Inhibitory Effect of TNF-α and IL-8 Secretion by Pimarane-Type Diterpenoids from *Acanthopanax koreanum*

Xing Fu CAI,[a] Guanghai SHEN,[a] Nguyen Tien DAT,[a] Ok Hwa KANG,[b] Jin A KIM,[b] Young Mi LEE,[b] Jung Joon LEE,[c] and Young Ho KIM*,[a]

[a] *College of Pharmacy, Chungnam National University; Daejeon 305–764, Korea:* [b] *Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University; Iksan, Cheonbuk 540–749, Korea: and* [c] *Korea Research Institute of Bioscience and Biotechnology; P.O.Box 115 Yusong, Daejeon 305–600, Korea.*

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A new pimarane-type diterpene compound, acanthokoreoic acid A together with three known compounds, acanthoic acid, acanthol, and sumogaside were isolated from a CH_2Cl_2 fraction of *Acanthopanax koreanum* by repeated column chromatography and reversed phase preparative HPLC. Acanthoic acid was isolated in high yields and showed potent inhibitory activity on the IL-8 secretion of the TNF-α-stimulated human colon adenocarcinoma cell line HT-29 and on the TNF-α secretion of the trypsin-stimulated human leukemic mast cell line HMC-1.

Key words *Acanthopanax koreanum*; IL-8; TNF-α; inhibitory effect; diterpenoid

Ulcerative colitis (UC) is an inflammatory bowel disease of undetermined etiology, characterized by chronic uncontrolled inflammation of the intestinal mucosa. In the colonic mucosa of patients with UC, the active inflammation is persistent and mucosal injury and repair is repeated. Recent studies have suggested that TNF-α and trypsin are found at high levels in the colon lumen of patients with UC,[1] and colonic epithelial cells[2,3] and mast cells[1] play an important role in the pathogenesis of UC. TNF-α induced the production of the potent neutrophil and T-lymphocyte chemoattractant cytokine IL-8 in colonic epithelial cells HT29.[2,3] Trypsin significantly induced the secretion of pro-inflammatory cytokine TNF-α in human leukemic mast cell line.[4]

Acanthopanax koreanum (Araliaceae) is a medicinal plant indigenous to Korea.[5] The root and stem barks of *Acanthopanax* species have been used as a tonic and sedative as well as in the treatment of rheumatism and diabetes.[6] In continuation of our studies on *A. koreanum* roots,[7—9] we isolated a new pimarane-type diterpene compound, acanthokoreoic acid A (**1**) together with three known compounds from the CH_2Cl_2 fraction of the MeOH extract. These were tested for inhibitory activity on IL-8 secretion in TNF-α-stimulated HT-29 and TNF-α secretion in trypsin-stimulated HMC-1. This paper describes the structural determination of the new diterpenoid compound, acanthokoreoic acid A (**1**), and compared the inhibitory activities of the four pimaradiene compounds isolated from the roots of *A. koreanum* on IL-8 and TNF-α secretion.

Results and Discussion

The four pimarane diterpenoids **1**—**4** were isolated from a CH_2Cl_2-soluble fraction of *A. koreanum* by repetitive column chromatography and preparative HPLC using a reverse-phase YMC J'sphere ODS-H80 column (YMC Co. Ltd., Japan). The structures of the known compounds were identified by comparing spectroscopic data with previously reported data as acanthoic acid (**2**), (−)-pimara 9(11),15-diene 19-ol, which was newly named as acanthol (**3**) and sumogaside (**4**), all of which had previously been isolated from the *A. koreanum* roots.[7,8)]

