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Bioactive Lignans from the Rhizomes of *Acorus gramineus*

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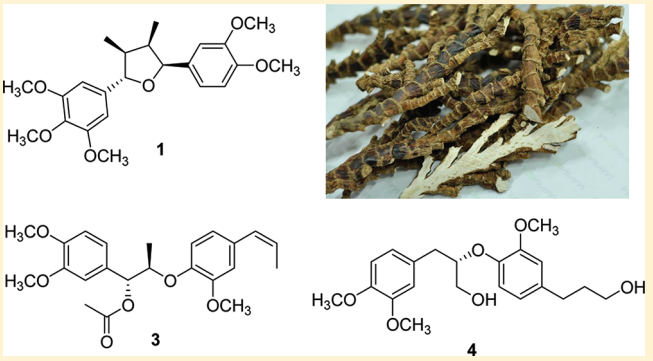
Supporting Information

ABSTRACT: As a part of our ongoing search for bioactive constituents from natural Korean sources, the investigation of rhizomes of *Acorus gramineus* afforded five new lignans, named ligraminols A–E (**1**–**5**), together with seven known ones (**6**–**12**). The structures of **1**–**5** were determined by a combination of 1D and 2D NMR, HRMS, CD, and enzymatic hydrolysis. Compounds **1**–**12** were tested for their antiproliferative activities toward a panel of human-derived normal and cancer cell lines. Moreover, compounds **1**–**12** were evaluated for their inhibitory activities on nitric oxide production in an activated murine microglial cell line.



Acorus gramineus (Araceae), which has the Japanese name "Japanese sweetflag", is an aquatic or wetland perennial with semi-evergreen grasslike foliage. It spreads aggressively by rhizomes, which have been used as a remedy for cognitive problems, sedation, and analgesia in Traditional Chinese Medicine.[1] This herb has long been used for the treatment of stomachache and edema and for the extermination of insects.[2,3] Previous phytochemical studies have led to reports of some pharmacologically active phenolics, such as β-asarone, α-asarone, and phenylpropenes.[4−6] β-Asarone was shown to have antibacterial and antifungal activities.[5,7] Anthelminthic and pesticidal activities of *A. gramineus* have been reported to be associated with the α- and β-asarones,[6] which are the major essential oil components in this plant.[5] These components also exhibited neuroprotective effects against the excitotoxicity induced by *N*-methyl-D-aspartate (NMDA) or glutamate (Glu) in cultured rat cortical cells.[8]

In a continuing search for bioactive constituents from Korean medicinal plants, we investigated a methanol extract of the rhizomes of *A. gramineus* and have isolated five new lignans (**1**–**5**), together with seven known ones (**6**–**12**). The compounds were evaluated for their antiproliferative activities toward a panel of human normal and cancer cell lines and for their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells, a microglial cell line.

The rhizomes of *A. gramineus* were collected in the Jeju Island area and extracted with 80% aqueous MeOH to give a MeOH extract that showed cytotoxic activity against A549, SK-OV-3, and SK-MEL-2 cells in a sulforhodamine B (SRB) bioassay. Purification of the MeOH extract led to the isolation of 12 lignans (**1**–**12**). The known compounds were identified as ganschisandrin (**6**),[9] veraguensin (**7**),[10] 5-methoxygalbelgin (**8**),[10] (7*S*,8*S*)-4,7,9,9′-tetrahydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan (**9**),[11] (7*R*,7*R*)-4,7,9,9′-tetrahydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan (**10**),[11] (7*R*,8*R*)-polysphorin (**11**),[12] and (−)-(7*R*,8*R*)-virolin (**12**),[13] by comparison of their spectroscopic and physical data with reported values. Their absolute configurations were established on the basis of their CD data.

