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Inhibitory Lignans against NFAT Transcription Factor from *Acanthopanax koreanum*

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Three lignans isolated from the roots of *A. koreanum* (Araliaceae), namely eleutheroside E (**1**), tortoside A (**2**), and hemiariensin (**4**), were evaluated for their ability to inhibit NFAT transcription factor. Of these compounds, compound **4**, possessing a diarylbutane skeleton, exhibited potent inhibitory activity against NFAT transcription factor (IC_{50}: 36.3 ± 2.5 μM). However, the activities of **1** (IC_{50}: > 500 μM) and **2** (IC_{50}: 136.1 ± 9.4 μM), which possess bisaryldioxabicyclooctane skeletons, were lower. As the lignan derivatives of the same skeletons, hinokinin (**5**) and (-)-yatein (**6**) with diarylbutane skeletons and (+)-syringaresinol (**3**) with a bisaryldioxabicyclooctane skeleton were also studied for their inhibitory effects on NFAT transcription factor.

Key words: *Acanthopanax koreanum*, Araliaceae, Lignan, NFAT transcription factor, Inhibitory effect

INTRODUCTION

NFAT, nuclear factor of activated T-cells, is a cytoplasmic protein and is activated by the stimulation of cell surface receptors coupled to Ca^{2+} mobilization. NFAT protein is dephosphorylated by the Ca^{2+}-activated phosphatase, calcineurin, and then migrates to the nucleus to induce transcription of genes required for T-cell activation, one of which is IL-2 (Winter *et al.*, 1993; Abbas *et al.*, 1997). The activation of NFAT normally plays a significant role in the induction of immune response. However, excessive activation provokes immunopathological reactions including autoimmunity, transplant rejection, and inflammation (Abbas *et al.*, 1997). Thus, the modulation of NFAT transcription factor ($NFAT^{TF}$) should be useful in the treatment of inflammations and immune diseases. Until now, only a few inhibitors of natural product origin have been reported to have inhibitory activity against $NFAT^{TF}$. Recently, lignans isolated from *Schisandra chinensis* (Lee *et al.*, 2003), and phthalides and an acetylenic component isolated from *Cnidium officinale* (Lee *et al.*, 2002), were reported to regulate $NFAT^{TF}$. In an ongoing screening study of $NFAT^{TF}$ regulators from medicinal plants, three lignans isolated from the roots of *Acanthopanax koreanum* (Araliaceae), which are used for the treatment of rheumatism in traditional Oriental medicine (Bensky *et al.*, 1986), and three lignan derivatives with the same skeleton were investigated in terms of their abilities to inhibit $NFAT^{TF}$.

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MATERIALS AND METHODS

General

Melting points were measured using a Yanaco micro melting point, optical rotation with a Jasco DIP-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 using a Waters HPLC system (600 pump, 600 controller, and a 996 photodiode array detector). NMR spectra were measured using a Bruker DRX 300 spectrometer (^1H, 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

A. koreanum roots were gratefully provided by Susin Ogapi Co. and identified by Professor Young Ho Kim at the College of Pharmacy, Chungnam National University. Voucher specimens (CNU 96076) were also deposited at the university's herbarium. Hinokinin (**5**) was obtained from the stem bark of *A. trifoliatus* (Phan *et al*, 2003), and (-)-yatein (**6**) was collected from the HPLC effluents of synthesized racemic yatein (Lim *et al.*, 2002).

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 H_2O layer was concentrated *in vacuo* yielding H_2O-soluble fraction (300 g), a portion of which (100 g) was chromatographed on MCI gel (Mitsubishi Chemical Corporation) by eluting with a H_2O-MeOH gradient to give six fractions (fr. A~F). Fr. B (21.0 g) was subjected to Sephadex LH-20 and silica gel column chromatography to afford **1** (550.0 mg). Fr. D (10.5 g) was subjected to silica gel column chromatography and successive MPLC using a LiChroprep® RP-18 (40-63 mm) column (Merck Art. 10624., Germany) to afford **2** (9.4 mg). The CH_2Cl_2 soluble fraction (200 g) of the MeOH extract of the root of *A. koreanum* was chromatographed on a silica gel column, and eluted with a hexane-EtOAc gradient, to give 5 TLC fractions (fr. a~e). Fr. d (13.0 g) was subjected to silica gel column chromatography and eluted with hexane-EtOAc to give nine subfractions (subfr. a~i). Subfr. d (2.9 g) was subjected to silica gel and YMC gel column chromatography to afford **4** (3.6 mg). The structures of the isolated compounds were identified as eleutheroside E (**1**), tortoside A (**2**), and hemiariensin (**4**) by comparing physicochemical and spectroscopic data with previously reported data (Takeshi *et al.*, 1983; Wang *et al.*, 1997; Lalit *et al.*, 1987).

