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A New Germacrane-Type Sesquiterpene from Fermented *Curcuma longa* L.

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Curcuma longa L. (Zingiberaceae), also known as turmeric, has been widely cultivated as a vegetable and spice in South and Southeastern Asia. In traditional medicine, it has been used extensively due to various beneficial properties, including anti-arthritic, reducing cholesterol level, and anti-inflammation.[1,2] Current studies on the pharmacological properties of *C. longa* showed anti-atherosclerotic, anti-diabetic, anti-mutagenic, antioxidant, and anti-cancer effects.[3,4] Essential oil and curcuminoids are known as the major active components of *C. longa*, and bisabolane-type sesquiterpenes were highly presented in the volatile oil.[5] *C. longa* is also used popularly in food as an active ingredient in curries and mustards.[6] However, its strong flavor and taste decreased the consumers' palatability, making it unsuitable for industrial applications. Currently, fermented *C. longa* L. is known to reduce bitterness and harsh taste, improving the flavor and taste and providing an increase of consumption.[7] So far, several pharmacological effects of fermented *C. longa*, including hepatoprotective[8] and anti-obesity[9] have been reported whereas little is known about the chemical components of fermented *C. longa* extract. In the present study, five compounds, including one new sesquiterpene (**1**) were isolated from the fermented *C. longa* extract by application of various chromatographic methods. Furthermore, cytoprotective, protein tyrosine phosphatase 1B (PTP1B) inhibitory, and nitrite inhibitory activities of the isolates were also evaluated.

Result and Discussion

Compound **1** was isolated as yellow viscous oil. The molecular formula of **1** was determined to be $C_{15}H_{24}O_5$ from a pseudomolecular ion *m/z* 249.1508 $[M + H - 2H_2O]^+$ (calcd. for $C_{15}H_{21}O_3$, 249.1491) in the HRESITOFMS. The ^{1}H NMR spectrum of **1** exhibited signals for one olefinic proton at δ 5.78 (1H, br s, H-9), two oxymethines [δ 4.29 (1H, m, H-1) and 3.52 (1H, dd, J = 2.0, 10.8 Hz, H-5)], one oxymethylene [δ 4.23 and 4.07 (each d, J = 12.4 Hz, H_2-12)], two vinylic methyls [δ 1.73 (3H, s, H_3-13) and 1.71 (3H, s, H_3-15)], and one tertiary methyl at δ 1.19 (3H, s, H_3-14)] (Table 1). The ^{13}C NMR and DEPT spectra displayed 15 carbon signals, including three methyls, four methylenes, three methines, and five quaternary carbons. Analysis of ^{13}C NMR spectrum revealed the presence of one carbonyl carbon [δ 207.8 (C-8)] and two olefinic group carbon signals [δ 145.0 (C-10), 140.7 (C-7), 133.6 (C-11), 126.1 (C-9)]. The positions of two double bonds at C-7 and C-9 were assigned by HMBC correlations from H_3-13 to C-7 and C-11, and from H_3-15 to C-9 and C-10, respectively (Fig. 2). The ^{13}C NMR spectrum further showed signals for an oxygenated quaternary carbon at δ87.7 (C-4), two oxymethines at δ 82.4 (C-1) and 78.9 (C-5), one oxymethylene at δ 63.2 (C-12), and three methylenes [δ 39.0 (C-3), 35.9 (C-6), and 31.9 (C-2)]. The HMBC correlations from H_3-14 to C-3, C-4, and C-5, and from H_3-15 to C-1, along with the ^{1}H-^{1}H COSY cross-peaks between H-1/H_2-2, H_2-2/H_2-3, and H5/H_2-6

Table 1. ^{1}H and ^{13}C NMR data for compound **1**

Position	δ_H[a,b] (mult., J in Hz)	δ_C[a,c]
1	4.29 (m)	82.4
2	2.21 (m)	31.9
	1.73 (m)	
3	2.05 (m)	39.0
	1.69 (m)	
4		87.7
5	3.52 (dd, 2.0, 10.8)	78.9
6	2.65 (dd, 10.8, 13.2)	35.9
	2.45 (br d, 13.2)	
7		140.7
8		207.8
9	5.78 (br s)	126.1
10		145.0
11		133.6
12	4.23 (d, 12.4)	63.2
	4.07 (d, 12.4)	
13	1.73 (s)	17.8
14	1.19 (s)	21.2
15	1.71 (s)	19.9