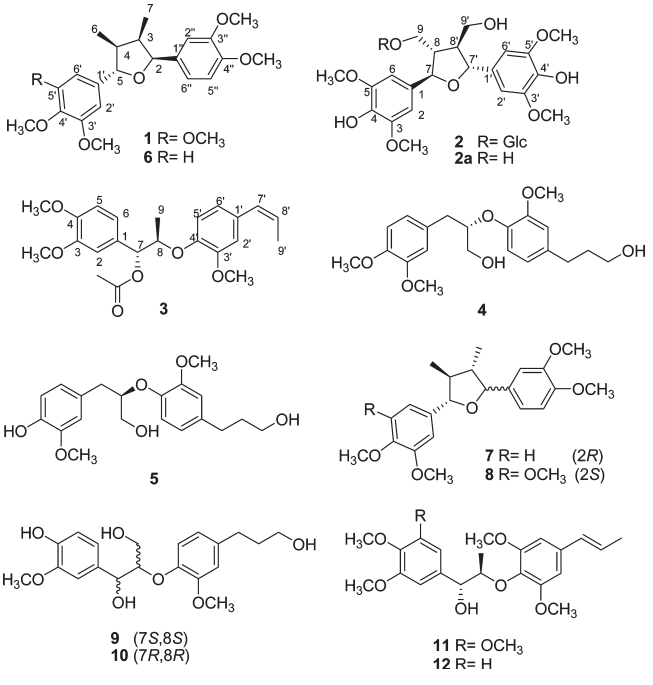


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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 HMz) Data of Ligraminols A (1) and B (2)

	1			2	
position	δ_C ($CDCl_3$)[a]	δ_H (J in Hz)	position	δ_C (CD_3OD)[a]	δ_H (J in Hz)
2	85.0	5.48, d (4.5)	1	133.0	
3	43.7	2.45, m	2	103.7	6.73, s
4	47.7	2.47, m	3	148.0	
5	86.1	4.65, d (9.0)	4	135.0	
6	12.2	1.05, d (6.5)	5	148.0	
7	9.6	0.63, d (7.0)	6	103.7	6.73, s
1′	139.1		7	83.4	5.03, d (8.0)
2′	103.2	6.62, s	8	53.4	2.46, m
3′	153.5		9	68.8	4.05, dd (10.0, 4.5)
4′	137.6				3.61, dd (10.0, 5.5)
5′	153.5		1′	133.0	
6′	103.2	6.62, s	2′	103.7	6.70, s
1″	133.3		3′	148.0	
2″	109.6	6.92, d (1.5)	4′	134.9	
3″	148.9		5′	148.0	
4″	148.0		6′	103.7	6.70, s
5″	111.1	6.86, d (8.0)	7′	83.2	4.95, d (8.5)
6″	118.2	6.87, dd (8.0, 1.5)	8′	50.7	2.32, m
3′-OMe	56.3	3.88, s	9′	61.5	3.82, dd (11.5, 4.5)
4′-OMe	61.0	3.84, s			3.62, dd (11.5, 6.0)
5′-OMe	56.3	3.88, s	3-OMe	55.6	3.82, s
3″-OMe	56.1	3.90, s	5-OMe	55.6	3.82, s
4″-OMe	56.1	3.91, s	3′-OMe	55.6	3.82, s
			5′-OMe	55.6	3.82, s
			Glc-1″	103.6	4.32, d (8.0)
			2″	73.9	3.17, m
			3″	76.8	3.25, m
			4″	70.4	3.25, m
			5″	77.0	3.33, m
			6″	60.0	3.77, br d (11.5)

[a] Assignments were based on HMQC and HMBC experiments.

Ligraminol A (**1**) was obtained as a colorless oil. The molecular formula was determined as $C_{23}H_{30}O_6$ from the molecular ion peak $[M + H]^+$ at m/z 403.2115 (calcd for $C_{23}H_{31}O_6$, 403.2121) in the positive-ion HR-ESIMS. The IR spectrum exhibited absorptions of phenyl (2946 and 1464 cm^{-1}) and ether (1270 cm^{-1}) groups. Inspection of the ^{1}H and ^{13}C NMR data (Table 1) revealed that the data of the 3,4-dimethyltetrahydrofuran part of the lignan skeleton of **1** were similar to those of **6** and the data of the phenolic moieties were similar to those of **8**.[9,10] The structure of **1** was supported by the cross-peaks in the $^{1}H-^{1}H$ COSY and HMBC spectra (Figure 1). The relative configuration of **1** was shown to be identical to **6**, having the *cis*-relation of H-5, the C-3 and C-4 methyl groups, and the C-2 aryl group by analysis of the NOESY experiment, showing correlations from H-7 to H-5, H-6, H-2″, and H-6″. The absolute configuration of **1** was established as 2*S*,3*R*,4*S*,5*S* by comparison of its CD curve with that of **6**.[9]