Eleutheroside E (1)

White powder, mp 250~252 °C, $[\alpha]_D^{25}$: -5.6 (*c* 0.09, H_2O); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.66 (4H, s, aromatic protons), 3.76 (12H, s, 4×-OCH_3).

Tortoside A (2)

Amorphous powder, mp 173~175 °C, $[\alpha]_D^{25}$: -10.0 (*c* 1, MeOH); ^{1}H-NMR (300 MHz, CD_3OD) δ: 6.73 (1H, s, H-2', 6'), 6.67 (1H, s, H-2", 6"), 4.88 (1H, m, H-1 of Glc), 4.78 (1H, d, *J* = 4.1 Hz, H-6), 4.73 (1H, d, *J* = 4.4 Hz, H-2), 4.29 (2H, m, H-4a, 8a), 3.91 (2H, m, H-4b, 8b), 3.87 (6H, s, H-3", 5"-OMe), 3.85 (6H, s, H-3', 5'-OMe), 3.79 (1H, dd, *J* = 11.9, 2.4 Hz, H-6b of Glc), 3.69 (1H, dd, *J* = 11.9, 5.1 Hz, H-6a of Glc), 3.47 (1H, m, H-2 of Glc), 3.43 (1H, m, H-5 of Glc), 3.41 (1H, m, H-4 of Glc), 3.21 (1H, m, H-3 of Glc), 3.14 (2H, m, H-1, 5). ^{13}C-NMR (75 MHz, CD_3OD) δ: 55.5 (d, C-1), 87.6 (d, C-2), 72.9 (t, C-4), 55.7 (d, C-5), 87.2 (d, C-6), 73.0 (t, C-8), 139.5 (s, C-1'), 104.5 (d, C-2', 6'), 154.4 (s, C-3', 5'), 135.6 (s, C-4'), 57.1 (q, 3',5'-OMe), 133.1 (s, C-1), 104.8 (d, C-2", 6"), 149.3 (s, C-3", 5"), 136.2 (s, C-4"), 56.8 (q, 3", 5"-OMe), 105.3 (d, glc C-1), 75.7 (d, glc C-2), 78.3 (d, glc C-3), 71.4 (d, glc C-4), 77.8 (d, glc C-5), 62.6 (t, glc C-6).

Hemiariensin (4)

White powder, mp 50~60 °C, FAB-MS *m/z*: 423.0 [M+Na]$^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.64 (2H, dd, *J* = 7.7, 1.9 Hz, H-5, 5'), 6.51 (4H, m, H-2, 2', 6, 6'), 5.85 [4H, s, (-OCH_2O-)$_2$], 4.01 (2H, m, H-9'), 3.54 (2H, d, *J* = 5.4 Hz, H-9), 2.55 (4H, m, H-7, 7'), 2.09 (1H, m, H-8'), 1.98 (3H, s, CH_3), 1.86 (1H, m, H-8). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 133.1 (s, C-1), 132.7 (s, C-1'), 107.1 (d, C-2, 2'), 146.6 (s, C-3, 3'), 144.8 (s, C-4, 4'), 108.2 (d, C-5, 5'), 120.7 (d, C-6. 6'), 34.0 (t, C-7), 33.7 (t, C-7'), 42.2 (d, C-8), 39.1 (d, C-8'), 61.3 (t, C-9), 63.5 (t, C-9'), 99.8 (-OCH_2O-), 19.9 (CH_3), 169.9 (CO).

Microbial transformation of 1

To identify the metabolites of **1** produced by human intestinal microflora, a reaction mixture containing 100 mg of **1** and 10 ml fresh human fecal suspension (250 mg) in a final volume of 50 ml of an anaerobic dilution medium (Hattori *et al.*, 1985) was incubated at 37 °C for 20 h. After the reaction, metabolites were extracted with EtOAc three times. The EtOAc phase was concentrated *in vacuo* to give an EtOAc extract (70 mg), which was subjected to preparative HPLC using a reverse-phase YMC J'sphere ODS-H80 column (YMC Co. Ltd., Japan) to afford **3** (22.3 mg). The structure of **3** was elucidated to be (+)-syringaresinol according to reported data (Takeshi *et al.*, 1983).

