Acylated Triterpenoid Saponins from *Schima noronhae* and Their Cell Growth Inhibitory Activity

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Two new acylated triterpenoid saponins were isolated from the branches of *Schima noronhae* by bioassay-guided purification. Their chemical structures were established on the basis of spectroscopic analysis and chemical means as 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloyl-A1-barrigenol (**1**) and 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloylerythrodiol (**2**). Compounds **1** and **2** showed cell growth inhibitory activity against both HeLa and DLD1 cells at a concentration of less than 10 μM.

Schima noronhae Reinw. ex Blume (Theaceae) (syn.: *S. wallichii* Choisy) is grown widely in Southeast Asia, and is known in Japan as "iju".[1] The plant is used as a source of timber, and its leaves have been used as a fodder crop in Nepal. In traditional medicine in Indonesia and Malaysia, its astringent corollas are used to treat uterine disorders and hysteria and also as an ointment to alleviate symptoms of smallpox.[2,3] The ethanol extract of *S. wallichi* has been found to exhibit a high level of dermatophytic activity.[4] In chemical investigations, several hydrolyzable tannins[1] and triterpenoid saponins[5] were isolated from this plant previously.

During a search for bioactive natural products in tropical plants,[6,7] we have investigated the chemical constituents of the MeOH extract of branches of *S. noronhae* collected in Thailand, as this extract showed potent cell growth inhibitory activity against human cervical carcinoma cells (HeLa cells) in a screening program. Herein we describe the isolation and structural elucidation of two new triterpenoidal saponins, 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloyl-A1-barrigenol (**1**) and 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloylerythrodiol (**2**). In addition, the cell growth inhibitory activities of **1** and **2** were evaluated against HeLa and human colon carcinoma DLD1 cells.

The MeOH extract of the branches of *S. noronhae* was partitioned between hexane and 10% aqueous MeOH, and the aqueous phase was further extracted with EtOAc and *n*-BuOH to give four fractions. Since the EtOAc-soluble fraction was found to be most active (IC_{50} values: EtOAc-soluble fraction, 15.3 μg/mL; other fractions, >25 μg/mL), it was subjected to repeated column chromatography over ODS and Sephadex LH-20 to afford two triterpenoid saponins (**1** and **2**).

Compound **1**, $[\alpha]^{20}_{D}$ –20 (*c* 1.1, MeOH), was obtained as a white powder, and its molecular formula was suggested to be $C_{59}H_{94}O_{26}$ on the basis of the HRFABMS data [*m/z* 1241.5922, $(M + Na)^+$, Δ –0.9 mmu]. The ^{1}H NMR spectrum of **1** (Table 1) showed characteristic proton signals due to seven tertiary methyl groups at δ_H 1.38 (3H, s), 1.06 (3H, s), 1.02 (3H, s), 1.00 (3H, s), 0.97 (3H, s), 0.89 (3H, s), and 0.87 (3H, s) of the triterpenoid moiety, one methyl group of a 6-deoxyhexapyranose sugar unit at δ_H 1.27 (d, J = 6.0 Hz), and four anomeric protons at δ_H 5.27 (1H, s), 5.17 (1H, d, J = 8.0 Hz), 4.87 (1H, d, J = 7.0 Hz), and 4.49 (1H, d, J = 8.0 Hz). In turn, the ^{13}C NMR spectrum of **1** showed 59 signals including those of four anomeric carbons at δ_C 105.8, 102.6, 102.1, and 100.9, suggesting **1** to be a triterpenoidal saponin having four sugar units.

