Cytotoxicity of Triterpenes Isolated from *Aceriphyllum rossii*

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Bioassay-guided fractionation of a MeOH extract of the whole plant of *Aceriphyllum rossii* (Saxifragaceae) led to the isolation of two new triterpenes, 3α,23-isopropylidenedioxyolean-12-en-27-oic acid (1) and 23-hydroxy-3-oxoolean-12-en-27-oic acid (2), together with six known triterpenes, 3-oxoolean-12-en-27-oic acid (3), 3α-hydroxyolean-12-en-27-oic acid (4), β-peltoboykinolic acid (5), aceriphyllic acid A (6), oleanolic acid (7), and gypsogenic acid (8). The structures of these compounds were elucidated on the basis of physicochemical and spectroscopic analyses. These compounds were evaluated for *in vitro* cytotoxicity against the K562 and HL-60 cell lines. Olean-12-en-27-oic acid derivatives (1—6) exhibited considerable cytotoxicity against K562 and HL-60 cell lines with IC_{50} values ranging from 12.2 to 28.7 μM and from 12.1 to 25.8 μM, respectively.

Key words *Aceriphyllum rossii*; Saxifragaceae; triterpene; cytotoxicity

Aceriphyllum rossii Engler (Saxifragaceae), an endemic species in Korea, is a perennial herb growing on damp rocks along valleys, usually to a height of 30 cm. It has deeply lobed maple-like leaves and thick brown rhizomes. Clusters of small white flowers bloom on an upright stem in spring. The young leaflets and stems have been used for food.[1] Only a few studies have been carried out on the chemical constituents and biological activities of this plant. Triterpenes and flavonol glycosides have been reported as constituents of this plant and have been found to inhibit ACAT (acyl-CoA: cholesterol acyltransferase) and to possess antioxidant activity.[2,3] In our continuing study for the discovery of novel antitumor agents from natural sources, we have found that a MeOH extract of the whole plant of *A. rossii* had cytotoxicity (>70% inhibition at 30 μg/ml) against the human leukemia HL-60 cell line. The cytotoxicity of the MeOH extract was concentrated in the hexane- and EtOAc-soluble fractions. Further phytochemical study on these fractions resulted in the isolation of two new triterpenes (**1**, **2**), together with six known triterpenes (**3**—**8**). The present paper reports the isolation and structural elucidation of these triterpenes and their *in vitro* cytotoxicity against the K562 and HL-60 cell lines.

Results and Discussion

The MeOH extract of the whole plant of *A. rossii* was suspended in H_2O and successively partitioned with hexane, EtOAc, and BuOH. The hexane- and EtOAc-soluble fractions, with cytotoxicity against both the K562 and HL-60 cell lines, were subjected to silica gel column chromatography to give several fractions that were further purified with column chromatography on silica gel to afford eight triterpenes, including the new compounds, 3α,23-isopropylidenedioxyolean-12-en-27-oic acid (**1**) and 23-hydroxy-3-oxoolean-12-en-27-oic acid (**2**), as well as six known triterpenes, 3-oxoolean-12-en-27-oic acid (**3**),[4] 3α-hydroxyolean-12-en-27-oic acid (**4**),[4] β-peltoboykinolic acid (**5**),[5] aceriphyllic acid A (**6**),[2] oleanolic acid (**7**),[6] and gypsogenic acid (**8**)[7] (Fig. 1). The structures of the known compounds were identified by comparing their physicochemical and spectral data (mp, $[\alpha]_D$, IR, ^{1}H- and ^{13}C-NMR) with those in the literature.

