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Inhibitors of Inducible Nitric Oxide Synthase Expression from *Arternisia iwayomogi*

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Nitric oxide (NO) is an important bioactive agent that mediates a wide variety of physiological and pathophysiological events. NO overproduction by inducible nitric oxide synthase (iNOS) results in severe hypotension and inflammation. This investigation is part of a study to discover new iNOS inhibitors from medicinal plants using a macrophage cell culture system. Two sesquiterpenes (**1** and **2**) were isolated from *Artemisia iwayomogi* (Compositae) and were found to inhibit NO synthesis (IC_{50} 3.64 μg/mL and 2.81 μg/mL, respectively) in lipopolysaccharide (LPS)-activated RAW 264.7 cells. Their structures were identified as 3-*O*-methyl-iso-secotanapartholide (**1**) and iso-secotanapartholide (**2**). Compounds **1** and **2** inhibited the LPS-induced expression of the iNOS enzyme in the RAW 264.7 cells. The inhibition of NO production via the down regulation of iNOS expression may substantially modulate the inflammatory responses.

Key words: *Artemisia iwayomogi,* Nitric oxide synthase, Sesquiterpene, Inhibitor

INTRODUCTION

L-Arginine-derived nitric oxide (NO) is an intracellular mediator produced in mammalian cells by two types of nitric oxide synthases (NOS). Constitutive NOS (cNOS) is Ca^{2+}-dependent and releases small amounts of NO, which are essential for some physiological functions (Bredt and Snyder, 1990). In contrast, inducible NOS (iNOS) is Ca^{2+}-independent (Lowenstein *et al.*, 1992). It is induced by either lipopolysaccharide (LPS) or the pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-γ (Kilbourn and Bellori, 1990), and produces large amounts of NO. The NO produced by iNOS as well as the NO derivatives, such as peroxynitrite and nitrogen dioxide, play a role in inflammation and possibly in the multistage process of carcinogenesis (Oshima and Bartsch, 1994). In addition, NO is also known to be responsible for the vasodilation and hypotension observed in septic shock (Kilbourn and Bellori, 1990; Thiemermann and Vane, 1990). Therefore, iNOS inhibitors may be useful for treating septic shock and inflammation. Recently, we reported the isolation of several iNOS inhibitors from the medicinal plants, *Saussurea lappa* (Jin *et al.*, 2000), *Tussilago farfara* (Ryu *et al.*, 1999), *Magnolia obovata* (Son *et al.*, 2000), and *Perilla frutescens* (Ryu *et al.*, 2001). This paper reports the isolation of two sesquiterpenes from *Artemisia iwayomogi* as well as their inhibitory effects on NO production via the inhibition of iNOS expression in the LPS-activated macrophages.

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MATERIALS AND METHODS

Reagents and instruments

DMEM was purchased from Gibco Laboratories (Detroit, MI), and LPS (*Escherichia coli*, 0127:B8) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Calbiochem Co. (San Diego, CA). The bovine serum albumin, sodium nitrite, naphthylethylene diamine, sulfanilamide, aminoguanidine, L-arginine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from the Sigma Chemical Co. (St. Louis, MO). The anti mouse iNOS and anti β-actin polyclonal antibodies were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

The NMR spectra were obtained using a Varian Inova-400 and a Bruker Avance-600 spectrometer. The EI mass

spectra were taken with a direct inlet and recorded using a Jeol JMS-700 mass spectrometer. The infrared spectra were recorded on a Jasco FT/IR-430. The optical rotations were determined using a Jasco DIP-1000 polarimeter and the optical density was measured with a Dynatech MR 5000 microplate reader.

Extraction and isolation

The aerial parts of *Artemisia iwayomogi* were collected from Pochun-Gun, the northern part of Kyung-Gi Province, Korea in November 1997. Vouchers were deposited in the laboratory of forest chemistry, the department of forest products, Kookmin University. Sungsik Kim, a researcher at Kwangnung Arboretum, Forest Research Institute, Korea, verified the voucher and living plants. The methanolic extract (183 g) of the dried and powdered plant materials (1.3 kg) was suspended in water and extracted with diethyl ether to give an ether soluble layer (40 g). The remaining water layer was again extracted with EtOAc to give an EtOAc soluble layer (14 g), which was used to isolate the inhibitory active compounds of NO production in the LPS-activated macrophages. The EtOAc layer was subjected to column chromatography on a silica gel (70-230 mesh, 500 g) using $CHCl_3$/MeOH mixtures (100:1, 50:1, 10:1, 1:1; 3.5 L of each) with increasing polarity as the eluents to yield the active fr. 2 (2.3 g, elution volume 6.0-7.5 L) and fr. 4 (1.2 g, elution volume 13.2-15.5 L). Fr. 2 was further chromatographed on silica gel (100 g) using *n*-hexane/acetone (10:1) as the eluent to afford compound **1** (6.0 mg, elution volume 1.3 L). Fr. 4 was also further chromatographed on silica gel (50 g) using *n*-hexane/EtOAc (5:1) as the eluent which afforded another compound **2** (13.8 mg, elution volume 1.1 L). Copies of the original spectra of compounds **1** and **2** can be obtained from the corresponding author.

