Antineuroinflammatory constituents from the root extract of *Paris verticillata*

Ki Hyun Kim, Kyu Ha Lee, Ho Kyung Kim, Eunjung Moon, Sung-Hoon Kim, Sun Yeou Kim, Kyung Ran Kim, and Kang Ro Lee

Abstract: Two new cyclopropanoic fatty acid glycosides, named parisveroside A (**1**) and parisveroside B (**2**), were isolated from a MeOH extract of the roots of *Paris verticillata* (Liliaceae) together with three other known compounds, salicin (**3**), 3-(β-D-glucopyranosyloxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxy-(2*R*,3*S*)-dihydrobenzofuran (**4**), and allantoin (**5**). Their structures were elucidated on the basis of spectroscopic data, including 1D and 2D NMR, HR-FAB-MS, and chemical evidence. To investigate the antineuroinflammatory effects of the isolated compounds (**1**–**5**), nitric oxide (NO) production was evaluated in the lipopolysaccharide-activated microglia cell line, BV-2. Compounds **2** and **4** significantly inhibited NO production with IC_{50} values of 74.8 and 60.5 μmol/L, respectively.

Key words: Paris verticillata, Liliaceae, fatty acid glycosides, parisveroside A, parisveroside B, neuroinflammation.

Résumé : Utilisant comme produit de départ le mélange obtenu par extraction des racines de *Paris verticillata* (Liliacée) à l'aide de méthanol, on a isolé deux nouveaux glycosides dérivés de l'acide gras cyclopropanoïque, soit le parisvéroside A (**1**) et le parisvéroside B (**2**), ainsi que trois autres produits connus, la salicine (**3**), le 3-(β-D-glucopyranosyloxyméthyl)-2-(4-hydroxy-3-méthoxyphényl)-5-(3-hydroxypropyl)-7-méthoxy-(2*R*,3*S*)-dihydrobenzofurane (**4**) et l'allantoïne (**5**). On a élucidé les structures en se basant sur des preuves chimiques et des données spectroscopiques, dont celles des RMN 1D et 2D et de la spectrométrie de masse à haute résolution avec bombardement avec des atomes rapides (SM-HR-BAR). Pour étudier les effets anti-neuroinflammatoires des composés isolés, on a évalué la production d'oxyde d'azote (NO) dans des cellules BV-2 de la lignée cellulaire de microglie activée par un lipopolysaccharide. Les composés **2** et **4** inhibent d'une façon significative la production de NO avec des valeurs de IC_{50} de 74,8 et 60,5 μmol/L respectivement.

Mots-clés : Paris verticillata, Liliacée, glycosides d'acides gras, parisvéroside A, parisvéroside B, neuroinflammation.

Introduction

The genus *Paris* consists of less than two dozen herbaceous plants that have 4- to 11-merous flowers.[1] Some *Paris* species are used in traditional Chinese medicine for their analgesic and anticoagulant properties, most notably as an ingredient of Yunnan Baiyao.[2] In Korea, the perennial herb *Paris verticillata* (Liliaceae) is widely distributed in valleys and its root has been used as a Korean traditional medicine against asthma, boils, and chronic bronchitis.[3] Few phytochemical and biological investigations on this plant have been previously conducted. The presence of several constituents, including saponins, sterols, and flavonoids, was reported from the aerial parts of *P. verticillata*.[4] In our continuing search for bioactive constituents from Korean medicinal plants, we investigated a MeOH extract from the roots of *P. verticillata*, which is in use as traditional medicine. The investigation led to the isolation of a phenolic amide, phenolics, and pyrrolizidine alkaloids with cytotoxic activity.[5,6] In our continuing investigation of bioactive constituents from the roots of *P. verticillata*, we further isolated two new cyclopropanoic fatty acid glycosides, named parisveroside A (**1**) and parisveroside B (**2**), together with three previously reported compounds (**3**–**5**). Compounds **3**–**5** were characterized for the first time from this plant. Here, we also investigated the antineuroinflammatory effects of isolated compounds (**1**–**5**) in lipopolysaccharide (LPS)-activated BV-2 cells, a microglia cell line.

