A Sphingolipid and Tyrosinase Inhibitors from the Fruiting Body of *Phellinus linteus*

Hye Sook Kang, Jin Ho Choi, Won Ki Cho, Jong Cheol Park[1], and Jae Sue Choi

Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Korea and [1]Dept. of Oriental Medicine Resources, Suncheon National University, Suncheon 540-742, Korea

This paper for the first time reports the isolation of 5 compounds from *Phellinus linteus*. A sphingolipid (1) and two tyrosinase inhibitory compounds (2, 3) along with two carboxylic acids (4, 5), were isolated from the fruiting body of *Phellinus linteus* (Berk & Curt) Aoshima. The structure of compound 1 was identified as 1-O-β-D-glucopyranosyl-(2S, 3R, 4E, 8E)-2-[(2R)-2-hydroxyhexadecanoylamino]-9-methyl-4,8-octadecadiene-1,3-diol, known as cerebroside B, based on spectroscopic methods such as 1D and 2D NMR as well as by acid hydrolysis. Compounds 2~5 were identified as protocatechualdehyde (2), 5-hydroxymethyl-2-furaldehyde (HMF) (3), succinic acid (4), and fumaric acid (5) based on the spectroscopic evidence. Compounds 2 and 3 inhibited the oxidation of L-tyrosine catalyzed by mushroom tyrosinase with an IC_{50} of 0.40 and 90.8 µg/mL, respectively. The inhibitory kinetics, which were analyzed by the Lineweaver-Burk plots, were found to be competitive and noncompetitive inhibitors with a K_i of 1.1 µM and 1.4 mM, respectively.

Key words: *Phellinus linteus*, Sphingolipid, Cerebroside B, Tyrosinase inhibitor

INTRODUCTION

Tyrosinase (EC 1.14.18.1), which is also known as polyphenol oxidase (PPO) (Mayer, 1987; Whitaker, 1995), is a multifunctional, copper-containing oxidase that catalyzes three distinct reactions of melanin synthesis, hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to dopaquinone and the conversion of 5,6-dihydroxyindole to melanochrome. Tyrosinase is found in microorganisms, animals and plants, and is known to cause the browning of some fruits, vegetables, and crustaceans, which significantly decreases their nutritional and market values. The enzymatic oxidation of L-tyrosine to melanin is of considerable importance because melanin has many functions, and alterations in melanin synthesis occur in many disease states. In addition, tyrosinase is a key enzyme in the insect molting process (Andersen, 1979). Therefore, tyrosinase its inhibitors might ultimately provide clues for controlling insect pests. Tyrosinase inhibitors have become increasingly important in cosmetic

and medical (Kim *et al.*, 2002; Pérez-Bernal *et al.*, 2000) products related to hyperpigmentation. Furthermore, it has been reported that tyrosinase might be central to dopamine neurotoxicity as well as contributing to the neurogeneration that is associated with Parkinsons disease (Xu *et al.*, 1997).

Phellinus linteus (Berk & Curt) Aoshima, which is commonly referred to as Sangwhang in Korea, is a fungus belonging to the Hymenochaetaceae basidiomycetes. *Phellinus linteus* is found mainly in tropical America and Africa (Dai and Xu, 1998). This mushroom has been long used as a traditional oriental medicine for treating stomachache and arthritis of the knee. The various bioactive properties of *P. linteus* have attached considerable attention since Ikekawa *et al.* (1968) first reported the anti-tumor effect of this mushroom. Several biological activities of *P. linteus* have been reported. These include antitumor activity (Sasaki *et al.*, 1971; Kim *et al.*, 1996; Cho *et al.*, 2002; Rhee *et al.*, 2000), antimutagenic activity (Shon and Nam, 2001), anti-angiogenic, antioxidant and xanthine oxidase inhibition (Song *et al.*, 2003) activity, inhibitory effects on the harmful intestinal bacterial enzymes, rat intestinal α-glucosidases (Kim *et al.*, 1998), and on tumor growth and metastasis (Han *et al.*, 1999).

Correspondence to: Jae Sue Choi, Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Korea
Tel: 82-51-620-6335, Fax: 82-51-620-6330
E-mail: choijs@pknu.ac.kr

There is little information on the compounds with biological activity, with the exception of polysaccharides, and the tyrosinase inhibitory activity of *P. linteus*. Therefore, this paper reports the isolation and structural elucidation of 5 compounds (1~5) from this mushroom, as well as the tyrosinase inhibitory activity of these compounds using L-tyrosine as a substrate and Lineweaver-Burk plots.

