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Synthesis and Biological Evaluation of Bergenin Analogues as Mushroom Tyrosinase Inhibitors

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In this manuscript, we synthesized a series of bergenin analogues, analyzed their structural importance for two biologic activities (anitioxidant activity (ORAC) and mushroom tyrosinase inhibitory activity). Among them, compound **5** which contains catechol moiety exhibited the most antioxidant activity (3.75 μmol of Trolox equiv. per μmol of **5**). Furthermore, compound **5** was found to be the most potent (IC_{50} value = 17.5 ± 0.04 μM) when compared with the standard tyrosinase inhibitors of arbutin (IC_{50} value = 221.8 ± 1.9 μM) and kojic acid (IC_{50} value = 46.6 ± 3.8 μM). The bergenin moiety, the ester linkage, and benzoic acid moiety of bergenin derivatives affected two biologic activities. Tyrosinase inhibitory activity was affected by substituents of benzoic acid moiety. This manuscript provides a good foundation for the design and development of new tyrosinase inhibitors.

Key words: Bergenin, Bergenin derivatives, Tyrosinase inhibitor, Antioxidant activity, Structure-activity relationship

INTRODUCTION

Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is widely distributed in microorganisms, animals, and plants (Song et al., 2006). Tyrosinase could catalyze two distinct reactions involving molecular oxygen in the hydroxylation of monophenols to *o*-diphenols (monophenolase) and in the oxidation of *o*-diphenols to *o*-quinones (diphenolase) (Chen et al., 2003). Due to their high reactivity, quinones could polymerize spontaneously to form a higher molecular weight brown pigments (melanins) or react with amino acids and proteins to enhance the brown color of the pigment produced (Nihei and Kubo, 2003; Matsuura et al., 2006). Previous reports confirmed that tyrosinase not only was involved in melanising in animals, but also was one of the main causes of quality loss of most fruits and vegetables during post harvest handling and processing, leading to faster degradation and shorter shelf life (Takahashi and Miyazawa, 2011; Lin et al., 2012). Recently, an investigation demonstrated that various dermatological disorders, such as age spots and freckles, were caused by the accumulation of an excessive level of epidermal pigmentation (Thanigaimalai et al., 2010). Tyrosinase has also been linked to Parkinson's and other neurodegenerative diseases (Xu et al., 1997). Therefore, tyrosinase inhibitors have become increasingly important in agriculture, the cosmetic industry, and medication, which makes the development and screening of potent inhibitors of tyrosinase extremely important. An important group of browning inhibitors is constituted by compounds structurally analogous to phenolic substrate. Recently, alkoxybenzoic acids have been targeted for the inhibition of the enzyme (Khan et al., 2010; Zhu et al., 2011).

However, antioxidant therapies have been increasingly recognized as a potential strategy for disorder development prevention, including but not limited to cancer, cardiovascular disease, inflammation, neurodegenerative diseases, and the aging processes (Laguerre et al., 2007; Bicas et al., 2011). In addition, applications of antioxidants such as preservatives in the food industry and skin-protective ingredients in cosmetics are also receiving increasing attention and interests. Re-

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cently, the oxygen radical absorbance capacity (ORAC) assay has gained much attention because it deals with peroxyl radicals, the most abundant radicals in biological systems. The ORAC assay was considered to be both the inhibition time and the inhibition degree as the reaction is completed and was evaluated directly as the chain-breaking antioxidant activity (Ou et al., 2002).

