Cyclooxygenase-2 Inhibitory Phenylbutenoids from the Rhizomes of *Zingiber cassumunar*

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Received April 25, 2005; accepted July 20, 2005

Phenylbutenoids 1—6 isolated previously from the $CHCl_3$ extracts of the rhizomes of *Zingiber cassumunar*, were evaluated for their cyclooxygenase-2 (COX-2) inhibitory activity along with a new isolate, 7 from the *n*-BuOH extracts of this plant. The COX-2 inhibitory assay was performed by measuring prostaglandin E_2 production in lipopolysaccharide-stimulated mouse macrophage RAW 264.7 cells. Two phenylbutenoid dimers, 1 and 2, exhibited considerable activity with IC_{50} values of 2.71 and 3.64 μM. Two phenylbutenoid monomers, 3 and 4, showed moderate activity (IC_{50} 14.97, 20.68 μM, respectively). The other three phenylbutenoids, 5—7, were found to be inactive. Compound 7 was elucidated as a new phenylbutenoid glycoside, namely, (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-*O*-β-D-glucopyranoside by spectral analysis including various 1D- and 2D-NMR experiments.

Key words *Zingiber cassumunar*; Zingiberaceae; cyclooxygenase-2; phenylbutenoid dimer; (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-*O*-β-D-glucopyranoside

Zingiber cassumunar Roxb. (Zingiberaceae) is the tropical ginger that has been widely distributed in Southeast Asia.[1] The ginger rhizomes have been traditionally used for gastrointestinal distress and to prevent motion sickness.[2] Previous phytochemical work on the rhizomes of *Z. cassumunar* has resulted in the isolation of various types of phenylbutenoids, curcuminoids, and sesquiterpenoids.[3—13] Some of these compounds were found to have diverse biological activities such as cytotoxic,[3,4] antioxidant,[10—12] anti-insecticidal,[14] anti-inflammatory,[15—17] and uterine relaxant activity.[18]

In our previous study, two phenylbutenoid dimers, (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**1**) and (±)-*trans*-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**2**), and four phenylbutenoids, 4-(2,4,5-trimethoxyphenyl)-but-1,3-diene (**3**), 4-(3,4-dimethoxyphenyl)but-1,3-diene (**4**),[3] (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol (**5**), and (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate (**6**)[4] were isolated from the $CHCl_3$ extract of the rhizomes of *Z. cassumunar*. Furthermore, extensive column chromatography of a *n*-BuOH extract of the same plant led to the isolation of a new phenylbutenoid glycoside, (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-*O*-β-D-glucopyranoside (**7**). All isolates were evaluated for their inhibitory activity of PGE_2 production (for COX-2 inhibitory activity) in a cell culture system, using LPS–activated murine macrophage RAW 264.7 cells. Herein, we describe the structural elucidation of **7** and the results of biological evaluations on compounds **1**—**7**.

Compound **7** was deduced to have an elemental formula of $C_{19}H_{28}O_7$ by HR-EI-MS which showed a molecular ion peak at *m/z* 370.1631. The IR spectrum showed a strong absorption band at 3401 cm^{-1} for one or more hydroxyl groups. The UV spectrum of **7** exhibited an absorption maximum at 260 nm, indicating the presence of one or more aromatic system(s). Two methoxy groups appeared at δ_H 3.81 (3H, s, OCH_3-3′)/δ_C 55.4 and 3.79 (3H, s, OCH_3-4′)/55.5 in the ^{1}H- and ^{13}C-NMR spectra of **7**. These methoxy groups were assigned to C-3′ and C-4′, respectively, by their three-bond connectivities of OCH_3-3′/C-3′ and OCH_3-4′/C-4′ in the HMBC experiment. Signals for an aromatic system appeared at δ_H 7.03 (1H, d, J=1.5 Hz, H-2′)/δ_C 109.6, 6.89 (1H, dd, J=8.1, 1.5 Hz, H-6′)/119.2, and 6.86 (1H, d, J=8.1 Hz, H-5′)/112.1. The ^{1}H- and ^{13}C-NMR spectra of **7** also showed signals for an olefinic group at δ_H 6.42 (1H, d, J=16.0 Hz, H-4)/δ_C 131.5, 6.31 (1H, dt, J=16.0, 6.9 Hz, H-3)/124.9, and 2.47 (2H, ddd, J=14.0, 6.9, 1.2 Hz, H-2)/33.5. The protons, H-3 and H-4 had a large coupling constant (J=16.0 Hz), indicating their *trans* configuration.[19] The olefinic group was positioned between C-1′ and C-2 by the ^{1}H–^{13}C HMBC correlation of H-4/C-1′, C-2′, C-6′, H-3/C-1′, C-2, C-1, and H-2/C-4, C-3, C-1 (Fig. 2). The presence of a β-D-glucopyranoside was identified by the ^{1}H- and ^{13}C-NMR data of **7**. Its stereochemistry was confirmed by the ^{1}H–^{1}H NOESY spectrum as shown in Fig. 1. The anomeric proton signal at δ_H 4.33 had a large coupling constant, J=7.8 Hz, indicating β configuration. As a result, the structure of **7** was determined