Ligraminol B (**2**) was obtained as a colorless gum. The molecular formula of **2** was established as $C_{28}H_{38}O_{14}$ by HR-ESIMS. Analysis of the ^{1}H and ^{13}C NMR data revealed similarity to those of 7*S*,7′*S*,8*R*,8′*R*-icariol A_2-9-*O*-*β*-D-glucopyranoside.[14] The structure of **2** was confirmed to be icariol A_2-9-*O*-*β*-D-glucopyranoside by 2D NMR data (HMQC, HMBC, and NOESY). However, the CD spectrum showed a positive (λ_{max} 244 nm) Cotton effect in discord with that of 7*S*,7′*S*,8*R*,8′*R*-icariol A_2-9-*O*-*β*-D-glucopyranoside,[14] indicating that the absolute configuration of **2** could be 7*R*,7′*R*,8*S*,8′*S*.[15,16] Enzymatic hydrolysis of **2** afforded the aglycone **2a** and D-glucose, which was identified by co-TLC confirmation and GC analysis.[17,18] The aglycone **2a** was identified as (+)-icariol A_2 by comparison of its ^{1}H NMR, the specific rotation of **2a** ($[\alpha]_D^{25}$ +23.5), and MS data.[14,15] The absolute configuration of **2a** was elucidated as 7*R*,7′*R*,8*S*,8′*S* by means of the CD data of **2a** showing a positive Cotton effect at 244 nm.[14]

Ligraminol C (**3**) was obtained as a colorless oil. The HR-ESIMS displayed a molecular ion peak $[M + Na]^+$ at m/z 423.1778 (calcd for $C_{23}H_{28}NaO_6$, 423.1784), consistent with a molecular formula of $C_{23}H_{28}O_6$. The ^{1}H and ^{13}C NMR data (Table 2) were similar to those of **12**, except for the presence of signals of an acetyl group [δ_H 2.01 (3H, s); δ_C 170.2 and 21.3].[13] This acetyl group was confirmed to be located at C-7 from the HMBC correlation between H-7 (δ_H 5.91) and the carbonyl carbon (δ_C 170.2) (Figure 1). In the ^{1}H NMR spectrum, signals of olefinic protons at δ_H 6.37 (H-7′) and 5.74 (H-8′) and the

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coupling constant (J = 11.5 Hz) indicated that **3** possessed a *Z*-olefinic functionality,[19] in contrast to reports of *E*-olefinic groups.[13] This structure was unambiguously confirmed by analysis of the 2D NMR data (HMQC, HMBC, and NOESY). The absolute configuration of **3** was determined as 7*R*,8*R* by the CD data showing a negative Cotton effect at 251 nm.

Ligraminol D (**4**) was obtained as a colorless oil with a molecular formula of $C_{21}H_{28}O_6$ based on the molecular ion peak $[M + Na]^+$ at m/z 399.1778 (calcd for $C_{21}H_{28}NaO_6$, 399.1784) by HR-ESIMS. The ^{1}H and ^{13}C NMR data (Table 2) were similar to those of (8*S*)-3-methoxy-8,4′-oxyneoligna-3′,4,9,9′-tetraol, with an apparent difference being the presence of signals for two methoxy groups [δ_H 3.87 (6H, s); δ_C 56.1 and 56.0].[20] The positions of these methoxy groups were determined as C-4 and C-3′ by the HMBC correlations from 4-OMe (δ_H 3.87) to C-4 (δ_C 149.1) and from 3′-OMe (δ_H 3.87) to C-3′ (δ_C 151.3) (Figure 1). The absolute configuration of **4** was elucidated as 8*S* by comparison of its CD data with that of (8*S*)-3-methoxy-8,4′-oxyneoligna-3′,4,9,9′-tetraol.[20]

