Anti-inflammatory Compounds from the Aerial Parts of *Aceriphyllum rossii*

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A new megastigmane glycoside, galloyl linarionoside A (1), together with 13 known compounds (2–14) were isolated from the aerial parts of *Aceriphyllum rossii* Engler. (Saxifragaceae). The chemical structures of the isolated compounds were established mainly by using nuclear magnetic resonance spectra, mass spectrometry, and modified Mosher's method. Among the isolates, compounds 4, 5, 6 and 7 showed potent inhibitory activity against the lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage cells with IC_{50} values of 12.5, 9.5, 10.5 and 9.3 μM, respectively. The anti-inflammatory effect of compound 7 was accompanied by dose-dependent decreases in the production of inducible nitric oxide synthase and cyclooxygenase-2 proteins not in the inhibitor kappa B (IκB)-dependent nuclear factor-kappa B activation.

Key words *Aceriphyllum rossii*; Saxifragaceae; megastigmane; anti-inflammatory activity

Inflammation is the normal physiological and immune response to tissue injury. Increased blood supply, enhanced vascular permeability and migration of immune cells occur at damaged sites. The inflammatory process is a protective response that occurs in response to trauma, infection, tissue injury or noxious stimuli.[1)] In this process, activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes and macrophages) secrete increased amounts of nitric oxide (NO), prostaglandin E_2 (PGE_2) and cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. These substances not only induce cell and tissue damage, but also activate macrophages in rheumatoid arthritis and chronic hepatitis.[2)] NO is a major product and its production is controlled by nitric oxide synthases (NOSs), which include inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS). Most importantly, iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. PGE_2 is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2).[3)] Inflammatory stimuli including lipopolysaccharide (LPS) and pro-inflammatory cytokines activate immune cells to up-regulate inflammatory states. Therefore, NO and PGE_2 production induced by LPS through iNOS and COX-2, respectively, can reflect the degree of inflammation, and the change in NO and PGE_2 level through the inhibition of iNOS and COX-2 activity provides a means of assessing the effect of agents on the inflammatory process.

In a preliminary study, a methanol extract of the aerial parts of *Aceriphyllum rossii* Engler. (Saxifragaceae) showed inhibitory effect against the LPS-induced NO production in RAW264.7 macrophage cells. *A. rossii*, an endemic species in Korea, is a perennial herb that grows on damp rocks along valleys in the central northern part of Korea. The roots of *A. rossii* contain oleanane-type triterpenoids, which are cytotoxic to MCF-7 and LLC cancer cells,[4)] acyl-CoA-cholesterol *O*-acyltransferase inhibition and antibacterial activity.[5,6)] Inhibition of protein tyrosine phosphatase 1B activity by triterpenes isolated from the seeds of *A. rossii* has also reported.[7)] In addition, flavonoids isolated from the aerial parts showed antioxidant activity.[8)] In our study, activity-guided fractionation lead to the isolation of a new megastigmane glycoside (**1**) together with 13 known compounds (**2**–**14**) (Fig. 1). The present paper reports the isolation and structural elucidation of these compounds as well as their anti-inflammatory activities.

Results and Discussion

Compound **1** was isolated as an amorphous powder, and had a molecular formula of $C_{26}H_{38}O_{11}$ based on the ion peak at *m/z* 549.2316 ($[M+Na]^+$) in the high resolution-fast atom bombardment mass spectrometry (HR-FAB-MS) spectrum. The infrared (IR) spectrum of **1** suggested the presence of hydroxyl group (3328 cm^{-1}), carbonyl group (1748 cm^{-1}) and aromatic absorption (1418 cm^{-1}). The ^{1}H-NMR spectrum of **1** displayed signals for four methyl groups at δ_H 0.88 (H-11), 0.93 (H-12), 1.15 (H-10) and 1.59 (H-13), eight methylene proton signals at δ_H 1.41 (H_{ax}-2), 1.77 (H_{eq}-2), 1.99 (H_{ax} −4), 2.26 (H_{eq}-4), 1.86 (H-7a), 2.14 (H-7b), 1.43 (H-8a), and 1.45 (H-8b), and two oxymethine proton signals at δ_H 3.93 (H-3) and 3.68 (H-9) as well as aromatic proton signal at δ_H 7.10 (H-2″, H-6″) (Table 1). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra showed the presence of a sugar moiety, four methyl groups at δ_C 23.3 (C-10), 28.8 (C-11), 30.2 (C-12), and 20.1 (C-13), four methylene groups at δ_C 47.7 (C-2), 39.9 (C-4), 25.6 (C-7), and 40.7 (C-8), two methines with oxygen function at δ_C 74.5 (C-3) and 69.3 (C-9), one quaternary carbon at δ_C 38.9 (C-1) and a tetrasubstituted double bond at δ_C 125.0 (C-5) and 138.7 (C-6) and remaining five signals of a galloyl unit which was characterized by δ_C 168.4 (C-7″), 110.4 (C-2″, C-6″), 146.7 (C-3″, C-5″), 139.9 (C-4″) and 121.5 (C-1″).[9)] These results suggested that **1** was a derivative of ionol (megastigmane) glycoside.[10)] The place of tetrasubstituted double bond in the megastigmane skeleton

