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Two unusual minor 18,19-*seco*-ursane glycosides from leaves of *Ilex cornuta*

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ABSTRACT

The leaves of *Ilex cornuta* is traditionally used as a functional tea in China. Two new minor 18,19-*seco*-ursane glycosides, named cornutaoside A (**1**) and B (**2**), were isolated from leaves of *I. cornuta*, along with two known compounds (**3** and **4**). Their structures were elucidated as (3β,12β)-3-[β-D-glucopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-12,21-dihydroxy-19-oxo-18,19-secours-13(18)-en-28-oic acid (**1**) and (3β,12β)-3-[β-D-glucopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-12,19,21-trihydroxy-18,19-secours-13(18)-en-28-oic acid (**2**), by chemical methods, 1D and 2D NMR experiments, and by comparison with known analogues. This is the first report of E-*seco* triterpenoids and diterpene skeletons (**4**) from this plant. In a preliminary cytotoxic test against U937, L1210, and B16 cell lines, **1** and **2** had no significant activities as compared to controls, with concentrations up to 443.61 and 346.25 μM/plate, for **1** and **2**, respectively.

1. Introduction

Leaves of some *Ilex* species are used to make a widely consumed beverage in China called "*Kudingcha*", bitter tea (Chau & Wu, 2006; Heck & Mejia, 2007; Jiangsu New Medical College, 1986). In addition to its common consumption, *Kudingcha* is also popular for its health benefits. *Ilex cornuta* Lindl. et Paxt. (Aquifoliaceae) is an evergreen shrub growing in Eastern and Southern China. The dried leaves of *I. cornuta* are traditionally employed as one of the staple *Ilex* species for "*Kudingcha*" or as an ingredient in dietary supplements. In addition, the aqueous decoction made from its leaves is commonly used as a contraceptive, cardiovascular or antibacterial agent (Jiangsu New Medical College, 1986; Qin et al., 1986; Zhang, Lin, Cen, & Shen, 2003). Previous phytochemical investigation of *I. cornuta* showed that leaves of this plant are a rich source of triterpenoids and flavonoids as well as their corresponding glycosides (Li, Wu, Cheng, & Wang, 2006; Nakanishi, Terai, Nasu, Miura, & Yoneda, 1982; Qin et al., 1986; Wu, Cheng, Liu, Li, & Wang, 2005). The saponin-containing fractions from leaves of this plant have been reported to possess various important pharmacological effects such as the increase of coronary blood flow and anti-hematoblastic coagulation activities (Qin, Zhao, & Fukuyama, 1988; Qin et al., 1986). Recently, our investigation of leaves of this plant demonstrated again that triterpenoids were the predominant components among its secondary metabolites (Li et al., 2006; Wu et al., 2005). As part of our continuous search for potentially active substances from *I. cornuta*, we report herein the isolation and structural elucidation of two unusual minor 18,19-*seco*-ursane glycosides along with a known triterpenoid saponin and a known diterpene. This is the first report of 18,19-*seco*-triterpenoids and diterpene skeletons from this species.

2. Materials and methods

2.1. General

Optical rotations were measured with a JASCO P-1030 polarimeter (Jasco Co., Tokyo, Japan). The IR spectra were obtained on a Perkin–Elmer Spectrum One FR-IR instrument (Perkin–Elmer Co., Norwalk, CT, USA). NMR spectra were recorded on Bruker ACF-500 spectrometers (Bruker Instruments, Inc., Billerica, MA, USA) [^{1}H (500 MHz) and ^{13}C (125 MHz)] in pyridine-d_5 as a solvent. Chemical shifts were reported using residual pyridine (δ_H 7.18 and δ_C 123.5) as internal standard. ESIMS and HR-Fourier Transformation ESIMS were obtained on a Bruker APIII MS instrument (Bruker Daltonics, Billerica, MA, USA) equipped with Microvip data system. HP-TLC was performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Reversed-phase preparative HPLC separation was carried out on an Agilent 1100 apparatus equipped with a 150 × 4.6 mm i.d. C_{18} silica gel column (ZORBAX SB-Aq, Agilent Co. Ltd., Palo Alto, CA, USA) at 25 °C and eluted with CH_3CN–H_2O (37:63, v/v, 1.0 mL/min) as mobile phase, using a G1315A DAD detector (Agilent Co. Ltd., Palo Alto, CA, USA).