Compound **1** was obtained as a white amorphous powder, $[\alpha]_D^{25}$ +172.3° (c=1.1, $CHCl_3$). The HR-FAB-MS spectrum of **1** exhibited a quasimolecular ion peak at m/z 535.3757 $[M+Na]^+$ (Calcd for $C_{33}H_{52}O_4Na^+$: 535.3765), corresponding to the molecular formula $C_{33}H_{52}O_4$. The EI-MS spectrum of **1** showed a typical fragment ion peak at m/z 248 $[C_{16}H_{24}O_2]^+$, which is the characteristic ion peak in the retro Diels–Alder fragmentation of a β-amyrin-type triterpene.[8] In addition, significant ion peaks at m/z 203 $[C_{16}H_{24}O_2-COOH]^+$ and 202 $[C_{16}H_{24}O_2-HCOOH]^+$ suggested that ring D or E in **1** is replaced by a carboxyl group. Its IR spectrum exhibited absorption bands for a carbonyl group (1710 cm^{-1})

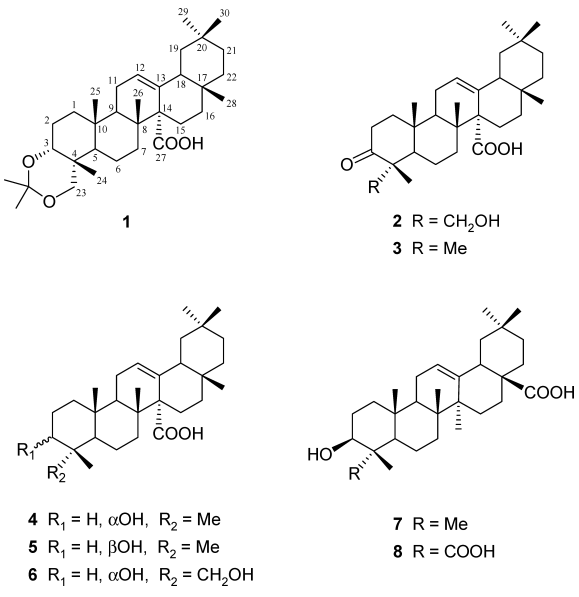

Fig. 1. Structures of Compounds **1**—**8** Isolated from *A. rossii*

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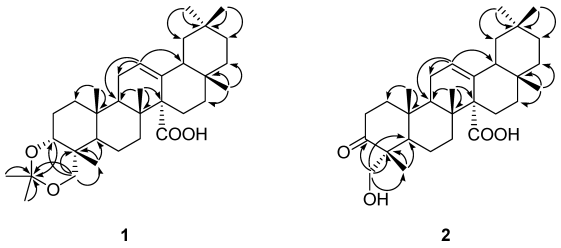
Table 1. ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) Data for Compounds **1** and **2** (in $CDCl_3$)[a]

C	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	36.7 t	1.22 m, 1.40 m	38.4 t	1.45 m, 1.87 m
2	23.4 t	1.44 m, 1.46 m	35.7 t	2.42 m, 2.50 m
3	72.7 d	3.63 br s	219.6 s	
4	40.0 s		52.4 s	
5	42.8 d	1.84 m	47.7 d	1.85 m
6	17.8 t	1.30 m, 1.42 m	19.5 t	1.32 m, 1.42 m
7	32.9 t	1.32 m, 1.34 m	34.5 t	1.25 m, 1.34 m
8	36.1 s		39.6 s	
9	49.2 d	2.01 m	49.2 d	1.98 m
10	35.0 s		36.5 s	
11	22.6 t	1.90 m, 1.94 m	23.2 t	1.90 m, 1.95 m
12	126.1 d	5.70 dd (2.4, 2.8)	125.3 d	5.60 dd (3.6, 3.9)
13	137.7 s		138.2 s	
14	55.9 s		55.9 s	
15	22.3 t	1.70 m, 1.74 m	22.3 t	1.67 m, 1.72 m
16	27.6 t	2.02 m, 2.05 m	27.8 t	2.01 m, 2.05 m
17	33.0 s		33.1 s	
18	47.2 d	2.25 m	46.0 d	2.28 m
19	44.0 t	1.01 m, 1.32 m	44.0 t	0.99 m, 1.30 m
20	31.1 s		31.2 s	
21	34.3 t	1.24 m, 1.27 m	35.8 t	1.22 m, 1.26 m
22	36.5 t	1.20 m, 1.40 m	36.7 t	1.17 m, 1.40 m
23	68.2 t	3.24 d (12.0), 3.65 d (12.0)	67.2 t	3.30 d (11.8), 3.55 d (11.8)
24	17.4 q	1.00 s	17.3 q	0.89 s
25	16.8 q	1.06 s	16.2 q	1.02 s
26	18.2 q	1.04 s	18.2 q	1.03 s
27	177.3 s		178.5 s	
28	28.3 q	0.86 s	28.3 q	0.80 s
29	33.2 q	0.84 s	33.3 q	0.78 s
30	23.6 q	0.85 s	23.7 q	0.79 s
$Me_2\underline{C}$	98.1 s			
Me	29.1 q	1.38 s		
Me	19.3 q	1.41 s		