[a]Spectra were recorded in CD_3OD. [b]400 MHz, [c]100MHz

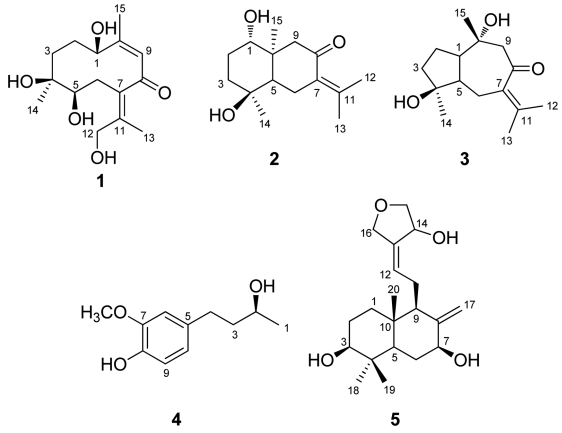

Figure 1. Structures of compounds **1-5**.

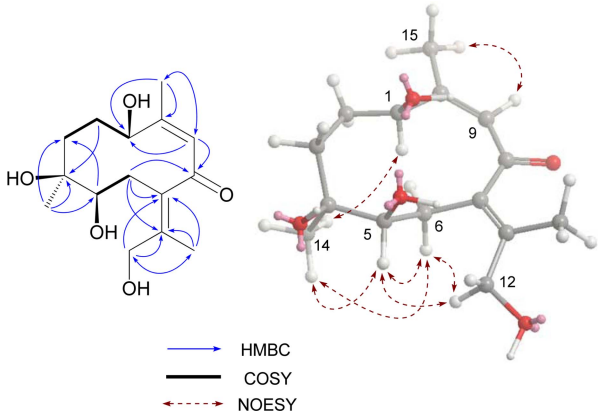

Figure 2. Selected HMBC, COSY, and NOESY correlations of compound **1**.

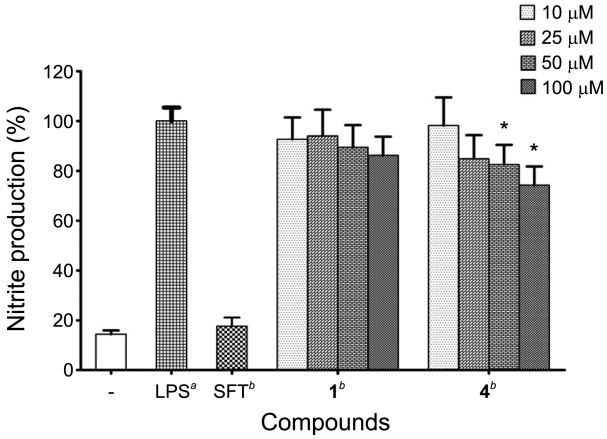

Figure 3. Effects of compounds **1** and **4** on the production of nitrite in LPS-stimulated RAW264.7 cells. The values are mean ±SD (n = 3). [a]Stimulated with LPS. [b]Stimulated with LPS in the presence of **1** and **4** (10, 25, 50, and 100 mM), and positive control, sulfuretin (SFT, 10 μM). Statistical significance is indicated as *($p < 0.05$) as determined by Dunnett's multiple comparison test.

allows to assign the positions of the two oxymethine groups (C-1 and C-5), three methylene groups (C-2, C-3, and C-6), and one oxygenated quaternary carbon (C-4). Furthermore, the HMBC cross peaks of H-6 with C-7, C-8, and C-11, along with HMBC correlation from H-9 to C-8 clearly indicated that the carbonyl group was located at C-8. The position of oxymethylene group (C-12) was suggested by HMBC correlation from H_3-13 to C-12. Based on these analyses, the planar structure of **1** was determined to be a germacrane-type sesquiterpene. The geometry of double bonds and relative configuration of **1** was established by NOESY spectrum. The NOE cross peaks between H_2-12/H-5 and H_2-12/H-6b confirmed the position of the oxymethylene group (C-12) and showed *E* geometry of the double bond at C-7. The NOESY correlation of H-9 with H_3-15 indicated *Z* configuration of the double between C-9 and C-10. The NOESY correlations between H-1/H_3-14 and between H-5/H_3-14 suggested that these groups are positioned at the same plane of the ring (Fig. 2). It is noted that the structure of **1** was similar to that of the reported compound, heyneanone A, exept for the presence of hydroxyl group at C-12 in **1**.[10] The similar NMR data and specific optical rotation values of these two compounds [**1**: $[\alpha]_D^{21}$ −34.2° (*c* = 0.19, $CHCl_3$); heyneanone A: $[\alpha]_D^{21}$ −21° (*c* = 0.23, $CHCl_3$)] suggested that both share the same configuration.[10] Thus, the gross structure of compound **1** was established as 7*E*,9*Z*-1*β*,4*β*,5*β*,12-tetrahydroxy-7(11),9-germacradien-8-one, named longanone A.

