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Enterolosaponins A and B, Novel Triterpene Bisdesmosides from *Enterolobium contortisiliquum*, and Evaluation for Their Macrophage-Oriented Cytotoxic Activity

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Abstract—Two novel triterpene bisdesmosides, designated as enterolosaponin A (**1**) and B (**2**), were isolated from *Enterolobium contortisiliquum*. The chemical structures of **1** and **2** were determined by analysis of their extensive spectroscopic data, as well as hydrolysis followed by chromatographic study. Enterolosaponins have a 2-amino-2-deoxy-D-glucosyl unit (D-glucosamine) as one of the monosaccharides constituting their oligosaccharide moieties, which have been rarely found in natural product research. Enterolosaponin A (**1**) exhibited a highly selective cytotoxicity against BAC1.2F5 mouse macrophages, and it should be notable that the macrophage death caused by **1** was shown to be neither necrotic nor due to induction of apoptosis from morphology of the died cells, whose cytosol occurred in vacuolation.
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Introduction

It is recognized that macrophages in peripheral tissues often proliferate under pathological conditions such as tumors, inflammation, and atherosclerosis.[1] Because the growth state of macrophages is believed to be a factor regulating the pathological process of disease, substances that regulate macrophage growth or survival may be useful for disease control. Previously, we have isolated two novel acylated triterpene saponins, securiosides A and B, from a Chinese crude drug, *Securidaca inappendiculata* roots.[2] Although securiosides A and B did not have any apparent cytotoxic activities against bone-marrow cells and lymphocytes, they induced apoptotic cell death of macrophages stimulated by M-CSF and are believed to be the primary compounds of new drugs for the treatment of pathological states in which macrophage proliferation occurs. In the course of our extensive studies on the plant constituents which exhibit selective sell growth inhibitory and/or cytotoxic activity against macrophages, we made a phytochemical screening of the pericarps of *Enterolobium contortisiliquum* (Vell.) Morong (Leguminosae), a triterpene saponin rich herbal medicine used in Brazil,[3] and isolated two novel bisdesmosidic triterpene saponins, named enterolosaponins A (**1**) and B (**2**), with up to seven monosaccharides including a 2-(acetylamino)-2-deoxy-D-glucosyl unit. Enterolosaponins A (**1**) was found to show a highly selective cytotoxicity against BAC1.2F5 mouse macrophages. This communication reports the isolation and structural determination of **1** and **2**, and the selective cytotoxic activity of **1** against macrophages.[1]

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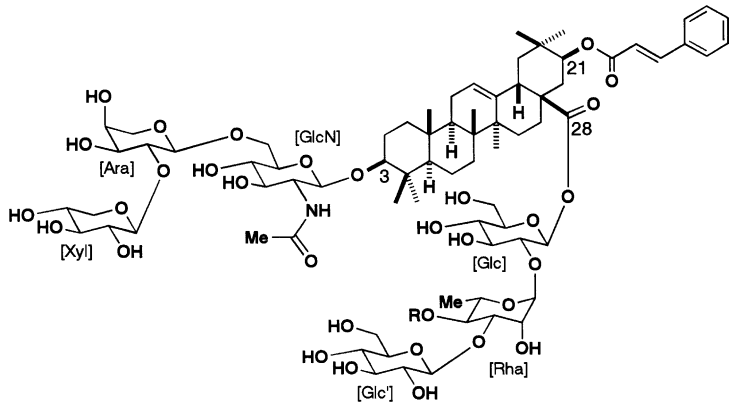

R
1 α-L-Ara*f*
2 H

Cell Culture Assay

Cells of the BAC1.2F5 murine macrophage cell line[4] were kindly provided by Dr. E. Richard Stanley, Einstein College of Medicine, NY, USA. The cells were maintained in RPMI 1640 medium (Nissui-Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Iwaki Glass, Chiba, Japan), penicillin (100 U/mL), kanamycin (60 μg/mL), and 20% L-cell-conditioned medium as a source of CSF-1.[5] For cytotoxicity assay, the cells were cultured in 96-well microplates (Iwaki Glass) at 1×10^4 cells/well with the samples to be tested, and simultaneously with 20% L-cell-conditioned medium for 72 h. EL-4 mouse lymphoma cells and L-929 mouse fibroblasts were maintained in RPMI 1640 medium supplemented with 5% FBS. The cells were cultured in 96-well microplates at 1×10^4 cells/well with the samples to be tested for 72 h.

