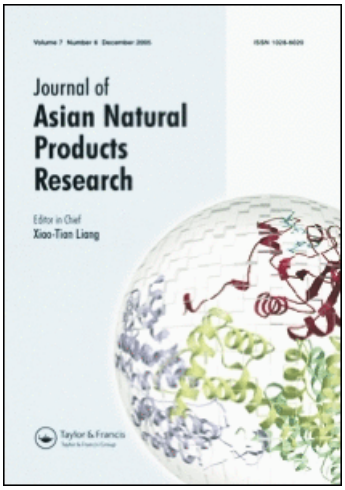

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THREE NEW SAPONINS FROM THE LEAVES OF *ILEX HYLONOMA*

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Three new triterpenoid saponins, hylonosides III–V (**1**–**3**) have been isolated, along with three known oleanolic acid saponins (**4**–**6**), from the methanol extract of leaves of *Ilex hylonoma*. The structures were elucidated using a combination of homo- and hetero-nuclear 2D NMR techniques (COSY, TOCSY, NOESY, HMQC and HMBC) and negative FAB-MS. The new compounds were characterized as 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucuronopyranosyl siaresinolic acid-28-*O*-β-D-glucopyranosyl ester (**1**), 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl siaresinolic acid-28-*O*-β-D-glucopyranosyl ester (**2**), 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl oleanolic acid-28-*O*-β-D-glucopyranosyl ester (**3**).

Keywords: *Ilex hylonoma*; Aquifoliaceae; Triterpenoid saponin

INTRODUCTION

In a continuation of our study on saponin constituents of medicinal plants of the Aquifoliaceae family [1–4], we have examined the saponin fraction of *Ilex hylonoma*. This plant is a well-known endemic herb in the Guangxi province of China as a substitute for the drug Die-Da-Wang and is utilized to treat bruises, wounds and rheumatism [5]. No previous phytochemical investigation has been reported on *I. hylonoma*. In this paper, we describe the isolation and structural elucidation of three new triterpenoid saponins named as hylonosides III–V (**1**–**3**) along with three known oleanolic acid saponins (**4**–**6**), previously isolated as compound **10**, **12** from *Ilex godajam* [3] and ladyginoside A from *Ladyginia bucharica* [8].

RESULTS AND DISCUSSION

The negative FAB-MS of **1** showed a quasi-molecular ion peak at *m/z* 971$[M-H]^-$, indicating a molecular weight of 972, compatible with the molecular formula $C_{48}H_{76}O_{20}$. Other significant ion peaks visible at *m/z* 809 $[(M-H)-162]^-$ and

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647 $[(M - H) - 162 - 162]^-$ corresponded to the loss of one hexose unit and two hexose units. The ^{13}C NMR spectrum of **1** showed an aglycone identified as siaresinolic acid by comparison with published data [1]. Acid hydrolysis of **1** with 5% H_2SO_4 afforded a mixture of sugars, which were identified as glucose and glucuronic acid. Alkaline hydrolysis of **1** performed with 3% KOH yielded a prosapogenin, which furnished glucuronic acid and glucose by acid hydrolysis. These chemical reactive results indicated that **1** must be a triterpene-bidesmosidic saponin in which glucuronic acid and glucose were bound to the aglycone by a glycosidic linkage at C-3, while the remaining sugars must be bound to the genin by a glycosidic ester linkage at C-28, which results were also confirmed by the signals observed in the ^{13}C NMR spectrum of **1** at δ_C 89.1 (downfield shift of C-3 of the aglycone) and δ_C 177.1 (upfield shift of C-28 of the aglycone).

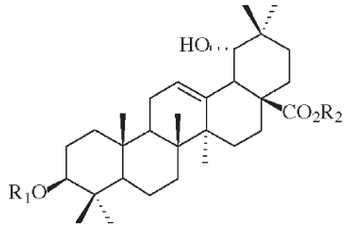
In the ^1H and ^{13}C NMR spectrum, **1** was shown to contain three sugar residues. In the HMQC, the anomeric proton signals at δ 6.24, 5.53 and 4.96 gave correlations with carbon signals at δ 95.9, 104.7 and 106.4, respectively. Assignments of all sugar proton signals were achieved by considering TOCSY and $^1H-^1H$ COSY spectra, while the carbon signals were assigned from HMQC and HMBC spectra (see Table I). Evaluation of spin–spin couplings and chemical shifts allowed the identification of one β-glucuronopyranosyl (GluA) and two β-glucopyranosyl (Glc) units. The correlations of the HMBC observed between the signals at δ_H 5.53 (d, $J = 7.0$ Hz) (Glc-1) and δc 84.2 (GluA-4), and the signals at δ_H 4.96 (d, $J = 7.2$ Hz) (GluA-1) and δc 89.1 (Agly-3), and the correlations in the NOESY experiment between signals at δ_H 5.53 (d, $J = 7.0$ Hz) (Glc-1) and δ_H 4.06 (GluA-4), and the signals at δ_H 4.96 (d, $J = 7.2$ Hz) (GluA-1) and 3.53 (Agly-3), showed that the bisaccharide

