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CHARACTERIZATION OF THREE NEW TRITERPENOID SAPONINS FROM *ARDISIA JAPONICA*

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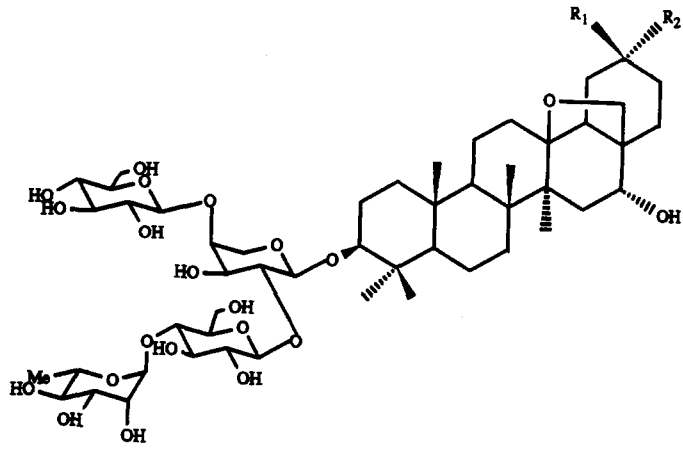
ABSTRACT.—The isolation and characterization of three novel triterpene glycosides **1–3** from the medicinal plant *Ardisia japonica* (Myrsinaceae) are described. The compounds are characterized by a branched oligosaccharide chain, composed of four sugar units. The oligosaccharide structures were determined by ^{1}H-^{1}H correlation spectroscopy (COSY, HOHAHA, ROESY) and ^{1}H-^{13}C heteronuclear correlation (HETCOR) nmr experiments. The aglycone moieties are the oleane-type triterpenes cyclamiretin A for **1** and the new 13,28-epoxy-30,30-dimethoxyolean-3β,16α-diol and 3β,16α-dihydroxy-13,28-epoxyolean-29-oic acid for **2** and **3**, respectively.

In China a decoction of *Ardisia japonica* (Thunb.) Bl. (Myrsinaceae) is taken to stop cough and uterine bleeding (1). Roots of *Ardisia crispa* (Thunb.) A. DC. are, in combination with other plants, used in Thai traditional medicine to "wash out dirty blood" in women who suffer from menstrual pains (1). In Burma, all parts of *Ardisia humilis* Vahl. are used to treat menstrual disorders. *Ardisia villosa* Roxb. is said to be a Chinese remedy for contusions and rheumatic and neuralgic pain, but pregnant women should avoid it. Medicinal use of 16 other species of *Ardisia* has also been reported (1). In a search for novel bioactive natural products from medicinal plants, we have isolated three new triterpene oligosaccharides **1–3** from the medicinal plant *A. japonica*. Structure elucidations were accomplished mainly on the basis of 2D proton-proton and proton-carbon shift correlation spectroscopy.

