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A NEW CYCLIC TRITERPENE SAPONIN FROM *PHYTEUMA JAPONICUM*

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Abstract — A new cyclic triterpene saponin, phyteujaposide (**1**) and eight known compounds (**2**-**9**) were isolated from the leaves of *Phyteuma japonicum*. The structure of the new compound (**1**) was elucidated by 1D and 2D data analysis, acid hydrolysis and GC-MS. Compounds **7** and **9** potently inhibited the NO production in LPS-activated BV-2 cells and compounds **5** and **6** showed a significant secretion induction of NGF in C6 glioma without cell toxicity.

Phyteuma japonicum Miquel (Campanulaceae), commonly known as horned rampion, is a perennial herb widely distributed throughout Korea, China, and Japan. The young leaves of this plant are as a salad raw consumed in Korea and many plant of Campanulaceae family were used as an herbal drug to treat bronchitis, inflammation, asthma, pulmonary tuberculosis, and ear-pains.[1-3] In previous phytochemical studies, phenolic compounds, flavonoids and triterpene saponins were isolated from *Phyteuma* genus, and some of them showed antioxidant activity.[4,5] However, no phytochemical and biological investigations have been reported on the secondary metabolites of this species.

The medicinal importance of the species and the lack of information with regards to its chemical constituents prompted this investigation of constituents of the leaves of *P. japonicum*. In our screening test, the MeOH extract of *P. japonicum* exhibited inhibitory activity for the nitric oxide (NO) production

in lipopolysaccharide (LPS)-activated BV-2 microglia cells. Through the repeated column chromatographic separation and purification, a new cyclic triterpene saponin, phyteujaposide (**1**) and eight known compounds (**2**-**9**) (Figure 1) were isolated from the $CHCl_3$ and EtOAc-soluble layers. The structure of the new compound (**1**) was elucidated by analysis of 1D and 2D NMR (^{1}H and ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY) data, acid hydrolysis and GC-MS. 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 of C6 glioma cells.

