Gymnasterkoreayne G, a New Inhibitory Polyacetylene against NFAT Transcription Factor from *Gymnaster koraiensis*

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A new polyacetylene, gymnasterkoreayne G (1) and seven known (2—8) constituents were isolated from the leaves of *Gymnaster koraiensis*. Base on extensive 1D and 2D NMR spectroscopic data, the structure of the new compound was identified as *erythro*-8(*S*)-9(*Z*),16-heptadecadiene-4,6-diyne-2,3,8-triol. Isolated compounds were evaluated for their ability to inhibit NFAT transcription factor. While other components did not show activity, most of polyacetylene components markedly inhibit NFAT transcription factor. Of these compounds, gymnasterkoreayne B (3) was the most potent (IC_{50} 1.44±0.59 μM). In term of the isomers, compound 1 (IC_{50} 43.9±2.24 μM) with an *erythro*-configuration showed less inhibition than 2 (IC_{50} 7.24±0.42 μM) with a *threo*-configuration.

Key words *Gymnaster koraiensis*; NFAT; polyacetylene; gymnasterkoreayne

Transcription factor called "nuclear factor of activated T cells" (NFAT) is a cytoplasmic protein, which is activated by cell surface receptor stimulation coupled to Ca^{2+} mobilization. The Ca^{2+} activated phosphatase, calcineurin, dephosphorylates NFAT protein, and thus promotes its nuclear translocation and activation.[1] However, the excessive activation of NFAT provokes immunopathological reactions including autoimmunity, transplant rejection and inflammation.[2] Therefore, the modulation of NFAT transcription factor could be useful in the therapy of immune diseases.

In our ongoing study to screen regulators from medicinal plants, potent activity was found in the MeOH extract of the leaves of *Gymnaster koraiensis* (NAKAI) KITAMURA (Asteraceae), which is a plant endemic to Korea. The polyacetylenic constituents and their anticancer activities had been reported from this plant.[3,4]

Based on activity-guided fractionation for inhibitory activity against NFAT transcription factor, a new compound named as gymnasterkoreayne G (**1**) was isolated from the leaves of *G. koraiensis* with seven known compounds: gymnasterkoreayne E (**2**), gymnasterkoreayne B (**3**), 2(*E*),9(*Z*),16-heptadecatriene-4,6-diyne-8-ol (**4**), gymnasterkoreayne F (**5**), friedelinol (**6**) , friedelin (**7**), and squalene (**8**).

Results and Discussions

During the screening for regulators of NFAT transcription factor in the MeOH extracts of several medicinal plants, we found that the aerial parts of *Gymnaster koraiensis* exhibited potent activity (IC_{50} 15.8±0.30 μg/ml). Thus, its MeOH extract was suspended in water and partitioned in dichloromethane and butanol, respectively. The dichloromethane fraction obtained, which showed potent inhibitory activity against NFAT transcription factor (IC_{50} 6.00±0.53 μg/ml), was chromatographed and yielded the new compound **1** and seven known compounds. As shown in Fig. 1, the structures of the known compounds were identified by comparing physicochemical and spectroscopic data with previously reported data; **2**, **3**,[3] **4**,[5,6] **5**,[3] **6**,[7] **7**,[8] and **8**.[9]

Compound **1** was obtained as an orange oil and its HR-FAB-MS spectrum revealed a $[M+Na]^+$ peak at *m/z* 299.3648, corresponding to molecular formula $C_{17}H_{24}O_3Na$. The ^1H- and ^{13}C-NMR spectra of **1** were similar to those of the known compound **2** except for chemical shifts change from C-1 to C-4 (Table 1). In the ^{13}C-NMR spectrum of **1**, the chemical shifts of C-3 and C-4 appeared at δ 67.8 and 77.4 *versus* δ 55.3 and 74.5 in **2**, while chemical shifts of C-