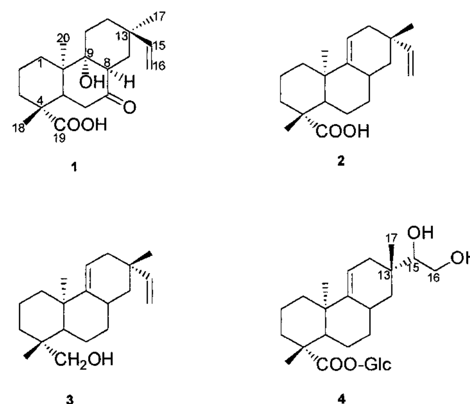

Fig. 1. Structure of Compounds Isolated from *Acanthopanax koreanum* Roots

Acanthokoreoic acid A (**1**) was isolated as white powder. HR-FAB-MS showed a quasi molecular ion at *m/z* 335.2222 (Calcd 335.2222) corresponding to a molecular formula $C_{20}H_{30}O_4$. The ^{1}H-NMR spectrum showed three tertiary methyl groups (δ 1.12, 1.22, 1.24) as a singlet and the characteristic pattern of a pimaradiene-type diterpene with an ABX system in the olefinic region (δ 4.82—5.95), which was assigned to a mono-substituted double bond (Table 1).[10] The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra indicated that **1** had a diterpene skeleton containing a carbonyl and a carboxyl group (δ 216.3, 181.1). Partial structures were linked by the HMBC experiments. Long-range correlations between H-5 (δ_H 1.47)/H-14 (δ_H 2.64) and C-7 (δ_C 216.3), and H-12 (δ_H 1.69, 2.28)/H-14 (δ_H 2.64) and C-9 (δ_C 62.9) confirmed that the carbonyl group and the hydroxyl group were connected to C-7 and C-9, respectively. The relative stereochemistry of **1** was determined by measuring the ROESY spectrum (Table 1). ROSEY correlations measured in $CDCl_3$ were observed between H_α-8 (δ_H 4.38)/$CH_{3\alpha}$-20 (δ_H 1.12); and H_α-8 (δ_H 4.38)/$CH_{3\alpha}$-17 (δ_H 1.24). In order to determine the stereochemistry of the hydroxyl group at C-9, the ROSEY spectra were measured again in DMSO-d_6. ROESY correlations

* To whom correspondence should be addressed. e-mail: yhk@cnu.ac.kr
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Table 1. ^{1}H- and ^{13}C-NMR Assignments, HMBC and ROESY Correlations of Compound **1** in $CDCl_3$

Position	^{1}H$^{a)}$	^{13}C$^{b)}$	HMBC	ROESY
1	1.37 (m)	36.4 (t)		
	1.82 (br d, 13.6)			
2	1.52 (m)	20.2 (t)		
	1.90 (m)			
3	1.01 (m)	38.7 (t)		
	2.13 (br d, 12.9)			
4		45.1 (s)	H-2, H-3, H-5, H-6, H-18	
5	1.47 (dd, 12.4, 3.0)	55.2 (d)	H-3, H-6, H-18,H-20	H-18
6	2.07 (dt, 14.1, 3.8)	38.8 (t)		
	1.38 (m)			
7		216.3 (s)	H-6, H-14	
8	4.38 (br d, 3.3)	79.8 (d)	H-6, H-14	9-OH$^{c)}$, H-17, H-20
9		62.9 (s)	H-11, H-12, H-14	
10		50.9 (s)	H-1, H-2, H-5, H-11, H-20	
11	1.93 (m)	21.4 (t)		
	2.32 (m)			
12	2.28 (dd, 14.1, 4.7)	48.4 (t)		
	1.69 (dd, 14.1, 1.8)			
13		42.4 (s)	H-8, H-15, H-16, H-17	
14	2.64 (d, 13.7)	48.7 (t)		
	1.41 (d, 13.6)			
15	5.95 (dd, 17.3, 10.6)	151.7 (s)		
16	4.93 (dd, 17.3, 1.1)	109.2 (t)		
	4.82 (dd, 10.6, 1.1)			
17	1.24 (s)	31.0 (q)		H-8
18	1.22 (s)	29.0 (q)		H-5
19		181.1 (s)		
20	1.12 (s)	16.6 (q)		H-8, 9-OH$^{c)}$

a) 600 MHz, *b*) 150 MHz, *c*) measured in DMSO-d_6.

were then observed between H$_\alpha$-8 (δ_H 4.56)/OH$_\alpha$-9 (δ_H 4.31); and CH$_{3\alpha}$-20 (δ_H 1.02)/OH$_\alpha$-9 (δ_H 4.31). On the basis of the above findings, acanthokoreoic acid A (**1**) was determined to be *ent*-7-oxo-9α-hydroxy-pimara-15-en-19-oic acid.