MATERIALS AND METHODS

General experimental procedures

The ^{1}H-, ^{13}C-NMR and DEPT spectra were recorded on a Varian UNITY-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The HMBC experiments were recorded using the pulsed field gradients. The EI-MS data were acquired using a JEOL JMS-700 spectrometer. The positive-ion LR and HR FAB-MS data were collected on a JEOL JMS-HX110/110A Tandem mass spectrometer (JEOL). GC-MS for the fatty acids were measured using a GC-MS QP-5050A gas chromatograph (Shimadzu, Japan): Conditions: GB-WAX capillary column (30 m×0.25 mm×0.2 μm); column temperature, 60°C-200°C, rate of temperature increase, 15°C/min; injector and detector (H$_2$ flame ionization detector) temperature, 240°C; H$_2$ flow rate, 0.7mL/min. Column chromatography was carried out using silica (Si) gel (Merck, 70-230 mesh) and Sephadex LH-20 and reversed phase C$_{18}$ (RP-18). Thin layer chromatography (TLC) was carried out on pre-coated Merck Kieselgel 60 F$_{254}$ plate (0.25 mm) and 50% H$_2$SO$_4$ was used as the spray reagent. The chemical shifts were referenced to the respective residual solvent peaks (δ_H 7.19, 7.56, and 8.70 and δ_C 123.5, 135.5, and 149.5 for pyridine-d_5, δ_H 3.30 and δ_C 49.0 for CD$_3$OD), recorded in values and expressed in ppm. The multiplicities of the ^{1}H-NMR signals are indicated as s (singlet), d (doublet), brs (broad singlet) and m (multiplet).

Plant materials

The *Phellinus linteus* used this experiment were kindly provided by the Cho-A Pharmaceutical Co., Ltd., and a specimen was deposited in our laboratory (No. 20020304).

Chemicals

The L-Tyrosine was purchased from Janssen Chimica (Geel Belgium), and Kojic acid and mushroom tyrosinase (EC 1.14.18.1) from the Sigma Chemical Co. (St. Louis, MO). K$_2$HPO$_4$ was obtained from Junsei Chemical Co. Ltd. (Tokyo, Japan), KH$_2$PO$_4$ from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan). All the solvents used for column chromatography were of reagent grade from commercial sources.

Isolation and identification of compounds from the fruiting body of *Phellinus linteus*

The lyophilized fruiting body of *P. linteus* (4.5 g) was powdered and refluxed with MeOH (39 L) for 3 h. The extract (838 g) was suspended in water and partitioned sequentially with CH$_2$Cl$_2$ (101.5 g), EtOAc (36.7 g), *n*-BuOH (366 g), H$_2$O (244 g). The EtOAc fraction exhibited tyrosinase inhibitory activity in a concentration-dependant manner. Therefore, the EtOAc (36 g) fraction was chromatographed over a Si gel column (880 Silica gel 60, Merck, 1.2 kg) eluted with CH$_2$Cl$_2$-MeOH 9:1 to yield 12 sub-fractions (F1~F12). The F3 (20.76 g) underwent further SiO$_2$ CC with hexane:EtOAc=3:2 to give compounds 2 (376.8 mg) and 3 (1.74 g). The F8 (6.33 g) was chromatographed over a Si gel column (5×45, Silica gel 60, Merck, 300 g) with EtOAc as the solvent to obtain 9 sub-fractions (F8-1~F8-9) and F8-4 (975 mg) was subjected to Sephadex LH-20 column chromatography with MeOH to obtain compounds 4 (47 mg) and 5 (36 mg). F8-5 (650 mg) was chromatographed over a Sephadex LH-20 column with MeOH and further purified over a C$_{18}$ sep-pak cartridge to afford compound 1 (18 mg).

Cerebroside B (1)

White amorphous powder, $[\alpha]_D^{20}$ +6° (*c* 0.43, MeOH). C$_{41}$H$_{77}$NO$_9$, negative-FABMS *m/z* 726 [M–H]$^+$, positive-FABMS *m/z* 750 [M+Na]$^+$, 710 [M–H$_2$O+H]$^+$, 548 [M-glucose+H]$^+$ HR-FABMS *m/z*: 750.5498 [M+Na]+ (Calcd for C$_{41}$H$_{77}$NO$_9$Na, *m/z* 750.5496, Δ +0.2 mmu), IR v_{max} (KBr) cm^{-1}: 3406 (OH), 2923, 2853, 1655, 1546 (amide), 1466, 1083 (glycosidic C-O), 878, 720 [(CH$_2$)$_n$], 637. ^{1}H-NMR (pyridine-d_5, 400 MHz) δ : 8.36 (1H, d, *J*=8.7 Hz, NH), 6.00 (1H, dd, *J*=15.4, 5.9 Hz, H-4), 5.92 (1H, dt, *J*=15.5, 5.2 Hz, H-5), 5.24 (1H, t, *J*=7.0 Hz, H-8), 4.91 (1H, d, *J*=7.8 Hz, H-1"), 4.80 (1H, m, H-2), 4.75 (1H, m, H-3), 4.71 (1H, dd, *J*=10.2, 3.9 Hz, H-1a), 4.56 (1H, m, H-2'), 4.50 (1H, dd, *J*=10.2, 2.5 Hz, H-6"a), 4.34 (1H, dd, *J*=11.7, 5.2 Hz, H-6"b), 4.23 (1H, dd, *J*=10.2, 3.8 Hz, H-1b), 4.21 (2H, m, H-3", 4"), 4.02 (1H, t, *J*=8.0 Hz, H-2"), 3.89 (1H, m, H-5"), 2.14 (2H, t, *J*=4.5 Hz, H-6), 2.01 (1H, m, H- 3'b),1.99 (2H, t, *J*=7.5 Hz, H-10), 1.78 (1H, m, H-4'b), 1.69 (1H, m, H-4'a), 1.60 (3H, s, H-19), 1.39 (2H, m, H-7), 1.26~1.23 [brs, (CH$_2$)$_n$], 1.21 (2H, m, H-15'), 0.84 (6H, t, *J*=6.6 Hz, H-18, 16'). ^{13}C-NMR (pyridine-d_5, 100 MHz) δ : 136.0 (C-9), 132.3 (C-5), 131.8 (C-4), 124.1 (C-8), 105.6 (C-1"), 78.5 (C-5"), 78.4 (C-3"), 75.0 (C-2"), 72.4 (C-2'), 72.3 (C-3), 71.4 (C-4'), 70.1 (C-1), 62.6 (C-6"), 54.5 (C-2), 39.9 (C-10), 35.6 (C-3'), 33.0 (C-6), 32.0 (C-16, 14'), 29.9~29.5 (C-11~15, 5'~13'), 28.1 (C-7), 25.8 (C-4'), 22.9 (C-17, 15'), 16.0 (C-19), 14.2 (C-18, 16').