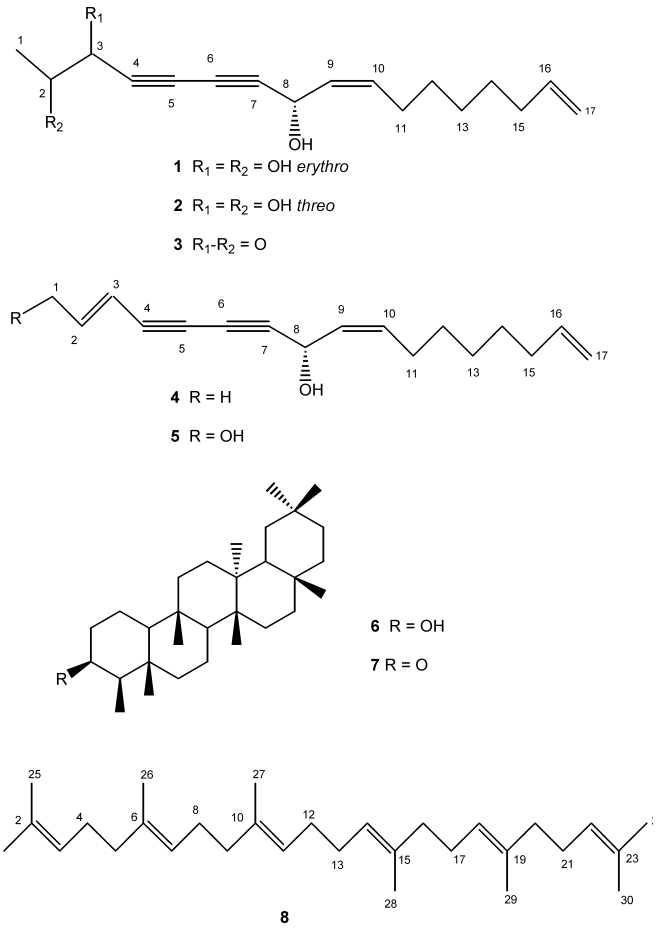

Fig. 1. Structure of Compounds Isolated from *G. koraiensis*

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Table 1. NMR Spectral Data for **1** and **2**

No.	**1**		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.30 (3H, d, J=6.3 Hz)	18.6	1.38 (3H, d, J=6.3 Hz)	19.2
2	3.93 (1H, dq, J=3.6, 6.3 Hz)	70.6	4.06 (1H, dq, J=4.2, 6.3 Hz)	71.0
3	4.38 (1H, d, J=3.6 Hz)	67.8	4.62 (1H, d, J=4.2 Hz)	55.3
4	—	77.4	—	74.5
5	—	71.0	—	72.4
6	—	69.0	—	68.7
7	—	79.7	—	81.3
8	5.21 (1H, d, J=7.8 Hz)	58.9	5.22 (1H, d, J=8.1 Hz)	58.9
9	5.53 (1H, dt, J=1.2, 8.1 Hz)	128.1	5.53 (1H, dt, J=1.2, 8.1 Hz)	127.9
10	5.62 (1H, m)	134.8	5.62 (1H, m)	135.1
11	2.13 (2H, m)	28.0	2.13 (2H, m)	28.0
12	1.36—1.43 (m)	29.4	1.36—1.43 (m)	29.4
13	1.36—1.43 (m)	29.0	1.36—1.43 (m)	29.0
14	1.36—1.43 (m)	29.0	1.36—1.43 (m)	29.0
15	2.07 (2H, m)	34.0	2.07 (2H, m)	34.0
16	5.83 (1H, m)	139.3	5.83 (1H, m)	139.3
17	4.93, 5.05 (2H, m)	114.8	4.93, 5.05 (2H, m)	114.8

1 and C-2 in **1** appeared at higher field than those in **2**. Both the ^{1}H-NMR of **1** and **2** showed identical peaks but the coupling constant between H-2 and H-3 in **1** (J=3.6 Hz) was smaller than that in **2** (J=4.2 Hz). These comparisons suggest that **1** and **2** are isomers. Moreover, empirically, the coupling constant of protons in the *erythro*-configuration is smaller than that in the *threo*-configuration.[10,11] Before gymnasterkoreayne E having large coupling constant (J=4.1 Hz) was reported[3] as *erythro*-configuration by only comparison of coupling constant by literature,[12] but it might be assigned incorrectly due to the lack of both isomers. This time both isomers were isolated from the same plant and determined to configuration having large coupling constant as *threo*- and small coupling constant as *erythro*-. By these reasons, compound **1** was identified as *erythro*-8(*S*)-9(*Z*),16-heptadecadiene-4,6-diyne-2,3,8-triol named gymnasterkoreayne G and compound **2** as gymnasterkoreayne E having *threo*-configuration.