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2.2. Plant material

The leaves of *I. cornuta* were collected from Shanghai, China, in August 2001, and identified by Prof. Zheng-tao Wang. A voucher specimen (Herbarium No. 0115) is on deposit at the herbarium of the present institute.

2.3. Extraction and isolation

The shade-dried and powdered leaves (10 kg) of *I. cornuta* were extracted with 85% aqueous ethanol (50 L × 2) at room temperature, and partitioned between *n*-butanol and H_2O. The *n*-butanol extract (117 g) was chromatographed over silica gel column by eluting with a gradient of CH_2Cl_2/CH_3OH (20:1–1:1, v/v) to afford five fractions I–V as described previously (Li et al., 2006). Compound **4** (88 mg) was obtained by separation of fraction I on silica gel with CH_2Cl_2/CH_3OH (15:1, v/v) as elutant. Separation of fraction V on silica gel column eluted by increasing amounts of CH_3OH in EtOAc afforded three subfractions: $subF_1$, F_2 and F_3. A portion of $subF_2$ was further purified by repeated silica gel column (CH_2Cl_2/MeOH, 95:4, v/v) followed by preparative HPLC on RP-18 (CH_3CN/H_2O, 37:63, v/v) to afford compounds **1** (40 mg) and **2** (35 mg). The $subF_3$ was subjected to repeated silica gel column eluting with CH_2Cl_2/MeOH (5:1, v/v) to afford compound **3** (100 mg).

2.4. Acidic hydrolysis and GC analysis of compounds *1* and *2*

Each compound (10 mg) was heated in 2 M trifluoroacetic acid at 120 °C for 2 h. The reaction mixture was partitioned with EtOAc. The aqueous layer was concentrated, and the residue was re-dissolved in MeOH and subjected to HP-TLC analysis with authentic sugars, applying silica gel HP-TLC plates, using EtOAc/H_2O/formaic acid/acetic acid (100:27:11:11, v/v/v/v) as the developing reagent. Spot of TLC was visualized by spraying with 10% H_2SO_4 in EtOH followed by heating at 110 °C for 5 min. Acidic hydrolysis of both **1** and **2** gave glucose and arabinose. The absolute configuration of glucose and arabinose was determined according to the method described by Cases, Cerezo, and Stortz (1995). Briefly, the hydrolyzates of **1** (7.0 mg), **2** (6.7 mg), or sugar standards (10.0 mg) were weighed into 25 mL glass-stoppered flasks. The following anhydrous methanolic solutions were sequentially added to the samples: (i) 11.11% (*S*)-1-amino-2-propanol (20 μL of solution/mg sugar); (ii) 20% glacial AcOH (17 μL of solution/mg sugar); and (iii) 3% $NaBH_3CN$ (13 μL of solution/mg sugar). The resulting mixture was heated and reacted for 1.5 h at 65 °C. Then the reaction mixture was cooled to room temperature, and 3 M aqueous CF_3COOH was added drop wise until the pH was 1–2. The mixture was evaporated, followed by co-evaporation with addition of water (3 × 0.5 mL) and MeOH (5 × 0.5 mL). Trace solvent was further removed overnight in a desiccator. Two mL of 50% pyridine in Ac_2O was added to the residue, and the mixture was heated for 45 min at 100 °C, followed by partition with EtOAc and washing with water (3 × 1.0 mL) and saturated $NaHCO_3$ (3 × 1.0 mL). The organic phase was dried with anhydrous Na_2SO_4 and subjected to GC analysis [TRACE GC ULTRA-DSQ (Thermo, San Jose, CA) equipped with a mass detector and a TRB-5MS capillary column (30 m × 0.25 mm × 0.25 μm)]. The oven temperature program was initiated at 130 °C (held for 2 min), then raised at 2 °C/min to 159 °C (held for 7 min), followed by a rise at 2 °C/min/ to 169 °C for 3 min, 175 °C for 2 min, 179 °C for 2 min, 185 °C for 2 min, 187 °C for 2 min, and finally, at 10 °C/min to 205 °C (held for 10 min). The identification of the sugars was achieved by comparison of their retention times (R_t: 59.84 and 60.25 min for L-arabinose and D-glucose, respectively) with those of authentic sugars. D-Glucose and L-arabinose were detected for compounds **1** and **2**.

