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Inducible Nitric Oxide Synthase Inhibitors from *Melia azedarach* var. *Japonica*

Hak Cheol Kwon, Byeong Gon Lee, Seung Hee Kim[1], Chil Mann Jung, Sung Youl Hong[2], Jeung Whan Han, Hyang Woo Lee, Ok Pyo Zee and Kang Ro Lee

College of Pharmacy, Sung Kyun Kwan University, Suwon 440-746, Korea, [1]National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-020, Korea, and [2]College of Life Science and Natural Resources, Sung Kyun Kwan University, Suwon 440-746, Korea

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In bioassay-guided search for inducible nitric oxide synthase (iNOS) inhibitory compounds from higher plants of South Korea, two β-carboline alkaloids, 4-methoxy-1-vinyl-β-carboline (**1**) and 4,8-dimethoxy-1-vinyl-β-carboline (**2**) have been isolated from the cortex of *Melia azedarach* var. *japonica*. The structures of these compounds were elucidated on the basis of spectroscopic data. Compounds **1** and **2** showed marked inhibitory activity of iNOS on LPS- and interferon-γ-stimulated RAW 264.7 cells.

Key words : *Melia azedarach* var. *japonica*, 4-Methoxy-1-vinyl--carboline, 4,8-Dimethoxy-1-vinyl-β-carboline, Inducible nitric oxide synthase

INTRODUCTION

The genus *Melia* (Meliaceae) comprises ca. 10 species and is distributed mainly over east Asia and Australia. In South Korea only 1 species is growing, and has been used in folk medicine for its antipyretic, anthelmintic and anti-inflammatory properties (Shanghai Science and Technologic Publisher et *al*. 1985). In previous phytochemical studies of the genus *Melia*, several cytotoxic limonoids were reported (Ahn, J. W. et *al*. 1994, Srivastava, S. D. *et al*. 1986, Takeya, K. *et al*. 1996a, Takeya, K. *et al*. 1996b). In our search for plant-derived inducible nitric oxide synthase (iNOS) inhibitory compounds from higher plants, we investigated *Melia azedarach* var. *japonica*. The MeOH extract of the cortex of this plant exhibited significant inhibitory activity of nitrite accumulation in LPS- and interferon-γ-stimulated RAW 264.7 cells, a murine macrophage cell line. And it was found that such activity of the MeOH extract was mainly concentrated in the EtOAc soluble fraction. Activity-guided column chromatography of the EtOAc fraction on the basis of the inhibitory activity of iNOS in RAW 264.7 cells, *in vitro*, afforded two active compounds. In this paper, we report the isolation and structural elucidation of two β-carboline alkaloids (**1**-**2**) as well as their iNOS inhibitory activity.

Correspondence to: Prof. Dr. Kang Ro Lee, Natural Product Laboratory, College of Pharmacy, Sung Kyun Kwan University, Chonchon-dong 300, Jangan-ku, Suwon 440-746, Korea
E-mail: krlee@yurim.skku.ac.kr

MATERIALS AND METHODS

General Experimental Procedures

Melting points were obtained on Gallenkamp melting point apparatus (uncorr.). IR spectra were recorded on a Shimadzu IR-435 spectrophotometer. UV spectra were recorded in MeOH on a Shimadzu UV 1601 UV-visible recording spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were determined on a Brucker AMX 500 or Varian UNITY INOVA-500 spectrophotometer in $CDCl_3$. MS were recorded on a VG70-VSEQ instrument. Analytical TLC was carried out on Merck aluminium plates precoated with Si gel 60 F_{254} and visualized by a UV lamp. Chromatography was performed on Merck Si gel 60 (230~400 mesh). LPLC was carried out on Duramat 80 equipped with Merck Lichroprep Si 60 (240×10 mm) or Merck Lichroprep RP-18 (24010 mm) columns.

Plant Material

Melia azedarach var. *japonica* was purchased from the Kyungdong herbal drug center, Seoul, Korea, in August 1998. A voucher specimen is deposited in the herbarium of College of Pharmacy, Sung Kyun Kwan University (SKKU-98-017).

