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Short communication

Anti-neuroinflammatory constituents from *Sinomenium acutum* rhizomes

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ABSTRACT

In our continuing search for bioactive constituents from Korean medicinal sources, an investigation of the *Sinomenium acutum* Rehder et Wilson rhizome afforded three new lignan derivatives (**1–3**) together with nine known compounds (**4–12**). The identification and structural elucidation of these new compounds were based on 1D and 2D-NMR (COSY, HMQC, HMBC and NOESY) and MS data. The absolute configurations were established based on the CD data. Nitric oxide production was evaluated in the BV-2 lipopolysaccharide-activated microglia cell line to investigate the anti-neuroinflammatory effects of the isolated compounds (**1–12**).

1. Introduction

Neuroinflammation is an important contributor to neuronal damage in neurodegenerative diseases such as stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, and Multiple Sclerosis (Stoll and Jander, 1999; Liu and Hong, 2003). Microglia are the resident immune cells in the central nervous system and play a key role in the inflammatory reaction (Dickson et al., 1993). Microglia serve the role of immune surveillance under normal conditions, but microglia activate and secrete pro-inflammatory and neurotoxic substances after brain damage or exposure to inflammation (McGeer and McGeer, 2004). Accumulation of proinflammatory and cytotoxic factors is deleterious to neurons, and these factors are thought to actively participate in the progression of neurodegenerative diseases (Jeohn et al., 1998). Therefore, controlling microglial activation and neuroinflammatory processes is an effective therapeutic target for treating various neurodegenerative diseases. We have reported recently that various natural lignans are useful anti-neuroinflammatory agents (Kim et al., 2011; Kim et al., 2012). In our ongoing screening of medicinal plants for potential anti-inflammatory compounds, lignan derivatives isolated from the *Sinomenium acutum* rhizome exhibited a potent anti-neuroinflammatory effect in lipopolysaccharide (LPS)-stimulated microglia cells

Sinomenium acutum Rehder et Wilson (Menispermaceae) is widely distributed on hilly regions in eastern Asia including Korea, Japan, and China. The plant's rhizomes and stems have been used as a traditional medicine in China for treating arthralgia due to pathogenic wind dampness, arthral paralysis, and swelling (Liu et al., 1991). Many alkaloids, such as sinomenine, disinoimenine, sinactine, sinoactine, acutumine, and magnoflorine, as well as the lignan syringaresinol have been isolated from this source (Tomita et al., 1971; Ichikawa et al., 1984; Bao et al., 2005; Wang et al., 2007; Jin et al., 2008; Kato et al., 2009). Among them, sinomenine (major alkaloid) possesses a variety of pharmacological effects, such as immunosuppression, conscious-sedation, anti-arrhythmia, analgesia, organ protection from damage caused by shock, and Ca^{2+}-antagonist (Vieregge et al., 1999; Candinas et al., 1996; Liu et al., 1996). We have recently reported the isolation of two new alkaloids from this source (Lee et al., 2012; Kim et al., 2013).

In continuing research on MeOH extracts of *S. acutum* rhizomes, three new lignans (**1–3**), along with nine known compounds (**4–12**) were isolated by repeated column chromatographic separation of the extract (Fig. 1). The compounds (**1–12**) were

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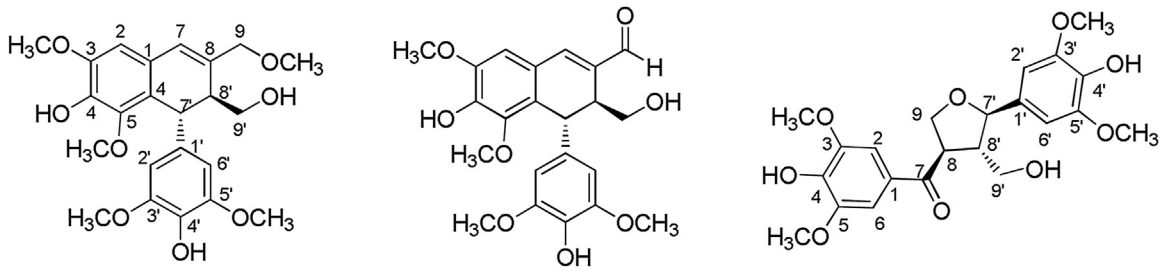

1 **2** **3**

Fig. 1. Chemical structures of compounds **1–3**.

evaluated for their inhibitory effects on nitric oxide (NO) production in LPS-activated BV-2 cells, a microglial cell line. Herein, we report the isolation and structural elucidation of the new compounds, as well as the anti-neuroinflammatory effects of the isolates (**1–12**).

