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Spirostanol and sesquiterpenoid glycosides from the rhizomes of *Trillium tschonoskii*

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ABSTRACT

Three new spirostanol glycosides, trilliumosides K–M (**1–3**), one new sesquiterpenoid glycoside, tritschsesuquiside A (**4**), along with three known analogues (**5–7**) were obtained from the rhizomes of *Trillium tschonoskii*. The structures of new glycosides were elucidated by spectroscopic analyses (HRMS and NMR) and chemical methods. Glycosides **5–7** displayed cytotoxicities against five human cancer cell lines with IC_{50} values ranging from 10.5 ± 1.0 to 1.0 ± 0.2 μM, with **7** being the most cytotoxic compound with IC_{50} values of 1.0 ± 0.2, 2.2 ± 1.2, and 3.4 ± 0.4 μM against Huh7, CCRF-CEM, and HeLa cell lines, respectively. The flow cytometric results revealed that both **5** and **6** could induce apoptosis of HCT116 and Huh7 cells.

1. Introduction

The genus *Trillium* (Melanthiaceae family), perennial herbaceous flowering plants, consists of approximately 40–50 species distributed in the regions of North America and Eastern Asia [1]. Previous phytochemical studies have revealed that plants of this genus are a rich resource of steroidal glycosides [2–9], some of which exhibited haemostatic [6], anti-fungal [6], cytotoxic [7,8], and anti-inflammatory [8,9] activities. The rhizomes of *T. tschonoskii* Maxim., known as "Toudingyikezhu" in Chines, has been historically used for the treatment of waist and leg pain and traumatic hemorrhage [10,11]. As part of our continuous search for bioactive steroidal glycosides of traditional medicinal plants [12–14], further chemical investigation of this plant led to the isolation of three new spirostanol glycosides, trilliumosides K–M (**1–3**), one new sesquiterpenoid glycoside, tritschsesuquiside A (**4**) (Fig. 1), along with three known glycosides, pennogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside (**5**) [15], pennogenin 3-*O*-*β*-chacotrioside (**6**) [15], and pennogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy1-(1 → 4)-[*α*-L-rhamnopyranosy1-(1 → 2)]-*β*-D-glucopyranoside (**7**) [15,16]. To systematically investigate the relationship between structurally different glycosides and their antitumor activity, the cytotoxic activities of all these isolates against Huh7, CCRF-CEM, HeLa, HCT116, and DU145 human cancer cell lines were evaluated *in vitro*. The apoptosis research of steroidal glycosides with potent cytotoxic properties was also performed. In this paper, we report the isolation, structural elucidation, and antitumor activities of these glycosides.

2. Results and discussion

The air-dried rhizomes of *T. tschonoskii* were extracted with 75% EtOH to a crude extract, which was successively partitioned with EtOAc and *n*-BuOH. The *n*-BuOH portion was fractionated by a series of chromatographic methods, to give glycosides **1–7**. Among them, compounds **1–3** were established as new spirostanol glycosides, and **4** was identified as a new sesquiterpenoid glycoside.

Trilliumoside K (**1**) was obtained as a white amorphous powder. Its molecular formula was determined as $C_{42}H_{62}O_{20}$ based on the HR-ESI-MS ion peak at *m/z* 909.3743 $[M+Na]^+$ (calcd. for $C_{42}H_{62}O_{20}Na$, 909.3727). The IR spectrum showed absorption bands at 3390, 1737, and 1515 cm^{-1} for the presence of hydroxyl, carbonyl, and olefinic functionalities, respectively. In the ^1H NMR spectrum (Table 1), one tertiary methyl signal at δ_H 1.25 (s, Me-19), two secondary methyl signals δ_H 1.04 (d, J = 6.4 Hz, Me-27) and 1.71 (d, J = 6.3 Hz, Me-6″), and an olefinic proton signal at δ_H 5.60 (brd, J = 5.4 Hz, H-6) were observed, as well as three anomeric protons at δ_H 4.60 (d, J = 7.5 Hz,

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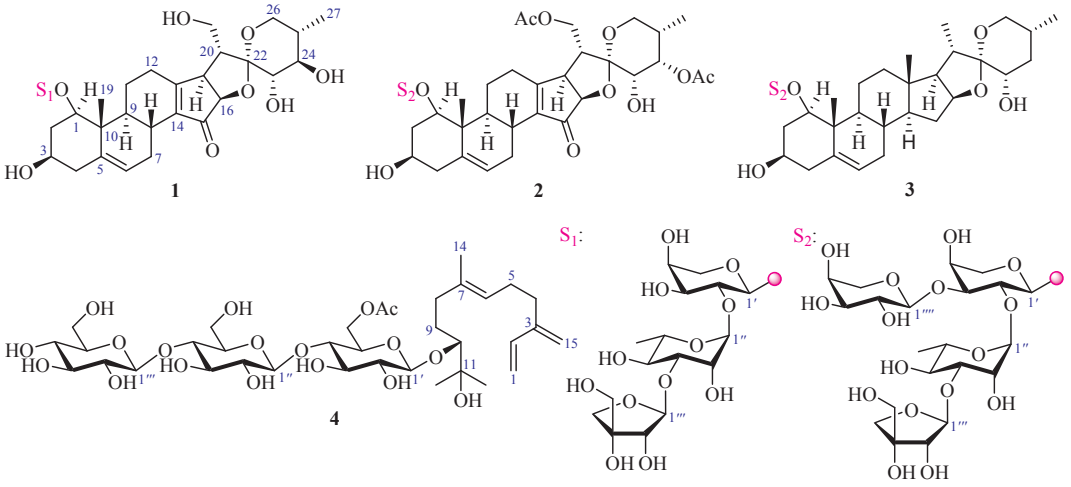

Fig. 1. Structures of glycosides **1–4** from *T. tschonoskii*.