The ^{1}H NMR spectrum of the aglycon moiety of **1** showed signals for seven methyl groups as singlets, one olefinic proton at δ_H 5.41 (1H, brt, J = 4.0 Hz), four oxygen-bearing methine protons at δ_H 5.43 (1H, dd, J = 12.0, 5.0 Hz), 3.90 (1H, d, J = 5.0 Hz), 3.75 (1H, m), and 3.20 (1H, dd, J = 13.0, 4.0 Hz), and one pair of hydroxymethylene protons at δ_H 3.31 (1H, overlapped) and 3.12 (1H, d, J = 12.0 Hz), suggesting that the aglycon moiety is an olean-12-ene skeleton with five oxygenated carbons,[8] which was confirmed on the basis of the 2D NMR spectroscopic data. The two oxymethylene proton signals at δ_H 3.31 and 3.12, which were correlated with a carbon signal at δ_C 63.9 in the HMQC spectrum, were assigned to H_2-28 from the HMBC correlations observed from H_2-28 (δ_H 3.31 and 3.12) to C-17 (δ_C 45.7). The presence of a trisubstituted olefin at C-12 and C-13 was revealed by the HMBC correlations observed for H_2-11/C-12, H_2-11/C-13, H-18/C-13, and H_3-27/C-13. In the COSY and TOCSY spectra, H-15 (δ_H 3.75) was coupled directly to the signal at δ_H 3.90 (H-16), and in the HMBC spectrum, cross-peaks observed for H_3-27/C-15 and H_2-28/C-16 showed the location of two secondary hydroxy groups at C-15 and C-16. Finally, the structure of the aglycon of **1** was determined as A1-barrigenol on the basis of the above-mentioned data as well as a detailed analysis of the COSY, ROESY, HMQC, and HMBC NMR spectra. This aglycon has been isolated previously and characterized from *Pittosporum undulatum*[9] and *Barringtonia asiatica*.[10] The NMR chemical shifts of the aglycon of **1** were consistent with data from the literature.[8] Also, characteristic signals for an angeloyl group were observed at δ_H 6.03 (1H, q-like, J = 7.0 Hz, H-3), 1.96 (3H, brd, J = 7.0 Hz, H-4′), and 1.89 (3H, d-like, J = 1.8 Hz, H-5′) in the ^{1}H NMR spectrum and at δ_C 169.6 (C-1′) 130.0 (C-2′), 138.0 (C-3′), 15.9 (C-4′), and 20.9 (C-5′) in the ^{13}C NMR spectrum. Additionally, the chemical shift of H-22 (δ_H 5.43) of the aglycon was shifted downfield by acylation, suggesting that the angeloyl group was attached to C-22 of the aglycon. This was confirmed by the HMBC correlation observed between H-22 (δ_H 5.43) of the aglycon and the carbonyl carbon (C-1′) of the angeloyl group (δ_C 169.6).

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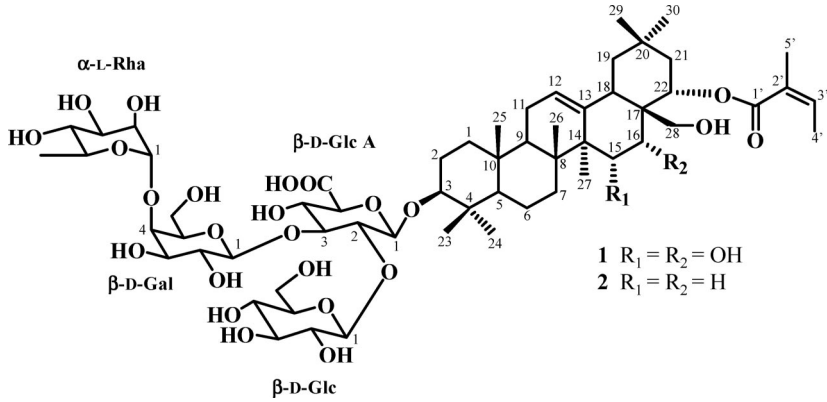

On acidic hydrolysis with 5% sulfuric acid, **1** gave a crude sugar mixture. The ^{1}H and ^{13}C NMR data (Table 1) for **1** suggested that it contained one glucose (Glc), one galactose (Gal), one rhamnose (Rha), and one glucuronic acid (GlcA) residue, which was confirmed by the HPLC analysis of the crude sugar mixture obtained by acid hydrolysis. The absolute configurations of the sugar residues were determined to be D-glucose, D-galactose, L-rhamnose, and D-glucuronic acid, respectively, on the basis of HPLC through comparison with authentic samples, using a combination of RI and optical rotation detectors. The configurations of the anomeric positions of one glucuronic acid, one glucose, and one galactose were assigned as β by judging from their large coupling constants between H-1 and H-2 of the sugar ring protons ($J_{1,2}$ values: GlcA, 8.0 Hz; Glc, 7.0 Hz; Gal, 8.0 Hz). The anomeric configuration for the one rhamnose was deduced as α from the one-bond coupling constant between C-1 and H-1 (Rha, $J_{C1,H1}$ values: 173.7 Hz; literature values:[11] α-anomer, 169 Hz; β-anomer, 160 Hz). The sequences of the tetrasaccharide unit and sugar−aglycon linkage were determined from the HMBC spectrum of **1**, which afforded key ^{1}H−^{13}C long-range correlations between H-1 of GlcA (δ_H 4.49) and C-3 of the aglycon (δ_C 92.2), H-1 of Glc (δ_H 4.87) and C-2 of GlcA (δ_C 79.2), H-1 of Gal (δ_H 5.17) and C-3 of GlcA (δ_C 81.0), and H-1 of Rha (δ_H 5.27) and C-4 of Gal (δ_C 75.9), respectively. Accordingly, the structure of **1** was deduced as 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloyl-A1-barrigenol.

