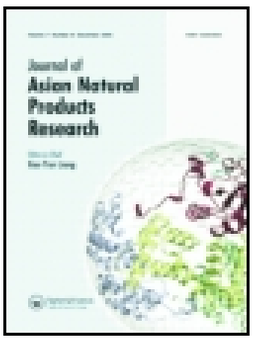

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Three new triterpenoid saponins from the stems of *Ilex asprella*

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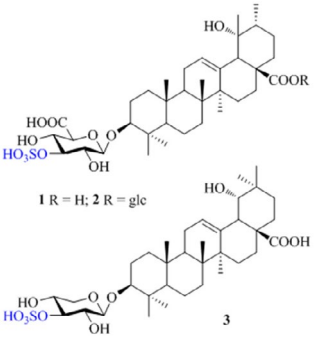
ABSTRACT

Three new triterpenoid saponins, namely asprellinoids A–C (**1**–**3**), featuring a sulfate substitution in sugar moiety, were isolated from the stems of *Ilex asprella* (Hook. et Arn.) Champ. ex Benth. Their structures were elucidated by the spectroscopic data analyses including HR-ESI-MS, IR, and NMR spectra, and chemical method.



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1. Introduction

The plant *Ilex asprella* (Hook. et Arn.) Champ. ex Benth. (Aquifoliaceae), is mainly distributed in Southern China. Its roots have been widely used as traditional Chinese medicine for the treatment of wind-heat cold, acute and chronic pharyngitis, and sore throat, and as a major material in the folk herbal drinking [1,2]. Previous phytochemical investigations on *I. asprella* revealed its main chemical constituents of triterpenoid saponins, especially

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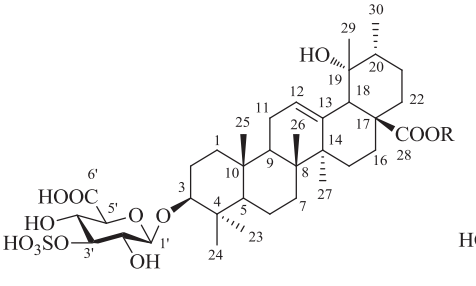
those sulfated [3]. As a part of research toward the pharmacological efficacy and quality evaluation, a chemical investigation on *I. asprella* stems was carried out. Herein, we describe the isolation and structural elucidation of three new sulfated triterpenoid saponins and anti-inflammation bioassays against NO production in RAW 264.7 and Bv-2 cells (Figure 1).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, with $[\alpha]_D^{25}$ +20 (c 0.1, MeOH). Its molecular formula $C_{36}H_{56}O_{13}S$, with nine degrees of unsaturation, was determined by a negative HR-ESI-MS ion at *m/z* 727.3369 $[M–H]^-$. The ^1H NMR data (Table 1) showed seven methyls at δ_H 0.79 (3H, s), 0.84 (3H, s), 0.93 (3H, d), 0.94 (3H, s), 1.07 (3H, s), 1.19 (3H, s) and 1.33 (3H, s), a typical axial H-3 at δ_H 3.22 (1H, dd, *J* = 4.0, 11.5 Hz), an anomeric sugar proton at δ_H 4,45 (1H, d, *J* = 7.5 Hz) and one olefinic proton at δ_H 5.28 (1H, br s). Its ^{13}C NMR data (Table 1) showed 30 resonances assignable to a triterpene skeleton and the remaining six carbon resonances from one hexosyl unit. Resonances of a double bond at δ_C 129.5 and 139.9, the typical signals of C-12 and C-13 of a Δ^{12}-ursane skeleton, and an anomeric carbon at δ_C 106.2 were observed also. In addition, two oxygenated carbons at δ_C 90.9 and 73.6 could be observed in the ^{13}C NMR spectrum. The ^1H and ^{13}C NMR data of **1** are similar to those of (3*β*)-19-hydroxy 28-oxours-12-en-3-yl *β*-D-glucopyranosiduronic acid n-butyl ester except for the sugar moiety [4]. According to the molecular weight of **1**, the S–O vibration (1233 cm^{-1}) in IR spectrum and the C-3′ data (δ_C 85.2), the 3′-OH in D-glucuronic acid was replaced by a sulfinic acid group [5]. HMBC correlations from H-1′ (δ_H 4.45, d, *J* = 7.5 Hz) to C-3 (δ_C 90.9) determined its glycosylation site at the 3-*O*-position, and the correlations from H-1′ (δ_H 4.45, d, *J* = 7.5 Hz) to C-3′ (δ_C 85.2) and from H-4′ (δ_H 3.59-3.64 m) to C-6′ (δ_C 176.3) showed the carbon connection of sugar moiety (Figure 2). Thus, the structure of compound **1** was determined and named as asprellinoid A.