2. Results and discussion

Compound **1** was obtained as a yellowish gum. The molecular formula was determined to be $C_{23}H_{28}O_8$ from the molecular ion peak $[M]^+$ at *m/z* 432.1786 (calcd for $C_{23}H_{28}O_8$, 432.1784) in the positive-ion HR-EIMS. The ^{1}H NMR spectrum of **1** (Table 1) showed signals for four olefinic protons at d_H 6.66 (1H, s), 6.36 (2H, s), and 6.46 (1H, s), two oxygenated methylene protons [d_H 3.74 (1H, d, J = 12.5 Hz), 4.02 (1H, dd, J = 12.5, 1.0 Hz) and d_H 3.19 (1H, t, J = 10.0 Hz), 3.60 (1H, m)], two methine protons [d_H 4.63 (1H, s) and 2.65 (1H, m)] and five methoxy protons [d_H 3.89 (3H, s), 3.70 (6H, s), 3.54 (3H, s), and 3.00 (3H, s)]. In the ^{13}C NMR spectrum, 23 carbon signals appeared, including twelve aromatic carbons [d_C 147.6, 147.5 (x2), 146.3, 139.3, 135.5, 133.3, 124.8, 121.4, 105.7, and 105.0 (x2)], two oxygenated carbons (d_C 74.5 and 61.8), two olefinic carbons (d_C 125.9, and 133.0), two methine carbons (d_C 46.8, and 37.5), and five methoxy carbons [d_C 59.4, 55.9, 55.3 and 55.2 (x2)], which were classified by HMQC experiment. These spectral data suggested that **1** was a dihydronaphthalene type lignan (Ahmed et al., 1973). The ^{1}H- and ^{13}C NMR spectral data (Table 1) of **1** were similar to those of (+)-magnoliadiol isolated from *Magnolia fargesii* (Ahmed et al., 1973), except for an additional methoxy group signal [d_H 3.00 (3H, s); d_C 55.9]. The position of the methoxy group was confirmed by HMBC experiment that showed a correlation between the methoxy proton signal (d_H 3.00) and C-9 (d_C 74.5) (Fig. 2). The stereochemistry of **1** was supposed to be same as that of (+)-magnoliadiol based on the J values in the ^{1}H NMR spectrum, which was reconfirmed by NOESY experiment (Fig. 3). The absolute configurations at C-7′ and C-8′ of **1** were determined to be 7′*R* and 8′*S*, respectively based on the CD data showing a positive Cotton effect at 257 nm and a negative Cotton effect at 353 nm (Nishizawa et al., 1990). Thus, the structure of **1** was determined to be (7′*R*,8′*S*)-3,3′,5,5′,9-pentamethoxy-9′-hydroxy-6,7-cyclolignan-7-ene.

Compound **2** was obtained as a yellowish gum, with the molecular formula of $C_{22}H_{24}O_8$ based on the positive-ion HR-FABMS (*m/z* 417.1549 $[M+H]^+$, calcd. for $C_{22}H_{25}O_8$, 417.1549). The ^{1}H- and ^{13}C NMR data (Table 1) were similar to those of **1**. The main differences were the appearance of a aldehyde proton and carbon signals [d_H 9.46 (H-9); d_C 193.2 (C-9)] in **2**, and the absence of an oxygenated methylene and methoxy signals in **1** (Han et al., 2008; Kashima et al., 2010). The position of the aldehyde group was C-9 by a HMBC experiment, which showed correlations between H-9 and C-7, C-8, and C-8′. The relative stereochemistry of **2** was the same as that of **1** based on the NMR data and a NOESY experiment (Fig. 3). The absolute configurations at C-7′ and C-8′ of **2** were established as 7′*R* and 8′*S*, respectively, by comparison of the CD data showing a negative Cotton effect at 353 nm and a positive Cotton effects at 249 (Nishizawa et al., 1990). Therefore, the structure of **2** was elucidated as (7′*R*,8′*S*)-3,3′,5,5′-tetramethoxy-9′-hydroxy-6,7-cyclolignan-7-ene-9-al.