2.5. Cytotoxic assay

The U937, L1210 and B16 cell lines were cultured, respectively, in Dulbecco's modified Eagle medium (180 μL; *Gibco BRL*) in 96-well micro-culture plates according to a previously described method (Wu et al., 2005). Medium (20 μL) containing different concentrations of compound **1** or **2** were added to all wells (in triplicate). Control cells received medium containing analogous dimethyl sulfoxide (DMSO) concentrations. After incubation at 37 °C in a 5% CO_2 atmosphere for three days, Alamar Blue (20 μL) was added to each well. After incubation for another 6 h, the color change of the solution was measured with an ELISA micro-autoreader at 570 and 600 nm. The activation rate was calculated according to the formula provided by the Alamar Blue assay (*Nivelles*, Belgium).

3. Results and discussion

3.1. Structure elucidation of the novel 18,19-ursane glycosides

Solvent extraction, followed by repeated silica gel column chromatography, and preparative high performance of liquid chromatography, of aqueous ethanol extract of *I. cornuta*, led to isolation of two 18,19-*seco*-ursane glycosides (Fig. 1) and two known compounds.

Compound **1** was obtained as colorless solids from MeOH, $[\alpha]_D^{20}$ – 29.1 (*c* 0.39, MeOH), and its molecular formula was determined as $C_{41}H_{66}O_{15}$ on basis of the quasi-molecular ion observed at *m*/*z* 803.4184 (HR–ESIMS, $[(M-H_2O)+Na]^+$, calcd. for $C_{41}H_{64}NaO_{14}$, 803.4188), and confirmed by ^{13}C NMR (DEPT) analysis. The IR spectrum exhibited strong absorptions at 3414, 1751, 1719 and 1624 cm^{-1}, indicating the existence of hydroxy, 2 × carbonyl, and olefinic functionalities. The ^1H NMR spectrum of **1** showed characteristic signals of triterpenoids with seven tertiary methyl signals at δ 0.89 (3H, s), 1.06 (3H, d, J = 7.1 Hz), 1.09 (3H, s), 1.31 (6H, s, overlap), 1.37 (3H, s) and 2.24 (3H, s), a typical 3α-H signal at δ 3.34 (1H, dd, J = 11.7, 4.3 Hz), two oxygen-bearing methine protons at δ 4.69 (1H, t, J = 6.8 Hz) and 4.92 (1H, m), and an olefinic proton at δ 6.24 (1H, s), as well as two anomeric signals at δ 5.06 (br s, overlap) and 5.27 (1H, d, J = 7.7 Hz). The ^{13}C NMR spectrum of **1** showed 41 carbon signals, with 30 attributed to the aglycone part and 11 to two monosaccharides (Table 1). Note that two diagnostic olefinic carbon signals observed at δ 151.16 and 119.64, the characteristic resonances of H-12 [δ_C 69.34, δ_H 4.69 (1H, t, J = 6.8 Hz)], and the C-30 methyl [δ_C 11.91, δ_H 1.06 (1H, d, J = 7.1 Hz)] geminal to a hydroxyl, were characteristics of 18,19-*seco*-ursane derivatives, which possess C-13(18) double bond and β-hydroxyls at C-12 or C-21 (Kakuno, Yoshikawa, Arihara, Takei, & Endo, 1991; Li et al., 2005). Moreover, existence of two hydroxyls attributed to HO-12 and HO-21 were supported by the fragments at *m*/*z* 803 $[(M-H_2O)+Na]^+$ and 785 $[(M-2H_2O)+Na]^+$ present in the positive ESI mass spectrum. Complete assignments of **1** based on a detailed analysis of ^1H, ^{13}C NMR, DEPT, HMQC, and HMBC allowed establishing the exact nature of the aglycone of **1**, which is in good agreement with that reported for 18,19-*seco*-ursane (Li et al., 2005). Further structural confirmation was performed by combinational analysis of HMQC and HMBC spectra. As shown in Fig. 2, the three-bond correlations of H-9 and H-18 with C-12 confirmed the position of HO-12, while the long-range correlations of H-22, H-20 and Me-30 with C-21 indicated the presence of HO-21. In addition, the determination of endocyclic double bond at C-13(18) and the C-19 ketone group were verified by HMBC correlations of H-18

