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TWO NEW STEROIDAL ALKALOIDS FROM THE BULBS OF *FRITILLARIA THUNBERGII*

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Abstract – In the quest for biologically active compounds from natural medicinal sources in Korea, the bulbs of *Fritillaria thunbergii* were investigated. Phytochemical analysis of the MeOH extracts resulted in the isolation and identification of ten steroidal alkaloids (**1**–**10**) including two new compounds, named frithunbol A and B (**1** and **2**). The structures of these new compounds were elucidated based on 1D, 2D NMR analyses and HR-FAB-MS. All the isolated compounds (**1**–**10**) were evaluated for their effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells and the neuroprotective effect was determined via induction of nerve growth factor (NGF) in C6 glioma cells. Compounds **2**, **3**, and **10** significantly inhibited NO levels with IC_{50} values of 16.35 μM, 11.45 μM and 18.02 μM, respectively. Also, compound **9** was a potent stimulant of NGF release, and enhanced the levels of NGF by 134.81 ± 3.66%.

The genus *Fritillaria* comprises a group of 130 species that contain mainly steroidal alkaloids.[1] Among them, *Fritillaria thunbergii*, known as “Jeol-Pae-Mo” is distributed in East Asia and traditionally used as anti-tussives, anti-asthmatics, and expectorant in China.[2] Studies have isolated steroidal alkaloids and diterpenoids from this species.[3,4] In the course of our ongoing quest for biologically active compounds from natural medicinal sources in Korea, we investigated the MeOH extracts of the bulbs of *F. thunbergii* and reported the identification of diterpenes.[5] Further purification of the $CHCl_3$ fractions led to the

isolation of two new steroidal alkaloids (**1** and **2**) and eight known compounds (**3**–**10**), which were identified on the basis of spectroscopic analyses. The structures of the new compounds (**1** and **2**) were elucidated via 1D and 2D NMR (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY) data. All the compounds were tested for their inhibitory effects on the NO production in LPS-activated murine microglial cells and their effects on nerve growth factor (NGF) secretion by C6 glioma cells.

Compound **1** was isolated as a colorless gum and tested positive with Dragendorff's reagent. The HR-FAB-MS spectrum revealed the $[M + H]^+$ peak at *m/z* 444.3113, suggesting a molecular formula of $C_{27}H_{41}NO_4$. The IR spectrum of **1** displayed absorption bands for *trans*-quinolizidine (2810 cm^{-1}),[6] carbonyl (1608 cm^{-1}) and hydroxyl (3260 cm^{-1}) groups. The ^{1}H NMR spectrum (Table 1) of **1** exhibited signals associated with a tertiary methyl group [δ_H 0.90 (3H, s, H-19)], two secondary methyl groups [δ_H 1.16 (3H, d, *J* = 7.5 Hz, H-21) and 1.07 (3H, d, *J* = 7.0 Hz, H-27)], and three oxygenated methine groups [δ_H 3.91 (1H, m, H-3), 3.74 (1H, td, *J* = 10.0, 4.5 Hz, H-6) and 4.40 (1H, br. t, *J* = 4.0 Hz, H-16)]. The ^{13}C NMR spectrum of **1** displayed 27 carbon signals including those associated with three methyl groups [δ_C 13.3 (C-19), 19.5 (C-21), and 17.7 (C-27)]; three oxygenated methine carbons [δ_C 71.6 (C-3), 70.3 (C-6), and 65.1 (C-16)]; two *N*-bearing carbons [δ_C 63.2 (C-22), and 50.3 (C-26)], one carbonyl carbon [δ_C 164.4 (C-18)], and double bond carbons [δ_C 147.5 (C-17), and 129.7 (C-13)]. The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of petilidine,[7] a cevane-type steroidal alkaloid, except for the presence of a hydroxyl group at C-16, a carbonyl carbon at C-18, and a double bond between C-13 and C-17 in the D/E ring junction. The planar structure of **1** was established by ^{1}H-^{1}H COSY, HMQC, and HMBC (Figure 2). The relative configuration of **1** was established by NOESY data and comparison of the previous reported ^{13}C chemical shift data,[8] indicating that CH_3-19, CH_3-27 and H-8 were *β*-oriented and CH_3-21, H-5, H-9, H-14, H-22, and CH_3-21 were in *α*-orientation. The NOESY cross-peaks of CH_3-21 (δ 1.16)/H-16 (δ 4.40) and H-22 (δ 3.10) showed the same orientation for CH_3-21, H-16, and H-22. Further, based on chemical shifts of δ_C 71.6 (C-3), and 70.3 (C-6), the hydroxyl groups located in **1** had *β*-, and *α*-configuration, respectively. Thus, the structure of **1** was established as 5α-cevanin-13-ene-3*β*,6*α*,16*β*-triol-18-one, and designated as frithunbol A.