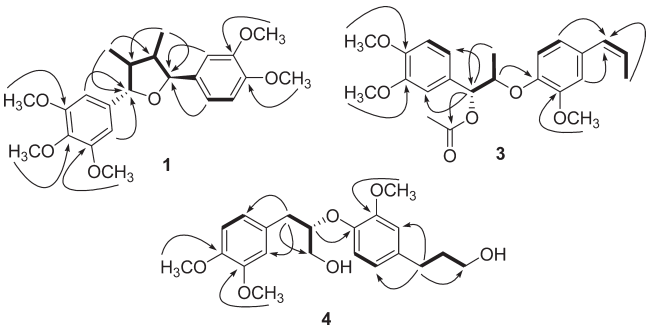

Figure 1. ^{1}H−^{1}H COSY (bold lines) correlations and key HMBC (arrows) for **1**, **3**, and **4**.

Ligraminol E (**5**), a colorless oil, exhibited a molecular formula of $C_{20}H_{26}O_6$ by the molecular ion peak $[M + Na]^+$ at m/z 385.1622 (calcd for $C_{20}H_{26}NaO_6$, 385.1627) by HR-ESIMS. The ^{1}H and ^{13}C NMR data (Table 2) were similar to those of **4**, except for the absence of signals for one methoxy group. The location of the two methoxy groups in **5** was confirmed by the HMBC experiment, showing correlations between 3-OMe (δ_H 3.78) and C-3 (δ_C 147.5) and between 3′-OMe (δ_H 3.81) and C-3′ (δ_C 150.6). Other noticeable differences between **4** and **5** were the chemical shift and splitting pattern of H-7 [δ_H 2.88 (2H, dd, J = 6.5, 2.0 Hz)] in **5** compared to those of H-7 in **4**, suggesting that they possess different C-7 configurations. The absolute configuration of **5** was determined as 8*R* by the CD data showing a negative Cotton effect at 231 nm.[20] A literature survey revealed that an enantiomer of **5** was isolated from *Picea jezoensis* without verifying its absolute configuration,[21] which may be 8*S* because of its similar chemical shift and splitting pattern of H-7 [δ_H 3.10 (1H, dd, J = 14.0, 6.0 Hz) and 2.88 (1H, dd, J = 14.0, 6.0 Hz)] to those of **4**, though other data such as ^{13}C NMR, specific rotation, and CD were not reported.

Table 2. ^{1}H (500 MHz) and ^{13}C NMR (125 HMz) Data of Ligraminols C (3), D (4), and E (5)

	3		4		5	
position	δ_C ($CDCl_3$)[a]	δ_H (J in Hz)	δ_C ($CDCl_3$)[a]	δ_H (J in Hz)	δ_C (CD_3OD)[a]	δ_H (J in Hz)
1	129.7		130.7		129.7	
2	111.0	6.93, d (1.5)	111.5	6.82, br s	113.1	6.81, d (1.5)
3	149.3		147.8		147.5	
4	149.1		149.1		144.8	
5	113.5	6.86, d (8.0)	113.0	6.82, br s	114.8	6.68, d (8.0)
6	120.3	6.95, dd (8.0, 1.5)	121.7	6.82, br s	121.8	6.68, dd (8.0, 1.5)
7	78.4	5.91, d (7.0)	37.6	3.09, dd (14.0, 6.5)	36.5	2.88, dd (6.5, 2.0)
				2.91, dd (14.0, 7.0)		
8	78.3	4.58, m	85.6	4.21, m	82.4	4.34, m
9	17.0	1.19, d (6.5)	63.7	3.70, dd (12.0, 3.0)	62.6	3.64, dd (11.5, 3.5)
				3.62, dd (12.0, 6.0)		3.62, dd (11.5, 6.5)
1′	132.1		137.5		136.5	
2′	111.1	6.87, d (1.5)	112.6	6.75, br s	112.9	6.81, d (1.5)
3′	150.3		151.3		150.6	
4′	146.8		145.8		145.7	
5′	116.9	6.94, d (8.0)	120.4	6.68, br s	117.6	6.82, d (8.0)
6′	121.7	6.86, dd (8.0, 1.5)	121.2	6.68, br s	120.6	6.70, dd (8.0, 1.5)
7′	130.1	6.37, dd (11.5, 1.5)	32.0	2.66, t (7.5)	31.4	2.61, t (7.5)
8′	125.9	5.74, dd (11.5, 7.5)	34.4	1.87, m	34.3	1.81, m
9′	14.8	1.92, dd (7.5, 1.5)	62.4	3.69, t (6.5)	61.0	3.58, t (6.5)
3-OMe	56.1	3.84, s	56.1	3.86, s	55.1	3.78, s
4-OMe	56.2	3.87, s	56.0	3.87, s		
3′-OMe	56.1	3.89, s	56.1	3.87, s	55.3	3.81, s
OAc	170.2					
	21.3	2.01, s				