Results and discussion

The 80% MeOH extract of the roots of *P. verticillata* was subjected to repeated column chromatography on silica gel to afford two new cyclopropanoic fatty acid glycosides (**1**–**2**), together with three known compounds (**3**–**5**) (Fig. 1). Their structures were elucidated by 1D and 2D NMR and HR-FAB-MS and compared with published data.

Parisveroside A (**1**) was isolated as a colorless gum with a molecular formula of $C_{17}H_{30}O_8$ on the basis of the $[M + H]^+$ peak at *m*/*z* 363.2021 (calcd. for $C_{17}H_{31}O_8$: 363.2019) in the

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Fig. 1. Chemical structures of compounds **1**–**5**.

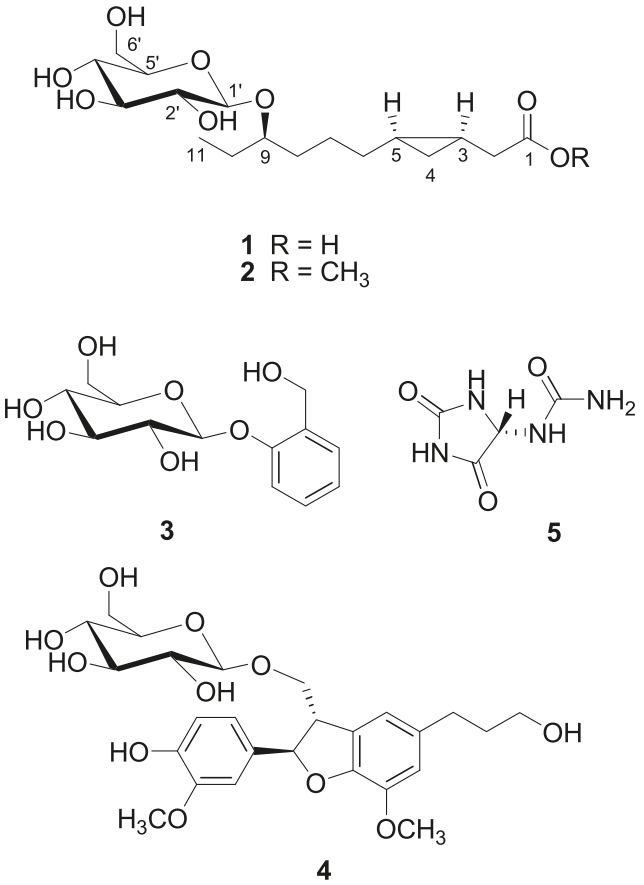
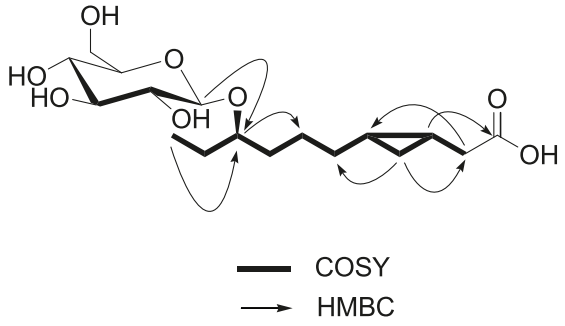


Fig. 2. Key ^{1}H–^{1}H COSY and HMBC correlations of **1**.