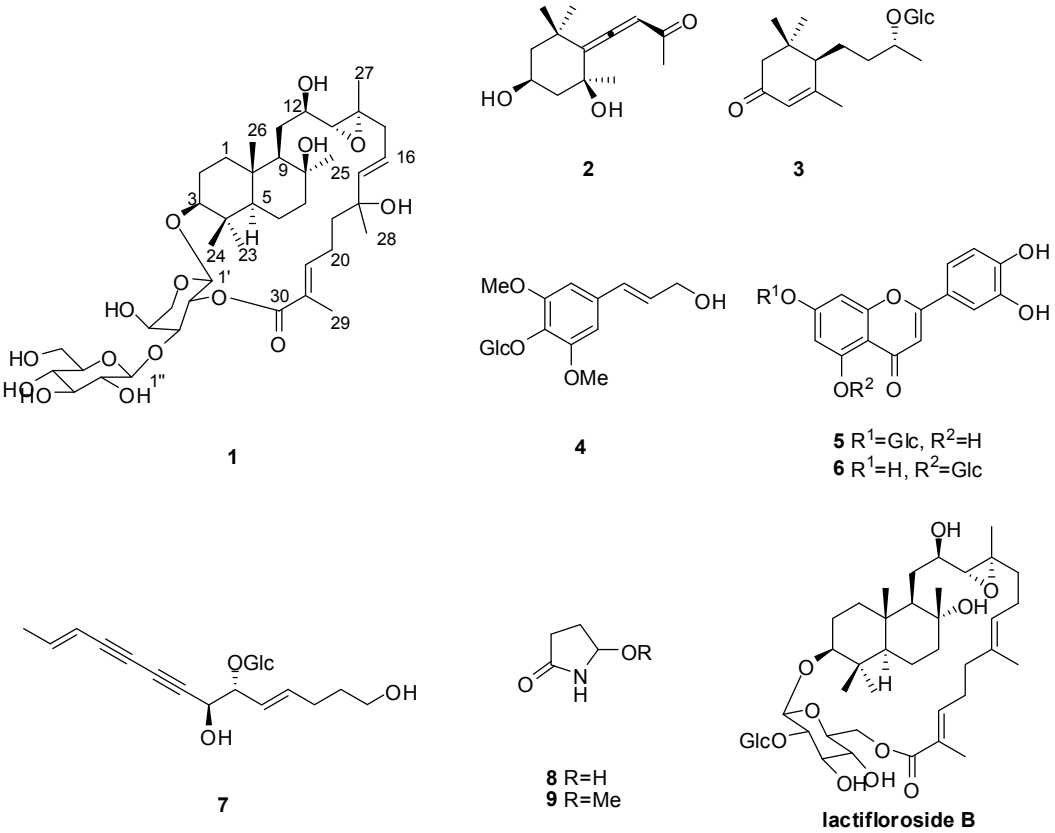

Figure 1. Structures of Compounds **1-9**

Compound **1** was isolated as a colorless gum with positive optical rotation ($[\alpha]_D^{25}$ +15.0, MeOH). The molecular formula of **1** was determined as $C_{41}H_{66}O_{15}$ from its positive mode HR-FAB-MS at *m/z* 821.4294 $[M + Na]^+$ (calcd for $C_{41}H_{66}O_{15}Na$, 821.4299). The ^{13}C NMR spectral data (Table 1) revealed in combination with an analysis of the DEPT and HMQC spectra 41 carbon signals composed of seven methyls, 10 methylenes, 17 methines, and seven quaternary carbons, of which 30 were assigned to the aglycone part and the remaining 11 carbons were ascribed to two sugars. The ^{1}H NMR spectral data showed three olefinic protons [δ_H 6.76 (1H, ddd, J = 9.0, 6.0, 1.0 Hz, H-21), 5.99 (1H, d, J = 16.0 Hz, H-17) and 5.43 (1H, ddd, J = 16.0, 10.5, 4.0 Hz, H-16)], three oxymethines [δ_H 3.55 (1H, ddd, J = 11.0, 9.0,

5.5 Hz, H-12), 3.09 (1H, dd, *J* = 12.0, 3.5 Hz, H-3) and 2.84 (1H, d, *J* = 9.0 Hz, H-13)] and seven methyl groups [δ_H 1.83 (3H, s, H-29), 1.35 (3H, s, H-28), 1.33 (3H, s, H-27), 1.26 (3H, s, H-25), 1.09 (3H, s, H-26), 0.81 (3H, s, H-24) and 0.80 (3H, s, H-23)] of aglycone part, and two anomeric protons [δ_H 4.61 (1H, d, *J* = 8.0 Hz, H-1′) and 4.31 (1H, d, *J* = 7.5 Hz, H-1″)] of two sugar moieties.

Table 1. ^{1}H and ^{13}C NMR [ppm, mult (*J* in Hz)] spectral data of compound **1** in CD_3OD