Compound **3** was obtained as a colorless gum. The molecular formula was determined to be $C_{22}H_{26}O_9$ from the molecular ion peak $[M]^+$ at *m/z* 434.1576 (calcd. for $C_{22}H_{26}O_9$, 434.1577) in the positive-ion HR-EIMS. The IR spectrum of **3** indicated the presence of hydroxyl (3424 cm^{-1}), carbonyl (1660 cm^{-1}), and aromatic groups (1610 and 1518 cm^{-1}). The ^{1}H NMR data (Table 2) of **3** showed the presence of four aromatic protons [d_H 7.39 (2H, s) and 6.72 (2H, s)], two oxygenated methylene protons [d_H 4.20 (1H, m), 4.19 (1H, m) and 3.66 (2H, m)], an oxygenated methine proton [d_H 4.64 (1H, d, J = 8.5 Hz)], two methine protons [d_H 4.26 (1H, m) and 2.65 (1H, m)] and four methoxy protons [d_H 3.91 (6H, s) and 3.85 (6H, s)]. The ^{13}C NMR data indicated 22 carbon resonances, which

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **1** and **2** in CD_3OD (d in ppm).[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		121.4		123.1
2	6.66, s	105.7	6.97, s	108.5
3		147.6		148.2
4		139.3		142.0
5		146.3		146.7
6		124.8		125.9
7	6.46, s	125.9	7.48, s	147.7
8		133.0		135.4
9	3.74, d (12.5) 4.02, dd (12.5, 1.0)	74.5	9.46, s	193.2
3-OCH_3	3.89, s	55.3	3.93, s	55.7
5-OCH_3	3.54, s	59.4	3.59, s	59.2
9-OCH_3	3.00, s	55.9		
1′		135.5		135.3
2′	6.36, s	105.0	6.28, s	104.9
3′		147.5		147.9
4′		133.3		133.9
5′		147.5		147.9
6′	6.36, s	105.0	6.28, s	104.9
7′	4.63, s	37.6	4.77, s	37.4
8′	2.65, m	46.8	3.18, m	42.5
9′	3.19, t (10.0) 3.60, m	61.8	3.09, t (10.0) 3.42, m	61.6
3′-OCH_3	3.70, s	55.2	3.69, s	55.5
5′-OCH_3	3.70, s	55.2	3.69, s	55.5

[a] Assignments were based on 2D NMR including COSY, HMQC, and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

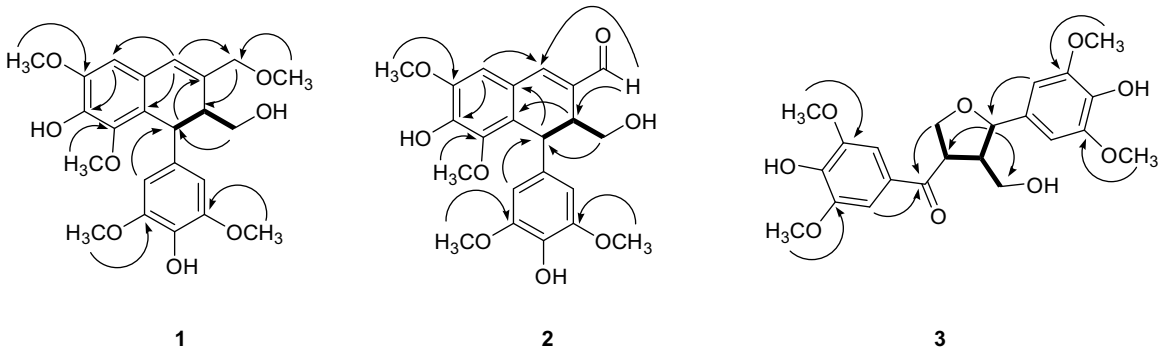

Fig. 2. Key ^{1}H–^{1}H COSY (—) and HMBC (→) correlations of **1**–**3**.

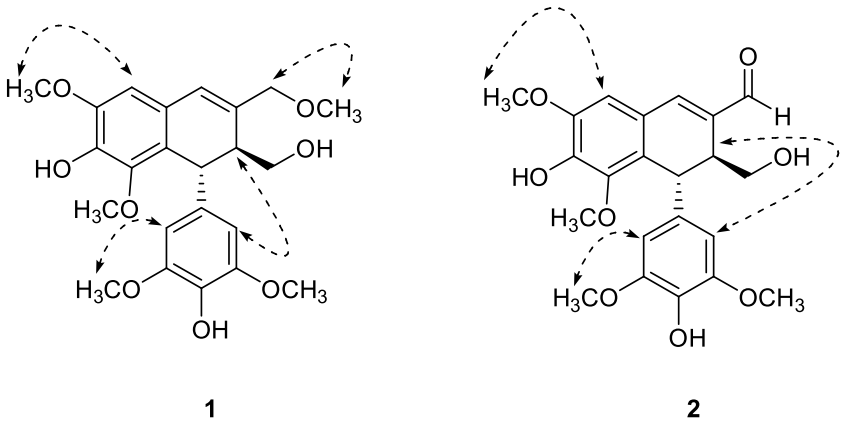

Fig. 3. Key NOESY (⇠⇢) correlations of **1**–**2**.

