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Steroidal saponins from *Trillium tschonoskii* rhizomes and their cytotoxicity against HepG2 cells

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ABSTRACT

A phytochemical study on the rhizomes of *Trillium tschonoskii* led to the isolation of fourteen new steroidal saponins, trillitschosides S1-S14 (**1–14**), along with ten known analogues (**15–24**). Their structures were established mainly by spectroscopic analyses as well as necessary chemical evidence. All isolated compounds were screened for the cytotoxicity against HepG2 cells, and the results demonstrated that only the known compounds **21–24** exhibited the remarkable cytotoxic activity against HepG2 cells which is much better than the positive control of 5-FU.

1. Introduction

Trillium tschonoskii Maxim (Liliaceae) is a perennial herb mainly distributed in Shan'xi, Sichuan, Yuannan, and Hubei provinces in China. In many regions, the dried rhizomes of *T. tschonoskii* were used as a kind of often-applied folk medicine (Yan-ling-cao in Chinese) for the purposes of treating neurasthenia, headache, traumatic injury and bleeding, cancer, and various inflammatory, *etc*. [1,2]. To Tujia nationality, *T. tschonoskii* is honored as one of four magical herbs due to its obvious curative effects for a series of diseases. *T. tschonoskii* and two other plants of same genus, *T. kamtschaticum* and *T. govanianum*, are only three species of genus Trillium distributed in China. Previous phytochemical investigations on these plants indicated that, all the time, there is lack of systematic chemical study on *T. tschonoskii*, which is not conductive to elucidate the active constituents of this herb medicine. Recently, a phytochemical study was carried out on *T. tschonoskii*, which let us finally obtain total twenty-four spirostanol saponins (**1–24**) from its rhizomes, of which **1–14** were new compounds (Fig. 1). A preliminary screening displayed that the extract of *T. tschonoskii* affect cytotoxic activity of HepG2 cells obviously after treatment for 24 h and in a dose-dependent manner (Supplementary Fig. S1). As our effort to search for anti-liver cancer bioactive compounds, all isolated compounds were tested for their individual cytotoxic activities *in vitro* using the human hepatocellular carcinoma (HepG2) cell line. Herein, we mainly described the isolation and structure elucidation of these compounds and their cytotoxicity against HepG2 cells.

2. Experimental

2.1. General methods

HRESIMS was recorded on a Synapt MS (Waters Corporation, USA). The NMR experiments were performed on Varian ^{UNITY} INOVA 600 spectrometer (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR). The optical rotations were measured with a JASCO J-810 polarimeter. IR spectra were recorded on a Bruker VERTEX 70 FT infrared spectrometer. HPLC analyses were performed on Agilent 1100 system equipped with an Alltech 2000 evaporative light scattering detector (Temperature: 110 °C, Gas: 2.4 L/min). Preparative HPLC was performed on an NP7000 module (Hanbon Co. Ltd., China) equipped with a Shodex RID 102 detector (Showa Denko Group, Japan). Silgreen HPLC C_{18} columns (4.6/10.0/20.0 × 250 mm, 5 μm, Greenherbs Science and Technology, China) were used for HPLC and preparative HPLC. TLC was performed on silica gel GF_{254} plates (Qingdao Marine Chemical, China). Macroporous resin SP825 (Mitsubishi Chemicals, Japan), silica gel H (Qingdao Marine Chemical, China), and MCI gel (Mitsubishi Chemicals, Japan) were applied for column chromatography.

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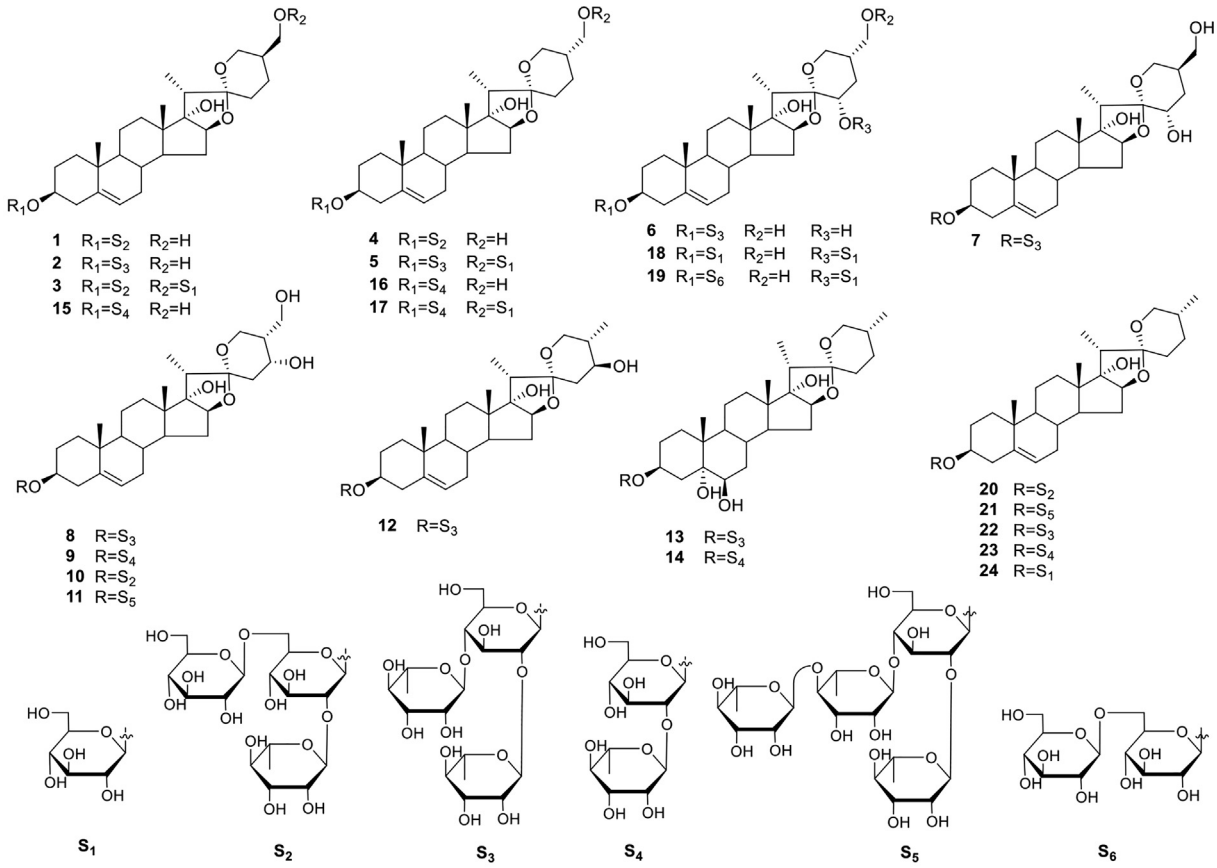

Fig. 1. Structures of 1–24.