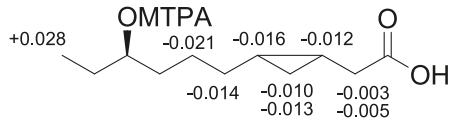
HR-FAB-MS. The IR spectrum of **1** indicated the presence of hydroxyl groups (3390 cm^{-1}) and a carbonyl group (1698 cm^{-1}). The ^{1}H NMR spectrum of **1** showed an anomeric proton at δ 4.34 (1H, d, J = 8.0 Hz, H-1′), which is a characteristic signal for a β-glucopyranosyl unit, and six oxygenated protons attributed to a sugar at δ 3.86–3.26. It also displayed signals for one methyl at δ 0.93 (3H, t, J = 6.5 Hz, H-11), 10 methylenes at δ 2.34–2.24 (2H, m, H-2), 1.62–1.33 (8H, m, H-6, 7, 8, 10), and one oxygenated methine at δ 3.75 (1H, m, H-9), as well as a cyclopropane ring at δ 1.08 (1H, m, H-3), 0.82 (1H, m, H-5), 0.73 (1H, m, H-4a), and –0.11 (1H, q, J = 5.5 Hz, H-4b). The ^{13}C NMR and DEPT spectra of **1** showed signals of 11 carbons of an aglycone attributable to one methyl, six methylenes, three methines (one oxygenated), and one quaternary carbon (carboxyl), along with signals for a β-glucopyranosyl unit (δ 102.3, 77.0, 76.5, 74.1, 70.6, and 61.7). These data suggested that compound **1** was a glycosidic fatty acid with a cyclopropane ring.[7,8] The glucose unit obtained after hydrolysis gave a positive optical rotation, $[\alpha]_D^{25}$ +48.6 (*c* 0.05, H_2O), indicating that it was D-glucose.

The proton and protonated carbon NMR signals of **1** were assigned unambiguously by the HSQC experiment. The cyclopropanoic fatty acid was further confirmed by the ^{1}H–^{1}H COSY correlations from H-2 to H-11 and the HMBC correlations H-2/C-5, H-3/C-1, H-4/C-2, 6, H-9/C-7, and H-11/C-9 (Fig. 2), together with their chemical shift values. The HMBC correlation of the anomeric proton H-1′ (δ 4.34) with C-9 (δ 80.7) indicated that the β-D-glucopyranosyl unit is located at C-9. The stereochemistry, including the absolute configuration at C-9, was established by a combination of analyses, including correlations in the NOESY experiment and the modified Mosher's method.[9] The proton signals for cyclopropylmethylene at δ 0.73 and –0.11 revealed the presence of a cis-1,2-disubstituted cyclopropane ring.[7,8] In the trans form, there are no signals for the cyclopropane proton above δ 0. The *cis*-cyclopropylmethine protons were also confirmed by the NOESY correlation H-3/H-5. On enzymatic hydrolysis of **1** with β-glucosidase, a new fatty acid, 9-hydroxy-*cis*-cascarillic acid (**1a**) was obtained. Compound **1a** was treated with (+)- and (–)-α-methoxy-α-trifluoromethylphenyl acetyl (MTPA) chlorides in NMR tubes to afford (*R*)-MTPA ester (**1r**) and (*S*)-MTPA ester (**1s**), respectively.[9] Diagnostic ^{1}H NMR chemical-shift differences between the MTPA esters of **1** ($\Delta\delta = \delta_S - \delta_R$) (Fig. 3) indicated the 9*R* configuration of **1**. Thus, the structure of **1** was determined as 9*R*-(β-D-glucopyranosyloxy)-*cis*-cascarillic acid, and it was named parisveroside A (Fig. 1).

Parisveroside B (**2**) was isolated as a colorless gum. Its molecular formula was determined to be $C_{18}H_{32}O_8$ from the $[M + Na]^+$ peak at *m*/*z* 399.2012 (calcd. for $C_{18}H_{32}NaO_8$: 399.1995) in the HR-FAB-MS spectrum. Its IR spectrum revealed similar absorption bands to **1**. The ^{1}H and ^{13}C NMR spectra of **2** were almost identical to those of **1**, except for the resonance of one additional methoxy signal. On the basis of the ^{1}H–^{1}H COSY and HSQC spectra, all protons and carbons could be assigned. The HMBC correlation between the methoxy proton at δ 3.69 and C-1 at δ 174.9 indicated that the methoxy group was located at C-1. Enzymatic hydrolysis of **2** yielded a new fatty acid, methyl-9-hydroxy-*cis*-cascarillate (**2a**) and glucose as the sugar component, having a β-D-configuration from the ^{1}H and ^{13}C NMR data and optical rotation analysis. The glycosidic linkage was determined from the HMBC correlation H-1′/C-9. The stereochemistry of **2** was assigned to be the same as that of **1** on the basis of the comparison of the NMR data and optical rotation values: $[\alpha]_D^{25}$ +26.6 (*c* 0.10, MeOH) of **2** with those of **1**. Thus, the structure of **2** was determined as methyl-9*R*-(β-D-glucopyranosyloxy)-*cis*-cascarillate, and it was named parisveroside B (Fig. 1).