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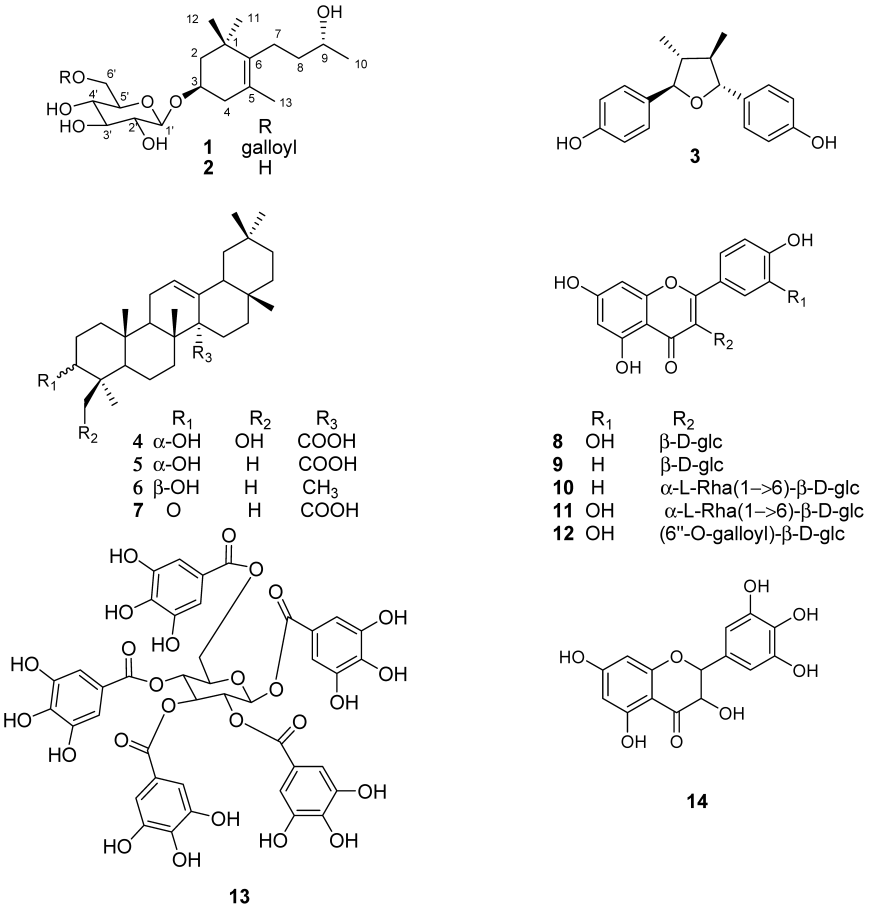

