Humulene Derivatives from *Zingiber zerumbet* with the Inhibitory Effects on Lipopolysaccharide-Induced Nitric Oxide Production

Dae Sik JANG,[a,e] Hye-Young MIN,[b] Moon-Sun KIM,[b] Ah-Reum HAN,[b] Tri WINDONO,[c] Gwang-Ho JEOHN,[d] Sam Sik Kang,[f] Sang Kook LEE,[b] and Eun-Kyoung SEO*,[b]

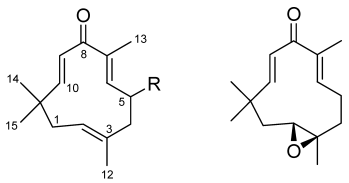
[a] *Division of Molecular Life Sciences, Ewha Womans University; and* [b] *College of Pharmacy, Ewha Womans University; 11–1 Daehyun-dong, Seodaemun-gu, Seoul 120–750, Korea:* [c] *Faculty of Pharmacy, University of Surabaya; Raya Kalirungkut, Surabaya 60293, Indonesia:* [d] *Faculty of Science, Brawijaya University; Malang 65145, Indonesia:* [e] *Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine; 461–24 Jeonmin-dong, Yuseong-gu, Daejeon 305–811, Korea: and* [f] *Natural Product Research Institute, Seoul National University; Seoul 110–460, Korea.* Received January 6, 2005; accepted March 25, 2005

A new humulene sesquiterpene, 5-hydroxyzerumbone (5-hydroxy-2*E*,6*E*,9*E*-humulatrien-8-one) (1) and a known compound, zerumboneoxide (2) were isolated from the rhizomes of *Zingiber zerumbet* (Zingiberaceae), and found to inhibit lipopolysaccharide-induced nitric oxide production in murine macrophage RAW 264.7 cells with IC_{50} values of 14.1 and 23.5 μM, respectively, by bioassay-guided fractionation (positive control: N^{ω}-monomethyl-L-arginine, IC_{50}=21.3 μM). The structure of 1 was determined by spectroscopic methods including 1D and 2D-NMR.

Key words 5-hydroxyzerumbone; zerumboneoxide; *Zingiber zerumbet*; nitric oxide production

Zingiber zerumbet (L.) J. E. SMITH (Zingiberaceae), a wild ginger, grows widely in Southeast Asia, and its rhizomes are used traditionally to treat inflammation.[1)] Previous phytochemical investigations on this plant resulted in the isolation of several sesquiterpenoids and flavonoids.[2—4)] In particular, a monocyclic sesquiterpene, zerumbone (2*E*,6*E*,10*E*-humulatrien-1-one), determined to be a major component of the essential oil of *Z. zerumbet*, has been studied intensively as a potential anti-inflammatory and chemopreventive.[2,5,6)] In our ongoing project directed toward the discovery of novel naturally occurring inducible nitric oxide synthase (iNOS) inhibitory agents in higher plants,[7)] the rhizomes of *Z. zerumbet* were chosen for detailed investigation, since the *n*-hexane-soluble fraction of its MeOH extract was found to significantly inhibit (IC_{50}=2.74 μg/ml) lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine macrophage RAW 264.7 cells. NO is a short-lived molecule that is required for many physiological functions, and is produced from L-arginine by NO synthase (NOS).[8)] Moreover, overproduced NO can stimulate tumor growth and metastasis by promoting the migratory, invasive, and angiogenic potentials of tumor cells.[9)] Thus, inhibitors or suppressors of iNOS have potential use as therapeutic agents in certain cancers. In the present study, an *in vitro* iNOS inhibition assay-guided fractionation of the *n*-hexane-soluble fraction of the rhizomes of *Z. zerumbet*, led to the isolations of a new (**1**) and a known (**2**) humulene derivative. Compound **2** was identified as zerumboneoxide, a compound previously isolated from *Z. zerumbet*.[10,11)] However, the iNOS inhibitory activity of zerumboneoxide (**2**) has not been previously reported. Herein, we describe the structural elucidation of **1** and the results of biological evaluations.

Compound **1** was obtained as a colorless oil and produced a molecular ion $[M]^+$ at *m*/*z* 234.1603 by HR-EI-MS, which is consistent with an elemental formula $C_{15}H_{22}O_2$. Its IR spectrum exhibited strong absorptions at 3412 and 1659 cm^{-1}, suggesting the presence of a hydroxyl group and an α,β-unsaturated carbonyl group, respectively. The ^{1}H-NMR spectrum of **1** showed two proton signals at δ 5.91 (1H, d, *J*=16.4 Hz) and 5.99 (1H, d, *J*=16.4 Hz) corresponding to a *trans*-double bond, two olefinic signals at δ 5.29 (1H, br d, *J*=12.0 Hz) and 5.80 (1H, d, *J*=9.4 Hz), an oxygenated methine signal at δ 4.62 (1H, dt, *J*=9.4, 5.4 Hz), two vinyl methyl signals at δ 1.56 (3H, s) and 1.90 (3H, s), and two single methyl signals at δ 1.08 (3H) and 1.20 (3H). The ^{13}C-NMR spectrum and a DEPT experiment on **1** showed 15 skeletal carbon signals, including; a carbonyl carbon (δ 204.7), six olefinic carbons (δ 126.8, 127.5, 133.3, 140.5, 146.2, 162.8), and a tertiary carbon (δ 64.9) with an adjoining oxygen. The COSY spectrum of **1** in combination with HMBC experimental data (Fig. 2) suggested that it is a humulene derivative with three double bonds, a hydroxyl group, and a conjugated carbonyl group. Comparisons of the above data with those in the literature indicated that **1** is closely re-