Three other known compounds obtained in this investigation were identified as salicin (**3**),[10] 3-(β-D-glucopyranosyloxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxy-(2*R*,3*S*)-dihydrobenzofuran (**4**),[11] and allantoin (**5**)[12] by comparison of their spectroscopic data with those published in the literature (Fig. 1). The known compounds **3**–**5** are reported from this plant source for the first time.

Fig. 3. Δδ Values ($\delta_S - \delta_R$) in ppm of the two α-methoxy-α-trifluoromethylphenyl acetyl (MTPA) esters derived from **1a**.



Microglia, the unique population of central nervous system resident immune cells, have been implicated in the pathogenesis of a variety of neurodegenerative diseases including Parkinson's disease and Alzheimer's disease.[13,14] Excessive production of nitric oxide (NO) and proinflammatory cytokines from activated microglia play an important role in neurodegenerative disorders. Therefore, regulation of the microglial activation will be an important strategy to prevent the progressive damage of the neurodegenerative diseases. Here, the antineuroinflammatory activities of the isolated compounds (**1**–**5**) were evaluated by assessing NO production in the BV-2 cells. BV-2 cells were pretreated with or without compounds **1**–**5** for 30 min prior to the addition of LPS (100 ng/mL). As shown in Fig. 4, compounds **2** and **4** significantly inhibited the NO production in LPS-stimulated BV-2 cells. Among tested compounds, compound **4** was the most potent. Pretreatment of BV-2 cells with compound **4** inhibited LPS-induced NO production in a dose-dependent manner with an IC_{50} value of 60.5 μmol/L. It revealed effective ratios of 23.9% ± 5.7%, 30.2% ± 6.2%, and 44.9% ± 3.5% at concentrations of 10, 20, and 50 μmol/L, respectively, in comparison with the control. The IC_{50} value of compound **2** was also 74.8 μmol/L. On the other hand, none of the compounds showed any cytotoxicity at concentrations used in the present study (data not shown).

Microglial cell activation plays a key role in mediating inflammatory processes in the CNS, which are associated with various neurodegenerative diseases. NO is one of the main proinflammatory mediators produced by activated microglia.[15] Therefore, compounds **2** and **4** from *P. verticillata* might be of therapeutic interest with respect to the treatment of neurodegenerative disease mediated by neuroinflammation.

Experimental

General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH or $CHCl_3$. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer. NMR spectra, including ^1H–^1H COSY, HSQC, HMBC, and NOESY experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C) with chemical shifts given in ppm (δ). Preparative HPLC was conducted using a Gilson 306 pump with a Shodex refractive index detector and an Apollo silica 5 μ column (250 mm × 10 mm). Silica gel 60 (Merck, 70–230 mesh and 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography (CC). Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (*v*/*v*).