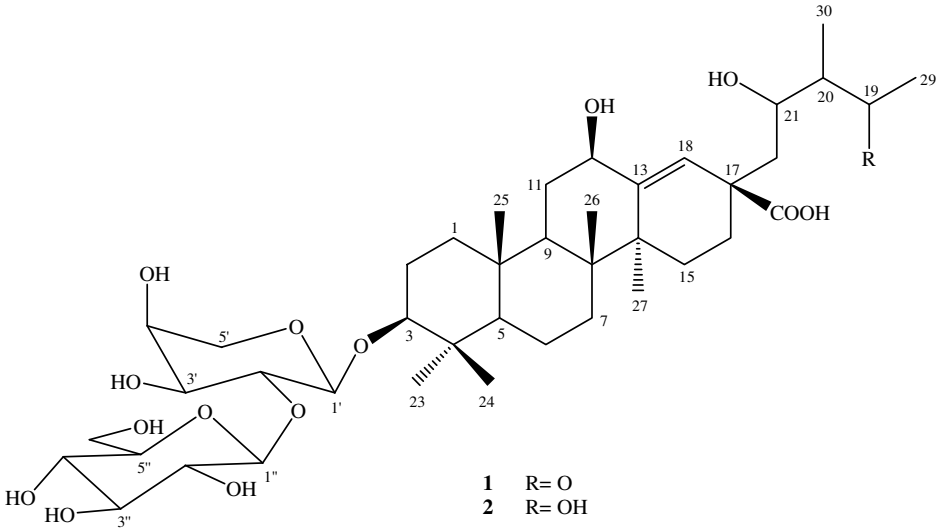

Fig. 1. Structures of compounds **1** and **2**.

Table 1
^{13}C NMR data of compounds **1** and **2** (125 MHz, pyridine-d_5)

No.	Aglycone moiety		No.	Sugar moiety	
	1	**2**		**1**	**2**
1	39.25	39.26	Inner arab-		
2	26.72	26.74	1′	104.98	104.98
3	88.89	88.91	2′	81.21	81.21
4	39.75	39.75	3′	73.54	73.55
5	56.17	56.19	4′	68.42	68.41
6	18.49	18.52	5′	65.08	65.08
7	35.20	33.16	Terminal glc-		
8	41.42	41.45	1″	106.15	106.16
9	49.92	49.98	2″	76.53	76.54
10	37.43	37.44	3″	78.29	78.31
11	33.10	35.24	4″	71.78	71.78
12	69.34	69.37	5″	78.35	78.36
13	151.16	150.66	6″	62.76	62.76
14	43.47	43.50			
15	27.72	27.82			
16	29.54	29.78			
17	45.29	45.78			
18	119.64	120.20			
19	209.45	68.38			
20	51.94	45.61			
21	77.62	77.97			
22	43.23	43.93			
23	28.34	28.35			
24	16.80	16.80			
25	16.88	16.90			
26	18.94	21.60			
27	22.33	22.39			
28	178.82	179.52			
29	29.65	19.01			
30	11.91	10.43			

with C-14, C-16, and C-17, and of Me-29, Me-30, and H-20 with C-19, respectively.

The nature of the sugars (glucose and arabinose) of **1** was first determined by the comparison of their ^{13}C NMR data (Table 1) with the corresponding monosaccharides (Yu & Yang, 1999), which was further confirmed by the high performance-TLC (HP-TLC) analysis with authentic samples after acidic hydrolysis. To verify their absolute configurations, **1** was subjected to acidic hydrolysis, followed by GC analysis in comparison with D-glucose and L-arabinose (Cases et al., 1995). By this procedure the sugars were identified as D-glucose and L-arabinose. The sequence of the monosaccharide residues was deduced from the presence of cross-peaks in HMBC spectra (Fig. 2). The glycosidic linkage at C-3 of the aglycone was shown by the HMBC correlation of the anomeric proton at δ 5.06 (br s, overlap) with C-3 at δ 88.89. The 1 → 2 interglycosidic linkage with a terminal D-glucose was assigned by the HMBC correlation between H-1″ of D-glucose and C-2′ of L-arabinose as well as the comparison of ^{13}C chemical shifts for each methylated monosaccharide (Yu & Yang, 1999). In addition, this assignment of the β-D-glucopyranosyl (1 → 2)-α-L-arabinopyranosyl unit was further supported by the reported ^{13}C NMR data of a similar disaccharide chain (Nakanishi et al., 1982).

