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# Inhibitory Effect of Kaurane Type Diterpenoids from *Acanthopanax koreanum* on TNF-α Secretion from Trypsin-Stimulated HMC-1 Cells

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Five known kaurane type diterpenoids, 16α*H*,17-isovaleryloxy-*ent*-kauran-19-oic acid (**1**), 16α-hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**2**), paniculoside-IV (**3**), 16α-hydroxy-*ent*-kauran-19-oic acid (**4**), and *ent*-kaur-16-en-19-oic acid (**5**) were isolated from the root of *Acanthopanax koreanum* by repeated column chromatography and reversed phase preparative HPLC. The structures of these compounds were established from physicochemical and spectral data. Among the isolated compounds 16α*H*,17-isovaleryloxy-*ent*-kauran-19-oic acid (**1**) showed potent inhibitory activity ($IC_{50}$ value, 16.2 uM) on TNF-α secretion from HMC-1, a trypsin-stimulated human leukemic mast cell line.

**Key words:** *Acanthopanax koreanum*, Kaurane type diterpenoid, Inhibitory effect, TNF-α secretion

## INTRODUCTION

The gastrointestinal tract is exposed to high levels of proteinases both physiologically and during diseases and it has been shown that trypsin play an important role in inflammation. Trypsin induces nitric oxide-dependent vasodilation (Kawabata *et al.*, 2001), the extravasation of plasma proteins, the neutrophil infiltration (Lindner *et al.*, 2000) and colonic inflammation (Cenac *et al.*, 2002). Trypsin also stimulates inflammatory mediator release from mast cells (Kang *et al.*, 2003), peritoneal macrophages (Lundberg *et al.*, 2000) and eosinophils (Miike *et al.*, 2001). TNF-α is expressed in human gastrointestinal mucosa, and this expression is strongly enhanced during the course of inflammatory bowel diseases (IBDs) such as Crohns disease or ulcerative colitis (Bischoff *et al.*, 1999). Moreover, human intestinal mast cells are a major source of TNF-α in the gastrointestinal tract (Bischoff *et al.*, 1999). Trypsin was found to significantly induce the secretion of the pro-inflammatory cytokine TNF-α in a human leukemic mast cell line (Kang *et al.*, 2003). The regulation of TNF-α production is critical for the maintenance of the homeostasis of the immune system and for the prevention and treatment of chronic inflammations of the intestinal mucosa.

*Acanthopanax koreanum* (Araliaceae) is a medicinal plant indigenous to Korea (Lee, 1996), and its roots and stem barks have been used as a tonic and sedative and for the treatment of rheumatism and diabetes (Perry *et al.*, 1980). As a result of our continued study of *A. koreanum* roots (Kim *et al.*, 1988; Kim *et al.*, 1990), we isolated five known kaurane type diterpenoid compounds from MeOH extract, which were tested for inhibitory activity on TNF-α secretion in trypsin-stimulated HMC-1 cells. This paper describes the inhibitory activities of the five known kaurane diterpenoids on HMC-1 TNF-α secretion.

## MATERIALS AND METHODS

### General

Melting points were measured using a Yanaco micro melting point apparatus, optical rotation with a Jasco DIP-

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370 automatic polarimeter, UV spectra with a Beckman Du-650 UV-VIS recording spectrophotometer and FT-IR spectra with a Jasco Report-100 infrared spectrometer. Preparative HPLC was carried out on a Waters HPLC system (600 pump, 600 controller, and a 996 Photodiode array detector). NMR spectra were measured using a Bruker DRX 300 spectrometer ($^1$H, 300 MHz; $^{13}$C, 75 MHz) and FAB-MS using a JEOL JMS-HX/HX110A tandem mass spectrometer. Column chromatography was performed using silica-gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and thin layer chromatography (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