2.2. Plant material

The rhizomes of *T. tschonoskii* were collected from the Shennongjia of Hubei province and identified by Professor Bao-Lin Guo (Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences). A voucher specimen (No. 151010) was deposited in the author's laboratory in the Beijing Institute of Radiation Medicine.

2.3. Extraction and isolation

T. tschonoskii rhizomes (5 kg) were crushed and extracted with 50% aq. EtOH at reflux three times (40 L, 30 L, and 30 L, each for 2 h). The filtered solution was concentrated in vacuo to get the supernatants and sediments. The supernatants were subjected to a SP825 macroporous resin column eluted with EtOH-H_2O (*v/v*, 5:95 → 30:70 → 50:50 → 75:25 → 95:5) to yield five factions (A ~ E). Fr.C (120 g) was subjected to silica-gel column chromatography (CC) eluted with a gradient mixture of $CHCl_3$-MeOH-H_2O (*v/v/v*, 5:1:0.1 → 2:1:0.1) to give five fractions (Fr.C1 ~ Fr.C5). Fr.C3 (35 g) was subjected to MCI CC eluted with $(CH_3)_2CO$-H_2O (*v/v*, 10:90 → 50:50). As a result, a total of 30 fractions were collected (Fr.C3/1 ~ Fr.C3/30). Fr.C3/6 was separated by preparative HPLC (pHPLC) with CH_3CN-H_2O (*v/v*, 20:80) to give seven subfractions (Fr.C3/6–1 ~ Fr.C3/6–7). Fr.C3/7–9 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 20:80) to yield three fractions (Fr.C3/7–9/1 ~ Fr.C3/7–9/3). Fr.C3/10–11 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to yield five fractions (Fr.C3/10–11/1 ~ Fr.C3/10–11/5). Fr.C3/12 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to give five subfractions (Fr.C3/12/1 − Fr.C3/12/5). Fr.C3/13–14 were separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to yield five subfractions (Fr.C3/13–14/1 ~ Fr. C3/13–14/5). Then, Fr.C3/6/2 together with Fr.C3/7–9/2 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 20:80) to give **7** (31.0 mg) and **10** (6.3 mg). Fr.C3/6/3, Fr.C3/7–9/3, Fr.C3/12/3 and Fr.C3/13–14/3 were together separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 22:78) to give **8** (49.0 mg) and **9** (19.5 mg). Fr.C3/6/4–6, Fr.C3/10–11/4, Fr.C3/12/2, and Fr.C3/13–14/2 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 22:78) to give **18** (25.0 mg). Fr.C3/12/4 and Fr.C3/13–14/4 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 24:76) to give **1** (26.0 mg) and **4** (62.0 mg). Fr.C4 (35 g) was subjected to a MCI gel column eluted with $(CH_3)_2CO$-H_2O (*v/v*, 10:90 → 15:85 → 20:80 → 30:70 → 50:50) to afford nine fractions (Fr.C4/1 ~ Fr.C4/9). Fr.C4/1 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 20:80) to give six fractions (Fr.C4/1/1 ~ Fr.C4/1/6). Fr.C4/2 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 20:80) to give six fractions (Fr.C4/2/1 ~ Fr.C4/2/6). Fr.C4/3 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 21:79) to give three fractions (Fr.C4/3/1 ~ Fr.C4/3/3). Fr.C4/4 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to give four fractions (Fr.C4/4/1 ~ Fr.C4/4/4). Fr.C4/5 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to give five fractions (Fr.C4/5/1 ~ Fr.C4/5/5). Fr. C4/6 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 23:77) to give five fractions (Fr. C4-6–1 − Fr.C4-6–5). Fr.C4/1/1–3 together with Fr.C4/2/2–4 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 23:77) to give **6** (12.3 mg) and **19** (56.5 mg). Fr.C4/1/6, Fr.C4/2/6 and Fr.C4/3/2 were together separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 25:75) to give **11** (16.0 mg). Fr.C4/5/5 and Fr.C4/6/5 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 28:72) to give **5** (10.0 mg) and **17** (49.0 mg). Fr.C5 (35 g) was subjected to ODS column with $(CH_3)_2CO$-H_2O (*v/v*, 24:76) to afford six fractions (Fr.C5/1 ~ Fr.C5/6). Fr.C5-2 was separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 22:78) to give four fractions (Fr.C/5/2/1 ~ Fr.C5/2/4). Fr.C5/3 was separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 22:78) to give five fractions (Fr.C5/3/1 ~ Fr.C5/3/5). Fr.C5/4 was separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 22:78) to give five fractions (Fr.C5/4/1 ~ Fr.C5/4/5). Fr.C5/5 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 20:80) to give six fractions (Fr.C5/5/1 ~ Fr.C5/

5/6). Fr.C5/6 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to give seven fractions (Fr.C5/6/1 ~ Fr.C5/6/7). Fr.C5/2/2 together with Fr.C5/3/2 and Fr.C5/4/1 were separated by pHPLC with CH_3CN-H_2O (*v/v*, 17:83) to give **3** (24.7 mg). The sediments (133 g) together with Fr.D (95 g) were separated on ODS CC with CH_3CN-H_2O (*v/v*, 45:55) to afford **23** (6.8 g), **22** (10.3 g) and **21** (1.2 g), along with a mix fraction (Fr.F). Fr.F was further subjected to ODS CC eluting with $(CH_3)_2CO$-H_2O (*v/v*, 40:60) to afford a five fractions (Fr.F1 ~ Fr.F5). Fr.F2 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 22:78) to give five fractions (Fr.F2/1 ~ Fr.F2/5). Fr.F3 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 24:76) to give six fractions (Fr.F3/1 ~ Fr.F3/6). Fr.F4 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 26:74) to give six fractions (Fr.F4/1 ~ Fr.F4/6). Fr.F5 was separated by pHPLC with CH_3CN-H_2O (*v/v*, 30:70) to give six fractions (Fr.F5/1 ~ Fr.F5/5). Fr.F2/2, Fr.F2/3 and Fr.F3/1 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 25:75) to give **13** (20.0 mg). Fr.F2/5, Fr.F3/2, Fr.F3/4 and Fr.F4/1 were together separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 29:71) to give **14** (37.0 mg) and **20** (25.0 mg). Fr.F3/5 together with Fr.F4/4, and Fr. F5/2 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 33:67) to give **2** (127.0 mg), **12** (120.0 mg), **16** (167.0 mg), and **15** (71.0 mg). Fr.F4/5, and Fr.F5/4 were separated by pHPLC with $(CH_3)_2CO$-H_2O (*v/v*, 36:64) to give **24** (21.8 mg).