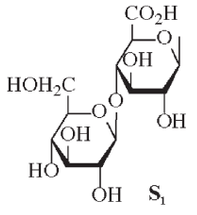
TABLE I ^{13}C NMR spectral data for compounds **1**–**3** (pyridine-d_5)

No.	***1***	***2***	***3***	*C-3*	***1***	***2***	***3***
1	39.0 CH_2	39.1 CH_2	39.2 CH_2	GluA-1	106.1 CH	105.3 CH	105.1 CH
2	26.7 CH_2	26.6 CH_2	28.9 CH_2	2	74.2 CH	81.1 CH	80.9 CH
3	88.3 CH	88.2 CH	88.4 CH	3	76.4 CH	78.4 CH	78.3 CH
4	39.7 C	39.7 C	39.7 C	4	84.0 CH	72.9 CH	72.8 CH
5	56.2 CH	56.1 CH	56.3 CH	5	75.3 CH	76.8 CH	76.6 CH
6	18.8 CH_2	18.8 CH_2	18.7 CH_2	6	175.1 C	174.7 C	174.4 C
7	33.1 CH_2	33.1 CH_2	32.4 CH_2	Glc-1′	104.4 CH	105.0 CH	104.8 CH
8	40.3 C	40.2 C	40.1 C	2′	75.9 CH	75.5 CH	75.4 CH
9	48.4 CH	48.4 CH	48.3 CH	3′	78.8 CH	77.9 CH	77.8 CH
10	37.3 C	37.2 C	37.2 C	4′	71.5 CH	83.4 CH	83.2 CH
11	24.2 CH_2	24.3 CH_2	23.9 CH_2	5′	79.0 CH	78.2 CH	78.0 CH
12	123.6 CH	123.6 CH	122.8 CH	6′	62.7 CH_2	62.5 CH_2	62.2 CH_2
13	144.4 C	144.5 C	144.4 C	Glc-1″		104.9 CH	104.8 CH
14	42.2 C	42.2 C	42.4 C	2″		76.5 CH	76.1 CH
15	29.2 CH_2	29.2 CH_2	28.3 CH_2	3″		79.2 CH	78.9 CH
16	28.1 CH_2	28.1 CH_2	23.6 CH_2	4″		71.8 CH	71.7 CH
17	46.6 C	46.7 C	47.3 C	5″		79.1 CH	79.0 CH
18	44.7 CH	44.6 CH	42.2 CH	6″		62.7 CH_2	62.7 CH_2
19	81.1 CH	81.0 CH	46.5 CH_2	Glc-1‴	95.6 CH	95.9 CH	95.7 CH
20	35.7 C	35.8 C	30.8 C	2‴	74.3 CH	74.3 CH	74.2 CH
21	29.0 CH_2	29.1 CH_2	34.2 CH_2	3‴	79.0 CH	79.1 CH	79.0 CH
22	33.3 CH_2	33.3 CH_2	33.3 CH_2	4‴	71.8 CH	71.3 CH	71.3 CH
23	28.2 CH_3	28.3 CH_3	28.3 CH_3	5‴	79.1 CH	79.3 CH	79.1 CH
24	17.1 CH_3	17.2 CH_3	17.2 CH_3	6‴	62.2 CH_2	62.6 CH_2	62.5 CH_2
25	15.7 CH_3	15.7 CH_3	15.9 CH_3				
26	17.7 CH_3	17.8 CH_3	17.6 CH_3				
27	24.8 CH_3	24.7 CH_3	26.1 CH_3				
28	177.5 C	177.4 C	176.6 C				
29	28.8 CH_3	28.8 CH_3	33.3 CH_3				
30	25.0 CH_3	25.1 CH_3	23.5 CH_3				

