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Steroidal saponins from *Smilax china* and their anti-inflammatory activities

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Abstract

Steroidal saponins, **1**, **2**, **3** and **4**, were isolated from the BuOH extract of *Smilax china* L., along with 13 known compounds, **5**–**17**. Their structures were elucidated on the basis of MS, 1D and 2D NMR spectroscopic analyses and chemical evidence. In the bioassay tests, all compounds showed inhibitory effects on cyclooxygenase-2 enzyme (COX-2) activities at final concentration of 10^{-5} M, and only compound **5** showed an inhibitory effect on production of TNFα (tumor necrosis factor α) in murine peritoneal macrophages at the same concentration.

Keywords: *Smilax china*; Liliaceae; Steroidal saponins; Smilaxchinoside A–D; Anti-inflammatory activities; Cyclooxygenase-2 enzyme; Tumor necrosis factor α

1. Introduction

Smilax (family Liliaceae; about 350 species) plants are distributed widely in tropical and temperate regions throughout the world, especially in East Asia and North America. Many of them have long been used as medicinal herbs. They are known to be rich in steroidal saponins (Bernardo et al., 1996; Ju and Jia, 1992, 1993, 1994; Jia and Ju, 1992; Woo et al., 1992; Kubo et al., 1992; Sautour et al., 2005). For example, the tuber of *Smilax china* L., known as "Ba Qia" (or "Jin Gang Teng") in Chinese, is used in traditional Chinese medicine (TCM) for treatment of diuretic, rheumatic arthritic, detoxication, lumbago, gout, tumor and inflammatory diseases (State Administration of Traditional Chinese Medicine of People's Republic of China, 1999). Recent pharmacological investigations showed that *S. china* has anti-inflammatory activity (Shu et al., 2006; Li and Zhou, 1996; Chen et al., 2000; Lu et al., 2003) and some steroidal saponins isolated from this plant exhibited significant cytotoxicity against several tumor cell lines (Hu and Yao, 2002; Hu et al., 1997). Previous phytochemical studies on this plant led to isolation of several saponins, including smilaxin, prosapogenin A of dioscin, gracillin, dioscin, pseudoprotodioscin, methylgracillin and methylprotodioscin (Sashida et al., 1992; Kim et al., 1989). However, a systematic phytochemical investigation of this plant has not been pursued, and the range of its bioactive compounds are unknown.

The present paper focusses on isolation and structural elucidation of saponins from the BuOH extract of *S. china*. In addition, their anti-inflammatory and cytotoxic activities are reported for the first time (see Fig. 1).

2. Results and discussion

The 95% EtOH and 50% EtOH extracts of *S. china* were suspended in water and extracted with petroleum ether, EtOAc and BuOH. The BuOH fraction was passed through a D_{101} macropore resin eluted successively with 30% EtOH, 50% EtOH, 70% EtOH and 95% EtOH, respectively. The

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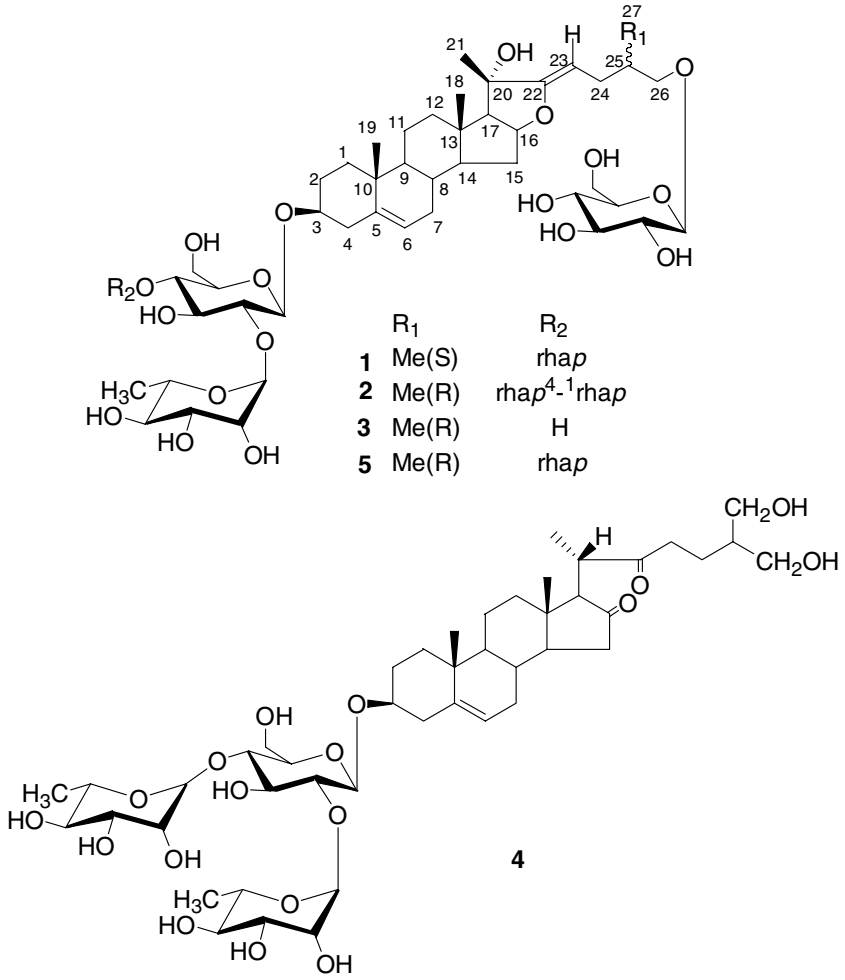

