A New Triterpene Glycoside from the Stems of *Akebia quinata*

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Akebiae caulis, dried stems of *Akebia quinata* (Lardizabalaceae), is an important crude drug used mainly as a diuretic agent for the treatment of edema and rheumatic pain.[1,2] Previous phytochemical investigations resulted in the isolation of triterpenes, triterpene glycosides, and phenylethanoid glycosides.[3–5] Regarding the biological activity of *A. quinata*, only the cytotoxic effect of oleanane disaccharides has been reported so far.[6] The anti-inflammatory activity of this plant has not been explored yet. We have recently reported the isolation of lignan glycoside derivatives with an anti-inflammatory effect from this plant.[7] In continuing research on this source, a new triterpene glycoside (**1**) and seven known triterpene glycosides (**2–8**) were further isolated from the *n*-BuOH soluble fraction. The structure of **1** was identified on the basis of 1D and 2D NMR, including ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY spectroscopic analyses. Here we report the isolation, structure elucidation of the new triterpene glycoside (**1**), and the inhibitory effects of all isolates on the NO production in lipopolysaccharides (LPS)-stimulated RAW 264.7 cells (Figure 1).

Compound **1** was obtained as a white amorphous powder, $[\alpha]_D^{25}$ + 19.4° (*c* 0.77, MeOH). Its molecular formula was identified as $C_{47}H_{74}O_{18}$ by the high-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOF-MS) data at *m/z* 949.4770 $[M + Na]^+$ ($C_{47}H_{74}O_{18}Na$, calcd. for 949.4773). In the infrared (IR) spectrum, absorption bands for hydroxyl (3350 cm^{-1}) and carbonyl (1730 cm^{-1}) groups were observed. The ^{1}H-NMR spectrum of **1** (Table 1) displayed signals due to four tertiary methyl groups at δ_H 0.81, 1.00, 1.14, and 1.16 (each 3H, s, H-24, 25, 26, 27), an olefinic proton at δ_H 5.47 (1H, br s, H-12), an oxygenated methine proton at δ_H 3.69 (1H, br s, H-3), an exomethylene proton each at δ_H 4.68 and 4.79 (each 1H, s, H-29a, and H-29b), one hydroxymethyl proton at δ_H 3.67 (1H, d, *J* = 10.6 Hz, H-23a) and 3.87 (1H, d, *J* = 10.6 Hz, H-23b), and three anomeric protons at δ_H 6.23 (1H, d, *J* = 7.6 Hz, H-1′), 5.88 (1H, br s, H-1‴), and 4.98 (1H, d, *J* =7.6 Hz, H-1″). The spectral features and physicochemical properties of **1** suggested that **1** would be a triterpene glycoside. The ^{13}C-NMR spectrum displayed 47 carbon signals; 29 of them were assigned to the aglycone part, and the others to the oligosaccharide. In the ^{13}C-NMR spectrum, four tertiary methyl groups at δ_C 16.3, 17.9, 18.9, and 26.4 (CH_3-24, 25, 26, and 27), two oxygenated carbons at δ_C 77.5 (C-3) and 71.6 (C-23), an olefinic carbon at δ_C 143.8 (C-12) and 123.4 (C-13), an exomethylene carbon at δ_C 107.6 (C-29), and a carbonyl carbon at δ_C 176.2 (C-28) were assigned to a 30-noroleanane type of triterpene.[8] Another 18 carbon signals were assigned to three sugar moieties. Based on the ^{1}H and ^{13}C NMR data, the structure of aglycone of **1** was closely related to quinatic acid, which was isolated from the callus tissue of *A. quinata*[8] except for the different substituent of C-23 in **1**. Meanwhile, three anomeric proton signals at δ_H 6.23 (1H, d, *J* = 7.6 Hz, glc-H1′), 5.88 (1H, br s, Rha-H1‴), and 4.98 (1H, d, *J* = 7.6 Hz, glc-H1″) showed HMQC correlations with anomeric carbon signals at δ_C 96.1 (glc-C1′), 103.0 (Rha-C1‴), and 105.3 (glc-C1″), indicating that **1** possessed three sugar units (Table 1).[9,10] Acid hydrolysis of **1** with 1M HCl in MeOH produced aglycone, as well as L-rhamnose and D-glucose as the sugar moieties. These sugars were identified by direct high-performance liquid chromatography (HPLC) analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column, and direct comparison with an authentic sample using co-TLC (sugars of Compound 1was confirmed by HPLC. And compound 1 direct comparison with an authentic sample using co-TLC.).[4] Furthermore, the configuration of the glucopyranosyl and rhamnopyranosyl were assigned to be β– and α–, respectively, according to the coupling constants of the anomeric protons.[11] In addition, the upfield chemical shift of C-28 (δ_C 176.2) indicated that sugar moieties should be located at C-28 in **1**. The sequence of sugar residues of **1** was assigned unambiguously on the basis of HMBC spectra. The location of the sugar chain at C-28 was identified as *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl from the following HMBC correlations: H-1‴ of rhamnose (δ_H 5.88) with C-4″ of glucose (δ_C 78.6), H-1″ of glucose (δ_H 4.98) with C-6′ of glucose (δ_C 69.7), and H-1′ of glucose (δ_H 6.23) with C-28 of aglycone (δ_C 176.2). Furthermore, the HMBC correlation between H-29 and C-19/C-21 indicated that 20(29)-exomethylene group was connected at C-20. The relative configuration of the