[a] Assignments were based on HMQC and HMBC experiments.

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Table 3. Cytotoxicity of Compounds 1, 3, and 4 against a Human Normal and Three Human Cancer Cell Lines Using the SRB Bioassay

	IC_{50} (μM)[a]			
compound	A549	SK-OV-3	SK-MEL-2	HUVEC
1	6.92	9.44	4.53	27.19
3	9.54	>10	>10	25.93
4	8.28	>10	>10	22.56
etoposide[b]	0.44	1.31	0.41	1.86
cisplatin[c]	1.67	1.31	1.08	0.94

[a] IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*. [b] Etoposide as a positive control. [c] Cisplatin as a reference compound.

Compounds **1**−**12** were evaluated for their antiproliferative activities against three human cancer cell lines including A549, SK-OV-3, and SK-MEL-2 using the SRB bioassay.[22] Compounds **1**, **3**, and **4** showed weak inhibitory activity against the proliferation of the tested cell lines with IC_{50} values in the range 4.53−9.54 μM (Table 3). In particular, compound **1** exhibited weak cytotoxicity against all tested cell lines, namely, A549, SK-OV-3, and SK-MEL-2 cells, with IC_{50} values of 6.92, 9.44, and 4.53 μM, respectively. All other compounds were inactive (IC_{50} >10 μM) for all cell lines. To establish whether the cytotoxicity of **1**, **3**, and **4** was selective between tumor and normal cells, these compounds were tested for a normal human cell line, HUVEC. The results (Table 3) showed that the cytotoxicity of **1**, **3**, and **4** was higher against tumor cells than normal cells. Compound **1** showed the highest selective cytotoxicity against the SK-MEL-2 cell line because it exhibited a selectivity index (SI) value of 6.0, greater than that of cisplatin, a well-known anticancer agent (SI 0.9). The SI value was obtained by dividing the IC_{50} value for the normal cell line (HUVEC) by the IC_{50} value for the tumor cell line (SK-MEL-2).[23]

Microglia have been proposed to play a role in homeostasis regulation and defense against injury.[24] However, activated microglia by various stimuli such as LPS, interferon-γ, and pathogens can produce and release a large variety of proinflammatory factors including nitric oxide, tumor necrosis factor α (TNF-α), and prostaglandin E_2 (PGE_2).[25,26] NO is involved in a number of physiological processes such as immune and inflammatory responses and neuronal transmission in the brain.[27] However, the overproduction of NO from activated microglia induces neuronal cell death via the formation of an extremely potent oxidizing and neurotoxic agent, peroxynitrite ($ONOO^-$).[28] Therefore, agents for inhibition of NO production induced by activated microglia may be potentially used as neuroprotection regulators. An investigation of components that exhibit neuroprotective effects from *A. gramineus* has already been reported.[8] Thus, we also investigated the neuroprotective activities of compounds (**1**−**12**) through the measurement of nitrite, a soluble oxidation product of NO, in the culture medium using the Griess reaction in LPS-activated BV-2 cells. As shown in Table 4, compounds **1**−**4** moderately inhibited NO production with IC_{50} values of 21.51, 22.96, 16.17, and 18.41 μM, respectively. Compounds **6**, **7**, **9**, and **10** also decreased NO levels in the medium with IC_{50} values in the range 48.38−75.97 μM. Some cell toxicity was observed in cells treated with compounds **1**−**3** and **8**, whereas other compounds had no influence on cell viability. The abnormal production of proinflammatory molecules by overactivated microglial cells can confuse activity of nerve terminals and cause dysfunction of synapses involved with cognitive and memory deficits.[29] Therefore, we suggest that the compounds **1**−**4** might be active components of *A. gramineus*, which has been used for improvement of cognitive problems.