Extraction and Isolation

The air-dried plant material (2 kg) was finely ground

and extracted with MeOH twice at 80°C for 3 h. The resultant MeOH extract (139 g) was suspended with water and followed by the solvent partitioning with EtOAc to give 61 g of EtOAc soluble fractions. The fraction (61 g) was applied over silica gel column chromatography using gradient solvent system of hexane-EtOAc (10:1→0:1) as eluents to give 22 subfractions. Guided by iNOS inhibition test by monitoring the accumulation of nitrite in culture medium using Griess assay, subfractions 12~15 were selected, concentrated and column chromatographed with silica gel eluting with chloroform-EtOAc (15:1), followed by hexane-CH_2Cl_2-EtOAc (1:1:1) to give five fractions (A~E). Fractions A and B were rechromatographed on LPLC (CH_2Cl_2-EtOAc, 8:1) to yield **1** (45 mg) and **2** (50 mg), respectively.

Compound (1): yellowish gum; mp 156°C; UV λ_{max}^{EtOH} nm: 231, 253, 270, 358; IR $\upsilon_{max}^{CHCl_3}$ cm^{-1}: 3450, 2900, 1620, 1590, 1500, 1250; EI-MS *m/z* (rel. int.): 254 (M^+, 100), 239 (30), 224 (35), 211 (20), 169 (11), 168 (9); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.01 (3H, s, 8-OCH_3), 4.14 (3H, s, 4-OCH_3), 5.59 (1H, dd, *J*=11.5, 1.0 Hz, *cis* H-B), 6.24 (1H, dd, *J*=17.5, 1.0 Hz, *trans* H-2'), 6.97 (1H, d, *J*=8.0 Hz, H-7), 7.16 (1H, dd, *J*=17.5, 11.5 Hz, H-1'), 7.23 (1H, t, *J*=8.0 Hz, H-6), 7.90 (1H, d, *J*=8.0 Hz, H-5), 8.07 (1H, s, H-3), 8.76 (1H, br.s, NH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 56.23 (8-OCH_3), 56.83 (4-OCH_3), 107.91 (C-7), 116.96 (C-5), 117.68 (C-2'), 119.78 (C-11), 121.65 (C-6), 122.02 (C-3), 122.75 (C-12), 130.64 (C-13), 133.46 (C-1'), 134.83 (C-1), 134.93 (C-10), 146.45 (C-8), 152.04 (C-4).

Compound (2): yellowish gum; mp 145; UV λ_{max}^{EtOH} nm: 225, 244, 267, 358; IR $\upsilon_{max}^{CHCl_3}$ cm^{-1}: 3400, 2900, 1630, 1580, 1500, 1270; EI-MS *m/z* (rel. int.): 224 (M^+, 100), 209 (28), 181 (50), 154 (28), 127 (16); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.17 (3H, s, 4-OCH_3), 5.59 (1H, dd, *J*=11.5, 1.0 Hz, *cis* H-2'), 6.22 (1H, dd, *J*=17.5, 1.0 Hz, *trans* H-2'), 7.16 (1H, dd, *J*=17.5, 11.5 Hz, H-1'), 7.32 (1H, td, *J*=8.0, 1.0 Hz, H-6), 7.50 (1H, td, *J*=8.0, 1.0 Hz, H-7), 7.51 (1H, dd, *J*=8.0, 1.0 Hz, H-8), 8.11 (1H, s, H-3), 8.33 (1H, dd, *J*=8.0, 1.0 Hz, H-5), 8.73 (1H, br.s, NH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 56.85 (4-OCH_3), 111.73 (C-8), 117.54 (C-2'), 119.37 (C-11), 121.25 (C-7), 121.89 (C-12), 122.39 (C-3), 124.87 (C-5), 128.22 (C-6), 133.72 (C-1'), 134.58 (C-1), 135.29 (C-10), 140.11 (C-13), 152.04 (C-4).