Plant material

The roots of *P. verticillata* were collected at Mount O-Dae, Gangwon Province, Korea, in August 2002, and the plants were identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2002-08) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

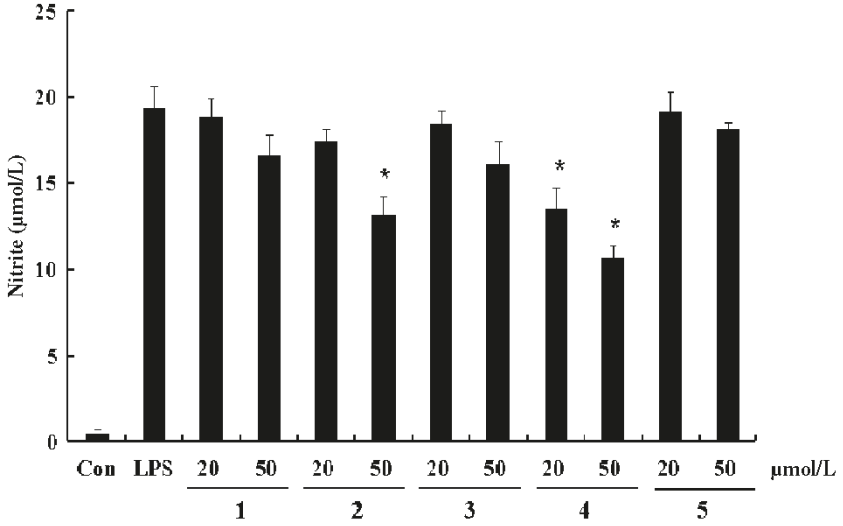
Extraction and isolation

The roots of *P. verticillata* (1.5 kg) were extracted using 80% aq MeOH at room temperature over a period of 3 days three times. The solvent was evaporated under reduced pressure to give a crude extract (180 g). The crude extract was dissolved in distilled water (800 mL) and successively extracted with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH to provide *n*-hexane (5.2 g), CH_2Cl_2 (3.5 g), EtOAc (4.4 g), and *n*-BuOH extracts (20 g). The EtOAc-soluble fraction (4.4 g) was subjected to normal-phase CC over a silica gel using $CHCl_3$–MeOH of increasing polarity (5:1–1:1) to give five fractions (E1–E5). Fraction E3 (1.2 g) was subjected to reversed-phase CC over a RP-C_{18} silica gel using MeOH–H_2O (2:3) to provide four subfractions (E31–E34). Subfraction E33 (120 mg) was separated by preparative normal-phase HPLC using a solvent system of *n*-hexane–$CHCl_3$–MeOH (3:5:1) over 30 min at a flow rate of 2.0 mL/min (Apollo silica 5 μ column; Shodex refractive index detector) to obtain parisveroside B (**2**, 7 mg). The *n*-BuOH-soluble fraction (10 g) was subjected to CC over HP-20 resin using 100% H_2O and 100% MeOH to give two fractions (B1, 100% H_2O, and B2, 100% MeOH). The B1 fraction (2 g) was washed using MeOH and recrystallized to give allantoin (**5**, 200 mg). The B2 fraction (3 g) was subjected to normal-phase CC over silica gel using $CHCl_3$–MeOH–H_2O (10:10:0.5) to give six fractions (B21–B26). The B21 fraction (180 mg) was further separated using silica gel CC with $CHCl_3$–MeOH–H_2O (10:4:0.5) and purified by preparative normal-phase HPLC using a solvent system of $CHCl_3$–MeOH–H_2O (9:4:0.5) to afford salicin (**3**, 6 mg) and 3-(β-D-glucopyranosyloxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxy-dihydrobenzofuran (**4**, 4 mg). The B22 fraction (70 mg) was purified using silica gel CC with $CHCl_3$–MeOH–H_2O (9:4:0.5) to yield parisveroside B (**1**, 5 mg).