Cell deaths of adherent BAC1.2F5 macrophages and L-929 fibroblasts were evaluated by crystal violet staining. The cells in 96-well plates were stained with 0.2% crystal violet for 15 min, washed with PBS and treated with 100 μg/mL sodium dodecyl sulfate. The optical density (595 nm) was measured with a microplate reader (Multiscan MS-UV, Labsystems, Helsinki, Finland). Cell death of EL-4 cells was evaluated by an MTT assay procedure.[6] The cells were added with 25 μL/well of MTT (5 mg/mL) and plates were incubated for an additional 3 h. Then, 150 μL of the supernatants was discarded, 100 μL of acid-isopropanol solution (0.04 M HCl in 2-propanol) was added to each well and the optical density (595 nm) was measured.

Extraction and Isolation

The dry pericarps of *E. contortisiliquum* (767 g)[7] was extracted with hot H_2O. The H_2O extract was partitioned between *n*-BuOH and H_2O. Column chromatography of the *n*-BuOH-soluble portion (31.6 g) on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (20:10:1; 7:4:1; 6:4:1), and finally MeOH alone, gave seven fractions (I–VII). Fraction II was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (7:4:1) and ODS silica gel with MeOH–H_2O (13:6) and MeCN–H_2O (4:7) to yield enterolosaponin A (**1**, 57.9 mg) and B (**2**, 16.5 mg).

Structural Elucidation

Enterolosaponin A (**1**)[8] was obtained as an amorphous solid that was analyzed for $C_{80}H_{121}NO_{36}$ by a combination of the negative-ion FABMS (*m*/*z* 1670 $[M-H]^-$), ^{13}C NMR spectrum (80 carbon signals), and elemental analysis data. The IR spectrum of **1** showed absorption bands of hydroxyl groups at 3375 cm^{-1}, two carbonyl groups at 1752 and 1705 cm^{-1}, and an amide carbonyl group at 1636 cm^{-1}. The UV absorption at 278 nm (log ε 4.38) was suggestive of a conjugated aromatic ring. The ^1H NMR spectrum of **1** in C_5D_5N contained seven three-proton singlet signals at δ 1.38, 1.22, 1.19, 1.06, 1.02, 1.00, and 0.95, and an olefinic proton signal at δ 5.49 (t-like, $J=3.3$ Hz), which were characteristic of the olean-12-ene skeleton, as well as signals for seven anomeric protons at δ 6.23 (d, $J=1.8$ Hz), 6.14 (d, $J=7.4$ Hz), 6.10 (br s), 5.37 (d, $J=7.8$ Hz), 5.17 (d, $J=5.0$ Hz), 5.05 (d, $J=8.3$ Hz), and 5.00 (d, $J=7.0$ Hz). The methyl carbon signal at δ 18.8 and proton signal at δ 1.85 (d, $J=6.0$ Hz) were indicative of **1** possessing one deoxy sugar. In addition, the presence of an acetyl group and an (*E*)-cinnamoyl group was shown by the following ^1H NMR, and ^{13}C NMR signals; δ_H 2.09 (3H, s)/δ_C 170.1 (C=O) and 23.7 (Me); δ_H 7.93 and 6.71 (ABq, $J=16.0$ Hz), 7.67 (2H, d-like, $J=6.9$ Hz, and 7.38 (3H, m)/δ_C 166.6 (C=O), 145.0 (CH), 135.0 (C), 130.6 (CH), 129.3×2 (CH×2), 128.6×2 (CH×2), and 119.1 (CH). Acid hydrolysis of **1** with 0.7 M HCl (dioxane–H_2O, 1:1) produced a triterpene aglycon with a cinnamoyl group (**1a**), identified as 21β-[(*E*)-cinnamoyloxy]-3β-hydroxyolean-12-en-28-oic acid,[9] and 2-amino-2-deoxy-D-glucose (D-glucosamine), L-arabinose, D-glucose, D-xylose, and L-rhamnose as the carbohydrate moieties. The monosaccharides, including their absolute configurations, were identified by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column using MeCN–H_2O (3:1) as solvent system, with detection being carried out

by using a combination of RI and optical rotation (OR) detectors.