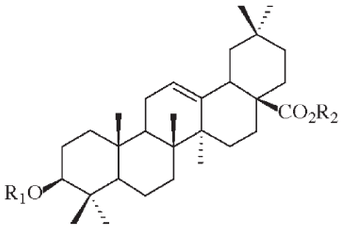
moiety of 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucuronopyranosyl was linked to the siaresinolic acid at C-3. Chemical shifts at δ_H 6.24 (d, $J = 7.9$ Hz) (Glc-1) and δc 95.9 (Glc-1) indicated that this sugar unit was involved in an ester linkage with the C-28 carboxylic group. This result was confirmed by the correlation in the HMBC spectrum between the signal at δ_H 6.24 (d, $J = 7.9$ Hz) (Glc-1) and δc 177.1 (Agly-28). Therefore, **1** was determined to be 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucuronopyranosyl siaresinolic acid-28-*O*-β-D-glucopyranosyl ester, named hylonoside III.

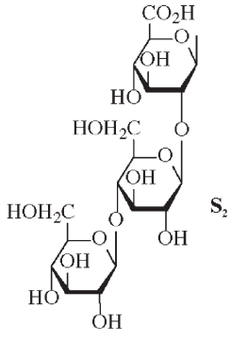
Compound **2** was obtained as an amorphous powder. The negative FAB-MS of **2** showed a quasi-molecular ion peak at *m/z* 1133 $[M - H]^-$, indicating a molecular weight of 1134, being compatible with the molecular formula $C_{54}H_{86}O_{25}$, and main fragment peaks at *m/z* 971 $[(M - H) - 162]^-$, 809 $[(M - H) - 162 - 162]^-$ and 647 $[(M - H) - 162 - 162 - 162]^-$. The ^{13}C NMR spectrum of **2** also showed a siaresinolic acid by comparison with published data [1].





1 $R_1 = S_1$ $R_2 = Glc$
2 $R_1 = S_2$ $R_2 = Glc$





3 $R_1 = S_2$ $R_2 = Glc$
4 $R_1 = S_1$ $R_2 = Glc$
5 $R_1 = S_1$ $R_2 = H$
6 $R_1 = GluA$ $R_2 = Glc$

Acid hydrolysis of **2** afforded a mixture of sugars, which were identified as glucose and glucuronic acid. Alkaline hydrolysis of **2** yielded a prosapogenin, which gave glucuronic acid and glucose on acid hydrolysis. These hydrolysis results indicated that **2** must be a triterpene-bidesmoside. Compound **2** was shown to contain four sugar residues from its HMQC spectrum. The anomeric proton signals at δ 6.27, 5.36, 5.08 and 4.81 gave correlations with carbon signals at δ 95.8, 104.9, 104.8 and 105.1, respectively. These anomeric signals and evaluation of their spin–spin couplings allowed the identification of one β-glucuronopyranosyl (GluA) and three β-glucopyranosyl (Glc) units. From the 2D NMR, HMQC and HMBC experiments, the proton and carbon NMR spectral data of **2** were obtained (see Table I). It revealed that the signals at δ 95.9 (Glc-1) and 177.4 (C-28) of **2** indicated that the glucosyl ester was connected to C-28. The sugar chain at C-3 was established from the following HMBC correlations: H-1 of terminal glucose at $\delta_{H\text{-}1}$ 5.08 (d, $J = 7.0$ Hz) (T-Glc) and C-4 of inner glucose at $\delta_{C\text{-}1}$ 83.3 (inn Glc), H-1 of inner glucose at $\delta_{H\text{-}1}$ 5.36

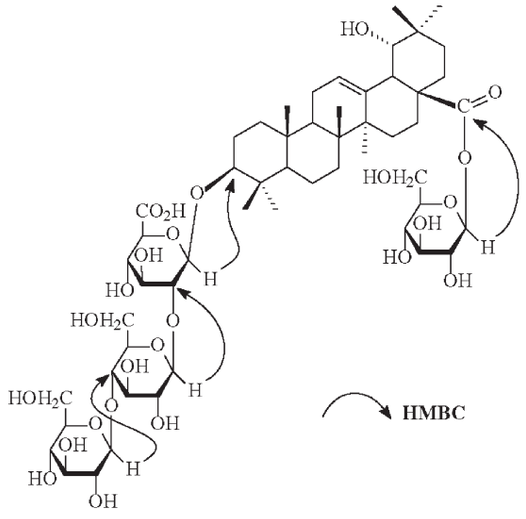


FIGURE 1 The HMBC correlations for compound **2**.