Fig. 1. The structures of compounds **1**–**5**.

concentrated 50% EtOH fraction was subjected to silica gel and Sephadex LH-20 column chromatography and finally purified by semi-preparative scale HPLC to afford four new compounds, **1**, **2**, **3** and **4**, along with 13 known compounds, **5**–**17**.

Compound **1** gave a pseudo-molecular ion peak $[M+Na]^+$ at *m/z* 1069.5184 (calculated for $C_{51}H_{82}O_{22}Na$, 1069.5195) in its high-resolution ESI-MS. Combined with the ^{13}C NMR spectroscopic data, its molecular formula was determined as $C_{51}H_{82}O_{22}$. Three tertiary methyl proton groups at δ 0.89, 1.04 and 1.70 (each *s*), a secondary methyl proton at δ 1.05 (1H, *d*, $J = 5.0$ Hz) and two trisubstituted olefinic protons at δ 5.29 (1H, *br s*, H-6) and δ 4.50 (1H, *m*, H-23), as well as protons attributable to an oxymethylene H-26 at δ 4.13 (1H, *dd*, $J = 9.5$, 6.0 Hz) and 3.50 (1 H, *dd*, $J = 9.5$, 7.5 Hz), observed in the ^1H NMR spectrum (Table 1). These data, when considered with the analysis of its ^{13}C NMR spectrum (three angular methyl groups at δ 13.5, 19.3 and 21.8, one secondary methyl group at δ 17.4, two trisubstituted double bonds at δ 140.8, 121.7 and δ 163.7, 91.3, and a methylene group linked to an oxygen atom at δ 75.2) (Table 2), indicated that the aglycone possessed a furost-5,22-diene skeleton. A comparison of the ^{13}C NMR spectroscopic signals of the aglycone moiety of **1** with the literature values (Chen et al., 2005), and an extensive gCOSY, HMQC and HMBC data analysis showed that the aglycone of **1** was 3β,20,26-trihydroxyfurost-5,22-diene. Its 25*S* configuration was deduced on the basis of differences in chemical shifts of the geminal protons at H_2-26 ($\delta a - \delta b = 0.63$ ppm), since the difference is usually >0.57 ppm for 25*S* compounds and <0.48 ppm for 25*R* compounds (Agrawal, 2004). The α-configuration of the C-20 hydroxyl group was also defined by the cross-peak between H-21 (δ 1.70) and H-18 (δ 0.89) in its NOESY spectrum. Hence, the aglycone of **1** was identified as (25*S*)-3β,20α,26-trihydroxy furost-5,22-diene.

Of the 51 carbon signals observed in the ^{13}C NMR spectrum of **1**, 27 were assigned to the aglycone part and the remaining 24 to the oligosaccharide moiety. The ^1H NMR and ^{13}C NMR spectra of **1** exhibited four sugar anomeric protons at δ 4.93 (1H, *d*, $J = 8.0$ Hz), 4.81 (1H, *d*, $J = 8.0$ Hz), 6.38 (1H, *brs*) and 5.83 (1H, *brs*) (Table 3), and carbon atoms at δ 100.2, 105.0, 102.0 and 102.8 (Table 2), respectively. Acid hydrolysis of **1** afforded D-glucose and L-rhamnose as revealed by HPLC analysis and comparison with authentic standards. The identity of the

Table 1
^{1}H NMR spectroscopic data for the aglycone moieties of compounds **1**–**4** (500 MHz, in pydine-d_5)[a]