H-1′), 6.21 (d, J = 2.3 Hz, H-1‴), and 6.34 (brs, H-1″). The ^{13}C NMR (Table 1) and HSQC spectra indicated the presence of 42 carbon resonances with 26 ones ascribed to the aglycone moiety, including two methyls, seven methylenes (two oxygenated), 11 methines (four oxygenated and an olefinic), four quaternary carbons (three olefinic), ketal carbon, and one carbonyl group, whereas the remaining 16 carbons could be assigned to those of two pentose and one hexose units.

Analysis of the 1D and 2D NMR spectra data (including ^{1}H–^{1}H COSY, HSQC, HMBC) allowed for the establishment of the aglycone structure of **1**. Its HSQC spectrum permitted the assignment of all the protons to their bonding carbons, respectively. The ^{1}H–^{1}H COSY spectrum disclosed that **1** had four partial structure units (Fig. 2). Further inspection of the HMBC spectrum enabled the connectivity of these spin coupling fragments and the other functional groups. As depicted in Fig. 2, the HMBC spectrum displayed correlations from: Me-19 to δ_C 84.4 (C-1), 139.5 (C-5), 47.9 (C-9), and 42.6 (C-10); δ_H 2.60 (m, H-4β) and 2.53 (dd, J = 11.8, 6.3 Hz, H-4α) to δ_C 125.0 (C-6); δ_H 2.22 (t, J = 11.3 Hz, H-8) to δ_C 138.9 (C-14); both δ_H 2.77 (brd, J = 18.6 Hz, H-12a) and 4.77 (d, J = 6.2 Hz, H-16) to δ_C 176.6 (C-13) and C-14; δ_H 3.17 (m, H-20), 4.31 (d, J = 9.6 Hz, H-23), and 3.74 (m, H-26α) to δ_C 114.7 (C-22). The aforementioned information revealed that the aglycone of **1** was a 18-norspirostanol featuring an α,β-unsaturated ketone moiety.

The relative configurations were unambiguously established by examination of the ROESY experiment (Fig. 3). The observed ROESY correlations of: H-1 with H-3/H-9α; H-9α with H-12α; and H-26α with H-16/H-24 indicated that these protons were co-facial and in an α-orientation. Likewise, the ROESY correlations of H-12β with H-20 and of H-25 with H-23/H-26β showed they were β-oriented. Based on the above evidence, the aglycone of **1** was thus determined to be trillenogenin [17].

Acid hydrolysis and further HPLC analysis of the L-cysteine methyl esters followed by conversion into *O*-tolyl isothiocyanate derivatives [18] yield L-arabinose, L-rhamnose, and D-apiose. The large coupling constant ($^{3}J_{1,2}$ = 7.5 Hz) of the anomeric proton for L-arabinopyranosyl suggested that it shared an α-orientation [19], whereas the α-orientation of the L-rhamnopyranosyl was indicated by the large coupling constant ($^{1}J_{C1,H1}$ = 170.8 Hz) of the L-rhamnose obtained by acid hydrolysis [20]. The β-orientation for the D-apiofuranosyl was determined by comparing its chemical shifts of δ_C 112.3 (C-1‴), 78.2 (C-2‴), 80.7 (C-3‴), 75.6 (t, C-4‴), and 66.1 (t, C-5‴) with those of corresponding carbons of α- and β-D-apiofuranosides [21,22]. The linkage site and the sequence of sugar moieties were determined by the HMBC correlations (Fig. 2) from: H-1′ to C-1, H-1″ to C-2′, and H-1‴ to C-3‴. Therefore, the structure of **1** was established as trillenogenin 1-*O*-β-D-apiofuranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside.

Trilliumoside L (**2**) was assigned a molecular formula of $C_{51}H_{74}O_{26}$ by its HR-ESI-MS ion peak at m/z 1125.4367 $[M+Na]^+$ (calcd. for $C_{51}H_{74}O_{26}Na$, 1125.4361). The ^{13}C NMR spectrum (Table 1) for the aglycone moiety of **2** exhibited the characterized signals for the presence of three carbonyl [δ_C 203.6 (C-15), 171.5 (Me*CO*-23), and 171.1 (Me*CO*-21)], four olefinic [δ_C 175.8 (C-13), 139.4 (C-14), 139.6 (C-5), and 124.9 (C-6)], one ketal [δc 111.8, (C-22)], and four methyl carbons [δc 12.3 (Me-27), 14.0 (Me-19), and 21.1 × 2 (*Me*CO-21/23)], suggesting that **2** was also a 18-norspirostanol derivative with two acetyl groups [23]. The observed HMBC correlations from δ_H 4.55 (dd, J = 11.1, 7.9 Hz, H-21) and 2.18 (s, *Me*CO-21) to Me*CO*-21 and from δ_H 5.76 (t, J = 2.9 Hz, H-24) and δ_H 1.87 (s, *Me*CO-24) to Me*CO*-24 enabled the establishment of the location for two acetyl groups at C-21 and C-24 (Fig. 2), respectively, and their α-orientation relative configurations were determined by the ROESY correlations of H-24 with δ_H 4.16 (brs, H-23)/2.02 (m, H-25β). Combined with four anomeric carbons [δ_C 101.1 (C-1′), 101.5 (C-1″), 111.8 (C-1‴), and 106.7 (C-1⁗)] observed in the ^{13}C NMR spectrum, the results of acid hydrolysis indicated that **2** had one more D-xylose than that of **1**. The coupling constant ($^{3}J_{1,2}$ > 7.0 Hz) of the anomeric proton for the additional D-xylopyranosyl suggested that it was β-oriented, and HMBC correlation from δ_H 4.98 (d, J = 7.6 Hz, H-1⁗) to δ_C 84.9 (C-3′) revealed that it was placed at OH-3′. Therefore, the structure of **2** was elucidated as 21,23-di-*O*-acetyl-epitrillenogenin 1-*O*-β-D-apiofuranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside.