Table 2
^{1}H (500 MHz) and ^{13}C— NMR (125 MHz) spectral data of **3** in $CDCl_3$ (d in ppm).[a]

Position	**3**	
	δ_H	δ_C
1		127.5
2	7.39, s	106.5
3		147.9
4		141.5
5		147.9
6	7.39, s	106.5
7		199.1
8	4.26, m	48.9
9	4.19, m	70.4
	4.20, m	
3-OCH_3	3.91, s	55.7
5-OCH_3	3.91, s	55.7
1′		131.7
2′	6.72, s	104.0
3′		148.0
4′		135.1
5′		148.0
6′	6.72, s	104.0
7′	4.64, d (8.5)	84.3
8′	2.65, m	53.9
9′	3.66, m	60.1
3′-OCH_3	3.85, s	55.6
5′-OCH_3	3.85, s	55.6

[a] Assignments were based on 2D NMR including COSY, HMQC, and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

were classified by HMQC experiments as four methoxy carbons [d_C 55.7 (x2) and 55.6 (x2)], twelve aromatic carbons [d_C 148.0 (x2), 147.9 (x2), 141.5, 135.1, 131.7, 127.5, 106.5(x2), and 104.0 (x2)], two oxygenated carbons (d_C 60.1 and 70.4), three methine carbons (d_C 84.3, 53.9 and 48.9), and a carbonyl carbon (d_C 199.1). A survey of the literature revealed that the ^{1}H- and ^{13}C NMR data of **3** were almost the same as those of 4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one, which was isolated from *Sinocalamus affinis* (Xiong et al., 2011), except for the optical rotation value {**3**: [α] +1.8 (*c* 0.13, MeCN); 4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one: [α] −1.5 (*c* 0.2, MeCN)} (Xiong et al., 2011). The relative configurations at C-7′, C-8, and C-8′ of **3** were supposed to be same as those of (+)-magnolone {[α] +31.0 (*c* 0.2, $CHCl_3$); **3**:[α] +14.0 (*c* 0.13, $CHCl_3$)}, based on the chemical shifts, coupling constants, and optical rotation data (Nakato and Yamauchi, 2007; Pandey et al., 2010). The CD spectrum of **3** exhibited a negative Cotton effect at 290 nm and a positive Cotton effect at 324 nm, whereas the CD spectrum of 4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one showed a positive Cotton effect at 286 nm and a negative Cotton effect at 324 nm (4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one: 7′*S*,8*R*,8′*S*), indicating that the absolute stereochemistry of **3** was the 7′*R*,8*S*,8′*R* form (Xiong et al., 2011; Liao et al., 2006). Thus, the structure of **3** was determined to be (+)-(7′*R*,8*S*,8′*R*)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one.

The known compounds were identified as (−)-lirioresinol A (**4**) (Lin et al., 1994), (−)-lyoniresinol (**5**) (Ohashi et al., 1994), *ent*-isolariciresinol (**6**) (Urones et al., 1987), buddlenol D (**7**) (Houghton, 1985), 2,6-dimethoxy-1,4-benzoquinone (**8**) (Kwon et al., 2001), 3-methoxy-4-hydroxyphenylethanol (**9**) (Kitagawa et al., 1981), β-hydroxypropiovanillone (**10**) (Karonen et al., 2004), ficusol (**11**) (Li and Kuo, 1998), and (hydroxymethyl)-2-furaldehyde (**12**) (Qui

Table 3
Inhibitory effect of compounds **1–12** on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells.

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]
1	11.23	105.1 ± 4.5
2	20.61	103.2 ± 4.4
3	57.79	100.2 ± 3.3
4	59.88	106.8 ± 6.0
5	42.67	97.0 ± 4.4
6	35.41	97.20 ± 2.0
7	13.04	96.7 ± 2.6
8	3.99	49.9 ± 7.7
9	74.49	100.4 ± 0.3
10	157.43	100.9 ± 5.2
11	89.85	101.0 ± 1.2
12	176.04	105.1 ± 3.3
NMMA[c]	18.29	96.7 ± 2.6

[a] IC_{50} values of each compound are defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability after treatment with 20 μM of each extract is expressed as a percentage (%) of the LPS-only treatment group. Results are means of three independent experiments, and data are mean ± standard deviation.
[c] NMMA was the positive control.

et al., 2007) by comparing experimental and literature spectroscopic data.