The relative configuration of **1** was proposed as shown in Fig. 1 by analysis of NOESY data and comparison of the documented data. As mentioned above, the superimposable NMR signals of the aglycone of **1** and the reported 18,19-*seco*-ursane derivatives (Kakuno, Yoshikawa, & Arihara, 1991; Li et al., 2005), indicated the β-orientation of Me-24, Me-25, Me-26, HO-12, and COOH-28, and α-orientation of H-3, H-5, H-9 and Me-27. However, limited cross-peak information was obtained in several trials of NOESY experiments, except that correlations between H-3 and H-5, H-3 and H-1′, H-5 and H-6α, and Me-25 and Me-26 confirmed that the configurations of H-3 and H-5 were α, and that of Me-25 and Me-26 were β. Note that the relative configurations at C-20 and C-21 were not ascertained yet due to the free rotation of the σ-bond between C-17 and C-22, as suggested by Li et al. (2005). Based on the above evidence, the structure of **1** was established as (3β, 12β)-3-[β-D-glucopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-12,21-dihydroxy-19-oxo-18,19-secours-13(18)-en-28-oic acid, named cornutaoside A. The detailed NMR spectra data of cornutaoside A are listed in Tables 1 and 2.

Compound **2** was obtained as a white amorphous powder, $[\alpha]_D^{20}$-26.3 (*c* 0.34, MeOH). It exhibited a pseudo-molecular ion peak at *m/z* 805.4392 ($[(M-H_2O)+Na]^+$, calcd. for $C_{41}H_{66}O_{14}Na$, 805.4404) in HR-ESIMS, consistent with a molecular formula of $C_{41}H_{68}O_{15}$ assigned by a combinational analysis of ^{1}H, ^{13}C NMR and DEPT. Inspection of ^{1}H, ^{13}C NMR data of **2** revealed that compounds **1** and **2** were closely comparable (Tables 1 and 2). The superimposable signals included seven tertiary methyl signals at δ 0.90 (3H, s, Me-25), 1.09 (6H, Me-24 and 30), 1.28 (6H, Me-23 and 27), 1.36 (3H, s, Me-26) and 1.41 (3H, d, *J* = 6.1 Hz, Me-29), a 3α-H signal at δ 3.33 (1H, dd, *J* = 11.0, 3.1 Hz), and H-18 olefinic proton at δ 6.29 (1H, s), two oxygen-bearing methine protons of HO-12 and HO-21 at δ 4.69 (1H, t, *J* = 6.2 Hz) and 5.16 (1H, m), as well as two anomeric signals at δ 5.05 (br s, overlap) and 5.27