2.3.1. Compound *1*

$C_{45}H_{72}O_{19}$; white amorphous powder; [α] = − 110.9 (*c* = 0.08, MeOH). IR (KBr, cm^{-1}): 3402, 2933, 1649, 1454, 1379, 1050, 898. HRESIMS (positive) *m/z*: 917.4803 $[M + H]^+$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.92 (3H, s, H-18), 1.06 (3H, s, H-19), 1.18 (3H, d, *J* = 7.2 Hz, H-21), 4.11 (1H, m, H-27a), 3.91 (1H, m, H-27b), 4.92 (1H, d, *J* = 6.6 Hz, H-1′ of 3-*O*-Glc), 6.32 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, *J* = 6.6 Hz, H-6″ of 2′-*O*-Rha), 5.04 (1H, d, *J* = 6.6 Hz, H-1‴ of 6′-*O*-Glc). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.2. Compound *2*

$C_{45}H_{72}O_{18}$; white amorphous powder; [α] = −129.7 (*c* = 0.10, MeOH). IR (KBr, cm^{-1}): 3404, 2931, 1639, 1452, 1379, 1220, 1128, 1069, 991, 917. HRESIMS (negative) *m/z*: 899.4671 $[M - H]^-$ (Calcd for $C_{45}H_{71}O_{18}$, 899.4640). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.98 (1H, m, H-3), 0.94 (3H, s, H-18), 1.07 (3H, s, H-19), 1.19 (3H, d, *J* = 7.2 Hz, H-21), 4.11 (1H, m, H-27a), 3.91 (1H, m, H-27b), 4.93 (1H, overlap, H-1′ of 3-*O*-Glc), 6.39 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, *J* = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, d, *J* = 6.6 Hz, H-1‴ of 4′-*O*-Rha), 1.62 (3H, d, *J* = 6.0 Hz, H-6‴ of 4′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.3. Compound *3*

$C_{51}H_{82}O_{24}$; white amorphous powder; [α] = − 100.5 (*c* = 0.11, MeOH). IR (KBr, cm^{-1}): 3416, 2934, 1637, 1454, 1383, 1221, 1044, 917. HRESIMS (negative) *m/z*: 1077.6578 $[M - H]^-$ (Calcd for $C_{51}H_{81}O_{24}$, 1077.6516). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.98 (1H, m, H-3), 0.89 (3H, s, H-18), 1.06 (3H, s, H-19), 1.17 (3H, d, *J* = 7.2 Hz, H-21), 4.96 (2H, m, H-27), 4.94 (1H, d, *J* = 7.2 Hz, H-1′ of 3-*O*-Glc), 6.32 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, *J* = 6.6 Hz, H-6″ of 2′-*O*-Rha), 5.08 (1H, d, *J* = 6.6 Hz, H-1‴ of 6′-*O*-Glc), 4.94 (1H, d, *J* = 7.8 Hz, H-1‴ of 27-*O*- Glc). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.4. Compound *4*

$C_{45}H_{72}O_{19}$; white amorphous powder; [α] = − 100.8 (*c* = 0.09, MeOH). IR (KBr, cm^{-1}): 3416, 2934, 1638, 1454, 1382, 1245, 1133, 1046, 911. HRESIMS (positive) *m/z*: 917.4801 $[M + H]^+$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.92 (3H, s, H-18), 1.06 (3H, s, H-19), 1.18 (3H, d, *J* = 7.2 Hz, H-21), 3.75 (1H, m, H-27a), 3.64 (1H, m, H-27b), 4.92 (1H, d, *J* = 6.6 Hz, H-1′ of 3-*O*-Glc), 6.32 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, *J* = 6.6 Hz, H-6″ of 2′-*O*-Rha), 5.04 (1H, d, *J* = 6.6 Hz, H-1‴ of 6′-*O*-Glc). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.5. Compound *5*

$C_{51}H_{82}O_{23}$; white amorphous powder; [α] = − 97.9 (*c* = 0.10, MeOH). IR (KBr, cm^{-1}): 3415, 2934, 1637, 1455, 1383, 1052, 906. HRESIMS (positive) *m/z*: 1063.5375 $[M + H]^+$ (Calcd for $C_{51}H_{83}O_{23}$, 1063.5325). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.93 (3H, s, H-18), 1.07 (3H, s, H-19), 1.18 (3H, d, *J* = 7.2 Hz, H-21), 3.76 (1H, m, H-27a), 3.70 (1H, m, H-27b), 4.94 (1H, d, *J* = 6.0 Hz, H-1′ of 3-

Table 1
^{13}C NMR data for aglycones of **1–14** (δ in pyridine-d_5).

POS.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	37.6	37.6	37.6	37.6	37.6	37.6	37.6	37.6	37.6	37.6	37.6	37.6	33.1	33.1
2	30.3	30.2	30.3	30.3	30.2	30.2	30.2	30.2	30.2	30.3	30.2	30.2	29.7	29.7
3	76.8	77.8	76.8	76.8	77.8	78.0	77.8	77.8	77.8	76.8	78.0	77.8	75.6	75.6
4	39.2	39.0	39.2	39.2	39.0	39.0	39.0	39.0	39.0	39.2	39.0	39.0	38.8	38.7
5	141.0	140.8	141.0	141.0	140.8	140.8	140.8	140.8	140.9	140.9	140.8	140.8	75.9	75.6
6	121.7	121.8	121.7	121.7	121.9	121.9	121.8	121.8	121.8	121.7	121.8	121.8	76.2	76.3
7	32.4	32.5	32.4	32.4	32.5	32.5	32.5	32.5	32.5	32.4	32.5	32.5	35.8	35.8
8	31.8	32.3	31.8	31.9	32.4	32.4	32.4	32.4	32.4	32.4	32.4	32.4	31.4	31.4
9	50.1	50.3	50.1	50.1	50.3	50.3	50.2	50.3	50.2	50.1	50.3	50.3	45.7	45.7
10	37.2	37.2	37.2	37.2	37.2	37.2	37.2	37.2	37.2	37.2	37.2	37.2	39.3	39.3
11	20.9	21.0	20.9	20.9	21.0	21.0	21.0	21.0	21.0	20.9	21.0	21.0	21.4	21.4
12	32.1	32.1	32.1	32.1	32.1	32.3	32.2	32.1	32.1	32.4	32.1	32.1	32.7	32.7
13	45.3	45.1	45.2	45.2	45.2	45.8	45.7	45.2	45.2	45.2	45.2	45.0	45.8	45.9
14	53.0	53.1	53.0	53.0	53.1	53.2	53.2	53.1	53.1	53.0	53.1	53.1	52.8	52.8
15	32.3	31.8	32.3	32.4	31.8	31.9	31.8	31.8	31.8	31.8	31.8	31.8	31.8	31.8
16	90.3	90.2	90.2	90.2	90.1	90.9	91.0	90.3	90.3	90.4	90.3	90.3	90.1	90.1
17	90.1	90.1	90.0	90.1	90.1	90.2	90.0	89.9	89.9	89.9	89.9	89.9	90.1	90.1
18	17.2	17.2	17.1	17.2	17.1	17.5	17.4	17.1	17.1	17.1	17.1	17.1	17.6	17.6
19	19.5	19.5	19.5	19.5	19.5	19.5	19.4	19.5	19.5	19.5	19.5	19.5	17.2	17.2
20	45.1	45.3	45.1	44.9	44.8	38.8	39.0	45.1	45.1	45.1	45.1	45.2	44.8	44.8
21	9.6	9.6	9.5	9.8	9.7	9.4	9.2	9.8	9.8	9.8	9.8	9.8	9.8	9.8
22	110.5	110.5	110.4	110.3	110.1	112.7	113.1	112.1	112.1	112.1	112.1	112.4	109.8	109.8
23	27.5	27.5	27.3	31.9	31.6	68.1	64.3	42.1	42.1	42.1	42.1	42.2	32.1	32.1
24	21.2	21.2	20.9	23.6	23.5	33.1	31.1	66.5	66.5	66.5	66.5	70.3	28.8	28.8
25	36.1	36.1	33.3	39.1	36.6	40.4	39.4	47.7	47.7	47.7	47.7	39.9	30.5	30.5
26	61.4	61.4	60.8	64.0	63.5	63.2	59.7	62.3	62.3	62.3	62.3	65.3	66.7	66.7
27	60.6	60.6	69.4	64.4	71.7	64.0	62.3	61.5	61.5	61.5	61.5	13.6	17.3	17.3

