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Pseudoguaianolides Isolated from *Inula britannica* var. *chinenis* as Inhibitory Constituents against Inducible Nitric Oxide Synthase

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Three pseudoguaianolide type sesquiterpenes, bigelovin **(1)**, 2,3-dihydroaromaticin **(2)**, and ergolide **(3)** were isolated as inhibitory constituents against inducible nitric oxide synthase (iNOS) from the flowers of *Inula britannica* var. *chinensis*. Bigelovin **(1)** exhibited a highly potent inhibitory activity on lipopolysaccharide (LPS)-induced iNOS in murine macrophage RAW 264.7 cells with an IC_{50} value of 0.46 mM, which is about 8 times more potent than the known selective inhibitor of iNOS, L-N^6-(1-iminoethyl)lysine (IC_{50} 3.49 μM). 2,3-Dihydroaromaticin **(2)** and ergolide **(3)** also exhibited potent inhibitory activities on LPS-induced iNOS with IC_{50} values of 1.05 and 0.69 μM, respectively.

Key words: *Inula britannica* var. *chinensis*, Bigelovin, 2,3-Dihydroaromaticin, Ergolide, iNOS inhibitor

INTRODUCTION

Nitric oxide (NO) is necessary for physiological activities, while massively produced NO and its derivatives (i.e., NO_2 and NO_3) exert cytotoxic effects on various target cells in humans. Following injury or certain inflammatory stimuli, the expression of an inducible NO synthase (iNOS), which produces a large amount of NO, can occur in a great variety of cells (Brosnan *et al.*, 1994). Therefore, an inhibitor of iNOS may be effective as a therapeutic agent for the pathological conditions related with NO.

In the traditional medical practices of East Asia, *Inula britannica* L. var. *chinensis* (Compositae) has been used to expel phlegm and stop vomiting (Bensky *et al.*, 1993). Our earlier study to identify inhibitory activity against iNOS from higher plants extracts, the ethyl acetate (EtOAc) extracts from the flowers of *I. britannica* var. *chinensis* demonstrated significant activity (IC_{50}:0.8 μg/ml). Thus, the EtOAc extracts were subjected to detailed phytochemical investigation. In the present study, activity guided fractionation using iNOS inhibitory assay system led to the isolation of three known pseudoguaianolide sesquiterpene lactones, bigelovin (**1**), 2,3-dihydroaromaticin (**2**), and ergolide (**3**) from *I. britannica* var. *chinensis* as active inhibitory constituents against iNOS.

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MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured with a DIP-1000 digital polarimeter (Jasco, Japan) at 25°C. UV and IR spectra were recorded on a U-3000 spectrophotometer (Hitachi, Japan) and a FTS 135 FT-IR spectrometer (Bio-Rad, CA), respectively. EIMS were recorded on an Autospec M393 mass spectrometer (Micromass, U.K.). The NMR experiments were performed on a Unity INOVA 400 MHz FT-NMR (Varian, CA), and TMS was used as an internal standard.

Plant materials

The flowers of *I. britannica* var. *chinensis* were purchased from an herb market in Seoul and voucher specimens (No. NPRI-A124) were deposited at the Natural Products Resource Depository of the Natural Products

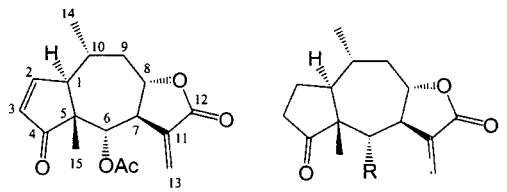
Research Institute, College of Pharmacy, Seoul National University.