1 R = OH **2**
3 R = H

Fig. 1. Structures of Compounds **1**—**3** Isolated from *Z. zerumbet*

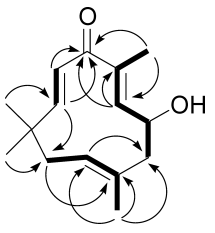


Fig. 2. Selected Correlations Observed in the COSY (−) and HMBC (→) Spectra of Compound **1**

* To whom correspondence should be addressed. e-mail: yuny@ewha.ac.kr

Table 1. The Inhibitory Effects of Compounds **1**—**3** from *Z. zerumbet* on LPS-Induced NO Production in RAW 264.7 Cells

Compound	iNOS activity	
	% Inhibition at 20 μg/ml	IC_{50} (μM)[a]
5-Hydroxyzerumbone (**1**)	100.0	14.1
Zerumboneoxide (**2**)	99.6	23.5
Zerumbone (**3**)	95.6	5.4
L-NMMA[b]	70.0	21.3

a) IC_{50} value represents the molar concentration (μM) that inhibited iNOS activity by 50% relative to the negative control. *b*) N^{ω}-monomethyl-L-arginine (L-NMMA) was used as a positive control.

lated structurally to zerumbone (**3**), which was also isolated during the present investigation; however, **1** was found to contain a hydroxyl group at C-5. The 2*E*,6*E* configuration of compound **1** was suggested by its ^{13}C-NMR spectrum since signals at δ 133.3 for C-3, δ 16.7 for C-12, δ 140.5 for C-7, and at δ 12.4 for C-13 lie at chemical shifts characteristic of related compounds with the *E*,*E* configuration.[12] This was supported by a NOESY NMR experiment, which showed a clear NOE correlation between H-2 (δ 5.29, 1H, br d, J=12.0 Hz) and H-6 (δ 5.80, 1H, d, J=9.4 Hz). Therefore, compound **1** was elucidated to be a new compound, namely, 5-hydroxyzerumbone (5-hydroxy-2*E*,6*E*,9*E*-humulatrien-8-one). However, the stereochemistry at C-5 of **1** could not be determined because it was isolated as a racemic mixture {$[\alpha]_D^{20}$ 0.0° (c=0.25, $CHCl_3$)}.

Compound **2** was identified as zerumboneoxide by comparing its NMR data with literature values.[13] Compounds **1** and **2** exhibited inhibitory activity against LPS-induced NO production with IC_{50} values of 14.1 and 23.5 μM, respectively (Table 1). The iNOS inhibitory activity of zerumboneoxide (**2**) has not been previously reported. Indeed, zerumbone (**3**), which is a known iNOS inhibitory sesquiterpene also from *Z. zerumbet*,[14] was found in the present study to be the principle iNOS inhibitory component, with an IC_{50} value of 5.4 μM. Further investigations are needed to determine the structure–activity relationships of humulene derivatives and their mechanism of action against LPS-induced NO production.

Experimental

General Experimental Procedures Melting points were measured on a J-923 melting point apparatus (Jisico, Korea) and are quoted uncorrected. Optical rotations were obtained using a DIP-1000 digital polarimeter (Jasco, Japan) at 25 °C. UV and IR spectra were recorded using a U-3000 spectrophotometer (Hitachi, Japan) and a FTS 135 FT-IR spectrometer (Bio-Rad, CA, U.S.A.), respectively. LR and HREIMS were recorded on an Autospec M393 mass spectrometer (Micromass, U.K.) operated at 70 eV. NMR experiments were conducted on a Unity INOVA 400 MHz FT-NMR (Varian, CA, U.S.A.), using TMS as an internal standard. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich) and then heat treated at 110 °C for 5—10 min. Silica gel (Merck 60A, 200—400 mesh ASTM) and Sephadex LH-20 (Amersham Pharmacia Biotech) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use.

Plant Material The rhizomes of *Z. zerumbet* (L.) J. E. Smith were collected in Surabaya, Indonesia, in June 2001 and were identified by one of the authors, Professor Tri Windono (University of Surabaya, Indonesia). A voucher specimen (no. 21/DT/VI/2001) has been deposited at the University of Surabaya, JL. Raya Kalirungkut, Surabaya 60293, Indonesia.