The roots of *A. koreanum* were provided by 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 dried 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 suspended in $H_2O$ and extracted with $CH_2Cl_2$. The resulting $CH_2Cl_2$ solution was concentrated *in vacuo* to yield the $CH_2Cl_2$-soluble fraction (470 g). The $CH_2Cl_2$ soluble fraction (200 g) was chromatographed on a silica gel column and eluted using a *n*-hexane-EtOAc gradient, to yield 5 fractions according to TLC (fr. SR-23-A~E). Fr. SR-23-A (108 g) was chromatographed on a silica gel column repeatedly and followed by preparative HPLC to yield **5** as one of its major components (35.2 g, 0.35% yield of the dried specimen by HPLC, data not shown). Fr. SR-23-C (12.6 g) was subjected to silica gel column chromatography to yield **1** (27 mg) as a white powder. Fr. SR-23-D (13.0 g) was eluted with *n*-hexane-EtOAc to give nine subfractions (subfr. SR-37-A~I), and subfr.SR-37-F (1.7 g) was subjected to silica gel and YMC gel column chromatography to afford **2** (133 mg) as white powder. Compound **4** (35 mg) was isolated from subfr. SR-37-G (7.2 g) by silica gel and YMC gel column chromatography as a white powder. The $H_2O$ layer was concentrated *in vacuo* to yield a $H_2O$-soluble fraction (300 g). A portion of which (100 g) was chromatographed on MCI gel (Mitsubishi Chemical Corporation) by elution using a $H_2O$-MeOH gradient to give six fractions (fr. SR-7-A~F). Fr. SR-7-E (1.1 g) was then subjected to silica gel and YMC gel column chromatography to give **3** (11 mg) as a white powder.

**16α*H*,17-Isovaleryloxy-*ent*-kauran-19-oic acid (1):** White powder, mp 148~150°C, FAB-MS m/z: 405.1 [M+H]$^+$; $^1$H-NMR (300 MHz, $CDCl_3$) δ: 0.85 (3H, s, H-20), 0.88 (6H, d, *J* = 6.6 Hz, H-4', H-5'), 1.16 (3H, s, H-18). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ: 40.9 (t, C-1), 19.3 (t, C-2), 38.0 (t, C-3), 43.8 (s, C-4), 57.2 (d, C-5), 22.6 (t, C-6), 41.7 (t, C-7), 45.1 (s, C-8), 55.5 (d, C-9), 39.8 (s, C-10), 18.9 (t, C-11), 31.4 (t, C-12), 38.7 (d, C-13), 37.3 (t, C-14), 45.2 (t, C-15), 39.8 (d, C-16), 68.5 (t, C-17), 29.1 (q, C-18), 183.9 (s, C-19), 15.7 (q, C-20), 173.6 (s, C-1'), 43.8 (t, C-2'), 25.9 (d, C-3'), 22.6 (q, C-4'), 22.6 (q, C-5').

**16α-Hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (2):** White powder, mp 185~187°C. $[\alpha]_D^{25}$: -69.9° (*c* 1, MeOH), FAB-MS m/z: 443.1 [M+Na]$^+$; $^1$H-NMR (300 MHz, $CDCl_3$) δ: 0.94 (3H, s, H-20), 0.96 (6H, d, *J* = 6.6 Hz, H-4', 5'), 1.22 (3H, s, H-18), 4.23 (2H, dd, *J* = 11.4, 15.3 Hz, H-17). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ: 39.5 (t, C-1), 17.9 (t, C-2), 36.7 (t, C-3), 42.6 (s, C-4), 55.7 (d, C-5), 21.0 (t, C-6), 40.7 (t, C-7), 43.7 (s, C-8), 54.6 (d, C-9), 38.6 (s, C-10), 17.4 (t, C-11), 25.1 (t, C-12), 44.9 (d, C-13), 36.0 (t, C-14), 51.7 (t, C-15), 79.1 (s, C-16), 67.1 (t, C-17), 27.8 (q, C-18), 182.4 (s, C-19), 14.5 (q, C-20), 172.2 (s, C-1'), 42.3 (t, C-2'), 24.7 (d, C-3'), 21.3 (q, C-4'), 21.3 (q, C-5').