Trilliumoside M (**3**) was found to have a molecular formula of $C_{43}H_{68}O_{19}$ by its HR-ESI-MS ion peak at m/z 1011.3753 $[M+Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$, 1011.3739). Apart from the same sugar chain comprising an α-L-arabinopyranosyl, a β-D-xylopyranosyl, an α-L-rhamnopyranosyl, and a β-D-apiofuranosyl units as revealed by the NMR data and acid hydrolysis, the ^{13}C NMR spectrum for the aglycone moiety of **3** showed characteristic signals ascribed to a double bond [δ_C 139.5 (C-5) and 124.9 (C-6)] and two oxygenated methines [δ_C 84.3 (C-1) and 68.3 (C-3)] for a ruscogenin derivative [24]. The ^{1}H–^{1}H COSY correlations between δ_H 2.10 (m, H-24α)/1.80 (m, H-24β) and 3.86 (m, H-23), along with the observed HMBC correlation from H-23 to δ_C 111.8 (C-22), verified that a hydroxyl group was located at C-23. The α-configuration of OH-23 was indicated by the ROESY correlation of H-25β with H-23. Therefore, the structure of **3** was determined as 23α-hydroxyruscogenin 1-*O*-β-D-apiofuranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside.

Tritschsesuquiside A (**4**) was shown to have a molecular formula of $C_{35}H_{58}O_{18}$ by its HR-ESI-MS ion peak at m/z 789.3527 $[M+Na]^+$ (calcd. for $C_{35}H_{58}O_{18}Na$, 789.3515). With the assistance of HSQC and

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data for **1–3** in pyridine-d_5.