We evaluated the inhibitory effects of the isolated compounds (**1–12**) isolated from *S. acutum*, based on the evaluation of the inhibitory activity on NO production in lipopolysaccharide (LPS)-activated murine microglia BV-2 cells (Table 3). Among these compounds, compound **8** inhibited NO levels in the medium with IC_{50} of 3.99 μM. However, compound **8** induced significant cytotoxicity at a concentration of 20 μM (49.9 ± 7.7%). Compound **1**, **2**, and 7 significantly decreased the production of NO, with an IC_{50} of 11.23, 20.61, and 13.04 μM, respectively, without evident cell toxicity. In particular, the activity of compounds **1** and **7** were more potent than that of positive control, L-NMMA, in inhibiting NO production with an IC_{50} of 18.29 μM. Therefore, we suggest that Compound **1**, and **7** isolated from the rhizomes of *S. acutum* may be potently active compounds that have anti-neuroinflammatory properties via inhibition of NO production.

In conclusion, the structures of three new lignans (**1–3**), together with nine known compounds (**4–12**) isolated from the rhizome of *S. acutum* were identified. The anti-inflammatory effects of the compounds isolated from this source were confirmed. In particular, lignan derivative compounds **1**, **2**, and **7** significantly decreased the production of NO in LPS-induced BV-2 cells, without high cell toxicity. Thus, compounds **1**, **2**, and 7 may have beneficial therapeutic potential for neuroinflammatory diseases by inhibiting microglial activation.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA). UV spectra were recorded with a Shimadzu UV-1601 UV–vis spectrophotometer (Shimadzu, Tokyo, Japan). HR-FAB and EI mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA, USA). Nuclear magnetic resonance (NMR) spectra, including ^{1}H–^{1}H COSY, HMQC, HMBC and NOESY experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Semi-preparative high performance liquid chromatography (HPLC) used a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA). 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. The packing material for size-exclusion column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F_{254} plates and RP–18 F_{254s} plates (Merck, Darmstadt, Germany) 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).

3.2. Plant material

The rhizomes of *Sinomenium acutum* Rehder et Wilson (20 kg) were collected on Jeju Island, Korea in November 2010, and the plant was identified by one of the authors (K.R.Lee). A voucher specimen (SKKU 2011-01) has been deposited at the herbarium in the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

3.3. Extraction and isolation

The dried and chopped rhizomes of *S. acutum* (20 kg) were extracted with 80% aqueous MeOH under reflux and then filtered. The filterate was evaporated under reduced pressure to give a residue (1200 g), which was dissolved in water (18 L) and then successively partitioned with $CHCl_3$ and *n*-BuOH after pretreatment with 2 N hydrochloric acid (HCl), yielding a $CHCl_3$-fraction (50 g), and *n*-BuOH-fraction (150 g), respectively. The $CHCl_3$-fraction (50 g) was separated over a silica gel column with a solvent system ($CHCl_3$:MeOH = 50:1–1:1) to give three fractions (C1-C3). Fraction C2 (35 g) was separated on a RP-C_{18} silica gel column with 50% aqueous MeOH to yield three subfractions
(C21-C23). Subfractions C21 (15 g) was separated on a Sephadex LH-20 column with a solvent system of 80% aqueous MeOH to give four subfractions (C211-C214). Subfraction C211 (2 g) was separated over an RP-C_{18} silica gel column with a solvent system of 50% aqueous MeOH and purified by semi-preparative normal-phase HPLC using an Apollo Silica column (250 mm × 10 mm, 2 mL/min, *n*-hexane-$CHCl_3$-MeOH; 7:20:1) to yield **8** (15 mg). Subfraction C211 (5 g) was separated over a silica gel column with $CHCl_3$-MeOH (50:1) and purified by semi-preparative reversed-phase HPLC using an Alltech Econosil RP-C_{18} column (250 mm × 10 mm, 2 mL/min; 70% aqueous MeOH) to yield **3** (34 mg) and **5** (36 mg). Subfraction C211 (2 g) was separated on a RP-C_{18} silica gel column with a solvent system of 60% aqueous MeOH and purified by semi-preparative normal-phase HPLC (2 mL/min, $CHCl_3$-MeOH; 10:1 and 7:1) to yield **9** (5 mg), **10** (6 mg), and **11** (7 mg). Subfraction C22 (4.3 g) was separated on a silica gel column ($CHCl_3$-MeOH, 50:1) to give five subfraction (C221- C225). Subfraction C221 (2 g) was separated on a Sephadex LH-20 column with a solvent system of 80% aqueous MeOH and purified with a semi-preparative reversed-phase HPLC (2 mL/min; 55% aqueous MeOH) to yield **6** (14 mg) and **7** (25 mg). The *n*-BuOH soluble fraction (150 g) was chromatographed on a Diaion HP-20, eluting a gradient solvent system of 100% H_2O and 100% MeOH to give two fractions (Fraction A-B). Fraction B (80 g) was subjected to column chromatography on an RP-C_{18} silica gel, using a solvent system of 30% aqueous MeOH to give three subfractions (B1-B3). Subfraction B1 (25 g) was separated over a silica gel column ($CHCl_3$:MeOH, 30:1) and purified with a semi-preparative reversed-phase HPLC (2 mL/min; 50% aqueous MeOH) to yield **12** (90 mg). Subfraction B3 (25 g) was separated over a silica gel column ($CHCl_3$-MeOH, 40:1) to give four subfractions (B31–B34). Subfraction B32 (2 g) was separated on a Sephadex LH-20 column with a solvent system of 80% aqueous MeOH and purified by semi-preparative reversed-