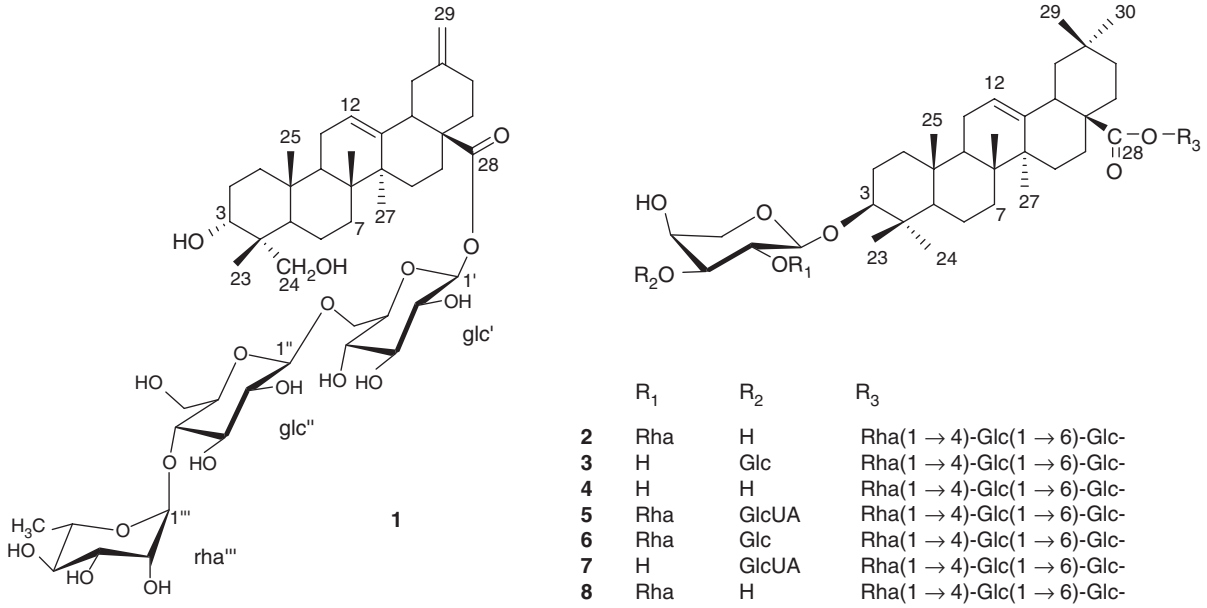

Figure 1. Structures of compounds **1–8** from *A. quinata.*

Table 1. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) data of **1** in pyridine-d_5 (δ in ppm)