Fig. 1. Chemical Structure of Isolated Compounds (**1**–**14**) from *Aceriphyllum rossii*

was between the 5- and 6-positions. This fact was confirmed by heteronuclear multiple bond connectivity (HMBC) between H-11, H-12 and C-6; H-13 and C-5, C-6 (Fig. 2A). The signals from the sugar moiety appeared at δ_C 103.1 (C-1′), 78.1 (C-3′), 75.5 (C-2′), 75.3 (C-5′), 72.2 (C-4′), 65.1 (C-6′) suggested the presence of D-glucopyranose.[10] The coupling constant J=7.6 Hz of the anomeric proton of D-glucopyranose indicated it to be the β-form.[10] The glycosidic and galloyl unit sites were determined base on the long-range correlation between the H-1′ (δ_H 4.42, d) and C-3 (δ_C 74.5) as well as H-6′ (δ_H 4.48, dd; 4.42, m) and C-7″ (δ_C 168.4) in the HMBC spectrum. Furthermore, the long-range correlations between the following protons and carbons (H-2, H-4, H-7, H-8, H-11, H-12, H-13 and C-6; H-2 and C-3; H-4, H-13 and C-5; H-2″ and C-1″, C-3″, C-4″, C-7″) were also observed in the HMBC spectrum (Fig. 2A). The glycosylated proton of H-3 at δ_H 3.93 showed couplings in the correlation spectroscopy (COSY) with H_{ax}-2, H_{eq}-2, H_{ax}-4 and H_{eq}-4. Thus, compound **1** was determined as 5-megastigmen-3,9-diol-3-*O*-(6′-galloyl)-β-D-glucopyranoside. To determine the absolute configuration of 3- and 9-positions, enzymatic hydrolysis of **1** with β-glucosidase yielded an aglycone as 5-megastigmen-3,9-diol, and then the modified Mosher's method was applied.[11] The (*R*)-α-methoxy-α-trifluoromethylphenyl acetyl (MTPA) and (*S*)-MTPA esters were prepared. The ^{1}H-NMR spectrum of (*S*)-MTPA ester showed the upfield chemical shift for H_2-2, H_3-11, and H_3-12 and downfield chemical shift for the H_2-4 relative to the same groups in the alternate diastereomer prepared from (*S*)-(+)-MTPA-Cl, so that the C-3 position has *R* configuration (Fig. 2B). In addition, the ^{1}H-NMR spectrum of (*S*)-MTPA ester showed the downfield chemical shift for H-10 and upfield chemical shift for H_2-8, H_2-7 compared with the same groups in other diastereomer obtained from (*S*)-(+)-MTPA-Cl, which also lead to determine the absolute configuration at C-9 was *R*[11] (Fig. 2B). From the above, **1** was identified as (3*R*,9*R*)-5-megastigmen-3,9-diol-3-*O*-(6′-galloyl)-β-D-glucopyranoside named galloyl linarionoside A.

The known compounds were identified as linarionoside A (**2**),[12] 2,3-*trans*-3,4-*trans*-4,5-*trans*-2,5-bis-(4-hydroxyphenyl)-3,4-dimethyltetrahydrofuran (**3**),[13] 3α,24-dihydroxyolean-12-en-27-oic acid (**4**),[14] 3α-hydroxyolean-12-en-27-oic acid (**5**),[15] β-amyrin (**6**),[16] 3-oxolean-12-en-27-oic acid (**7**),[15] isoquercitrin (**8**), astragalin (**9**), kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**10**), rutin (**11**),[8] quercetin-3-*O*-(6′-galloyl)-β-D-glucopyranoside (**12**),[9] 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranoside (**13**)[17] and dihydromyricetin (**14**)[18] on basis of spectroscopic analysis, chemical evidence and the literature data.

The cytotoxic effects of isolated compounds (**1**–**14**) were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[19] These compounds did not affect the cell viabilities of RAW264.7 cells in either the present or absence of LPS, even at a dose of 30 μM after a period of 24 h (data not shown). In murine RAW264.7 macrophages, LPS stimulation alone can induce iNOS transcription and protein synthesis and subsequent NO production.[20] Therefore, this cell system is an excellent model for evaluating topical agents and screening potential inhibitors of the pathways that induce