Compound **2** was isolated as a colorless gum, testing positive with Dragendorff's reagent. Its molecular formula was determined as $C_{27}H_{41}NO_3$ based on the molecular ion peak $[M + H]^+$ at *m/z* 428.3167 (calcd for $C_{27}H_{42}NO_3$, 428.3165) in the positive-ion HRFABMS. The IR spectrum indicated absorption bands of hydroxyl (3370 cm^{-1}) and carbonyl (1715 cm^{-1}) groups. The ^{1}H NMR spectrum (Table 1) showed the signals due to two tertiary methyl groups [δ_H 0.66 (3H, s, H-19), and 1.71 (3H, s, H-18)], two secondary methyl groups [δ_H 1.30 (3H, d, *J* = 6.0, H-21) and 0.84 (3H, d, *J* = 5.5, H-27)], and two oxygenated methine groups [δ_H 3.96 (1H, t, *J* = 9.0, H-23), and 3.85 (1H, m, H-3)]. The ^{13}C NMR spectrum showed

Table 1. ^{1}H and ^{13}C NMR [ppm, mult (*J* in Hz)] spectral data of compounds **1** and **2** in pyridine-d_5

Pos.	**1**		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.12 m	39.1	1.37 m	37.4
	1.49 m		1.53 m	
2	1.76 m	32.4	1.70 m	31.5
	2.00 m		2.05 m	
3	3.91 m	71.6	3.85 m	70.3
4	1.64 m	33.9	1.85 m	31.2
	2.97 brd (12.0)		2.35 brd (12.5)	
5	1.36 m	53.2	2.28 m	56.8
6	3.74 td (10.0, 4.5)	70.3	-	210.2
7	2.71 m	42.1	2.25 m	45.9
			2.63 dd (13.0, 4.5)	
8	2.28 m	43.6	1.45 m	46.6
9	1.21 m	57.7	1.75 m	54.4
10	-	35.6	-	38.5
11	1.28 m	33.5	1.95 m	28.8
	2.65 m		2.25 m	
12	3.35 q (8.5)	37.1	-	127.5
13	-	129.7	-	142.3
14	1.91 m	40.6	1.80 m	48.5
15	2.00 m	34.2	169 m	24.5
	2.08 m			
16	4.40 t (4.0)	65.1	1.95 m	31.5
17	-	147.5	-	86.1
18	-	164.5	1.71 s	13.3
19	0.90 s	13.3	0.66 s	12.5
20	2.74 m	34.2	2.90 m (overlap)	38.5
21	1.16 d (7.5)	19.5	1.30 d (6.0)	11.4
22	3.10 brd (11.0)	63.2	3.45 t (8.5)	63.9
23	1.19 m	27.3	3.96 t (9.0)	72.8
	1.97 m			
24	1.40 m	30.4	2.20 m	37.7
			1.40 m	
25	1.44 m	31.3	2.21 m	28.4
26	4.77 d (13.0)	50.3	2.90 m (overlap)	51.2
	2.85 dd (13.0, 3.5)		3.62 m	
27	1.07 d (7.0)	17.7	0.84 d (5.5)	18.1