Structures of the known compounds were identified as: 1*α*,4*β*-dihydroxyeudesman-8-one (**2**),[11] zedoarondiol (**3**),[12] 4-(4-hydroxy-3-methoxyphenyl)-2*S*-butanol (**4**),[13] and cononarin I (**5**)[14] by analyses of their NMR and MS data, and comparison with those of reported compounds (Fig. 1).

Cytoprotective effects of the metabolites **1-5** were assessed using glutamate-induced cytotoxicity in mouse hippocampal HT-22 cells.[15] In addition, PTP1B inhibitory effects of compounds **1-5** were also evaluated.[16] As the result, all the metabolites showed no significant cytoprotective and PTP1B inhibitory effects (data not shown).

Nitrite production, a stable end product of NO oxidation process, was used as a measure of iNOS activity. The inhibitory effects of the isolated compounds on nitrite production were also evaluated in LPS-stimulated RAW264.7 cells (Fig. 3).[17] Among the five compounds, compound **1** showed nitrite inhibitory effect at the concentrations of 50 and 100 μM, with the inhibition of 10.6 and 13.8%, respectively. Compound **4** inhibited the nitrite production at the concentrations of 25 μM, 50 μM, and 100 μM in a dose-dependent manner, with the inhibition of 15.2, 17.5, and 25.8%, respectively. Compounds **2**, **3**, and **5** showed no inhibitory effects on nitrite production in LPS-stimulated RAW264.7 cells (data not shown).

Experimental

General Procedures. Optical rotations were recorded using a Jasco P-2000 digital polarimeter. NMR spectra (1D and 2D) were recorded in CD_3OD or $CDCl_3$ using a JEOL JNM ECP-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to the corresponding residual solvents signals (CD_3OD: δ 4.80 and

3.30/49.0; $CDCl_3$: δ 7.25/77.0). HMQC and HMBC experiments were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively. ESIMS data were obtained using a Q-TOF micro LC-MS/MS instrument (Waters) at Korea University, Seoul, Korea. TLC was performed on Kieselgel 60 F_{254} (1.05715; Merck, Darmstadt, Germany) or RP-18 F_{254s} (Merck) plates. Spots were visualized by spraying with 10% aqueous H_2SO_4 solution, followed by heating. Column chromatography was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and YMC octadecyl-functionalized silica gel (C_{18}).

Plant Material and Fermentation. Raw *C. longa* L. was harvested and obtained from Jindo, Jellanamdo, Korea, and was authenticated by Dr. Humyoung Baek at Korea INS Pharm Research Institute (Hwasun, Korea). The voucher specimen was deposited in the same institute. The powder of *C. longa* L. was fermented with 2% (wt/wt) of *Aspergillus oryzae* at 25 °C for 36 h and dried.

