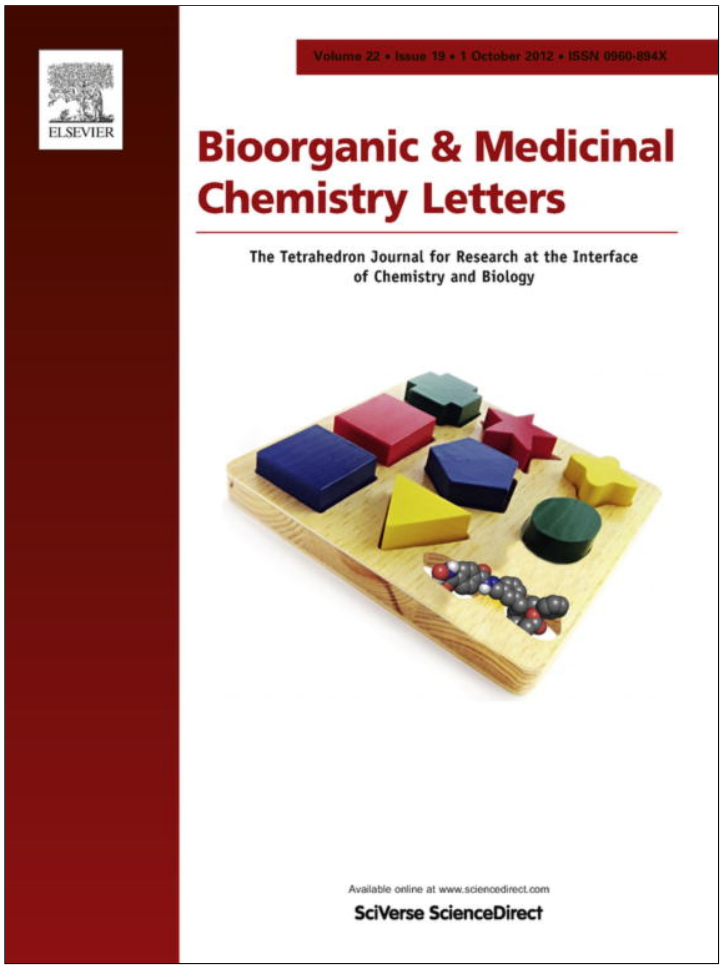

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Phenolic constituents from the rhizomes of *Acorus gramineus* and their biological evaluation on antitumor and anti-inflammatory activities

Ki Hyun Kim [a], Eunjung Moon [b], Ho Kyung Kim [a], Ju Yeoun Oh [a], Sun Yeou Kim [c], Sang Un Choi [d], Kang Ro Lee [a,*]

[a] *Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, 300 Chonchon-dong, Jangan-ku, Suwon, Gyeonggi-do 440-746, Republic of Korea*
[b] *Graduate School of East-West Medical Science, Kyung Hee University Global Campus, #1732 Deogyeong-daero, Giheung-gu, Yongin, Gyeonggi-do 446-701, Republic of Korea*
[c] *College of Pharmacy, Gachon University, #191 Hambakmoero, Yeonsu-gu, Incheon 406-799, Republic of Korea*
[d] *Korea Research Institute of Chemical Technology, Teajeon 305-600, Republic of Korea*

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ABSTRACT

On the search for anti-cancer compounds from natural Korean medicinal sources, a bioassay-guided fractionation and chemical investigation of the MeOH extract from the rhizomes of *Acorus gramineus* resulted in the isolation and identification of thirteen phenolic derivatives (**1**–**13**) including two new 8-*O*-4′-neolignans, named surinamensinols A (**1**) and B (**2**) and a new phenolic compound, named acoramol (**9**). The structures of these new compounds were elucidated on the basis of 1D and 2D NMR spectroscopic data analyses as well as circular dichroism (CD) spectroscopy studies. The cytotoxic activities of the isolates (**1**–**13**) were evaluated by determining their inhibitory effects on human tumor cell lines. The new 8-*O*-4′-neolignans, compounds **1** and **2**, showed moderate antiproliferative activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values in the range of 4.17–26.18 μM. On the basis of the expanded understanding that inflammation is a crucial cause of tumor progression, anti-inflammatory activities of these compounds were determined by measuring nitric oxide (NO) levels in the medium using murine microglia BV-2 cells. Compounds **1**, **2**, **4**, **7** and **10** inhibited NO production in BV-2 stimulated by lipopolysaccharide with IC_{50} values of 8.17–18.73 μM via NO scavenging, inhibition of iNOS activity, and/or suppression of iNOS expression.