Position	**1**		**2**		**3**	
	δ_C, type	δ_H (mult, *J* in Hz)	δ_C, type	δ_H (mult, *J* in Hz)	δ_C, type	δ_H (mult, *J* in Hz)
1	84.4, CH	3.75, dd (11.8, 3.8)	84.8, CH	3.73, dd (11.8, 3.8)	84.3, CH	3.73, dd (11.8, 3.4)
2	37.7, CH_2	*α* 2.67, m	37.9, CH_2	*α* 2.68, m	37.7, CH_2	*α* 2.75, m
		β 2.44, q-like (11.8)		*β* 2.49, q-like (11.8)		*β* 2.44, q-like (11.8)
3	68.3, CH	3.83, m	68.3, CH	3.85, m	68.3, CH	3.86, m
4	43.3, CH_2	*α* 2.53, dd (11.8, 6.3)	43.2, CH_2	*α* 2.54, dd (11.9, 5.0)	43.9, CH_2	*α* 2.56, dd (11.8, 3.9)
		β 2.60, m		*β* 2.60, m		*β* 2.68, t (11.8)
5	139.5, C		139.6, C		139.5, C	
6	125.0, CH	5.60, brd (5.4)	124.9, CH	5.61, brd (5.7)	124.9, CH	5.56, brd (5.5)
7	29.6, CH_2	*α* 1.66, m	29.5, CH_2	*α* 1.70, m	32.1, CH_2	*α* 1.84, m
		β 3.26, m		*β* 3.28, m		*β* 1.53, m
8	32.0, CH	2.22, t (11.3)	32.0, CH	2.12, t (11.3)	33.0, CH	2.05, m
9	47.9, CH	1.73, m	47.8, CH	1.72, m	50.4, CH	1.53, m
10	42.6, C		42.4, C		43.0, C	
11	25.3, CH_2	*α* 3.19, m	25.4, CH_2	*α* 3.22, m	24.1, CH_2	*α* 2.94, m
		β 0.98, m		*β* 0.94, m		*β* 1.58, m
12	28.3, CH_2	*α* 2.62 m	28.0, CH_2	2.62, 2H, m	40.8, CH_2	*α* 1.57, m
		β 2.77 brd (18.6)				*β* 1.33, td (12, 6, 3.3)
13	176.6, C		175.8, C		40.8, C	
14	138.9, C		139.4, C		56.9, CH	1.18, m
15	204.3, C		203.6, C		32.4, CH_2	*α* 2.06, m
						β 1.54, m
16	81.5, CH	4.77, d (6.2)	81.8, CH	4.77, d (6.2)	81.7, CH	4.64, m
17	49.0, CH	3.32, t (7.0)	49.0, CH	3.17, t (6.8)	62.6, CH	1.85, m
18					17.0, CH_3	1.04, s
19	14.0, CH_3	1.25, s	14.0, CH_3	1.24, s	15.1, CH_3	1.40, s
20	49.5, CH	3.17, m	49.0, CH	3.17, brt (6.8)		
21	61.7, CH_2	4.35, dd (11.1, 7.0)	64.3,	4.55, dd (11.1, 7.9)	14.8, CH_3	1.16, d (7.0)
		4.21, dd (11.1, 7.0)		4.51, dd (11.1, 7.9)		
22	114.7, C		111.8, C		111.8, C	
23	74.6, CH	4.31, d (9.6)	68.6, CH	4.16, brs	67.5, CH	3.86, m
24	75.2, CH	4.03, t (9.6)	72.9, CH	5.76, t (2.9)	38.8, CH_2	*α* 2.10, m
						β 1.80, m
25	39.1, CH	2.03, m	34.5, CH	2.02, m	31.8, CH	1.83, m
26	65.1, CH_2	*α* 3.74, m	61.6, CH_2	*α* 3.98, t (11.4)	66.0, CH_2	*α* 3.54, dd (10.8, 2.2)
		β 3.63, m		*β* 3.37, dd (11.4, 4.5)		*β* 3.48, t (10.8)
27	13.1, CH_3	1.04, d (6.4)	12.3, CH_3	0.77, d (6.8)	17.0, CH_3	0.71, d (5.9)
*Me*CO-21			171.1, C			
Me*CO*-21			21.1, CH_3	2.18, s		
*Me*CO-24			171.5, C			
Me*CO*-24			21.1, CH_3	1.87, s		
Ara-1′	100.8, CH	4.60, d (7.5)	101.0, CH	4.59, d (7.2)	100.8, CH	4.69, d (7.2)
2′	76.0, CH	4.15, m	73.4, CH	4.61, m	73.6, CH	4.66, m
3′	74.6, CH	4.32, t (8.4)	84.9, CH	4.05, dd (8.4, 4.1)	84.8, CH	4.10, dd (8.9, 3.0)
4′	70.4, CH	4.16, m	69.8, CH	4.42, m	69.7, CH	4.44, m
5′	67.7, CH_2	4.23, brd (12.5)	67.1, CH_2	4.20, brd (9.6)	67.1, CH_2	4.26, dd (12.1, 1.7)
		3.66, brd (12.2)		3.60, brd (12.2)		3.87, m
Rha-1″	101.7, CH	6.34, brs	101.5, CH	6.40, brs	101.5, CH	6.41, brs
2″	71.9, CH	4.94, brs	71.9, CH	4.97, brs	71.9, CH	4.97, brs
3″	80.3	4.67, t (4.2)	79.9, CH	4.66, dd (9.6, 2.7)	79.8, CH	4.70, m
4″	72.5, CH	4.44, t (9.4)	72.6, CH	4.41, m	72.6, CH	4.67, m
5″	69.6, CH	4.88, m	69.5, CH	4.87, m	69.6, CH	4.88, m
6″	19.1, CH_3	1.71, d (6.3)	19.1, CH_3	1.69, d (6.1)	19.1, CH_3	1.69, d (6.1)
Api-1‴	111.9, CH	6.21, d (2.3)	111.8, CH	6.25, d (2.3)	111.8, CH	6.26, d (2.3)
2‴	77.8, CH	4.84, d (2.3)	77.8, CH	4.86, d (2.3)	77.8, CH	4.86, d (2.3)
3‴	80.3, C		80.4, C		80.3, C	
4‴	75.2, CH_2	a 4.56, d (9.4)	75.2, CH_2	a 4.64, d (9.4)	75.2, CH_2	a 4.65, d (9.4)
		b 4.30, d (9.4)		b 4.29, d (9.4)		b 4.30, d (9.4)
5‴	65.7, CH_2	4.18, 2H, brs	65.7, CH_2	4.17, 2H, d (9.7)	65.7, CH_2	4.17, 2H, d (9.7)
Xyl-1⁗′			106.8, CH	4.98, d (7.6)	106.7, CH	5.01, d (7.6)
2⁗′			74.7, CH	3.88, brd (7.9)	74.7, CH	3.92, t (8.0)
3⁗′			78.6, CH	4.12, overlapped	78.5, CH	4.14, overlapped
4⁗′			71.1, CH	4.12, overlapped	71.1, CH	4.14, overlapped
5⁗′			67.2, CH_2	a 4.29, brd (11.6)	67.2, CH_2	a 4.30, brd (11.6)
				b 3.70, brd (11.6)		b 3.71, brd (11.6)

DEPT spectra, the ^{13}C NMR (Table 2) showed a total of 35 carbon signals, 15 of which were assigned to the aglycone moiety [25] including six olefinic [δ_C 113.6 (C-1), 116.4 (C-15), 124.9 (C-6), 135.9 (C-7), 139.5 (C-2), and 146.5 (C-3)], one oxygenated methine [δ_C 90.3 (C-10)], four methylene [δ_C 26.9 (C-5), 30.9 (C-9), 31.7 (C-4), and 36.3 (C-8)], and three quaternary methyl [δ_C 16.1 (Me-14), 25.5 (Me-12), and 26.7 (Me-13)] carbons, while the remaining signals were characteristic to three glucosyl moieties and an acetyl group [δ_C 20.9 (*Me*CO-6′) and 170.9 (Me*CO*-6′)]. Compared with (2,3-*S*-*trans*,10*R*,6*E*)-7,11-dimethyl-3-methylene-1,6-dodecadien-10,11-diol 10-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-glucopyranoside [26], the acetyl group was placed at C-6′ as suggested by the downfield chemical