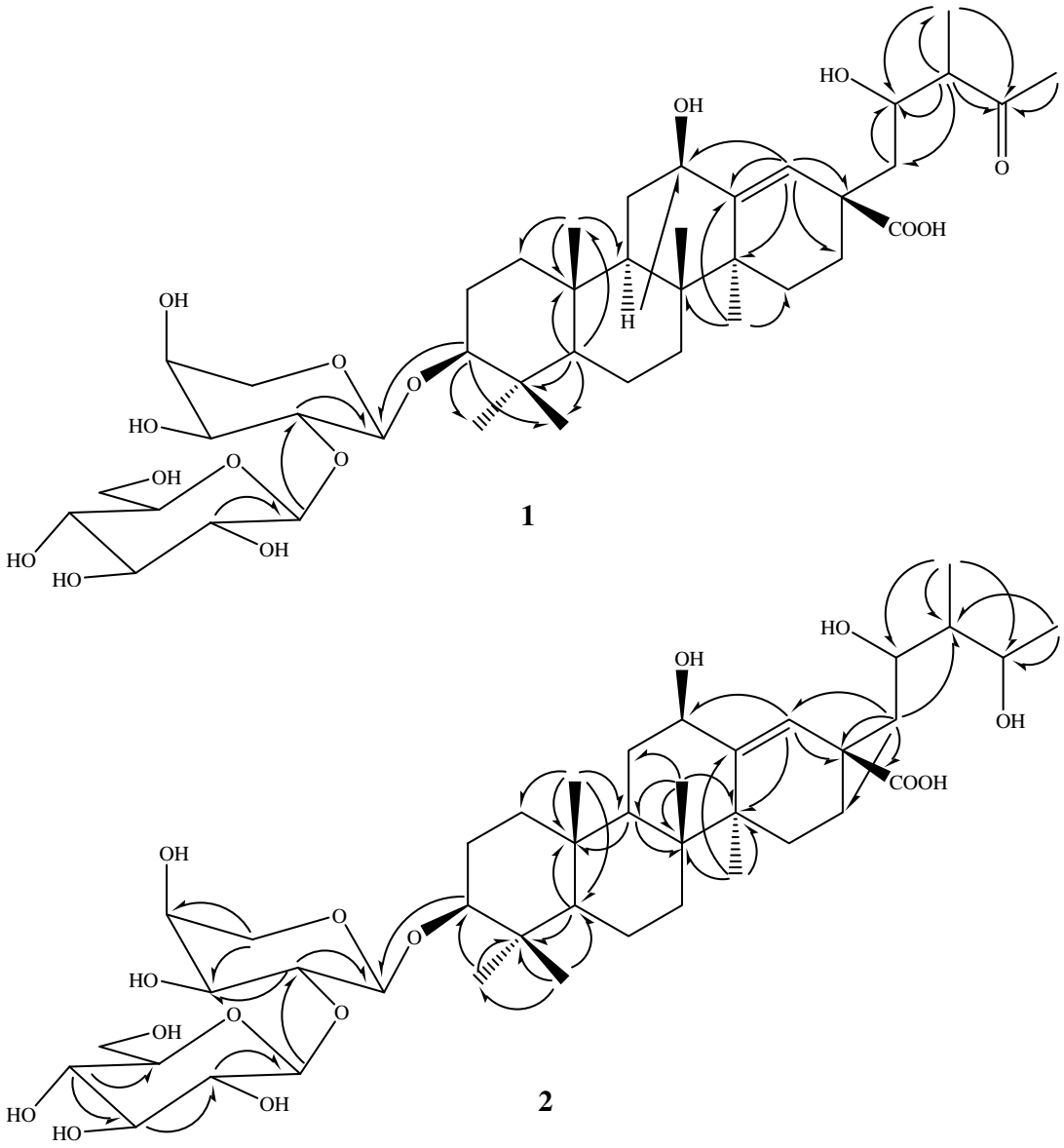

Fig. 2. Key HMBC correlations for cornutaoside A (**1**) and cornutaoside B (**2**).

(1H, d, J = 7.7 Hz). The significant difference which appeared in ^{1}H NMR was that the ketone group in **1** was replaced with a hydroxyl group in **2** at C-19 position (δ 4.10). The comparison of ^{13}C NMR data of **1** and **2** showed that differences appeared only in the resonances associated with the 18,19-*seco*-ursane section at C-19 to C-22, which replacement of the ketone group at C-19 in **1** by an additional hydroxyl at δ 68.38 in **2** indicated a 19,21-diol structure of that chain. This assignment was established by ^{13}C NMR signals of C-19, C-20, C-21, and C-22, observed at δ 68.38, 45.61, 77.97, and 43.93, respectively. Additional evidence for this assignment was obtained by the HMBC correlations of C-19 with Me-29 and Me-30 (Fig. 2), and an ESI quasi-molecular ion peak at m/z 805 $[(M-H_2O)+Na]^+$, differing from **1** by **2** amu. The sugar part of **2** was determined as the same with that of **1** by the comparison of the NMR data (Table 1) and the methods applied to **1** including HMBC experiments, chemical hydrolysis, and GC analysis (Fig. 2). The similar cross-peak correlations as for **1** observed in the NOESY spectrum gave evidence for the relative stereochemistry of **2** at C-3, C-5, H-9, C-25, and C-26. Combining the comparison of NMR data among **1**, **2** and the 18,19-*seco*-ursane derivatives (Li et al., 2005), compound **2** was established as (3β,12β)-3-[β-D-glucopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-12,19,21-trihydroxy-18,19-secours-13(18)-en-28-oic acid, named cornutaoside B. The detailed NMR spectra data of cornutaoside B were listed in Tables 1 and 2.

