An 18-Norspirostanol Saponin with Inhibitory Action against COX-2 Production from the Underground Part of *Trillium tschonoskii*

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A novel 18-norspirostanol saponin (1), along with Trillenoside A (2), was obtained from the underground parts of *Trillium tschonoskii* MAXIM., collected in Shennongjia Forest District, China. Based on the chemical and spectroscopic evidences, their structures were determined as shown in Fig. 1. 1 and 2 displayed marked inhibitory action towards COX-2 production in macrophagocytes of the mouse abdominal cavity stimulated by LPS at 10 μg/ml.

Key words *Trillium tschonoskii*; 18-norspirostanol saponin; COX-2; folk medicine; trillenoside A

The genus *Trillium* (Trilliaceae) contains about 48 related species in temperate eastern Asia and eastern North America, as well as western North America.[1)] The majority of *Trillium* species are associated with the ancient Arcto-Tertiary deciduous forests, which have persisted with dramatic changes in geographical ranges since the early Tertiary period in the northern hemisphere but especially in the North American continent during the Pleistocene Ice Age Today, each species of *Trillium* is restricted to one of three geographical areas: eastern Asia, western and eastern North America. *T. tschonoskii* MAXIM. grows in Shennongjia Forest District of central China, and its dried underground parts were used as a folk medicine to activate blood, to remove carbuncles, and to ameliorate pains, *etc*.[2)] This medicine was also used as an anit-inflammatory agent in the folk of Zhejiang and Shanxi districts of China.[3)] Although several novel steroidal saponins were isolated from its fresh aerial and underground parts,[4—7)] their bioactivities were little revealed before and most studies on this species of plants were carried out twenty years ago.[8—13)] In the course of our studies on the native folk medicines of Shennongjia Forest District,[14,15)] a new 18-nor steroidal saponin was obtained from the water soluble part of *Trillium tschonoskii* rhizomes. This paper deals with the structural elucidation of a novel saponin and a known compound[16)] isolated from this plant, and their inhibitory effect on COX-2 production was observed.

1 was obtained as a colorless powder from water-soluble part, $[\alpha]_D^{20}$ −45° (c=0.65, 15% acetonitrile). Its IR spectrum showed absorption maxima at 3431 cm^{-1}, 1690 cm^{-1}, 1649 cm^{-1}, due to a hydroxyl and an α,β-unsaturated ketone system, respectively. Its HR-FAB-MS showed a quasi-molecular ion peak at m/z 1057.4110 $[M+Na]^+$, which indicated a formula $C_{47}H_{70}O_{25}Na$ (Calcd: 1057.4104). Positive coloration reactions for Molish and Liebermann–Buchard tests indicated a steroidal saponin skeleton for **1**. The ^{1}H-NMR spectrum of **1** showed one singlet methyl signal at δ 1.23, one doublet methyl signal at δ 0.998 (d, J=6.5 Hz) and one olefinic signal at δ 6.00 (d, J=5.5 Hz), ascribable to a steroidal sapogenin moiety. The ^{13}C-NMR spectrum of **1** showed 27 carbon signals, including one carbonyl carbon signal at δ 204.50, four olefinic carbon signals at δ 177.46, 141.97, 137.39, 128.29, one acetal carbon signal at δ 114.55, two oxygenated methylene carbon signals at δ 64.98, 61.56, and six oxygenated methine carbon signals at δ 84.11, 81.49, 75.37, 74.61, 67.72, 61.88. In additon to signals attributable to a steroidal aglycone, four anomeric proton signals were observed in the ^{1}H-NMR spectrum of **1** at δ 6.39 (1H, s), 6.22 (1H, d, J=2.4 Hz), 4.97 (1H, d, J=7.6 Hz), 4.46 (1H, d, J=7.5 Hz), and four anomeric carbon signals in the ^{13}C-NMR spectrum of **1** at δ 101.38, 111.72, 106.62, 100.86. Upon acid hydrolysis of **1** with 2.0 M HCl, rhamnose, apiose, xylose and arabinose were detectd in the supernatant on the paper chromatography and TLC. The proton and carbon resonances due to sugar moieties (Table 1) are identical with those of corresponding sugar moieties in trillenoside A.[16)] In comparison of carbon signals due to aglycone of **1** with those of trillenoside A, downfield shifts of 3.3 ppm for C-6, 32.2 ppm for C-7 and 8.2 ppm for C-8 were observed in the ^{13}C-NMR spectrum of **1**, respcetively. The downfield shifts of 0.41 ppm for H-6, 2.15 ppm for H-7b and 0.28 ppm for H-8 were also observed in the ^{1}H-NMR spectrum of **1**, respectively. These showed that a hydroxy group was substituted at C-7 of aglycone in **1**, which was consistent with the HMBC results of **1**. The correlations of H-7 at δ 5.40 (1H, br s) and H-8 at δ 2.49 (1H, br d, J=4.9 Hz), H-7 at δ 5.40 (1H, br s) and H-6 at δ 6.00 (d, J=5.5 Hz) in the NOESY spcetrum of **1**, indicated a β-orientation of hydroxy group at C-7, which was agreeable with the couple constants between H-7 and H-8, as well as H-7 and H-6. Therefore, **1** was identified as 7-β-hydroxy trillenogenin 1-*O*-β-D-apiofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