a) Assignments made on the basis of ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

and trisubstituted double bond (1665, 850 cm^{-1}). The ^{1}H-NMR spectrum of **1** displayed the characteristic signals of an olefinic proton at δ_H 5.70 (dd, J=2.4, 2.8 Hz), a hydroxymethine proton at δ_H 3.63 (br s), a pair of hydroxymethylene protons at δ_H 3.65 and 3.24 (each d, J=12.0 Hz), and eight tertiary methyl protons at δ_H 1.41, 1.38, 1.06, 1.04, 1.00, 0.86, 0.85, and 0.84. The ^{13}C-NMR and DEPT spectra of **1** revealed 33 carbon signals including a carboxyl carbon at δ_C 177.3, olefinic carbons at δ_C 137.7 and 126.1, a hydroxymethine at δ_C 72.7, a hydroxymethylene at δ_C 68.2, and eight methyls at δ_C 33.2, 29.1, 28.3, 23.6, 19.3, 18.2, 17.4, and 16.8 (Table 1), which were typical for the olean-12-en-27-oic acid series.[4,8] The difference in **1** compared with **6** was the carbon signals for a dioxymethylene at δ_C 98.1 and two additional methyls at δ_C 29.1 and 19.3. The HMBC experiment showed long-range correlations between the dioxymethylene carbon (δ_C 98.1) and H-23 (δ_H 3.65, 3.24), H-3 (δ_H 3.63), and two tertiary methyls (δ_H 1.41, 1.38) (Fig. 2), indicating that the acetonide moiety is connected with C-3 and C-23. On the other hand, a broad singlet signal at H-3 (δ_H 3.63) in **1** indicated the α-orientation of the hydroxyl group,[9,10] which was further supported by the observation of a cross-peak between H-3 (δ_H 3.63) and Me-24 (δ_H 1.00) in the NOESY spectrum. Thus the structure of **1** was determined to be 3α,23-isopropylidenedioxyolean-12-en-27-oic acid, an acetonide derivative of **6**, which might be an artifact produced during the isolation procedure.