Compound **2** was obtained as a yellow amorphous powder, with $[\alpha]_D^{25}$ +20 (c 0.1, MeOH). Its molecular formula $C_{42}H_{66}O_{18}S$, with 10 degrees of unsaturation, was determined by a negative HR-ESI-MS ion at *m/z* 889.3897 $[M–H]^-$. Its ^1H and ^{13}C NMR data (Table 1) are similar to **1,** except that **2** has six glycosylation signals more than **1**. HMBC correlations (Figure 2) from H-1′ (δ_H 4.49, d, *J* = 7.5 Hz) to C-3 (δ_C 91.1) and from H-1″ (δ_H 5.28-5.32 m) to C-28 (δ_C 178.6) established its glycosylation sites. The 28-*O*- glycoside moiety was supposed to be D-glucose by comparison with the ^{13}C NMR data of monepaloside F [6]. HPLC profile comparison with authentic sample, acidic hydrolysis of **2** gave D-glucose.



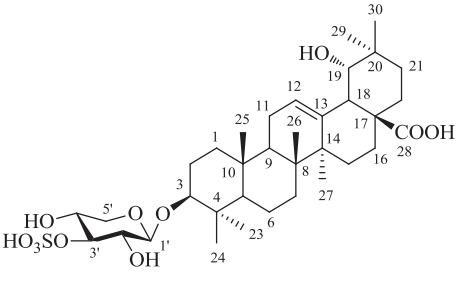


Figure 1. Chemical structures of compounds **1**–**3.**

Table 1. The ^{1}H NMR and ^{13}C NMR spectral data (500/125 MHz) of **1**–**2** (CD_3OD) and **3** (C_5D_5N, *J* in Hz).