Pos.	Aglycone		Pos.	Sugar	
	^{1}H	^{13}C		^{1}H	^{13}C
1ax	0.87, td (13.0, 2.5)	38.6, CH_2	1' (Ara)	4.61, d (8.0)	99.4, CH
1eq	1.68, overlap		2'	5.21, dd (10.0, 8.0)	73.1, CH
2ax	1.64, overlap	21.7, CH_2	3'	3.89, dd (10.0, 3.5)	80.6, CH
2eq	1.81, overlap		4'	4.09, m	68.8, CH
3	3.09, dd (12.0, 3.5)	88.0, CH	5'a	3.90, dd (13.0, 2.0)	66.4, CH_2
4		38.2, C	5'b	3.70, dd (13.0, 1.0)	
5	0.66, dd (12.0, 1.5)	57.0, CH	1" (Glc)	4.31, d (7.5)	104.4, CH
6ax	1.68, overlap	17.9, CH_2	2"	3.23, overlap	73.4, CH
6eq	1.51, brd (14.0)		3"	3.28, overlap	76.2, CH
7ax	1.38, td (13.5, 4.0)	42.5, CH_2	4"	3.29, overlap	69.8, CH
7eq	1.88, overlap		5"	3.23, overlap	76.5, CH
8		71.7, C	6"a	3.84, dd (11.5, 2.5)	61.0, CH_2
9	0.73, dd (6.0, 1.5)	53.7, CH	6"b	3.67, dd (11.5, 5.5)	
10		38.5, C			
11a	1.87, overlap	30.8, CH_2			
11b	1.80, overlap				
12	3.55, ddd (11.0, 9.0, 5.5)	69.3, CH			
13	2.84, d (9.0)	66.7, CH			
14		61.7, C			
15a	2.61, dd (13.0, 10.5)	42.4, CH_2			
15b	1.95, dd (13.0, 4.0)				
16	5.43, ddd (16.0, 10.5, 4.0)	122.3, CH			
17	5.99, d (16.0)	143.3, CH			
18		71.4, C			
19a	1.66, overlap	43.6, CH_2			
19b	1.58, td (12.5, 5.5)				
20a	2.51, overlap	22.6, CH_2			
20b	2.26, overlap				
21	6.76, ddd (9.0, 6.0, 1.0)	142.3, CH			
22		128.7, C			
23	0.80, s	26.6, CH_3			
24	0.81, s	15.5, CH_3			
25	1.26, s	30.7, CH_3			
26	1.09, s	14.6, CH_3			
27	1.33, s	18.0, CH_3			
28	1.35, s	26.0, CH_3			
29	1.83, s	11.2, CH_3			
30		167.3, C			

These spectroscopic data of the aglycone part were similar to those of lactifloroside B[6] except for the presence of an additional oxygenated carbon (δ_C 71.4). The location of the oxygenated carbon was assigned by the HMBC correlation of H-28/C-17, C-18, and C-19. The planar structure of **1** of aglycone was elucidated by analysis of 1D and 2D NMR (^{1}H and ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, and HMBC) data (Figure 2). The NOESY cross peaks of H-3/H-23, H-5/H-6eq, H-7ax/H-9 and H-25, H-9/H-

12, H-13/H-15 and H-24/H-26 confirmed the relative stereochemistry of **1** (Figure 2). The *erythro* configuration of C-12/C-13 was corroborated by a small difference in ^{13}C NMR ($\Delta\delta = \delta_{C13} - \delta_{C14}$; Δδ 5.0, **1**; Δδ 7.8, *erythro*-(2*R*,3*R*,4*R*)-6,6-diethoxy-4-methyl-3,4-epoxyhexan-2-ol; Δδ 10.0, *threo*-6,6-diethoxy-4-methyl-3,4-epoxyhexan-2-ol).[7] However, the configuration of the OH group at C-18 was not determined. The sugar parts were established to be β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl unit by analysis of ^{1}H and ^{13}C NMR spectra which were in agreement with those of assamicoside A,[8] and the linkages among two sugars and aglycone part were confirmed by HMBC correlations of H-1′/C-3, H-1″/C-3′, and H-2′/C-30 (Figure 2). The acid hydrolysis of **1** gave aglycone **1a**, which was identified by ^{1}H NMR, HR-FAB-MS and optical rotation, and L-arabinose and D-glucose. Absolute configurations of two sugars were confirmed by GC-MS analysis.[9-11] Thus, the structure of **1** was established as 3β-*O*-[β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl]-13α,14α-epoxy-8β,12β,18-trihydroxy-(16*E*,21*E*)-16,21-campanuldien-1′(30)-olide, named phyteujaposide.