Acetylation of Cerebroside B hexaacetate (1)

A mixture of compound 1 (8 mg), acetic anhydride (0.3 mL), and pyridine (0.2 mL) was allowed to stand at room temperature for 24 h. The solvent in the reaction mixture was then removed by evaporation with a N$_2$ gas stream to

afford compound **1a** (14.9 mg). ^{1}H-NMR (CDCl$_3$, 400 MHz) δ : 6.33 (1H, d, J=9.1 Hz, N-H), 5.82 (1H, d, J=15.1 Hz, H-4), 5.39 (1H, dd, J=15.2, 7.4 Hz, H-5), 5.31 (1H, t, J=6.9 Hz), 5.18 (1H, t, J=9.5 Hz), 5.15 (1H, dd, J=7.3, 4.9 Hz), 5.07 (2H, t, J=9.7 Hz), 4.95 (1H, dd, J=8.0, 9.5 Hz), 4.47 (1H, dd, J=8.0 Hz, H-1"), 4.30 (1H, m), 4.23 (1H, dd, J=12.3, 4.5 Hz), 4.13 (1H, dd, J=12.4, 2.3 Hz), 3.93 (1H, dd, J=10.3, 3.9 Hz), 3.69 (1H, ddd, J=10.0, 4.5, 2.4 Hz), 3.61 (1H, dd, J=10.4, 4.5 Hz), 2,05 (3H, t, J=3.1), 2.17 (COC$\underline{H}_3$), 2.10 (COC$\underline{H}_3$), 2.03 (COC$\underline{H}_3$), 2.02 (2×COC$\underline{H}_3$), 2.00 (COC$\underline{H}_3$), 1.94 (2H, t, J=7.2 Hz), 1.79 (2H, m), 1.57 (3H, s, C$\underline{H}_3$), 1.25 [(C$\underline{H}_2)_n$], 0.87 (6H, t, J=6.8 Hz, 2×C$\underline{H}_3$). ^{13}C-NMR (CDCl$_3$, 100 MHz) δ : 170.6, 170.2, 169.8, 169.7, 169.6, 169.4, 169.2 (all Me$\underline{C}$O, NH$\underline{C}$O), 136.8, 136.2, 124.5, 122.9, 100.5, 73.9, 73.1, 72.7, 71.9, 71.2, 68.2, 67.2, 61.8, 50.6, 39.7, 32.6, 31.9, 31.9, 31.8, 29.7~29.3, 28.0, 27.4, 24.7, 22.7, 21.0 (CO$\underline{C}$H$_3$), 20.9 (CO$\underline{C}$H$_3$), 20.7 (CO$\underline{C}$H$_3$), 20.6 (3×CO$\underline{C}$H$_3$), 16.0, 14.1, 14.1.

Methanolysis of compound 1a

Fourteen mg of compound **1a** was refluxed with 0.9 N HCl in 82% aqueous MeOH (50 mL) for 18 h. The reaction mixture was suspended with H$_2$O and extracted with *n*-hexane. The *n*-hexane soluble fraction was then dried over Na$_2$SO$_4$ and concentrated to obtain a fatty acid methyl ester (4.4 mg). The fatty acid methyl ester of compound **1a** was recrystallized with MeOH and analyzed by GC-MS (t$_R$ 22.183 min, hydroxypalmitic acid methyl ester), GC-MS (*m/z*, %): 286 ([M]$^+$, 5.2), 254 ([M–CH$_3$OH]$^+$, 3.2), 227 ([M–COOMe]$^+$, 15.2), 208, 145, 127 ([C$_9$H$_{19}$]$^+$, 5.2), 111 ([C$_8$H$_{15}$]$^+$, 10.0), 97 ([C$_7$H$_{13}$]$^+$, 23.2), 90 ([CH$_3$OC(OH)= CH$_2$]$^+$, 23.2); 83 [C$_6$H$_{11}$$^+$], 69 [C$_5H_9$$^+$], 55; [α]$_D^{20}$ 1.7° (*c* 0.3, CHCl$_3$).