RESULTS AND DISCUSSION

The aerial parts of *A. japonica* were extracted successively with petroleum ether, $CHCl_3$, and MeOH. The MeOH extract was partitioned into a mixture of *n*-BuOH and H_2O to afford the *n*-BuOH-soluble portion, which was subjected to Sephadex LH-20 cc and then to dccc [$CHCl_3$–MeOH–H_2O–*n*-PrOH–EtOH (9:6:8:1:8), descending mode] to give three saponins **1–3**. ^{1}H- and ^{13}C-nmr spectra indicated that saponins **1–3** had identical saccharide chains but differed in the aglycone portion. The molecular formulae ($C_{53}H_{86}O_{22}$ for **1**, $C_{55}H_{92}O_{23}$ for **2**, $C_{53}H_{86}O_{23}$ for **3**) were determined by ^{13}C, ^{13}C DEPT nmr data and negative ion fab mass spectra. The fabms spectrum of **3** showed the $[M-H]^-$ ion at *m/z* 1089 and prominent fragments at *m/z* 943 $[(M-H)-146]^-$, *m/z* 927 $[(M-H)-162]^-$ (cleavage of a deoxyhexose unit with or without the glycosidic oxygen), and *m/z* 781 $[(M-H)-(146+162)]^-$ due to the subsequent loss of an hexose unit. Also observed was a fragment at *m/z* 1045 $[(M-H)-44]^-$ corresponding to loss of a carboxylic group from the *m/z* 1089 peak. The ^{13}C and DEPT ^{13}C-nmr spectra showed 53 signals, of which 23 were assigned to the saccharide portion and 30 to a triterpenic moiety. The ^{1}H-nmr spectrum of **3** showed, in addition to six singlets assignable to tertiary methyls at δ 0.87–1.40, two signals at δ 3.00 and 3.52 (d, J=10 Hz each) ascribable to a -CH_2OH group. The 3β-OH substitution was evident from the chemical shift and the J value of the proton ascribable to C-3 at δ 3.17 (dd, J=11 and 4.5 Hz). The signal at δ 3.95 (br s) indicated the presence of a C-16α hydroxyl group



1	R_1=-CHO	R_2=Me
2	R_1=-CH(OMe)$_2$	R_2=Me
3	R_1=Me	R_2=-COOH

which was further supported by ^{13}C-nmr data (Table 1) and by the C-27 methyl which resonated at δ 1.32, i.e., downfield from its usual position (2). The ^{13}C nmr of **3** also showed a quaternary signal at δ 178.00 ppm, indicative of a carboxylic group, which was located at C-29 on the basis of the upfield shifts exhibited by C-19, C-21, and C-30 (Table 1) and the downfield shift experienced by C-20 when compared with model compounds reported in the literature (3,4). The presence of two signals at δ 88.01 (C) and 78.00 (CH_2) in the ^{13}C-nmr spectrum suggested the 13,28 epoxy group.

The aglycone of **3**, 3β,16α-dihydroxy-13,28-epoxyolean-29-oic acid, has not been reported previously. ^{13}C-nmr signals assigned to the pentacyclic nucleus of the aglycone of compound **1** (Table 1) were similar to those reported for cyclamiretin A (5), an oleane-related natural triterpene with an aldehydic group at C-30. The fabms of compound **2** showed an $[M-H]^-$ ion at *m/z* 1119, which was 46 mass units higher than that of **1**. The ^{1}H-nmr spectrum was very similar to that of **1**; main differences were the absence of the signal at δ 9.50 assigned in **1** to an aldehydic proton and the presence of a signal at δ 5.11 for one acetalic proton and of one signal at δ 3.24 (6H, s) assigned to two -OMe groups. The ^{13}C-nmr data of **2** in comparison with **1** (see Table 1) showed an acetalic carbon a 109.30 ppm and two signals at 58.00 and 57.80 for -OMe groups. Therefore the aglycone of compound **2** was established as the new dimethyl acetal at C-30 of cyclamiretin A (5): compound **2** could be an artifact due to the MeOH used during the isolation.

Attachment of the glycosidic chain at C-3 was indicated by the significant downfield shift (δ_C 90.86) observed for this carbon resonance in **1**, relative to the corresponding signal in cyclamiretin A (5) (δ_C 78.00), and was subsequently confirmed by 2D nmr experiments.

Structural elucidation of the sugar chain of these compounds began with the most abundant compound **1**. On anhydrous acid methanolysis, **1** gave methyl arabinoside, methyl rhamnoside, and methyl glucoside in a 1:1:2 ratio.

The structure of the oligosaccharide unit was determined by 2D nmr spectroscopy. The positions of interglycosidic linkages were determined using a combination of ^{1}H-^{1}H Correlation Spectroscopy (COSY), Rotating-Frame Overhauser Enhancement Spec-

TABLE 1. ^{13}C-nmr Data of the Aglycones of Compounds **1–3** in CD_3OD.