	1		**2**		**3**	
No.	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	0.97–0.99, 1.61–1.63 m	39.8	0.97–1.00, 1.60–1.63 m	39.8	0.83–0.86, 1.43–1.46 m	38.7
2	1.53–1.55, 1.73–1.75 m	26.8	1.63–1.67, 1.90–1.92 m	26.8	1.75–1.80, 1.96–1.99 m	26.8
3	3.22 dd 4.0, 11.5	90.9	3.20 dd 3.5, 11.5	91.1	3.18 d 7.0	88.9
4	–	40.1	–	40.1	–	39.7
5	0.79–0.82 m	57.0	0.79–0.82 m	56.9	0.70–0.80 m	56.1
6	1.41–1.42, 1.64–1.66 m	19.4	1.32–1.35, 1.49–1.53 m	19.4	1.29–1.33, 1.47–1.51 m	18.9
7	1.28–1.29, 1.54–1.56 m	34.2	1.28–1.29, 1.53–1.56 m	34.1	1.31–1.36, 1.47–1.52 m	33.6
8	–	41.1	–	41.2	–	40.3
9	1.70–1.73 m	48.8[a]	1.63–1.66 m	49.0[a]	1.77–1.83 m	48.5
10	–	37.8	–	37.8	–	37.4
11	1.10–1.12, 2.00–2.02 m	24.7	1.71–1.73, 1.94–1.98 m	24.7	1.94–1.98, 2.00–2.06 m	24.4
12	5.28 br s	129.5	5.30 br s	129.7	5.56 s	123.7
13	–	139.9	–	139.5	–	145.1
14	–	42.5	–	42.6	–	42.5
15	0.98–1.02, 1.76–1.84 m	29.6	0.96–0.98, 1.87–1.92 m	29.6	1.26–1.30, 2.11–2.15 m	29.5
16	1.53–1.56 m, 2.57 dt 4.0, 7.0	26.6	1.86–1.88 m, 2.60 dt 4.0, 8.5	26.5	2.01–2.04 m, 2.84 br s	28.7
17	–	48.7[a]	–	48.7[a]	–	46.4
18	2.50 s	55.1	2.51 s	54.9	3.63 br s	45.2
19	–	73.6	–	73.6	3.63 br s	81.6
20	1.31 s	43.0	1.36–1.39 m	42.6	–	36.0
21	1.22–1.26, 1.73–1.76 m	27.1	1.21–1.97, 1.67–1.70 m	27.2	1.27–1.31, 2.14–2.19 m	29.5
22	1.63–1.65, 1.73–1.76 m	39.0	1.65–1.69, 1.72–1.76 m	38.2	2.01–2.07, 2.15–2.21 m	34.0
23	1.07 3H s	28.5	1.06 3H s	28.5	1.20 3H s	29.2
24	0.84 3H s	17.0	0.83 3H s	17.0	0.84 3H s	17.2
25	0.94 3H s	16.0	0.95 3H s	16.0	0.93 3H s	15.7
26	0.79 3H s	17.5	0.77 3H s	17.6	1.03 3H s	17.8
27	1.33 3H s	24.8	1.32 3H s	24.7	1.68 3H s	25.2
28	–	182.4	–	178.6	–	181.2
29	1.19 3H s	27.3	1.20 3H s	27.1	1.20 3H s	28.4
30	0.93 3H d 6.5	16.6	0.93 3H d 6.5	16.6	1.12 3H s	25.2
1′	4.45 d 7.5	106.2	4.49 d 7.5	106.2	4.78 d 5.5	106.0
2′	3.45 t 8.5	74.3	3.42–3.46 m	74.1	3.98 br s	74.6
3′	4.28 t 8.5	85.2	4.30 t 9.0	84.9	5.37 t 7.5	81.9
4′	3.59–3.64 m	72.3	3.63–3.68 m	72.1	5.08 br s	74.4
5′	3.64–3.67 m	76.9	3.72–3.77 m	76.6	3.84 t 10.0, 4.84 br s	64.4
6′	–	176.3	–	175.2		
1″			5.28–5.32 m	95.7		
2″			3.32–3.35 m	73.8		
3″			3.41–3.43 m	78.1		
4″			3.37–3.42 m	71.1		
5″			3.35–3.37 m	78.4		
6″			3.67–3.71, 3.77–3.83 m	62.4		

[a]Overlapped.

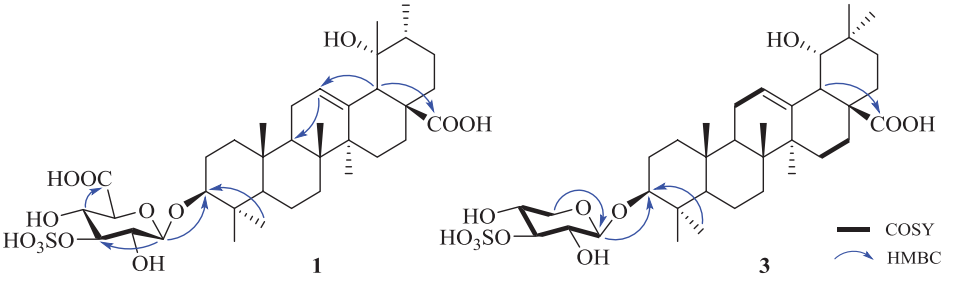


Figure 2. Key HMBC and COSY correlations of compounds **1** and **3.**

The characteristic absorption (1241.68 cm^{-1}) due to an S–O band stretching vibration in its IR spectrum, supporting the existence of a sulfinic acid group in **2** [5]. Thus, the structure of **2** was elucidated and named as asprellinoid B.