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data for Compound **1**

Position	δ_H (J in Hz)[a]	δ_C[b]
1		38.9
2ax	1.41 (m)	47.7
2eq	1.77 (brd, 12.5)	
3	3.93 (m)	74.5
4ax	1.99 (dd, 9.6,16.0)	39.9
4eq	2.26 (brdd, 4.8, 16.0)	
5		125.0
6		138.7
7a	1.86 (td, 6.4, 11.2)	25.6
7b	2.14 (td, 6.4, 11.2)	
8a	1.43 (m)	40.7
8b	1.45 (m)	
9	3.68 (sext, 6.4)	69.3
10	1.15 (d, 6.4)	23.3
11	0.88 (s)	28.8
12	0.93 (s)	30.2
13	1.59 (s)	20.1
Glc		
1′	4.42 (d, 7.6)	103.1
2′	3.19 (t, 7.6)	75.5
3′	3.38 (m)	78.1
4′	3.34 (m)	72.2
5′	3.58 (m)	75.3
6′	4.48 (dd, 4.8, 11.2), 4.42 (m)	65.1
Galloyl		
1″		121.5
2″	7.10 (s)	110.4
3″		146.7
4″		139.9
5″		146.7
6″	7.10 (s)	110.4
7″		168.4

a) ^{1}H-NMR (CD_3OD, 400 MHz, δ value) spectroscopic data. *b*) ^{13}C-NMR (CD_3OD, 100 MHz) spectroscopic data.

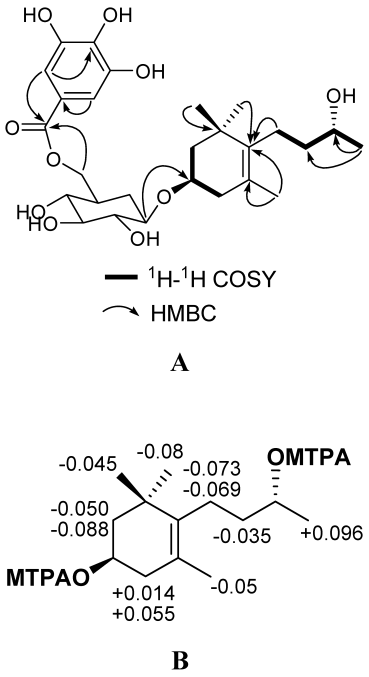

Fig. 2. Selected ^{1}H–^{1}H COSY and Key HMBC Correlations of Compound **1** (A) and Chemical Shift Difference for the (*S*)-MTPA Ester (**1c**) and (*R*)-MTPA Ester (**1b**) in ppm (δ_S–δ_R) (B)

Table 2. Inhibition of NO Production in Macrophage RAW264.7 Cells by Compounds **1**–**14**

Compound	IC_{50} (μM)[a]
1	>30
2	>30
3	>30
4	12.5±3.0
5	9.5±2.0
6	10.5±1.8
7	9.3±2.4
8	>30
9	>30
10	>30
11	>30
12	>30
13	17.2±2.8
14	>30
Celastrol[b]	1.0±0.1

a) The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean±S.D.). *b*) Positive control for NO production.

iNOS and NO production. Due to the rapid half-life of NO *in vivo*, we used nitrite production as a biomarker of NO production in LPS-stimulated RAW264.7 cells. To investigate the effect of compounds **1**–**14** on NO production, the Griess assay was applied.[19] RAW264.7 cells were stimulated with LPS in the presence or absence of **1**–**14** for 24 h. Nitrite levels in LPS-stimulated cells increased significantly compared to control. As shown in Table 2, compounds **4**, **5**, **6** and **7** showed potent inhibitory activity with IC_{50} values of 12.5, 9.5, 10.5 and 9.3 μM, respectively. Compound **13** displayed moderate inhibitory effect with IC_{50} value of 17.2 μM, but the others were inactive. In this experiment, celastrol, a positive inhibitor, significantly inhibited LPS-induced NO production with IC_{50} value of 1.0 μM.[21–23]

In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of large amounts of PGE_2 at inflammatory sites.[3] Among the tested compounds, **7** was most effective on the LPS-induced production of NO in RAW264.7 cells. Therefore, Western blot was performed to determine the inhibitory effects of compound **7** on the modulation of iNOS and COX-2 expression. In unstimulated RAW264.7 cells, iNOS and COX-2 proteins were not detected, but LPS markedly upregulated their protein levels, and pre-treatment with **7** inhibited these up-regulations. Compound **7** (1–10 μg/mL) dose-dependently reduced the LPS-induced iNOS and COX-2 expressions, but did not change the α-tubulin expression. Particularly, the LPS-induced COX-2 was changed clearly as the concentration of compound **7** increased from 3 to 10 μg/mL (Fig. 3).