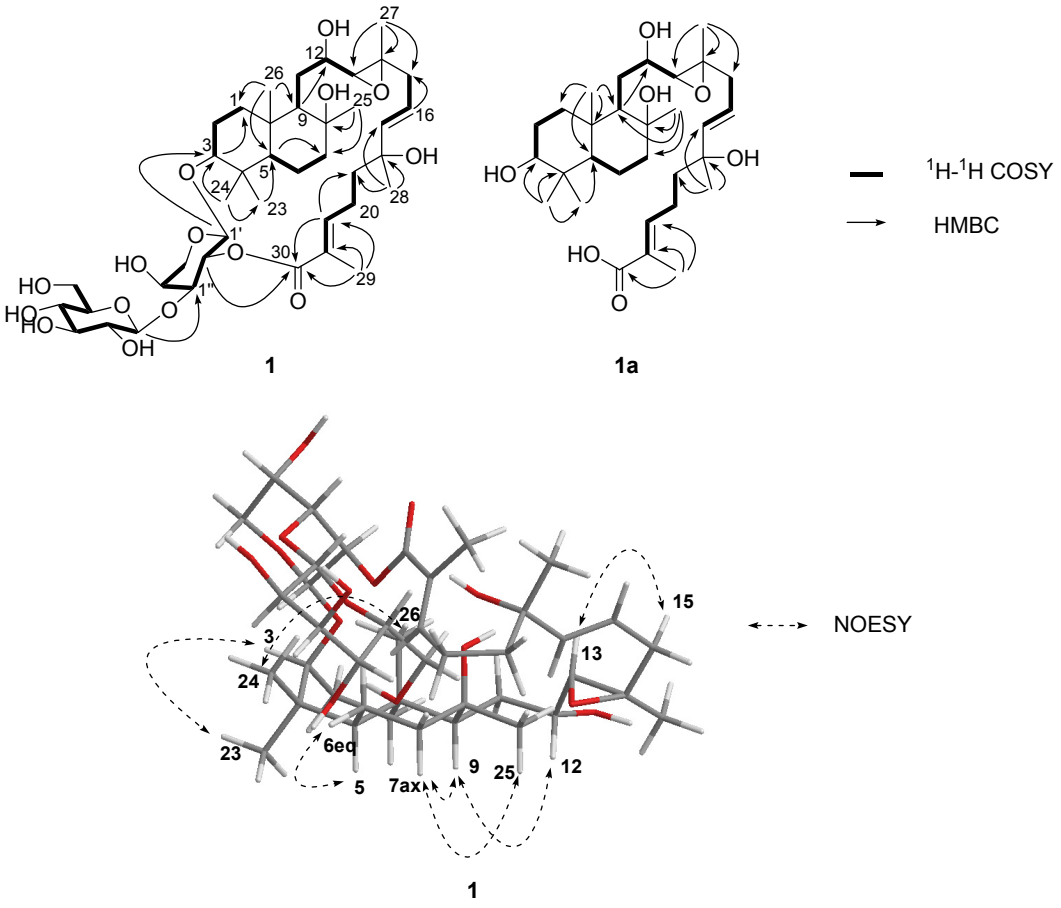

Figure 2. ^{1}H-^{1}H COSY, HMBC and NOESY correlations of compounds **1** and **1a**

The known compounds were identified as 3*S*,5*R*-dihydroxy-6*S*,7-megastigmadien-9-one (**2**),[12] byzantionoside B (**3**),[13] syringin (**4**),[14] luteolin 7-*O*-β-D-glucoside (**5**),[15] luteolin 5-*O*-β-D-glucoside (**6**),[16] lobetyolin (**7**),[17] 5-hydroxypyrrolidin-2-one (**8**)[18] and pterolactam (**9**)[18] by comparing the physicochemical

and spectroscopic data with previously reported data. To the best of our knowledge, all the known compounds (**2**-**9**) were isolated from this plant for the first time.

Table 2. Effects of compounds **1**-**9** and L-NMMA on LPS-induced NO production in BV-2 microglia cells

Compounds	IC_{50}[a] (μM)	Cell viability[b] (%)
1	39.96	128.93 ± 11.78
2	45.37	125.01 ± 3.21
3	21.02	110.57 ± 5.34
4	38.52	111.89 ± 0.57
5	26.59	116.87 ± 2.82
6	124.15	108.81 ± 6.07
7	17.03	110.39 ± 8.54
8	74.82	122.73 ± 4.40
9	11.36	111.49 ± 2.41
L-NMMA[c]	19.24	101.54 ± 3.59

[a]The IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.

[b]The cell viability after the treatment of each compound with 20 μM was determined by MTT assay and is expressed as percentage (%). The results are averages of three independent experiments and the data are expressed as mean ± SD.

