Lupane Triterpene Glycosides from Leave of *Acanthopanax koreanum* and Their Cytotoxic Activity

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One new lupane-triterpene glycoside, acankoreoside I (1), and four known compounds, acankoreoside A (2), acankoreoside D (3), acankoreoside F (4), and acantrifoside A (5), were isolated from the leaves of *Acanthopanax koreanum* (Araliaceae). Their chemical structures were elucidated by mass, 1D- and 2D-NMR spectroscopy. The structure of new compound 1 was determined to be 3α,11α,30-trihydroxylup-23-al-20(29)-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester. The cytotoxic activities of these compounds were evaluated with four cancer cell lines such as A-549 (lung), HL-60 (acute promyelocytic leukemia), MCF-7 (breast), and U-937 (leukemia). Compound 1 showed growth inhibitory effect in A-549, HL-60, and MCF-7 cell lines with the IC_{50} values of 8.2 μM, 12.1 μM, and 28.6 μM, respectively, whereas it was less active in the U937 cell line (the IC_{50} >100 μM).

Key words *Acanthopanax koreanum*; Araliaceae; lupane-triterpene glycoside; acankoreoside I; cytotoxic activity

Acanthopanax koreanum NAKAI is deciduous scrub of the Araliaceae family and an endemic species in Korea. The roots and stem bark are used to treat sinew, bone pains, rheumatism, hepatitis and diabetes in traditional oriental medicine.[1)] Several studies on the triterpenoid constituents of *A. koreanum* have been reported.[2—8)] There are many studies specially citing cytotoxic and anticancer activities of the lupane triterpenoids.[9—11)] A number of derivatives bearing the lupane skeleton have been found to possess potential anticancer capacity.[11)] Interestingly, our extensive phytochemical study led to isolate one new lupan triterpenoid glycoside, as well as four known triterpenoid glycosides. However, there have no studies with regard to the anticancer activity of these isolated compounds. For these reasons, evaluation of the cytotoxic activities of these isolated compounds was performed against four specific cancer cell lines with A-549, HL-60, MCF-7, and U-937 using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Results and Discussion

Compound **1** was obtained as a white amorphous powder from the methanolic extract of *A. koreanum* leaves by various chromatographic techniques. Its basic ion peak at *m/z* 973 $[M+H]^+$ was observed in the positive-ion electrospray ionization (ESI)-MS, and HR-FAB-MS analysis revealed the molecular formula to be $C_{48}H_{76}O_{20}$, with a cluster ion peak at *m/z* 995.4831 $[M+Na]^+$ (Calcd for $C_{48}H_{76}O_{20}Na$: 995.4827). The ^{1}H-NMR spectrum of **1** (in methanol-d_4) showed the following signals: four tertiary methyl groups at δ 0.74, 0.99, 1.09, and 1.11 (each 3H, s); one secondary methyl group at δ 1.27 (3H, d, J=6.0 Hz), assigned to H″-6 of the rhamnose; and three anomeric protons were at δ 4.40 (1H, d, J=8.0 Hz), 4.85 (1H, br s), and 5.45 (1H, d, J=8.0 Hz), suggesting the appearance of three sugar units. The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra revealed 48 carbon signals, of which, 30 signals were assigned to a triterpenoid sapogenol moiety and 18 signals belong to three monosaccharide moieties. The aglycone of **1** was recognized to be a lupane triterpene type by ^{1}H- and ^{13}C-NMR analysis (see Table 1), with the typical olefinic carbons at δ 155.9 (C-20) and 107.7 (C-29), five quaternary methyl carbons at δ 15.1, 15.2, 17.2, 18.0, and 18.3, one oxymethine carbon at δ 73.8, one oxymethylene carbon at δ 65.5, and carboxyl and aldehydic signals at δ 176.3 and δ 211.2, respectively. Assignment of the α-hydroxyl group at C-3 was performed by comparing its spectral data with literature values.[12—15)] The chemical shifts of C-1 (δ 35.9), C-5 (δ 44.5), and C-24 (δ 15.2) in the aglycone further confirmed the *axial* configuration of the 3-hydroxyl group by comparing with the corresponding data of the 3β-hydroxy-lup-20(29)-en-23-al-28-oic acid[16)] [δ values for 38.9 (C-1), 47.9 (C-5), and 9.4 (C-24)]. Nevertheless, the carbon chemical shifts of C-11 (δ 71.1) and C-23 (δ 211.2) were also similar to those of acankoreoside D suggesting that the α-hydroxyl group was at C-11 and the aldehydic group was at C-23.[4)] In the heteronuclear multiple bond connectivity (HMBC) spectrum, the aldehydic proton signal at δ 9.48 (1H, s) correlated with carbons C-3 (δ 73.8), C-4 (δ 53.7), and C-5 (δ 44.5); the proton signal at δ 0.99 (3H, s, H-24) correlated with carbons C-3 (δ 73.8), C-4 (δ 53.7), and C-23 (δ 211.2). In the rotating frame Overhauser effect spectroscopy (ROESY) spectrum, cross-peaks between H-24 and H-25 as well as H-3, which indicated that the methyl group (H-24) was *axial*, which in turn suggested that the aldehyde group at C-4 was α-positioned. Furthermore, in the HMBC spectrum, the C-30 signal at δ 65.5 had correlations with proton signals at δ 4.95 (1H, br s, H_a-29) and 5.01 (1H, br s, H_b-29), which also correlated with C-19 (δ 43.7) and C-20 (δ 155.9). This evidence confirmed that the double bond was at C-20/C-29 and the other hydroxyl group was at C-30. Acid hydrolysis of **1** (see Experimental) provided the monosaccharide components of rhamnose and glucose (identified as trimethylsilylimidazole (TMS) derivatives). Moreover, the HMBC correlations between the inner glc H-1 (δ 5.45) and C-28 of the