Acorus gramineus Soland (Araceae), which is an aquatic perennial herbaceous plant, is widely distributed in Korea, Japan, and China. The plant's rhizomes have been used as a traditional medicine in China for the treatment of various disorders including cognitive problems, sedation, stomach ache, and edema.[1–3] The major bioactive components with medicinal value in *A. gramineus* rhizomes are known as β-asarone, α-asarone, and phenylpropenes, which are associated with various pharmacological activities including antibacterial, antifungal, anthelminthic, and pesticidal activities.[4–7] Our preliminary studies confirmed that the MeOH extract from the rhizomes of *A. gramineus* had excellent cytotoxic activity against A549, SK-OV-3, and SK-MEL-2 cells in a sulforhodamine B (SRB) bioassay. This result allowed us to investigate the bioactive compounds from *A. gramineus* rhizomes and our group already had reported the isolation of 12 bioactive lignans.[8]

Recent preliminary studies also confirmed that the MeOH extract from the rhizomes of *A. gramineus* have a significant inhibitory effect on NO production in the lipopolysaccharide (LPS)-stimulated BV-2 microglial cell line. Our interest in further research on bioactive constituents from this plant source led us to investigate bioactive metabolites of *A. gramineus* rhizomes. The rhizomes of *A. gramineus* collected in the Jeju island area were extracted with 80% MeOH. The MeOH extract was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. To identify the bioactive constituents responsible for the anti-cancer activity, each fraction was evaluated for its cytotoxicity against human tumor cell lines using the SRB assay.[9] The active fractions were the *n*-hexane-soluble and $CHCl_3$-soluble fractions, which also inhibited NO production in LPS-stimulated BV-2 microglial cells. The active fractions were separated using repeated silica gel column chromatography, followed by preparative HPLC to afford thirteen phenolic derivatives (**1**–**13**) including two new 8-*O*-4′-neolignans, named surinamensinols A (**1**) and B (**2**), and a new phenolic compound, named acoramol (**9**) (Fig. 1). On the search for anti-cancer compounds from natural Korean medicinal sources, herein we report the isolation and structural elucidation of isolates (**1**–**13**) and their antitumor and anti-inflammatory activities.

Surinamensinol A (**1**) was obtained as a colorless oil with negative optical rotation ($[\alpha]_D^{25}$ −62.1). The molecular formula of **1** was determined to be $C_{22}H_{30}O_7$ by HR-ESIMS data at *m*/*z* 429.1893

* Corresponding author. Tel.: +82 31 290 7710; fax: +82 31 290 7730.
E-mail address: krlee@skku.ac.kr (K.R. Lee).

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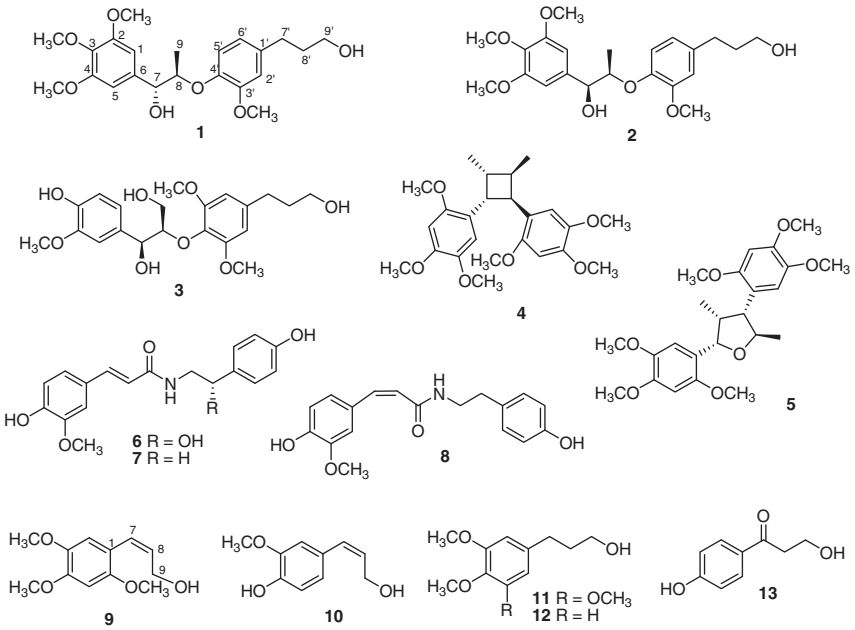

Figure 1. The structures of compounds **1**–**13**.