The eight compounds isolated were investigated for their abilities to inhibit NFAT transcription factor. The triterpenes (**6**, **7**) and the polyene (**8**) components had no inhibitory effect (IC_{50} >50 μM). In a previous NFAT transcription factor inhibitory study of triterpenes isolated from *Liquidambar formosana*, liquidambaric acid and oleanolic acid, which lack an oxy methylene group at C-25 showed no activity, whereas 3α-acetoxy-25-hydroxy-olean-12-en-28-oic acid and lantanolic acid, which have this group, exhibited activity.[13] Thus, this report is consistent with the result of **6** and **7** in the present study, which also lack the oxy methylene group at C-25. However, the polyacetylenic compounds (**1**—**5**) showed strong inhibition (Table 2). In term of the isomeric polyacetylene compounds (**1**, **2**), the activity of **1** (IC_{50} 43.9±2.24 μM) with *erythro*-isomer was lower than that of **2** (IC_{50} 7.24±0.42 μM) with *threo*-isomer (p<0.05). This result indicates that the *threo*-configuration is more effective than the *erythro*-configuration at regulating the NFAT transcription factor. However, compound **3** (IC_{50} 1.44±0.59 μM) with an epoxy group between C-2—C-3 strongly inhibited the NFAT transcription factor as compared to **1** (p<0.01). This result suggests that the introduction of an epoxy group between C-2—C-3 increases activity than the isomeric hydroxy groups. Compounds **4** and **5** possess the same polyacetylene skeleton with **1**—**3** containing a triene-component but differ with respect to an additional double bond between C-2—C-3. These two compounds showed potent inhibitory activities against NFAT transcription factor. However, the inhibitory activity of **5** (IC_{50} 10.6±0.46 μM), which contains a hydroxy group at C-1, was significantly (p<0.01) lower than that of **4** (IC_{50} 4.95±0.24 μM). These findings indicate that the introduction of hydroxy group in polyacetylene and triterpene compounds tend to reduce activity against NFAT transcription factor. From these results, the potent activity of *G. koraiensis* against NFAT transcription factor is due to polyacetylene constituents, and that the introduction of an epoxy group considerably increases activity. Therefore, *G. koraiensis* and its polyacetylenic components should be further studied for their therapeutic applications for the treatment of autoimmunity, transplant rejection, and inflammation caused by the excessive NFAT activation.

Table 2. Inhibitory Activity against NFAT Transcription Factor of Compounds Isolated from *G. koraiensis*

Compound	IC_{50} (μM)[a]
MeOH extract	15.8±0.30 (μg/ml)
CH_2Cl_2 fraction	6.00±0.53 (μg/ml)
Gymnasterkoreayne G (**1**)	43.9±2.24
Gymnasterkoreayne E (**2**)	7.24±0.42
Gymnasterkoreayne B (**3**)	1.44±0.59
2(*E*),9(*Z*),16-heptadecatriene-4,6-diyne-8-ol (**4**)	4.95±0.24
Gymnasterkoreayne F (**5**)	10.6±0.46
Epifriedelanol (**6**)	>50
Friedelin (**7**)	>50
Squalene (**8**)	>50
Cyclosporin A[b]	0.31±0.01

a) Values of IC_{50} are presented as mean±S.E. of three experiments. *b*) Cyclosporin A was used as the positive control.

Experimental

General Melting points were measured using a Yanagimoto micro hot-stage melting point apparatus and were uncorrected. IR spectra were obtained from a Jasco 100 IR spectrophotometer; UV spectra from a Jasco V-550 UV/VIS spectrometer; ^{1}H- and ^{13}C-NMR spectra from a Bruker DRX-300 and 600-NMR spectrometer; and FAB-MS spectra from a JMS-HX/HX 110A tandem mass spectrometer.

Plant Material The aerial parts of *G. koraiensis* were collected in a plant garden at Chungnam National University (Korea) in October 2001 and identified by Prof. Young Ho Kim. A voucher specimen (CNU01004) was deposited at the herbarium in College of Pharmacy, Chungnam National University.

Extraction and Isolation The aerial parts of *G. koraiensis* (2.9 kg) were dried at room temperature, powdered, and extracted with hot methanol (6 l×3 times). The MeOH extract (162 g) was then suspended in water (2 l) and extracted with dichloromethane (700 ml×3 times) and butanol (700 ml×3 times), respectively. The dichloromethane fraction (60.8 g), which had potent NFAT transcription inhibitory activity, was chromatographed on silica gel (300 g) with gradient solvent hexane–ethyl acetate (100 : 1 to 1 : 1, 23 l) as eluant to give 15 fractions (Fr. 2-A—P). Compound **1** (45.0 mg) was separated from Fr. 2-N (3.3 g) using a silica-gel column (200 g) with chloroform–methanol (12 : 1, 2 l). The Fr. 2-C (4.7 g) was subjected on silica gel column (150 g) with hexane–chloroform–acetone (4 : 1 : 1, 1 l) as eluant to afford compound **2** (81.0 mg). Compound **3** (102.0 mg) was obtained from Fr. 2-F (1.5 g) using a reverse phase column (100 g YMC powder) with methanol–water (10 : 1, 800 ml) as eluant. Compounds **4** (20.8 mg) and **6** (4.2 mg) were obtained from Fr. 2-D (2.6 g) using a silica gel column (200 g) with solvent hexane–ethyl acetate (15 : 1, 1.2 l) as eluant. Fr. 2-I (0.94 g) was chromatographed on silica gel (150 g) with