The inhibitory actions of **1** and **2** against COX-2 production were observed in macrophagocytes of the mouse abdominal cavity stimulated by LPS at 10 μg/ml. The activities of 9.7×10^{-6} mol/l of **1** and 9.8×10^{-6} mol/l of **2** were approximately equivalent to that of 1.1×10^{-4} mol/l of aspirin (Table 2).

Experimental

Optical rotations were recorded with a Perkin-Elmer 241 spectropolarimeter. IR spectra were measured on a Nicolet FT360 instrument as samples in pressed KBr disks. 1D and 2D NMR spectra were recorded using Bruker

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Table 1. ^{13}C- and ^{1}H-NMR Data for Compound **1** (125 and 500 MHz in Pyridine-d_5)

Position	**1**		Position	**1**	
	δ_C	δ_H J (Hz)		δ_C	δ_H J (Hz)
1	84.11	3.77, dd (4.0, 11.7)	arap		
2	37.55	2.60, m; 2.46, m	1	100.86	4.46, d (7.5)
3	67.72	3.76, m	2	73.31	4.59, m
4	43.17	2.59, m	3	84.76	4.01, dd (3.3, 7.8)
5	141.97		4	69.71	4.38, m
6	128.29	6.00, d (5.5)	5	67.08	4.09, m; 3.52, m
7	61.88	5.40, br s	xyl		
8	40.18	2.49, br d (4.9)	1	106.62	4.97, d (7.6)
9	49.11	3.28, br t (7.3)	2	74.55	3.91, t (8.0)
10	42.92		3	78.39	4.13, m
11	25.27	3.23, m	4	70.97	4.13, m
12	28.04	2.77, br d (18.2); 2.68, m	5	67.08	3.70, d (11.5); 4.31, br s
13	177.46		rham		
14	137.39		1	101.38	6.39, s
15	204.5		2	71.75	4.94, m
16	81.49	4.73, d (6.3)	3	79.82	4.64, m
17	49.43	3.18, m	4	72.51	4.40, m
19	12.65	1.23, s	5	69.42	4.83, m
20	38.62	2.46, m	6	19.06	1.68, d (6.1)
21	61.56	4.31, m; 4.15, m	api		
22	114.55		1	111.72	6.22, d (2.4)
23	74.61	4.28, d (9.5)	2	77.72	4.82, d (2.4)
24	75.37	4.00, dd (9.2, 9.5)	3	80.18	
25	38.94	1.99, m	4	75.09	4.63, m; 4.27, m
26	64.98	3.68, dd (12.3,11.5); 3.55, d (12.3)	5	65.57	4.15, m; 4.18, m
27	13.30	0.998, d (6.5)			

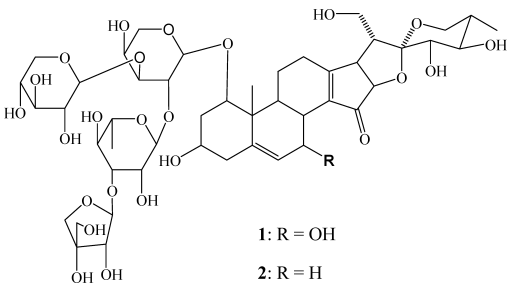

Fig. 1. The Strucutures of Compounds **1** and **2**

Table 2. The Effects of Compounds **1** and **2** on the COX-2 Production in Macrophagocytes from Abdominal Cavity in Mouse Induced by LPS (X±S)

Groups	Culture pores (n)	Dosage (μg/ml)	PGE_2 content (pg/ml)
Blank	8	—	417.18±42.28**
Model	8	—	675.35±51.39
Aspirin	8	20	473.16±62.83**
1	8	10	589.74±69.25**
1	8	20	521.57±77.91*
2	8	10	563.18±52.41**
2	8	20	480.94±64.22**

Compared with model group. * p<0.05, ** p<0.01.