In the ^{13}C NMR spectrum of **1**, the signals assignable to C-3 and C-28 were observed at δ 89.1 and 174.9, respectively, indicating that the sugar linkages were formed at both C-3 and C-28. The severe overlapping of the proton signals for the sugar moieties excluded the possibility of a complete assignment in a straightforward way using the conventional 2D NMR techniques such as ^{1}H–^{1}H COSY, 2D TOCSY, and HMQC spectra in **1**. Analysis of the 1D TOCSY spectra followed by interpretation of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra allowed us to solve the exact sugar sequences of the sugar moieties and their linkage positions to the aglycon. Because of the selectivity of the multistep coherence transfer, 1D TOCSY method allowed a sub-spectrum of a single monosaccharide unit to be extracted from the crowded overlapped region. The isolated anomeric proton signals and methyl doublet signal of the rhamnosyl moiety, which resonated in an uncrowded region of the spectrum, were used for the starting points of the 1D TOCSY experiments. As a result, the sub-spectrum of each sugar residue was obtained with high digital resolution. Subsequent analysis of the ^{1}H–^{1}H COSY spectrum resulted in the sequential assignments of all the proton resonances for the individual monosaccharides (Table 1). The HMQC and HSQC-TOCSY spectra correlated the proton resonances with those of the corresponding one-bond coupled carbons, leading to the unambiguous assignments of the carbon shifts (Table 1). Comparison of the carbon chemical shift thus assigned with those of the reference methyl glycosides,[10] taking into account the known effects of *O*-glycosylation, indicated that **1** contained an α-L-arabinofuranosyl unit (Ara*f*), a β-D-glucopyranosyl unit (Glc′), and a β-D-xylopyranosyl unit (Xyl) as the terminal glycosyl moieties, and a 2-amino-2-deoxy-β-D-glucopyranosyl unit (GlcN), an α-L-arabinopyranosyl unit (Ara), a β-D-glucopyranosyl unit (Glc), and an α-L-rhamnopyranosyl unit (Rha) as the substituted sugar moieties.[11] Finally, the $^{3}J_{C,H}$ correlation from each anomeric proton across the glycosidic bond to the carbon of another substituted monosaccharide revealed the sugar sequences. In the HMBC spectrum, the anomeric proton signals at δ 6.23 (Ara*f*), 6.14 (Glc), 6.10 (Rha), 5.37 (Glc′), 5.17 (Ara), 5.05 (GlcN), and 5.00 (Xyl) showed correlations with the carbon signals at δ 79.2 (C-4 of Rha), 174.9 (C-28 of aglycon), 76.6 (C-2 of Glc), 82.0 (C-3 of Rha), 69.5 (C-6 of GlcN), 89.1 (C-3 of aglycon), and 80.5 (C-2 of Ara), respectively. The amide proton signal at δ 8.84 had a spin-coupling correlation with H-2 of GlcN ($J = 8.9$ Hz) and showed long-range correlations with the carbonyl carbon signal at δ 170.1 and methyl carbon signal at δ 23.7 of the acetyl group (Fig. 1). Accordingly, **1** was elucidated as 21β-[(*E*)-cinnamoyloxy]-3β-[(*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl)oxy]-olean-12-en-28-oic acid 28-*O*-α-L-arabinofuranosyl-(1→4)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Table 1. ^{1}H and ^{13}C NMR spectral data for the sugar moieties of **1** in C_5D_5N