Table 4. Inhibitory Effect on NO Production of Compounds 1−12 in LPS-Activated BV-2 Cells

compound	IC_{50} (μM)[a]	cell viability[b]	compound	IC_{50} (μM)[a]	cell viability[b]
1	21.51	88.6 ± 4.7*	**8**	125.34	87.3 ± 8.2*
2	22.96	84.4 ± 5.5*	**9**	53.03	96.7 ± 1.3
3	16.17	90.5 ± 4.1*	**10**	52.00	97.5 ± 2.3
4	18.41	98.4 ± 2.8	**11**	>200	101.1 ± 3.4
5	>200	97.1 ± 1.4	**12**	176.06	102.2 ± 1.4
6	75.97	103.5 ± 3.9	NMMA[c]	15.41	98.7 ± 3.6
7	48.38	103.6 ± 6.4			

[a] 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] Cell viability was expressed as a percentage (%) of the LPS-only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD (*: p-value <0.05) [c] NMMA as a positive control.

In this study, compounds **1**, **3**, and **4** showed not only selective cytotoxicity among tumor and normal cells but also anti-neuroinflammatory activity. Therefore, our results may demonstrate that new lignans ligraminols A, C, and D suppress the survival of cancer cells and the neuroinflammation of activated microglia.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. CD spectra were measured on a JASCO J-810 spectropolarimeter. UV spectra were recorded using an Agilent 8453 UV−visible spectrophotometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively. ESIMS and HR-ESIMS spectra were recorded on a Micromass QTOF2-MS. Preparative HPLC was performed using 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. TLC was performed using Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). Low-pressure liquid chromatography was performed over a Merck Lichroprep Lobar-A (240 × 10 mm) column with an FMI QSY-0 pump (ISCO).

Plant Material. The rhizomes of *A. gramineus* were collected on Jeju Island, Korea, in March 2009, and the plant was identified by one of the authors (K.R.L.). A voucher specimen (SKKU-NPL-0910) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. The rhizomes of *A. gramineus* (15 kg) were extracted at room temperature with 80% aqueous MeOH and filtered. The filtrate was evaporated under vacuum to obtain a MeOH extract (825 g), which was suspended in distilled H_2O (2 L) and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 166, 14, 5, and 47 g of residues, respectively. The *n*-hexane-soluble