AM 500 and instruments with Me_4Si as the intestinal standard. FAB and ESI mass spectra were obtained using a VG AUTO Spec-300 mass spectrometer and Finnigan-MAT LCQ DECA XP plus mass spectrometer, respectively. HPLC was performed using a Varian ProStar 1510 system for analytical (YMC-Pack ODS-AQ column: 5 μm, 60 Å, 150×3.9 mm i.d.) HPLC. Macroporous resins (AB-8, Nankai), Silica gel (10—40 μm, 200—300 mesh, Qingdao), Sephadex LH-20 (Pharmacia), RP C_{18} Silica Gel (100—200 mesh, YMC) were used as packing materials for column chromatography. Standard sugars and PGE_2 reagent kids were purchased from Sigma Co. Ltd.

Plant Material The rhizomes of *Trillium tschonoskii* were purchased at Muyu, a town of Shennongjia Forest District of China in May 2005 and identified by Professor Chen Faju. A voucher specimen (Herbarium No.: 2005ZW03128) has been deposited in the Herbarium of Department of Medicinal Plants, College of Chemistry and Life Science, China Three Gorges University, Yichang.

Extraction and Isolation Air-dried powdered rhizomes (6.4 kg) was extracted with methanol under reflux. After the removal of solvent *in vacuo* and freeze-drying, the methanol extract (2427 g) was obtained. The extract was suspended in water (2.2 l), and then extracted with $CHCl_3$, EtOAc and *n*-BuOH, successively. The rest solution was reduced *in vacuo* to a small volume (1.5 l), and then subjected to macroporous resin column chromatography with gradient elution (100% water→100% methanol). The 30% methanol eluate (9.3 g) was separated by repeated Rp-C_{18} silica gel column chromatography in elution with gradient solvent system (100% water→50% acetonitrile). The 13% and 15% acetonitrile eluates were further separated by repeated Sephadex LH-20 column chromatography in elution with water, giving rise to compound **1** (20.8 mg) and **2** (650 mg).

Bioassay 1.0 ml of 1% soluble starch for each mouse was injected into the abdominal cavity of C57BL/6 mice. Once the mice were decapitated after 3 d, 8.0 ml of Hanker's solution for each mouse was injected into their abdominal cavities to obtain the cell solution under aseptic condition. The cell solution was centrifugated for 8 min at 1000 rpm, and the prepititates were purged with Hanker's solution. The concentration of cells was adjusted to 10^6 cell/ml using DMEM culture medium which contains 10% calf serum. Then, the cells was inoculated to a culture plate with 96 pores (0.2 ml for each pore) for cultivation for 6 h under 37 °C and 5% CO_2. The suspended cells were washed away with Hanker's solution and the culture medium without calf serum was added. After 12 h, the culture solution was taken away and the LPS solutioin was added to the plates, making LPS concentration be 1.0 μg/ml. After they were incubated for 6 h, samples were added and cultivated for 1 h according to the groups list in Table 2. The arachidonic acid (AA) was added and its concentration was kept at 10 μmol/l in culture solutions. The solutions were cultivated for 30 min under 37 °C. The content of PGE_2 in supernatant was measured by using PGE_2 reagent kids.

Acid Hydrolysis Compound **1** (3.5 mg) was refluxed with 2.0 M HCl, respectively. The hydrolysate was neutralized with $NaHCO_3$, and then extracted with chloroform. The chloroform-insolvable part was detected on PC

and TLC according to the procedures.[17)]

Compound **1**: A colorless powder, $[\alpha]_D^{20}$ −45° (c=0.65, 15% acetonitrile). HR-FAB-MS (positive mode) m/z: 1057.4110 $[M+Na]^+$ (Calcd for $C_{47}H_{70}O_{25}Na$: 1057.4104). ESI-MS (positive ion mode) m/z: 1073 $[M+K]^+$, 1057 $[M+Na]^+$, (negative ion mode) m/z: 1033 $[M-H]^-$, 901 $[M-api-H]^-$, 769 $[M-api-xyl-H]^-$. IR (KBr) ν_{max}: 3430, 2922, 1690, 1649, 1050 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Table 1.

Compound **2**: A white powder, $[\alpha]_D^{20}$ −78° (c=2.5, 15% acetonitrile). ^{1}H- and ^{13}C-NMR data were well agreeable with those of trillenoside A.[16)]

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