Position	δ_C	δ_H	Position	δ_C	δ_H
1	33.7	1.40 (2H, m)	Glc-1′	96.1	6.23 (d, *J* = 8.1)
2	26.9	1.85 (1H, m), 1.11 (1H, m)	2′	74.1	4.12 (dd, *J* = 9.0, 8.1)
3	77.5	3.94 (1H, br s)	3′	79.0	4.22 (dd, *J* = 9.0, 9.0)
4	43.8		4′	71.2	4.31 (dd, *J* = 9.0, 9.0)
5	41.0	1.69 (1H, m)	5′	78.3	4.08 (m)
6	18.5	1.72 (2H, m)	6′a	69.6	4.65 (dd, *J* = 11.7, 3.0)
7	33.1	1.35 (2H, m)	6′b		4.29 (m)
8	40.4		Glc-1″	105.3	4.98 (d, *J* = 7.8)
9	48.3	1.93 (1H, m)	2″	75.6	3.91 (dd, *J* = 9.2, 7.8)
10	37.6		3″	76.1	4.12 (dd, *J* = 9.2, 9.2)
11	23.8	1.96 (2H, m)	4″	78.5	4.36 (dd, *J* = 9.2, 9.2)
12	123.4	5.48 (1H, br s)	5″	76.9	3.63 (dd, *J* = 9.2, 3.2)
13	143.8		6″a	61.6	4.18 (br d, *J* = 11.9)
14	42.5		6″b		4.07 (dd, *J* = 11.9, 3.2)
15	28.5	1.17 (1H, m), 2.31 (1H, m)	Rha-1‴	103.0	5.88 (br s)
16	24.1	2.00 (2H, m)	2‴	72.9	4.66 (br d, *J* = 3.3)
17	47.7		3‴	73.1	4.53 (dd, *J* = 9.3, 3.3)
18	47.9	3.14 (dd, *J* =14.5, 4.0)	4‴	74.3	4.31 (dd, *J* = 9.3, 9.3)
19	42.0	2.19 (2H, m)	5‴	70.6	4.91 (dd, *J* = 9.3, 6.2)
20	148.7		6‴	18.7	1.73 (d, *J* = 6.2)
21	30.4	2.18 (1H, m), 2.08 (1H, m)			
22	38.0	2.02 (1H, m),1.75 (1H, m)			
23	71.6	3.87, 3.67 (each, 1H, d, *J* = 10.5)			
24	18.9	0.81 (3H, s)			
25	16.3	1.00 (3H, s)			
26	17.9	1.14 (3H, s)			
27	26.4	1.16 (3H, s)			
28	176.1				
29	107.6	4.74 (1H, br s), 4.68 (1H, br s)			

Assignments were based on 2D NMR including COSY, HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hertz in parentheses.

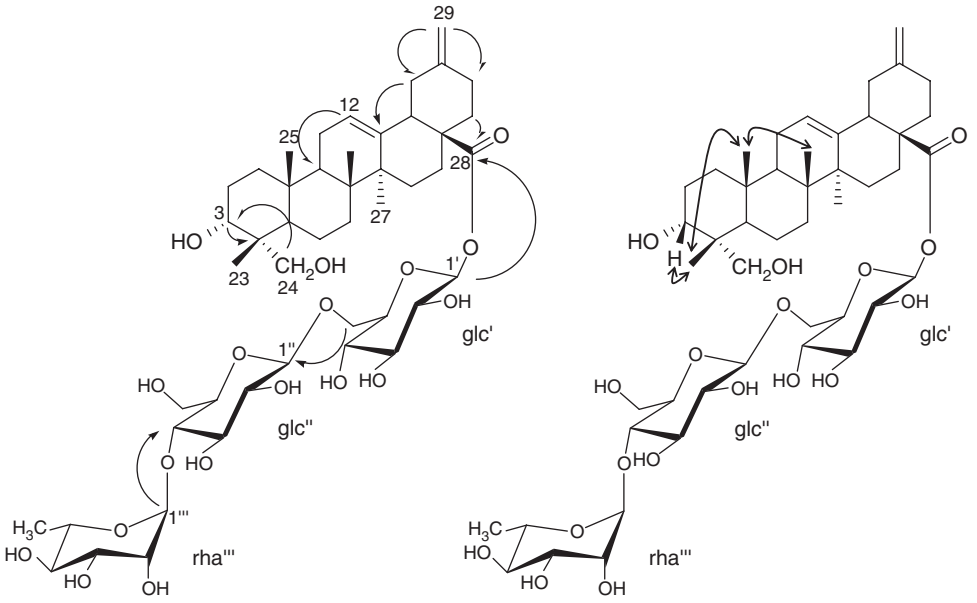

Figure 2. Key HMBC and NOESY correlations of compound.