Extraction and Isolation. Fermented *C. longa L.* was sterilized at 121 °C for 15 min, and was extracted with distilled water at room temperature. The dried extracts (600 g) was dissolved then applied to open column chromatography filled with amberlite XAD-2 resin, eluting with a stepwise gradient of 100% H_2O, 25% MeOH/H_2O, 50% MeOH/H_2O, 75% MeOH/H_2O, and 100% MeOH. The MeOH 25% fraction was concentrated (10 g) and suspended in H_2O, and then partitioned successively with CH_2Cl_2, EtOAc, and BuOH to give CH_2Cl_2, EtOAc, BuOH, and aqueous extracts, respectively. The CH_2Cl_2, EtOAc, BuOH extracts were combined and concentrated *in vacuo* to give a fraction FCLA (900 mg). Fraction FCLA was subjected to reversed phase C_{18} column chromatography (CC), stepwise eluted with MeOH-H_2O (1:3 to 2:1) to provide 12 fractions: FCLA1 (160 mg), FCLA2 (45 mg), FCLA3 (32 mg), FCLA4 (64 mg), FCLA5 (66 mg), FCLA6 (63 mg), FCLA7 (79 mg), FCLA8 (50 mg), FCLA9 (110 mg), FCLA10 (52 mg), FCLA11 (38 mg), and FCLA12 (31 mg). Fraction FCLA6 was chromatographed over silica gel column, eluting with CH_2Cl_2-MeOH (10:1 to 3:1) to give 3 subfractions, FCLA61-FCLA63. Subfraction FCLA62 was further purified by preparative TLC using CH_2Cl_2-MeOH (5:1) as eluent to obtain compound **5** (1.2 mg). Fraction FCLA7 was subjected to silica gel CC, eluting with *n*-Hexane:Acetone (2:1) to give compound **3** (15 mg) and five subfractions FCLA7A-E. Fraction FCLA7E was further chromatographed over silica gel column, using CH_2Cl_2-MeOH (15:1) to obtain compound **1** (1.5 mg). Fraction FCLA9 was subjected to Sephadex LH-20 CC, eluted with CH_2Cl_2-MeOH (5:1) to give 5 subfractions, FCLA91-FCLA95. From subfraction FCLA92, by using silica gel CC and eluting with *n*-Hexane-EtOAc (1:1 to 1:5), compound **4** (1.5 mg) was isolated along with three subfractions, FCLA922-FCLA924. Subfraction FCLA923 was further purified by silica gel CC, using *n*-Hexane-EtOAc-MeOH (1:5:0.1) as eluent to give compound **2** (1.4 mg).

Longanone A (1): Yellow viscous oil; $[\alpha]_D^{25}$ −34.2 (*c* 0.19, $CHCl_3$). HR-ESITOFMS: *m/z* 249.1508 $[M+H-2H_2O]^+$ (calcd. for $C_{15}H_{21}O_3$, 249.1491). ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see Table 1.

1α,4β-Dihydroxyeudesman-8-one (2): Colorless gum; $[\alpha]_D^{25}$ −9.9 (*c* 0.18, MeOH). ESIMS *m/z* 253 $[M+H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 3.37 (m, H-1), 3.03 (dd, *J* = 4.4, 15.6 Hz, H-6a), 2.54 (d, *J* = 15.6 Hz, H-6b), 1.97 (d, *J* = 1.6 Hz, H_3-12), 1.86 (d, *J* = 1.6 Hz, H_3-13), 1.19 (s, H_3-14), 0.87 (d, *J* = 1.2 Hz, H_3-15). ^{13}C NMR (100 MHz, CD_3OD) δ 79.0 (C-1), 29.2 (C-2), 41.6 (C-3), 72.3 (C-4), 51.6 (C-5), 27.1 (C-6), 132.1 (C-7), 205.4 (C-8), 58.1 (C-9), 42.8 (C-10), 145.7 (C-11), 22.4 (C-12), 22.6 (C-13), 23.5 (C-14), 13.2 (C-15).

Zedoarondiol (3): Colorless gum; $[\alpha]_D^{25}$ −40.7 (*c* 0.9, MeOH). ESIMS *m/z* 253 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.93 (d, *J* = 12.8 Hz, H-9a), 2.56 (d, *J* = 12.8 Hz, H-9b), 1.79 (s, H_3-12), 1.89 (s, H_3-13), 1.14 (s, H_3-14), 1.16 (s, H_3-15). ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.9 (C-1), 22.9 (C-2), 28.5 (C-3), 80.0 (C-4), 51.9 (C-5), 39.7 (C-6), 134.7 (C-7), 203.2 (C-8), 59.9 (C-9), 72.7 (C-10), 142.2 (C-11), 21.9 (C-12), 22.2 (C-13), 22.6 (C-14), 20.0 (C-15).