Table 1
^{1}H and ^{13}C NMR data of **1**–**2** in $CDCl_3$. (δ in ppm, 500 MHz for ^{1}H and 125 MHz for ^{13}C)[a]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1		135.8		135.9
2	6.61 s	104.6	6.59 s	103.5
3		153.3		153.4
4		138.0		137.4
5		153.3		153.4
6	6.61 s	104.6	6.59 s	103.5
7	4.60 d (8.5)	79.0	4.81 d (3.5)	74.0
8	4.08 dq (8.5, 6.0)	84.4	4.33 dq (6.5, 3.5)	82.5
9	1.20 d (6.0)	17.4	1.18 d (6.5)	13.8
1′		137.4		137.6
2′	6.78 d (2.0)	112.6	6.78 d (2.0)	112.6
3′		150.9		151.6
4′		144.9		146.0
5′	6.93 d (8.0)	119.5	6.94 d (8.0)	120.2
6′	6.75 dd (8.0, 2.0)	120.9	6.77 dd (8.0, 2.0)	121.1
7′	2.68 t (7.5)	32.0	2.68 t (7.5)	32.0
8′	1.90 m	34.4	1.90 m	34.4
9′	3.70 t (6.5)	62.4	3.70 t (6.5)	62.4
3,5-OCH_3	3.86 s	56.3	3.86 s	56.3
4-OCH_3	3.83 s	61.0	3.83 s	61.0
3′-OCH_3	3.88 s	56.0	3.87 s	56.0

[a] J values are in parentheses and reported in Hz; the assignments were based on ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

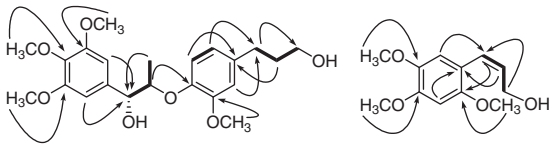

Figure 2. Key ^{1}H–^{1}H COSY (—) and HMBC (→) correlations of **1** and **9**.

$[M+Na]^+$ (calcd for $C_{22}H_{30}NaO_7$, 429.1889). The IR spectrum of **1** showed the presence of hydroxyl (3390 cm^{-1}) and aromatic functions (1601 and 1512 cm^{-1}). In the UV spectrum of **1**, absorption maxima were observed at 231 and 282 nm. The ^{13}C NMR spectrum (Table 1) of **1** showed 18 carbon signals except for 4 methoxy signals which were classified by the HMQC experiment as two aromatic rings, a methyl, two methylenes, an oxygenated methylene, and two oxygenated methines, suggesting **1** to be a neolignan.[10] Overall, the proton and carbon signals in the ^{1}H and ^{13}C NMR data of **1** were similar to those of surinamensin,[11] except that the proton and carbon resonances of a double bond and a methyl group in the side chain of surinamensin were absent, and instead, the resonances of two methylene groups at δ_H 2.68 (2H, t, J = 7.5 Hz); δ_C 32.0 and 1.90 (2H, m); δ_C 34.4 and an oxygenated methylene group at δ_H 3.70 (2H, t, J = 6.5 Hz); δ_C 62.4 were present in **1**. This functional group was confirmed by the ^{1}H–^{1}H COSY and HMBC correlations (Fig. 2). The gross planar structure of **1** was established on the basis of the above consideration and analysis of 2D NMR experiments (^{1}H–^{1}H COSY, HMQC, and HMBC) (Fig. 2). The absolute configuration of **1** was clarified by the CD spectroscopic study in combination with the physical and NMR spectral characteristics of **1**. The absolute configuration of C-8 was determined as 8*R* by the CD data which showed a negative Cotton effect at 253 nm causing by an exciton coupling interaction between the two aryl moieties (positive Cotton effects in the range of 230–290 nm for the 8*S* configuration of the 8-*O*-4′-neolignans regardless of the substituent at C-7).[8,10] The negative optical rotation value ($[\alpha]_D^{25}$ −62.1) and the relatively large coupling constant (J = 8.5 Hz) between H-7 and H-8 of **1** were in good agreement with the values for the related (7*R*,8*R*)-enantiomer.[8,10] Thus, the structure of **1** was elucidated as shown in Figure. 1, and named surinamensinol A.