Compounds **1**—**4** were tested for their inhibitory activity on IL-8 secretion in TNF-α-stimulated HT-29 and TNF-α secretion in trypsin-stimulated HMC-1. In the TNF-α-stimulated HT-29, compounds **2** and **4** significantly inhibited IL-8 secretion at concentration of 1, 10 and 100 μM and at concentration of 10 and 100 μM, respectively. In contrast, compounds **1** and **3** did not show significant inhibitory effect (Table 2). In the trypsin-stimulated HMC-1, compound **2** also showed significant inhibition at concentration of 10 and 100 μM, whereas compound **1**, **3**, and **4** did not show a significant inhibitory effect (Table 3).

The gastrointestinal tract includes a unique lymphoid component designated the gut-associated lymphoid tissue (GALT), which contributes to overall immune homeostasis. In addition to GALT, intestinal epithelial cells and mast cells can also act as immune cells. Recent studies showed that TNF-α induced the production of IL-8, a strong chemoattractant for neutrophils, in HT29.[2,3] The chemokine increase capillary permeability and migration of neutrophils to the sites of gastrointestinal mucosal inflammation from peritoneal blood and bone marrow. Furthermore, trypsin is founded at high levels in the colon lumen of patients with UC.[1] Trypsin induced the secretion of pro-inflammatory cytokine TNF-α from HMC-1.[4] Acanthoic acid (**2**) from *A. koreanum* significantly inhibited secretion of the pro-inflammatory mediators such as IL-8 and TNF-α in HT29 and HMC-1. These results may have clinical applicability of acanthoic acid to the disease such as ulcerative colitis.

Table 2. Effect of Compounds on IL-8 Secretion from TNF-α-Stimulated HT29

Treatment	IL-8 (pg/ml)	Inhibition (%)
None	10.6±1.5	—
TNF-α	252.3±17.1	—
Acanthokoreoic acid A (**1**), μM		
1	221.6±18.2	12.7
10	207.3±14.7	18.6
100	242.9±15.8	3.9
Acanthoic acid (**2**), μM		
1	194.5±13.5*	23.9*
10	162.6±12.8*	37.1*
100	78.0±6.2*	72.1*
Acanthol (**3**), μM		
1	250.8±20.8	0.4
10	251.3±23.4	0.6
100	249.6±21.3	1.1
Sumogaside (**4**), μM		
1	218.5±21.5	14.0
10	168.4±11.1*	34.7*
100	149.6±10.8*	42.5*

HT29 (3×10^5 cells/well) were pre-incubated with the compounds for 30 min and then stimulated with TNF-α (50 ng/ml) for 6 h. IL-8 in the supernatant was measured by ELISA. Results are expressed as the mean±S.E. from three separate experiments. * $p<0.05$ compared with TNF-α-treated value.

Table 3. Effect of Compounds on TNF-α Secretion from Trypsin-Stimulated HMC-1

Treatment	TNF-α (pg/ml)	Inhibition (%)
None	1.3±1.5	—
Trypsin	285.0±39.7	—
Acanthokoreoic acid A (**1**), μM		
1	283.3±28.4	0.6
10	279.0±26.7	2.1
100	258.9±23.8	9.2
Acanthoic acid (**2**), μM		
1	276.2±25.8	3.1
10	100.6±8.4*	65.0*
100	74.8±6.3*	74.1*
Acanthol (**3**), μM		
1	282.4±25.9	0.9
10	250.7±24.2	12.1
100	233.4±22.4	18.2
Sumogaside (**4**), μM		
1	284.4±29.5	0.2
10	262.6±24.9	7.9
100	256.1±26.8	10.2

HMC-1 (5×10^5 cells/well) were pre-incubated with the compounds for 30 min and then stimulated with trypsin (100 nM) for 8 h. TNF-α in the supernatant was measured by ELISA. Results are expressed as the mean±S.E. from three separate experiments. * $p<0.05$ compared with trypsin-treated value.