Extraction and isolation

The dried flowers of *I. britannica* var. *chinensis* (3 kg) were ground and extracted with methanol (MeOH; 6 l × 6) for 24 h by percolation. The MeOH extracts were concentrated *in vacuo*, suspended in water, and partitioned with hexane (1 l × 1) and EtOAc (1 l × 3), successively. A silica gel column chromatography of the EtOAc extract (70 g) using a gradient solvent system of hexane-EtOAc (9:1 → 1:1), afforded ten fractions. Fraction 2 eluted with hexane-EtOAc (4:1) from the first column chromatography was further fractionated using chloroform-MeOH (50:1) as a solvent system, providing three fractions including compounds **1-3**. Compounds **1-3** were purified by a preparative HPLC (column: Inertsil 10 × 250 mm Prepsil, detector: Shimadzu SPD-6AV; 254 nm, pump: Shimadzu LC-9A; flow rate 1 ml/min) using a solvent system of hexane-ethanol (9:1), affording three pure compounds **1** (3.0 mg, t_R 11.5 min), **2** (25.2 mg, t_R 6.1 min), and **3** (17.2 mg, t_R 7.1 min).

The structures of compounds **1-3** (Fig. 1) were identified as bigelovin (Park and Kim, 1998; Wang *et al.*, 1996), 2,3-dihydroaromaticin (Bohlmann and Mahanta, 1979), and ergolide (Park and Kim, 1998; Wang *et al.*, 1996), respectively, by analysis of the HMQC, HMBC, and ROESY NMR experiments as well as EIMS data for each compound and a comparison of their physical (UV, $[a]_D$ and IR) and spectral data with those of literature values.

Bigelovin (1): Physical and spectral data were comparable to the literature values (Park and Kim, 1998; Wang *et al.*, 1996). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.21 (3H, *s*, H_3-15), 1.29 (3H, *d*, *J* = 5.7 Hz, H_3-14), 1.55 (1H, *ddd*, *J* = 12.4, 12.4, 12.4 Hz, H-9), 1.97 (3H, *s*, H-17), 2.06 (1H, *m*, H-10), 2.57 (1H, *dt*, *J* = 12.4, 3.6 Hz, H-9), 3.03 (1H, *br d*, *J* = 10.8 Hz, H-1), 3.08 (1H, *m*, H-7), 4.61 (1H, *td*, *J* = 12.4, 3.0 Hz, H-8), 5.60 (1H, *d*, *J* = 7.4 Hz, H-6), 5.92 (1H, *d*, *J* = 3.0 Hz, H-13), 6.11 (1H, *dd*, *J* = 6.0, 2.4 Hz, H-3), 6.22 (1H, *d*, *J* = 3.0 Hz, H-13), 7.71 (1H, *d*, *J* = 6.0 Hz, H-2). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 19.8 (C-14, q), 21.1 (C-17, q), 22.7 (C-15, q), 27.2 (C-10, d), 44.4 (C-9, t), 52.2 (C-1, d), 53.9 (C-7, d), 56.3 (C-5, s), 73.1 (C-6, d), 76.1 (C-8, d), 122.2 (C-13, t), 130.8 (C-3, d), 137.2 (C-11, s), 162.6 (C-2, d), 168.9 (C-12, s), 169.5 (C-16, s), 209.2 (C-4, s)



1

2 R = H

3 R = OAc

Fig. 1. Structures of compounds **1-3** isolated from the flowers of *I. britannica* var. *chinensis*

2,3-Dihydroaromaticin (2): Physical and spectral data were comparable to the literature values (Bohlmann and Mahanta, 1979). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.04 (3H, *s*, H_3-15), 1.09 (3H, *d*, *J* = 6.0 Hz, H_3-14), 1.42 (1H, *m*, H-9), 1.51 (1H, *m*, H-3), 1.61 (1H, *m*, H-2), 1.92 (1H, *m*, H-1), 1.95 (1H, *m*, H-10), 2.10 (1H, *m*, H-2), 2.18 (1H, *m*, H-6), 2.43 (1H, *m*, H-9), 2.47 (1H, *m*, H-6), 2.50 (1H, *m*, H-3), 2.81 (1H, *m*, H-7), 4.28 (1H, *ddd*, *J* = 11.6, 8.8, 2.8 Hz, H-8), 5.51 (1H, *d*, *J* = 3.2 Hz, H-13), 6.17 (1H, *d*, *J* = 3.2 Hz, H-13) ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 20.0 (C-14, q), 22.0 (C-15, q), 24.1 (C-2, t), 29.6 (C-10, d), 34.5 (C-3, t), 35.2 (C-6, t), 44.1 (C-9, t), 44.7 (C-7, d), 48.7 (C-1, d), 50.0 (C-5, s), 80.8 (C-8, d), 120.0 (C-13, d), 140.3 (C-11, s), 169.8 (C-12, s), 222.5 (C-4, s).