Surinamensinol B (**2**), obtained as a colorless oil, has the molecular formula $C_{22}H_{30}O_7$, as determined by the HR-ESIMS data at m/z 429.1890 $[M+Na]^+$ (calcd for $C_{22}H_{30}NaO_7$, 429.1889). This compound showed UV maxima at 281 and 232 nm and similar IR bands to **1**. The ^{1}H and ^{13}C NMR spectra of **2** were almost identical to those of **1**, except for the proton and carbon resonances attributable to C-7 and C-8 at δ_H 4.81 (1H, d, J = 3.5 Hz, H-7); δ_C 74.0 (C-7) and 4.33 (1H, dq, J = 6.5, 3.5 Hz, H-8); δ_C 82.5 (C-8) in **2** [δ_H 4.60 (1H, d, J = 8.5 Hz, H-7); δ_C 79.0 (C-7) and 4.08 (1H, dq, J = 8.5, 6.0 Hz, H-8); δ_C 84.4 (C-8) in **1**]. These data suggested that compound **2** is a stereoisomer of **1**. Likewise, the gross planar structure of **2** was

confirmed by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectroscopic data. In a similar manner as described for **1**, the absolute configuration of **2** was determined on the basis of the CD data showing a negative Cotton effect at 260 nm in combination with the optical rotation value ($[\alpha]_D^{25}$ −11.7) and the relatively small coupling constant ($J_{7,8}$ = 3.5 Hz) of **2**,[8,10] which proved the 7*S*,8*R*-configuration. Thus, the structure of **2** was established as shown in Figure 1, and named surinamensinol B.

The known compounds were identified as (1*R*,2*S*)-*rel*-1-(4′-hydroxy-3′-methoxyphenyl)-2-[4″-(3-hydroxypropyl)-2″,6″-dimethoxyphenoxy]-1,3-propanediol (**3**),[12] magnosalin (**4**),[13,14] magnosalicin (**5**),[15] (*S*)-*N*-*trans*-feruloyloctopamine (**6**),[16] *N*-*trans*-feruloyl tyramine (**7**),[17] and *N*-*cis*-feruloyl tyramine (**8**)[17] by comparison of their spectroscopic data with previously reported values. The relative configurations of the above known lignans (**3**–**5**) were established on the basis of their ^{1}H NMR coupling constant and optical rotation values. To the best of our knowledge, this is the first time that the above known compounds **3** and **5**–**8** were isolated from this genus *Acorus*. Compound **4** was discovered from this genus but a different species.

Acoramol (**9**) was isolated as a colorless oil. The molecular formula of **9** was determined to be $C_{12}H_{16}O_4$ by positive mode HR-ESIMS data at *m*/*z* 247.0943 $[M+Na]^+$ (calcd for $C_{12}H_{16}NaO_4$, 247.0946). Analysis of the ^{1}H and ^{13}C NMR data (Table 2) of **9** revealed that the part of the molecule comprising the aromatic ring was nearly identical to those of magnosalin (**4**) and magnosalicin (**5**),[12–15] and the side chain was similar to that of (*Z*)-coniferyl alcohol (**10**).[18] The locations of three methoxy groups at C-2, C-4, and C-5 were confirmed by the HMBC experiment illustrating the correlations between 2-OCH_3 (δ_H 3.85) and C-2 (δ_C 152.1), between 4-OCH_3 (δ_H 3.91) and C-4 (δ_C 148.7), and between 5-OCH_3 (δ_H 3.82) and C-5 (δ_C 142.4) (Fig. 2). The *Z*-olefinic functionality at C-7 and C-8 was unambiguously determined by the chemical shifts of olefinic protons at δ_H 6.64 (H-7) and 5.87 (H-8) and their coupling constant value (J = 11.5 Hz),[8,18,19] in contrast to the reports of *E*-olefinic groups.[10] Analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC correlations led to the establishment of the gross structure for **9** (Figure. 2). In conclusion, the structure of **9** was assigned as shown in Fig. 1, and named acoramol.