4-(4-Hydroxy-3-methoxyphenyl)-2*S*-butanol (4): Colorless viscous liquid; $[\alpha]_D^{25}$ +33.9 (*c* 0.15, MeOH). ESIMS *m/z* 197 $[M+H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 1.17 (d, *J* = 6.0 Hz, H_3-1), 3.71 (m, H-2), 6.76 (d, *J* = 2.0 Hz, H-6), 6.68 (d, *J* = 8.0 Hz, H-9), 6.61 (dd, *J* = 2.0, 8.0 Hz, H-10), 3.82 (s, 7-OMe). ^{13}C NMR (100 MHz, CD_3OD) δ 23.5 (C-1), 67.9 (C-2), 32.7 (C-3), 42.3 (C-4), 135.2 (C-5), 113.1 (C-6), 148.8 (C-7), 145.4 (C-8), 116.0 (C-9), 121.7 (C-10), 56.3 (7-OMe).

Conorarin I (5): Colorless viscous liquid; $[\alpha]_D^{25}$ −9.5 (*c* 0.11, $CHCl_3$). ESIMS *m/z* 337 $[M+H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 3.20 (m, H-3), 1.17 (m, H-5), 3.88 (m, H-7), 1.65 (m, H-9), 5.47 (d, *J* = 6.0 Hz, H-12), 4.67 (m, H-14), 3.62 (m, H-15a), 3.51 (m, H-15b), 4.11 (d, *J* = 12.4 Hz, H-16a), 3.97 (d, *J* = 12.4 Hz, H-16b), 5.21 (br s, H-17a), 4.67 (br s, H-17b), 0.99 (s, H_3-18), 0.77 (s, H_3-19), 0.72 (s, H_3-20). ^{13}C NMR (100 MHz, CD_3OD) δ 38.2 (C-1), 28.7 (C-2), 79.4 (C-3), 40.0 (C-4), 53.6 (C-5), 34.2 (C-6), 74.4 (C-7), 151.1 (C-8), 56.5 (C-9), 40.1 (C-10), 23.2 (C-11), 131.7 (C-12), 138.9 (C-13), 72.0 (C-14), 66.3 (C-15), 64.3 (C-16), 105.5 (C-17), 28.9 (C-18), 16.2 (C-19), 15.0 (C-20).

Cell Culture. RAW264.7 and HT22 cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air at 5×10^5 cells/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM).

Cytoprotective Assay. HT22 cells were pre-treated for 3 h with indicated concentrations of compounds, and then incubated for 12 h with glutamate (5 mM). The effects of various experimental modulations on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into fomazan crystals. The synthesis of formazan is proportional to the number of functional mitochondria in living cells. For the determination of cell viability, 50 mg/mL of MTT was added to 1 mL of cell suspension (1×10^5

cells/mL in 96-well plates) for 4 h. The formazan synthesized was dissolved in acidic 2-propanol and the optical density was measured at 590 nm. The optical density of the formazan formed in control (untreated) cells was considered as 100% viability.

Nitrite Production Determination. RAW264.7 macrophages were pretreated for 3 h with indicated concentrations of compounds, and treated 24 h with LPS (1 μg/mL). The nitrite concentration in the medium was measured as an indicator of NO production as per the Griess reaction. The nitrite present in the conditioned media was determined spectrophotometrically using the Griess reaction. An aliquot (100 μL) of each supernatant was mixed with an equal volume of Griess reagent (0.1% [w/v] *N*-(1-naphthyl)-ethylenediamine and 1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid) for 10 min at room temperature. The absorbance of the final reactant at 525 nm was measured using an ELISA plate reader, and the nitrite concentration was determined using a standard curve of sodium nitrite prepared in DMEM without phenol red.

PTP1B Inhibitory Activity Assay. PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc. A reaction mixture containing 0.05 μg PTP1B and 2 mM *p*-nitrophenyl phosphate (*p*-NPP) in a buffer consisting of 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol with or without a tested compound solution (prepared in the above buffer solution containing 3% DMSO) was incubated at 37 °C for 30 min. The reaction was terminated by addition of 10 N NaOH. The amount of produced *p*-nitrophenol was evaluated by measuring the increase in absorbance at 405 nm. The blank was measured in the same way except for adding buffer solution instead of the enzyme.

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Supporting Information. The HR-ESITOFMS, ^{1}H, ^{13}C, HMQC, and HMBC NMR spectra of compound **1**.

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