Carbon	Compound			
	1	DEPT[a]	**2**	**3**
C-1	40.15	CH_2	40.03	40.24
C-2	27.00	CH_2	27.00	27.19
C-3	90.86	CH	90.90	90.05
C-4	39.50	C	40.00	38.33
C-5	56.73	CH	56.78	56.81
C-6	18.64	CH_2	18.69	18.77
C-7	32.42	CH_2	32.60	31.00
C-8	43.18	C	43.00	43.34
C-9	51.30	CH	51.00	51.38
C-10	37.00	C	37.50	37.83
C-11	19.92	CH_2	20.00	19.85
C-12	32.42	CH_2	31.70	31.00
C-13	88.00	C	88.00	88.10
C-14	45.16	C	45.00	45.23
C-15	37.50	CH_2	36.90	37.00
C-16	77.71	CH	77.37	77.70
C-17	44.36	C	43.00	46.00
C-18	53.95	CH	54.00	49.00
C-19	33.95	CH_2	33.30	37.00
C-20	48.00	C	47.70	40.24
C-21	34.87	CH_2	34.85	23.40
C-22	30.78	CH_2	30.85	35.10
C-23	28.54	Me	28.80	28.46
C-24	16.72	Me	16.72	16.60
C-25	16.72	Me	16.72	16.69
C-26	18.24	Me	18.28	18.45
C-27	19.90	Me	18.70	19.85
C-28	78.50	CH_2	78.30	78.00
C-29	24.31	Me	24.39	178.00
C-30	209.00	CH	109.30	18.77
-OMe			58.00	
-OMe			57.80	

[a]DEPT assignments for compound **1**.

troscopy (ROESY) (6,7), 2D-Homonuclear Hartmann Hahn (HOHAHA) (8), and of ^{1}H-^{13}C Correlation Spectroscopy (HETCOR). The COSY experiment allowed the sequential assignment of most resonances for each sugar ring, starting from the anomeric signals. Nevertheless, not all proton resonances could be successfully assigned with confidence, because even at high field (500 MHz) the 1D sugar spectral region of **1** was complex. Most of the signals were found between δ 5.31 and 3.00 and were overlapped also by the aglycone signals. Complete assignments were achieved by a combination of COSY and 2D HOHAHA results. 2D HOHAHA spectroscopy was used to resolve the overlapped spectra of oligosaccharides into a subset of individual monosaccharide spectra. In a 2D HOHAHA spectrum of **1** (Table 2) the anomeric proton signal ascribable to a α-L-arabinose (H-1′, δ 4.52) showed connectivities to three methines (δ 3.87, 3.90, and 4.09, respectively). The coherence transfer to methylene H-5′ was not obtained because of the small coupling constants H-4′, H-5′ (9).

These observations combined with those of the COSYexperiment permitted the following assignments for this sugar fragment: H-1 (δ 4.52), H-2 (δ 3.87), H-3 (δ 3.90), H-4 (δ 4.09), H_a-5 (δ 4.11), H_b-5 (δ 3.60).

TABLE 2. ^{1}H-nmr[a] Data for the Oligosaccharide Moiety of **1** in CD_3OD.

Proton	Arabinose	Glucose II	Glucose I	Rhamnose
H-1	4.52, d, J=5.2	4.65, d, J=7.5	4.70, d, J=7.5	5.31, d, J=1.5
H-2	3.87, dd, J=5.2, 8.2	3.22, dd, J=7.5, 9.5	3.43, dd, J=7.5, 9.0	3.95, dd, J=1.5, 2.5
H-3	3.90, dd, J=8.2, 3.0	3.48, t, J=9.5, 9.5	3.45, t, J=9.0, 9.0	3.79, dd, J=2.5, 9.0
H-4	4.09, m	3.44, t, J=9.5, 9.5	3.31, t, J=9.0, 9.5	3.40, dd, J=9.0, 9.0
H_a-5	4.11, dd, J=2.0, 12.0	3.36, m	3.26, m	4.15, dd, J=9.0, 6.5
H_b-5	3.60, dd, J=12.0, 2.5			
H_a-6		3.90, dd, J=12.0, 3.5	3.85, dd, J=3.5, 12.0	1.30, d, J=6.5
H_b-6		3.68, dd, J=12.0, 5.0	3.66, dd, J=12.0, 5.0	

[a]From 2D COSY, 2D HOHAHA, and HETCOR experiments. ^{1}H-^{1}H coupling constants in the sugar spin system were measured from COSY and HOHAHA spectra and are reported in Hz.

