-Rha-1), 6.21 (1H, d, $J = 2.5$ Hz, H-Api-1) as well as two methyl protons at $\delta_H$ 1.71 (3H, d, $J = 6.1$ Hz, H-Rha-6) and 1.54 (3H, d, $J = 6.3$ Hz, H-Fuc-6) were observed, which were correlated with five anomic carbon signals at $\delta_C$ 107.4 (C-Glc-1), 106.6 (C-Fuc-1), 101.0 (C-Ara-1), 102.0 (C-Rha-1), 112.3 (C-Api-1), 19.5 (C-Rha-6) and 18.0 (C-Fuc-6), respectively. Acid hydrolysis of I resulted in the production of apiose (Api), arabinose (Ara), rhamnose (Rha), fucose (Fuc) and glucose (Glc), which were confirmed by GC analysis of the trimethylsilyl-L-cysteine derivatives of the hydrolysate of I and the authentic sugars. Coupling constants of the anomic proton signals suggested $\beta$-configuration of D-glucose, D-fucose and D-apiose, and $\alpha$-configuration of L-arabinose, respectively. The $\alpha$-configuration of the rhamnose unit was deduced from the absence of intraresidual NOESY correlations between H-1$_{\text{rha}}$ and H-3$_{\text{rha}}$/H-5$_{\text{rha}}$[12]. Furthermore, the $^{13}$C NMR spectra exhibited 55 carbon signals, of which the distinctive quaternary carbon signal at $\delta_C$ 112.3 (C-22) led to the hypothesis that I was a spirostanol saponin [13].

For the aglycone of I, the $^1$H NMR spectrum (Table 1) showed three methyl protons at $\delta_H$ 0.96 (3H, s, Me-18), 1.43 (3H, s, Me-19) and 1.09 (3H, d, $J = 6.9$ Hz, Me-21), and two exomethylene protons at $\delta_H$ 5.22 (1H, br s, H-27a) and 5.11 (1H, br s, H-27b), as well as one olefinic proton at $\delta_H$ 5.57 (1H, d, $J = 5.4$ Hz, H-6). In addition, three methyl groups at $\delta_C$ 17.3 (C-18), 15.6 (C-19), and 15.3 (C-21) were observed in the $^{13}$C NMR spectra (Table 1). The presence of a terminal olefinic bond was deduced by a quaternary carbon signal at $\delta_C$ 144.4 (C-25), as well as a methylene carbon signal at $\delta_C$ 114.3, which exhibited correlations with two olefinic proton signals at $\delta_H$ 5.22 (H-27a) and 5.11 (H-27b) in the HSQC spectrum. HSQC spectrum also displayed the correlation from the olefinic proton at $\delta_H$ 5.57 (1H, d, $J = 5.4$ Hz, H-6) to $\delta_C$ 125.1 (C-6). $^1$H-$^1$H COSY correlations from H-1/H$_2$-2/H-3/H$_2$-4, from H-6/H$_2$-7/H-8/H$_2$-9/H$_2$-11/H$_2$-12, from H-8/H-14/H$_2$-15/H-16/H-17/H-20/H$_2$-21, and from H-23/H-24, accompanied with HMBC correlations (Figure 2) from H-3/C-2, C-4, and C-5, from H-19/C-1, C-5, C-9 and C-10, from H-6/C-4, C-7, C-8 and C-10, from H$_2$-21/C-17, C-20 and C-22, from H$_2$-18/C-12, C-13, C-14 and C-17, from H-16/C-13, C-14, C-17, C-20 and C-22, from H-24/C-22, C-23, C-25 and C-26, from H$_2$-27/C-24, C-25 and C-26, and from H$_2$-26/C-22, C-24, C-25 and C-27 demonstrated a planar structure of the aglycone moiety as 1,3,23,24-tetraol-spirost-5,25(27)-diene. In addition, in the NOESY spectrum (Figure 2) of I, the NOE correlations of H-1/H-3/H-9 and Me-19/H-2a/H-4a/H-8/Me-18, indicated $\alpha$-axial configurations of H-1 and H-3, and $\beta$-orientation of Me-19, 1-OH and 3-OH; Furthermore, the configurations of C-23 and C-24 were determined to be S by a small coupling constant between H-23 and H-24 ($J = 3.5$ Hz) and the NOESY correlations of H-23/H-20, H-23/Me-21/H$_{27\beta}$, and H-24/H$_{27\alpha}$[15-18]. Comparison of the $^1$H and $^{13}$C NMR spectroscopic data of the aglycone moiety of I with those of 3, along with the above analysis, the structure of the aglycone of I was elucidated as (23S,24S)-1/$\beta$, 3/$\beta$,23,24-tetrahydroxy-spirosta-5,25(27)-diene.