Other known compounds were identified as (*Z*)-coniferyl alcohol (**10**),[18] 3-(3,4,5-trimethoxyphenyl)propan-1-ol (**11**),[20] 3-(3,4-dimethoxyphenyl)propan-1-ol (**12**),[21] and 3-hydroxy-1-(4-hydroxyphenyl)-1-propanone (**13**),[22] by comparison of their spectroscopic data with literature values. To the best of our knowledge, above known compounds **10**–**13** were isolated from this genus *Acorus* for the first time.

In this study, we investigated whether compounds **1**–**13** had cytotoxic activities against various tumor cell lines and anti-inflammatory effects in stimulated microglia on the basis of the fact that the MeOH extract of *A. gramineus* rhizomes had significant cytotoxic and anti-inflammatory activities in our screening test. The cytotoxic activities of the isolates (**1**–**13**) were evaluated by determining their inhibitory effects on four human tumor cell lines, namely A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) using the SRB bioassay.[9] The results (Table 3) showed that the new 8-*O*-4′-neolignans, compounds **1** and **2**, showed moderate antiproliferative activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values ranging from 4.17 to 26.18 μM. In particular, they exhibited potent cytotoxicity against the A549 cell line with IC_{50} values of 4.17 and 5.41 μM. However, compound **3** showed little cytotoxicity against the tested cell lines despite its similar structure to **1** and **2**, suggesting that the presence of an OH group at C-9 and substitution pattern of aromatic ring in the 8-*O*-4′-neolignans may be a critical point in exerting the cytotoxic activity against the tested cell lines. Compound **4** had potent cytotoxicity against the SK-MEL-2 cell line with IC_{50} values of 8.70 μM. The compounds **6**–**8**, as a group of feruloyl tyramine derivatives, were inactive against the tested cell lines (IC_{50} >30.0 μM). Among the simple phenolic compounds (**9**–**13**), compounds **10** and **12** showed cytotoxicity against the four tested tumor cell lines with IC_{50} values ranging from 11.03 to 26.64 μM. Based on the obtained data of compounds **9**–**13**, the 3,4-disubstituted aromatic ring in simple phenolic compounds **10** and **12** seem to increase the cytotoxic activity against the tested cell lines, compared with the results of other compounds with different substitution patterns (**9** with 2,4,5-trisubstituted aromatic ring, **11** with 3,4,5-trisubstituted aromatic ring, and **13** with 4-monosubstituted aromatic ring).

Cancer and inflammation are related by epidemiology, histopathology, and inflammatory profiles.[23] On the basis of the expanded understanding that inflammation plays a crucial role in tumor progression, we also evaluated anti-inflammatory activities of the isolates (**1**–**13**) through the measurement of produced NO levels in the medium using murine microglia BV-2 cells. Compounds **1**, **2**, **4**, **7** and **10** significantly inhibited NO levels (IC_{50} values <20 μM) in LPS-stimulated BV-2 (Table 4). In particular, compound **2** exhibited the highest inhibitory activity with an IC_{50} of 8.17 μM. Among the active compounds, compounds **1** and **2** also had little cell toxicity at a concentration of 20 μM. We assume that reduction of BV-2 cell viability by treatment with compounds **1** and **2** may be associated with high cytotoxic

Table 2
^{1}H and ^{13}C NMR data of **9** in $CDCl_3$. (δ in ppm, 500 MHz for ^{1}H and 125 MHz for ^{13}C)[a]

Position	**9**	
	δ_H	δ_C
1		118.3
2		152.1
3	6.54 s	97.8
4		148.7
5		142.4
6	6.76 s	114.0
7	6.64 d (11.5)	129.9
8	5.87 dt (11.5, 6.5)	127.0
9	4.31 d (6.5)	60.2
2-OCH_3	3.85 s	56.8
4-OCH_3	3.91 s	56.8
5-OCH_3	3.82 s	56.3