Table 2
^{13}C NMR data for sugar units of **1–14** (δ in pyridine-d_5).

POS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
3-*O*-*β*-D-Glc*p*-1′	100.7	100.3	100.7	100.7	100.3	100.3	100.3	100.3	100.4	100.7	100.4	100.3	100.6	100.5
2′	79.6	78.1	79.5	79.6	78.1	78.2	78.1	78.1	79.7	79.6	77.8	78.1	78.1	79.8
3′	77.5	78.7	77.5	77.5	78.6	78.7	78.7	78.7	78.0	77.5	77.0	78.7	78.5	77.9
4′	71.7	78.0	71.7	71.7	78.0	78.0	78.0	78.0	71.9	71.7	78.1	78.0	77.9	71.7
5′	78.4	76.9	78.4	78.3	76.9	77.0	76.9	76.9	78.3	78.4	77.8	76.9	76.9	78.2
6′	69.9	61.3	69.9	69.9	61.3	61.4	61.3	61.3	62.7	69.9	61.2	61.3	61.2	62.6
2′-*O*-*α*-L-Rha*p*-1″	102.0	102.0	102.0	102.0	102.0	102.1	102.0	102.0	102.1	102.0	102.3	102.0	101.8	101.9
2″	72.6	72.6	72.6	72.6	72.6	72.6	72.5	72.6	72.6	72.6	72.7	72.6	72.6	72.7
3″	72.8	72.8	72.8	72.8	72.8	72.8	72.8	72.8	72.9	72.8	72.9	72.8	72.8	72.5
4″	74.2	73.9	74.2	74.2	74.0	74.0	73.9	74.0	74.2	74.2	74.2	73.9	73.9	74.5
5″	69.5	69.5	69.5	69.5	69.5	69.6	69.5	69.5	69.5	69.5	69.6	69.5	69.3	69.3
6″	18.7	18.7	18.7	18.7	18.7	18.7	18.6	18.7	18.7	18.7	18.7	18.7	18.6	18.7
6′-*O*-*β*-D-Glc*p*/4′-*O*-*α*-L-Rha*p*-1‴	105.5	102.9	105.5	105.5	102.9	103.0	102.9	102.9		105.5	103.3	102.9	102.9	
2‴	75.2	72.6	75.2	75.2	72.6	72.6	72.6	72.6		75.2	73.3	72.6	72.4	
3‴	78.5	72.8	78.5	78.5	72.9	72.9	72.8	72.8		78.5	72.9	72.8	72.7	
4‴	71.7	74.2	71.8	71.7	74.2	74.2	73.9	74.2		71.7	80.4	74.2	74.4	
5‴	78.5	70.4	78.6	78.5	70.4	70.5	70.4	70.4		78.5	68.4	70.4	70.4	
6‴	62.8	18.5	62.8	62.8	18.5	18.6	18.5	18.5		62.8	18.9	18.5	18.5	
27-*O*-*β*-D-Glc*p*/4‴-*O*-*α*-L-Rha*p*-1⁗			105.0		105.1						102.2			
2⁗			75.3		75.2						72.5			
3⁗			78.4		78.6						72.9			
4⁗			71.7		71.9						74.1			
5⁗			78.6		78.6						70.4			
6⁗			63.0		62.9						18.5			

O-Glc), 6.41 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, d, J = 6.6 Hz, H-1‴ of 4′-*O*-Rha), 1.62 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha), 4.73 (1H, d, J = 6.6 Hz, H-1⁗ of 27-*O*-Glc). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.6. Compound **6**

$C_{45}H_{72}O_{19}$; white amorphous powder; [α] = − 98.7 (c = 0.101, MeOH). IR (KBr, cm^{-1}): 3406, 2935, 1640, 1456, 1384, 1130, 1043, 911. HRESIMS m/z: 917.4785 [M + H]$^+$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 1.01 (3H, s, H-18), 1.16 (3H, s, H-19), 1.30 (3H, d, J = 7.2 Hz, H-21), 3.76 (1H, m, H-27a), 3.70 (1H, m, H-27b), 4.92 (1H, d, J = 6.0 Hz, H-1′ of 3-*O*-Glc), 6.26 (1H, s, H-1″ of 2′-*O*-Rha), 1.74 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.83 (1H, d, J = 6.6 Hz, H-1‴ of 4′-*O*-Rha), 1.60 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.7. Compound **7**

$C_{45}H_{72}O_{19}$; white amorphous powder; [α] = −119.5 (c = 0.08, MeOH). IR (KBr, cm^{-1}): 3416, 2935, 1735, 1642, 14556, 1381, 1248, 1136, 1046, 915. HRESIMS (positive) m/z: 917.4785 [M+H]$^+$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 1.01 (3H, s, H-18), 1.13 (3H, s, H-19), 1.26 (3H, d, J = 7.2 Hz, H-21), 4.18 (1H, m, H-27a), 4.01 (1H, m, H-27b), 4.94 (1H, d, J = 6.0 Hz, H-1′ of 3-*O*-Glc), 6.38 (1H, s, H-1″ of 2′-*O*-Rha), 1.75 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, d, J = 6.6 Hz, H-1‴ of 4′-*O*-Rha), 1.59 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.8. Compound **8**

$C_{45}H_{72}O_{19}$; white amorphous powder; [α] = −86.2 (c = 0.09, MeOH). IR (KBr, cm^{-1}): 3416, 2933, 1636, 1454, 1383, 1245, 1050, 898. HRESIMS (positive) m/z: 917.4769 [M + H]$^+$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.93 (3H, s, H-18), 1.07 (3H, s, H-19), 1.28 (3H, d, J = 7.2 Hz, H-21), 4.38 (1H, m, H-27a), 4.03 (1H, m, H-27b), 4.94 (1H, d, J = 6.0 Hz, H-1′ of 3-*O*-Glc), 6.41 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, d, J = 6.6 Hz, H-1‴ of 4′-*O*-Rha), 1.59 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.9. Compound **9**