1 **2**

Fig. 2. Selected HMBC Correlations (H→C) of Compounds **1** and **2**

Compound **2** was obtained as a white amorphous powder, $[\alpha]_D^{25}$ +113.1° (c=1.1, $CHCl_3$). The HR-FAB-MS spectrum of **2** exhibited a quasimolecular ion peak at m/z 493.3286 $[M+Na]^+$ (Calcd for $C_{30}H_{46}O_4Na^+$: 493.3295), corresponding to the molecular formula $C_{30}H_{49}O_4$. Its EI-MS fragment ion peak at m/z 248 $[C_{16}H_{24}O_2]^+$ was typical of the retro Diels–Alder fragmentation of a β-amyrin-type triterpene,[8] and other significant ion peaks at m/z 203 $[C_{16}H_{24}O_2-COOH]^+$ and 202 $[C_{16}H_{24}O_2-HCOOH]^+$ suggested that **2** is an olean-12-ene derivative with a hydroxyl group and a keto function on ring(s) A and/or B, and a carboxyl group on ring D or E. The ^{1}H-NMR spectrum of **2** displayed the characteristic signals of an olefinic proton at δ_H 5.60 (dd, J=3.6, 3.9 Hz), a pair of hydroxymethylene protons at δ_H 3.55 and 3.30 (each d, J=11.8 Hz), and six tertiary methyl protons at δ_H 1.03, 1.02, 0.89, 0.80, 0.79, and 0.78. Thirty carbon signals appeared in the ^{13}C-NMR and DEPT spectra, including a ketone at δ_C 219.6, a carboxyl carbon at δ_C 178.5, olefinic carbons at δ_C 138.2 and 125.3, a hydroxymethylene at δ_C 67.2, and six methyls at δ_C 33.3, 28.3, 23.7, 18.2, 17.3, and 16.2 (Table 1), which are typical of the olean-12-en-27-oic acid series.[4,8] Except for the hydroxymethylene at δ_C 67.2 in **2**, its ^{13}C-NMR data were similar to those of 3-oxoolean-12-en-27-oic acid (**3**). The position C-23 for the hydroxymethylene was confirmed by comparing the chemical shifts of ^{1}H- and ^{13}C-NMR data, as well as analysis of HMBC data, where correlations of the H-23 (δ_H 3.55, 3.30) with C-3 (δ_C 219.6), C-4 (δ_C 52.4), C-5 (δ_C 47.7), and C-24 (δ_C 17.3) were observed (Fig. 2). Furthermore, the NOE cross-peak observed between Me-24 (δ_H 0.89) and Me-25 (δ_H 1.02) indicated that the hydroxymethylene was in an α-orientation. Thus the structure of **2** was determined to be 23-hydroxy-3-oxoolean-12-en-27-oic acid.

All the isolates were evaluated for *in vitro* cytotoxicity against the human leukemia K562 and HL-60 cell lines using the MTT assay method,[11] and the results are presented in Table 2. Of the triterpenes tested, compounds **1**—**6** with a carboxyl group at C-27 exhibited considerable cytotoxicity against the K562 and HL-60 cell lines, with IC_{50} values ranging from 12.2 to 28.7 μM and from 12.1 to 25.8 μM, respectively, while the olean-type triterpenoids (**7**, **8**) with a carboxyl group at C-28 were inactive (IC_{50}>100 μM) against both cell lines. Previous studies showed that the pentacyclic triterpenes bearing a carboxyl group at C-27 have cytotoxic and antitumor activities,[12,13] and inhibit ACAT (acyl-CoA: cholesterol acyltransferase) and protein tyrosine phosphatase

Table 2. Cytotoxicity of Compounds **1**—**8** from *A. rossii*

Compound	IC_{50} (μM)[a]	
	K562	HL-60
1	28.7±2.5	25.8±2.0
2	21.2±1.8	14.8±1.5
3	15.3±2.0	13.3±0.4
4	12.2±1.1	12.1±0.1
5	12.5±2.4	12.3±0.8
6	16.4±1.7	13.1±0.9
7	>100	>100
8	>100	>100
AM[b]	3.0±0.2	5.1±0.1

a) The values represent the mean±S.D. of three individual experiments. *b*) Adriamycin was used as a positive control.

1B.[2,14] Accordingly, our results indicate that olean-12-en-27-oic acid derivatives (**1**—**6**) may be the cytotoxic principles of *A. rossii*.