Protocatechualdehyde (2)

Yellowish needles. EI-MS *m/z* (R. int): 138 (92) [M$^+$], 137 (100) [M–H], 109 (73), 81 (65), 63 (33), 53 (34). IR v_{max} (KBr) cm^{-1}: 3222, 3329, 1652, 1596, 1535, 1445, 1297, 1165. ^{1}H-NMR (CD$_3$OD) δ: 9.68 (C$\underline{H}$O), 7.30 (1H, dd, J=1.70, 9.48 Hz), 7.28 (1H, d, J=1.41 Hz), 6.90 (1H, d, J=8.1 Hz). ^{13}C-NMR (CD$_3$OD) δ: 193.8 ($\underline{C}$HO), 154.5 (C-4), 148.0 (C-3), 131.6 (C-1), 127.2 (C-6), 117.0 (C-5), 116.2 (C-2).

5-Hydroxymethyl-2-furaldehyde (3)

Yellow oil. EI-MS *m/z*: 126 [M$^+$]. UV λ_{max}^{MeOH} nm (log ε): 279 (4.72). IR v_{max} (KBr) cm^{-1}: 3380 (OH), 2848, 1655 (C=O). ^{1}H-NMR (CD$_3$OD) δ: 9.52 (1H, brs, -CHO), 7.37 (1H, d, J=3.5 Hz, H-3), 6.67 (1H, d, J=3.5 Hz, H-4), 4.60 (2H, s, CH$_2$OH). ^{13}C NMR (CD$_3$OD) δ: 180.2 (-CHO), 164.0 (C-5), 154.7 (C-2), 125.6 (C-3), 111.7 (C-4), 58.4 (CH$_2$OH).

Succinic acid (4)

White crystals, C$_4$H$_6$O$_4$, ^{1}H-NMR (MeOH-d_4, 400 MHz) δ:

2.55 (4H, s, CH$_2$), ^{13}C-NMR (MeOH-d_4, 100 MHz) δ: 30.6 (CH$_2$), 176.9 (COOH).

Fumaric acid (5)

White crystals, C$_4$H$_4$O$_4$, ^{1}H-NMR (MeOH-d_4, 400 MHz) δ: 6.75 (2H, s, CH). ^{13}C-NMR (MeOH-d_4, 100 MHz) δ: 135.9 (CH), 168.8 (COOH).

Enzyme assay

The tyrosinase activity using L-tyrosine as a substrate, was determined spectrophotometrically using a slight modification of a method reported by No *et al.* (No *et al.*, 1999). Ten μL of each sample solution at different concentrations (1-500 μg/mL) and 20 μL of mushroom tyrosinase (1000 units/mL) in a 50 mM phosphate buffer (pH 6.5) were added to 170 μL of a assay mixture containing the ratio 10:10:9 of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5) and distilled water in a 96-well microplate. The samples dissolved in DMSO were diluted 30 times with H$_2$O prior to the experiments. After incubation of the reaction mixture at 25°C for 30 min, the absorbance of the mixture was measured at 490 nm (ε = 3.3×10^3 M^{-1}cm^{-1}) using a microplate reader (VERSA max, Molecular Device, CA). The extent of the inhibition due to the samples is expressed as the concentration necessary for 50% inhibition (IC$_{50}$). The percent inhibition of the tyrosinase activity was calculated using the following equation: the % inhibition = [{1 − (A$_a$ − A$_b$)/A$_c$}×100] where A$_a$ is the absorbance at 490 nm with the test sample and the enzyme, A$_b$ is the absorbance at 490 nm with the test sample and without the enzyme, and A$_c$ is the absorbance at 490 nm with the enzyme and without the test sample. One unit (U) of enzymatic activity was defined as the amount of enzyme needed to increase the absorbance at 280 nm by 0.001 per min, in a 3 mL reaction mixture containing L-tyrosine at pH 6.5 at 25°C.

Kinetic analysis

The reaction mixture consisted of four different L-tyrosine concentrations (0.5 to 2 mM) as substrate and mushroom tyrosinase in 50 mM potassium phosphate buffer. Each sample at several concentrations was added to the reaction mixture. The Michaelis constant (K$_m$) and maximum velocity (V$_{max}$) of the tyrosinase were determined by the Lineweaver-Burk plots. The velocity equation for the competitive inhibition in reciprocal form is: $1/V$=K$_m$/V$_{max}$ (1 + [I]/K$_i$)$1/$[S]$+1/V_{max}$. The inhibition constants (K$_i$) of the competitive inhibitors were calculated by the following equation: K$_{mapp}$=K$_m$[1+([I]/K$_i$)] where K$_{mapp}$ is the apparent K$_m$ in the presence of an inhibitor. The reciprocal equation for the noncompetitive inhibition is: $1/V$=K$_m$/V$_{max}$(1+[I]/K$_i$)$1/$[S]$+1/V_{max}$(1+[I]/K$_i$). K$_i$ of the noncompetitive inhibitors were calculated using the following equation: $1/V_{maxapp}$=(1+[I]/K$_i$)

N_{max} where V_{maxapp} is the apparent V_{max} in the presence of an inhibitor.