hexane–chloroform–acetone (4 : 1 : 1, 1 l) to give compound **5** (10.5 mg). Compounds **7** (9.6 mg) and **8** (289.0 mg) were obtained from Fr. 2-C (4.7 g) by silica gel column chromatography (200 g) with hexane–ethyl acetate (100 : 1, 1.8 l) as eluant.

Gymnasterkoreayne G (**1**): Orange oil; $[\alpha]_D^{20}$ +40.0° (c=0.3, $CHCl_3$); UV (KBr) λ_{max} nm (log ε): 235 (0.81), 250 (0.45); IR (CH_3Cl) ν_{max} cm^{-1}: 3400 (OH), 1620 (C=C), 1210 (C–O). FAB-MS m/z: 299 $[M+Na]^+$; HR-FAB-MS m/z: 299.3648 (Calcd for $C_{17}H_{24}O_3Na$; 299.3651); ^{1}H-NMR ($CDCl_3$, 600 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz): see Table 1.

Gymnasterkoreayne E (**2**): $C_{17}H_{24}O_3$, $[\alpha]_D^{20}$ +87.9° (c=0.3, $CHCl_3$).

Gymnasterkoreayne B (**3**): $C_{17}H_{22}O_2$, $[\alpha]_D^{20}$ +163.0° (c=0.3, $CHCl_3$).

2(*E*),9(*Z*),16-Heptadecatriene-4,6-diyne-8-ol (**4**): $C_{17}H_{22}O$, $[\alpha]_D^{20}$ +173.5° (c=0.3, $CHCl_3$).

Gymnasterkoreayne F (**5**): $C_{17}H_{22}O_2$, $[\alpha]_D^{20}$ +134.0° (c=0.3, $CHCl_3$).

Epifriedelanol (**6**): $C_{30}H_{52}O$, mp 278—281 °C, $[\alpha]_D^{20}$ +28.9° (c=0.5, $CHCl_3$).

Friedelin (**7**): $C_{30}H_{50}O$, mp 264—266 °C, $[\alpha]_D^{20}$ −35.5° (c=0.5, $CHCl_3$).

Squalene (**8**): $C_{30}H_{50}$. FAB-MS: m/z 411 $[M+H]^+$.

Selection of an NFAT Dependent Reporter Cell Line The sense and antisense oligonucleotides containing the NFAT binding site were synthesized, annealed, and ligated to obtain a repeated NFAT binding site. Digested DNA fragment was transfected into T Jurkat cells, and then the growing clones resistant to G418 (0.8 mg/ml) were selected.

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 μg/ml) and ionomycin (0.5 μM), a stimulator, were dissolved in DMSO, and *p*-nitrophenylphosphate (120 mM) used as substrate, was dissolved in SEAP buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 10 mM homoarginine).

Preparation of Cells and Samples The selected Jurkat T cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Harvested cells were re-suspended in RPMI 1640 without phenol red. Each sample was dissolved in DMSO and diluted in RPMI 1640 without phenol red.

Determination of Inhibitory Activity against NFAT Transcription Factor NFAT transcription factor inhibition was determined using the modified secreted alkaline phosphatase (SEAP) assay, as described previously.[14] 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. The reaction mixture was centrifuged and 100 μl of supernatant was heated at 65 °C for 1 h to eliminate nonspecific alkaline phosphatase. This was then incubated with 50 μl of SEAP buffer and 50 μl of substrate at 37 °C for 4 h. The optical density of specific alkaline phosphatase was measured at 405 nm. NFAT transcription factor inhibition is expressed as a percentage of the inhibition of the control and statistical significance was determined by using the Student's *t*-test. The positive control was used with cyclosporin A, which blocks the phosphatase activity of calcineurin, thus preventing the subsequent dephosphorylation of NFAT and its translocation to the nucleus. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation Kit (1465007, Roche).

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