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Table 1. The NMR Spectral Data of Compound **1**

Position	**1**	
	δ_H[a,c] (mult., Hz)	δ_C[a,b]
Aglycon		
1	1.39 (m)	35.9
2	1.43(m), 1.90 (m)	27.0
3	3.61 (m)	73.8
4	—	53.7
5	2.02 (m)[d]	44.5
6	1.50 (m)[d]	22.0
7	1.31, 1.59 (m)[d]	36.2
8	—	41.6
9	1.67 (d, 10.5)	56.3
10	—	38.2
11	3.60 (m)	71.1
12	1.29 (m)	38.3
13	2.70 (m)	39.5
14	—	44.3
15	1.18 (d, 13.5)	30.8
	1.49 (m)	
16	2.36 (t, 9.5)	32.8
17	—	57.9
18	1.84 (m)	50.9
19	2.90 (dt, 4.5, 11.0)	43.7
20	—	155.9
21	2.08 (m)	33.5
22	1.52, 1.95 (m)	37.3
23	9.48 (s)	211.2
24	0.99 (s)	15.2
25	1.09 (s)	17.2
26	0.74 (s)	18.3
27	1.11 (s)	15.1
28	—	176.3
29	4.95 (br s)	107.7
	5.01 (br s)	
30	4.09 (s)	65.5
C-28 O-glc		
1	5.45 (d, 8.0)	95.3
2	3.33 (m)[d]	74.0
3	3.43 (m)[d]	78.2
4	3.41 (m)[d]	71.0
5	3.43 (m)[d]	78.3
6	3.80 (m)[d]	69.4
	4.12 (d, 10.5)	
glc′(1→6)glc		
1′	4.40 (d, 8.0)	104.4
2′	3.24 (m)[d]	75.4
3′	3.47 (m)[d]	76.7
4′	3.54 (m)[d]	79.6
5′	3.30 (m)[d]	76.9
6′	3.66 (m)[d]	62.0
	3.82 (d, 6.5)	
rha(1→4)glc′		
1″	4.85 (br s)	103.0
2″	3.64 (dd, 3.5, 9.5)	72.2
3″	3.84 (m)[d]	72.5
4″	3.41 (t, 9.5)	73.8
5″	3.98 (dd, 6.0, 9.5)	70.7
6″	1.27 (d, 6.0)	18.0

a) Measured in CD_3OD, *b*) 125 MHz, *c*) 500 MHz, *d*) Overlapped signals, assignments were done by HMQC, HMBC, COSY and ROESY experiments; glc, β-D-glucopyranosyl; rha, L-rhamnopyranosyl.

aglycone (δ 176.3), between outer glc H-1′ (δ 4.40) and inner glc C-6 (δ 69.4), between rha H-1″ (δ 4.85) and glc C-4′ (δ 79.6) were observed. This evidence suggests the sequence of sugar linkages of **1**. The carbon signals of the sugar moiety were superimposable on those of characteristic triterpene glycosides isolated from *Acanthopanax* species.[2—8,12—18] Consequently, the structure of **1** was determined as 3α,11α,30-trihydroxylup-23-al-20(29)-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**1**).

Table 2. The Effects of Lupane Triterpene Glycosides on the Growth of Human Cancer Cells

Compounds	IC_{50} [μM][a]			
	A-549 (Lung)	HL-60 (Leukemia)	MCF-7 (Breast)	U-937 (Leukemia)
1	8.2	12.1	28.6	>100
2	12.1	18.9	16.9	16.5
3	9.2	>100	12.5	>100
4	32.1	33.2	16.5	21.5
5	21.5	22.5	16.5	18.5
MX[b]	7.2	8.4	10.3	12.1

a) IC_{50} (concentration that inhibits 50% of cell growth). Compounds were tested at a maximum concentration of 100 μM. Data are presented as the mean of experiments performed in triplicate. *b*) Mitoxantrone (MX), an anticancer agent, was used as reference compound.