Moreover, HMBC correlations of H-Api-1/C-Rha-3, H-Rha-1/C-Ara-2 and H-Ara-1/C-1 disclosed that the D-apiose unit was linked at C-3 of the L-rhamnose, L-rhamnose unit was linked at C-2 of the inner L-arabinose unit, then the L-arabinose unit was linked at C-1 of the aglycone. In addition, correlations of H-Glc-1/C-Fuc-4 and H-Fuc-1/C-24 disclosed that the terminal D-glucose unit was linked at C-4 of the inner D-fucose unit, then the D-fucose unit was linked at C-24 of the aglycone. Therefore, the structure of I was characterized as (23S,24S)-24-{$\beta$-D-glucopyranosyl-
Thibetanoside K \( (2) \) was obtained as a white amorphous powder. A [M - H] peak at \( m/z \) 1087.4911 in the HR-ESI-MS indicated that the molecular formula was \( C_{52}H_{80}O_{24} \). Comparison of the NMR data of \( 2 \) and \( 1 \) (Table 1), indicated almost similar NMR spectroscopic features, except an increase of the acetyl linked at C-4 of Rha and an absence of the terminal apiose unit in compound \( 2 \). The proton and carbon NMR signals of \( \delta H \) 4.68 (1H, m, H-Rha-3) and \( \delta C \) 80.6 (C-Rha-3) and \( \delta H \) 4.42 (1H, m, H-Rha-4) and \( \delta C \) 73.0 (C-Rha-4) in \( 1 \), were replaced by \( \delta H \) 4.76 (1H, m, H-3), \( \delta C \) 70.5 (C-Rha-3) and \( \delta H \) 5.83 (1H, \( J = 9.6 \) Hz, H-Rha-4) and \( \delta C \) 76.9 (C-Rha-4) in \( 2 \), which was supported by HSQC, HMBC and NOESY spectrums. The presence of an acetyl group in \( 2 \) was shown by the signals at \( \delta H \) 2.03 (3H, s) and \( \delta C \) 171.3 (C=O) and 21.5 (methyl). Moreover, HMBC correlations of H-Rha-1/C-Ara-2 and H-Ara-1/C-1 disclosed that the L-rhamnose unit was linked at C-2 of the inner L-arabinose unit, then the L-arabinose unit was linked at C-1 of the aglycone. In addition, correlations of H-Glc-1/C-Fuc-4 and H-Fuc-1/C-24 disclosed that the terminal D-glucose unit was linked at C-4 of the inner D-fucose unit, then the D-fucose unit was linked at C-24 of the aglycone. Similarly as compound \( 1 \), the results of the acid hydrolysis procedure and subsequent GC analysis of the hydrolysates and showed the structure of \( 2 \) was defined as (23S,24S)-24-\{[O-\beta-D-glucopyranosyl(1\rightarrow4)-\beta-D-fucopyranosyl]oxy\}-3ß,23-dihydroxyspirosta-5,25(27)-diene-1ß-ylo-(4-O-acetyl-\alpha-L-rhamnopyranosyl)-(1\rightarrow2)-O-\alpha-L-arabinopyranoside.

Figure 2. Key \(^1\)H-\(^1\)H COSY, HMBC and NOESY correlations of compound \( 1 \)
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**Table 1.** $^1$H-NMR (400 MHz, in pyr-$d_5$) and $^{13}$C-NMR (100 MHz, in pyr-$d_5$) spectral data of compounds 1 and 2