RESULTS AND DISCUSSION

Identification of the compounds from *P. linteus*

The solvent soluble fractions including CH_2Cl_2, EtOAc, n-BuOH, and the H_2O layers derived from *P. linteus*, were evaluated in order to identify the bioactive principles. As summarized in Table I, the EtOAc soluble fraction of the MeOH extract from the fruiting body of *P. linteus* strongly inhibited the oxidation of L-tyrosine catalyzed by tyrosinase with an IC_{50} value of 21.9 µg/mL. The bioassay-guided fractionation of the EtOAc soluble fraction led to the isolation of two active compounds (2, 3) and together with the sphingolipid (1), and two carboxylic acids (4, 5) (Fig. 1).

Compound 1 was isolated as an amorphous white powder with a molecular formula of $C_{41}H_{77}NO_9$, as determined by the HR-FAB mass spectrum data exhibiting an [M + Na]$^+$ ion peak at m/z 750.5498 (Calcd for $C_{41}H_{77}NO_9Na$, m/z 750.5496, +0.2 mmu) as well as the ^{13}C-NMR and DEPT spectrum. The ^1H-NMR spectral data of compound 1 showed signals corresponding to three terminal methyl groups [δ_H 1.60 (3H, s) and 0.87 (6H, t, J = 6.6 Hz)], two aliphatic long chains [δ_H 1.26 (brs) and 1.23 (brs)], an amide linkage [δ_H 8.36 (1H, d, J=8.7 Hz, NH)], and an anomeric proton [δ_H 4.91 (1H, d, J=7.8 Hz)], which indicated the presence of glycosphingolipid. The ^{13}C-NMR spectrum indicated a carbon attached to nitrogen at δ_C 54.5, three terminal methyl groups at δ_C 16.0 and 14.2, four olefinic carbons at δ_C 136.0, 132.3, 131.8 and 124.1, and two oxygenated carbons at δ_C 72.4 and 72.3. In addition, six carbon signals corresponding to a β-D-glucopyranoside moiety appeared at δ_C 105.6 (C-1"), 78.5 (C-5"), 78.4 (C-3"), 75.0 (C-2"), 71.4 (C-4"), and 62.6 (C-6") (Agrawal, 1992). In the HMBC experiment (Fig. 2), the anomeric proton peak of glucose at δ 4.91 (d, J = 7.8 Hz, H-1") correlated with the methylene carbon at δ 70.1 (C-1). The

Table I. Tyrosinase inhibitory effects of various fractions obtained from the methanolic extract of *P. linteus*

Samples	IC_{50} [a] (µg/mL)
MeOH ex.	846
CH_2Cl_2 fr.	339
EtOAc fr.	21.9
BuOH fr.	770
H_2O fr.	> 1000
Kojic acid	3.1

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determines obtained by interpolation of concentration-inhibition curve.
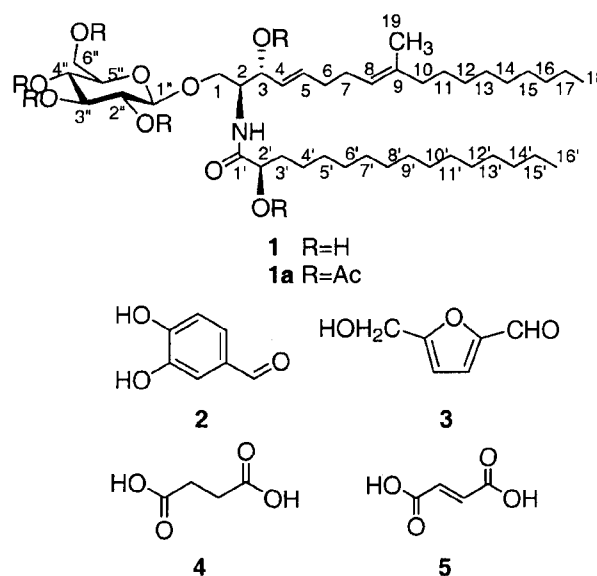