iNOS inhibition activity *in vitro*

RAW 264.7 cells were maintained in high glucose (4,500 mg/L)-DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). RAW 264.7 cells were washed twice with phosphate-buffered saline and incubated in fresh DMEM without phenol red, and stimulated with LPS (1 μg/ml) and interferon-γ (10 U/ml) with or without test compounds for the indicated periods. NO release was determined spectrophotometrically by accumulation of nitrite, a stable end-product of NO, in the media of cultured cells. Nitrite was determined with Griess reaction (Green *et al.*, 1982) by mixing of 100 μl of culture supernatant with 100 μl of Griess reagent, equal volume of 1% (w/v) sulfanylamide in 5% (w/v) phosphoric acid and 0.1% (w/v) of N-(1-naphthyl)ethylene-diamine solution. The absorbance was measured in a microplate reader (Bio-Tek Elx 800, Bio-Tek Instrument Inc. Winooski, VT. USA) at 540 nm against a calibration curve with sodium nitrite standards. For the iNOS assay, RAW 264.7 cells were plated into 100 mm-culture dish (1.0×10^7 cells/dish) and stimulated with LPS (1 μg/ml) and interferon-γ (10 U/ml) with or without test compounds for 16~18 hrs. The cell-free extracts were prepared using the method of Hayashi *et al* (1997). The cell pellets were resuspended with 300 μl of 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA, aprotinin, leupeptin, pepstatin each at 10 μg/ml, as well as 1 mM PMSF. Cells were lysed by sonication for 3 seconds and centrifuged at 20,000×g for 30 min at 4°C. The supernatants were used as cell-free extracts and the protein content was determined by Bradford protein assay using bovine serum albumin as a standard. Specific activity of iNOS was determined in cell-free extracts by monitoring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline, using a modified procedure based on the method of Vodobotz *et al* (1993). Briefly, the reaction mixture (total 200 μl) containing 50 mM Tris buffer (pH 7.4), 1 mM NADPH, 20 μM tetrahydrobiopterin, 5 μM FAD, 5 μM FMN, 1 mM DTT, 25 μM L-[^{3}H] arginine (200,000 dpm), and 20~40 μg of cell lysate protein was incubated for 10 min at 37°C. The reaction was stopped by the addition of 1 ml of 20 mM sodium acetate buffer (pH 5.5) containing 1 mM citrulline, 2 mM EDTA, and 2 mM EGTA. The mixture was loaded onto a 1 ml of DOWEX 50W-X8 cation exchange column, and the quantity of labelled [^{3}H] citrulline in the collected eluate was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Repeated colum chromatography of the MeOH extract guided by iNOS activity yielded two alkaloids. Compounds **1** and **2** have very similar pattern in their UV, IR and NMR spectra. The compounds were positive (orange) in Dragendorff test.

Compound **1** was isolated as a yellowish gum. Its molecular formula was established as $C_{15}H_{14}N_2O_2$ by EI-MS (*m/z* 254. M^+) and DEPT spectral data (2 methyls, 1 methylene, 5 methines, 7 quaternary carbons). From the UV data (λ_{max}^{EtOH} nm 231, 253, 270, 358) and MS fragmentation pattern (*m/z* 239 ([M-CH_3]$^+$), *m/z* 224 ([M-$(CH_3)_2$]$^+$), *m/z* 211 ([M-CH_3-C_2H_4]) and *m/z* 168), compound **1** supposed to be β-carboline type alkaloid

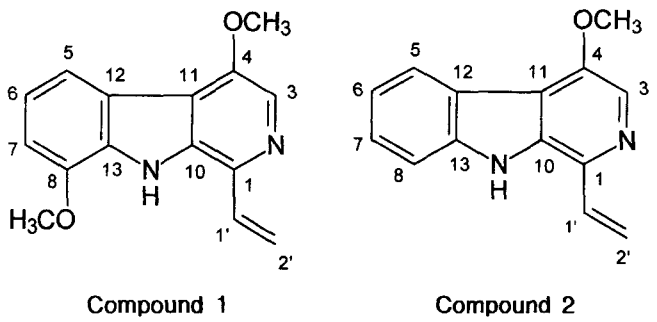

Fig. 1. Structures of compounds **1** and **2**

(Ohmoto, T. *et al.* 1982). ^{1}H-NMR spectrum exhibits signals at δ 4.01 and 4.14 for the presence of two methoxy groups, a vinyl group at 5.59 (1H, dd, *J*=11.5, 1.0 Hz), 6.24 (1H, dd, *J*=17.5, 1.0 Hz) and 7.16 (1H, dd, *J*=17.5, 11.5 Hz), an aromatic ring at δ 6.97 (1H, d, *J*=8.0 Hz), 7.23 (1H, t, *J*=8.0 Hz), 7.90 (1H, d, *J*=8.0 Hz), and a secondary amine (NH) group at δ 8.76. ^{13}C-NMR and DEPT spectra show 15 carbon signals; δ 56.23 and 56.83 ppm indicated two methoxy groups, δ 107.91, 116.96, 121.65, 122.02, 122.75 and 130.64 ppm an aromatic ring, δ 117.68 and 133.46 ppm a vinyl group, respectively.