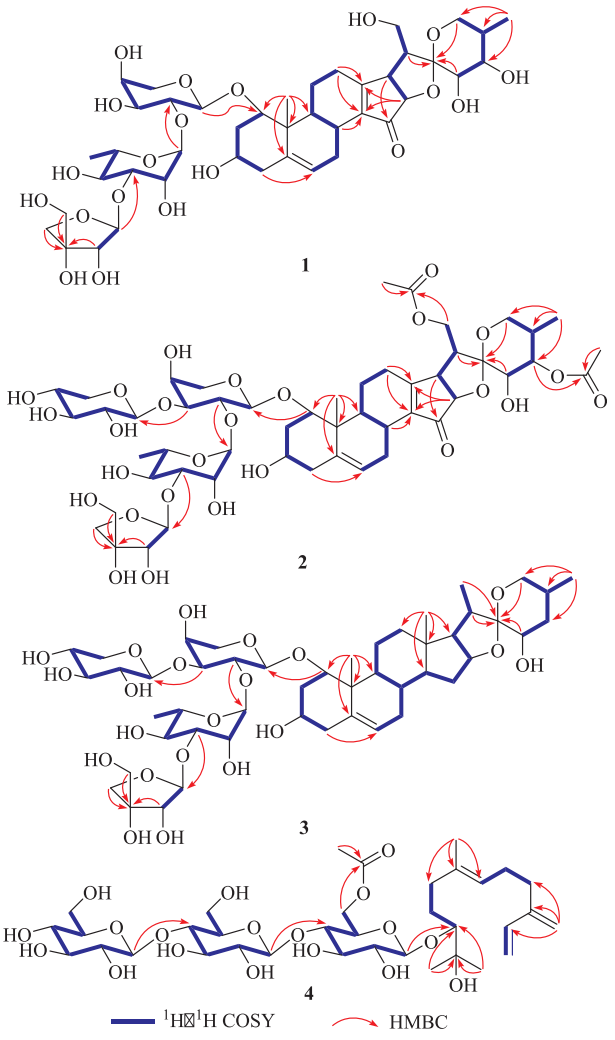

Fig. 2. Key ^{1}H-^{1}H COSY and HMBC correlations of **1**–**4**.

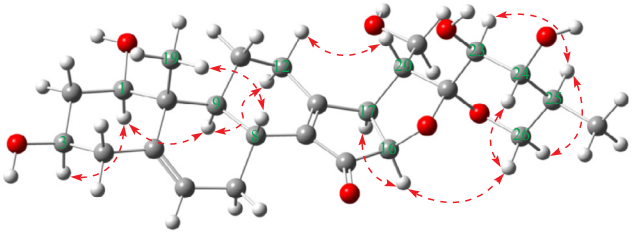

Fig. 3. Key ROESY correlations for the aglycone moiety of **1**.

shift at δ_C 64.2, and this was further supported by an examination of its ^{1}H–^{1}H COSY and HMBC experiments (Fig. 2). Therefore, the structure of **4** was established as (2,3-*S*-*trans*,10*R*,6*E*)-7,11-dimethyl-3-methylene-1,6-dodecadien-10,11-diol 10-*O*-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 4)-6-*O*-acetyl-*β*-D-glucopyranoside.

Glycosides **1**–**7** were evaluated for their cytotoxicities against the Huh7, CCRF-CEM, HeLa, HCT116, and DU145 cancer cell lines using the MTT method. Compared with the positive control VP-16, **5**–**7** exhibited significant cytotoxicities against various cancer cell lines with IC_{50} values ranging from 10.0 to 1.0 μM (Table 3), whereas others were inactive (IC_{50} > 20 μM). Among them, glycoside **7** was the most cytotoxic compound against Huh7, CCRF-CEM, and HeLa cell lines with IC_{50} values of 1.0 ± 0.2, 2.2 ± 1.2, and 3.4 ± 0.4 μM, respectively. Moreover, both pennogenin glycosides **5** and **6** were found to display potent antiproliferative effects against HCT116 and Huh7 cells by inducing apoptosis in a dose-dependent manner (Fig. 4).

3. Experimental

3.1. General experimental procedures

Optical rotations were obtained by a Jasco P-1020 polarimeter. UV spectra were taken on a Shimadzu UV2401 PC spectrophotometer. IR spectra were determined on a Bruker FT-IR Tensor-27 infrared spectrophotometer with KBr discs. HRESIMS were measured using an Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. NMR spectra were measured on Bruker Avance III-500 and Bruker Avance III-600 spectrometer. Sephadex LH-20 (GE Chemical Corporation), Si gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), and RP-18 (50 μm, Merck, Germany) were used for column chromatography (CC). Thin-layer chromatography was carried out with $HSGF_{254}$ (0.2 mm, Qingdao Marine Chemical Co., China) or RP-18 F_{254} (0.25 mm, Merck) plates. Semi-preparative HPLC was performed using an Agilent 1260 liquid chromatography system equipped with a ZORBAX SB-C18 column (5 μm, 9.4 × 250 mm).