fraction (62 g) was separated over a silica gel column with *n*-hexane−EtOAc (11:1) to yield eight fractions (H1−H8). Fraction H6 (606 mg) was separated on a Sephadex LH-20 column (CH_2Cl_2−MeOH, 1:1) and separated further on a LiChroprep Lobar-A RP-18 column (40% MeOH(aq)) to give seven subfractions (H61−H67). Subfraction H64 (135 mg) was separated on a Lichroprep Lobar-A Si gel 60 column (*n*-hexane−EtOAc, 4:1) to give three subfractions (H641−H643). Compounds **1** (7 mg), **6** (6 mg), **7** (7 mg), and **8** (4 mg) were obtained from subfraction H642 (56 mg) by separation with preparative HPLC (*n*-hexane−EtOAc, 3:1). Subfraction H65 (30 mg) was subjected to passage over a Waters Sep-Pak Vac 6 cm^3 (*n*-hexane−EtOAc, 6:1) column and purified by preparative HPLC (*n*-hexane−EtOAc, 2:1) to yield compound **3** (3 mg). Fraction H7 (495 mg) was separated over an RP-C_{18} silica gel column (50% MeOH(aq)) and subjected to passage over a Waters Sep-Pak Vac 6 cm^3 (*n*-hexane−$CHCl_3$−EtOAc, 6:8:1) column to give two subfractions (H71 and H72). Subfraction 71 (36 mg) and subfraction 72 (52 mg) were purified by preparative HPLC (70% MeOH(aq)) to give compounds **11** (5 mg) and **12** (8 mg), respectively. The $CHCl_3$-soluble fraction (14 g) was separated over an RP-C_{18} silica gel column (50% MeOH(aq)) to afford seven fractions (C1−C7). Fraction C1 (800 mg) was separated on a silica gel column with $CHCl_3$−MeOH (40:1) to give five subfractions (C11−C15). Subfraction C14 (114 mg) was separated on a Sephadex LH-20 column (CH_2Cl_2−MeOH, 1:1) and purified by preparative HPLC (55% MeOH(aq)) to obtain compounds **9** (28 mg) and **10** (10 mg). Fraction C3 (544 mg) was separated on a silica gel column with *n*-hexane−EtOAc−MeOH (10:1:1) to give five subfractions (C31−C35). Subfraction C33 (161 mg) was separated on a Lichroprep Lobar-A Si gel 60 column (*n*-hexane−$CHCl_3$−MeOH, 10:5:1) and purified by preparative HPLC (55% MeOH(aq)) to yield compound **5** (5 mg). Fraction C4 (675 mg) was separated on a silica gel column with *n*-hexane−$CHCl_3$−MeOH (20:10:1) to give seven subfractions (C41−C47). Subfraction C45 (40 mg) was separated on a Sephadex LH-20 column (70% MeOH(aq)) and purified by preparative HPLC ($CHCl_3$−MeOH, 50:1) to give compound **4** (5 mg). The *n*-BuOH-soluble fraction (47 g) was separated over a silica gel column with $CHCl_3$−MeOH (5:1) to afford four fractions (B1−B4). Fraction B2 (3.8 g) was separated on an RP-C_{18} silica gel column (25% MeOH(aq)) to give five subfractions (B21−B25). Compound **2** (15 mg) was obtained from subfraction B24 (60 mg) by separation with preparative HPLC ($CHCl_3$−MeOH, 9:1).

Ligraminol A (1): colorless oil; $[\alpha]_D^{25}$ +17.3 (*c* 0.15, MeOH); IR (KBr) ν_{max} 2946, 1590, 1464, 1270, 1130, 699 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 300 (+1.7), 282 (−1.0), 258 (+1.3), 240 (−9.1) nm; UV (MeOH) λ_{max} (log ε) 277 (1.7), 238 (2.5), 217 (4.5) nm; ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; positive HR-ESIMS *m*/*z* 403.2115 $[M + H]^+$ (calcd for $C_{23}H_{31}O_6$, 403.2121).

Ligraminol B (2): colorless gum; $[\alpha]_D^{25}$ +2.4 (*c* 0.35, MeOH); IR (KBr) ν_{max} 3387, 2906, 1645, 1594, 1504, 1427, 1235, 1130 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 244 (+7.7) nm; UV (MeOH) λ_{max} (log ε) 273 (1.5), 241 (2.7), 218 (4.3) nm; ^1H (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; positive HR-ESIMS *m*/*z* 621.2154 $[M + Na]^+$ (calcd for $C_{28}H_{38}NaO_{14}$, 621.2159).

Ligraminol C (3): colorless oil; $[\alpha]_D^{25}$ −20.2 (*c* 0.05, MeOH); IR (KBr) ν_{max} 2945, 1702, 1574, 1451, 1275, 1131, 700 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 298 (−1.2), 251 (−5.3) nm; UV (MeOH) λ_{max} (log ε) 287 (1.2), 257 (1.7), 233 (2.8), 204 (4.5) nm; ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 2; positive HR-ESIMS *m*/*z* 423.1778 $[M + Na]^+$ (calcd for $C_{23}H_{28}NaO_6$, 423.1784).