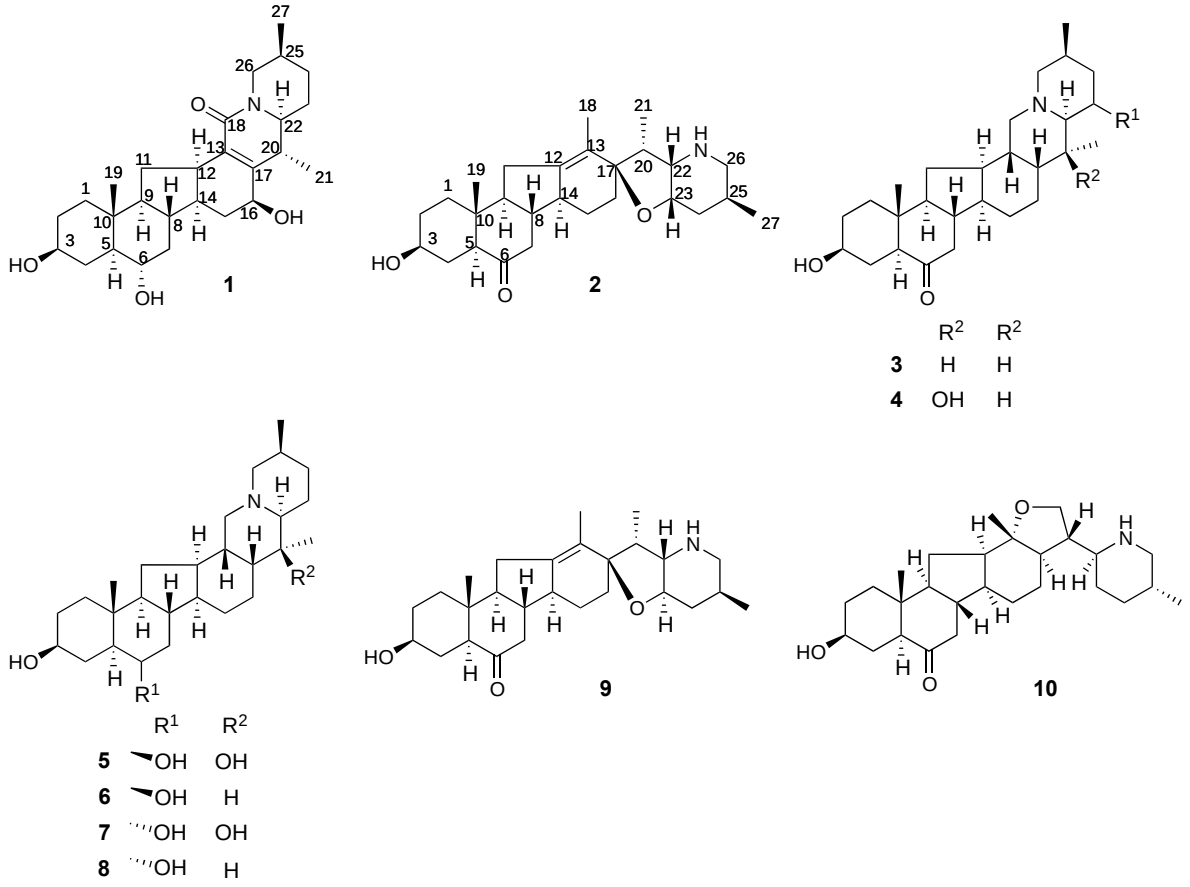

Figure 1. The structures of compounds **1**–**10**

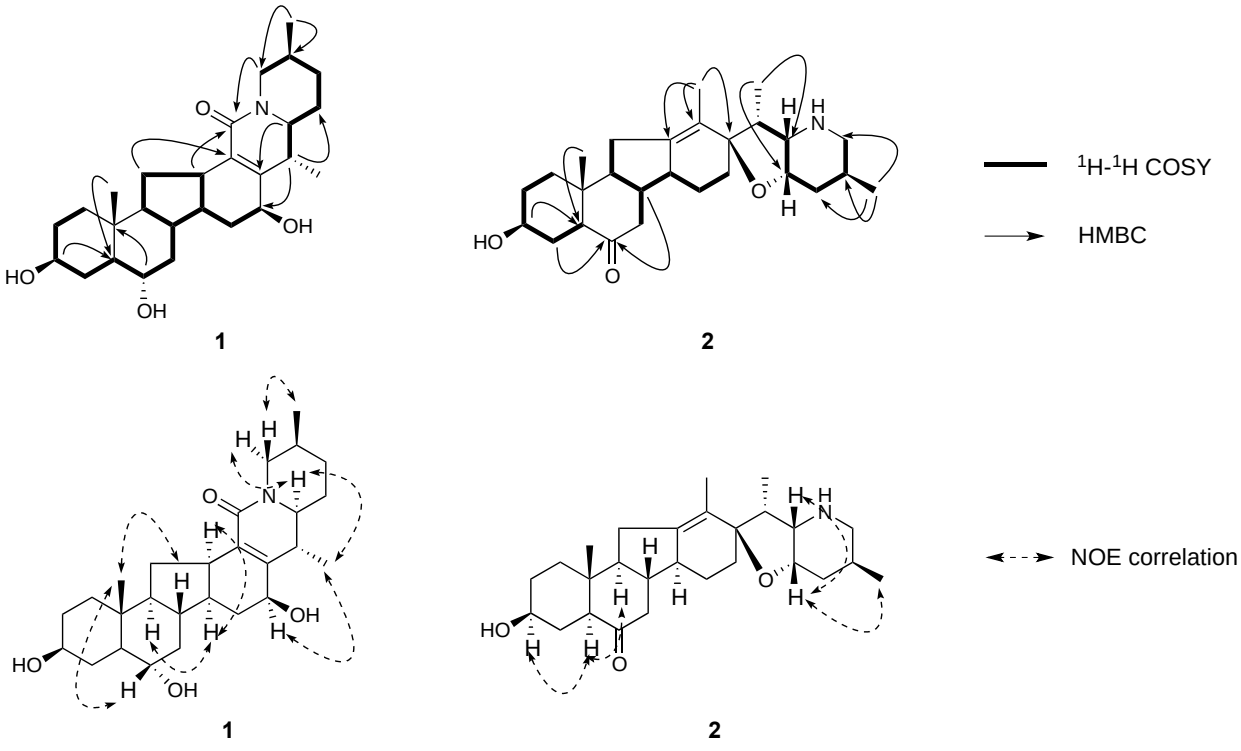

Figure 2. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **1** and **2**

the presence of four methyl groups [δ_C 11.4 (C-21), 12.5 (C-19), 13.3 (C-18), and 17.7 (C-27)]; three oxygenated carbons [δ_C 70.3 (C-3), 72.8 (C-23), and 86.1 (C-17)]; and two nitrogenated carbons [δ_C 51.2 (C-26), and 63.9 (C-22)], and two olefinic carbons [δ_C 127.5 (C-12), and 142.3 (C-13)]. The ^{1}H, and ^{13}C NMR data of **2** were quite similar to those of **9**, except the chemical shifts of furan ring part. Thus, compound **2** was expected to be a stereoisomer of **9**. The planar structure of **2** was determined through 2D NMR, including ^{1}H-^{1}H COSY, HMQC, and HMBC spectra. The NOESY cross-peaks of H-3 (δ 3.85)/H-5 (δ 2.28) and H-9 (δ 1.75) and CH_3-27 (δ 0.84)/H-22 (δ 3.45) and H-23 (δ 3.96) confirmed the relative stereochemistry of **2** (Figure 2). Also, the stereochemistry of furan ring in **2** was determined by comparison with the ^{13}C chemical shift data of 23-isokuroyurinidine,[9] which was isolated from *Fritillaria maximowiczii* [**2**: δ_C 63.9 (C-22), 72.8 (C-23), 37.7 (C-24), 28.4 (C-25), 51.2 (C-26); **23-isokuroyurinidine**: δ_C 64.3 (C-22), 72.9 (C-23), 37.9 (C-24), 28.4 (C-25), 51.5 (C-26)]. Thus, the structure of **2** was established as 3*β*-hydroxy-5*α*-jerv-12-en-6-one, and designated as frithunbol B.