Experimental

General Experimental Procedures Melting points were measured on an Electrothemal apparatus. Optical rotation was measured in $CHCl_3$ on a JASCO DIP-370 digital polarimeter. UV and IR spectra were recorded on JASCO V-550 UV/VIS and JASCO 100 IR spectrometers, respectively. HR-FAB-MS and EI-MS data were recorded on JEOL JMS-DX 300 and Hewlett-Packard 5989B spectrometers, respectively. ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) spectra were obtained on Bruker DRX-300 spectrometer with tetramethylsilane (TMS) as an internal standard. Two-dimensional (2D) NMR experiments (HMQC, HMBC, and NOESY) were performed on a Bruker Avance 500 spectrometer. Column chromatography was carried out using silica gel (Kieselgel 70—230 and 230—400 mesh, Merck), and thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates (0.25 mm, Merck).

Plant Material Whole plants of *A. rossii* were collected at Mt. Seolak, Kangwondo, Korea, in August 2000, and identified by Prof. KiHwan Bae. A voucher specimen (CNU-908) has been deposited in the herbarium of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation The air-dried whole plant (3.7 kg) was extracted three times with MeOH at room temperature for 1 month, filtered, and concentrated to give a MeOH extract (382 g). The MeOH extract was suspended in H_2O (2.5 l) and then partitioned successively with hexane (2.5 l×3), EtOAc (2.5 l×3), and BuOH (2.5 l×3) to afford hexane- (106.4 g), EtOAc- (42.1 g), and BuOH-soluble fractions (35.2 g), respectively. Since the hexane- and EtOAc-soluble fractions exhibited considerable cytotoxicity (79.8% and 75.2% inhibition at 30 μg/ml, respectively) against the HL-60 cell line, these fractions were investigated extensively. The hexane-soluble fraction (106.4 g) was chromatographed on a silica gel column (30×10.0 cm) eluted with a gradient of hexane–EtOAc (20 : 1→1 : 1) to afford six fractions (fr. A—F). Fr. B (15.4 g) was rechromatographed on a silica gel column (50×5.0 cm) with a gradient of hexane–EtOAc (10 : 1→5 : 1) to give three subfractions (fr. B1—B3). Fr. B2 (3.2 g) was further purified on a silica gel column (50×3.5 cm) using hexane–EtOAc (8 : 1) to yield compound **1** (360 mg). Repeated silica gel column chromatography (50×5.0 cm) of fr. C (6.5 g) using hexane–EtOAc (6 : 1) gave compound **3** (850 mg). Fr. E (10.5 g) was further chromatographed on a silica gel column (50×5.0 cm) with a gradient of hexane–EtOAc (8 : 1→4 : 1) to afford compounds **4** (140 mg) and **5** (250 mg), respectively. The EtOAc-soluble fraction (42.1 g) was chromatographed on a silica gel column (80×10.0 cm) using a gradient of hexane–EtOAc (5 : 1→0 : 1) to afford six fractions (fr. G—M). Fr. G (3.5 g) was rechromatographed on a silica gel column (50×3.5 cm) using hexane–EtOAc (4 : 1) to give compound **2** (80 mg). Fr. H (5.2 g) was further purified on silica gel column chromatography (50×3.5 cm) using hexane–EtOAc (3 : 1) to afford compound **6** (800 mg). Fr. J (6.4 g) was rechromatographed on a silica gel column (50×5.0 cm) with a gradient of hexane–EtOAc (5 : 1→1 : 1) to afford three subfractions (fr. J1—J3). Compounds **7** (6 mg) and **8** (15 mg) were isolated from fr. J2 (2.1 g) using silica gel column chromatography (50×3.5 cm) with hexane–EtOAc (2 : 1).

3α,23-Isopropylidenedioxyoelan-12-en-27-oic Acid (**1**): White amorphous powder, mp 231—232 °C. $[\alpha]_D^{25}$ +172.3° (c=1.1, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 2950, 1710, 1665, 1445, 1380, 1245, 850. HR-FAB-MS *m/z*: 535.3757 $[M+Na]^+$ (Calcd for $C_{33}H_{52}O_4Na^+$: 535.3765). EI-MS *m/z* (rel. int.): 512 $[M]^+$ (4), 497 (10), 454 (23), 436 (74), 424 (29), 248 (40), 206 (100), 203 (14), 202 (9), 191 (21), 175 (22), 121 (19). ^{1}H- and ^{13}C-NMR: see Table 1.