[c]L-NMMA as a positive control.

The isolated compounds **1**-**9** were evaluated by examining NO production in LPS-activated microglia BV-2 cells (Table 2).[19,20] Of them, compounds **3**, **5**, **7** and **9** significantly inhibited the LPS-stimulated NO production with IC_{50} values of 21.02, 26.59, 17.03 and 11.36 μM and of them displayed compounds **7** and **9** more activity than N^G-monomethyl-L-arginine (L-NMMA), a well-known NOS inhibitor. The new compound **1** showed moderate activity (39.96 μM) and all isolates (**1**-**9**) had no influence on cell viability at concentrations up to 20 μM.

Table 3. Effects of compounds **1-6** on NGF secretion in C6 cells.[a]

Compounds	NGF secretion (%)	Cell viability[b] (%)
1	131.75 ± 4.68	113.10 ± 2.64
2	151.08 ± 6.02	50.08 ± 11.91
3	124.32 ± 0.31	117.43 ± 30.64
4	113.54 ± 2.81	105.50 ± 3.16
5	155.27 ± 1.39	90.84 ± 10.24
6	147.06 ± 4.44	91.04 ± 11.10
7	117.27 ± 5.43	112.68 ± 6.51
8	136.83 ± 2.29	75.97 ± 8.44
9	135.84 ± 5.95	66.66 ± 2.64
6-shogaol[c]	135.21 ± 3.01	108.49 ± 4.79

[a]The C6 cells were treated with 20 μM of compounds **1-9**. After 24 h, the content of NGF secretion in C6-conditioned media was measured by ELISA. The level of secreted NGF cells of the untreated control is expressed as percentage. The data shown represent the means ± SD of three independent experiments performed in triplicate.

[b]The cell viability after the treatment of each compound with 20 μM was determined by MTT assay and is expressed as percentage (%). The results are averages of three independent experiments and the data are expressed as mean ± SD.

[c]6-Shogaol as positive control.

Also, we tested the NGF secretion effects of compounds **1-9** using an enzyme-linked immunosorbant assay (ELISA) development kit of C6 glioma cells to measure the NGF release into the medium in a cell viability by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.[21] As shown in Table 3, compounds **5** and **6** exhibited a significant induction of NGF secretion (155.27 ± 1.39% and 147.06 ± 4.44%, respectively) without cell toxicity, which displayed more activity than 6-shogaol (135.21 ± 3.01%) as positive control. The new compound **1** showed weak activity (131.75 ± 4.68%) and we suggest the NGF secretion effect of **2**, **8** and **9** may be influenced by its high cytotoxicity.

In conclusion, we isolated and identified a new cyclic triterpene saponin, phyteujaposide (**1**), together with eight known compounds (**2-9**) from the leaves of *P. japonicum.* All the isolates were evaluated by NO production examination in LPS-activated microglia BV-2 cells and NGF secretion in C6 glioma cells. The present study indicates the compounds **5**, **6**, **7** and **9** as good candidates to be further investigated as anti-inflammatory agents.

EXPERIMENTAL

General. Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH. IR spectra were

recorded on a Bruker IFS-66/S FT-IR spectrometer. HRFABMS were obtained on a JEOL JMS700 mass spectrometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) with chemical shifts given in ppm (δ). Semi-preparative HPLC was conducted using a Gilson 306 pump with Shodex refractive index detector and Alltech Econosil RP-18 column (250 × 10 mm i.d.). Silica gel 60 and RP-C_{18} silica gel (Merck, 230-400 mesh) was used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). TLC was performed using Merck precoated silica gel F_{254} plates. Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in EtOH (v/v).