The translocation of nuclear factor-kappa B (NF-κB) to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of inhibitor kappa B-alpha (IκB-α).[24] We determined whether the inhibition of LPS-induced NF-κB activation by compound **7** was caused by inhibition

of IκB-α degradation. Cells were exposed to LPS for various times. Cytoplasmic extracts were analyzed for the presence of IκB-α using Western blots. IκB-α was almost completely degraded in 15 min after stimulation with LPS and resynthesized in 30 min (Fig. 4A). We pretreated cells with difference concentrations of **7** (3–30 μg/mL) and then exposed them to LPS for 10 min. However, compound **7** could not significantly prevent LPS-induced degradation and resynthesis of IκB-α (Fig. 4B). Thus, this study could be combined suggesting that compound **7** produced anti-inflammatory activity through reducing inducible nitric oxide synthase and cyclooxygenase-2 proteins, not through IκB-α dependent nuclear factor-kappa B pathway.

Experimental

Materials and Methods The aerial parts of *A. rossii* were collected at Jeongbong-san, Kangwondo, Korea, in June 2007 and identified by Dr. Hyeong Kyu Lee. A voucher specimen (PB-1636) was deposited at the herbarium of the Korea Research Institute of Bioscience and Biotechnology, Korea. The aerial parts (3.0 kg) were extracted reflux with MeOH (5 L×3 times). After evaporation of the solvent under reduced pressure, the crude MeOH extract (290 g) was obtained and suspended in hot water and partitioned with *n*-hexane, $CHCl_3$, EtOAc and BuOH, successively, to afford *n*-hexane- (39.5 g), $CHCl_3$- (3.2 g), EtOAc- (53.8 g), BuOH- (67.2 g) soluble fractions, respectively. By the activity-guided fractionation, the $CHCl_3$ soluble fraction (3.2 g) was chromatographed over a silica gel column (6×60 cm, 63–200 μm particle size, Merck) and eluted with *n*-hexane–EtOAc (20 : 1→1 : 1, each 1 L) yielded eight fractions (F1–F8). Subfraction F4 (430.0 mg) was subjected to a silica gel column (4×50 cm, 40–63 μm particle size, Merck), eluted with $CHCl_3$–EtOAc (15 : 1→2 : 1, each 0.5 L) to obtain compounds **3** (3.4 mg) and **7** (50.0 mg). Subfraction F6 (280 mg) was applied to a silica gel column (3×40 cm, 40–63 μm particle size, Merck), eluted with $CHCl_3$–EtOAc (25 : 1→5 : 1, each 0.5 L) to afford six subfractions (F6.1–F6.6). The subfraction F6.2 (55.6 mg) was chromatograped over a silica gel column (2×40 cm, 40–63 μm particle size) and eluted with $CHCl_3$–MeOH (30 : 1→5 : 1, each 0.4 L) to obtain compounds **4** (7.0 mg) and **5** (7.2 mg). Compound **6** (7.1 mg) was crystallized from subfraction F6.4 (18 mg) with $CHCl_3$–MeOH (10 : 1). The EtOAc-soluble fraction (53.8 g) was also subjected to a silica gel column (12×60 cm, 63–200 μm particle size), eluted with $CHCl_3$–MeOH–H_2O (9 : 1 : 0.1→1 : 1 : 0.02, each 2 L) to obtain nine subfractions (E1–E9). Subfraction E3 (3.5 g) was applied to RP-C_{18} silica gel column (6×60 cm, 40–63 μm particle size), eluted with MeOH–H_2O (1 : 4→1 : 1) to yield ten subfractions (E3.1–E3.10). Subfraction E3.4 (72.0 mg) was applied to one more RP-C_{18} silica gel column (2×60 cm, 40–63 μm particle size) and eluted with ACN–H_2O (1 : 8→2 : 3, each 1 L) to afford compounds **1** (15.0 mg) and **8** (25.0 mg). Subfraction E3.6 (85.0 mg) was purified by HPLC [eluting with MeOH–H_2O (75 : 25→55 : 45) over 90 min; flow rate: 5 mL/min; UV detection at 210 nm; column COSMOSIL (20×250 mm, 5 μm)] to obtain compounds **9** (8.0 mg, t_R=29.8 min), **10** (3.4 mg, t_R=42.6 min) and **11** (7.1 mg, t_R=51.3 min). Subfraction E8 (2.2 g) was subjected to RP-C_{18} silica gel column (6×60 cm, 40–63 μm particle size), eluted with ACN–H_2O (1 : 5→2 : 3, each 1.2 L) to afford 8 subfractions (E8.1–E8.8). Subfraction E8.4 (65.0 mg) was applied to RP-C_{18} silica gel column (2×40 cm, 40–63 μm particle size) using MeOH–H_2O (1 : 4→2 : 3, each 0.6 L) to obtain compounds **12** (20.0 mg) and **14** (10.8 mg). Compound **2** (30.0 mg) was crystallized from subfraction E8.6 (45.0 mg). Subfraction E8.5 (48.0 mg) was chromatogaphed over RP-C_{18} silica gel column (2×40 cm, 40–63 μm particle size), eluted with ACN–H_2O (1 : 5→1 : 2, each 0.5 L) to afford compound **13** (32.0 mg).