[a] J values are in parentheses and reported in Hz; the assignments were based on ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

Table 3
Cytotoxic activities of compounds (**1**–**13**) isolated from *A. gramineus*

Compound	IC_{50}[a] (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	4.17	26.18	14.21	24.33
2	5.41	21.42	10.39	18.83
3	>30.0	>30.0	29.08	>30.0
4	19.64	27.38	8.70	23.05
5	>30.0	26.37	11.29	>30.0
6	>30.0	>30.0	>30.0	>30.0
7	>30.0	>30.0	>30.0	>30.0
8	>30.0	>30.0	>30.0	>30.0
9	>30.0	>30.0	25.23	>30.0
10	11.03	13.04	14.70	20.35
11	>30.0	>30.0	>30.0	>30.0
12	23.10	11.75	21.86	26.64
13	>30.0	>30.0	>30.0	>30.0
Doxorubicin[b]	0.001	0.004	0.004	0.082

[a] IC_{50} value of compounds against each cancer cell line. IC_{50} value was defined as concentration (μM) causing 50% inhibition of cell growth in vitro.
[b] Doxorubicin as a positive control.

Table 4
Inhibitory effect on NO production of compounds (**1**–**13**) isolated from *A. gramineus* in LPS-activated BV-2 cells

Compound	IC_{50} (μM)[a]	Cell viability[b] (%)	Compound	IC_{50}[a] (μM)	Cell viability[b] (%)
1	17.91	88.8 ± 4.0*	**8**	32.06	100.4 ± 5.1
2	8.17	76.2 ± 1.7*	**9**	126.45	104.7 ± 3.4
3	37.18	106.0 ± 3.8	**10**	14.08	98.4 ± 4.5
4	18.73	93.1 ± 5.0	**11**	141.74	96.3 ± 2.2
5	59.88	100.6 ± 6.0	**12**	>500	103.3 ± 3.7
6	54.79	98.3 ± 4.6	**13**	58.1	107.2 ± 3.7*
7	17.36	97.8 ± 2.8	NMMA[c]	18.25	98.8 ± 2.6

[a] IC_{50} value of each compound was defined as concentration (μM) causing 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability at 20 μM was expressed as 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.

activities of these compounds against various human tumor cells. In this study, compound **3** (IC_{50} value of 37.18 μM) was less active than compounds **1** and **2** in the inhibitory activity on NO production. Therefore, the presence of an OH group at C-9 and substitution pattern of aromatic ring in the 8-*O*-4′-neolignans may also play a critical part in mediating anti-inflammatory activity in LPS-stimulated BV-2 cells. To investigate the precise mechanisms of active compounds (**1**, **2**, **4**, **7**, and **10**) on NO regulation, we performed NO radical scavenging assay. As shown in Fig. 3A, compounds **2**, **4**, and **7** (10 and 20 μM) reduced the accumulation of nitrite upon decomposition of NO-donor, sodium nitroprusside (SNP). NO production in microglia is regulated primarily by the inducible nitric oxide synthase (iNOS) enzyme.[24] Therefore, to investigate the effects of isolated compounds on regulation of iNOS, we also performed iNOS activity assay, and evaluated the levels of iNOS protein by Western blot analysis. As shown in Fig. 3B, compounds **1**, **7** and **10** significantly reduced iNOS activity of BV-2 cells at 20 μM. Moreover, compounds **4**, **7** and **10** reduced iNOS pretein expression, significantly (Fig. 3C). Therefore, compounds **1**, **2**, **4**, **7** and **10** may exert their anti-inflammatory effects by regulation of NO via various mechanisms including NO scavenging, inhibition of iNOS activity, and suppression of iNOS expression. In this study, compound **1** reduced iNOS activity, and compound **2** had the NO scavenging activity. Compound **4** and **10** suppressed the expression of iNOS protein, significantly. However, compound **4** also had the effects on NO scavenging, while compound **10** also inhibited iNOS enzyme activity. Compound **7** exerted all the above-mentioned NO-related activities.