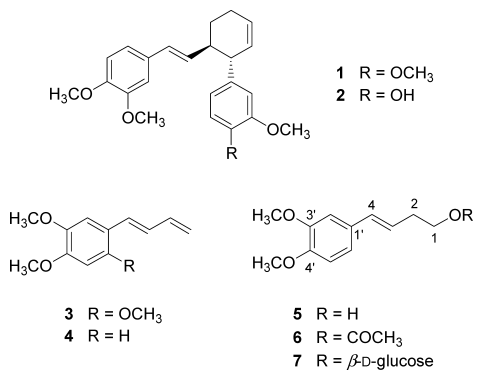

Fig. 1. Structures of Compounds **1**—**7** Isolated from *Z. cassumunar*

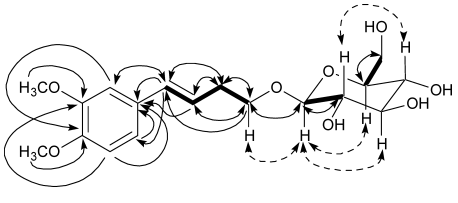

Fig. 2. Important ^{1}H–^{1}H COSY (—), ^{1}H–^{1}H NOESY (···), and ^{1}H–^{13}C HMBC (—) Correlations of Compound **7**

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Table 1. The Inhibitory Effects of Isolates **1**—**7** from *Z. cassumunar* on LPS-Induced PGE_2 Production in RAW 264.7 Cells

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Celecoxib[a)]
IC_{50} (μM)[b)]	2.71	3.64	14.97	20.68	>50	>50	>50	0.52 (nM)

a) Celecoxib was used as a positive control. *b*) IC_{50} value represents the molar concentration (μM) giving 50% inhibition.

as a new compound, namely, (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-*O*-β-D-glucopyranoside.

The COX-2 inhibitory assay was performed by measuring prostaglandin E_2 (PGE_2) production in LPS-activated murine macrophage RAW 264.7 cells. Prostaglandins (PGs) are formed from arachidonic acid by the action of COX and involved in various pathophysiological processes including inflammation and carcinogenesis. It has been known that two forms of the COX enzyme exist, designated COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and cells and is thought to control synthesis of those PGs important for normal cellular functions such as gastrointestinal integrity and vascular homeostasis. In contrast, COX-2 is not normally present, but is inducible in certain cells in response to inflammatory stimuli and control of cell growth.[20,21)] Thus, compounds that inhibit the activity of COX-2 might be an important target for anti-inflammation or cancer chemoprevention. In the present study, phenylbutenoid dimers, **1** and **2**, showed the most potent COX-2 inhibitiory activity with the IC_{50} values of 2.71 and 3.64 μM, respectively. This type of phenylbutenoid dimers might be a good candidate for the COX-2 inhibitory agents because the two similar compounds exhibited similar potency, although it seems hard to conclude only with these two compounds. The phenylbutenoid monomers, **3** and **4**, containing butadiene functionalities, exhibited the moderate COX-2 inhibitory activity with the IC_{50} values of 14.97 and 20.68 μM, respectively. The other three phenylbutenoid monomers, **5**—**7**, containing butene groups, were found to be inactive in the present COX-2 assay system. From these results, we can assume that a conjugated system in the butadiene side chain might be very important for the COX-2 inhibitory activity in the phenylbutenoid monomers. Further study needs to establish the structure–activity relationship between phenylbutenoids and COX-2 inhibition. To the best of our knowledge, this is the first report on the evaluation of compounds, **1**—**7** for their COX-2 inhibitory activity.

Experimental

General Experimental Procedures Optical rotations were measured with a P-1010 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, U.S.A.), respectively. 1D and 2D NMR experiments were performed on a UNITY INOVA 400 MHz FT-NMR instrument (Varian, CA, U.S.A.) with tetramethylsilane (TMS) as internal standard. EI-MS was obtained on a JMS 700 Mstation HR-MS spectrometer (JEOL, Japan) operating at 70 eV. Silica gel (230—400 mesh, Merck, Germany) and RP-18 (YMC-GEL ODS-A, 12 nm, S-150 μm, YMC, Japan) were used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (silica gel, 0.25 mm layer thickness, Merck, Germany) and RP-18 F_{254s} (Merck, Germany) plates, with visualization under UV light (254, 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120 °C, 5 min).

Plant Material The rhizomes of *Zingiber cassumunar* Roxb. (Zingiberaceae) were collected in Surabaya, Indonesia, in June 2001 and were identified by Prof. Tri Windono (University of Surabaya, Indonesia). The voucher specimen (No. 20/DT/VI/2001) has been deposited at the University of Surabaya.