The known compounds were identified as ebeiedinone (**3**),[10] verticinone (**4**),[4] isoverticine (**5**),[11] ebeiedine (**6**),[10] verticine (**7**),[12] eduardinine (**8**),[13] 3β-hydroxy-5α-jervanin-12-en-6-one (**9**),[7] and suchengbeisine (**10**)[14] based on comparison of their spectroscopic data with those in the literatures.

Table 2. Inhibitory effect of compounds **1**–**10** on NO production in LPS-activated BV-2 cells

Compound	IC_{50} (μM)[a]	Cell Viability (%)[b]
1	99.63	144.09 ± 6.27
2	16.35	140.69 ± 5.97
3	11.45	142.04 ± 5.33
4	103.26	129.55 ± 5.33
5	83.00	127.08 ± 5.91
6	31.61	138.63 ± 5.81
7	45.28	139.30 ± 5.05
8	31.76	128.29 ± 4.61
9	20.19	131.17 ± 5.33
10	18.02	137.62 ± 6.48
L-NMMA[c]	19.45	120.10 ± 4.44

[a]IC_{50} value of each compound was defined as the concentration (μM) associated with 50% inhibition of NO production in LPS-activated BV-2 cells.
[b]Cell viability after treatment with 20 μM of each compound was determined by the MTT assay and is expressed as a percentage (%). Results represent means of three independent experiments, and the data are expressed as mean ± SD.
[c]L-NMMA as a positive control.

Anti-neuroinflammatory effects of these isolated compounds (**1**–**10**) were evaluated by measuring NO levels in LPS-stimulated murine microglia BV2 cells (Table 2). Among these isolated compounds, compounds **2**, **3**, and **10** significantly inhibited NO levels with IC_{50} values of 16.35, 11.45 and 18.02 μM, respectively. They exhibited higher NO-inhibition than N^G-monomethyl-L-arginine (L-NMMA), the positive control (IC_{50} 19.45 μM), without any cytotoxicity at a concentration of 20 μM. However, other compounds showed weak NO-inhibiting activity.

Table 3. Effects of compounds **1**–**10** on NGF secretion in C6 cells

Compound	NGF secretion (%)[a]	Cell Viability (%)[b]
1	116.41 ± 4.23	104.82 ± 5.21
2	116.38 ± 3.36	103.21 ± 0.93
3	121.61 ± 1.56	106.84 ± 0.56
4	103.97 ± 3.46	101.07 ± 4.20
5	104.04 ± 9.25	97.65 ± 1.35
6	99.90 ± 1.32	101.18 ± 0.43
7	111.53 ± 2.24	102.66 ± 1.83
8	120.84 ± 3.09	99.76 ± 7.12
9	134.81 ± 3.66	97.12 ± 6.80
10	127.95 ± 9.63	105.22 ± 0.93
6-Shogaol[c]	134.08 ± 2.21	88.72 ± 3.72

[a]C6 cells were treated with 20 μM of the compounds. After 24 h, the content of NGF secreted in C6-conditioned media was measured by ELISA. The level of secreted NGF is expressed as a percentage of the untreated control (set as 100%). Data are means ± SD of three independent experiments performed in triplicate.
[b]Cell viability after treatment with 20 μM of each compound was determined by MTT assay and is expressed as a percentage (%). Results are means of three independent experiments, and the data are expressed as mean ± SD.
[c]6-Shogaol as a positive control.