Our results suggest that the new 8-*O*-4′-neolignans, surinamensinols A (**1**) and B (**2**), isolated from the rhizomes of *A. gramineus* may be bioactive molecules which exhibit both antitumor and anti-inflammatory properties. These compounds should be further studied for their beneficial therapeutic potential against various cancers and inflammation-related diseases.

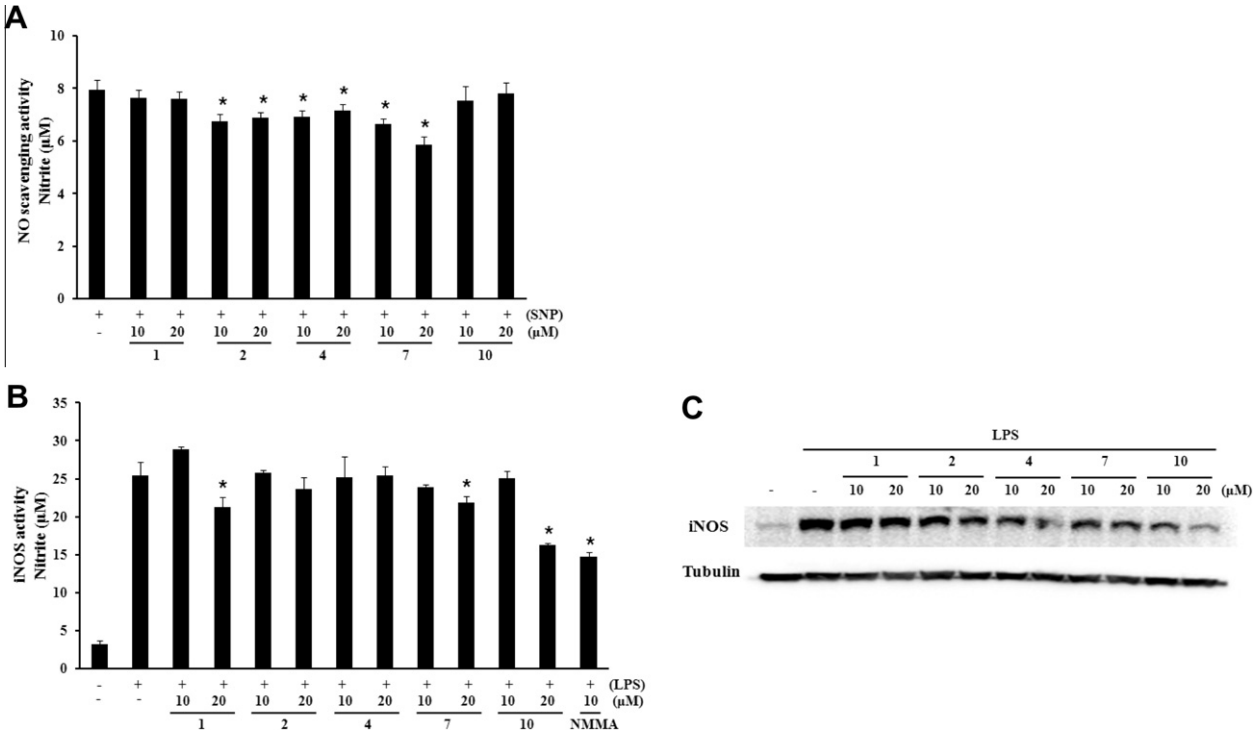

Figure 3. Effects on NO regulation of compounds (**1**, **2**, **4**, **7**, and **10**) isolated from *A. gramineus*. (A) Effect of compounds (**1**, **2**, **4**, **7**, and **10**) on NO scavenging. Sodium nitroprusside (SNP) was used as a NO-donor. (B) Effect of compounds on iNOS activity in BV-2 cells. After stimulation by LPS for 12 h, BV-2 cells were treated with compounds for 12 h. Then, the levels of produced NO were measured by Griess reaction. NMMA was used as a positive control. (C) Effect of compounds on LPS-induced iNOS expression in BV-2 cells. BV-2 cells were pretreated with compounds for 30 min and then stimulated with LPS for another 6 h. And protein levels of iNOS were evaluated by Western blotting analysis. All data are presented as the mean ± S.E.M of three independent experiments. *p <0.05 indicate statistically significant differences compared to treatment with SNP or LPS alone.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.08.016.

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