$C_{39}H_{62}O_{15}$; white amorphous powder; [α] = −115.7 (c = 0.09, MeOH). IR (KBr, cm^{-1}): 3416, 2933, 1636, 1454, 1383, 1056, 976. HRESIMS (positive) m/z: 771.4196 [M + H]$^+$ (Calcd for $C_{39}H_{63}O_{15}$, 771.4167). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.88 (1H, m, H-3), 0.93 (3H, s, H-18), 1.07 (3H, s, H-19), 1.27 (3H, d, J = 7.2 Hz, H-21), 4.38 (1H, m, H-27a), 4.03 (1H, m, H-27b), 4.93 (1H, d, J = 6.6 Hz, H-1′ of 3-*O*-Glc), 6.32 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.6 Hz, H-6″ of 2′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.10. Compound **10**

$C_{45}H_{72}O_{20}$, white amorphous powder, [α] = −100.8 (c = 0.11, MeOH). IR (KBr, cm^{-1}): 3404, 2933, 1642, 1454, 1382, 1253, 1136, 1046, 905. HRESIMS (positive) m/z: 933.4767 [M+H]$^+$ (Calcd for $C_{45}H_{73}O_{20}$, 933.4695). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.93 (3H, s, H-18), 1.07 (3H, s, H-19), 1.28 (3H, d, J = 7.2 Hz, H-21), 4.38 (1H, m, H-27a), 4.03 (1H, m, H-27b), 4.93 (1H, d, J = 6.6 Hz, H-1′ of 3-*O*-Glc), 6.32 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.6 Hz, H-6″ of 2′-*O*-Rha), 5.07 (1H, d, J = 6.6 Hz, H-1‴ of 6′-*O*-Glc); ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.11. Compound **11**

$C_{51}H_{82}O_{23}$; white amorphous powder; [α] = −114.3 (c = 0.08, MeOH). IR (KBr, cm^{-1}): 3415, 2934, 1637, 1455, 1383, 1129, 1052, 906. HRESIMS (positive) m/z: 1063.5375 [M+H]$^+$ (Calcd for $C_{51}H_{83}O_{23}$, 1063.5325). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.97 (1H, m, H-3), 0.93 (3H, s, H-18), 1.07 (3H, s, H-19), 1.27 (3H, d, J = 7.2 Hz, H-21), 4.38 (1H, m, H-27a), 4.03 (1H, m, H-27b), 4.94 (1H, d, J = 6.0 Hz, H-1′ of 3-*O*-Glc), 6.38 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.6 Hz, H-6″ of 2′-*O*-Rha), 5.81 (1H, s, H-1‴ of 4′-*O*-Rha), 1.58 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha), 6.27 (1H, s, H-1⁗ of 4″-*O*-Rha), 1.60 (3H, d, J = 6.0 Hz, H-6⁗ of 4″-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.12. Compound **12**

$C_{45}H_{72}O_{18}$; white amorphous powder; [α] = −112.2 (c = 0.08, MeOH). IR (KBr, cm^{-1}): 3417, 2933, 1637, 1455, 1380, 1044, 981.

HRESIMS (negative) *m/z*: 899.4603 $[M-H]^-$ (Calcd for $C_{45}H_{71}O_{18}$, 899.4640). ^{1}H NMR (600 MHz, pyridine-d_5): δ 3.98 (1H, m, H-3), 0.95 (3H, s, H-18), 1.07 (3H, s, H-19), 1.26 (3H, d, J = 7.2 Hz, H-21), 1.09 (1H, d, J = 6.0 Hz, H-27), 4.93 (1H, overlap, H-1′ of 3-*O*-Glc), 6.39 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, s, H-1‴ of 4′-*O*-Rha), 1.62 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha); ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.13. Compound 13

$C_{45}H_{74}O_{19}$; white amorphous powder; [α] = −84.2 (*c* = 0.10, MeOH). IR (KBr, cm^{-1}): 3415, 2932, 2879, 1654, 1455, 1383, 1051, 979. HRESIMS (negative) *m/z*: 917.4775 $[M-H]^-$ (Calcd for $C_{45}H_{73}O_{19}$, 917.4746). ^{1}H NMR (600 MHz, pyridine-d_5): δ 4.10 (1H, m, H-3), 1.02 (3H, s, H-18), 1.66 (3H, s, H-19), 1.22 (3H, d, J = 7.2 Hz, H-21), 0.65 (1H, d, J = 6.0 Hz, H-27), 4.83 (1H, d, J = 7.2 Hz, H-1′ of 3-*O*-Glc), 6.41 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.0 Hz, H-6″ of 2′-*O*-Rha), 5.84 (1H, s, H-1‴ of 4′-*O*-Rha), 1.62 (3H, d, J = 6.0 Hz, H-6‴ of 4′-*O*-Rha). ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.3.14. Compound **14**

$C_{39}H_{64}O_{15}$; white amorphous powder; [α] = −164.8 (*c* = 0.12, MeOH). IR (KBr, cm^{-1}): 3415, 2933, 2874, 1658, 1456, 1382, 1050, 979, 919. HRESIMS (negative) *m/z*: 771.4222 $[M-H]^-$ (Calcd for $C_{39}H_{63}O_{15}$, 771.4167). ^{1}H NMR (600 MHz, pyridine-d_5): δ 4.10 (1H, m, H-3), 1.02 (3H, s, H-18), 1.66 (3H, s, H-19), 1.22 (3H, d, J = 7.2 Hz, H-21), 0.65 (1H, d, J = 6.0 Hz, H-27), 4.83 (1H, d, J = 7.2 Hz, H-1′ of 3-*O*-Glc), 6.39 (1H, s, H-1″ of 2′-*O*-Rha), 1.76 (3H, d, J = 6.6 Hz, H-6″ of 2′-*O*-Rha); ^{13}C NMR (150 MHz, pyridine-d_5) spectroscopic data see Tables 1 and 2.

2.4. Acid hydrolysis and absolute configuration determination

Standard monosaccharides, L-rhamnose (5 mg), D-glucose (5 mg) and L-cysteine methyl ester hydrochloride (5 mg) was dissolved in pyridine (5 mL) and heated to 60 °C for 1 h. And then o-tolyl isothiocyanate (10 μL) was added to the mixture and refluxed on a water bath further for 1 h. The reaction mixture (1 μL) was analyzed by UPLC-Q/TOFMS on a Acquity UPLC HSS T3 C18 column (2.1 × 100 mm, 1.7 μm) at 40 °C with gradient elution of CH_3CN-H_2O (containing 0.1% FA) (*v/v*, 20:80 → 30:70) for 8 min at a flow rate of 0.6 mL/min. Compounds **1**–**14** (each 1.0 mg) were individually hydrolyzed by heating in 6 M TFA (1 mL) at 90 °C for 2 h. After cooling, the reaction mixture was extracted with $CHCl_3$. Then, each aqueous layer was evaporated to dryness, and the residue was dissolved in pyridine (1 mL) containing L-cysteine methyl ester hydrochloride (1 mg) and heated at 60 °C for 1 h. Following, o-tolyl isothiocyanate (5 μL) was added to each mixture, and heated at 60 °C for another 1 h. The reaction mixture (1 μL) was analyzed following the above procedure. As a result, the sugars in the test compounds were identified as D-glucose and L-rhamnose, respectively, by comparing their molecular weight and retention time with the standards (t_R 3.18 min for D-glucose; t_R 5.38 min for L-rhamnose).