H	1	2	3	4
H-1a	1.70 *o*	1.72 *o*	1.71 *o*	1.69 *o*
H-1b	0.95 *o*	0.96 *o*	0.95 *m*	0.97 *d* (3.5)
H-2a	2.05 *o*	2.05 *o*	2.10 *m*	2.07 *d* (8.0)
H-2b	1.83 *o*	1.83 *o*	1.87 *o*	1.84 *o*
H-3	3.85 *m*	3.86 *m*	3.93 *o*	3.87 *m*
H-4a	2.76 *br d* (10.5)	2.77 *br d* (10.0)	2.78 *br d* (10.0)	2.79 *dd* (13.0, 3.5)
H-4b	2.70 *br t* (12.0)	2.70 *br t* (12.0)	2.72 *br t* (12.0)	2.71 *o*
H-6	5.29 *br s*	5.29 *br s*	5.28 *br s*	5.28 *br s*
H-7a	1.83 *o*	1.83 *o*	1.84 *o*	1.71 *m*
H-7b	1.43 *m*	1.44 *m*	1.44 *m*	1.44 *o*
H-8	1.50 *t* (4.0)	1.49 *m*	1.52 *m*	1.41 *d* (4.0)
H-9	0.84 *m*	0.88 *m*	0.84 *m*	0.99 *o*
H-11	1.40 *m*	1.40 *m*	1.40 *m*	1.44 *o*
				1.36 *o*
H-12a	1.86 *o*	1.90 *m*	1.88 *o*	1.83 *o*
H-12b	1.17 *d* (7.0)	1.16 *m*	1.16 *d* (7.0)	1.31 *o*
H-14	0.95 *o*	0.96 *o*	0.97 *d* (6.5)	1.33 *m*
H-15a	2.05 *o*	2.06 *o*	2.06 *o*	2.03 *d* (7.5)
H-15b	1.54 *d* (5.0)	1.53 *d* (4.0)	1.50 *m*	1.69 *o*
H-16	5.19 *dd* (10.5, 7.0)	5.20 *dd* (10.0, 7.0)	5.19 *dd* (9.5, 6.5)	
H-17	2.21 *d* (6.0)	2.22 *d* (6.0)	2.22 *d* (6.0)	2.73 *o*
H-18	0.89 *s*	0.90 *s*	0.89 *s*	0.66 *s*
H-19	1.04 *s*	1.04 *s*	1.03 *s*	1.04 *s*
H-20				2.70 *o*
H-21	1.70 *s*	1.72 *s*	1.71 *s*	1.02 *d* (6.5)
H-23a	4.50 *o*	4.50 *o*	4.49 *o*	3.13 *m*
H-23b				3.01 *m*
H-24a	2.49 *m*	2.40 *m*	2.38 *m*	2.21 *o*
H-24b	2.13 *q* (7.0)	2.26 *q* (7.5)	2.25 *t* (7.5)	
H-25	2.07 *o*	2.05 *o*	2.03 *o*	2.20 *o*
H-26a	4.13 *dd* (9.5,6.0)	3.96 *dd* (9.5,7.5)	3.93 *dd* (9.5,7.5)	4.14 *br s*
H-26b	3.50 *dd* (9.5,7.5)	3.68 *dd* (9.5,6.0)	3.66 *dd* (9.5,6.0)	
H-27	1.05 *d* (5.0)	1.05 *d* (6.5)	1.04 *d* (5.5)	4.16 *br s*

The chemical shifts (δ values) are given in parts per million (ppm).
The coupling constants (J values) are reported in hertz (Hz).
[a] *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *br*, broad; *m*, multiplet; *o*, overlapped.

monosaccharides and the sequence of the oligosaccharide chains in **1** were determined by combined analyses of 1D TOCSY, gCOSY, HMQC, HMBC and NOESY experiments. The individual spin systems could be discerned from the spectra corresponding to either the anomeric protons or methyl groups (for the 6-deoxy sugar) in the 1D TOCSY analysis. A HMQC experiment then gave the corresponding carbon assignments, which were confirmed by a HMBC study. These analysis demonstrated the presence of two β-glucopyranosyl ($^3J_{H1,H2} = 8.0$ Hz) and two α-rhamnopyranosyl (chemical shifts of C-5 at δ 69.5 and 70.4) units, respectively. The downfield shift exhibited by C-3 and C-26 of the aglycone (δ 78.0 and 75.2) allowed the deduction that both carbon atoms were the glycosyl sites. The sequence of the trisaccharide chain at C-3, which was the same as the known compounds **6**, **7**, **10**–**12**, was established from the following HMBC correlations: H-1 (δ 4.93) of GlcI with C-3 (δ 78.0) of the aglycone, H-1 (δ 6.38) of RhaI with C-2 (δ 77.7) of GlcI and H-1 (δ 5.83) of RhaII with C-4 (δ 78.5) of GlcI. The presence of glucose connected to C-26 was proved by the correlation between H-1 (δ 4.81) of GlcII with C-26 (δ 75.2) of the aglycone in the HMBC experiment. The NOESY analyses also confirmed the above deductions. Therefore, structure **1** was identified as (25*S*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L- rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside, and named smilaxchinoside A.