Neuroprotective activities of these isolated compounds (**1**–**10**) were examined by measuring NGF secretion in C6 cells (Table 3). The level of NGF released into the medium was measured and cell viability was determined using MTT assay. Among the compounds isolated, compound **9** stimulated NGF release by 134.81 ± 3.66% (134.08 ± 2.21% for 6-shogaol, a positive control) without cytotoxic effect at a concentration of 20 μM.

EXPERIMENTAL

General. Optical rotations were measured on a Jasco P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer using methanol as a solvent. UV spectra were recorded with a

Shimadzu UV-1601 UV-Visible spectrophotometer using MeOH as a solvent. HR-FAB mass spectra were determined using JEOL JMS700 mass spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HMQC, HMBC, NOESY experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts expressed in ppm (δ). Semi-preparative HPLC used a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (Merck, 230-400 mesh) and RP-C_{18} silica gel (Merck, 230-400 mesh) were used for column chromatography. Low-pressure LC was performed using a LiChroprep Lobar-A RP-18 (240 × 10 mm i.d.) column with a FMI QSY-O pump (ISCO). Sephadex LH-20 (Pharmacia Co. Ltd) was used as the packing material for molecular sieve column chromatography. Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates were used for TLC. Spots were detected using TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Plant material. The bulbs of *F. thunbergii* (11 kg) were purchased at Naemome Dah, Korea in January 2012. The plant was authenticated by one of the authors (K. R. Lee). A voucher specimen of the plant (SKKU-NPL-1201) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation. The dried and chopped bulbs of *F. thunbergii* (11 kg) were extracted with 80% MeOH under reflux and then filtered. The filtrate was evaporated under reduced pressure to yield a residue (760 g), which was dissolved in water (2 L) and successively partitioned with $CHCl_3$, and *n*-BuOH after pre-treatment with 1 N hydrochloric acid (HCl), yielding a $CHCl_3$-fraction (21 g), and *n*-BuOH-fraction (230 g), respectively. The $CHCl_3$-soluble layer (21 g) was separated over a silica gel column (600 g) with a $CHCl_3$-MeOH (20:1 to 1:1, v/v) to give yield 10 fractions (C1-C10). Fraction C4 (500 mg) was subjected to the Sephadex LH-20 column using a solvent system of CH_2Cl_2-MeOH (1:1) to obtain two fractions (C41 and C42). Fraction C41 (250 mg) was separated by RP-C_{18} silica gel column chromatography with MeOH-H_2O (3:2) and further purified by semi-preparative reversed-phase HPLC system with 60% MeOH in 0.05% trifluoroacetic acid to afford compound **3** (35 mg). Fraction C6 (3 g) was applied to a Sephadex LH-20 column using a solvent system of CH_2Cl_2-MeOH (1:1) to obtain two fractions (C61 and C62). Fraction C61 (1.2 g) was subjected to RP-C_{18} silica gel column chromatography with MeOH-H_2O (1:1) and further purified by semi-preparative reversed-phase HPLC system with 55% MeOH in 0.05% TFA to generate compound **2** (20 mg). Fraction C62 (400 mg) was purified by semi-preparative reversed-phase HPLC system with 60% MeOH in 0.05% TFA to afford compounds **9** (7 mg) and **10** (35 mg). Fraction C7 (2.5 g) was applied to a Sephadex LH-20 column using a solvent system of CH_2Cl_2-MeOH (1:1) to obtain two fractions (C71 and C72). Fraction C71 (1.1 g) was subjected to