2.5. HepG2 cell proliferation assay

Cell proliferation was measured by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) method. HepG2 cells in logarithmic phase were trypsinized and seeded into 96 well plates at a density of 1 × 10^5 cells/mL. 100 μL of cell suspension was added to each well (1 × 10^4 cells per well) and the plates were incubated at 37 °C for 24 h in a 5% CO_2 incubator. After pre-incubation, 100 μL of the corresponding drug-containing medium was added to each well. At the same time, a negative control group, a vehicle control group, and a positive control group were set up, and 5 duplicate wells in each group; 96-well plates were placed at 37 °C, and cultured in a 5% CO_2 incubator for 48 h. 10 μL CCK-8 solutions was added into each well, and further cultured for 4 h at 37 °C. The absorbance of each group at 450 nm was detected (n = 5) using an absorbance microplate reader (Sunrise, Tecan, Germany). The optical density values of each well represented the survival/proliferation of cells. The inhibition rates of all test compounds on HepG2 were calculated.

3. Results and discussion

Through series of purification steps over macroporous resin, MCI gel, silica gel, ODS and preparative HPLC, twenty-four steroidal saponins were isolated from 50% ethanol extract of *T. tschonoskii* rhizomes. Detailed comparison of NMR data with the reported values in the literatures allowed the ten known analogues to be identified as trikamsteroside A (**15**) [3], (25*S*)-27-hydroxypenogenin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside (**16**) [3], (25*S*)-27-[(β-D-glucopyranosyl)oxy]-17α-hydroxyspirost-5-en-3β-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside (**17**) [4], chonglouside SL-17 (**18**) [5], chonglouside SL-18 (**19**) [5], trikamsteroside B (**20**) [3], saponin Tg (**21**) [6], pennogenin 3-*O*-β-chacotrioside (**22**) [7], pennogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**23**) [3,7], and pennogenin 3-*O*-β-D-glucopyranoside (**24**) [3].

Compound **1** had a molecular formula $C_{45}H_{72}O_{19}$ as determined by HRESIMS. In the ^{1}H NMR spectrum, three typical methyl proton signals at δ 0.92 (3H, s), 1.07 (3H, s), 1.18 (3H, d, J = 7.2 Hz), one olefinic proton signal at δ 5.27 (1H, d, J = 6.0 Hz), and three anomeric proton signals at δ 6.32 (1H, br s), 5.04 (1H, d, J = 6.6 Hz) and 4.92 (1H, d, J = 6.6 Hz) were observed. The ^{13}C NMR spectrum exhibited total 54 carbon resonances including 27 ones due to the aglycone part and 18 ones attributed to the sugar moiety consisting of three hexoses (Tables 1 and 2). Comparison of the NMR data of **1** with those of trikamsteroside A [3] indicated that they shared the identical spirostanol skeleton of (25*R*)-5-en-spirost-3β,17α,27-triol, which was further confirmed by detailed analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra of **1**. Starting from three anomeric proton signals, all proton signals of each sugar unit were delineated using ^{1}H–^{1}H COSY analysis, and then the carbon signals were assigned depending on the HSQC correlations. Thus, the structures of all sugar units could be established, and the NMR data suggested them to be two glucoses and a rhamnose. Acid hydrolysis experiment further proved that the sugar moiety of **1** comprised of D-glucopyranose (Glc*p*) and L-rhamnopyranose (Rha*p*). The large coupling constants ($J^{1,2}$ greater than 7 Hz) indicated the β-configurations of two D- Glc*p*, and the α-configurations of the L-Rha*p* was determined by carbon signals of δ 72.8 (C-3″) and δ 69.5 (C-5″), respectively [8]. The connectivity and the sequence of the sugar moiety was determined due to the HMBC correlations between δ 4.92 (H-1′ of 3-*O*-Glc*p*) and 76.8 (C-3), between δ 6.32 (H-1″ of 2′-*O*-Rha*p*) and 79.6 (C-2′), and between δ 5.04 (H-1‴ of 6′-*O*-Glc*p*) and 69.6 (C-6′). Consequently, the structure of **1** was elucidated as (25*R*)-5-en-spirost-3β,17α,27-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)]-β-D-glucopyranoside, named trillitschoside S1.

Compound **2** had a molecular formula $C_{45}H_{72}O_{18}$ as determined by HRESIMS. The NMR data suggested that **2** had the same aglycone as **1**. In the ^{1}H NMR spectrum, the observed three anomeric carbon resonances at δ 4.83 (1H, d, J = 7.2 Hz), 6.41 (1H, s) and 5.84 (1H, s) suggested the presence of three sugar units. Comparison of the NMR data of sugar moiety between **2** and pennogenin 3-*O*-β-chacotrioside [7] suggested they had the same sugar chain. By detailed analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, the structure of **2** was confirmed to be (25*R*)-5-en-spirost-3β,17α,27-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosy1-(1 → 2)-β-D-glucopyranoside, named trillitschoside S2.

Compound **3** had a molecular formula $C_{51}H_{82}O_{24}$ as determined by HRESIMS. The NMR data suggested it had the same aglycone and C-3 sugar chain with **1**, but one glucose more than **1**. In the ^{13}C NMR

spectrum, the chemical shift of C-27 (δ 69.4) shifting to a lower field suggested the linkage of the additional glucose to the C-27, which was supported by the HMBC correlation of δ 4.94 (H-1‴ of 27-*O*-Glc) and δ 69.4 (C-27). The structure of **3** were finally confirmed by detailed analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, and elucidated as (25*R*)-27-*O*-*β*-D-glucopyranosyl-5-en-spirost-3*β*,17*α*,27-triol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-[*β*-D-glucopyranosyl-(1 → 6)]-*β*-D-glucopyranoside, named trillitschoside S3.

Compound **4** had a molecular formula $C_{45}H_{72}O_{19}$ as determined by HRESIMS, which was the same with **1**. The NMR data of **4** showed the pattern analogous to **1** except for the obvious differences in F-ring (C-22 to C-27), suggesting **4** was a pair of C-25 epimer of **1**. Further comparison of the NMR data of **4** with those of (25*S*)-27-hydroxypenogenin-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*O*-*β*-D-glucopyranoside [3] confirmed that the aglycone of **4** was (25*S*)-5-en-spirost-3*β*,17*α*,27-triol. Finally, confirmed by detailed analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, the structure of **4** were elucidated as (25*S*)-5-en-spirost-3*β*,17*α*,27-triol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-[*β*-D-glucopyranosyl-(1 → 6)]-*β*-D-glucopyranoside, named trillitschoside S4.