Compound **2** displayed a $[M-H]^-$ ion at m/z 1191.5831 (calculated for $C_{57}H_{91}O_{26}$, 1191.5804) in the high-resolution FABMS, thus being 146 mass units higher than **1**. This led to the determination of its molecular formula as $C_{57}H_{92}O_{26}$. Comparison of the ^{1}H NMR spectra of the aglycone parts of both **2** and **1** revealed that all signals were the same except for the protons of H_2-26 (Table 1). Thus, the absolute configuration of C-25 was deduced to be R on the basis of the differences in chemical shifts of the geminal protons at H_2-26 ($\delta a - \delta b = 0.28$ ppm < 0.48 ppm) (Agrawal, 2004). The ^{13}C NMR spectroscopic data of the sugar parts of **2** (Table 2) showed the presence of an additional rhamnose (RhaIII) when compared to those of **1**. The downfield shift exhibited by C-4 (δ 80.4) of RhaII

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**4** (125 MHz, in Pydine-d_5)

Aglycon	1	2	3	4	3-*O*-sugar	1	2	3	4
C-1	37.4 *t*	37.5 *t*	37.5 *t*	37.2 *t*	GlcI-1	100.2 *d*	100.3 *d*	100.3 *d*	100.3 *d*
C-2	30.1 *t*	30.2 *t*	30.2 *t*	30.1 *t*	2	77.7 *d*	77.9 *d*	77.7 *d*	78.5 *d*
C-3	78.0 *d*	78.0 *d*	77.8 *d*	78.0 *d*	3	77.9 *d*	77.7 *d*	78.2 *d*	77.0 *d*
C-4	38.9 *t*	39.0 *t*	38.9 *t*	38.9 *t*	4	78.5 *d*	77.7 *d*	71.7 *d*	78.0 *d*
C-5	140.8 *s*	140.8 *s*	140.8 *s*	140.9 *s*	5	76.9 *d*	77.0 *d*	79.6 *d*	77.7 *d*
C-6	121.7 *d*	121.7 *d*	121.6 *d*	121.4 *d*	6	61.2 *t*	61.2 *t*	62.6 *t*	61.3 *t*
C-7	32.0 *t*	32.1 *t*	32.0 *t*	31.9 *t*					
C-8	31.1 *d*	31.1 *d*	31.1 *d*	30.9 *d*	RhaI-1	102.0 *d*	102.2 *d*	102.0 *d*	102.0 *d*
C-9	50.1 *d*	50.1 *d*	50.1 *d*	50.0 *d*	2	72.5 *d*	72.7 *d*	72.5 *d*	72.5 *d*
C-10	37.0 *s*	37.0 *s*	37.0 *s*	37.0 *s*	3	72.8 *d*	72.9 *d*	72.7 *d*	72.9 *d*
C-11	20.5 *t*	20.6 *t*	20.5 *t*	20.7 *t*	4	74.1 *d*	74.1 *d*	74.1 *d*	74.1 *d*
C-12	39.2 *t*	39.3 *t*	39.3 *t*	38.6 *t*	5	69.5 *d*	69.5 *d*	69.4 *d*	69.5 *d*
C-13	40.3 *s*	40.4 *s*	40.3 *s*	41.6 *s*	6	18.6 *q*	18.7 *q*	18.6 *q*	18.7 *q*
C-14	56.9 *d*	57.0 *d*	56.9 *d*	51.1 *d*					
C-15	33.5 *t*	33.5 *t*	33.5 *t*	37.4 *t*	RhaII-1	102.8 *d*	103.3 *d*		102.9 *d*
C-16	84.2 *d*	84.2 *d*	84.2 *d*	217.6 *s*	2	72.5 *d*	72.9 *d*		72.5 *d*
C-17	67.8 *d*	67.8 *d*	67.8 *d*	66.4 *d*	3	72.7 *d*	73.3 *d*		72.7 *d*
C-18	13.5 *q*	13.5 *q*	13.5 *q*	12.8 *q*	4	73.8 *d*	80.4 *d*		73.9 *d*
C-19	19.3 *q*	19.4 *q*	19.4 *q*	19.4 *q*	5	70.4 *d*	68.3 *d*		70.4 *d*
C-20	76.7 *s*	76.7 *s*	76.7 *s*	43.7 *d*	6	18.4 *q*	18.4 *q*		18.5 *q*
C-21	21.8 *q*	21.9 *q*	21.8 *q*	15.6 *q*					
C-22	163.7 *s*	163.7 *s*	163.6 *s*	213.4 *s*	RhaIII-1		102.2 *d*		
C-23	91.3 *d*	91.7 *d*	91.6 *d*	40.5 *t*	2		72.5 *d*		
C-24	29.6 *t*	29.9 *t*	29.8 *t*	22.9 *t*	3		72.9 *d*		
C-25	34.8 *d*	35.0 *d*	34.9 *d*	44.0 *d*	4		74.0 *d*		
C-26	75.2 *t*	75.2 *t*	75.2 *t*	63.7 *t*	5		70.4 *d*		
C-27	17.4 *q*	17.6 *q*	17.6 *q*	63.6 *t*	6		18.9 *q*		
					26-*O*-sugar				
					GlcII-1	105.0 *d*	104.9 *d*	104.8 *d*	
					2	75.2 *d*	75.2 *d*	75.2 *d*	
					3	78.5 *d*	78.6 *d*	78.5 *d*	
					4	71.6 *d*	71.7 *d*	71.6 *d*	
					5	78.4 *d*	78.5 *d*	78.4 *d*	
					6	62.7 *t*	62.8 *t*	62.7 *t*	

Multiplicities were assigned from DEPT spectra.

enabled deduction that this carbon atom is the glycosyl site. Additionally, the correlation between H-1 (δ 6.28) of RhaIII with C-4 (δ 80.4) of RhaII in the HMBC experiment further confirmed this tetrasaccharide, with the tetrasaccharide chain in compound **2** the same as that in compound **9**. Therefore, compound **2** was elucidated as (25*R*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside, and named smilaxchinoside B.