Similarly results of the HOHAHA and COSY experiments for all the other sugar residues (Table 2) allowed complete sequential assignment for all proton resonances. A HETCOR correlated all proton resonances with those of the corresponding carbons (Table 3). Data from the above experiment determined the position of the interglycosidic linkages by comparison of the carbon chemical shifts observed with those of the corresponding methyl pyranosides and taking into account the known effects of glycosidation (10).

Thus, glucosyl and rhamnosyl residues were the terminal units as suggested by the absence of any ^{13}C glycosidation shift for these sugars, while glycosidation shifts on C-2 (+7 ppm) and C-4 (+7 ppm) of arabinose and on C-4 (+6 ppm) of an inner glucose established the presence of both a nodal arabinopyranosyl residue glycosylated at C-2 and C-4 and a C-4 glycosylated glucopyranosyl unit.

These data left two possible sequences for the tetraglycoside chain of compound **1**: cyclamiretin A 3β-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside or cyclamiretin A 3β-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside.

A ROESY (7) experiment allowed us to differentiate between the two proposed structures. Key correlation peaks were obtained (Table 4) between anomeric protons and protons linked to glycosylated carbons.

Chemical shifts, multiplicity of the signals, absolute values of the coupling constants and their magnitude in the ^{1}H-nmr spectrum as well as ^{13}C-nmr data (Table 3) indicated the β configuration at the anomeric positions for both glucopyranosyl units ($J_{H1\text{-}H2}$=7.5 Hz) and the α configuration for the rhamnopyranosyl unit (Table 2) ($J_{H1\text{-}H2}$=1.5 Hz). L-Arabinose in the pyranose form was evident from ^{13}C-nmr data (11). No further support for the anomeric configuration of the L-arabinopyranose unit could be drawn from the ^{1}H- and ^{13}C-nmr data. In fact, the value of its $^3J_{H1\text{-}H2}$ coupling constant (5.2 Hz) was midway between that observed for methyl-β-L-arabinopyranoside (4 Hz)

TABLE 3. ^{13}C-nmr Data for the Oligosaccharide Moieties of Compound **1** in CD_3OD[a].

Carbon	Arabinose	Glucose II	Glucose I	Rhamnose
C-1	105.30	104.53	103.71	101.70
C-2	79.55	76.40	77.00	71.80
C-3	72.38	77.70	79.10	72.20
C-4	76.68	77.90	71.40	74.20
C-5	64.00	77.70	77.90	70.30
C-6		62.90	62.90	16.75

[a]Assignments based on 2D COSY, 2D HOHAHA and HETCOR experiments.

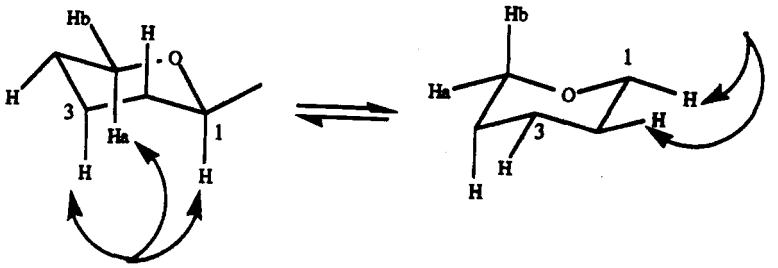
TABLE 4. Selected Data from ROESY Experiments of **1** in CD_3OD.[a]