Positions		δ_H		*J* (Hz)	δ_C	Positions		δ_H		*J* (Hz)	δ_C
GlcN	1	5.05	d	8.3	104.8	Glc	1	6.14	d	7.4	95.4
	2	4.54	ddd	9.4, 8.9, 8.3	57.9		2	4.20	dd	8.6, 7.4	76.6
	3	4.38	dd	9.4, 9.4	75.7		3	4.21	dd	8.6, 8.6	78.4
	4	4.39	dd	9.4, 9.4	73.0		4	4.19	dd	8.6, 8.6	71.3
	5	4.06	m		76.2		5	3.96	m		79.0
	6a	4.65	br d	10.2	69.5		6a	4.37	br d	11.0	62.1
	b	4.21	br d	10.2			b	4.26	br d	11.0	
	NH	8.84	d	8.9							
Ac		2.09	s		170.1						
					23.7						
Ara	1	5.17	d	5.0	102.3	Rha	1	6.10	br s		101.7
	2	4.53	dd	6.4, 5.0	80.5		2	5.19	d	3.1	70.9
	3	4.40	dd	6.4, 2.9	72.6		3	4.92	dd	9.4, 3.1	82.0
	4	4.41	br s		67.4		4	4.52	dd	9.4, 9.4	79.2
	5a	4.32	dd	11.9, 2.7	64.2		5	4.58	dq	9.4, 6.0	69.0
	b	3.76	dd	11.9, 2.6			6	1.85	d	6.0	18.8
Xyl	1	5.00	d	7.0	106.3	Glc′	1	5.37	d	7.8	105.8
	2	4.04	dd	8.5, 7.0	75.4		2	4.01	dd	9.5, 7.8	75.4
	3	4.07	dd	8.5, 8.5	77.9		3	4.14	dd	9.5, 9.5	78.3
	4	4.16	ddd	11.3, 8.5, 4.8	70.9		4	4.21	dd	9.5, 9.5	71.8
	5a	4.39	dd	11.3, 4.8	67.2		5	3.99	m		78.1
	b	3.61	dd	11.3, 11.3			6a	4.49	br d	10.8	62.8
							b	4.24	dd	10.8, 2.4	
						Ara*f*	1	6.23	d	1.8	111.0
							2	4.97	dd	4.7, 1.8	84.9
							3	4.80	dd	7.9, 4.7	78.3
							4	4.74	m		84.7
							5a	4.31	br d	12.0	62.4
							b	4.15	br d	12.0	

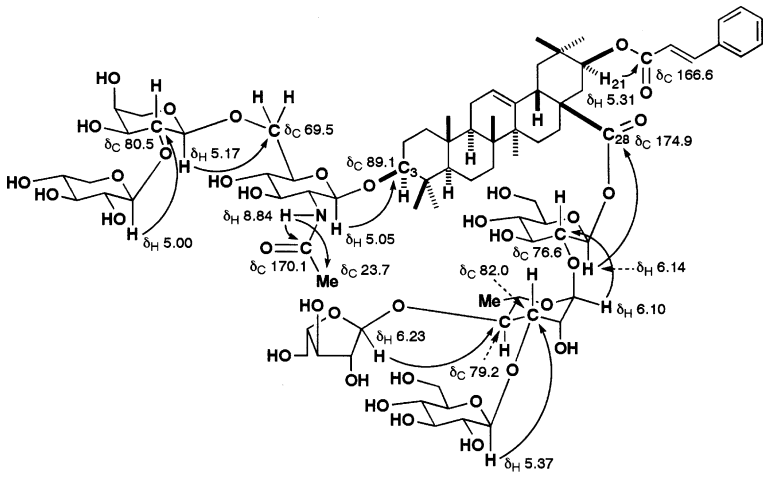

Figure 1. HMBC correlations of the sugar moieties of **1**.

Enterolosaponin B (**2**)[12] was isolated as an amorphous solid. On the basis of the negative-ion FABMS (*m*/*z* 1538 [M–H]$^-$), ^{13}C NMR spectrum (75 carbon signals), and elemental analysis data, its molecular formula was determined as $C_{75}H_{113}NO_{32}$, which was less than that of **1** by $C_5H_8O_4$, corresponding to the lack of one pentose. The ^{1}H NMR spectrum of **2** exhibited signals for six anomeric protons at δ 6.40 (br s), 6.16 (d, J = 8.1 Hz), 5.47 (d, J = 7.8 Hz), 5.18 (d, J = 5.0 Hz), 5.06 (d, J = 8.3 Hz), and 5.00 (d, J = 7.0 Hz), as well as signals for seven methyl groups arising from the aglycon moiety at δ 1.30, 1.25, 1.16, 1.07, 1.01×2, and 0.93. The presence of an acetyl group and an (*E*)-cinnamoyl group in **2** as in **1** was readily revealed by the ^{1}H and ^{13}C NMR spectra. Acid hydrolysis of **2** with 0.7 M HCl (dioxane-H_2O) gave **1a**, 2-amino-2-deoxy-D-glucose, L-arabinose, D-glucose, D-xylose, and L-rhamnose. When the ^{13}C NMR spectrum of **2** was compared with that of **1**, the signals assignable to the terminal α-L-arabinofuranosyl group could not be detected, and the resonance for C-4 of the rhamnosyl moiety was shifted upfield by 6.3 ppm and observed at δ 72.9 in **2**. All other signals appeared at almost the same positions between the two compounds. These chemical and spectral data confirmed that **2** was closely related to **1**, but differed in structure lacking the terminal α-L-arabinofuranosyl group. Thus, the structure of **2** was formulated as 21β-[(*E*)-cinnamoyloxy]-3β-[(*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl)oxy]-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Triterpene saponins are widely distributed in not only the plant kingdom but also the marine organism, and more than 2600 saponins have been reported up to now.[13] However, saponins having a 2-amino-2-deoxy-D-glucosyl unit (D-glucosamine) as one of the monosaccharides constituting their oligosaccharide moieties such as **1** and **2** have been rarely found in natural product research.