Extraction and Isolation The dried rhizomes of *Z. cassumunar* (500 g) were extracted with MeOH (3×2 l) overnight at room temperature. The solvent was evaporated *in vacuo* to afford a concentrated MeOH extract, which was then suspended in water (200 ml). It was partitioned with *n*-hexane (4×200 ml), $CHCl_3$ (3×200 ml), and *n*-BuOH (2×200 ml), subsequently. The *n*-BuOH extract (3.3 g) was separated by a silica gel flash column chromatography (ϕ 5.5 cm; 230—400 mesh, 500 g) with a gradient of CH_2Cl_2–MeOH–H_2O (9 : 1 : 0.05→1 : 1 : 0.1, 5 l each) as a mobile phase, affording 15 fractions (FI—FXV). FII (33.8 mg) eluted with CH_2Cl_2–MeOH–H_2O (9 : 1 : 0.05), was subjected to a reversed-phase column chromatography (ϕ 2 cm; ODS-A, 12 nm, S-150 μm, 30 g) with a gradient of MeOH–H_2O (1 : 2→1 : 1, 1 l each) as a solvent system, yielding compound **7** (12 mg, RP-18 TLC R_f 0.3, MeOH–H_2O=1 : 1).

(*E*)-4-(3,4-Dimethoxyphenyl)but-3-en-1-*O*-β-D-glucopyranoside (**7**): Pale yellow gum. $[\alpha]_D^{25}$ −21.4° (*c*=0.23, MeOH). UV λ_{max} (MeOH) nm (log ε): 260 (2.51), 212 (4.28). IR ($CHCl_3$) cm^{-1}: 3401, 2935, 1603, 1515, 1264, 1024. ^{1}H-NMR (400 MHz, acetone-d_6) δ: 7.03 (1H, d, *J*=1.5 Hz, H-2′), 6.89 (1H, dd, *J*=8.1, 1.5 Hz, H-6′), 6.86 (1H, d, *J*=8.1 Hz, H-5′), 6.42 (1H, d, *J*=16.0 Hz, H-4), 6.31 (1H, dt, *J*=16.0, 6.9 Hz, H-3), 4.33 (1H, d, *J*=7.8 Hz, H-1″), 3.95 (1H, dt, *J*=14.0, 4.8 Hz, H-1a), 3.84 (1H, dd, *J*=11.6, 1.8 Hz, H-6″a), 3.81 (3H, s, OCH_3-3′), 3.79 (3H, s, OCH_3-4′), 3.67 (1H, dd, *J*=11.6, 5.2 Hz, H-6″b), 3.62 (1H, dt, *J*=14.0, 4.8 Hz, H-1b), 3.41 (1H, t, *J*=8.6 Hz, H-3″), 3.35 (1H, t, *J*=8.6 Hz, H-4″), 3.31 (1H, m, H-5″), 3.19 (1H, dd, *J*=8.6, 7.8 Hz, H-2″), 2.47 (2H, ddd, *J*=14.0, 6.9, 1.2 Hz, H-2). ^{13}C-NMR (100 MHz, acetone-d_6) δ: 149.8 (s, C-3′), 149.2 (s, C-4′), 131.5 (d, C-4), 131.1 (s, C-1′), 124.9 (d, C-3), 119.2 (d, C-6′), 112.1 (d, C-5′), 109.6 (d, C-2′), 103.5 (d, C-1″), 77.4 (d, C-3″), 76.8 (d, C-5″), 74.2 (d, C-2″), 71.1 (d, C-4″), 69.1 (t, C-1), 62.4 (t, C-6″), 55.5 (q, OCH_3-4′), 55.4 (q, OCH_3-3′), 33.5 (t, C-2). LR-EI-MS *m/z* (rel. int.): 370 ($[M]^+$, 15), 267 (28), 177(50), 164 (100). HR-EI-MS *m/z*: 370.1631 $[M]^+$ (Calcd for $C_{19}H_{28}O_7$, 370.1628).

Measurements of PGE_2 Accumulation by COX-2 in Cultured LPS-Induced RAW264.7 Cells RAW264.7 macrophage cells were maintained in DMEM supplemented with penicillin–streptomycin and 10% FBS at 37 °C, 5% CO_2 in humidified air. For evaluating the inhibitory activity of test materials on COX-2, the cells were allowed to adhere for 2 h in the presence of aspirin (250 μM) to inactivate endogenous COX in 96-well culture plate, washed three times with media, and then incubated in the fresh medium with 1 μg/ml of LPS. Test materials were simultaneously added to each well. After the additional 16 h incubation, the media were removed and analyzed by PGE_2 enzyme immunometric assay (EIA). PGE_2-EIA was performed according to Pradelles (1985) with the minor modification.[22)] In these assays, 100% activity was defined as the difference between PGE_2 accumulation in the absence and in the presence of LPS for 16 h in triplicate determination. % Inhibition was expressed as [1−(PGE_2 level of sample)÷PGE_2 level of vehicle treated-control]×100.

Acknowledgments This study was supported by the Korea Science and Engineering Foundation for the National R&D Program for Enhancing R&D Infrastructure of Women's University [R06-2002-011-01002-0(2004)]. We are grateful to late Dr. Gwang-Ho Jeohn for plant collection and this paper is dedicated to him.

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