3-Oxo-23-hydroxyolean-12-en-27-oic Acid (**2**): White amorphous powder, mp 246—248 °C. $[\alpha]_D^{25}$ +113.1° (c=1.1, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3440, 2955, 1710, 1690, 1650, 1450, 1382, 1240, 840; HR-FAB-MS *m/z*: 493.3286 $[M+Na]^+$ (Calcd for $C_{30}H_{46}O_4Na^+$: 493.3295). EI-MS *m/z* (rel. int.): 470 $[M]^+$ (3), 440 (17), 422 (20), 395 (15), 341 (23), 248 (100), 247 (35), 203 (22), 202 (7), 191 (26), 137 (14). ^{1}H- and ^{13}C-NMR: see Table 1.

Cytotoxicity Assay The cancer cell lines (K562 and HL-60) were maintained in RPMI 1640 that included L-glutamine (JBI) with 10% FBS (JBI) and 2% penicillin–streptomycin (GIBCO). Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxicity was measured using a modified MTT assay.[11] Viable cells were seeded in the growth medium (180 μl) into 96-well microtiter plates (1×10^4 cells per well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 100 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After standing for 2 h, 20 μl of the test sample was added to each well. The same volume of DMSO was added to the control wells. Forty-eight hours after the test sample was added, MTT 20 μl was also added to the each well (final concentration, 5 μg/ml). Two hours later, the plate was centrifuged for 5 min at 1500 rpm, the medium was removed, and the resulting formazan crystals were dissolved with DMSO 150 μl. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.

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References

1) Lee C. B., "Illustrated Flora of Korea," Hyangmunsa, Seoul, 1989, p. 409.
2) Han J. T., Kim H. Y., Park Y. D., Lee Y. H., Lee K. R., Kwon B. M., Baek N. I., *Planta Med.*, **68**, 558—561 (2002).
3) Han J. T., Bang M. H., Chun O. K., Kim D. O., Lee C. Y., Baek N. I., *Arch. Pharm. Res.*, **27**, 390—395 (2004).
4) Chen T. K., Ales D. C., Baenziger N. C., Wiemer D. F., *J. Org. Chem.*, **48**, 3525—3531 (1983).
5) Kim C. M., Kang S. S., *Kor. J. Pharmacogn.*, **17**, 195—198 (1986).
6) Shao C. J., Kasai R., Xu J. D., Tanaka O., *Chem. Pharm. Bull.*, **37**, 42—45 (1989).
7) Oshima Y., Ohsawa T., Hikino H., *Planta Med.*, **50**, 254—258 (1984).
8) Takahashi K., Kanayama K., Tanabe Y., Takani M., *Chem. Pharm. Bull.*, **20**, 2106—2111 (1972).
9) Deepak M., Handa S. S., *Phytochemistry*, **49**, 269—271 (1998).
10) Su B. N., Kang Y. H., Pinos R. E., Santarsiero B. D., Mesecar A. D., Soejarto D. D., Fong H. H. S., Pezzuto J. M., Kinghorn A. D., *Phytochemistry*, **64**, 293—302 (2003).
11) Mosmann T. J., *J. Immunol. Methods*, **65**, 55—63 (1983).
12) Sun H., Zhang J., Ye Y., Pan Y., Shen Y., *Helv. Chim. Acta*, **86**, 2414—2423 (2003).
13) Arisawa M., Bai H., Shimizu S., Koshimura S., Tanaka M., Sasaki T., Morita N., *Chem. Pharm. Bull.*, **40**, 3274—3276 (1992).
14) Na M., Cui L., Min B. S., Bae K., Yoo J. K., Kim B. Y., Oh W. K., Ahn J. S., *Bioorg. Med. Chem. Lett.*, **16**, 3273—3276 (2006).