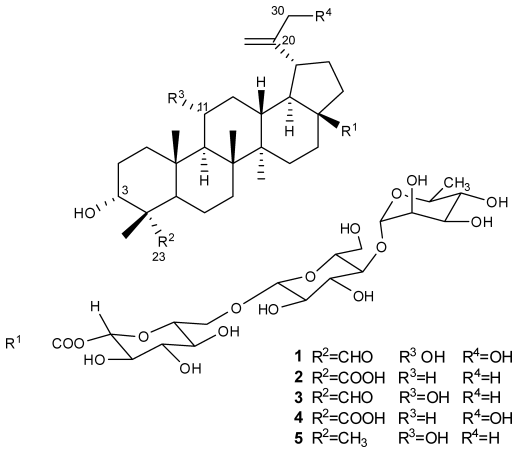

Fig. 1. Structures of Compounds **1**—**5**

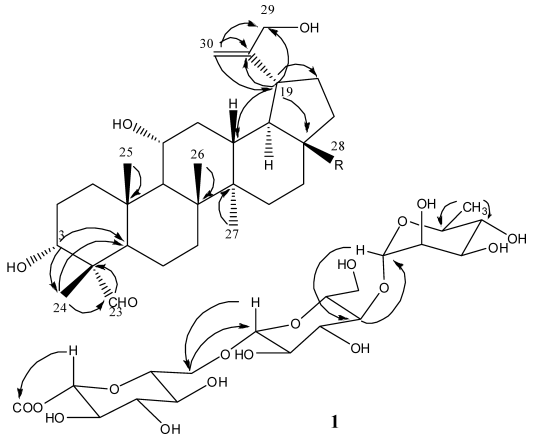

Fig. 2. Selected HMBC Spectrum of Compound **1**

Compounds **2**—**5** were identified as acankoreoside A,[3] acankoreoside D,[4] acankoreoside F,[8] and acantrifoside A,[2] respectively, by comparison the NMR and mass spectral data with the literature values.

Compounds **1**—**5** were screened for their cytotoxicity

against A-549, HL-60, MCF-7, and U-937 cell lines, after continuous exposure for 72 h (see Table 2). Compounds **2**, **4**, and **5** showed moderate cytotoxic activity against A-549, HL-60, MCF-7, and U-937 cell lines with the IC_{50} values ranging from 12.1 to 33.2 μM. Whereas, compound **1** exhibited considerable cytotoxic activity against A-549 and HL-60 cell lines with IC_{50} values of 8.2 μM and 12.1 μM, respectively, comparing to these of positive control (mitoxantrone, IC_{50}: 7.2 and 8.4 μM, respectively), except for MCF-7 (moderate activity with IC_{50} of 28.6 μM) and U-937 (inactive effect at the concentration value of 100 μM). In addition, compound **3** exhibited no cytotoxic activity against HL-60 and U-937 at the concentration value of 100 μM, except for the significant cytotoxicity against A-549 and MCF-7 cell lines with IC_{50} values of 9.2 and 12.5 μM, respectively. This is the first report on the cytotoxic effect against A-549, HL-60, MCF-7, and U-937 cell lines with these compounds. Until now, a number of studies have been reported on the cytotoxicity of lupan type against different tumor and cancer cell lines.[19—22] This has led to betulinic acid, the best-known representative of lupane-derived compound exhibited broader cytotoxicity against different cancer cell lines.[20,23] In the study on relationship between structure and activity of lupan triterpenoids reported that C-3 derivatives and ring A modified compounds, C-28 derivatives and ring E modified compounds, and derivatives *via* the isopropylidene moiety played an important role on the cytotoxic potency against cancer cells.[22,24] In the current study, the presence of a carbonyl group at C-23 of compounds **1** and **3** may influence the cytotoxicity against selective cancer cell lines, comparing to those of **2**, **4**, and **5**. Moreover, the presence a sugar moiety at C-28 does not affect the cytotoxic potency when comparing to those of lupan aglycon-type.[22] In conclusion, this is noteworthy for further study in order to discover new, effective and low side effect drugs.

Experimental

General Experimental Procedures Optical rotations were determined on a Jasco DIP-370 automatic polarimeter. The FT-IR spectra were obtained from a Jasco Report-100 infrared spectrometer. Preparative HPLC was carried out using a Waters HPLC system (600 pump, 600 controller, and a 996 photodiode array detector). The NMR spectra were recorded using a Bruker DRX 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz), and the FAB-MS using a JEOL JMS-HX/HX110A tandem mass spectrometer. The ESI-MS was obtained on an AGILENT 1200 SERIES LC-MSD Trap spectrometer. GC spectra were recorded on a Shimadzu-2010 spectrometer. Column chromatography was performed using a silica-gel (Kieselgel 60, 70—230 mesh and 230—400 mesh, Merck) or YMC RP-18 resins (30—50 μm, Fujisilisa Chemical Ltd.), and thin layer chromatography (TLC) using a pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

Plant Material The leaves of *A. koreanum* were collected in Susin Ogapi, Cheonan City, Korea and identified by one of authors (Prof. Young Ho Kim). A voucher specimen (CNU 070614) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Korea.