Compound **5** had a molecular formula $C_{51}H_{82}O_{23}$ as determined by HRESIMS. The NMR data of **5** suggested it had same C-3 sugar chain with **2**. And the aglycone together with the C-27-*O*-glucosyl was same as (25*S*)-27-[(*β*-D-glucopyranosyl)oxy]-17*α*-hydroxyspirost-5-en-3*β*-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*O*-*β*-D-glucopyranoside [4] by detailed comparison of their NMR data. Though combined use of ^{1}H–^{1}H COSY, HSQC and HMBC experiments, the structure of **5** was further confirmed, and elucidated as 27-*O*-*β*-D-glucopyranosyl-(25*S*)-5-en-spirost-3*β*,17*α*,27-triol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschoside S5.

Compound **6** had a molecular formula $C_{45}H_{72}O_{19}$ as determined by HRESIMS. The NMR data suggested that it showed the pattern analogous to **2** except for the obvious differences in F-ring. **6** had 16 Da mass units more than of **2**, deducing the presence of an additional hydroxyl group at its F-ring. Further comparison of the NMR data of **6** and borassoside B [9] confirmed that they had the same F-ring structure, attributing to verify the presence of 23*α*-OH group and the C-25*S* configuration for **6**. Consequently, the structure of **6** was elucidated as (23*S*, 25*S*)-5-en-spirost-3*β*,17*α*,23*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschonide S6.

Compound **7**, same with **6**, also had a molecular formula $C_{45}H_{72}O_{19}$ as determined by HRESIMS. The NMR data suggested that it showed the pattern analogous to **6** except for the differences in F-ring, suggesting **7** and **6** was a pair of C-25 epimers. By comparing of the NMR data of **7** and borassoside C [9], the identical chemical shifts of F-ring verified the C-25*R* configuration of **7**. Subsequently, the structure of **7** was elucidated as (25*R*)-5-en-spirost-3*β*,17*α*,23*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschoside S7.

Compound **8** had a molecular formula $C_{45}H_{72}O_{19}$ as determined by HRESIMS, same with that of **6**. When compared the NMR data of **8** with those of **6**, it was suggested that they had the almost identical structure except for the slightly different in F-ring. The ^{1}H–^{1}H COSY correlations of H-24 (δ 4.55)/H-25 (δ 2.25) and the HMBC correlation of H-27 (δ 4.18) and C-24 (δ 66.5) deduced the presence of a 24-OH group. Comparison of the NMR data of **8** with those of (24*R*, 25*S*)-spirost-5-ene-3*β*,17*α*,24,27-tetraol-3-O-*β*-D-glucopyranoside [10] deduced that they had the same F-ring structure, attributing to further verify the location of 24-OH group and the 24*R*, 25*S* configurations of **8**. Therefore, the structure of **8** was elucidated as (24*R*, 25*S*)-spirost-5-ene-3*β*,17*α*,24*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschonide S8.

Compound **9** had a molecular formula $C_{39}H_{62}O_{15}$ as determined by HRESIMS. When comparing the NMR data, it was verified that of **9** to have same spirotanol skeleton with **8**. On the other hand, the NMR data further suggested that **9** had the same sugar chain with trikamsteroside A [3]. By detailed analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, the structure of **9** was confirmed to be (24*R*, 25*S*)-spirost-5-ene-3*β*,17*α*,24*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschonide S9.

Compound **10** had a molecular formula $C_{45}H_{72}O_{20}$ as determined by HRESIMS. The NMR data of **10** suggested it had the same spirotanol skeleton with **8**, but had the identical sugar chain with **1**. Therefore, structure of **10** was confirmed to be (24*R*, 25*S*)-spirost-5-ene-3*β*,17*α*,24*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside though combined analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, named trillitschonide S10.

Compound **11** had a molecular formula $C_{51}H_{82}O_{23}$ as determined by HRESIMS. The NMR data suggested that **11** had an identical spirotanol skeleton with **8**, and had a same sugar chain with saponin Tg [6]. Thus, by combined analyses of ^{1}H–^{1}H COSY, HSQC and HMBC spectra, the structure of **11** was confirmed to be (24*R*, 25*S*)-spirost-5-ene-3*β*,17*α*,24*α*,27-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 4)-[*α*-L-rhamno-pyranosyl-(1 → 2)]-*β*-D-glucopyranoside, named trillitschonide S11.

Compound **12** possessed the molecular formula $C_{45}H_{72}O_{18}$ determined by HRESIMS. The ^{1}H NMR spectrum of **12** showed four methyl proton signals at δ 0.95 (3H, s), δ 1.07 (3H, s), 1.09 (3H, d, J = 6.0 Hz), 1.26 (3H, d, J = 7.2 Hz), and three anomeric proton signals at δ 4.93 (1H, overlap), 5.84 (1H, br s) and 6.39 (1H, br s). By comparing of the NMR data, it was verified that **12** had the similar structure with **8** except for the F-ring. The HMBC correlations of H-27 (δ 1.09) and C-24 (δ 70.3), C-25 (δ 39.9), and C-26 (δ 65.2) suggesting the presence of 24-OH group in **12**. When compared the NMR data of **12** with those of (24*S*,25*S*)-3*β*,17*α*,24-trihydroxy-5*α*-spirostan-6-one 3-*O*-[*α*-L-rhamnopyranosyl-(1 → 2)]-*β*-D-glucopyranoside [11], their F-ring structure was verified to be identical. Therefore, the structure of **12** was elucidated as (24*S*, 25*R*)-5-en-spirost-3*β*,17*α*,24*β*-triol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 2)-*β*-D-glucopyranoside, named trillitschoside S12.

Compound **13** possessed the molecular formula $C_{45}H_{74}O_{19}$ determined by HRESIMS. The ^{1}H NMR spectrum of **13** showed four typical methyl signals of steroidal saponin at δ 0.65 (3H, d, J = 6.0 Hz), 1.02 (3H, s) 1.22 (3H, d, J = 7.2 Hz), and 1.66 (3H, s). Moreover, three anomeric proton signals at δ 4.82 (1H, d, J = 7.2 Hz), 5.84 (1H, br s), and 6.41 (1H, br s) due to three sugar unites in the molecule were obviously observed. The NMR data indicated that **13** had the identical structure with pennogenin 3-*O*-*β*-chacotrioside [7] except for the obviously differences in C-5 and C-6 positons. The HMBC correlations of H-19 (δ 1.66)/C-5 (δ 75.4) and in the ^{1}H–^{1}H COSY spectrum a spin system correlations of H-6 (δ 4.81) → H-7 (δ 1.75, 2.15) → H-8 (δ 2.15) → H-9 (δ 1.97) indicated that **13** presented two hydroxyl groups at C-5 and C-6, respectively. The α- and *β*-orientations for 5-OH and 6-OH, respectively, were determined by comparing the chemical shifts of C-5 (δ 75.9) and C-6 (δ 75.6) with those of (3*β*,5*α*,6*β*,25*R*)-spirostane-3,5,6-triol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside [12]. By detailed analyses of the ^{1}H–^{1}H COSY, HSQC and HMBC spectra of **13**, its structure were further confirmed. Consequently, the structure of **13** was established as (25*R*)-spirost-3*β*,5*α*,6*β*,17*α*-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-[*α*-L-rhamnopyranosy1-(1 → 2)]-*β*-D-glucopyranoside, named trillitschoside S13.