Ergolide (3): Physical and spectral data were comparable to the literature values (Park and Kim, 1998; Wang *et al.*, 1996). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.08 (3H, *s*, H_3-15), 1.11 (3H, *d*, *J* = 6.4 Hz, H_3-14), 1.45 (1H, *m*, H-2), 1.51 (1H, *q*, *J* = 12.4 Hz, H-9), 1.86 (1H, *m*, H-10), 2.13 (1H, *m*, H-3), 2.18 (1H, *m*, H-2), 2.29 (1H, *ddd*, *J* = 11.2, 11.2, 5.2 Hz, H-1), 2.41 (1H, *dd*, *J* = 17.8, 7.8 Hz, H-3), 2.51 (1H, *ddd*, *J* = 13.0, 4.8, 2.8 Hz, H-9), 3.04 (1H, *ddd*, *J* = 13.6, 7.6, 3.2 Hz, H-7), 4.49 (1H, *ddd*, *J* = 12.0, 10.2, 3.2 Hz, H-8), 5.50 (1H, *d*, *J* = 8.0 Hz, H-5), 5.85 (1H, *d*, *J* = 3.4 Hz, H-13), 6.20 (1H, *d*, *J* = 3.4 Hz, H-13). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 18.4 (C-15, q), 20.0 (C-14, q), 21.1 (C-17, q), 24.5 (C-2, t), 130.0 (C-10, d), 37.9 (C-3, t), 44.3 (C-9, t), 46.7 (C-1, d), 52.7 (C-7, d), 56.1 (C-5, s), 74.7 (C-6, d), 76.2 (C-8, d), 122.1 (C-13, t), 137.3 (C-11, s), 169.1 (C-12, s), 169.4 (C-16, s), 218.5 (C-4, s)

Assay methods

For the determination of iNOS activity, RAW 264.7 murine macrophage cells (ATCC TIB-71) were seeded in 96-well plates (2×10^5/200 μl), cultured for 24 h and incubated with lipopolysaccharide (LPS; 1 μg/ml) in the presence of various plant extracts (final 0.5% dimethyl sulfoxide as a vehicle solvent) for an additional 24 h. Nitrite concentrations produced in the medium were determined by measuring the absorbance at 540 nm based on the Griess reaction (Green *et al.*, 1982). Cytotoxicity was measured by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

Table I. The inhibitory effects of isolates from the flowers of *I. britannica* var. *chinensis* against the LPS-induced iNOS activity in RAW 264.7 macrophages

Compounds	IC_{50}[a] (μM)	
	iNOS activity	Cytotoxicity
Bigelovin (**1**)	0.46	6.84
2,3-Dihydroaromaticin (**2**)	1.05	28.71
Ergolide (**3**)	0.69	15.29
L-N^6-(1-Iminoethyl)lysine (control)	3.49	>50

[a]IC_{50} value represents the molar concentration (μM) giving 50% inhibition relative to the negative control.

bromide] to formazan (Mosmann, 1983) using the same treatment procedure as for the determination of the iNOS enzyme activity. The inhibitory effects are represented as the molar concentration (μM) yielding 50% inhibition (IC_{50}) relative to the negative control.