Plant material. Leaves of *P. japonicum* (5 kg) were collected in Yangju City, Korea, in July 2011. The plant was identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL 0911) of the plant has been deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation. Leaves of *P. japonicum* (5 kg) were extracted with 80% MeOH at room temperature and filtered. The resulting extract (360 g) was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding each 17, 30, 7 and 53 g of residues, respectively. The $CHCl_3$ soluble layer (30 g) was separated over a silica gel column (600 g) with a $CHCl_3$-MeOH (60:1 to 1:1, v/v) to give six fractions (C1-C6). Fraction C5 (7 g) was chromatographed on a RP-C_{18} silica gel column (100 g), eluting with MeOH-H_2O (1:1) to give six subfractions (C51-C56). Fraction C52 (590 mg) was further separated over a silica gel column (10 g) with $CHCl_3$-MeOH (15:1) and purified by semi-preparative HPLC, using an Alltech Econosil RP-18 column (250 mm × 10 mm i.d., 10 μm; MeCN-H_2O, 1:4) equipped with a Shodex refractive index detector to afford **1** (7 mg), **2** (5 mg) and **7** (12 mg). Fraction C53 (210 mg) was separated over a silica gel column (7 g) with $CHCl_3$-MeOH (20:1) and purified by a semi-preparative HPLC system (MeCN-H_2O, 1:3) to give **3** (10 mg) and **8** (9 mg). The EtOAc soluble layer (7 g) was separated over a RP-C_{18} silica gel column (100 g) with MeOH-H_2O (7:3) to give eight fractions (E1-E8). Fraction E6 (400 mg) was subjected to the Sephadex LH-20 column (MeOH-H_2O, 7:3) and further purified by a semi-preparative HPLC system (MeCN-H_2O, 1:3) to give **4** (5 mg) and **9** (4 mg). Compounds **5** (3 mg) and **6** (3 mg) were isolated from fraction E8 (500 mg) by passage through a Sephadex LH-20 column (MeOH-H_2O, 7:3) and separation by the same HPLC system (MeCN-H_2O, 1:3).

Phyteujaposide (1): Colorless gum (7 mg); $[\alpha]_D^{25}$ +15.0 (*c* 0.04, MeOH); IR (KBr) ν_{max} 3382, 2948,

2833, 1660, 1452, 1031, 697 cm^{-1}; UV (MeOH) λ_{max} (log ε) 235 (4.0) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR (see Table 1); HR-FAB-MS (positive-ion mode) *m/z*: 821.4294 $[M + Na]^+$ (calcd for $C_{41}H_{66}O_{15}Na$, 821.4299).

Acid hydrolysis of 1 and sugar determination. Compound **1** (2.0 mg) was refluxed with 1 mL of 1 N HCl for 4 h at 100 °C. The hydrolysate was extracted with $CHCl_3$ and the extract was evaporated in vacuo to yield the aglycone (**1a**, 1.0 mg). The H_2O layer was neutralized by passage through an Amberlite IRA-67 column (Rohm and Haas) and was repeatedly evaporated to give D-glucose and L-arabinose detected by co-injection of the hydrolysate with standard silylated samples, giving a single peak at 9.712 min (D-glucose) and 7.877 min (L-arabinose) by analysis of GC-MS under the following conditions: capillary column, HP-5MS UI (30 m × 0.25 mm × 0.25 μm, Agilent), column temperature, 230 °C; injection temperature, 250 °C; carrier gas, N_2. Authentic samples (Sigma) treated in the same way showed a single peak at 9.743 min (D-glucose) and 7.851 min (L-arabinose).

13α,14α-Epoxy-8β,12β,18-trihydroxy-(16*E*,21*E*)-16,21-campanuldien-30-oic acid (1a): Colorless gum; $[\alpha]_D^{25}$ +80.0 (*c* 0.01, MeOH); ^1H NMR (CD_3OD, 500 MHz) δ 6.80 (1H, m, H-21), 5.95 (1H, d, *J* = 16.0 Hz, H-17), 5.50 (1H, m, H-16), 2.03 (3H, s, H-29), 1.30 (6H, s, H-27 and H-28), 1.24 (3H, s, H-25), 1.01 (3H, s, H-26), 0.90 (3H, s, H-24), 0.83 (3H, s, H-23); HR-FAB-MS (positive-ion mode) *m/z*: 523.3636 $[M + H]^+$ (calcd for $C_{30}H_{51}O_7$, 523.3635).