Fig. 1. Structures of the compounds isolated from the fruiting body of *P. linteus*

proton signal at δ 1.60 (3H, s) indicated that a methyl group was attached to one of the double bonds (Yue et al., 2001). The 4,5 alkene bond was found to be *trans*, which was evidenced by the large vicinal coupling constants ($J_{4,5}$ = 15.5 Hz). The *trans* geometry of this double bond was also supported by the chemical shift of C-6 (δ 33.0). Usually, the signals of the carbons adjacent to a *trans* double bond appear at δ 32-33, while those of a *cis* double bond appear at δ 27-28 (Stothers, 1972). The characteristic steric structure of compound 1 was determined to be a 2S, 3R, 4E, 8E, 2'R configuration by a comparison of its ^{13}C-NMR spectral data with those of the known glucosphingolipids (Jung et al., 1996; Kang et al., 1999; Sang et al., 2002; Shibuya et al., 1990; Yue et al., 2001). The lengths of the two aliphatic fatty acids were confirmed by acid hydrolysis of compound 1, followed by GC-MS analysis (Gaver and Sweely, 1966; Kang et al., 1999). The molecular ion peak at m/z 286 (GC/MS) suggested the fatty acid methyl ester of compound 1 is a hydroxypalmitic acid methyl ester. Moreover, the molecular weight of compound 1 at m/z 750.5498 ($C_{41}H_{77}NO_9Na$) indicated the presence of an additional terminal methyl (-CH_3) unit, by a comparison with that of soya-cerebroside I at m/z 714.5489 ($C_{40}H_{76}NO_9$ [M+H]$^+$) in the HR-FAB mass spectrum (Shibuya et al., 1990). Based on these spectral results as well as the GC-MS spectrum with chemical modification, as represented by methanolysis, compound 1 was confirmed to be 1-O-β-D-glucopyranosyl-(2S, 3R, 4E, 8E)-2-[(2R)-2-hydroxyhexadecanoylamino]-9-methyl-4, 8-octadecadiene-1,3-diol, named cerebroside B (Sitrin et al., 1988), which was first reported from *Phellinus* species.
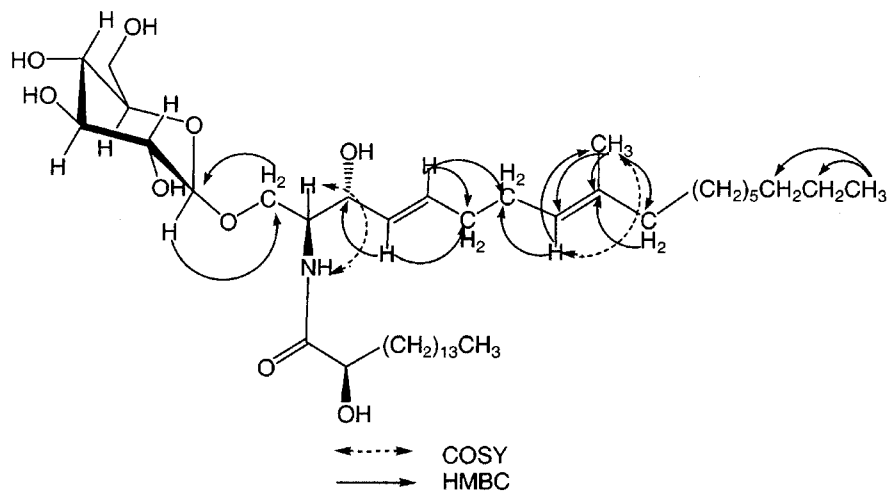
Glycosphingolipids comprise of a sphingoid base skeleton,

◄----► COSY
────► HMBC

Fig. 2. Long-range correlations of compound 1 in the ^{1}H-^{1}H COSY and HMBC spectrum

an amide linked aliphatic long chain fatty acid and a β-glucopyranose moieties. The nonpolar portion of glycos-phingolipids, such as a fatty acid moiety, varies according to the presence or absence of a hydroxy group on the carbon atom. In addition, they are distributed widely in most foods with a mammalian origin in a similar manner to that in lipid-rich structures such as cellular membranes and lipoproteins (Vesper *et al.*, 1999). Recently, it was reported that these compounds exhibit an ionophoretic activity (Shibuya *et al.*, 1990), repellent activity (Yoshioka *et al.*, 1990), anticancer (Dillehay *et al.*, 1994), anti-hepatotoxic (Kim *et al.*, 1997), anti-hyperlipidemic (Kobayashi *et al.*, 1997), and cyclooxygenase-2 (COX-2) inhibitory activities (Kang *et al.*, 2001). It was reported that the cerebrosides A, B, C, and D isolated from *Pachybasium* species, exhibited cell wall-active antifungal activity based on a synergy with the known glucan synthetase inhibitor, aculeacin (Sitrin *et al.*, 1988).

Compound 2 was obtained as light yellow needles. The molecular formula of compound 2 was determined to be $C_7H_6O_3$ based on the NMR and EIMS data [M]+, m/z: 138. ^{1}H-NMR spectral data of compound 2 indicated the presence of a 1,3,4-trisubstituted benzene ring at δ 7.30 (1H, dd, J=1.70, 9.48 Hz), 7.28 (1H, d, J=1.41 Hz) and 6.90 (1H, d, J=8.1 Hz), and showed a singlet signal of an aldehyde group at δ 9.68. A direct comparison with the spectra of an authentic sample identified compound 2 as protocatechualdehyde. Compound 2 had been isolated from the insect cuticle (Atkinson *et al.*, 1973), *Salvia miltiorrhiza* (Liu *et al.*, 1990), grapevine leaves (Webera *et al.*, 1995), and grape seeds (Murga *et al.*, 2002). In addition, several activities reported for protocatechualdehyde such as protective action against peroxidative damage to the biomembranes (Liu *et al.*, 1992), antirheumatic (Watanabe *et al.*, 1993), radical scavenging (Kikuzaki *et*

al., 2001), and antiproliferative activities (Wang *et al.*, 2001).