(d, $J = 7.1$ Hz) (inn-Glc) and C-2 of inner glucuronic acid at $\delta_{C\text{-}1}$ 81.0 (inn GluA), H-1 of inner glucuronic acid at $\delta_{H\text{-}1}$ 4.81 (d, $J = 7.9$ Hz) (inn-GluA) and C-3 of the genin at $\delta_{C\text{-}3}$ 89.0. These linkages were confirmed by the NOESY correlations between the signal at δ_H 5.08 (T-Glc-1) and δ_H 4.08 (inn-Glc-4), δ_H 5.36 (inn-Glc-1) and δ_H 4.18 (inn-GluA-2), δ_H 4.81 (inn-GluA-1) and δ_H 3.55 (Agly-3). On the basis of the above results, compound **2** is 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl siaresinolic acid-28-*O*-β-D-glucopyranosyl ester (**2**), and named hylonoside IV (Fig. 1).

Compound **3** showed the same sugar chain as hylonoside IV (**2**) by comparison of its ^{1}H and ^{13}C NMR with those of **2**. The main differences concerned the genin parts. **3** afforded oleanolic acid by comparing the ^{13}C NMR data [6] and a mixture of sugars of glucose and glucuronic acid on acid hydrolysis. Its negative ion FAB-MS displayed an ion at *m/z* 1117 $[M - H]^-$ (to give a molecular formula of $C_{54}H_{86}O_{24}$ combined with DEPT spectrum) and main fragment ions at *m/z* 955, 793, 631, and 455, which were attributed to the losses of glucose, glucose, glucose and glucuronic acid, successively. The signal of C-3 resonated at δ 88.4 and C-28 resonated at δ 176.6 in **3** instead of δ 78.7 and 181.0 in oleanolic acid, showing the glycosidating positions and presence of a bisdesmosic saponin. Thus, **3** was 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl ester, and named hylonoside V.

EXPERIMENTAL SECTION

General Experimental Procedures

The 1D and 2D NMR spectra were obtained using Bruker AM-400 and DRX-500 spectrometers and the solvent was pyridine-d_5. FAB-MS were taken on a VG Autospec 3000 system spectrometer. Optical rotations were measured on a JASCO-20C digital polarimeter and gas chromatography (GC) was run on a Hitachi G-3000 gas chromatograph. Chromatographic materials were RP-8 (40–60 μm, Merck), silica gel (160–200 mesh), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd.) and MCI-gel CHP20P

(75–150 μm, Mitsubishi Chemical Industries, Ltd.). The following solvent systems were used for separating saponins, $CHCl_3$–MeOH–H_2O (7:3:0.5), $CHCl_3$–MeOH–H_2O (65:35:9) and MeOH–H_2O (0–100%), and for detecting sugars, $CHCl_3$–MeOH–H_2O (7:3:1) lower-layer 9 ml + 1 ml HOAc. TLC spots were detected by spraying with 5% H_2SO_4 followed by heating. Sugars were detected by spraying with aniline–phthalate reagent.

Plant Material

The leaves of *I. hylonoma* Hu et Tang were collected at the Plant Garden of the Guangxi Institute of Botany, Chinese Academy of Sciences in July 1999. A voucher specimen (No. 13521) was deposited in the Herbarium of the Guangxi Institute of Botany. The plant was identified by Professor C.H. Li.

Extraction and Isolation

The dry leaves of *I. hylonoma* Hu et Tang (1050 g) were extracted (2 × 10 l) with MeOH. The methanol extract was evaporated under reduced pressure, then was treated with water and filtered. The water soluble fraction was passed through a D_{101} column and eluted with water and methanol. Evaporation of the methanol eluate yielded 5 g of a brown fraction (A). The fraction (A) was chromatographed on silica gel to give five fractions. The fractions were chromatographed on RP-8 gel column (solvent: MeOH–H_2O, 10–70%) and silica gel column ($CHCl_3$–MeOH–H_2O, 100:10:1–80:20:1) to yield **1** (74 mg), **2** (90 mg), **3** (205 mg), **4** (281 mg), **5** (120 mg), **6** (170 mg).