Measurement of NO production and cell viability in LPS-activated BV-2 cells. BV-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To measure NO production, BV-2 cells were dispensed in wells of a 96-well plate (3×10^4 cells/well). After 24 h, the cells were pretreated with compounds for 30 min and stimulated with 100 ng/mL LPS for 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant was harvested and mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using an Emax microplate reader (Molecular Devices). Sodium nitrite was used as a standard to calculate the nitrite concentration. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. N^G-Monomethyl-L-arginine (L-NMMA), a well-known NO synthase inhibitor, was tested 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 medium and 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 one day. Media supernatant was used for the NGF assay using an ELISA development kit (R&D Systems). Cell viability was assessed by the MTT assay.

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REFERENCES AND NOTES

1. J. Ren, Z. Lin, and Z. Yuan, *Phytochem. Lett.,* 2013, **6**, 567.
2. T. W. Kim, I. B. Song, H. K. Lee, J. H. Lim, E. S. Cho, H. Y. Son, S. J. Park, J. W. Kim, and H. I. Yun, *Food Chem. Toxicol.,* 2012, **50**, 4254.
3. M. U. Dumlu, E. Gurkan, and E. Tuzlaci, *Nat. Prod. Res.,* 2008, **22**, 477.
4. C. Abbet, I. Slacanin, M. Hamburger, and O. Potterat, *Food Chem.,* 2013, **136**, 595.
5. C. Abbet, M. Neuburger, T. Wagner, M. Quitschau, M. Hamburger, and O. Potterat, *Org. Lett.,* 2011, **13**, 1354.
6. N. Yayli, A. Usta, H. Yaşar, O. Üçüncü, C. Güleç, and M. Küçükislamoğlu, *Turk. J. Chem.,* 2006, **30**, 21.
7. A. A. Nikitenko, B. M. Arshava, I. G. Tara, I. E. Mikerin, V. I. Shvets, and Y. E. Raifeld, *Tetrahedron,* 1998, **54**, 11731.
8. J. M. Tian, X. Y. Fu, Q. Zhang, H. P. He, J. M. Gao, and X. J. Hao, *Biochem. Syst. Ecol.*, 2013, **48**, 288.
9. C. S. Kim, S. Y. Kim, E. Moon, M. K. Lee, and K. R. Lee, *Bioorg. Med. Chem. Lett.,* 2013, **23**, 1771.
10. C. S. Kim, J. Y. Oh, S. U. Choi, and K. R. Lee, *Carbohyd. Res.,* 2013, **381**, 1.
11. C. S. Kim, O. W. Kwon, S. Y. Kim, and K. R. Lee, *J. Nat. Prod.,* 2013, **76**, 2131.
12. M. Dellagreca, C. D. Marino, A. Zarrelli, and B. D'Abrosa, *J. Nat. Prod.,* 2004, **67**, 1492.
13. K. Matsunami, H. Otsuka, and T. Takeda, *Chem. Pharm. Bull.*, 2010, **25**, 438.
14. M. Sugiyama, E. Nagayama, and M. Kikuchi, *Phytochemistry,* 1993, **33**, 1215.
15. F. Orhan, Ö. Bariş, D. Yanmiş, T. Bal, Z. Güvenalp, and M. Güllüce, *Food Chem.,* 2012, **135**, 764.
16. M. Veit, H. Geiger, F. C. Czygan, and K. R. Markham, *Phytochemistry,* 1990, **29**, 2555.
17. K. Ishimaru, H. Yonemitsu, and K. Shimomura, *Phytochemistry,* 1991, **30**, 2255.

18. S. W. Chang, K. H. Kim, I. K. Lee, S. U. Choi, S. Y. Ryu, and K. R. Lee, *Nat. Prod. Sci.,* 2009, **15**, 234.
19. M. Colasanti, T. Persichini, T. Di Pucchio, F. Gremo, and G. M. Lauro, *Neurosci. Lett.,* 1995, **200**, 144.
20. D. W. Reif and S. A. McCreedy, *Arch. Biochem. Biophys*., 1995, **1**, 170.
21. T. Mosmann, *J. Immunol. Methods,* 1983, **65**, 55.