phase HPLC (2 mL/min; 50% aqueous MeOH) to yield **4** (410 mg). Subfraction B34 (3 g) was separated over a silica gel column ($CHCl_3$-MeOH, 10:1) and purified with a semi-preparative reversed-phase HPLC (2 mL/min; 50% aqueous MeOH) to yield **1** (24 mg) and **2** (5 mg).

*3.3.1. (7′R,8′S)-3′,5′,3,5,9-pentamethoxy-9′-hydroxy-6,7-cyclolignan-7-ene (**1**)*

Yellowish gum; [α] +15.8 (*c* 0.06, MeOH); IR (KBr) ν_{max} cm^{-1}: 3433, 2243, 1699, 1620, 1457, 1290; UV (MeOH) λ_{max} (log ε) 356 (3.56), 255 (3.88), 220 (4.14) nm; CD (MeOH) λ_{max} (Δε) 353 (-3.30), 257 (+8.87) nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive-ion mode) *m*/*z* 432.1786 $[M]^+$ (calcd for $C_{23}H_{28}O_8$, 432.1784).

*3.3.2. (7′R,8′S)-3′,5′,3,5-tetramethoxy-9′-hydroxy-6,7-cyclolignan-7-ene-9-al (**2**)*

Yellowish gum; [α] +28.0 (*c* 0.10, MeOH); IR (KBr) ν_{max} cm^{-1}: 3418, 2951, 2838, 1653, 1452, 1115, 1019, 719; UV (MeOH) λ_{max} (log ε) 260 (3.6), 350 (3.3) nm; CD (MeOH) λ_{max} (Δε) 249 (+1.3), 353 (-1.8) nm; ^1H and ^{13}C NMR spectra, see Table 1; HR-FABMS (positive-ion mode) *m*/*z* 417.1549 $[M+H]^+$ (calcd for $C_{22}H_{25}O_8$, 417.1549).

*3.3.3. (+)-(7′R,8S,8′R)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-7′,9-epoxylignan-9′-ol-7-one (**3**)*

Colorless gum; [α] + 14.0 (*c* 0.13, $CHCl_3$), [α] + 1.8 (*c* 0.13, MeCN); IR (KBr) ν_{max} cm^{-1}: 3423, 2941, 1660, 1610, 1518, 1453, 1166, 1033, 800, 709; UV (MeOH) λ_{max} (log ε) 227 (4.2), 302 (3.5) nm; CD (MeOH) λ_{max} (Δε) 290 (-1.0), 324 (4.3) nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS (positive-ion mode) *m*/*z* 434.1576 $[M]^+$ (calcd for $C_{22}H_{26}O_9$, 434.1577).

3.4. Measurement of NO production and cell viability in LPS-activated BV-2 cells

BV-2 microglia cells were stimulated with 100 ng/mL of LPS in the presence or absence of samples for 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was measured by the Griess reaction. The supernatant (50 mL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, U.S.A.). N^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a well-known nitric oxide synthase inhibitor, was tested as a positive control (Reif and McCreedy, 1995).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.07.012.

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