Compound **2**, $[\alpha]^{20}_D$ –15 (*c* 1.1, MeOH), was isolated as a white powder. The molecular formula was suggested to be $C_{59}H_{94}O_{24}$ on the basis of the HRFABMS data [*m/z* 1263.5361, $(M + 2K + H)^+$, Δ +3.0 mmu]. Acid hydrolysis of **2** afforded D-glucose, D-galactose, L-rhamnose, and D-glucuronic acid as sugar residues, as identified by HPLC analysis. The ^{1}H NMR and ^{13}C NMR chemical shifts of **2** were similar to those of **1** except for the signals of positions 15 and 16 of the aglycon. Thus, it was observed that the signals of H-15 (δ_H 1.59, 1.33) and H_2-16 (δ_H 1.27) of the aglycon of **2** were shifted upfield from those of **1** (H-15: δ_H 3.75, H-16: δ_H 3.90) in the ^{1}H NMR spectrum. Similarly, C-15 (δ_C 34.0) and C-16 (δ_C 29.6) of the aglycon of **2** resonated upfield when compared to **1** (C-15: δ_C 68.6, C-16: δ_C 75.4) in the ^{13}C NMR spectrum, suggesting that the aglycon of **2** does not possess hydroxyl groups at positions 15 and 16. Thus, the aglycon of **2** was assigned as 22α-hydroxyerythrodiol from the COSY, ROESY, HMQC, and HMBC NMR spectra and by comparison of its spectroscopic data with those reported in the literature.[12] This aglycon has been previously isolated and characterized from *Harpullia pendula.*[13,14] Also, a comparison of the NMR data indicated that **2** has the same sugar chain at C-3 as **1**. Therefore, the structure of **2** was determined as 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl22-*O*-angeloylerythrodiol.

The cell growth inhibitory activity of the isolated compounds (**1** and **2**) was examined by a fluorometric microculture cytotoxicity assay (FMCA).[15] As shown in Table 2, **1** and **2** exhibited inhibitory activity against human cervical carcinoma HeLa cells, with IC_{50} values of 5.5 μM (6.7 μg/mL) and 3.8 μM (4.5 μg/mL), respectively. Also, they were active against human colon carcinoma DLD1 cells. Kartal et al. reported cell growth inhibitory activity of triterpenoid saponins with an angeloyl group at C-22.[8] These compounds showed weak activity against HCT116 and HT-29 with IC_{50} values between 40 and 100 μg/mL. It was found that **1** and **2** were more active than the aforementioned compounds. Moreover, the activity of **1** and **2** was nearly equal to that of the positive control, mitomycin C.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured on ATR in a JASCO FT-IR 230 spectrophotometer. NMR spectra were recorded on JEOL JNM ECA 800 and ECP600 spectrometers. High-resolution fast atom bombardment mass spectra (HRFABMS) were obtained on a JMS HX-110 mass spectrometer. HPLC was performed using a JASCO HPLC system equipped with a Shodex RI-72 refractive index detector and a JASCO OR-1590 optical rotation detector.

Plant Material. Branches of *Schima noronhae* were collected in Khon Kaen, Thailand, in June 2005 and were identified by T.K. A voucher specimen (6-693) is maintained at the Faculty of Agriculture, Khon Kaen University.

Extraction and Isolation. The air-dried branches (330 g) were extracted with MeOH. The MeOH extract (21 g) was dissolved in 10% aqueous MeOH (400 mL) and was partitioned successively between hexane (400 mL × 3), EtOAc (400 mL × 3), and *n*-BuOH (400 mL × 3), to obtain four fractions (hexane phase, 1.3 g; EtOAc phase, 2.7 g; *n*-BuOH phase, 13.7 g; aqueous phase, 6.2 g). The EtOAc-soluble fraction (13.7 g) was subjected to ODS column chromatography (40 × 220 mm) eluted with a gradient of 60–100% MeOH in H_2O to give 18 fractions: A (1.4 g), B (341.0 mg), C (44.6 mg), D (37.9 mg), E (36.0 mg), F (15.2 mg), G (14.7 mg), H (14.4 mg), I (31.3 mg), J (27.9 mg), K (47.8 mg), L (32.1 mg), M (246.0 mg), N (52.0 mg), O (9.8 mg), P (31.6 mg), Q (215.0 mg), and R (55.2 mg). Fraction K, eluted with 70% MeOH, was purified by Sephadex LH-20 column chromatography (13 × 420 mm) eluted with MeOH to give compound **1** (14.7 mg). Fraction M, eluted with 80% MeOH, was separated by ODS column chromatography (27 × 310 mm) with a gradient of 60–70% MeOH in H_2O to yield compound **2** (9.0 mg).