hydroxyl groups at C-3 and C-23 was proposed from the NOESY experiment (Figure 2) and from the comparison of the observed and reported NMR data.[3,12] In the ^{1}H-NMR spectrum, the coupling pattern of H-3 (δ_H 3.69, br s) supported the β-orientation of H-3. In addition, in the NOESY spectrum, correlations between H-3 and Me-24, Me-24, and Me-25/Me-26 indicated that these are on the same side (β). Based on these results, the relative configuration of the hydroxyl groups at C-3 and C-23 were identified as the α form. Accordingly, compound **1** was identified as 3α,23α-dihydroxy-30-norolean-12,20(29)-dien-28-oic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl ester, named, akeqintoside E, as shown in Figure 1.

The structures of the other known compounds (**2–8**) were identified as akeboside St$_h$ (**2**),[13] begoniifolide A (**3**),[14] akebia saponin P$_{J2}$ (**4**),[15] 3β-[(*O*-GlcUA-(1–3)-*O*-[Rha-(1–2)]-Ara) oxy]olean-12-en-28-oic acid *O*-Rha-(1–4)-Glc-(1–6)-Glc ester (**5**),[4] kalopanax saponin D (**6**),[13] 3β-[(*O*-GlcUA-(1–3)-Ara)oxy]olean-12-en-28-oic acid *O*-Rha-(1–4)-Glc-(1–6)-Glc ester (**7**),[4] and akebia saponin P$_{J3}$ (**8**)[5] by comparing their spectroscopic data with those reported in the literature. All isolated compounds were examined for their inhibitory effects on LPS-induced NO production in RAW 264.7 cells. The IC$_{50}$ values of the isolated compounds (**1–8**) were more than 100 μM, while that for compound **8** was 65.59 μM (data not shown). All isolated triterpene glycosides (**1–8**) were inactive.

Experimental

General Procedures. Optical rotations were measured using an Autopol-IV polarimeter. IR spectra were recorded on an IMS 85 (Bruker). HR-ESI-MS spectra were obtained on a Q-TOF (Synapt HDMS system, Waters, USA) mass spectrometer. NMR spectra, including NOESY, COSY, heteronuclear multiple quantum coherence (HMQC) and HMBC experiments, were recorded on a Varian VNMRS 600 MHz spectrometer (KBSI-Gwangju center) operating at 600 (^{1}H) and 150 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ). TLC was carried out on precoated Kieselgel 60 F$_{254}$ (art. 5715, Merck, Germany) and RP-18 F$_{254}$s (art. 15389, Merck, Germany) plates. Column chromatography (CC) was performed on silica gel 60 (40–63 and 63–200 μM, Merck, Germany), Diaion HP 20 (75–150 μM, Mitsubishi Chemical Co., USA), and Sephadex LH-20 (25–100 μM, Sigma, Sweden). Silver carbonate (Ag_2CO_3, Aldrich Co., Koenigs–Knorr glucosylation, USA), D-(+)glucose ($C_6H_{12}O_6$, Sigma), and L-(+)rhamnose ($C_6H_{12}O_5$, Sigma) were used as neutralization reagents, and standard sugar on acid hydrolysis experiment, respectively. HPLC was performed using a Waters HPLC system equipped with Waters 600 Q-pumps, an ELSD detector, and a Capcell Pak (NH_2) UG 80 column (250 × 4.6 mm, 5 μM, Shiseido, Japan) (flow rate 1.0 mL/min, solvent (MeCN/H_2O = 17:3)). Low-pressure liquid chromatography was carried out over a Merck Lichroprep Lobar®-A RP-18 (240 × 10 mm) column with an FMI QSY-0 pump (ISCO).