Compound **3** was obtained as a white amorphous powder, with $[\alpha]_D^{25}$ +60 (c 0.1, MeOH). Its molecular formula $C_{35}H_{56}O_{11}S$, with eight unsaturation, was determined by the negative HR-ESI-MS ion at *m/z* 683.3471 $[M-H]^-$. The ^1H NMR data (Table 1) of **3** showed signals due to seven methyls at δ_H 0.84 (3H, s), 0.93 (3H, s), 1.03 (3H, s), 1.12 (3H, s), 1.20 (6H, s) and 1.68 (3H, s), a typical signal at δ_H 3.18 (1H, d, J = 7.0 Hz) ascribable to an axial H-3, an anomeric sugar proton at δ_H 4.78 (1H, d, J = 5.5 Hz) and one olefinic proton at δ_H 5.56 (1H, s). The ^{13}C NMR data of **3** (Table 1) exhibit 30 resonances assignable to a triterpene skeleton and the remaining 5 carbon resonances from one pentose sugar unit. There are two C=C carbons at C-12 (δ_C 123.7) and C-13 (δ_C 145.1), and an anomeric carbon at C-1′ (δ_C 106.0) defined. The ^1H and ^{13}C NMR data of **3** are similar to those of a known 19-α-hydroxyoleanolic acid 3-*O*-β-D-glucuronopyranoside expect for the sugar part [7]. Acidic hydrolysis of **3** gave L-arabinose, which was confirmed by HPLC comparison with an authentic sample. According to the molecular weight and the S–O (1241.68 cm^{-1}) in IR spectrum, a hydroxy hydrogen in the L-arabinose was replaced by the sulfonic acid [8]. The connection of the carbons in sugar moiety was also confirmed through a COSY data between H-1′ (δ_H 4.78, d, J = 5.5 Hz) and H-2′ (δ_H 3.98), H-2′ (δ_H 3.98) and H-3′ (δ_H 5.37), H-3′ (δ_H 5.37) and H-4′ (δ_H 5.08), H-4′ (δ_H 5.08) and H-5′ (δ_H 3.84, 4.84) (Figure 2). The HMBC spectrum showed correlations from H-1′ (δ_H 4.78, d, J = 5.5 Hz) to C-3 (δ_C 88.9), determined its glycosylation sites. Thus compound **3** was elucidated, named as asprellinoid C.

Considering that a significant *in vivo* anti-inflammatory effect of *I. asprella* aqueous extract (publish elsewhere) that was pharmacologically evaluated using xylene-induced ear edema in mice and carrageenan-induced paw edema in rats, these isolated triterpenoid saponins were evaluated the nitrogen oxide (NO) production inhibition lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and Bv-2 cells *in vitro*, however, compounds **1**–**3** did not inhibit NO production in these cells at concentration of 40 μM. Related biological assay is needed.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a Rudolph Autopol IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, U.S.A.). IR spectra were measured on a Thermo Nicolet Nexus 470 FT-IR spectrophotometer using KBr pellets (Nicolet Company, Madison, U.S.A.). NMR spectra were obtained on a Varian-500 spectrometer (Varian Inc., Santa Clara, U.S.A.). HR-ESI-MS were recorded by a Shimadzu LC-MS-IT-TOF (Shimadzu, Tokyo, Japan). Column chromatography (CC) separations were performed using macroporous resins (Mitsubishi Chemical Corp., Tokyo, Japan), silica gel (Qingdao Haiyang Chem. Co. Ltd., Qingdao, China), and Sephadex LH-20 (GE Healthcare, Sweden). Thin layer chromatography (TLC) was conducted on silica gel GF254 (Qingdao Haiyang Chem. Co. Ltd., China) and RP C_{18} plates (Merck, Darmstadt, Germany). Analytical HPLC was performed on a LC-20A chromatography system composed of a diode array detector (DAD) (Shimadzu, Tokyo, Japan), Agilent XDB C_{18} column (5 μm, 4.6 × 250 mm). Thin-layer

chromatography (TLC) was performed on pre-coated silica gel GF254 (Qingdao Haiyang Chem. Co. Ltd, China).