Bergenin (**1**) is a C-glucoside of 4-*O*-methylgallic acid that occurs naturally in several plant genera. Different studies have shown the various pharmacological effects of **1**, such as anti-inflammatory (Swarnalakshmi et al., 1984), hypolipidemic (Jahromi et al., 1992), anti-HIV (Piacente et al., 1996), antiarrhythmic (Pu et al., 2002), hepatoprotective (Lim et al., 2000), neuroprotective (Takahashi et al., 2003), gastroprotective, antitussive, and antinociceptive (DeOliveira et al 2011). Furthermore, bergenin and its esterified derivatives occur widely in several plants and have been found as ingredients in plant extracts (Yoshida et al., 1982; Saijo et al., 1990; Jia et al., 1995; Fuji et al., 1996; Lee et al., 2005). Owing to such a broad spectrum of biological activities associated with bergenin, a number of studies have been devoted to either derivatizes of the molecule or to synthesize its related compounds to optimize it as a lead molecule. Bergenin contains five hydroxyl groups which are considered to be potentially active. Though bergenin and its esterified derivatives (**1**-**10**) are a new class of potent tyrosinase inhibitors and antioxidants, to our knowledge, there is no report on the correlation between tyrosinase inhibition and structure of bergenin derivatives in detail. In this study, therefore, bergenin and its derivative (**1**-**10**) were prepared from naturally occurring bergenin (Fig. 1) and the structural importance of two biologic activities was analyzed. This is first report of an antioxidant activity evaluation using the ORAC assay and the structure-activity relationships of tyrosinase inhibitory activity exhibited by bergenin and its derivatives.

MATERIALS AND METHODS

Plant

Roots of *Bergenia ligulata* were provided by Koei Kogyo Co., Ltd. of Tokyo in Japan.

Materials

A thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F_{254}, 0.25 mm, Merk). Column chromatography was carried out using 70-230 mesh silica gel (Kieselgel 60, Merk). Optical rotations were measured on a Japan Spectroscopic Co. LTDDIP-1000. The melting points (m.p.) were measured on a Yanaco MP-5000D melting-point apparatus. The infrared (IR) spectra were recorded with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer (JASCO Co., Ltd). ^{1}H- and ^{13}C-NMR data were all obtained on JEOL ECA-400 (400 MHz) spectrometers in DMSO-d_6 or $CDCl_3$ with TMS as the internal standard (chemical shift in δ, ppm). *J* values are reported in Hertz. EI-MS spectra were obtained on a JEOL JMS-700 Tandem MS station (Japan Electron Optics Laboratory

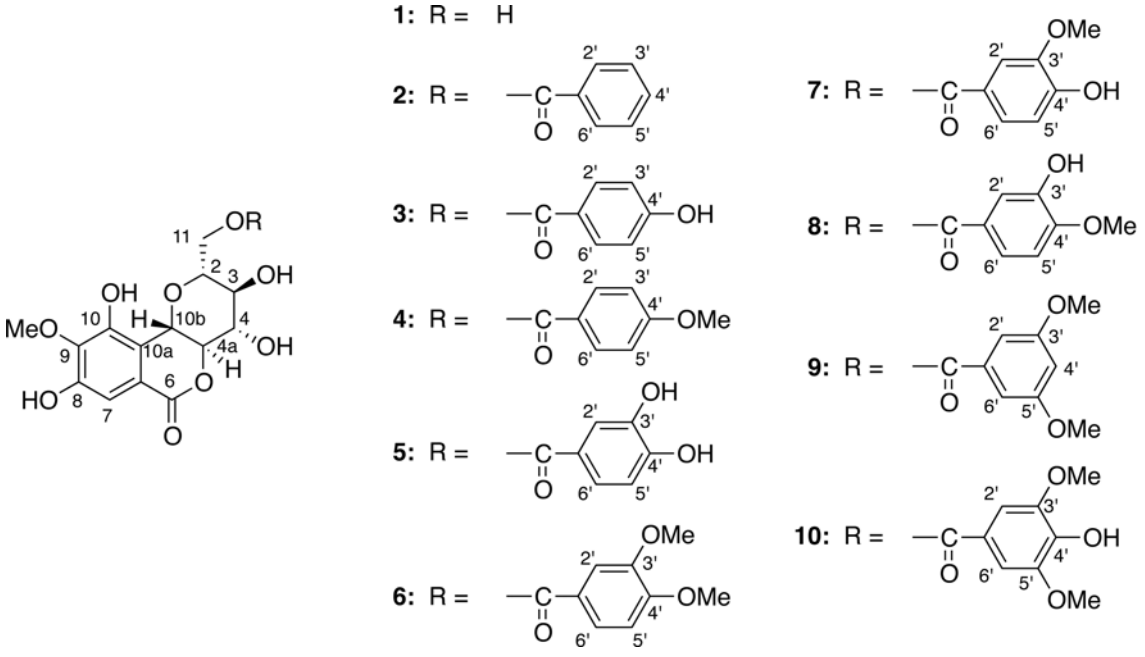

Fig. 1. Structures of bergenin (**1**) and bergenin derivatives (**2**-**10**).