3.2. Plant material

The rhizomes of *Trillium tschonoskii* identified by Dr. Yun-Heng Ji (Kunming Institute of Botany) were collected in October 2016 from Tacheng country, Weixi city of Shangri-La district, Yunnan Province, China. A voucher specimen was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3. Extraction and isolation

Air-dried and powdered rhizomes of *T. tschonoskii* (3.0 kg) were extracted with 75% EtOH, under conditions of reflux for a total of 9 h (3 × 3 h). The crude extract (510 g) was suspended in H_2O and then extracted with EtOAc and *n*-BuOH to yield EtOAc and *n*-BuOH moieties, respectively. The *n*-BuOH extract (180 g) was subjected to a silica gel column (200–300 mesh) with gradients of $CHCl_3$–MeOH–H_2O (9:1:0.2 → 0:1:0, v/v) to give eight fractions (Fr.1–Fr.8). Glycoside **5** (18.4 mg) was obtained via a recrystallization method from Fr.1 (50.0 mg). Fr. 2 (35.0 mg) was subjected to MPLC (MeOH–H_2O, 35:65 → 90:10) to give **6** (12.6 mg). Fr. 4 (13.5 g) was subjected to MPLC (MeOH–H_2O, 45:55 → 90:10) to give six subfractions (Fr. 4.1–Fr. 4.6). Fr. 4.2 (600 mg) was purified by semi-preparative HPLC with a mobile phase of CH_3CN–H_2O (40:60 → 70:30, v/v) to yield **2** (33.3 mg). **7** (28.0 mg) was obtained by a recrystallization method from Fr. 4.6 (44 mg). Fr. 6 (15.4 g) was subjected to MPLC (MeOH–H_2O, 40:60 → 90:10, v/v) to give eight subfractions (Fr. 6.1–Fr. 6.8). Fr. 6.8 (300 mg) was further purified by semi-preparative HPLC with a mobile phase of CH_3CN–H_2O (35:65 → 50:50, v/v) to yield **3** (5.5 mg). Likewise, Fr. 8 (46.7 g) was subjected to MPLC (MeOH–H_2O, 40:60 → 90:10, v/v) to give seven subfractions (Fr. 8.1–Fr. 8.7). Fr. 8.6 (200 mg) was purified by semi-preparative HPLC with a mobile phase of CH_3CN–H_2O (25:75 → 40:60, v/v) to give **1** (7.1 mg) and **2** (10.0 mg).

3.4. Physical and spectroscopic data of new glycosides

3.4.1. Trilliumoside K (1)

White, amorphous powder; $[\alpha]_D^{20}$ −106.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 247 (3.8), 230 (3.2) nm; IR (KBr) ν_{max} 3390, 3180, 3012, 2955, 1737, 1515, 1370 1202, 939, 876 cm^{-1}; ^{1}H (pyridine-d_5, 600 MHz) and ^{13}C (pyridine-d_5, 150 MHz) NMR data, see Table 1; HRESIMS *m/z* 909.3473 $[M+Na]^+$ (calcd for $C_{42}H_{62}O_{20}Na$, 909.3727).

3.4.2. Trilliumoside L (2)

White, amorphous powder; $[\alpha]_D^{20}$ −152.6 (*c* 0.10, MeOH); UV

Table 2
^{1}H (500 MHz) and ^{13}C (120 MHz) NMR spectral data for **4** in pyridine-d_5.

Position	δ_C, type	δ_H (mult, *J* in Hz)	Position	δ_C, type	δ_H (mult, *J* in Hz)
1	113.6, CH_2	5.31, d (17.6),	Glc-1′	105.6, CH	4.89 d (7.9)
		5.06, d (10.9)	2′	74.3, CH	4.00, overlapped
2	139.5, CH	6.47, dd (17.6, 10.9)	3′	76.5, CH	4.19, brt (8.8)
3	146.5, C		4′	81.5, CH	4.00, overlapped
4	31.7, CH_2	2.27, 2H, m	5′	73.3, CH	4.13, brt (9.1)
5	26.9, CH_2	2.26, 2H, m	6′	64.2, CH	5.13, overlapped
6	124.9, CH	5.45, t (6.4)			4.79 dd (11.8, 7.3)
7	135.9, C		Glc-1″	105.0, CH	5.15, d (7.8)
8	36.3, CH_2	2.81, ddd (14.2, 9.0, 4.6),	2″	74.7, CH	4.04, m
		2.47, m	3″	76.8, CH	4.24, m
9	30.9, CH_2	1.90, m	4″	81.2, CH	4.25, m
		1.77, ddd (14.2, 9.8, 9.8, 4.5)	5″	78.5, CH	4.03, m
10	90.3, CH	3.75, brd (9.8)	6″	62.0, CH_2	4.59, dd (11.6, 1.3)
11	71.9, C				4.45, dd (11.6, 4.9)
12	25.5, CH_3	1.42, s	Glc-1‴	104.7, CH	4.98, d (7.8)
13	26.7, CH_3	1.33, s	2‴	74.8, CH	4.06, t (8.5)
14	16.1, CH_3	1.63, s	3‴	78.3, CH	4.22, brd (8.8)
15	116.4, CH_2	5.11, s	4‴	71.6, CH	4.16, t (9.2)
		5.08, s	5‴	76.6, CH	4.01, m
*Me*CO-6′	20.9, CH_3	2.04, s	6‴	62.5, CH_2	4.55, dd (11.4, 1.3)
Me*CO*-6′	170.9, C				4.28, dd (11.4, 5.9)

Table 3
Cytotoxicities of glycosides **1–7** (μM).