Acid Hydrolysis

A solution of each compound (10 mg) was heated at 100°C in 5% H_2SO_4 and 50% EtOH for 10 h. The reaction mixture was diluted with water, neutralized with 2% NaOH and evaporated *in vacuo* to dryness. The sugars were extracted with pyridine from the residue. The extracts were analyzed by comparing with authentic sugars on silica gel ($CHCl_3$–MeOH–H_2O–AcOH, 7:3:0.5:1) using 4% α-naphthol–EtOH–5% H_2SO_4 as spray reagent, in which the presence of glucose and glucuronic acid were detected. The pyridine extract was derivatized with thiazolidine as described previously [7]. Monosaccharides were detected by GC and conditions: column, SupelcoSPB-1, 0.25 mm × 27 m; column temperature, 230°C; carrier gas, N_2; t_R, L-glucose (13.3 min), D-glucose (13.8 min), L-glucuronic acid (10.6 min), D-glucuronic acid (10.8 min). D-glucose and D-glucuronic acid were detected in all compounds **1**–**6**.

Alkaline Hydrolysis

Each saponin (8 mg) was refluxed in 0.5 N aq. KOH (2 ml) for 2 h. The mixture was adjusted to pH 6 with 1 N aq. HCl and then the extract was concentrated to dryness, which was extracted with pyridine and was analyzed by HPTLC to detect sugars.

Hylonoside III (**1**) white amorphous powder, $C_{48}H_{76}O_{20}$, $[\alpha]_D^{21}$ +21 (*c* 0.7, MeOH); FAB-MS *m/z* 971 $[M - H]^-$, 809 $[M - H\text{-}162]^-$, 647 $[M - H\text{-}2 \times 162]^-$; ^{1}H NMR δ 0.87, 0.97, 1.10, 1.12, 1.14,1.19,1.64 (3H, s, $CH_3 \times 7$), 3.30 (1H, dd, $J = 11.2, 4.3$ Hz), 4.96 (1H, d, $J = 7.2$ Hz, H-1 of GlcA), 5.53 (1H, d, $J = 7.0$ Hz, H-1 of Glc), 6.24 (1H, d, $J = 7.9$ Hz, H-1 of Glc); and ^{13}C NMR, see Table I.

Hylonoside IV (**2**) amorphous powder, $C_{54}H_{86}O_{25}$, $[\alpha]_D^{21}$ +17 (*c* 0.8, MeOH); FAB-MS *m/z* 1133 $[M - H]^-$, 971 $[M - H\text{-}162]^-$, 809 $[M - H\text{-}2 \times 162]^-$, 647 $[M - H\text{-}3 \times 162]^-$; ^{1}H NMR δ 0.87, 0.97, 1.10, 1.12, 1.14, 1.18, 1.64 (3H, s, $CH_3 \times 7$), 3.28 (1H, dd, $J = 11.3$, 4.3 Hz, 3-H), 5.50 (br.s, H-12), 4.81 (1H, d, $J = 7.3$ Hz, H-1 of GlcA), 5.08 (1H, d, $J = 7.0$ Hz, H-1 of Glc), 5.36 (1H, d, $J = 7.1$ Hz, H-1 of Glc), 6.27 (1H, d, $J = 8.0$ Hz, H-1 of Glc); and ^{13}C NMR, see Table I.

Hylonoside V (**3**) $C_{54}H_{86}O_{24}$, $[\alpha]_D^{21}$ +9 (*c* 0.8, MeOH); FAB-MS *m/z* 1117 $[M - H]^-$, 955 $[M - H\text{-}162]^-$, 793 $[M - H\text{-}2 \times 162]^-$, 631 $[M - H\text{-}3 \times 162]^-$, 455 $[M - H\text{-}3 \times 162\text{–}176]$; ^{1}H NMR δ 0.80, 0.83, 0.88, 1.07, 1.11, 1.18, 1.24 (3H, s, $CH_3 \times 7$), 3.21 (1H, dd, $J = 11.2$, 4.0 Hz, 3-H), 5.16 (br.s, H-12), 4.82 (1H, d, $J = 7.3$ Hz, H-1 of GluA), 5.08 (1H, d, $J = 7.0$ Hz, H-1 of Glc), 5.35 (1H, d, $J = 7.1$ Hz, H-1 of Glc), 6.29 (1H, d, $J = 8.0$ Hz, H-1 of Glc); and ^{13}C NMR, see Table I.

Acknowledgements

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