3.2. Plant material

The stems of *Ilex asprella* were collected from Meizhou City Grass Lake Planting Base, Guangdong Province, China, in Sep 2016. The plant was identified by Prof. Zheng-Zhou Han (SANJIU Medical & Pharmaceutical Co.). A voucher specimen (2016GM901) is deposited at the Modern Research Center for TCM, Beijing University of Chinese Medicine, China.

3.3. Extraction and isolation

The dried and powdered stems (19.0 kg) were extracted with water (2 × 190 L) for 2 h. After removal of water under reduced pressure, the aqueous extract (4.0 kg) was subjected to macroporous resin column chromatography (CC) with a gradient of aqueous ethanol (EtOH-H_2O). Fraction from 95% EtOH (1.5 kg) was chromatographed on silica gel, eluting with a gradient of CH_2Cl_2-MeOH (10:1→0:1) to yield 12 fractions (Fr. I–Fr. XII). Fr. VIII (27.1 g) was chromatographed on silica gel, eluting with a gradient of EtOAc-MeOH-H_2O-Formic acid (60:9:3.5:0.7–0:1:0:0) to yield nine subfractions (Frs. $VIII_1$–$VIII_9$), then Fr. $VIII_3$ (2.1 g) was applied to Sephadex LH-20 CC (MeOH) to yield compound **1** (208 mg), and Fr. $VIII_5$ (3.2 g) was applied to Sephadex LH-20 CC (MeOH) to yield compound **2** (317 mg). Fr. VII (34.0 g) was subjected to silica gel CC, using a gradient of EtOAc-MeOH-Water-Formic acid (90:10:0.8:0.1) to yield 10 fractions (Frs. VII_1–VII_{10}) and from Fr. VII8 (4.3 g) compound **3** (465 mg) was obtained by precipitation in MeOH.

3.3.1. Asprellinoid A (1)

White amorphous powder, $[\alpha]_D^{25}$ +20 (c 0.1, MeOH). IR (KBr) ν_{max}: 3444, 2936, 2878, 1694, 1619, 1449, 1414, 1389, 1233, 1157, 1069, 1021, 998 cm^{-1}. ^1H and ^{13}C NMR spectral data: see Table 1. HR-ESI-MS :*m/z* 727.3369 $[M–H]^-$ (calcd for $C_{36}H_{55}O_{13}S$, 727.3371).

3.3.2. Asprellinoid B (2)

Yellow amorphous powder, $[\alpha]_D^{25}$ +20 (c 0.1, MeOH). IR (KBr) ν_{max}: 3436, 2934, 1731, 1620, 1455, 1417, 1389, 1259, 1228, 1163, 1073, 1025, 997 cm^{-1}. ^1H and ^{13}C NMR spectral data: see Table 1. HR-ESI-MS: *m/z* 889.3897 $[M–H]^-$ (calcd for $C_{42}H_{65}O_{18}S$, 889.3910).

3.3.3. Asprellinoid C (3)

White amorphous powder, $[\alpha]_D^{25}$ +60 (c 0.1, MeOH). IR (KBr) ν_{max}: 3456, 2943, 2877, 1696, 1636, 1459, 1389, 1241, 1173, 1051, 1019, 914 cm^{-1}. ^1H and ^{13}C NMR spectral data: see Table 1. HR-ESI-MS: *m/z* 683.3471 $[M–H]^-$ (calcd for $C_{35}H_{55}O_{11}S$, 683.3472).

3.4. Acid hydrolysis

Compounds **1**–**3** were disposed with HCl (2 mol/L) and refluxed at 80 °C for 4 h, respectively. The reaction mixture was diluted with water and extracted exhaustively with $CHCl_3$. After drying under reduced pressure, the residue and authentic samples were dissolved in anhydrous pyridine and then mixed with a pyridine solution of L-cysteine methyl ester

hydrochloride reaction for 1 h at 60 °C. Then o-tolyl isothiocyanate was added, and the mixture was warmed at 60 °C for another 1 h [9–13].

3.5. NO production inhibitory assays

The procedures used for *in vitro* anti-inflammatory activity against NO production in BV-2 and RAW 264.7 were same as those previously reported [14].

Disclosure statement

No potential conflict of interest was reported by the authors.

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