RESULTS AND DISCUSSION

The known compound, 2,3-dihydroaromaticin **(2)** was identified for the first time in this plant, whereas bigelovin **(1)** and ergolide **(3)** have been reported previously along with other sesquiterpene lactones and flavonol glycosides from *I. britannica* (Park and Kim, 1998; Han *et al.*, 2001). Recently, the iNOS inhibitory activity of ergolide isolated from *I. britannica* has been reported (Han *et al.*, 2001). Herein, we report the significant inhibitory activity of pseudoguaianolides **1** and **2** against iNOS for the first time with IC_{50} values of 0.46 and 1.05 μM, respectively. These pseudoguaianolides **1-3** also showed weak cytotoxic effects on Raw 264.7 cells, however, the inhibition of iNOS activity was not due to the direct cytotoxic effect of the compounds (Table I). It has been reported that NO production by iNOS is initiated from inflammatory signals such as cytokines or endotoxins (Hukkanen *et al.*, 1995). Therefore, these results show that bigelovin (**1**), 2,3-dihydroaromaticin (**2**), and ergolide (**3**) may be potential candidates for the treatment of human diseases associated with iNOS such as inflammation (Ohshima and Bartsch, 1994), ischemia (Escott *et al.*, 1998), and aging (Licinio *et al.*, 1999).

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REFERENCES

Bensky, D., Gamble, A., Kaptchuk, T. J., and Bensky, L. L., *Chinese Herbal Medicine: Materia Medica*. Eastland Press, Seattle, pp. 193-194, (1993).

Bohlmann, F. and Mahanta, P. K., Zwei neue pseudoguajanolide aus *Telekia speciosa*. *Phytochemistry*, 18, 887-888 (1979).

Brosnan, C. F., Battistini, L., Raine, C. S., Dickson, D. W., Casadevall, A., and Lee, S. C., Reactive nitrogen intermediates in human neuropathology: an overview. *Dev. Neurosci.*, 16, 152-161 (1994).

Escott, K. J., Beech, J. S., Haga, K. K., Williams, S. C., Meldrum, B. S., and Bath, P. M., Cerebroprotective effect of the nitric oxide synthase inhibitors, 1-(2-trifluoromethylphenyl) imidazole and 7-nitro indazole, after transient focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.*, 18, 281-287 (1998).

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [^{15}N]-nitrite in biological fluids. *Anal. Biochem.*, 126, 131-138 (1982).

Han, J. W., Lee, B. G., Kim, Y. K., Yoon, J. W., Jin, H. K., Hong, S., Lee, H. Y., Lee, K. R., and Lee, H. W., Ergolide, sesquiterpene lactone from *Inula britannica*, inhibits inducible nitric oxide synthase and cyclo-oxygenase-2 expression in RAW 264.7 macrophages through the inactivation of NF-kB. *Br. J. Pharmacol.*, 133, 503-512 (2001).

Hukkanen, M., Hughes, F. J., Buttery, L. D., Gross, S. S., Evans, T. J., Seddon, S., Riveros-Moreno, V., Macintyre, I., and Polak, J. M., Cytokine-stimulated expression of inducible nitric oxide synthase by mouse, rat, and human osteoblast-like cells and its functional role of osteoblast metabolic activity. *Endocrinology*, 136, 5445-5453 (1995).

Licinio, J., Prolo, P., McCann, S. M., and Wong, M. L., Brain iNOS: current understanding and clinical implications. *Mol. Med. Today*, 5, 225-232 (1999).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 16, 55-63 (1983).

Ohshima, H. and Bartsch, H., Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.*, 305, 253-264 (1994).

Park, E. J. and Kim J., Cytotoxic sesquiterpene lactones from *Inula Britannica*. *Planta Med.*, 64, 752-754 (1998).

Wang, Q., Zhou, B. N., Zhang, R. W., Lin, Y. Y., Lin, L. Z., Gil, R. R., and Cordell, G. A., Cytotoxicity and NMR spectral assignments of ergolide and bigelovin. *Planta Med.*, 62, 166-168 (1996).