(+)-Syringaresinol (3)

Colorless needles, mp 180.5~183.6 °C, $[\alpha]_D^{25}$: +44.0 (*c* 0.1, $CHCl_3$); ^{1}H-NMR (300 MHz, CD_3OD) δ: 6.67 (4H, s, H-2', 2", 6', 6"), 4.80 (2H, d, *J* = 4.0 Hz, H-2, 6), 4.34 (2H, dd, *J* = 6.9, 9.0 Hz, H-4, 8), 3.86 (12H, s, 4×OCH_3), 3.64 (2H, dd, *J* = 3.6, 9.0 Hz, H-4, 8), 3.14 (2H, m, H-1, 5). ^{13}C-NMR (75 MHz, CD_3OD) δ: 54.5 (d, C-1, 5), 86.6 (d, C-2, 6), 71.7 (t, C-4, 8), 132.2 (s, C-1', 1"), 103.7 (d, C-2', 2", 6', 6"), 148.4 (s, C-3', 3", 5', 5"), 135.4 (s, C-4', 4"), 55.9 (q, 3', 3", 5', 5"- OMe).

Preparation of buffers and reagents

RPMI 1640 without phenol red (11835-030, Gibco. BRL) was mixed with 0.5% fetal bovine serum and 1% penicillin-streptomycin. Phorbol 12-myristate 13-acetate

(25 ng/mL) and ionomycin (0.5 μM), as a stimulator, were dissolved in DMSO. *p*-Nitro-phenylphosphate (120 mM), as a substrate, was dissolved in a modified secreted alkaline phosphatase (SEAP) buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 10 mM homoarginine).

Preparation of cells and samples

The Jurkat T-cell line, which contains an NFAT-dependent transcriptional reporter gene, pCMV·SEAP, was maintained in RPMI 1640 medium containing phenol red supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were harvested by centrifugation, washed once in PBS and resuspended in RPMI 1640 without phenol red. Each sample was dissolved in DMSO and diluted in phenol red free RPMI 1640.

Inhibitory activity upon NFAT transcription

The inhibitory activities of the samples on $NFAT^{TF}$ were determined by using a modified SEAP assay (Yang *et al.*, 1997), as previously described (Lee *et al.*, 2002; Lee *et al.*, 2003). For this assay, 100 μL of cells (1×10^4 cells/well) were incubated with 50 μL of sample and 50 μL of stimulator at 37 °C for 18 h, and centrifuged. 100 μL of the supernatant obtained was then heated at 65 °C for 1 h, and then incubated with 50 μL of SEAP buffer and 50 μL of the substrate at 37 °C for 4 h. Optical density was measured at 405 nm. The inhibitory activity of a sample on $NFAT^{TF}$ was expressed as a percentage of the uninhibited control. Cyclosporin A was used as a positive control, because it blocks the phosphatase activity of calcineurin, thereby preventing the subsequent dephosphorylation and translocation of NFAT to the nucleus (Jain *et al.*, 1995). Cell viability was determined using an MTT cell proliferation kit (1465007, Roche).