Compound **3** had a molecular formula $C_{45}H_{72}O_{18}$, as deduced from its pseudo-molecular ion peak $[M+Na]^+$ at *m*/*z* 923.4628 (calculated for $C_{45}H_{72}O_{18}Na$, 923.4616), 146 mass units less than **1**, in its high-resolution FABMS. Comparison of the ^1H NMR spectra of the aglycone parts of **3** and **2** (Table 1) indicated that they were the same. The ^{13}C NMR spectra of the sugar parts of **3** (Table 2) revealed all signals almost at the same position, except for the absence of a rhamnose moiety, when compared with those of **1**. On the basis of the above analysis, compound **3** was characterized as (25*R*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside, and named smilaxchinoside C.

Compound **4** had the molecular formula $C_{45}H_{72}O_{18}$, as determined from its molecular ion peak $[M-H]^-$ ion at *m*/*z* 899.4660 (calculated for $C_{45}H_{71}O_{18}$, 899.4645), in its high-resolution FABMS. The two tertiary methyl proton groups at δ 0.66, 1.04, a secondary methyl proton at δ 1.02 (1H, *d*, $J = 6.5$ Hz) and a trisubstituted olefinic proton at δ 5.28 (1H, *br s*) (Table 1) observed in the ^1H NMR spectrum coupled with information from the ^{13}C NMR spectrum (two angular methyl groups at δ 12.8, 19.4, a secondary methyl group at δ 15.6 and a trisubstituted double bond at δ 140.9, 121.4, as well as two carbonyl signals at δ 217.6 and 213.4) (Table 2) indicated that the aglycone possessed a cholest-5-ene-16, 22-dione skeleton. The α-configuration of the C-21 methyl group was defined by the cross-peaks between H-20 and H-18 in the NOESY spectrum. After a comparison of the ^{13}C NMR signals of the aglycone moiety of **4** with the literature (Honbu et al., 2002) and an extensive gCOSY, HMQC and HMBC data analysis, the aglycone of **4** was identified as 3β,26,27-trihydroxycholest-5-ene-16,22-dione.

Table 3
^{1}H NMR spectroscopic data for the sugar parts of compounds **1**–**4** (500 MHz, in Pydine-d_5)[a]

H	1	2	3	4
GlcI-1	4.93 *d* (8.0)	4.94 *d* (7.0)	4.96 *d* (7.5)	4.95 *d* (7.5)
2	4.20 *o*	4.22 *o*	4.26 *o*	4.24 *o*
3	4.20 *o*	4.22 *o*	4.23 *o*	4.24 *o*
4	4.36 *t* (9.5)	4.39 *t* (9.0)	4.10 *t* (9.0)	4.41 *t* (9.0)
5	3.62 *m*	3.59 *m*	3.87 *m*	3.66 *m*
6a	4.19 *o*	4.18 *o*	4.46 *br d* (12.0)	4.22 *o*
6b	4.08 *br d* (12.0)	4.02 *br d* (12.0)	4.31 *br d* (11.5)	4.09 *br d* (11.5)
RhaI-1	6.38 *br s*	6.40 *br s*	6.36 *br s*	6.42 *br s*
2	4.80 *br s*	4.89 *br s*	4.78 *br s*	4.84 *br s*
3	4.60 *dd* (9.0, 2.5)	4.64 *dd* (9.0, 2.5)	4.60 *dd* (10.0, 2.5)	4.64 *br d* (6.0)
4	4.33 *t* (9.0)	4.36 *t* (9.0)	4.32 *t* (9.5)	4.37 *t* (9.5)
5	4.93 *m*	4.94 *m*	4.96 *m*	4.97 *m*
6	1.74 *d* (5.5)	1.76 *d* (6.5)	1.75 *d* (6.0)	1.77 *d* (6.5)
RhaII-1	5.83 *br s*	5.84 *br s*		5.87 *br s*
2	4.66 *br s*	4.55 *o*		4.69 *br s*
3	4.50 *dd* (8.5, 2.5)	4.54 *o*		4.55 *d* (6.5)
4	4.30 *t* (8.5)	4.43 *t* (9.5)		4.36 *t* (9.0)
5	4.89 *m*	4.97 *m*		4.95 *m*
6	1.60 *d* (6.0)	1.58 *d* (6.0)		1.63 *d* (6.0)
RhaIII-1		6.28 *br s*		
2		4.90 *br s*		
3		4.54 *dd* (9.0, 2.5)		
4		4.29 *t* (9.0)		
5		4.35 *m*		
6		1.58 *d* (5.5)		
GlcII-1	4.81d (8.0)	4.83 *d* (8.0)	4.80 *d* (8.0)	
2	4.00 *t* (8.5)	4.01 *t* (9.0)	4.00 *t* (8.5)	
3	4.20 *o*	4.22 *o*	4.20 *o*	
4	4.21 *o*	4.22 *o*	4.20 *o*	
5	3.91 *m*	3.92 *m*	3.90 *m*	
6a	4.52 *dd* (11.5, 3.0)	4.52 *dd* (12.0, 3.5)	4.52 *dd* (11.5, 2.5)	
6b	4.36 *dd* (11.5, 4.5)	4.38 *dd* (12.0, 4.5)	4.36 *t* (11.5, 5.0)	