| No. | 1          | 2          |
|-----|------------|------------|
|     | $\delta_c$ | $\delta_H (J \text{ in Hz})$ | $\delta_c$ | $\delta_H (J \text{ in Hz})$ |
| 1   | 84.3       | 3.78 (dd, 11.8, 3.5) | 84.1       | 3.80 (dd, 12.0, 4.6) |
|     | 2.71 (m, H-2a) | | 2.73 (m, H-2a) | |
| 2   | 37.9       | 2.37 (dd, 12.0, 12.0, H-2b) | 38.0       | 2.32 (dd, 13.4, 11.7, H-2b) |
|     | 2.72 (m, H-4a) | | 2.75 (m, H-4a) | |
|     | 2.58 (m, H-4b) | | 2.67 (m, H-4b) | |
| 3   | 68.7       | 3.87, m | 68.5       | 3.90, m |
| 4   | 44.3       | 2.71 (m, H-2a) | 44.5       | 2.32 (dd, 13.4, 11.7, H-2b) |
|     | 2.75 (m, H-4a) | | 2.67 (m, H-4b) | |
|     | 2.67 (m, H-4b) | | 2.67 (m, H-4b) | |
| 5   | 140.2      | — | 140.1      | — |
| 6   | 125.1      | 5.57 (d, 5.4) | 125.3      | 5.64 (d, 4.7) |
|     | 1.82 (m, H-7a) | | 1.84 (m, H-7a) | |
| 7   | 32.4       | 1.48 (m, H-7b) | 32.5       | 1.53 (m, H-7b) |
| 8   | 33.4       | 1.46, m | 33.5       | 1.53, m |
| 9   | 50.9       | 1.47, m | 50.8       | 1.54, m |
| 10  | 43.4       | — | 43.4       | — |
| 11  | 24.4       | 2.96 (m, H-11a) | 24.4       | 2.92 (m, H-11a) |
|     | 1.60 (m, H-11b) | | 1.57 (m, H-11b) | |
| 12  | 40.9       | 1.51 (m, H-12a) | 40.9       | 1.54 (m, H-12a) |
|     | 1.27 (m, H-12b) | | 1.26 (m, H-12b) | |
| 13  | 41.3       | — | 41.3       | — |
| 14  | 57.2       | 1.06, m | 57.2       | 1.09, m |
| 15  | 32.8       | 1.81 (m, H-15a) | 32.9       | 1.82 (m, H-15a) |
|     | 1.37 (m, H-15b) | | 1.37 (m, H-15b) | |
| 16  | 83.5       | 4.62, m | 83.5       | 4.65, m |
| 17  | 62.0       | 1.74, m | 62.1       | 1.72 (dd, 7.2, 7.5) |
| 18  | 17.3       | 0.96, s | 17.3       | 1.02, s |
| 19  | 15.6       | 1.43, s | 15.4       | 1.41, s |
| 20  | 37.9       | 2.89, m | 38.0       | 2.92, m |
| 21  | 15.3       | 1.09 (d, 6.9) | 15.3       | 1.1 (d, 6.6) |
| 22  | 112.3      | — | 112.3      | — |
| 23  | 70.8       | 3.98 (d, 3.5) | 70.8       | 3.98 (d, 2.8) |
| 24  | 82.8       | 4.79 (d, 3.5) | 82.8       | 4.82 (d, 2.8) |
| 25  | 144.4      | — | 144.4      | — |
| 26  | 62.0       | 4.83 (d, 10.9, H-26a) | 62.0       | 4.87 (d, 11.7, H-26a) |
|     | 4.01 (m, H-26b) | | 4.06 (m, H-26b) | |
| 27  | 114.3      | 5.22 (s, H-27a) | 114.3      | 5.23 (s, H-27a) |
|     | 5.11 (s, H-27b) | | 5.12 (s, H-27b) | |
| 1-O-Ara | | | | |
| 1   | 101.0      | 4.68 (d, 7.9) | 100.9      | 4.7 (d, 7.9) |
| 2   | 75.8       | 4.58, m | 74.7       | 4.57, m |
3.2. Cytotoxicity Assay

The cytotoxic activity assay toward three human tumor cell lines (HCT116, A549 and HepG2) were measured following the procedures that we reported previously [18-20], the details were listed in the Supporting Information.
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**Table 2. Cytotoxicity of compounds 1-5 (IC$_{50}$ values expressed in µM)**

| Compounds | HCT116 (µM) | A549 (µM) | HepG2 (µM) |
|-----------|-------------|-----------|------------|
| 1         | >100        | 7.69 ± 1.13 | 8.32 ± 2.63 |
| 2         | 20.67 ± 1.06 | >100       | >100       |
| 3         | >100        | >100       | >100       |
| 4         | >100        | >100       | 80.54 ± 1.62 |
| 5         | >100        | >100       | >100       |
| 5-FU$^a$  | 24.13 ± 2.44 | 18.92 ± 2.79 | 41.68 ± 1.58 |

$^a$ 5-fluorouracil (5-Fu) as positive control.

3.3. **Sugar Analysis of Compounds 1 and 2**

Sugar moieties of compounds 1 and 2 were confirmed by using the $t_R$ of D-Glc (45.2 min), D-Fuc (35.2 min), D-Api (11.2 min), L-Ara (12.2 min), and L-Rha (14.5 min), following the procedures that we reported previously [21-23], the details were listed in the Supporting information file of the article.

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**Supporting Information**

Supporting Information accompanies this paper on [http://www.acgpubs.org/journal/records-of-natural-products](http://www.acgpubs.org/journal/records-of-natural-products).

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