A New Bisabolene Epoxide from *Tussilago farfara,* and Inhibition of Nitric Oxide Synthesis in LPS-Activated Macrophages

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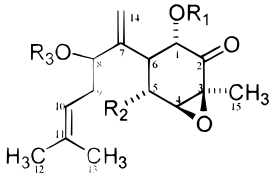
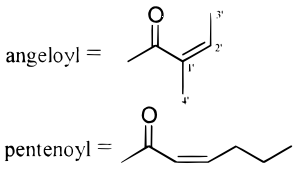
A new bisabolene epoxide was isolated from the flower buds of *Tussilago farfara*, and the structure was determined by spectroscopic methods to be 1α,5α-bisacetoxy-8-angeloyloxy-3*β*,4*β*-epoxy-bisabola-7(14),-10-dien-2-one (**1**). Compound **1** showed inhibition of nitric oxide synthesis in lipopolysaccharide-activated macrophages.

The flower buds of *Tussilago farfara* L. (Compositae) are used in traditional Chinese medicine for the treatment of bronchitic and asthmatic conditions.[1] Biological activities reported for this plant include inhibition of arachidonic acid metabolism,[2] inhibition of platelet-activating factor receptor,[3,4] and cardiovascular and respiratory stimulation.[5] In the course of research to find antiinflammatory agents from plants,[6,7] we observed that the MeOH extracts of *T. farfara* showed strong inhibitory activity of nitric oxide (NO) synthesis in lipopolysaccharide (LPS)-stimulated macrophages. NO is derived from L-arginine by nitric oxide synthase (NOS), and the overproduction of NO by inducible nitric oxide synthase (i-NOS) is responsible for vasodilation in septic shock and inflammation.[8,9] Therefore, an inhibitor of i-NOS may be effective as a therapeutic agent for the treatment of septic shock and inflammation. Activity-guided fractionation and repeated column chromatography revealed a new bisabolene epoxide to be an active compound.

The structure of compound **1** was deduced from comparison of its ^{1}H NMR spectrum with the spectra of similar bisabolene derivatives (**2** and **3**).[10,11] Two methyl groups (δ 1.97 and 1.89) and one olefinic proton (δ 6.08) coupled together indicated the presence of an angeloyl group. One methylene carbon at δ 114.4 (C-14) correlated with two terminal methylene protons (δ 5.24 and 5.33) in ^{1}H−^{13}C COSY, and these two protons correlated together in ^{1}H−^{1}H COSY. The proton peak at δ 2.87 (H-6) correlated with two protons at δ 5.68 (H-1) and 5.35 (H-5) in ^{1}H−^{1}H COSY spectra. In comparison with the NMR spectra of **2** and **3**, **1** showed a characteristic oxymethine proton signal (δ 5.35) assigned to H-5. From the correlations of one carbonyl carbon (δ 170.4) with H-5 and with acetyl protons at δ 2.15 in the HMBC spectra, the position of an acetyl group was assigned to C-5. In the same way the another acetyl group was identified at C-1. The position of angeloyl was also confirmed from the correlations of carbonyl carbon at δ 166.9 with δ 5.23 (H-8) and methyl peak of angeloyl group at δ 1.89 (H-4′) in the HMBC spectra. The other NMR peaks were fully assigned by using ^{1}H−^{1}H COSY, ^{1}H−^{13}C COSY, and HMBC spectra. The configurations at C-1 and C-5 were deduced from the large coupling constant ($J_{1,6}$ = 12.77 Hz) of H-1[7] and the weak coupling ($J_{4,5}$ < 1 Hz) between H-4 and H-5 in ^{1}H−^{1}H COSY. These configurations were also confirmed by the correlations between H-1 and H-6, H-5 and H-6, H-4 and H-15, respectively, in NOESY spectra. This compound is the first bisabolene epoxide oxygenated at C-5.





	R_1	R_2	R_3
1	acetyl	O-acetyl	angeloyl
2	angeloyl	H	angeloyl
3	acetyl	H	pentenoyl

In an LPS-activated macrophage cell culture system, **1** showed dose-dependent inhibition on NO synthesis, and the IC_{50} was 8.9 μM. Accumulation of NO was also inhibited by treatment with 0.1 M N^G-monomethyl-L-arginine (L-NMMA, Calbiochem Co., San Diego, CA), an inhibitor of NOS through substrate competition, and the production of NO was restored by addition of 1 mM arginine from 7.53 ± 0.29 to 14.15 ± 0.10 μM. However, inhibition of NO production by **1** was not restored by addition of arginine (Table 1). These data implied that inhibition of NO production by **1** was not due to substrate competition for NOS. Inhibition of NO synthesis was very weak (11.1% inhibition comparing with LPS-control, Table 1) when **1** was treated after induction of i-NOS by activated macrophages. And the inhibition of NO synthesis was proportional to the incubation time of **1** during LPS-activation (data not shown). Aminoguanidine exhibited 78.8% inhibition of NO production by the i-NOS, which was expressed by LPS-stimulation of RAW 264.7 cells. Thus, **1**

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Table 1. Inhibition of NO Synthesis in LPS-Activated Macrophages (RAW 264.7)[a]

group	NO concentration (μM)	inhibition (%)[b]
LPS control[c]	19.07 ± 1.32	0.0
compound **1**[c]	5.51 ± 0.37	71.1
L-NMMA[c]	7.53 ± 0.29	60.5
compound **1** + arginine[c]	5.93 ± 1.27	68.9
L-NMMA + arginine[c]	14.15 ± 0.10	25.8
LPS control[d]	38.22 ± 1.76	0.0
compound **1**[d]	33.96 ± 1.41	11.1
L-NMMA[d]	17.83 ± 0.97	53.3
aminoguanidine[d]	8.10 ± 0.29	78.8