Multiplicities and the coupling constants were assigned from 1D TOCSY spectra.
The coupling constants (*J* values) are reported in hertz (Hz).
The chemical shifts (δ values) are given in parts per million (ppm).
[a] *s*, singlet; d, doublet; *t*, triplet; *q*, quartet; *br*, broad; *m*, multiplet; *o*, overlapped.

Of the 45 carbon signals observed in the ^{13}C NMR spectrum of **4**, 27 were assigned to the aglycone part and the remaining 18 to the oligosaccharide moiety. Acid hydrolysis of **4** gave D-glucose and L-rhamnose by HPLC analysis compared with the authentic standards. The spectroscopic data of **4** showed that the structure of the trisaccharide unit was the same as the trisaccharide unit of **1**. Thus, compound **4** was elucidated as 3β,26,27-trihydroxycholest-5-ene-16,22-dione 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside, and named smilaxchinoside D.

Additionally, 13 known compounds were isolated. On the basis of the NMR spectroscopic data and comparison with the literatures or authentic samples, their structures were determined to be (25*R*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside (**5**) (Chen et al., 2005), methylprotodioscin (**6**) (Aquino et al., 1986), dioscin (**7**) (Han et al., 1999), prosapogenin B of dioscin (**8**) (Han et al., 1999), (25*R*) spirostan-5-ene 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside (**9**) (Yu et al., 2000), protodioscin (**10**) (Hu et al., 1997), isonarthogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside (**11**) (Sashida et al., 1992), pseudoprotodioscin (**12**) (Sashida et al., 1992), diosgenin (**13**) (Agrawal et al., 1992), β-sitosterol (**14**), daucosterol (**15**), ethyl caffeate (**16**) (Cheng et al., 2002) and helonioside A (**17**) (Nakano et al., 1986), respectively.

The anti-inflammatory activities of **1**–**9** were evaluated by inhibitory effects on COX-2 activity and TNFα production induced by lipopolysaccharide (LPS) in murine peritoneal macrophages. The rates of inhibition by these nine compounds at a final concentration of 10^{-5} M of the production of PGE_2 induced by LPS in murine peritoneal macrophages are 76.1%, 78.5%, 75.9%, 82.0%, 59.1%, 82.5%, 81.5%, 76.5% and 81.7%, respectively. Indomethacin was used as the positive control. This primary screening results indicated that all compounds showed significant inhibition of COX-2. In addition, these compounds showed mild inhibition of the production of TNFα in murine peritoneal macrophages. The rates of inhibition by these nine compounds at final concentration of 10^{-5} M

of TNFα production stimulated with LPS in murine peritoneal macrophages are 42.3%, 34.0%, 48.0%, 46.0%, 53.2%, 25.9%, 43.7%, 40.7% and 18.0%, respectively. Resveratrol was used as the positive control. These results suggested that inhibition of COX-2 activity and TNFα production were probably the partial explanation of the anti-inflammatory activities of *Smilax china*.

The cytotoxic activities of compounds **1**–**5** were evaluated against leukemia HL-60 and human gastric cancer BGC cell lines, in which taxol was used as the reference substance. The results showed that none of the five compounds had cytotoxic activity against these two cell lines ($IC_{50} > 100$ μM). However, there are reports (Hu and Yao, 2002; Hu et al., 1997) that some furostanol saponins isolated from this plant, such as protodioscin, exhibited significant cytotoxicity against several cancer cell lines. This indicated that there might exist cell line selectivity for the Smilax saponins. Therefore, the wider cancer cell lines for steroidal saponins are necessary to thoroughly investigate the cytotoxicities of steroidal saponins.