Ligraminol D (4): colorless oil; $[\alpha]_D^{25}$ +9.5 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3392, 2945, 1601, 1510, 1275, 1032 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 236 (+2.1) nm; UV (MeOH) λ_{max} (log ε) 280 (1.7), 232 (2.5), 206 (3.6) nm; ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 2; positive HR-ESIMS *m*/*z* 399.1778 $[M + Na]^+$ (calcd for $C_{21}H_{28}NaO_6$, 399.1784).

Ligraminol E (5): colorless oil; $[\alpha]_D^{25}$ +18.3 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3395, 2940, 1600, 1507, 1274, 1030 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 231 (−1.7) nm; UV (MeOH) λ_{max} (log ε) 280 (1.8), 236 (2.2), 210 (3.6) nm; ^1H (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 2; positive HR-ESIMS *m*/*z* 385.1622 $[M + Na]^+$ (calcd for $C_{20}H_{26}NaO_6$, 385.1627).

Enzymatic Hydrolysis of 2. Compound **2** (3.6 mg) was treated with cellulase (from *Aspergillus niger*, Nagase Biochemical Co., 6.5 mg) in a HOAc−NaOAc buffer solution (0.02 mol/L, pH 4.6, 4.5 mL). The mixture was stirred at 37 °C for 3 days and extracted with an equal amount of EtOAc (×3), and the EtOAc layer evaporated under reduced pressure. The residue was dried and purified by a silica gel Waters Sep-Pak Vac 6 cm^3 ($CHCl_3$−MeOH, 25:1) to give the aglycone **2a** (1.7 mg). The sugar fraction, obtained by concentration of the H_2O layer, was analyzed by silica gel co-TLC with an authentic sample (solvent system $CHCl_3$−MeOH−H_2O, 8:5:1; R_f of glucose, 0.30).[17,18] The absolute configuration of the glucose was determined by a GC experiment according to reported procedures.[17,18]

***7R,7′R,8S,8′S*-Icariol A_2 (2a):** amorphous powder; $[\alpha]_D^{25}$ +23.5 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3385, 2901, 1644, 1594, 1502, 1427, 1235, 1130 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 244 (+6.5) nm; UV (MeOH) λ_{max} (log ε) 273 (1.4), 243 (2.5), 215 (4.3) nm; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.73 (4H, s, H-2, H-6, H-2′, H-6′), 4.95 (2H, d, *J* = 8.5 Hz, H-7, H-7′), 3.86 (12H, s, OCH_3), 3.72 (2H, dd, *J* = 11.5, 3.5 Hz, H-9a, H-9′a), 3.62 (2H, dd, *J* = 11.5, 5.5 Hz, H-9b, H-9′b), 2.32 (2H, m, H-8, H-8′); positive ESIMS *m*/*z* 459 $[M + Na]^+$.

Cytotoxicity Testing. The cell lines used were A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HUVEC (human umbilical cord endothelial cells). The cancer cell lines A549, SK-OV-3, and SK-MEL-2 were provided by the National Cancer Institute (NCI). The normal cell line HUVEC was purchased from American Type Culture Collection. A sulforhodamine B bioassay was used to determine the cytotoxicity of each compound against the cell lines mentioned above.[22] The assays were performed at the Korea Research Institute of Chemical Technology. Etoposide was used as a positive control.

Measurement of NO Production and Cell Viability. Murine microglia BV-2 cells were plated into a 96-well plate (3×10^4 cells/well.) After 24 h, cells were pretreated with samples for 30 min and then stimulated with 100 ng/mL of LPS for another 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, 0.1% *N*-1-napthylethylenediamine 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 a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. N^G-Monomethyl-L-arginine (L-NMMA, Sigma, USA), a well-known NOS inhibitor, was tested as a positive control.[30]

■ ASSOCIATED CONTENT

Supporting Information. 1D, 2D NMR and HRESIMS data of **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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