[a] Results are expressed as mean ± SD of three experiments. [b] Inhibition is expressed as % compared with LPS-treated control. [c] RAW 264.7 cells were activated by LPS (1 μg/mL) for 18 h with effectors (30 μM compound **1**, 0.1 mM L-NMMA, 0.1 mM dexamethasone), and media were exchanged for fresh. After another 18-h incubation with or without 1 mM arginine, the concentration of NO was determined by Griess reagent. [d] After 18 h of LPS-activation, media were exchanged with fresh ones. RAW 264.7 cells were treated with effectors as above for another 18 h, and then the concentration of NO was determined.

is an inhibitor of i-NOS expression rather than an enzyme inhibitor of i-NOS, such as aminoguanidine. This new inhibitor of i-NOS expression may have potential in the treatment of endotoxemia and inflammation accompanied by the overproduction of NO.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker AMX 400 or 500 NMR spectrometer with TMS as internal standard. CIMS and EIMS (ionization voltage, 70 eV) were measured with a Hewlett−Packard 5890-JMS AX505WA spectrometer. IR spectra were recorded on a JASCO FT-IR-430 spectrometer in $CHCl_3$ solution. UV spectra were obtained on a Pharmacia Biotech Ultraspec 4000 UV−vis spectrophotometer. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter.

Plant Material. Flower buds of *T. farfara* were purchased from the Kyungdong oriental drug market in Seoul and authenticated by Prof. T. H. Kim at the College of Pharmacy, Sookmyung Women's University. A voucher specimen was deposited in the herbarium of the Sookmyung Women's University, Seoul, Korea.

Extraction and Isolation. The dried flower buds of *T. farfara* (2 kg) were extracted three times with MeOH, and the combined extracts were concentrated and partitioned between *n*-hexane and H_2O. A portion of the *n*-hexane-soluble fraction (24 g) was subjected to column chromatography on silica gel, *n*-hexane−EtOAc (25:1) to yield subfractions. Fraction 7 (1.6 g) containing compound **1** was rechromatographed on silica gel eluting with *n*-hexane−acetone (25:1) and finally purified by reversed-phase HPLC (μ-Bondapak C-18 column, 10 × 300 mm; 70% MeOH, 2.0 mL/min; UV 254 nm) to yield **1** (4.4 mg).

1α,5α-Bisacetoxy-8-angeloyloxy-3*β*,4*β*-epoxy-bisabola-7(14),10-dien-2-one (1): colorless oil; $[\alpha]_D$ +4.64° (*c* 0.22, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 203.4 (4.20) nm; IR ν_{max} ($CHCl_3$) 2924, 1736, 1650, 1228 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.08 (1H, qq, J = 7.11, 1.59, H-2′), 5.68 (1H, d, J = 12.77, H-1), 5.35 (1H, d, J = 8.67, H-5), 5.33 (1H, s, H-14a), 5.24 (1H, s, H-14b), 5.23 (1H, dd, J = 7.78, 4.32, H-8), 5.04 (1H, br t, J = 7.22, H-10), 3.40 (1H, s, H-4), 2.87 (1H, dd, J = 12.77, 8.67, H-6), 2.33 (2H, m, H-9), 2.15 (3H, s, 5-OCOCH_3), 2.06 (3H, s, 1-OCOCH_3), 1.97 (3H, dq, J = 7.11, 1.49, H-3′), 1.89 (3H, dq, J = 1.59, 1.49, H-4′), 1.68 (3H, br s, 12-CH_3), 1.62 (3H, br s, 13-CH_3), 1.48 (3H, s, 15-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.9 (s, C-2), 170.4 (s, 5-O*C*OCH$_3$), 169.9 (s, 1-O*C*OCH$_3$), 166.9 [s, 8-O*C*OC(CH$_3$)CHCH$_3$], 146.1 (s, C-7), 139.0 (d, C-2′), 134.9 (s, C-11), 128.2 (s, C-1′), 119.4 (d, C-10), 114.4 (t, C-14), 75.3 (d, C-8), 73.0 (d, C-5), 72.9 (d, C-1), 66.1 (d, C-4), 61.8 (s, C-3), 48.9 (d, C-6), 32.3 (t, C-9), 26.1 (q, C-13), 21.2 (q, C-4′), 20.7 (q, 5-OCO*C*H$_3$), 21.0 (q, C-3′), 18.4 (q, C-12), 16.1 (q, 1-OCO*C*H$_3$), 14.8 (q, C-15); positive CIMS m/z 449 $[M + 1]^+$; EIMS m/z 448 $[M]^+$; positive HRCIMS m/z 449.2169 (calcd for $C_{24}H_{33}O_8$, 449.2175).

Cell Culture. The murine macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM (Gibco Laboratories, Gaithersburg, MD) containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/mL), and streptomycin (10 μg/mL). The cells were cultured at 37 °C, 5% CO_2 in fully humidified air, and were split twice a week. RAW 264.7 cells were seeded at 8 × 10^5 cells/mL in 24-well plates and were activated by incubating in the medium containing LPS (*Escherichia coli*, 0127:B8) (Sigma Chemical Co., St. Louis, MO), and various concentrations of test compounds dissolved in H_2O or DMSO. The supernatant was collected as a source of secreted NO. The final concentration of DMSO in culture media was 0.1%, and this concentration showed no effect on the assay systems.

Nitrite Assay. NO from macrophages was assessed by determining the NO_2-concentration in the culture supernatant. Samples (100 μL) of culture media were incubated with 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution) at room temperature for 10 min in a 96-well microplate.[12] Absorbances at 540 nm were read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as a standard.

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