3-*O*-α-L-Rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloyl-A1-barrigenol (1): white powder; $[\alpha]^{20}_D$ –20 (*c* 1.1, MeOH); IR ν_{max} (ATR) 3371 and 1641 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; FABMS *m/z* 1241 $(M + Na)^+$ and *m/z* 1257 $(M + K)^+$; HRFABMS *m/z* 1241.5922, calcd for $C_{59}H_{94}O_{26}Na$, 1241.5931.

3-*O*-α-L-Rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloylerythrodiol (2): white powder; $[\alpha]^{20}_D$ –15 (*c* 1.1, MeOH) ; IR ν_{max} (ATR) 3378 and 1641 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; FABMS *m/z* 1225 $(M + K)^+$; HRFABMS *m/z* 1263.5361, calcd for $C_{59}H_{93}O_{24}K_2$, 1263.5331.

Acid Hydrolysis of Compounds 1 and 2 and Determination of the Absolute Configuration of Sugars. Compound **1** (5.4 mg) in 1,4-dioxane (4.5 mL) and 5% aqueous H_2SO_4 (3 mL) was heated at 95 °C for 1.5 h. After cooling to room temperature, water was added to the

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2** in Methanol-d_4

		compound **1**		compound **2**	
	position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
aglycon	1	1.00 m, 1.63 br d (13.0)	40.1	1.00 m, 1.63 br d (13.8)	40.0
	2	1.70 m, 1.91 m	27.0	1.69 m, 1.94 m	27.0
	3	3.20 dd (13.0, 4.0)	92.2	3.19 brd (5.5)	92.1
	4		40.5		40.6
	5	0.78 d (12.0)	56.7	0.78 d (12.0)	57.0
	6	1.42 m, 1.54 m	19.5	1.41 m	19.3
	7	1.68 m, 1.72 m	37.2	1.72 m	35.3
	8		42.3		41.8
	9	1.57 m	48.1	1.67 dd (11.0, 7.5)	48.0
	10		37.9		37.8
	11	1.83 m, 1.98 m	24.8	1.92 m	24.6
	12	5.41 t (4.0)	126.3	5.33 brt (3.7)	124.6
	13		144.4		143.9
	14		48.5		41.0
	15	3.75 m	68.6	1.33 brd (12.8), 1.59 dd (12.8, 4.0)	34.0
	16	3.90 d (5.0)	75.4	1.27 m	29.6
	17		45.7		45.3
	18	2.51 dd (13.5, 5.0)	42.4	2.51 brd (13.8)	42.4
	19	2.41 t (13.5), 1.04 m	47.6	1.03 m, 2.46 t-like (13.4)	48.0
	20		32.4		32.5
	21	1.52 brd (12.0), 2.20 t (12.0)	41.9	1.55 m, 2.26 t (12.0)	41.8
	22	5.43 dd (12.0, 5.0)	73.4	5.43 dd (12.0, 5.0)	73.8
	23	1.06 s	28.4	1.07 s	28.3
	24	0.87 s	16.3	0.88 s	16.9
	25	0.97 s	16.3	0.98 s	16.2
	26	1.00 s	17.9	0.95 s	17.3
	27	1.38 s	20.9	1.47 s	27.7
	28	3.12 d (11.0), 3.31[a]	63.9	3.05 d (12.0), 3.25 d (12.0)	64.8
	29	0.89 s	33.5	0.90 s	33.6
	30	1.02 s	25.1	1.04 s	25.2
angeloyl	1′		169.6		169.7
	2′		130.0		130.0
	3′	6.03 q-like (7.0)	138.0	6.04 dq (7.3, 1.8)	137.9
	4′	1.96 brd (7.0)	15.9	1.96 dd (7.3, 1.8)	15.9
	5′	1.89 d-like (1.8)	20.9	1.89 d-like (1.8)	20.9
GlcA	1	4.49 d (8.0)	105.8	4.48 d (8.0)	105.8
	2	3.92 t-like (8.0)	79.1	3.91 brd (9.0)	79.2
	3	4.04 t (9.0)	81.0	4.04 t (9.0)	81.1
	4	3.62 brd (9.2)	71.8	3.60 brd (9.5)	71.9
	5	3.77 brd (9.0)	76.1	3.77 brd (8.5)	76.2
	6		172.0[b]		173.4[b]
Glc	1	4.87 d (7.0)	102.6	4.87 d (7.0)	102.6
	2	3.23 t-like (9.0)	76.0	3.23 t-like (7.5)	76.1
	3	3.34 t (9.0)	77.9	3.33 t (9.0)	77.9
	4	3.07 t (9.0)	72.6	3.05 t (9.0)	72.6
	5	3.39 m	78.2	3.38 m	78.2
	6	3.55 dd (12.0, 5.0)	63.6	3.52 dd (12.0, 4.0)	63.5
		3.86 dd (12.0, 8.0)		3.84 brd (12.0)	
Gal	1	5.17 d (8.0)	100.9	5.18 d (8.0)	100.8
	2	3.77 brd (9.0)	76.0	3.77 brd (8.0)	76.1
	3	3.69 dd (9.0, 3.0)	72.3	3.69 m	72.3
	4	3.71 brd (3.0)	75.9	3.71 brd (4.0)	75.9
	5	3.51 dd (8.0, 4.0)	77.0	3.51 m	77.0
	6	3.61 dd (12.0, 4.0)	62.9	3.61 dd (12.0, 4.5)	62.8
		3.79 dd (12.0, 8.0)		3.79 dd (12.0, 8.0)	
Rha	1	5.27 s	102.1	5.27 s	102.1
	2	3.94 brd (1.0)	72.6	3.94 brs	72.6
	3	3.70 dd (9.0, 3.0)	71.7	3.70 m	71.8
	4	3.41 t (9.0)	73.8	3.40 t (9.0)	73.8
	5	4.09 dq (9.0, 6.0)	70.2	4.09 dq (9.0, 6.0)	70.3
	6	1.27 d (6.0)	17.9	1.27 d (6.0)	17.9