RP-C_{18} silica gel column chromatography with MeOH-H_2O (2:3) to obtain nine fractions (C71-1 and C71-9). Fraction C71-2 (95 mg) was purified by semi-preparative reversed-phase HPLC system with 60% MeOH in 0.05% TFA to afford compound **6** (23 mg). Fraction C71-9 (29 mg) was purified by semi-preparative reversed-phase HPLC system with 60% MeOH in 0.05% TFA to afford compound **1** (5 mg). Fraction C72 (600 mg) was purified by semi-preparative reversed-phase HPLC system with 60% MeOH in 0.05% TFA to afford compound **8** (130 mg). Fraction C9 (2 g) was applied to a Sephadex LH-20 column using a solvent system of CH_2Cl_2-MeOH (1:1) to obtain two fractions (C91 and C92). Fraction C91 (800 mg) was subjected to RP-C_{18} silica gel column chromatography with MeOH-H_2O (1:1) and further purified by semi-preparative reversed-phase HPLC system with 55% MeOH in 0.05% TFA to afford compound **5** (40 mg). The *n*-BuOH fraction (230 g) was separated on a silica gel column chromatography using a gradient solvent system of $CHCl_3$-MeOH (20:1–1:1) to give eight subfractions (B1-B8). Fraction B6 (20 g) was subjected to RP-C_{18} silica gel column chromatography with MeOH-H_2O (3:7) to obtain six fractions (B61-B66). Fraction B64 (5.2 g) was purified by semi-preparative reversed-phase HPLC system with 45% MeOH in 0.05% TFA to afford compound **4** (1.2 g). Fraction B7 (38 g) was subjected to RP-C_{18} silica gel column chromatography with MeOH-H_2O (1:1) to obtain four fractions (B71-B74). Fraction B72 (15 g) was purified by semi-preparative reversed-phase HPLC system with 40% MeOH in 0.05% TFA to afford compound **7** (3 g).

Frithunbol A (1): Colorless gum (5 mg); $[\alpha]_D^{25}$ -18.8 (*c* 0.02, MeOH); IR (KBr) ν_{max} 3346, 2942, 2810, 1608, 1450, 1023 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR (see Table 1); HR-FAB-MS (positive-ion mode) *m/z*: 444.3113 $[M + H]^+$ (calcd for $C_{27}H_{41}NO_4$, 444.3114).

Frithunbol B (2): Colorless gum (20 mg); $[\alpha]_D^{25}$ +0.8 (*c* 0.09, MeOH); IR (KBr) ν_{max} 3370, 2944, 2834, 1715, 1677, 1449, 1202, 1136, 1023, 838, 801, 722 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR (see Table 1); HR-FAB-MS (positive-ion mode) *m/z*: 428.3167 $[M + H]^+$ (calcd for $C_{27}H_{42}NO_3$, 428.3165).

Measurement of NO Production and Cell Viability. The BV-2 mouse microglial cell line has been extensively used in published studies as an *in vitro* culture system for the investigation of primary microglial function. In this study, BV-2 cells were maintained in DMEM supplemented with 5% FBS and 1% PS. To measure NO production, BV-2 cells were seeded into a 96-well plate (3×10^4 cells/well) and treated with 100 ng/mL lipopolysaccharide (LPS) in the presence or absence of isolates (**1**–**10**) for 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media via Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to

calculate the NO_2^- concentration. Cell viability was assessed by the MTT assay. In this study, N^G-monomethyl-L-arginine (L-NMMA, Sigma-Aldrich), a well-known nitric oxide synthase (NOS) inhibitor, was used as a positive control.

NGF and cell viability assays. C6 glioma cells were used to measure NGF release into the medium. C6 cells were purchased from the Korean Cell Line Bank and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2. To measure NGF content in the medium and determine cell viability, C6 cells were seeded into 24-well plates (1×10^5 cells/well). After 24 h, the cells were treated with DMEM containing 2% FBS and 1% penicillin-streptomycin with 20 μM of each sample for a single day. Media supernatant was used for the NGF assay using an ELISA development kit (R&D Systems). Cell viability was assessed via MTT assay.

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