Experimental

Melting points were measured using a Yanaco micro melting point apparatus, optical rotation with a Jasco DIP-370 automatic polarimeter, and UV and FT-IR spectra using a Beckman Du-650 UV–VIS recording spectrophotometer and a Jasco Report-100 infrared spectrometer, respectively. Preparative HPLC was carried out on a Waters HPLC system (600 pump, 600 controller, 996 Photodiode array detector). NMR spectra were measured with a Bruker DMX 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz), FAB-MS using a JEOL JMS-HX/HX110A tandem mass spectrometer, column chromatography on silica-gel (Kieselgel 60, 70—230 mesh and 230—400 mesh, Merck), and TLC on pre-coated Silica-gel 60 F_{254} (0.25 mm, Merck) and

RP-18 F_{254S} plates (0.25 mm, Merck).

Plant Materials *A. koreanum* roots were gratefully provided by Kwang Su Soung at the Susin Ogapi Co. and identified by Professor Young Ho Kim at the College of Pharmacy, Chungnam National University. Voucher specimens (CNU 96076) were deposited at the herbarium in the College of Pharmacy, Chungnam National University.

Extraction and Isolation The roots of *A. koreanum* (10 kg) were extracted with MeOH three times under reflux for 15 h yielding 960 g of a dark solid extract, 950 g of which was then suspended in H_2O and extracted with CH_2Cl_2. The resulting CH_2Cl_2 solution was concentrated *in vacuo* to yield a CH_2Cl_2-soluble fraction (470 g). This CH_2Cl_2 soluble fraction (200 g) was chromatographed on a silica gel column, and eluted with a hexane–EtOAc gradient [20 : 1 (2 l), 10 : 1 (4 l), 5 : 1 (3 l), 3 : 1 (3 l), 1 : 1 (4 l), EtOAc (1 l)] and finally MeOH (2 l), which yielded 5 fractions according to TLC [fr. SR-23-A: 108.6 g, fr. SR-23-B: 7.4 g, fr. SR-23-C: 12.6 g, fr. SR-23-D: 16.0 g, and fr. SR-23-E: 32.0 g, respectively]. Fr. SR-23-D (13.0 g) was subjected to silica gel column chromatography and eluted with hexane–EtOAc [5 : 1 (4 l), 4 : 1 (1.5 l), 3 : 1 (1 l), 2 : 1 (1 l), 1 : 1 (1 l), 1 : 2 (3 l)] to give nine fractions [fr. SR-37-A: 0.2 g, fr. SR-37-B: 0.4 g, fr. SR-37-C: 1.2 g, fr. SR-37-D: 2.9 g, fr. SR-37-E: 1.5 g, fr. SR-37-F: 1.8 g, fr. SR-37-G: 1.2 g, fr. SR-37-H: 1.0 g, fr. SR-37-I: 1.0 g, respectively]. Fr. SR-37-H (1.0 g) was subjected to silica gel column chromatography and was eluted with $CHCl_3$–Acetone [8 : 1 (2 l)] to give three fractions [fr. SR-54-A: 0.5 g, fr. SR-54-B: 0.3 g, fr. SR-54-C: 0.1 g, respectively]. Fr. SR-54-C (0.1 g) was subjected to YMC gel column chromatography and eluted with MeOH–H_2O [3 : 1 (1 l)] to afford compound **1** (31.3 mg) as a white powder. Compound **2** (32.4 g, total yield by HPLC: 0.32% dried specimen, data not shown) was isolated as major component from Fr. SR-23-A and compound **3** (10.0 mg) from SR-23-C using a previously described method with slight modification[8] and this was followed by preparative HPLC. Compound **4** (3.9 mg) was isolated as white crystal from the H_2O-soluble fraction, and was chromatographed on silica gel and MCI gel. This was followed by MPLC using a LiChroprep® RP-18 (40—63 μm) column (Merck, Germany), and eluted with MeOH–H_2O [6 : 5 (0.3 l)].