Compound 3, which was obtained as a yellow oil, exhibited a MS peak at m/z 126 corresponding to the molecular formula $C_6H_6O_3$. The infrared spectrum of compound 3 showed absorption bands due to hydroxyl (3380 cm^{-1}) and conjugated aldehyde (1655 cm^{-1}) functions. The ^{1}H- and ^{13}C-NMR spectral data suggested the structure of compound 3 to be a two-substituted furan derivative. Therefore, compound 3 was identified as 5-hydroxymethyl-2-furaldehyde (HMF) from the above results and the physical and published spectral data (Shimizu *et al.*, 1993). HMF, which is a common product of the Maillard reaction, occurs in many foods in high concentrations, some times exceeding 1 g/kg (in certain dried fruits and caramel products). It was isolated from the Rehmanniae Radix Preparata samples (Hwang *et al.*, 2001), Kampo medicine (Shimizu *et al.*, 1993), and *Prunus mume* (Ichikawa *et al.*, 1989). HMF was reported aldose reductase inhibitory (Shimizu *et al.*, 1993), Ca^{2+} antagonist activities (Ichikawa *et al.*, 1989), and cytotoxicity at high concentrations (Ulbricht *et al.*, 1984). However, this does not appear to pose a serious health risk, even though the highest concentrations in some foods approach the biologically effective concentration range in cell systems (Janzowski *et al.*, 2000).

Compound 4 was obtained as white crystals, and identified as succinic acid based on the spectral evidence, which was also confirmed by that of an authentic sample. Fumaric acid and maleic acid are *trans-* and *cis-*olefinic isomers with the same molecular formula of $C_4H_4O_2$. They showed slightly different chemical shifts on the ^{1}H- and ^{13}C NMR spectra, in which the former is at δ_H 6.75 and δ_C 135.9, and the latter at δ_H 6.31 and δ_C 132.4, respectively. The spectrum of compound 5, which was obtained as a

white powder, agreed well with that of the *trans*-isomer of fumaric acid. These compounds 1~5 were isolated from *P. linteus* for the first time and were identified as cerebroside B (1), protocatechualdehyde (2) 5-hydroxymethyl-2-furaldehyde (3), succinic acid (4), and fumaric acid (5) based on the chemical and physicochemical evidence.

Tyrosinase inhibitory activities of the compounds from *P. linteus*

The tyrosinase inhibitory activity of compounds 1~5, as shown in Table II, was determined using L-tyrosine as a substrate. Compounds 2 and 3 exhibited tyrosinase inhibitory activity with an IC$_{50}$ of 0.4 and 90.8 μg/mL, respectively. Compound 2 was a stronger tyrosinase inhibitor than kojic acid with an IC$_{50}$ of 3.1 μg/mL as a positive control. The high content of compound 3 (1.74 g) may contribute to the inhibitory activity of the EtOAc soluble fraction of *P. linteus*, which was similar to that of compound 2 (376.8 mg).

The Lineweaver-Burk plots shown in Fig. 3 and 4 show the inhibitory kinetics of compounds 2 and 3, respectively. The lines, which were obtained from the uninhibited enzyme and from the three different concentrations of compound 2, intersect on the vertical axis (Fig. 3). This demonstrates that compound 2 is a competitive inhibitor for the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. A competitive inhibitor is a substance that combines with a free enzyme in a manner that prevents substrate binding. This means that the inhibitor and the substrate are mutually exclusive, often the result of true competition for the same site. A competitive inhibitor might be a non-metabolic analog, a derivative of the true substrate, an alternate substrate of the enzyme, or a product of the reaction (Segel, 1976).

The aldehyde group is generally known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. The formation of a Schiff base is largely governed by factors affecting the

Table II. Tyrosinase inhibitory effects of compounds 1~5 isolated from the EtOAc soluble fractions of the methanolic extract of *P. linteus*

Samples	IC$_{50}$[a] (μg/mL)
Cerebroside B (1)	> 500.0
Protocatechualdehyde (2)	0.4
5-Hydroxymethyl-2-furaldehyde (3)	90.8
Succinic acid (4)	> 500.0
Fumaric acid (5)	> 500.0
Kojic acid	3.1

[a] Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determines obtained by interpolation of concentration-inhibition curve.