Biological Activities of 1 and 2

The cytotoxic activities of **1** and **2** against BAC1.2F5 mouse macrophages, EL-4 mouse lymphoma cells, and L-929 mouse fibroblasts, were examined. Although **2** and the de-(*E*)-cinnamoyl derivative of **1** did not show any apparent cytotoxic activities against all the cell lines, **1** exhibited a highly selective cytotoxicity against BAC1.2F5 mouse macrophages with an LD_{50} value of

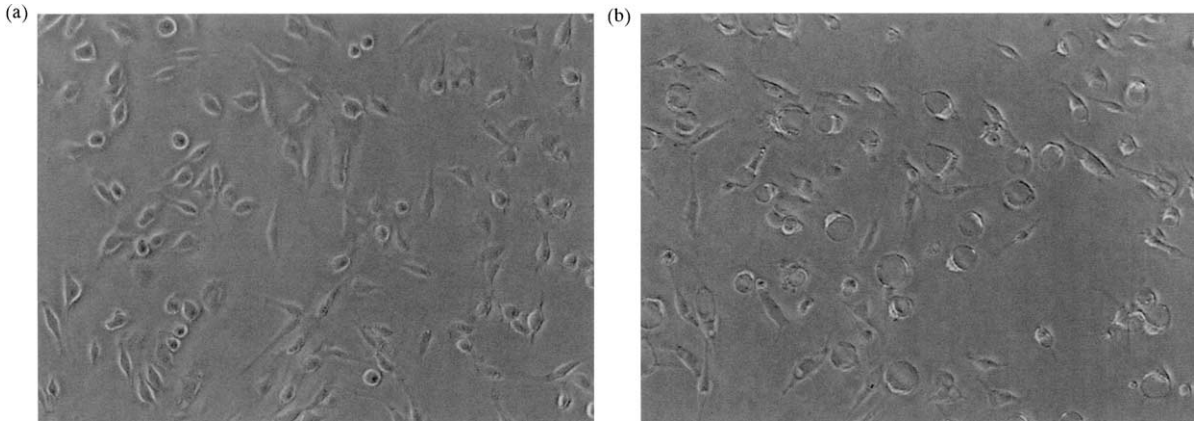

Figure 2. Photomicrographs of macrophages cultured without (a) or with (b) 10 μg/mL of enterolosaponin A (**1**) in the presence of 20% L-cell supernatant for 72 h. Original magnification: ×200 (phase contrast microscopy).

about 3 μM. The cinnamoyl group attached at the C-21β-hydroxyl group and the terminal α-L-arabinofuranosyl group were considered to be essential for the selective cytotoxicity. It should be notable that the macrophage death caused by **1** was shown to be neither necrotic nor due to induction of apoptosis from morphology of the died cells, whose cytosol occurred in vacuolation (Fig. 2). Although the precise mechanism is unknown, one possible could be raised that **1** caused fusion of endosomal membranes to make the large vacuole structure after it internalized by macrophages. Further investigation into the detailed mechanism on the activity of **1** is now under way.