Acanthokoreoic Acid A (**1**): White powder, mp 60—62 °C. $[\alpha]_D^{20}$ +3.5° (c=1.0, MeOH). IR (KBr) cm^{-1}: 3215 (OH), 1710, 1685 (C=O), 1639 (C=C). UV λ_{max} (EtOH) nm (log ε): 207 (3.92). FAB-MS m/z: 335 $[M+H]^+$. HR-FAB-MS m/z $[M+H]^+$: 335.2222 (Calcd for $C_{20}H_{31}O_4$: 335.2222). ^1H-NMR ($CDCl_3$): see Table 1. ^1H-NMR (DMSO-d_6) δ: 5.89 (1H, dd, J=17.4, 10.6 Hz, H-15), 4.89 (1H, dd, J=17.4, 1.1 Hz, H-16a), 4.78 (1H, dd, J=10.6, 1.1 Hz, H-16b), 4.56 (1H, d, J=3.4 Hz, H-8), 4.31 (1H, s, OH-9), 1.02 (3H, s, CH_3-20). ^{13}C-NMR ($CDCl_3$): see Table 1.

Acanthoic Acid (**2**): Amorphous powder, mp 135—136 °C. $[\alpha]_D^{20}$ −55.7° (c=1.0, MeOH). Acanthol (**3**): White powder, mp 73—74 °C. $[\alpha]_D^{20}$ −14.9° (c=0.2, $CHCl_3$). Sumogaside (**4**): white crystal, mp 210—214 °C. Copies of the original spectra are obtainable from the author of correspondence.

Assay of IL-8 and TNF-α Secretion Human colon adenocarcinoma cell line HT-29 was a gift from Dr. Y. I. Yeom (KRIBB, Korea) and was maintained in RPMI 1640 (Gibco BRL, U.S.A.) with 10% fetal bovine serum (Gibco BRL, U.S.A.) at 37 °C under humidified 5% CO_2 in air. HT-29 (3×10^5 cells/well) were pre-incubated with the sample for 30 min and then stimulated with recombinant human TNF-α (50 ng/ml, Sigma, St. Louis, MO, U.S.A.) for 6 h.

The human leukemic mast cell line HMC-1 cell was a gift from Professor Yukihiko Kitamura (Osaka University, Japan). Cells were maintained in Iscove's Modified Dulbecco's Medium (Gibco BRL, U.S.A.) with 10% fetal bovine serum (Gibco BRL, U.S.A.) at 37 °C under humidified 5% CO_2 in air. HMC-1 (5×10^5 cells/well) were pre-incubated with the sample for 30 min and then stimulated with trypsin (100 nM, Sigma, St. Louis, U.S.A.) for 8 h. IL-8 and TNF-α levels in supernatant were measured by using a modified enzyme-linked immunosorbent assay (ELISA) as instructed by the manufacture (Pharmingen, SanDiego, CA, U.S.A.). The ELISA was performed by coating 96 well plates with human monoclonal antibody specific for human IL-8 or TNF-α. Before performing the next assay steps, the coated plates were washed with PBS (phosphate-buffered saline) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant IL-8 or TNF-α were diluted and used as a standard. Assay plates were exposed sequentially to biotinylated human IL-8 or TNF-α, avidin peroxidase, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution containing 30% H_2O_2, and the plates were read at 405 nm. The inhibition percentages of IL-8 or TNF-α secretion were calculated using the following equation: Inhibition (%)=$(A-B)\times100/A$ [Where A is the IL-8 or TNF-α concentration when the TNF-α or trypsin only was treated, and B is the IL-8 or TNF-α concentration when the compounds were pre-treated]. A and B values were obtained by subtraction of the none-treated value from the TNF-α or compounds-treated value. Data were analysed by Dunnett's test. Results with $p<0.05$ were considered statistically significant.

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