3.2. Significance of 18,19-seco-ursanes in genus Ilex

The two known compounds were identified as pomolic acid 3-*O*-β-D-glucopyranosyl (1 → 2)-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranoside (**3**) (Nakanishi et al., 1982) and darutoside (**4**) (Son, Giang, & Taylor, 2005) by spectroscopic analysis, and comparison with literature data. Compound **4** was the first diterpene skeleton isolated from the genus *Ilex*. To the best of our knowledge, the isolation and characterization of **1** and **2** also showed it to be the first example of an 18,19-*seco*-ursane skeleton from this species, though several 18,19-*seco*-ursane derivatives were previously isolated from *I. aculeolata* (Ouyang, Zhang, & Li, 2002) and *I. crenata* (Kakuno, Yoshikawa, Arihara, 1991; Kakuno, Yoshikawa, Arihara, Takei et al., 1991) with different substitutions at E-ring. The possible biogenetic pathway of 18,19-*seco*-ursanes was recently pro-

Table 2
Selective ^{1}H NMR data of compounds **1** and **2** (500 MHz, pyridine-d_5)[a]

No.	1	2
H-3	3.34 (1H, dd, *J* = 11.7, 4.3 Hz)	3.33 (1H, dd, *J* = 11.0, 3.1 Hz)
H-5	0.82 (1H, d, *J* = 11.6 Hz)	0.82 (1H, br d, *J* = 11.6 Hz)
H-11	1.48 (m)	1.48 (2 H, br s)
H-12	4.69 (1H, t, *J* = 6.8 Hz)	4.69 (1H, t, *J* = 6.2 Hz)
H-18	6.24 (1H, br s)	6.29 (1H, s)
H-19	–	4.10 (1H, m)
H-20	2.84 (1H, m)	1.93 (1H, m)
H-21	4.92 (1H, m)	5.16 (1H, m)
H-22	2.08 (m), 2.20 (m)	2.22 (m), 2.41 (m)
Me-23	1.31 (overlap, s)	1.31 (3H, s)
Me-24	1.09 (3H, s)	1.09 (3H, s)
Me-25	0.89 (3H, s)	0.90 (3H, s)
Me-26	1.37 (3H, s)	1.36 (3H, s)
Me-27	1.31 (overlap, s)	1.28 (overlap, s)
Me-29	2.24 (3H, s)	1.41 (3H, d, *J* = 6.1 Hz)
Me-30	1.06 (3H, d, *J* = 7.1 Hz)	1.11 (3H, d, *J* = 6.5 Hz)
H-1′	5.06 (1H, s)	5.05 (1H, s)
H-2′	4.69 (1H, t, *J* = 6.8 Hz)	4.69 (1H, t, *J* = 6.2 Hz)
H-3′	4.42 (m)	4.45 (m)
H-4′	4.45 (m)	4.45 (m)
H-5′	3.86 (m), 4.37 (m)	3.88 (m), 4.41 (m)
H-1″	5.27 (1H, d, *J* = 7.7 Hz)	5.27 (1H, d, *J* = 7.7 Hz)
H-2″	4.19 (1H, t, *J* = 8.3 Hz)	4.19 (1H, t, *J* = 8.1 Hz)
H-3″	4.30 (1H, t, *J* = 9.0 Hz)	4.29 (1H, t, *J* = 9.1 Hz)
H-4″	4.39 (m)	4.41 (m)
H-5″	3.92 (m)	3.91 (m)
H-6″	4.52 (2H, d, *J* = 3.1 Hz)	4.52 (2H, br s)

[a] Assignments were confirmed by HMQC and HMBC experiments.

posed by Li et al. (2005), in which ursane derivatives of closed E-ring might be converted to 18,19-*seco*-ursanes via a series of oxidation, reduction and rearrangement reactions. In the case of *I. cornuta*, the occurrence of pomolic acid, a predominant aglycone found in leaves, might act as the precursor for this transformation.

3.3. Cytotoxic activities of the 18,19-seco-ursane compounds

Compounds **1** and **2** were evaluated *in vitro* against two mouse cancer cell lines (L1210 and B16) and one human cell line (U937). 5-Fluorouracil (5-Fu) was used as a positive control. Slight inhibition of cell growth, achieving corresponding 23.75%, 19.99%, and 13.89% decreases in L1210, B16 and U937 cell lines at a concentration of 346.25 μM, was observed with **2**, whereas significant decreases of 73.76%, 25.73%, and 67.87% were observed with 5-Fu. As for **1**, a similar trend was observed for the above three cell lines with the inhibition values of 23.13%, 11.48% and 12.00% at a concentration of 443.61 μM. Results from this cytotoxic test showed that compounds **1** and **2** were found to have no potential cytotoxic activities under the current experiment conditions.

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