Extraction and Isolation Dried and milled plant material (5 kg) was extracted with 10 l of MeOH three times by maceration. Extracts were combined and concentrated *in vacuo* at 40 °C, and concentrated extract was suspended in H_2O (2 l) and partitioned with 2 l of *n*-hexane three times to afford a *n*-hexane-soluble syrup on drying. This *n*-hexane-soluble extract (165 g) was then column chromatographed through silica gel (12×42 cm, 230—400 mesh) using a combinatorial solvent system [cyclohexane (20 l), cyclohexane–CH_2Cl_2 (1 : 1 v/v, 10 l), CH_2Cl_2 (6 l), CH_2Cl_2–MeOH (19 : 1 v/v, 6 l), MeOH (6 l)] to afford 10 pooled fractions (F001—F010). With the exception of fractions F01, F02, and F010, all *n*-hexane-soluble extract fractions showedsignificant iNOS inhibitory activity (IC_{50} <10 μg/ml). Fraction F09 [eluted with CH_2Cl_2–MeOH (19 : 1 v/v); 32 g] was chromatographed through silica gel (7×30 cm, 230—400 mesh; *n*-hexane–EtOAc gradient from 19 : 1 to 1 : 2 v/v, final elution with 100% MeOH) to produce eighteen subfractions (F0901—F0918). Compound **2** (980 mg, 0.0196%) was obtained from a combined fraction [F0909+F0910; eluted with *n*-hexane–EtOAc (4 : 1 v/v); 4.0 g] by recrystallization from *n*-hexane. Fractions F0913 and F0914 [eluted with *n*-hexane–EtOAc (3 : 2 v/v); 2.8 g] were combined, and then purified through a silica gel column (4.5×24 cm, 230—400 mesh) using a CH_2Cl_2–acetone (from 49 : 1 to 3 : 1 v/v) solvent system, to yield the new compound **1** (68 mg, 0.00136%).

5-Hydroxyzerumbone (**1**): Colorless oil. $[\alpha]_D^{20}$ 0.0° (c=0.25, $CHCl_3$); IR (NaCl) cm^{-1}: 3412, 2961, 2931, 2866, 1659, 1450, 1364, 1110, 1015, 971; UV λ_{max} (MeOH) nm (log ε): 203 (4.14), 225 (3.97), 248 (3.90); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 5.99 (1H, d, J=16.4 Hz, H-9), 5.91 (1H, d, J=16.4 Hz, H-10), 5.80 (1H, d, J=9.4 Hz, H-6), 5.29 (1H, br d, J=12.0 Hz, H-2), 4.62 (1H, br dt, J=9.4, 5.4 Hz, H-5), 2.74 (1H, dd, J=11.4, 5.4 Hz, H-4a), 2.36 (1H, t, J=12.0 Hz, H-1a), 2.17 (1H, br t, J=11.4 Hz, H-4b), 1.91 (overlapped, 1b), 1.90 (3H, s, H-13), 1.56 (3H, s, H-12), 1.20 (3H, s, H-15), 1.08 (3H, s, H-14); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 204.7 (C-8), 162.8 (C-10), 146.2 (C-6), 140.5 (C-7), 133.3 (C-3), 127.5 (C-9), 126.8 (C-2), 64.9 (C-5), 49.4 (C-4), 42.7 (C-1), 38.7 (C-11), 29.4 (C-15), 24.4 (C-14), 16.7 (C-12), 12.4 (C-13); EI-MS *m*/*z* (rel. int.): 234 ([M]$^+$, 4), 166 (83), 151 (100), 123 (34); HR-EI-MS *m*/*z*: 234.1603 ([M]$^+$, Calcd for $C_{15}H_{22}O_2$: 234.1620).

Zerumboneoxide (**2**) Colorless crystals. mp 96—97 °C; $[\alpha]_D^{20}$ 0.0° (c=0.24, $CHCl_3$); EI-MS: *m*/*z* (rel. int.): 234 ([M]$^+$, 9), 219 (6), 191 (24), 147 (68), 135 (100), 107 (85), 96 (57); ^{1}H- and ^{13}C-NMR data were in agreement with literature values.[13]

Measurements of NO Production by LPS-Stimulated Macrophage Cells NO formation by iNOS was followed in cultured RAW 264.7 macrophages.[7] Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin–streptomycin and 10% fetal bovine serum (FBS) at 37 °C, in 5% CO_2 humidified air. To determine the inhibitory effects of test materials on LPS-induced NO production, cells in 10% FBS-DMEM (without phenol red) were plated in 24-well plates (5×10^5 cells/ml), incubated for 24 h, washed with PBS, placed in fresh medium, and then incubated in medium containing 1 μg/ml of LPS in the presence or absence of test samples. After an additional incubation for 20 h, media were collected and analyzed for nitrite (an indicator of NO production) using the Griess reaction. Absorbance was measured at 540 nm using a microplate reader, and nitrite concentrations were determined by comparison with a standard sodium nitrite curve. Percentage inhibition was expressed as [1-(NO level of test samples/NO level of vehicle-treated control)]×100. IC_{50} values, defined as the sample concentrations resulting in a 50% inhibition of NO production, were determined by non-linear regression analysis of percent inhibition versus concentration. N^{ω}-monomethyl-L-arginine (L-NMMA) was used as a positive control.

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