stability of the carbon-nitrogen double bond. It has been suggested that there is a strong correlation between the stability of the Schiff base the inhibitory activity. More specifically, a more hydrophobic electron donor group such as methoxy or isopropyl at the *para* position in benzaldehyde produces a more potent tyrosinase inhibitor (Kubo *et al.*, 1998a). On the other hand, *p*-hydroxybenzaldehyde binds a hydroxy group to the coupled binuclear copper
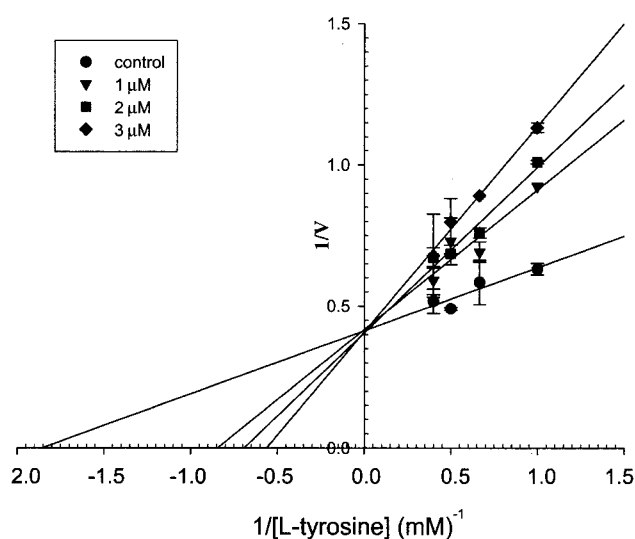

Fig. 3. Lineweaver-Burk plots of tyrosinase and L-tyrosine without (●) and with [1 μM (▼), 2 μM (■) and 3 μM (◆)] protocatechualdehyde (1). V$_{max}$ = 2.4 μmole/mL, K$_m$ = 0.54 mM. The inhibitory constant of the compound was determined as K$_i$ = 1.1 μM. 1/V: 1/[Dopachrome] (μmol/min/mL)$^{-1}$.
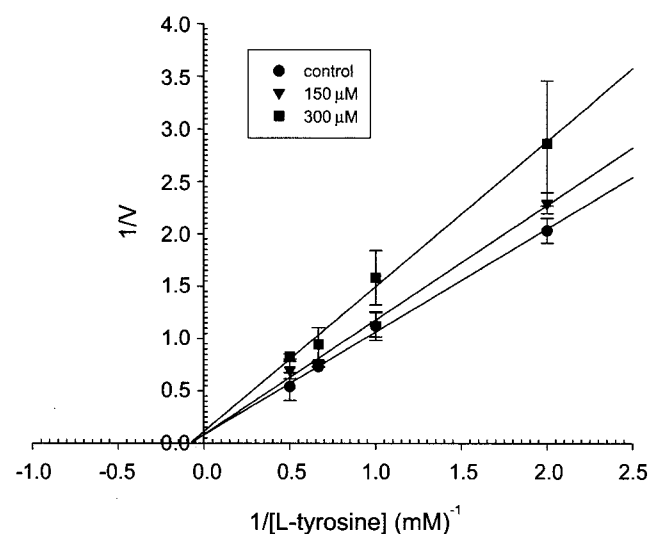

Fig. 4. Lineweaver-Burk plots of tyrosinase and L-tyrosine without (●) and with [150 μM (▼) and 300 μM (■)] 5-hydroxymethyl-2-furaldehyde (2). V$_{max}$ = 13.3 μmole/mL, K$_m$ = 1.0 mM. The inhibitory constant of the compound was determined as K$_i$ = 1.4 mM. 1/V: 1/[Dopachrome] (μmol/min/mL)$^{-1}$.

active site, rather than forming a Schiff base with the aldehyde group (Kubo et al., 1998b). Protocatechualdehyde (2) binds the coupled binuclear copper active site with hydroxy groups, rather than forming a Schiff base with the aldehyde group. This is supported by the observation that the inhibition kinetics analyzed by the Lineweaver-Burk plot shows that protocatechualdehyde is a competitive inhibitor with respect to L-tyrosine. Nerya et al. (2003) suggested that most competitive inhibitors have the ability to chelate copper in this enzyme, suggesting a possible inhibitory mechanism. Phenol with an electron-withdrawing aldehyde group in the para position did not serve as a substrate. The aromatic ring is apparently sufficiently deactivated, which prevents electrophilic attack by oxygen (Conrad et al., 1994).

As shown in Fig. 4, the lines obtained from the uninhibited enzyme and from the three different concentrations of compound 3, intersected on the horizontal axis. This demonstrates that compound 3 is a noncompetitive inhibitor for tyrosinase. A classical noncompetitive inhibitor has no effect on substrate binding and vice versa. S and I bind reversibly, randomly, and independently at different sites.

As a noncompetitive inhibitor, 5-hydroxymethyl-2-furaldehyde (3) may form a Schiff base with primary amino groups in the enzyme rather than by binding to the binuclear copper active center.

Two aldehydes (2, 3) exhibiting tyrosinase inhibitory activity along with a sphingolipid (1) and two carboxylic acids (4, 5) were isolated from the fruiting body of Phellinus linteus (Berk & Curt) Aoshima, for the first time. In addition, this work showed that two tyrosinase inhibitory aldehydes might be involved in the control of pigmentation in plants as well as other organisms by inhibiting the tyrosinase activity using L-tyrosine as a substrate.

ACKNOWLEDGEMENT

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