Parisveroside A (1)

Colorless gum; $[\alpha]_D^{25}$ +36.5 (*c* 0.05, MeOH). IR (KBr, cm^{-1}) ν_{max}: 3390, 2949, 1698, 1522, 1450, 1026, 674. ^1H NMR (CD_3OD, 500 MHz) δ: 4.34 (1H, d, *J* = 8.0 Hz, H-1′), 3.86 (1H, dd, *J* = 11.0, 1.0 Hz, H-6′a), 3.77 (1H, dd, *J* = 11.0, 3.5 Hz, H-6′b), 3.75 (1H, m, H-9), 3.46–3.35 (3H, m, H-2′, 3′, 4′), 3.26 (1H, m, H-5′), 2.34–2.24 (2H, m, H-2), 1.62–1.58 (4H, m, H-8, 10), 1.43–1.33 (4H, m, H-6, 7), 1.08 (1H, m, H-3), 0.93 (3H, t, *J* = 6.5 Hz, H-11), 0.82 (1H, m, H-5), 0.73 (1H, m, H-4a), –0.11 (1H, q, *J* = 5.5 Hz, H-4b). ^{13}C NMR (CD_3OD, 125 MHz) δ: 177.5 (C-1), 102.3 (C-1′), 80.7 (C-9), 77.0 (C-3′), 76.5 (C-5′), 74.1 (C-2′), 70.6 (C-4′), 61.7 (C-6′), 34.1 (C-2), 33.1 (C-8), 28.8 (C-6), 27.4 (C-10), 25.2 (C-7), 15.4 (C-5), 11.7 (C-3), 10.2 (C-4), 8.8 (C-11). HR-FAB-MS *m*/*z* 363.2021 $[M + H]^+$ (calcd. for $C_{17}H_{31}O_8$: 336.2019).

Fig. 4. Inhibitory effects of compounds **1**–**5** on nitric oxide (NO) production in lipopolysaccharide (LPS)-activatied BV-2 cells.



Parisveroside B (2)

Colorless gum; $[\alpha]_D^{25}$ +26.6 (*c* 0.10, MeOH). IR (KBr, cm^{-1}) ν_{max}: 3395, 2948, 1702, 1515, 1455, 1026, 674. ^1H NMR (CD_3OD, 500 MHz) δ: 4.33 (1H, d, *J* = 8.0 Hz, H-1′), 3.86 (1H, dd, *J* = 11.5, 1.5 Hz, H-6′a), 3.69 (3H, s, OCH_3), 3.68 (1H, dd, *J* = 11.5, 3.5 Hz, H-6′b), 3.65 (1H, m, H-9), 3.38–3.33 (3H, m, H-2′, 3′, 4′), 3.25 (1H, m, H-5′), 2.39–2.30 (2H, m, H-2), 1.64–1.56 (4H, m, H-8, 10), 1.50–1.35 (4H, m, H-6, 7), 1.08 (1H, m, H-3), 0.93 (3H, t, *J* = 6.5 Hz, H-11), 0.83 (1H, m, H-5), 0.73 (1H, m, H-4a), –0.10 (1H, q, *J* = 5.5 Hz, H-4b). ^{13}C NMR (CD_3OD, 125 MHz) δ: 174.9 (C-1), 102.4 (C-1′), 80.7 (C-9), 77.1 (C-3′), 76.6 (C-5′), 74.2 (C-2′), 70.7 (C-4′), 61.8 (C-6′), 50.9 (OCH_3), 33.3 (C-2), 33.1 (C-8), 28.8 (C-6), 27.5 (C-10), 25.1 (C-7), 15.4 (C-5), 11.4 (C-3), 10.2 (C-4), 8.9 (C-11). HR-FAB-MS *m/z* 399.2012 $[M + Na]^+$ (calcd. for $C_{18}H_{32}NaO_8$: 399.1995).