Plant Material. The stems of *A. quinata* were collected in Gyeongju, Gyeongbuk province, Korea, in August 2011 and identified by Professor J.H. Lee, one of the authors. A voucher specimen (CSU-877-17) is deposited in the Herbarium of the College of Pharmacy, Chosun University.

Extraction and Isolation. The air-dried stems of *A. quinata* (11 kg) were cut and extracted with MeOH three times for 4 h at 80 °C. The resultant MeOH extract (480 g) was suspended in water (1.5 l × 3) and then partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and *n*-butanol. Each fraction was evaporated *in-vaccuo* to yield the residues of CH_2Cl_2 (45.2 g), EtOAc (11.0 g), *n*-BuOH (57.0 g), and

water (150.3 g) extracts. The *n*-BuOH soluble fraction (22.0 g) was subjected to CC over a Diaion HP 20 column and eluted with H_2O/MeOH (100:0 → 0:100) gradient system to yield six subfractions (B1–B6). Subfraction B5 (2.6 g) was purified by Sephadex LH 20 CC (MeOH/H_2O, 1:1 → 2:1) to yield three subfractions (B51–B53). Subfraction B51 (1.6 g) was purified by Lichroprep RP 18 CC (MeOH/H_2O, 3:2) to yield 10 subfractions (B511–B5110). Subfraction B514 (0.14 g) was purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 4:1:0.2 → 2:1:0.2) to yield **8** (85.2 mg). Subfraction B515 (0.13 g) was purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 2:1:0.2) to yield **3** (18.7 mg), **4** (46.6 mg), and **1** (9.3 mg). In addition, subfraction B516 (0.13 g) was purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 2:1:0.2) to yield **5** (13.1 mg) and **6** (20.7 mg). Subfraction B517 (0.13 g), and B519 (0.12 g) were then purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 2:1:0.2 and 3:1:0.2) to yield **2** (35.9 mg) and **7** (22.4 mg), respectively.

Acidic Hydrolysis of Compound 1. Compound **1** (3 mg) was dissolved in 1N HCl (1 mL) and MeOH (1 mL), and refluxed at 90 °C for 90 min.[16] The reaction solution was evaporated under reduced pressure, and the hydrolysate was extracted with EtOAc (3 × 3 mL). The aqueous fraction was neutralized with Ag_2CO_3 and filtered. The filtrate was then concentrated under reduced pressure. The residue was compared with a standard sugar using TLC ($CHCl_3$:MeOH:H_2O, 6/4/1), which revealed the sugar to be D-(+)glucose (R_f = 0.26), and L-(+) rhamnose (R_f = 0.46) in **1**. In addition, HPLC of the sugar fraction showed the presence of L-rhamnose and D-glucose; t_R (min) 7.6 and 15.0, respectively.

Akeqintoside E (1). Colorless gum; $[\alpha]_D^{25}$ +19.4° (*c* 0.77, MeOH); IR (KBr) ν_{max} 3350, 2950, 2830, 1730, 1660, 1452, 1115, 1032 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; HR-ESI-TOF-MS (positive mode) *m/z*: 949.4770 $[M + Na]^+$ (calcd. for $C_{47}H_{74}O_{18}Na$, 949.4773).

Measurement of NO Production and Cell Viability Assay. Nitric oxide production was determined by measuring the amount of nitrite from cell culture supernatant as previously described.[17] Briefly, RAW 264.7 cells (1 × 10^5 cells/well) were cultured in a flat-bottom 96-well microtiter plate in quadruplicate for 12 h. Thereafter, 100 μL of the medium was replaced with fresh medium containing either compound and 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, USA), and further cultured for 24 h. The culture supernatant was collected at the end of culture for nitrite assay, which was used as a measure of NO production. The culture supernatant (100 μL) was mixed with equal volume of Griess reagent at room temperature for 10 min. The absorbance was measured at 540 nm by a microplate reader. The cells remaining after Griess assay were used for cell viability with the MTT-based colorimetric assay.

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Supporting Information. Spectral data of compound **1**, general experimental procedures, and the isolation details are available upon request from the corresponding author.

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