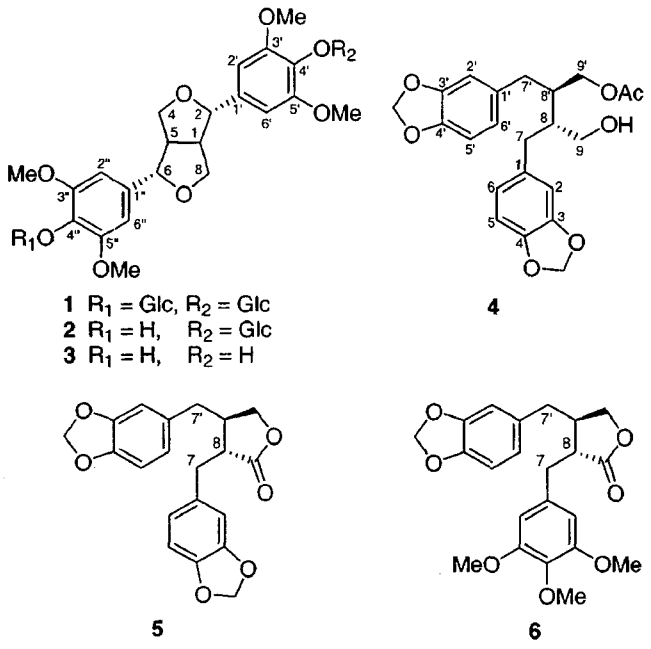

Fig. 1. *Structures of compounds* **1-6**

RESULTS AND DISCUSSION

$NFAT^{TF}$ is required for the expression of a group of proteins that collectively coordinate immune response. However, the excessive activation of $NFAT^{TF}$ provokes immunopathological reactions including autoimmunity, transplant rejection, and inflammation (Abbas *et al.*, 1997). Thus, modulators of $NFAT^{TF}$ are felt likely to be useful in the treatment of inflammations and immune diseases. Recently, we described several $NFAT^{TF}$ inhibitors with natural plant origins (Lee *et al.*, 2002; Lee *et al.*, 2003; Nguyen *et al.*, 2004). In an ongoing screening of $NFAT^{TF}$ inhibitors in medicinal plants, three lignans (**1**, **2**, and **4**) were isolated by repeated column chromatography from the dichloromethane and H_2O fractions of *A. koreanum*, and their $NFAT^{TF}$ inhibitory activities were assayed. Of these compounds, compound **4**, with a diarylbutane skeleton, exhibited potent inhibitory activity (IC_{50}: 36.3 ± 2.5 μM) on $NFAT^{TF}$. Thus, the inhibitory activity of **4** was compared with those of hinokinin [**5**, mp 92~95 °C, $[\alpha]_D^{25}$: -27.0°, (*c* 0.5, MeOH), Phan *et al.*, 2003] and (-)-yatein [**6**, $[\alpha]_D^{25}$: -33.3°, (*c* 0.1, $CHCl_3$), Lim *et al.*, 2003], both of which contain a diarylbutane skeleton. Interestingly, compound **5**, having a butylolactone group and two dioxabicyclo ring moieties, did not inhibit $NFAT^{TF}$ activity (IC_{50}: > 500 μM), whereas **6**, having a butylolactone group and three methoxy groups on one aryl ring, showed a moderate inhibitory effect (IC_{50}: 66.8 ± 1.7 μM). These results suggest that diarylbutane skeleton lignans with a functional group, e.g., a methoxy, acetoxy, or hydroxy might have enhanced $NFAT^{TF}$ inhibitory activity.

In the case of the lignan type of bisaryldioxabicyclooctane skeleton, compound **2** (IC_{50}: 136.1 ± 9.4 μM), with a hydroxy group at C-4, had a higher activity than **1** (IC_{50}: > 500 μM), with two sugar moieties at C-4 instead. In order

Table I. Inhibitory activity on NFAT transcription of lignan components

Compounds	IC_{50} value (mM)[a]
Eleutheroside E (**1**)	>500
Tortoside A (**2**)	136.1 ± 9.4
(+)-Syringaresinol (**3**)	329.4 ± 8.7
Hemiariensin (**4**)	36.3 ± 2.5
Hinokinin (**5**)	>500
(-)-Yatein (**6**)	66.8 ± 1.7
Cyclosporin A[b]	0.29 ± 0.1

[a] Values of IC_{50} are presented as mean ± SE of three experiments.
[b] This compound was used as the positive control.

to compare the inhibitory effect of the aglycone type of bisaryldioxabicyclooctane skeleton, compound **1** was hydrolyzed by human intestinal microflora to produce a lignan with a bisaryldioxabicyclooctane skeleton and two hydroxy groups at C-4. Compound **3** was isolated by preparative HPLC using a reverse-phase column from the culture broth of intestinal microflora. However, the inhibitory effect of **3** against NFAT^{TF} (IC_{50}: 329.4 ± 8.7 μM) was not enhanced although it possesses two hydroxy groups at C-4. From these results, it is presumed that the lignan type of the bisaryldioxabicyclooctane skeleton does not contribute to NFAT^{TF} inhibition, and that this is independent of the number of sugar moieties or hydroxy groups. Therefore, the inhibition of NFAT^{TF} by lignans isolated from *A. koreanum* was attributed to the diarylbutane skeleton, and this activity was found to be increased by functional groups such as methoxy, acetoxy, and hydroxy.

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