Connectivities observed between	
Proton	ROESY (^{1}H-anomeric)
3.17 (H-3 Aglycone)	4.52 (H-1 Arabinose)
4.09 (H-4 Arabinose)	4.70 (H-1 Glucose I)
3.87 (H-2 Arabinose)	4.63 (H-1 Glucose II)
3.44 (H-4 Glucose II)	5.31 (H-1 Rhamnose)

[a]The experiments were optimized for dipolar couplings with a mixing times of 200 msec.

and methyl-α-L-arabinopyranoside (8 Hz) (11,12). The value of this coupling constant has been reported not to be diagnostic on its own, owing to the high conformational mobility of arabinopyranosides ($^4C_1 \leftrightarrow {}^1C_4$) (13). Evidence supporting an α-L-arabinopyranoside configuration in rapid conformational exchange was obtained from ROESY experiments in **1**.

NOe's were observed from C_{ara}-1 to C_{ara}-2 and C_{ara}-1 to C_{ara}-3 as expected for $^1C_4$ and $^4C_1$ conformations respectively. The nOe C_{ara}-1–C_{ara}-3 would not be expected for both $^1C_4$-$^4C_1$-β-L-arabinopyranosides. An nOe was also observed between C_{ara}-1 and C_{ara}-5 as expected for an α-L-arabinopyranoside in a $^4C_1$ conformation (Figure 1). Further evidence for the β configuration at the anomeric center of both glucose units and for the α configuration at each of the other two sugars was suggested by consideration of the molecular rotation values in the light of Klyne's rule (see Experimental) (14–16). On the basis of the foregoing data the structures of the three compounds are proposed to be cyclamiretin A 3β-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]α-L-arabinopyranoside [**1**]; 3β-*O*-{α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]α-L-arabinopyranoside}-16α-hydroxy-13,28-epoxyolean-29-oic acid [**3**], and 3β-*O*-{α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]α-L-arabinopyranoside}-16α-hydroxy-13,28-epoxy-30,30-dimethoxyoleane [**2**].



$^4C_1$ α-L-Arabinopyranosyl (-OH groups not reported) $^1C_4$

FIGURE 1. Observed nOe's for $^1C_4$ and $^1C_4$ conformations of α-L-arabinopyranosyl unit.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—For nmr, a Bruker WH-250 Spectroscopin or Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer using the UXNMR software package was used. Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional pulse sequence. The COSY spectrum was obtained using a data set (t1×t2) of 1024×1024 points for a spectral width of 1165 Hz (relaxation delay 1 sec). The data matrix was processed

using an unshifted sine bell window function, followed by transformation to give a magnitude spectrum with symmetrization (digital resolution in both F2 and F1 dimensions 1.13 Hz per point). The 2D HOHAHA (8) experiment was performed in the phase-sensitive mode (TPPI) using an MLEV-17 sequence for mixing (14). The spectral width (t2) was 1002 Hz; 512 experiments of 40 scans each (relaxation delay 1.5 sec, mixing time 100 msec) were acquired in both dimensions before transformation. The resulting digital resolution in F2 was 0.48 Hz per point. The ROESY (7) experiment was performed in the phase-sensitive mode (TPPI). The spectral width (t2) was 1002 Hz; 512 experiments of 80 scans each (relaxation delay 1.5 sec, mixing time 300 msec) were acquired in 2 K data points. For processing, a sine bell window function was applied in both dimensions before transformation. The HETCOR experiment was performed on a data matrix 512×1024, using a CH coupling of 135 Hz and relaxation delay 1.5 sec. The data matrix was processed using a q sine window function.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. Fabms was recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (XE atoms of energy of 2–6 kV). Dccc was performed on an apparatus manufactured by Buchi, equipped with 300 tubes. Glc analysis was performed on a Supelco SP200 capillary column (30 m, i.d. 0.32 mm, film thickness 0.25 mm, carrier gas He, 5 ml·min^{-1}, 156°).