**Paniculoside-IV (3):** White powder, mp 152~155°C, FAB-MS m/z: 521.1 [M+Na]$^+$; $^1$H-NMR (600 MHz, $CD_3OD$) δ: 0.97 (3H, s, H-20), 1.21 (3H, s, H-18), 5.40 (1H, d, *J* = 7.9 Hz, H-1 of glc). $^{13}$C-NMR (125 MHz, $CD_3OD$) δ: 41.8 (t, C-1), 20.1 (t, C-2), 39.1 (t, C-3), 45.1 (s, C-4), 58.5 (d, C-5), 23.2 (t, C-6), 43.4 (t, C-7), 45.8 (s, C-8), 57.3 (d, C-9), 40.9 (s, C-10), 19.6 (t, C-11), 27.2 (t, C-12), 46.2 (d, C-13), 38.1 (t, C-14), 53.7 (t, C-15), 83.0 (s, C-16), 66.9 (t, C-17), 29.0 (q, C-18), 178.2 (s, C-19), 16.4 (q, C-20), 95.6 (d, glc, C-1), 74.1 (d, glc, C-2), 78.7 (d, glc, C-3), 71.1 (d, glc, C-4), 78.7 (d, glc, C-5), 62.4 (t, glc, C-6).

**16α-Hydroxy-*ent*-kauran-19-oic acid (4):** White powder, mp 254~255°C. FAB-MS m/z: 343.1 [M+Na]$^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 0.97 (3H, s, H-20), 1.16 (3H, s, H-17), 1.32 (3H, s, H-18). $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 41.9 (t, C-1), 20.2 (t, C-2), 39.2 (t, C-3), 44.6 (s, C-4), 58.1 (d, C-5), 23.3 (t, C-6), 43.2 (t, C-7), 46.4 (s, C-8), 57.4 (d, C-9), 40.8 (s, C-10), 19.3 (t, C-11), 27.8 (t, C-12), 49.5 (d, C-13), 38.4 (t,C-14), 58.4 (t, C-15), 79.8 (s,C-16), 24.4 (q, C-17), 29.4 (q, C-18), 181.6 (s, C-19), 16.2 (q, C-20).

***Ent*-kaur-16-en-19-oic acid (5):** Amorphous white powder, mp 162~163°C. $[\alpha]_D^{25}$: -104.2° (*c* 1, MeOH), $^1$H-NMR (300 MHz, $CD_3OD$) δ: 4.73(2H, br. s, H-17), 1.19 (3H, s, H-18), 1.00 (3H, s, H-20). $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 42.2 (t, C-1), 20.4 (t, C-2), 39.4 (t, C-3), 44.9 (s, C-4), 58.4 (d, C-5), 23.2 (t, C-6), 42.6 (t, C-7), 45.5 (s, C-8), 56.6 (d, C-9), 40.9 (s, C-10), 19.5 (t, C-11), 34.2 (t, C-12), 45.3 (d, C-

13), 40.3 (t, C-14), 50.2 (t, C-15), 156.9 (s, C-16), 103.6 (t, C-17), 29.6 (q, C-18), 186.3 (s, C-19), 16.4 (q, C-20).

### Assay of TNF-α secretion

The human leukemic mast cell line HMC-1 was a gift from Professor Yukihiko Kitamura (Osaka University, Japan). Cells were maintained in Iscove's Modified Dulbecco's Medium (Gibco BRL, USA) containing 10% fetal bovine serum (Gibco BRL, USA) at 37°C under humidified 5% $CO_2$ in air. HMC-1 ($5\times10^5$ cells/well) were pre-incubated with the sample for 30 min and then stimulated with trypsin (100 nM, Sigma, St. Louis, USA) for 8 h. TNF-α levels in the supernatant were measured by using a modified enzyme-linked immunosorbent assay (ELISA), as instructed by the manufacturer (Pharmingen, San Diego, CA). The ELISA was performed by coating 96 well plates with human monoclonal antibody specific for TNF-α, and 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 TNF-α was diluted and used as a standard. Assay plates were exposed sequentially to biotinylated 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 inhibitory activity on TNF-α secretion was calculated using the following equation: Inhibition (%) = (A − B)×100/A [where A is the TNF-α concentration when the only trypsin was treated, and B is the TNF-α concentration when compounds were pre-treated]. A and B values were obtained by subtracting the none-treated values from the trypsin with compounds treated values. The $IC_{50}$ value was defined as the final concentration (1.0~100 μM) of the inhibitor required to inhibit TNF-α secretion by 50%. Statistical significance was determined using the Student *t*-test.