Glycosides	IC_{50} ± SD				
	Huh7	CCRF-CEM	HeLa	HCT116	DU145
1	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
2	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
3	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
4	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
5	4.7 ± 0.3	8.6 ± 2.1	7.0 ± 0.4	9.6 ± 1.4	2.6 ± 0.4
6	1.7 ± 0.3	5.0 ± 0.6	3.9 ± 0.3	7.5 ± 0.9	2.5 ± 0.5
7	1.0 ± 0.2	2.2 ± 1.2	3.4 ± 0.4	10.5 ± 1.0	6.2 ± 2.7
VP-16	7.4 ± 1.3	5.8 ± 1.9	8.0 ± 1.6	28.2 ± 0.9	2.6 ± 1.1

(MeOH) λ_{max} (log ε) 248 (3.1), 223 (3.6) nm; IR (KBr) ν_{max} 3390, 3180, 3012, 2955, 1737, 1515, 1419, 1370, 1343, 1324, 1300, 1202, 939, 876 cm^{-1}; ^{1}H (pyridine-d_5, 600 MHz) and ^{13}C (pyridine-d_5, 150 MHz) NMR data, see Table 1; HRESIMS *m/z* 1125.4367 $[M+Na]^+$ (calcd for $C_{51}H_{74}O_{26}Na$, 1125.4361).

3.4.3. Trilliumoside M (3)

White, amorphous powder; $[\alpha]_D^{20}$ –81.8 (*c* 0.10, MeOH) ; IR (KBr) ν_{max} 3425, 2971, 2930, 2906, 1632, 1551, 1453, 1378, 1156, 1041, 985, 910, 875, 835 cm-^{1}; ^{1}H (pyridine-d_5, 600 MHz) and ^{13}C (pyridine-d_5, 150 MHz) NMR data, see Table 1; HRESIMS *m/z* 1011.4774 $[M+Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$, 1011.4771).

3.4.4. Tritschsesuquiside A (4)

White, amorphous powder; $[\alpha]_D^{20}$ −79.8 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 196.0 nm (4.1); IR (KBr) ν_{max} 3390, 2956, 1721, 1704, 1657, 1646, 1580, 1542, 1516, 1454, 1443, 1168, 949, 931, 886 cm^{-1}; ^{1}H (pyridine-d_5, 500 MHz) and ^{13}C (pyridine-d_5, 125 MHz) NMR data, see Table 2; HRESIMS *m/z* 789.3527 $[M+Na]^+$ (calcd. for $C_{35}H_{58}O_{18}Na$, 789.3515).

3.5. Acid hydrolysis of compounds

The absolute configurations of the sugar moieties were determined by the method described in the literature. Compounds **1–3** (2.0 mg, each) were refluxed with 6.0 M CF_3COOH (1,4-dioxane/H_2O 1:1, 2.0 mL) on a water bath for 2.0 h at 90 °C, the reaction mixture was then extracted with EtOAc (3 × 5.0 mL). The aqueous layer was evaporated to dryness using a rotary evaporator. The dried residue was dissolved in 1.0 mL pyridine mixed with L-cysteine methyl ester hydrochloride (1.5 mg) (Aldrich, Japan) and heated at 60 °C for 1.0 h. Then, *O*-tolyl isothiocyanate (5.0 μL) (Tokyo Chemical Industry Co., Ltd., Japan) was added to the mixture, which was heated at 60 °C for 1.0 h. Similarly, the standard monosaccharides, L-Ara (1.0 mg), L-Rha (1.0 mg), D-Api (1.0 mg), and D-Xyl (1.0 mg) were subjected to L-cysteine methyl ester hydrochloride (5.0 mg) with pyridine (1.0 mL), respectively. After heated at 60 °C for 1.0 h, *O*-tolyl isothiocyanate (20.0 μL) was added to each and kept at 60 °C for 1.0 h. Analytical HPLC was performed on a ZORBAX SB-C18 column (250 × 4.6 mm i.d., 5 μm, Aglient, U.S.A) at 35 °C with gradient elution of 20%→50% CH_3CN for 30 min at a flow rate of 1.0 mL/min. Peaks were detected by a UV detector at 254 nm. The sugar moieties of new glycosides were identified as L-Ara (t_R = 12.29 min), L-Rha (t_R = 16.36 min), D-Api (t_R = 16.18 min), and D-Xyl (t_R = 12.70 min), respectively, by comparison their retention time of detected peaks with those of the standard monosaccharide derivatives.

3.6. Cytotoxicity assay

The cytotoxicity of isolated glycosides was evaluated against five human tumor cell lines, Huh7 (hepatocarcinoma), CCRF-CEM (leukaemic), HeLa (cervical), HCT116 (colon), and DU145 (prostate), using the MTT method in 96-well plates [27]. The compounds were tested in a five-dose assay ranging from 10^{-8} to 10^{-4} M concentration. After incubation for 72 h at 37 °C, the 96-well plates were measured using a microplate reader (Bio-Tek) at 570 nm [28]. The IC_{50} value of each compound was calculated by nonlinear regression analysis (GraphPad Prism). The experiments were conducted for three independent replicates, and etoposide (VP-16) was used as a positive control.