EXTRACTION AND ISOLATION.—The plant *A. japonica* was collected at Suzhou, Jiang-Sou province China. A voucher sample of the plant is deposited at the herbarium of this department. The air-dried leaves (300 g) were defatted with petroleum ether and $CHCl_3$ and then extracted with MeOH to give 21 g of residue. Part of the MeOH extract (10 g) was partioned between *n*-BuOH and H_2O to afford an *n*-BuOH-soluble portion (4 g) which was chromatographed on a Sephadex LH-20 column (100×5 cm) with MeOH as eluent. Fractions (8 ml) were collected and checked by tlc [Si gel plates, *n*-BuOH–HOAc–H_2O (60:15:25)]. Fractions 18–27 (737 mg) containing the crude glycosidic mixture were further purified by dccc with $CHCl_3$–MeOH–H_2O–*n*-PrOH–EtOH (9:6:8:1:8) in which the stationary phase consisted of the higher phase (descending mode, flow 10 ml/h). Fractionation of each glycoside was achieved by hplc on C18 μ-Bondapak column (30 cm×7.8 mm id) with MeOH-H_2O (65:35) to yield pure **1** (33 mg, Rt 15 min), **2** (10 mg, Rt 19 min), and **3** (16 mg, Rt 13 min).

METHANOLYSIS OF COMPOUNDS **1–3**, CARBOHYDRATE CONSTITUENTS.—A solution of each compound (2 mg) in anhydrous 2N HCl/MeOH (0.5 ml) was heated at 80° in a stoppered reaction vial for 12 h. After cooling, the solution was neutralized with Ag_2CO_3 and centrifuged. The supernatant was evaporated to dryness under N_2. The residue was reacted with TRISIL-Z (Pierce) and analyzed by glc. Retention times were identical to those of the authentic methyl sugars.

Compound **1**.—$[\alpha]_{25D}$ −8.7 (*c*=1, MeOH); ^{1}H nmr for aglycone (500 MHz), Me-23 (δ 1.12), Me-24 (δ 0.87), Me-25 (δ 0.99), Me-26 (δ 1.20), Me-27 (δ 1.33), Me-29 (δ 1.12), H_a-28 (δ 3.00, d, *J*=12 Hz), H_b-28 (δ 3.52, d, *J*=12 Hz), H-16 (δ 3.97, br s), H-3 (δ 3.17, dd, *J*=10 and 4.5 Hz), H-30 (δ 9.50, s); fabms *m/z* $[M-H]^-$ 1073. M_D of **1** = −94.5; calculated contribution to the M_D: (M_D cyclamiretin = +123°) + [M_D of sugar chain (α-L-rha + α-L-ara + β-D-glu + βD-glu) = −226°] = −103; all other possible combinations give much more positive values.

Compound **2**.—$[\alpha]_{25D}$ −10.5 (*c*=1, MeOH); ^{1}H nmr for aglycone (500 MHz) Me-23 (δ 1.12), Me-24 (δ 0.87), Me-25 (δ 0.95), Me-26 (δ 1.20), Me-27 (δ 1.30), Me-29 (δ 1.11), H_a-28 (δ 3.00, d, *J*=12 Hz), H_b-28 (δ 3.52, d, *J*=12 Hz); H-16 (δ 3.97, br s), H-3 (δ 3.17, dd, *J*=10 and 4.5 Hz), -OMe (δ 3.24, s); fabms *m/z* $[M-H]^-$ 1119.

Compound **3**.—$[\alpha]_{25D}$ −2.4 (*c*=1, MeOH); ^{1}H nmr of aglycone (500 MHz) Me-23 (δ 1.10), Me-24 (δ 0.85), Me-25 (δ 0.94), Me-26 (δ 1.20), Me-27 (δ 1.36), Me-30 (δ 1.42), H_a-28 (δ 3.28, d, *J*=12 Hz), H_b-28 (δ 3.62, d, *J*=12 Hz), H-16 (δ 3.97, br s), H-3 (δ 3.17, dd, *J*=10 and 4.5 Hz).

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