## RESULTS AND DISCUSSION

The four kaurane type diterpenoids (**1, 2, 4** and **5**) were isolated from the $CH_2Cl_2$-soluble fraction and one kaurane type glycoside (**3**) from the $H_2O$-soluble fraction of *A. koreanum* by repeated column chromatography and preparative HPLC using a reverse-phase YMC J'sphere ODS-H80 column (YMC Co. Ltd., Japan). The structures of these compounds were identified by comparing physicochemical and spectroscopic data with previously reported results (Kim *et al.*, 1995; Zhang *et al.*, 1999; Yamasaki *et al.*, 1977; Toshiko *et al.*, 1984; Choudhury *et al.*, 1982) as 16α*H*, 17-isovaleryloxy-*ent*-kauran-19-oic acid (**1**), 16α-hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**2**), paniculoside (**3**), 16á-hydroxy-*ent*-kauran-19-oic acid (**4**), *ent*-kaur-16-en-19-oic acid (**5**). Of these compounds, compounds **2-4** were identified for the first time in *A. koreanum*.

Compounds **1-5** were tested for their inhibitory activity on TNF-α secretion in trypsin-stimulated HMC-1 cell (Table I). Compound **1** exhibited a significant inhibitory effect on TNF-α secretion in trypsin-stimulated cells with an $IC_{50}$ value of 16.2 μM. However, its inhibitory activity on TNF-α secretion was reduced by six times when a hydroxyl group was introduced at C-16. The inhibitory activity of **5** was similar to that of **2** which has a hydroxy group at C-16 and an isovaleryloxy group at C-17. It was interesting that **3** having a hydroxy group at C-16 and **4** having a hydroxy group at C-16 and a glucose at C-19 did not inhibit TNF-α secretion. Although the structure activity relationships of the kaurane type diterpenoids was not conclusively elucidated, it was found that the inhibitory effect on TNF-α secretion is increased by the presence of an isovaleryloxy group at C-17 and decreased by the introduction of a hydroxy moiety at C-16 and a glucose at C-19. Trypsin induces colonic inflammation (Cenac *et al.*, 2002) and the secretion of pro-inflammatory cytokine TNF-α from mast cells (Kang *et al.*, 2003). Moreover, TNF-α is a multifunc-

**Table I.** Effect of compounds on TNF-α secretion from trypsin-stimulated HMC-1

| Compounds | $IC_{50}$(μM)* |
|---|---|
| 16α*H*,17-isovaleryloxy-*ent*-kauran-19-oic acid (**1**) | 16.2 |
| 16α-Hydroxy-17-isovaleryloxy-*ent*-kauran-19-oic acid (**2**) | 98.0 |
| Paniculoside-IV (**3**) | >1000 |
| 16α-Hydroxy-*ent*-kauran-19-oic acid (**4**) | >1000 |
| *Ent*-kaur-16-*en*-19-oic acid (**5**) | 85.8 |
| Luteolin[a] | 12.6 |

*HMC-1 ($5\times10^5$ 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. [a]This compound was used as positive control.

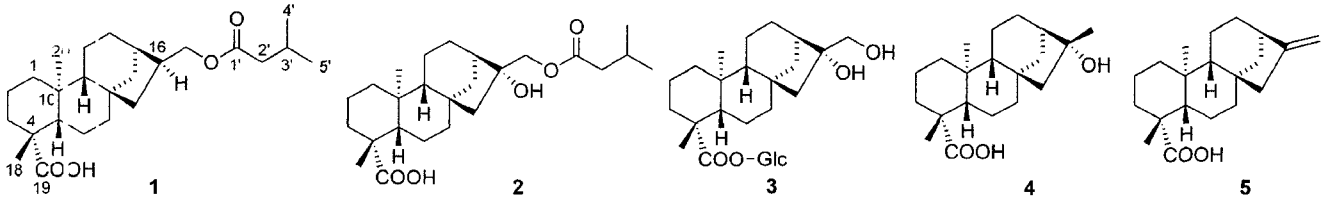


**Fig. 1.** Structures of compounds **1-5**

tional cytokine with proinflammatory and immunoregulatory activity. Therefore, the inhibitory activity of kauran type diterpenoids from *A. koreanum* may be responsible for the therapeutic efficacy of the crude drug in TNF-α related chronic anti-inflammatory diseases and upon the immune system.

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