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8. Enterolosaponin A (**1**): $[\alpha]_D$ −28.0° (*c* 0.10, MeOH); anal. C 52.34%, H 7.40%, N 0.47%, calcd for $C_{80}H_{121}NO_{36}\cdot 9H_2O$, C 52.36%, H 7.64%, N 0.76%; ^{13}C NMR of the aglycon moiety (C_5D_5N) δ 38.9 (C-1), 26.6 (C-2), 89.1 (C-3), 39.4 (C-4), 55.9 (C-5), 18.8 (C-6), 33.5 (C-7), 40.0 (C-8), 48.1 (C-9), 37.1 (C-10), 23.9 (C-11), 123.3 (C-12), 142.8 (C-13), 42.4 (C-14), 28.7 (C-15), 24.8 (C-16), 48.7 (C-17), 41.4 (C-18), 46.6 (C-19), 35.4 (C-20), 75.6 (C-21), 36.5 (C-22), 28.1 (C-23), 17.1 (C-24), 15.7 (C-25), 17.4 (C-26), 25.8 (C-27), 174.9 (C-28), 28.8 (C-29), 18.5 (C-30).
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11. The α-orientation of the anomeric center of the Ara moiety, and β-orientations of the GlcN, Glc, Glc′, and Xyl moieties were indicated by the relatively large $^3J_{H\text{-}1,H2}$ values of their anomeric protons. For the Rha moiety, the large $^1J_{C,H}$ values (171.4 Hz) and three-bond coupled strong HMBC correlations from the anomeric proton to the C-3 and C-5 carbons (the dihedral angles between H-1 and C-3, and between H-1 and C-5 about 180°), indicated that the anomeric proton was equatorial thus possessing an α-pyranoid anomeric form. The ^{13}C NMR shift of C-1 of the Ara*f* moiety at δ 111.0 was consistent with the α-anomeric orientation.
12. Enterolosaponin B (**2**): $[\alpha]_D$ −4.0° (*c* 0.10, MeOH); FABMS (negative) *m*/*z* 1538 $[M - H]^-$; anal. C 53.62%, H 7.68%, N 0.48%, calcd for $C_{75}H_{113}NO_{32}\cdot 8H_2O$, C 53.47%, H 7.72%, N 0.83%; UV (MeOH) λ_{max} 278 (log ε 4.27) nm; IR (film) ν_{max} 3363 (OH), 2935 (CH), 1750 and 1709 (C=O), 1636 (CONH) cm^{-1}; ^1H NMR (C_5D_5N) δ 8.86 (1H, d, *J* = 9.0 Hz, NH), 7.93 (1H, d, *J* = 16.0 Hz, H-7 of Cin), 7.66 (2H, dd, *J* = 7.6, 1.6 Hz, H-2 and H-6 of Cin), 7.37 (3H, m, H-3, H-4, and H-5 of Cin), 6.72 (1H, d, *J* = 16.0 Hz, H-8 of Cin), 6.40 (1H, br s, H-1 of Rha), 6.16 (1H, d, *J* = 8.1 Hz, H-1 of Glc), 5.48 (1H, t-like, *J* = 3.5 Hz, H-12), 5.47 (1H, d, *J* = 7.8 Hz, Glc'), 5.32 (1H, dd, *J* = 11.9, 4.6 Hz, H-21), 5.18 (1H, d, *J* = 5.0 Hz, H-1 of Ara), 5.06 (1H, d, *J* = 8.3 Hz, H-1 of GlcN), 5.00 (1H, d, *J* = 7.0 Hz, H-1 of Xyl), 3.46 (1H, dd, *J* = 11.6, 4.3 Hz, H-3), 3.21 (1H, dd, *J* = 13.7, 4.3 Hz, H-18), 2.11 (3H, s, Ac), 1.79 (3H, d, *J* = 6.1 Hz, Me-6 of Rha), 1.30 (3H, s, Me-27), 1.25 (3H, s, Me-23), 1.16 (3H, s, Me-26), 1.07 (3H, s, Me-24), 1.01 (3H×2, s, Me-29 and Me-30), 0.93 (3H, s, Me-25); ^{13}C NMR (C_5D_5N) δ 104.8, 58.0, 75.8, 73.1, 76.4, 69.5 (C-1–C-6 of GlcN), 102.3, 80.6, 72.6, 67.5, 64.3 (C-1–C-5 of Ara), 106.4, 75.5, 77.9, 70.9, 67.2 (C-1–C-5 of Xyl), 95.3, 76.0, 79.2, 71.4, 79.1, 62.1 (C-1–C-6 of Glc), 101.8, 71.5, 83.9, 72.9, 69.9, 18.6 (C-1–C-6 of Rha), 107.0, 75.8, 78.4, 71.4, 78.4, 62.5 (C-1–C-6 of Glc′), 170.1 and 23.7 (Ac). The ^{13}C NMR resonances for the aglycon moiety of **2** were in good agreement with those of **1**.
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