Extraction and Isolation The dried leaves of *A. koreanum* (8.0 kg) were extracted with MeOH three times under reflux for 15 h to yield 960 g of a dark solid extract, which was then suspended in H_2O and then extracted with ethyl acetate giving the EtOAc (400 g) and water (300 g) extracts after removal solvent *in vacuo*. The H_2O soluble fraction (300 g) was chromatographed on a Dianion HP-20P column (Mitsubishi Chem. Ind. Co., Japan) eluting with water containing increasing concentrations of MeOH (100% H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, and 100% MeOH). The 20% and 40% MeOH fractions were combined and chromatographed on a silica gel column eluting with $CHCl_3$–MeOH–H_2O (75 : 20 : 3, v/v/v) to give 4 fractions, F1—F4, with amounts as 5.0 g, 15.0 g, 7.3 g, and 5.2 g, respectively. Sub-fraction F2 was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH–H_2O (75 : 20 : 3, v/v/v) to obtain compound **3** (120.9 mg). Sub-fraction F3 was further chromatographed on a silica gel column eluting with CH_2Cl_2–MeOH–H_2O (60 : 20 : 2, v/v/v) to give five smaller fractions, F3A—F3E. Fraction F3B was chromatographed on a YMC RP-18 column eluting with acetone–water (1 : 1, v/v) to yield **4** (20.7 mg). Fraction F3E was chromatographed on a silica gel column using CH_2Cl_2–MeOH–H_2O (40 : 20 : 3, v/v/v) as an eluent to obtain **2** (6.5 mg) and **5** (10.4 mg). Sub-fraction F4 was further separated on a YMC RP-18 column eluting with acetone–methanol–water (1 : 2 : 2, v/v/v) to give four fractions F4A—F4D. The new compound **1** (6.0 mg) was purified from fraction F4A through chromatography on a YMC RP-18 column eluting with acetone–water (1 : 2, v/v).

Acankoreoside I (**1**): An amorphous white powder, $[\alpha]_D^{25}$ −46.3° (c=0.5 in MeOH); IR (KBr) ν_{max} cm^{-1}: 3423 (OH), 1732 (HC=O), 1215 (C–O); positive ESI-MS *m/z*: 973 $[M+H]^+$, 995 $[M+Na]^+$, HR-FAB-MS found *m/z*: 995.4831 $[M+Na]^+$; Calcd $C_{48}H_{76}O_{20}Na$ for 995.4827, ^{1}H- and ^{13}C-NMR: see Table 1.

Acid Hydrolysis of 1 Compound **1** (2.0 mg) was dissolved in 1.0 N HCl (dioxane–H_2O, 1 : 1, v/v, 1.0 ml) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 ml of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 ml) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 ml of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 ml, each), and the organic layer was analyzed by gas liquid chromatography (GC): Column: column SPB-1 (0.25 mm×30 m); detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (2.0 ml/min). The retention times of persilylated glucose and rhamnose were found to be 14.11 and 4.50 min, respectively, when compared with the standard solutions prepared by the same reaction from the standard monosaccharides. (The retention times of persilylated D-glucose, L-glucose and L-rhamnose were 14.11, 14.26, and 4.50 min, respectively.)

Cytotoxicity Tests Compounds **1**—**5** on the growth of human cancer cells were determined by measuring the cytotoxic activity using a MTT assay.[25] Four human cancer cell lines were used. The A-549 (human lung cancer), HL-60 (human acute promyelocytic leukemia), MCF-7 (human breast cancer), and U-937 (human leukemia) cell lines were obtained from the Korea Cell Line Bank (KCLB) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 g/ml, respectively) at 37 °C in a humidified 5% CO_2 atmosphere. The exponentially growing cells were used throughout the experiments.

The MTT assays were performed as follows: human cancer cells (1.5—2.5×10^5 cells/ml) were treated for 3 d with 1, 10, 30 and 100 μM of compounds. Mitoxantrone was used to final concentrations of 1, 3, 10 and 20 μM as a reference. After incubation, 0.1 mg (50 μl of a 2 mg/ml solution) MTT (Sigma, St. Louis, MO, U.S.A.) was added to each well and the cells were then incubated at 37 °C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. Dimethylsulfoxide (150 μl) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., U.S.A.). All the experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of crude extract or solvent fractions compared to the untreated controls. A dose–response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound as well as each cell line.

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