Cell culture and nitrite assay

The murine macrophage, RAW 264.7 cells, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM containing 10% fetal bovine serum, glutamine (2 mM), pyruvate (1 mM), penicillin (100 U/mL), and streptomycin (10 μg/mL). The cells were grown at 37°C, 5% CO_2 in fully humidified air, and were split twice a week. The RAW 264.7 cells were treated with various concentrations of the test samples dissolved in either EtOH or dimethyl sulfoxide (DMSO). The final concentration of EtOH or DMSO in the culture media was 0.1%. This concentration did not show any effect on the assay systems.

The RAW 264.7 cells were seeded at 8×10^5 cells/mL in 24 well plates and were activated by incubation in the medium containing LPS (1 μg/mL) and various concentrations of the test compounds. The NO, which was released from the macrophages, was assessed by measuring the NO_2^- concentration in the culture supernatant. Samples (100 μL) of the culture media were incubated with 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplates (Green *et al.*, 1982). The absorbance at 540 nm was measured using an ELISA plate reader. Standard calibration curves were prepared from sodium nitrite.

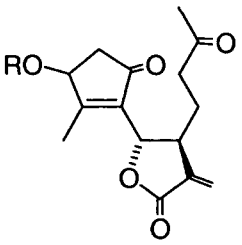
Immunoblot analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method reported by Laemmli (Laemmli, 1970). Immunoblot analysis was performed according to the previously published procedure (Son *et al.*, 2000). Briefly, the protein concentrations of the cell lysates were determined using the Bradford method (Bio-Rad, Hercules, CA), and equal amounts of the protein were loaded on 8% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF, Bio-Rad, Hercules, CA) membranes, probed with anti mouse iNOS antibodies and visualized using an enhanced chemiluminescnece (ECL) system (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instruction. β-actin was used as an internal loading control.

RESULTS

The inhibitory activity of NO production in the LPS-stimulated RAW 264.7 cells was screened in order to discover new iNOS inhibitors from medicinal plants. Among the tested plants that have been used to treat inflammation in oriental medicine, the methanolic extract of *Artemisia iwayomogi* exhibited an 84 % inhibition of the LPS-induced NO production at a concentration of 50 μg/mL in the cell culture media. Activity-guided fractionation of the EtOAc soluble fraction of *A. iwayomogi* resulted in two active sesquiterpenes (compounds **1** and **2**) being isolated. The structures of compounds **1** and **2** were elucidated by NMR analyses and compared with those reported in the literature. Compound **1** was identified as the 3-*O*-methyl-iso-secotanapartholide isolated from *Tanacutum cilicium* (Oksuz, 1990) and compound **2** identified to be the iso-secotanapartholide isolated from *Artemisia rutifolia* (Huneck *et al.*, 1986) (Fig. 1). This is the first report of these compounds in *A. iwayomogi.*

The iNOS activity in the RAW 264.7 cells was stimulated by treating with LPS (1 μg/mL). The LPS concentration and incubation time for cell stimulation were optimized as reported previously (Ryu *et al.*, 2001). Treatment of the cells with **1** and **2** during cell stimulation for 18 h resulted in the inhibition of NO production (Fig. 2A and B). The IC_{50}



1 R = CH_3
2 R = H

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

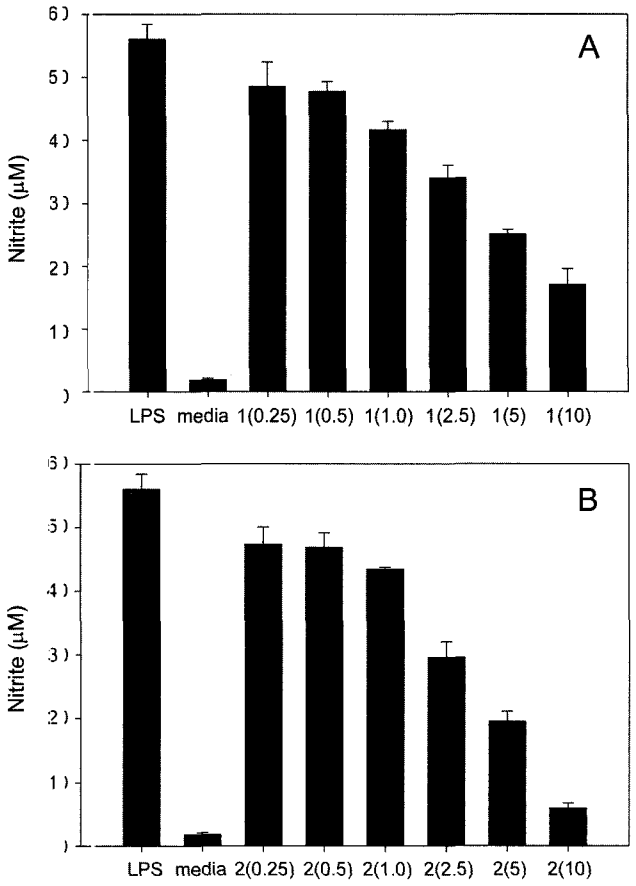


Fig. 2. Dose dependent inhibition of NO synthesis in the LPS-activated macrophages by compound **1** (A) and **2** (B). Compounds (0.25-10 μg/mL) were treated 2 h before LPS-stimulation and the nitrite assay was performed after 18 h incubation with LPS (1 μg/mL). The results are expressed as a mean ± S.D. of three experiments.