Enzymatic hydrolysis of 1 and 2

Compounds **1** and **2** (3 mg each) were dissolved in H_2O (3 mL) and β-glucosidase (emulsin, 9 mg) was added to the solution and kept at 37 °C for 3 days, in each case. The reaction mixtures were extracted with $CHCl_3$, and the aqueous phases compared with an authentic sugar sample using co-TLC (MeCN–H_2O, 6:1, R_f 0.33 for glucose). Identification of D-glucose in the aqueous layer was carried out by comparing the optical rotation of the liberated glucose ($[\alpha]_D^{25}$ +48.6 (*c* 0.05, H_2O) from **1** and $[\alpha]_D^{25}$ +45.3 (*c* 0.05, H_2O) from **2**) with that of an authentic sample of D-glucose.[16] The $CHCl_3$ layers of **1** and **2** were followed by purification on a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$–MeOH, 15:1) to afford new compounds **1a** (1.4 mg) and **2a** (1.1 mg). **1a**: colorless gum; $[\alpha]_D^{25}$ –12.5 (*c* 0.04, $CHCl_3$). ^1H NMR (C_5D_5N, 500 MHz) δ: 3.95 (1H, m, H-9), 2.62–2.48 (2H, m, H-2), 1.64–1.57 (4H, m, H-8, 10), 1.30 (2H, m, H-7), 1.24 (2H, m, H-6), 1.17 (1H, m, H-3), 1.06 (3H, t, *J* = 6.5 Hz, H-11), 0.85 (1H, m, H-5), 0.76 (1H, m, H-4a), –0.03 (1H, q, *J* = 5.5 Hz, H-4b). HR-FAB-MS *m/z* 201.1485 $[M + H]^+$ (calcd. for $C_{11}H_{21}O_3$: 201.1491). **2a**: colorless gum; $[\alpha]_D^{25}$ –20.7 (*c* 0.03, $CHCl_3$). ^1H NMR (C_5D_5N, 500 MHz) δ: 3.91 (1H, m, H-9), 3.70 (3H, s, OCH_3), 2.65–2.47 (2H, m, H-2), 1.65–1.58 (4H, m, H-8, 10), 1.28 (2H, m, H-7), 1.23 (2H, m, H-6), 1.19 (1H, m, H-3), 1.05 (3H, t, *J* = 6.5 Hz, H-11), 0.87 (1H, m, H-5), 0.77 (1H, m, H-4a), –0.01 (1H, q, *J* = 5.5 Hz, H-4b). HR-FAB-MS *m/z* 215.1652 $[M + H]^+$ (calcd. for $C_{12}H_{23}O_3$: 215.1647).

Preparation of the Mosher ester derivatives of 1a

Compound **1a** (0.7 mg) was transferred to a clean and completely dry NMR tube. Deuterated pyridine (0.7 mL) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, (*S*)-MTPA-Cl, (5 μL) were added immediately to the NMR tube and then the NMR tube was shaken carefully to mix the sample and (*S*)-MTPA-Cl evenly. The reaction NMR tube was then permitted to stand at room temperature overnight. The completed reaction afforded the (*R*)-MTPA ester derivative (**1r**) of **1a**. The (*S*)-MTPA ester derivative (**1s**) of **1a** was obtained in the same manner as described for **1r**. The ^1H NMR spectra for **1r** and **1s** were directly measured with the reaction NMR tubes. **1s**: ^1H NMR (C_5D_5N, 500 MHz) δ: 5.184 (1H, m, H-9), 2.537 (1H, m, H-2a), 2.485 (1H, m, H-2b), 1.762–1.559 (4H, m, H-8, 10), 1.257 (2H, m, H-7), 1.200 (2H, m, H-6), 1.156 (1H, m, H-3), 1.042 (3H, t, *J* = 6.5 Hz, H-11), 0.801 (1H, m, H-5), 0.686 (1H, m, H-4a), –0.078 (1H, q, *J* = 5.5 Hz, H-4b). **1r**: ^1H NMR (C_5D_5N, 500 MHz) δ: 5.192 (1H, m, H-9), 2.540 (1H, m, H-2a), 2.490 (1H, m, H-2b), 1.772–1.560 (4H, m, H-8, 10), 1.278 (2H, m, H-7), 1.214 (2H, m, H-6), 1.168 (1H, m, H-3), 1.014 (3H, t, *J* = 6.5 Hz, H-11), 0.817 (1H, m, H-5), 0.696 (1H, m, H-4a), –0.065 (1H, q, *J* = 5.5 Hz, H-4b).