3. Experimental

3.1. General

Optical rotations were measured with a Polatronic D polarimeter. IR spectra were recorded in KBr with an Avatar 360 FT-IR spectrophotometer. HRFAB-MS experiments were recorded on a Bruker APEX II FT-ICR mass spectrometer and HRESI-MS experiment were obtained on a JMS-T100CS mass spectrometer. ^{1}H, ^{13}C, DEPT, gCOSY, HMBC, HMQC, NOESY and 1D TOCSY NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in pyridine-d_5 at ambient temperature with TMS as the internal standard. For the further purification of samples, a Spectra SERIES HPLC system (Thermo Quest) equipped with a P100 pump and a UV100 detector was used. The column was Senshu Pak PEGASIL ODS packing (5 μm, 9.4 mm I.D × 250 mm; SSC Co., Japan). The flow rate was 2.0 ml/min, and the detection wavelength was 203 nm. HPLC analysis was carried out on an Agilent 1100 system equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. The column was Inertsil ODS-3 (5 μm, 4.6 mm I.D × 250 mm; GL sciences Inc.). Silica gel 60 (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Corporation, Qingdao, China. Sephadex LH-20 was from Pharmacia. D_{101} macropore resin was from Nankai University, Tianjin, China. All chemical solvents used for isolation were of analytical grade or higher.

3.2. Plant material

The tubers of *S. china* L. were collected from Hubei Province, PR China, in August 2002 and identified by Professor Dean Guo. A voucher specimen (NO. BMU020817A) has been deposited in the Division of Pharmacognosy Biotechnology, School of Pharmaceutical Sciences, Peking University.

3.3. Extraction and isolation

The air-dried and powdered tuber (9 kg) was extracted with 95% EtOH and with EtOH–H_2O (1:1) at room temperature. It was concentrated in vacuo and then extracted with petrol (60–90 °C fraction), EtOAc and *n*-BuOH. The *n*-BuOH fraction (500 g) was passed through D_{101} macropore resin and eluted with 30% EtOH, 50% EtOH, 70% EtOH and 95% EtOH in H_2O, successively. The concentrated EtOH–H_2O (1:1) fraction (75 g) was then subjected to chromatography on a silica gel column and eluted with a gradient of 8:2:0.2–6:4:0.2 (by vol.) $CHCl_3$/MeOH/H_2O. Four fractions were collected. Fractions 2, 3, and 4 were subjected to chromatography on a Sephadex LH-20 (MeOH) column to yield three subfractions. Each subfraction was finally purified by semi-preparative scale HPLC to yield compounds **1** (15 mg), **2** (10 mg), and **5** (50 mg) eluted with MeCN/H_2O (23:77, v/v), compound **3** (35 mg) eluted with MeCN/H_2O (25:75, v/v), compound **4** (5 mg) eluted with MeCN/H_2O (22:78, v/v) and compounds **6** (150 mg), **10** (10 mg), and **12** (10 mg) eluted with MeCN/H_2O (28:72, v/v). Compound **11** (20 mg) was obtained by recrystallization in $CHCl_3$/MeOH from fraction 1. Fraction 1 was passed through an ODS open column to yield compound **17** (2 mg) by elution with MeOH–H_2O (30:70). The concentrated EtOH–H_2O (7:3) fraction (9 g) was then applied to a silica gel column eluted with a gradient of 9:1:0.2–6:4:0.2 (by vol.) $CHCl_3$/MeOH/H_2O. Fractions 1, 2, and 3 were obtained. Compounds **7** and **9** were obtained by recrystallization in $CHCl_3$/MeOH from fraction 1, which was passed through a silica gel column to yield compound **16** (5 mg) by eluting with $CHCl_3$/MeOH/H_2O (10:1:0.1). Compound **8** was obtained by recrystallization in $CHCl_3$/MeOH from the EtOAc fraction. Compounds **13** (40 mg), **14** (400 mg), and **15** (300 mg) were obtained by recrystallization in petrol/EtoAc from the petrol (60–90 °C) fraction.

3.3.1. Smilaxchinoside A (*1*)

White amorphous powder; $[\alpha]_D^{26}$ – 60 (H_2O; c 0.10); IR ν_{max}(KBr) cm^{-1}: 3420, 2927, 1041. For ^{1}H NMR (500 MHz, pyridine-d_5), see Tables 1 and 2. For ^{13}C NMR (125 MHz, pyridine-d_5), see Table 3; HRESI–MS *m*/*z*: 1069. 5184 ([M+Na]$^+$), (calculated for $C_{51}H_{82}O_{22}$ Na, 1069.5195).

3.3.2. Smilaxchinoside B (*2*)

White amorphous powder; $[\alpha]_D^{26}$ – 73 (H_2O; c 0.10); IR ν_{max}(KBr) cm^{-1}: 3377, 2937, 1043. For ^{1}H NMR (500 MHz, pyridine-d_5), see Tables 1 and 2. For ^{13}C NMR (125 MHz, pyridine-d_5), see Table 3; HRFAB–MS *m*/*z*: 1191.5831 ([M−H]$^-$, calculated for $C_{57}H_{91}O_{26}$, 1191.5804).

*3.3.3. Smilaxchinoside C (**3**)*

White amorphous powder; $[\alpha]_D^{26}$ – 49 (H_2O; c 0.10); IR ν_{max}(KBr) cm^{-1}: 3402, 2929, 1045. For ^1H NMR (500 MHz, pyridine-d_5), see Tables 1 and 2. For ^{13}C NMR (125 MHz, pyridine-d_5), see Table 3; HRFAB–MS *m/z*: 923.4628 ($[M+Na]^+$, calculated for $C_{45}H_{72}O_{18}Na$, 923.4616).