values of compounds **1** and **2**, which were calculated based on the nitrite concentrations released into the culture media, were 3.64 μg/mL and 2.81 μg/mL, respectively. Compound **2**, without a methoxy group in the structure, showed more potent activity than compound **1**. The cell viability was assessed by the MTT method at the sample concentrations up to 10 μg/mL to be >90%. N^G-monomethyl-L-arginine (L-NMMA, 0.1 mM) and aminoguanidine (0.1 mM) were used as the positive controls for iNOS inhibition (data not shown).

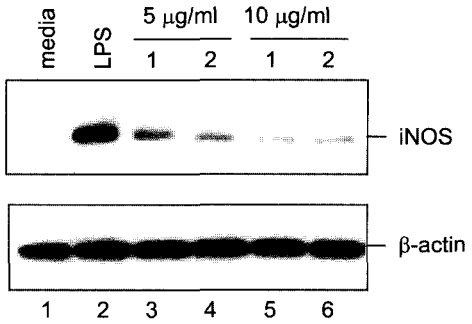


Fig. 3. Western blot analyses of iNOS in the cytosol of RAW 264.7 cells. The cells were treated for 18 h with LPS (1 μg/mL) with or without compound **1** or **2** pretreatment (*i.e.*, 2 h before LPS). Lane 1: media control, lane 2: LPS alone, lane 3: LPS + 5 μg/mL of compound **1**, lane 4: LPS + 5 μg/mL of compound **2**, lane 5: LPS + 10 μg/mL of compound **1**, lane 6: LPS + 10 μg/mL of compound **2**.

The inhibition of NO production by compounds **1** and **2** was shown to be resulted from the suppression of iNOS induction by LPS stimulation. Western blot analyses (Fig. 3) showed that the cytosol of the RAW 264.7 cells activated by LPS in the presence of compounds **1** and **2** contained dose dependently reduced amounts of the iNOS protein compared to the LPS control groups.

DISCUSSION

Nitric oxide is an important bioactive agent, which mediates a variety of physiological and pathophysiological events. Increased iNOS expression and NO overproduction results in the severe inflammation and hypotension observed in septic shock. Recent studies have suggested that NO has a variety of effects on the tumor biology. High NOS activity was observed in the various malignant tissues and the NO exerted effects on angiogenesis (Gallo *et al.*, 1998), differentiation (Thomsen *et al.*, 1995) and metastasis (Dong *et al.*, 1994) of tumors. There are many reports regarding the iNOS inhibitors that exhibit anti-inflammatory (Brouet and Ohshima, 1995) and anti-cancer activity (Rao *et al.*, 2002). Many of them originate from medicinal plants, which can be lead compounds for the development of therapeutic medicine. As part of our ongoing study, plant extracts have been screened for iNOS inhibitory activity and the structures of many inhibitors have been elucidated (Ryu *et al.*, 1999; Son *et al.*, 2000; Ryu *et al.*, 2001).

The medicinal plants of the Artemisia species have been used to treat inflammation, diarrhea and circulatory disorders in oriental medicine. Several iNOS inhibitors have been isolated from this species (Ryu *et al.*, 1998; Kang *et al.*, 1999), and it was found that these biological

activities were related to the anti-inflammatory and chemopreventive activities (Seo *et al.*, 2002). In this report, two sesquiterpenes from *A. iwayomogi* were found to inhibit the NO synthesis of LPS-activated macrophages through the downregulation of iNOS expression. These compounds did not inhibit the NO synthesis when added to the macrophages in which the synthesis of the iNOS enzyme had already been induced by LPS (data not shown). Therefore, these compounds from *A. iwayomogi* are not enzyme inhibitors of iNOS, but are inhibitors of iNOS induction in response to LPS by macrophages. The reaction mechanisms of these compounds such as the effects on the nuclear transcription factor kappa B (NFκB) activation were not fully disclosed in this report. It is expected that these compounds may also have inhibitory activity against cyclooxygenase-2 expression, which is also regulated by NFκB activation. These two compounds can be the active principles, which can explain the biological activities of *A. iwayomogi*.

In summary, two sesquiterpenes isolated from *A. iwayomogi* were identified as inhibitors of iNOS expression in LPS-activated macrophages. These sesquiterpenes may have potential use for treating the endotoxemia and inflammation that accompany NO overproduction.

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