In the HMBC spectrum, the vinyl proton signal H-1' (7.16) showed ^{1}H-^{13}C long range correlation with C-1 (δ 134.83). And the methoxy signals (δ 4.01 and δ 4.14) correlated with C-8 (δ 146.45) and C-4 (δ 152.04), respectively. By 2D NMR (^{1}H-^{1}H COSY, HMQC and HMBC) and DEPT experiments the gross structure was revealed and especially, by HMBC the positions of vinyl and methoxy groups were confirmed. From the above data the structure of compound **1** was finally determined as 4,8-dimethoxy-1-vinyl-β-carboline (Fig. 1).

Compound **2** was obtained as a yellowish gum. EI-MS (*m/z* 224, M^+) revealed a molecular formula of $C_{14}H_{12}N_2O$. The ^{1}H- and ^{13}C-NMR data of compound **2** were nearly identical with those of **1**, except for the absence of methoxy signal at δ 4.01 in compound **2**. A proton signal at δ 7.51 appeared instead of the methoxy signal at δ 4.01. Thus, compound **2** was determined as 4-methoxy-1-vinyl-β-carboline.

Compounds **1** and **2** were reported from plant sources (Ohmoto, T. *et al.* 1983), but not yet isolated in the *Melia* genus.

Compounds **1** and **2** caused a dose-dependent inhibition of NO production (Table I; IC_{50} values for compound **1** and **2** are 1.5 μM) and iNOS activity (Table II) in RAW 264.7 cells induced by LPS and interferon-γ. These inhibitons were not due to toxicity, as certified by MTT assay (data are not shown). The Inhibitory effect of compound **2** on the iNOS activity in cell-free cellular extract from stimulated RAW 264.7 cells was slightly higher than compound **1**. These inhibition activities of compounds **1** and **2** were more potent than hydrocortisone (IC_{50} > 10 μM, Amin, *et al.*, 1996).

Table I. Effect of compounds **1** and **2** on the nitrite accumulation in RAW 264.7 cells stimulated with LPS and interferon-γ

Test Materials[a] (M)	Nitrite Concentration[b] (μM)	Inhibition (%)
No Test Materials	17.34±1.12	
Compound **1**		
6	1.67±0.32	89
3	5.48±0.51	64
1.5	7.49±2.83	51
0.75	11.16±0.94	27
0.38	13.45±1.29	12
Compound **2**		
4	2.73±0.35	84
2	7.10±0.07	59
1	10.03±0.84	42
0.5	11.89±0.59	31
0.25	12.60±0.67	27

[a]RAW 264.7 cells were seeded at 1.0×10^5 cells in per well of a 96-well plate and incubated for 2 hrs. After media change with fresh DMEM media supplemented with 10% FCS, LPS (1 μg/ml), IFN-γ (10 Unit/ml) and test compounds were added, and then, the cells were further incubated for 16 hrs.
[b]Mean±SD values of nitrite concentration in culture supernatant.

Table II. Effect of compounds **1** and **2** on the iNOS activity in RAW 264.7 cells stimulated with LPS and interferon-γ

Test Materials[a] (M)	iNOS specific activity (pmole/mg protein/min)[b]	Inhibition (%)
No Test Materials	170.2±1.2	1.2
Compound **1**		
3	27.4±0.7***	84
1.5	73.4±1.1***	57
0.75	181.6±3.3	-
Compound **2**		
3	9.9±1.6**	94
1.5	46.0±1.3**	73
0.75	127.5±1.9*	25

[a]RAW 264.7 cells were seeded at 1.0×10^7 cells per 100 mm-culture dish and incubated for 2 hrs. Compounds **1** or **2** was added to the cells just prior to addition of LPS and IFN-γ after culture media change with new high-glucose DMEM supplemented with 10%-FCS. The cells were incubated for 16 hrs, and iNOS activity was measured by [^{3}H]-L-citrulline formation assay with cell-free extracts.
[b]Mean±SD values were obtained from duplicated experiments. *P<0.05, **P<0.01, and ***P<0.001 significantly different from LPS- and IFN-γ-induced cells (DMSO control), analyzed by Student's *t* test.

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