Galloyl Linarionoside A (**1**): Amorphous powder; $[\alpha]_D^{25}$ 0 (c=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 277 (3.98), 216 (4.36); IR ν_{max} (KBr) 3328, 2943, 2831, 2527, 2356, 1748, 1449, 1418, 1116, 1024 cm^{-1}; HR-FAB-MS m/z 549.2316 $[M+Na]^+$ (Calcd for $C_{26}H_{38}O_{11}Na$, 549.2312). ^1H- and ^{13}C-NMR spectroscopic data, see Table 1.

Enzymatic Hydrolysis of 1 Compound **1** (10.0 mg) was dissolved in AcOH–NaOAc buffer (pH 5.0, 5 mL) with naringinase (Sigma, EC 232-962-4, β-glucosidase activity: 69 units/g) (25.0 mg) and incubated at room temperature for 14 h. The crude reaction mixture was extracted with EtOAc

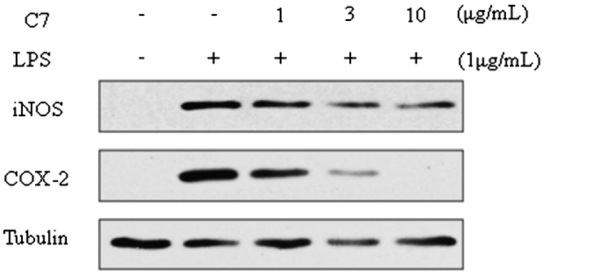

Fig. 3. Inhibition of LPS-Induced iNOS and COX-2 Expression in RAW264.7 Cells by Compound **7** (C7)

RAW264.7 cells were pretreated for 30 min indicated concentrations of **7**, followed by stimulation with LPS (1 μg/mL) for 18 h. Total lysates were prepared, the expression levels of iNOS and COX-2 were determined by western blot analysis. Histograms show densitometry analyses of relative iNOS, COX-2 expression levels normalized against tubulin.

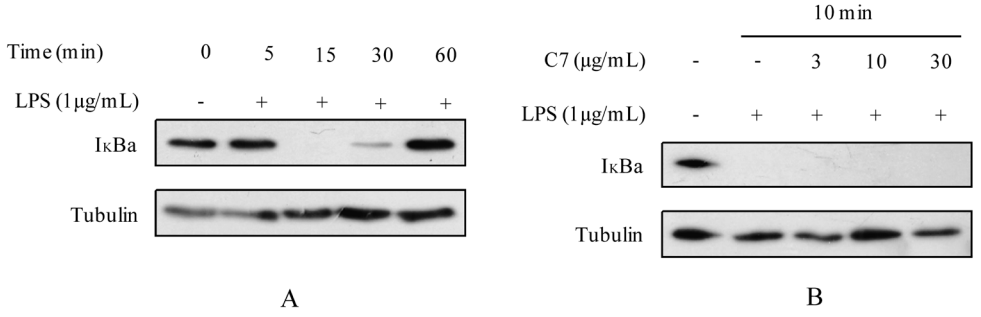

Fig. 4. Effect of Compound **7** on the LPS-Induced IκB-α Degradation

RAW264.7 cells were exposed with 1 μg/mL LPS for the various time periods (A). Cytoplasmic extracts were analyzed for the presence of IKB-α with Western blot analysis. Preincubation with **7** and then exposed them to 1 μg/mL LPS for the indicated time (B).