[a] Overlapped with CD_3OD. [b] Signals were detected from the HMBC spectrum.

Table 2. Cell Growth Inhibitory Activities of Compounds **1** and **2** against HeLa and DLD1 Cells

	IC_{50} (μM)	
compound	HeLa	DLD1
1	5.5	7.5
2	3.8	7.1
mitomycin C	5.1	4.2

reaction mixture, and the mixture was partitioned with EtOAc. The aqueous layer containing the saccharide mixture was neutralized by passage through an Amberlite IRA-96SB column, then analyzed by HPLC (Capcell Pak NH_2 UG80, 4.6 × 250 mm; eluent, 85% MeCN; flow rate, 0.7 mL/min; column temperature, 40 °C; detection, RI and optical rotation) according to literature conditions,[16] to identify L-rhamnose (t_R 7.78 min, negative peak in optical rotation detector), D-galactose (t_R 12.3 min, positive peak in optical rotation detector), and D-glucose (t_R 13.1 min, positive peak in optical rotation detector). D-Glucuronic acid (t_R 19.42 min, positive peak in optical rotation

detector) was identified by HPLC analysis (Capcell Pak NH_2 UG80, 4.6 × 250 mm; eluent, 65% MeCN/35% KH_2PO_4, flow rate, 1.0 mL/min; column temperature, 40 °C; detection, RI and optical rotation). Sugar residues of compound **2** were also identified as L-rhamnose, D-galactose, D-glucose, and D-glucuronic acid by HPLC.

Cell Culture. DLD1 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). HeLa cells were the same as described previously.[6,7] DLD1 cells were cultured in RPMI 1640 (Wako) supplemented with 10% fetal bovine serum (FBS). HeLa cells were also cultured in IMDM (Wako) supplemented with 5% FBS. Cells were cultured at 37 °C under an atmosphere of 5% CO_2.

Cell Growth Inhibitory Activity. The assay procedure was the same as previously described.[6,7] Briefly, HeLa or DLD1 cells (6×10^3 per well) were treated with different concentrations of each isolated compound for 24 h at 37 °C. After the medium containing the samples was removed, cell growth inhibitory activity was determined by the fluorometric microculture assay (FMCA) method using a fluorescence plate reader. The ratio of living cells was determined as the fluorescence in sample wells expressed as a percentage of that in the control wells, and inhibitory activity was indicated as IC_{50} values. Each experiment was conducted in triplicate.

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