Measurement of NO production and cell viability

The BV-2 mouse microglial cell line was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS). To measure NO production, BV-2 cells were plated into a 96 well plate (3×10^4 cells/well) and treated with 100 ng/mL LPS in the presence or absence of chrysin for 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phos-

phoric 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 a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.[17] N^G-Monomethyl-L-arginine (L-NMMA, Sigma-Aldrich), a well-known NOS inhibitor, was tested as a positive control. The IC_{50} value for the positive control, L-NMMA, was 18.0 μmol/L.

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References

(1) Farmer, S. B.; Schilling, E. E. *Syst. Bot.* **2002**, *27*, 674.
(2) Liu, H.; Huang, Y.; Wang, Q.; Zhang, T.; Song, Y. *Planta Med.* **2006**, *72* (9), 835. doi:10.1055/s-2006-946637.
(3) Ahn, D. K. *Illustrated Book of Korean Medicinal Herbs;* Kyo-Hak Pub.: Seoul, 1998; p 107.
(4) Nakano, K.; Murakami, K.; Nohara, T.; Tominatsu, T.; Kawasaki, T. *Chem. Pharm. Bull. (Tokyo)* **1981**, *29*, 1445.
(5) Lee, K. H.; Yang, M. C.; Kim, K. H.; Kwon, H. C.; Choi, S. U.; Lee, K. R. *Molecules* **2008**, *13* (1), 41. doi:10.3390/molecules13010041.
(6) Kim, K. H.; Lee, K. H.; Choi, S. U.; Kim, K. R.; Lee, K. R. *Heterocycles* **2010**, *81* (6), 1493. doi:10.3987/COM-10-11942.
(7) Wilson, S. R.; Prodan, K. A. *Tetrahedron Lett.* **1976**, *17* (47), 4231. doi:10.1016/0040-4039(76)80081-0.
(8) Jiao, Y.; Yoshihara, T.; Ishikun, S.; Uchino, H.; Ichihara, A. *Tetrahedron Lett.* **1996**, *37* (7), 1039. doi:10.1016/0040-4039(95)02342-9.
(9) Su, B. N.; Park, E. J.; Mbwambo, Z. H.; Santarsiero, B. D.; Mesecar, A. D.; Fong, H. H. S.; Pezzuto, J. M.; Kinghorn, A. D. *J. Nat. Prod.* **2002**, *65* (9), 1278. doi:10.1021/np0202475.
(10) Khatoon, F.; Khabiruddin, M.; Ansari, W. H. *Phytochemistry* **1988**, *27* (9), 3010. doi:10.1016/0031-9422(88)80716-7.
(11) Kikuzaki, H.; Kayano, S.; Fukutsuka, N.; Aoki, A.; Kasamatsu, K.; Yamasaki, Y.; Mitani, T.; Nakatani, N. *J. Agric. Food Chem.* **2004**, *52* (2), 344. doi:10.1021/jf034954v.
(12) Yin, F.; Hu, L.; Pan, R. *Chem. Pharm. Bull. (Tokyo)* **2004**, *52* (12), 1440. doi:10.1248/cpb.52.1440.
(13) Wilms, H.; Zecca, L.; Rosenstiel, P.; Sievers, J.; Deuschl, G.; Lucius, R. *Curr. Pharm. Des.* **2007**, *13* (18), 1925. doi:10.2174/138161207780858429.
(14) McGeer, P. L.; McGeer, E. G. *Brain Res. Brain Res. Rev.* **1995**, *21* (2), 195. doi:10.1016/0165-0173(95)00011-9.
(15) Iadecola, C. *Trends Neurosci.* **1997**, *20* (3), 132. doi:10.1016/S0166-2236(96)10074-6.
(16) Kim, K. H.; Choi, S. U.; Lee, K. R. *J. Nat. Prod.* **2009**, *72* (6), 1121. doi:10.1021/np900101t.
(17) Sargent, J. M.; Taylor, C. G. *Br. J. Cancer* **1989**, *60*, 206.