(5 mL×3 times). The organic layer was dried under reduced pressure. The residue (5.1 mg) was applied to a RP-C_{18} silica gel column (2×20 cm, 40–63 μm particle size, Merck), eluted with acetone–H_2O (2 : 3, 0.5 L) to yield **1a** (3.2 mg).[25]

Preparation of (*R*)- and (*S*)-MTPA Esters (1b, c) from 1a A solution of **1a** (1.0 mg) in dry pyridine (50 μL) was reacted with 5 μL of (*S*)-(−)-MTPA-Cl and 2 mg of 4-DMAP at room temperature for 30 min. The reaction mixture was dried by N_2 gas. The dried product was partitioned with CH_2Cl_2 and H_2O. The organic layer was dried using Na_2SO_4 and concentrated under reduced pressure. The residue was purified by preparative TLC silica gel (0.25 mm thickness) developed with CH_2Cl_2, and the product was eluted with CH_2Cl_2–MeOH (10 : 1, 0.2 L) to furnish (*R*)-MTPA ester, **1b** (1.3 mg). In a similar manner, **1a** (1.7 mg) yielded (*S*)-MTPA ester (**1c**, 1.1 mg).[26]

5-Megastigmen-3,9-di-(*R*)-MTPA Ester (**1b**): Colorless oil; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H 1.571 (1H, m, H_{ax}-2), 1.796 (1H, ddd, *J*=2, 3.6, 12 Hz, H_{eq}-2), 5.228 (1H, m, H-3), 2.283 (1H, dd, *J*=6.4, 16.8 Hz, H_{eq}-4), 2.114 (1H, m, H_{ax}-4), 1.899 (1H, td, *J*=4.4, 12.4 Hz, H-7a), 2.051 (1H, m, H-7b), 1.617 (2H, m, H-8a, H-8b), 5.090 (1H, sext, *J*=5.6 Hz, H-9), 1.271 (3H, d, *J*=6.4 Hz, H-10), 1.045 (3H, s, H-11), 0.990 (3H, s, H-12), 1.520 (3H, s, H-13).

5-Megastigmen-3,9-di-(*S*)-MTPA Ester (**1c**): Colorless oil; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H 1.521 (1H, m, H_{ax}-2), 1.708 (1H, ddd, *J*=1.2, 3.2, 12 Hz, H_{eq}-2), 5.207 (1H, m, H-3), 2.338 (1H, dd, *J*=5.6, 16.4 Hz, H_{eq}-4), 2.128 (1H, m, H_{ax}-4), 1.826 (1H, td, *J*=5.2, 16.1 Hz, H-7a), 1.982 (1H, m, H-7b), 1.582 (2H, m, H-8a, H-8b), 5.117 (1H, sext, *J*=6.0 Hz, H-9), 1.340 (3H, d, *J*=6.4 Hz, H-10), 1.000 (3H, s, H-11), 0.910 (3H, s, H-12), 1.470 (3H, s, H-13).

Cell Culture The murine macrophage cell line (RAW264.7) was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Inc., NY, U.S.A.) supplemented with 100 U/mL of penicillin, 100 U/mL of streptomycin and 10% fetal bovine serum (FBS, GIBCO, Inc., NY, U.S.A.). The cells were incubated in an atmosphere of 5% CO_2 at 37°C and were subcultured every 3 d.

Determination of NO Production and the Cell Viability Assay The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants as described previously.[19,23] Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 24 h in the presence or absence of the test compounds (0.5−25 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled H_2O). The absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, U.S.A.), and the absorption coefficient was calibrated using a $NaNO_2$ solution standard. The amount of TNF-α in the culture supernatant was measured using the enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.). Cell viability was measured with MTT-based colorimetric assay. For this experiment, celastrol was used as a positive control.

Immunoblot Analysis Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethyl sulfonyl fluoride, 1 μg/mL leupeptin, 1 mM sodium vanadate, 150 mM NaCl). Fifty micrograms of protein (for iNOS) per lane was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.). The membrane was blocked with 5% skim milk, and then incubated with the corresponding antibody. The antibody for iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The antibody for α-tubulin was obtained from Sigma. Antibody for IκB-α was obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotec, Buckinghamshire, U.K.).[23,27]

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