Compound **14** possessed the molecular formula $C_{39}H_{64}O_{15}$ determined by HRESIMS. By comparing of the NMR data, it was verified that **14** had the similar structure with **13** except for the sugar chain. In the ^{1}H NMR spectrum, the observed only two anomeric proton signals at δ 4.83 (1H, d, J = 7.2 Hz) and 6.39 (1H, br s) suggested that **14** had a rhamnose less than **13**. Comparison of NMR data of **14** and **9** suggested they had the same sugar chain. Therefore, the structure of **14** was elucidated as (25*R*)-spirost-3*β*,5*α*,6*β*,17*α*-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2) -*β*-D-glucopyranoside, named trillitschoside S14.

Compounds **1–24** were tested for their cytotoxic activities *in vitro* using the HepG2 cell line. A preliminary screening displayed that, after

Table 3
HepG2 cell survival rate after treatment of **1–24** and 5-Fu (each 100 μM).

Compounds	Cell survival rate (%)	Compounds	Cell survival rate (%)
1	98.36 ± 6.10	**14**	92.41 ± 0.44
2	97.42 ± 6.13	**15**	96.61 ± 4.08
3	97.85 ± 3.91	**16**	94.37 ± 2.54
4	90.99 ± 6.99	**17**	92.09 ± 2.27
5	91.65 ± 8.36	**18**	102.96 ± 4.62
6	101.36 ± 6.09	**19**	101.54 ± 0.86
7	94.70 ± 0.48	**20**	103.30 ± 5.80
8	97.71 ± 3.75	**21**	1.45 ± 0.08
9	107.33 ± 1.71	**22**	0.98 ± 0.16
10	90.88 ± 7.95	**23**	0.69 ± 0.06
11	98.16 ± 5.32	**24**	4.74 ± 1.95
12	99.33 ± 1.02	5-FU	21.09 ± 0.49
13	95.42 ± 3.92		

treatment with **21–24** (100 μM), the average survival rate of HepG2 cells was 0.69% to 4.74%, which were better than the positive control 5-FU (5-Fluorouracil), suggesting their remarkable cytotoxic activities against HepG2 cells, while the other compounds displayed little inhibitory effects (Table 3). **21–24** have much better cytotoxic activity against HepG2 cells than other compounds, which presumed that the aglycone of pennogenin is critical for the cytotoxic activity, and the structural changes on pennogenin due to substituents or the configuration difference could result in the activity disappeared. Not surprisingly, HepG2 cells treated with **21** and **24** for 24 h could significantly induce apoptosis (Supplementary Fig.S2). While it should be noticed that **20** is also a pennogenin glycoside but displaying no activity and it is speculated that this is caused by its different sugar chain, which further indicated that the sugar moiety of steroidal saponin is also important for their activity.

4. Conclusion

This work presented a phytochemical study on *T. tschonoskii* rhizomes, resulting in isolation of total twenty-four steroidal saponins with multiple structural characteristics, which was helpful to understand the structural composition of steroidal saponins as main constituents in this medicine. The following cytotoxic activity assay showed that pennogenin glycosides had the remarkable cytotoxic activities against HepG2 cells overall, suggesting the pennogenin glycosides, especially the high-content ones, should be the basis of active material of this herb medicine for playing an anti-tumor role. *T. tschonoskii* have various pharmacological activities, therefore more biological activities of these isolated compounds such as hemostasis, anti-inflammation and neuroprotection, etc. should be tested in the next, so as to illuminate the material foundation of *T. tschonoskii* for its multiple activity.

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Statement clarifying

There are no conflicts of interests of all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2020.108587.

References

[1] Q. Li, M. Xiao, L. Guo, L. Wang, L. Tang, Y. Xu, Genetic diversity and genetic structure of an endangered species, *Trillium tschonoskii*, Biochem. Genet. 43 (2005) 445–458.
[2] L. Fu, China Plant Red Data Book Vol. 1 Science Publishing House Press, Beijing, Rare and Endangered Plants, 1992.
[3] M. Ono, C. Takamura, F. Sugita, C. Masuoka, H. Yoshimitsu, T. Ikeda, T. Nohara, Two new steroid glycosides and a new sesquiterpenoid glycoside from the underground parts of *Trillium kamtschaticum*, Chem. Pharm. Bull. 55 (2007) 551–556.
[4] A. Yokosuka, Y. Mimaki, Steroidal glycosides from the underground parts of *Trillium erectum* and their cytotoxic activity, Phytochemistry 69 (2008) 2724–2730.
[5] X.J. Qin, M.Y. Yu, W. Ni, H. Yan, C.X. Chen, Y.C. Cheng, L. He, H.Y. Liu, Steroidal saponins from stems and leaves of *Paris polyphylla* var. *yunnanensis*, Phytochemistry 121 (2016) 20–29.
[6] Y. Hira, S. Sanada, Y. Ida, Study on the constituents of Palmae plants III. The constituents of *Chamaerops humilis* L. and *Trachycarpus wagnerianus* BECC, Chem. Pharm. Bull. 34 (1986) 82–87.
[7] T. Nohara, K. Miyahara, T. Kawasaki, Steroid saponins and sapogenins of underground parts of *Trillium kamtschaticum* Pall. II. Pennogenin- and kryptogenin 3-*O*-glycosides and related compounds, Chem. Pharm. Bull. 23 (1975) 872–885.
[8] P.K. Agrawal, NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides, Phytochemistry 31 (1992) 3307–3330.
[9] M. Yoshikawa, F. Xu, T. Morikawa, Y. Pongpiriyadacha, S. Nakamura, Y. Asao, A. Kumahara, H. Matsuda, Medicinal flowers. XII.[1)] New spirostane-type steroid saponins with antidiabetogenic activity from *Borassus flabellifer*, Chem Pharm Bull 55 (2007) 308–316.
[10] Y. Chen, W. Ni, H. Yan, X.J. Qin, A. Khan, H. Liu, T. Shu, L.Y. Jin, H.Y. Liu, Spirostanol glycosides with hemostatic and antimicrobial activities from *Trillium kamtschaticum*, Phytochemistry 131 (2016) 165–173.
[11] Carbohyd. Res. 361 (2012) 19–26, https://doi.org/10.1016/j.carres.2012.07.027.
[12] X. Wu, L. Wang, H. Wang, Y. Dai, W.C. Ye, Y.L. Li, Steroidal saponins from *Paris polyphylla* var. yunnanensis, Phytochemistry 81 (2012) 133–143.