3.7. Flow cytometry assay

HCT116 and Huh7 cells (3.0×10^5 cells/mL) were grown in culture medium on a 6-well plate treated with each compound (1.0, 3.0, and 9.0 μM) or untreated for 24 h. The cells were then harvested from the medium and washed with cold PBS, resuspended in 1 × binding buffer, and then stained with 5.0 μL FITC Annexin V and 10.0 μL propidium-miodide (KeyGen Biotech, China) for 15 min in the dark. The stained cells were analyzed by using flow cytometry (BD, FACSCalibur, USA) within 1.0 h [29]. The experiments were repeated independently for three times.

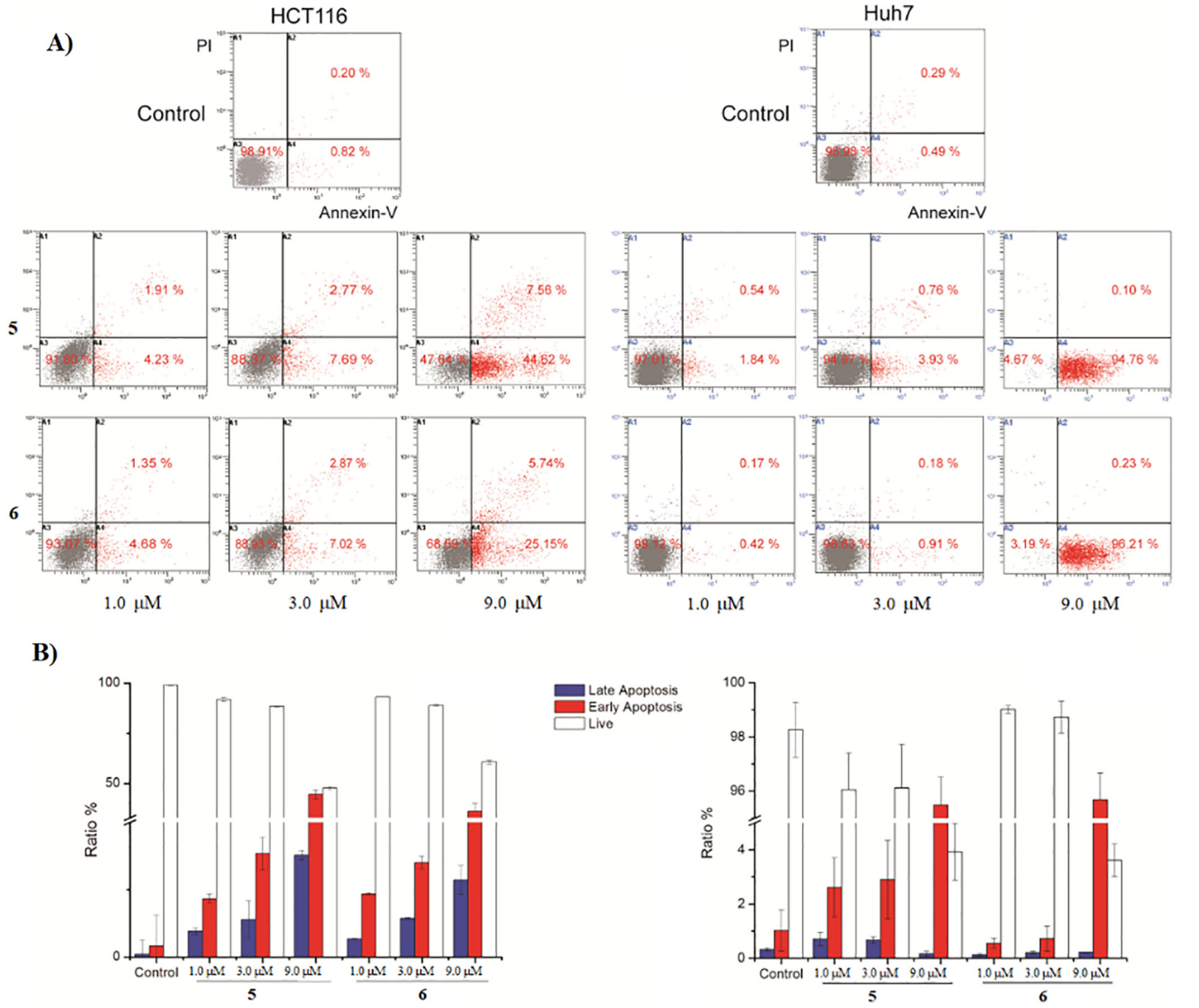

Fig. 4. A) Flow cytometry histograms and apoptosis of HCT116 and Huh7 cells treated with **5** and **6**; (B) The quantitation of the apoptosis of HCT116 and Huh7 cells treated with **5** and **6**.

4. Conclusions

Further phytochemical investigation of the *n*-BuOH portion partitioned from the 75% EtOH extract of the rhizomes of *T. tschonoskii* yielded three new spirostanol glycosides (**1**–**3**), one new sesquiterpenoid glycoside (**4**), and three known analogues (**5**–**7**). Glycosides **5**–**7** exhibited significant cytotoxicities against five human cancer cell lines, whereas **1**–**4** were inactive. Furthermore, both **5** and **6** could induce apoptosis of Huh7 and HCT116 cells. These data suggested that pennogenin-type glycosides from *T. tschonoskii* could be used as candidates for the further development of antitumor agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.108569.

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