*3.3.4. Smilaxchinoside D (**4**)*

White amorphous powder; $[\alpha]_D^{26}$ – 34 (H_2O; c 0.10); IR ν_{max}(KBr) cm^{-1}: 3413, 2926, 1736, 1710, 1042. For ^1H NMR (500 MHz, pyridine-d_5), see Tables 1 and 2. For ^{13}C NMR (125 MHz, pyridine-d_5), see Table 3; HRFAB–MS *m/z*: 899.4660 ($[M-H]^-$, calculated for $C_{45}H_{71}O_{18}$, 899.4645).

*3.4. Acid hydrolysis of compounds **1**, **2**, **3** and **4***

Compounds **1**, **2**, **3** and **4** (1.5 mg, each) were hydrolyzed with 2 M CF_3COOH (5 ml) at 100 °C for 9 h in a sealed tube. After cooling, the reaction mixture was extracted with $CHCl_3$ (3 × 5 ml). The aqueous layer was evaporated to dryness with MeOH until neutral, the residue was dissolved in 1 ml of water, to which L-(−)-α-methyl-benzylamine (5 mg) and $NaBH_3CN$ (8 mg) in EtOH (1 ml) were added. After being stirred at 40 °C for 4 h followed by addition of glacial HOAc (0.2 ml) and evaporated to dryness, the reaction mixture was acetylated with Ac_2O (0.3 ml) in pyridine (0.3 ml) for 24 h at room temperature. After evaporation, H_2O (1 ml) was added to the residue and the solution was passed through a Sep-Pak C_{18} cartridge washed with H_2O, H_2O/MeCN (4:1, 1:1, v/v each 5 ml), successively. The H_2O/MeCN (1:1) eluate was analyzed and the l-[(*S*)-*N*-acetyl-α-methyl benzylamino]-l-deoxyglucitol acetate derivatives were identified by HPLC analysis with the derivative of standard sugar prepared under the same conditions (Guo et al., 2004). The derivative of D-glucose with t_R of 23.43 min and the derivative of L-rhamnose were detected with t_R of 31.81 min.

HPLC conditions: Inertsil ODS-3, 4.6 mm × 25 0 mm; solvent, MeCN/H_2O (2:3, v/v); flow rate, 0.8 ml/min; detection, UV absorbance at 230 nm.

3.5. Assay of COX-2 activity

According to the method of Chen et al. (2004), peritoneal macrophages were harvested from male C57BL-6J mice (the Experimental Animal Center, Institute of Experimental Animal, Chinese Academy of Medical Sciences & Peking Union Medical College) 3 days after the injection (i.p.) of Brewer's thioglycollate medium, washed twice in D-Hanks' buffer and resuspended in RPMI-1640 (GIBCO/BRL, Gaithersburg, MD, USA). Compounds **1**–**9** were added to murine peritoneal macrophages at final concentrations of 10 μM. After incubation at 37 °C in 5% (v/v) CO_2 for 1 h, they were stimulated with LPS (Sigma Chemical Co.) at a final concentration of 1 μg/ml, followed by incubation at 37 °C in 5% CO_2 for an additional 12 h. The amount of prostaglandin E_2 (PGE_2) in the supernatant was measured by radioimmunoassay (RIA) (Hou et al., 2000) using the PGE_2RIA kit (PLA General Hospital, Beijing, China). Indomethacin ($IC_{50} = 7.1 \times 10^{-9}$ M) was used as the reference substance.

3.6. Assay of TNFα production

Compounds **1**–**9** at a final concentration of 10^{-5} M and LPS (0.5 μg/ml) were added to murine peritoneal macrophages (prepared by the method described in Section 3.5). The supernatant was collected and incubated at 37 °C in 5% CO_2 for 24 h for later use. L929 cell (5×10^4 cells/ml) was inoculated in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h. Then 100 μl of the supernatant prepared as described above or RPMI-1640 and 100 μl of actinomycin D (0.5 μg/ml) were added. After incubation at 37 °C in 5% CO_2 for 20 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/ml) was added (Mizutani et al., 1995; Carmicheal et al., 1987), followed by incubation for an additional 4 h. The cytotoxicity was assayed by measuring the absorbance at 570 nm and presented as the TNFα activity of murine peritoneal macrophages treated with test compounds. Resveratrol ($IC_{50} = 5.2 \times 10^{-5}$ M) was used as the reference substance.

3.7. Assay of cytotoxicity

Human gastric cancer BGC-823 cells and human leukemia HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and cultured in 96-well plates for the assay. Appropriate dilutions (1–10^3 μM) of the test compounds were added to the cultures. After incubation at 37 °C in 5% CO_2 for 72 h, the survival rates of the cancer cells were evaluated by the MTT method. The activity is shown as the IC_{50} value. Taxol was used as the reference substance.

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