Co., Ltd.). The absorbance was measured with a MTP-800 Lab microplate reader. Mushroom tyrosinase (EC 1.14.18.1), Tween® 20 Sigma ultra, fluorescein sodium salt, trolox, and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich, L-tyrosinse, benzoic acid, *p*-hydroxy benzoic acid, *p*-anisic acid, protocatechuic acid, 3,4-dimethoxy benzoic acid, vanillic acid, isovanillic acid, 3,5-dimethoxy benzoic acid, syringic acid, diisopropyl azodicarboxylate (DIAD), tetrakis (triphenyl phosphine) palladium ($Pd(PPh_3)_4$), triphenylphosphine (Ph_3P), sodium iodide (NaI), morpholine, allylbromide, and dibutylhydroxytoluene (BHT) were purchased from Wako Pure Chemistry, arbutin and kojic acid were purchased from Tokyo Kasei Kogyo, all solvents were purchased from Kanto Chemical.

Extraction and isolation

The bark (5.0 kg) of *B. ligilata* was extracted once with MeOH at room temperature. The solution was evaporated until dry *in vacuo* to create methanol extract (480 g). The residue was re-extracted successively with *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH and water. Each fraction was concentrated to dryness *in vacuo* to give *n*-hexane extract (12.5 g), CH_2Cl_2 extract (40.5 g), EtOAc extract (88.2 g), *n*-BuOH extract (98.1 g) and water fraction (240.7 g), respectively.

The *n*-BuOH extract (98.1 g) was fractionated to fraction 1-3 by silica gel column chromatography with CH_2Cl_2-MeOH (9:1, 4:1, 7:3, v/v) as eluents. Fraction 2 was recrystallized from CH_2Cl_2-MeOH (12:1, v/v), thereby bergenin (**1**) (3.7 g) was isolated. The structure of **1** was identified by the comparison of its physical and spectral data with those described in the literature (Jia et al., 1995; Wang et al., 2005).

Synthesis of bergenin derivatives 1a, 2-10

Synthesis of 8,10-diallyloxy-bergenin (1a)

First, the phenolic hydroxy groups in bergenin (**1**) (700 mg, 2.13 mmol) was selectively allylated. Allyl bromide and NaI were added to the solution bergenin and K_2CO_3 in anhydr. DMF (5 mL). After the mixture was stirred 2 h at 55°C under nitrogen, it was concentrated *in vacuo*. The residue was treated with water (15 mL) and extracted with EtOAc (3 × 10 mL). The organic extract was washed successively with brine (3 × 15 mL). The extract was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel to afford **1a** (554 mg, 84%) as a white powder.

Synthesis of bergenin derivatives (2-4, 6-10)

First, to a solution of **1a** in anhydrous tetrahydrofuran (anhydr. THF) (2 mL/mmol), various benzoic acids and Ph_3P (2 equiv) were dissolved. DIAD (1.7 equiv) was added in a drop wise manner when the temperature dropped below 0 degrees Celsius. The mixture was stirred for 2 h with nitrogen. It was concentrated *in vacuo*. The residue was chromatographed on silica gel to afford benzoic acid ester of **1a** (yield 79-92%) as a white powder.

Secondly, the allyl protected esters and Pd $(PPh_3)_4$ (1 mol%, freshly prepared) was dissolved in degassed anhydr. THF (2 mL/mmol) and morpholine (10 equiv per allylgroup to be cleaved) was added dropwise. The mixture was stirred at room temperature and concentrated *in vacuo*. The residue was taken up in EtOAc. The organic layer was washed several times with small amounts of 1 N HCl, dried and concentrated. The crude material was purified by silica gel column chromatography. The purified material was bergenin derivatives (**2**-**4**, **6**-**10**).

Synthesis of 11-*O*-protocatechuoylbergenin (5)

First, the phenolic hydroxy groups in protocatechualdehyde were selectively allylated. Secondly, 3,4-diallyloxy-protocatechuic acid was accomplished according to previous study (Pearl, 1963). Then, the same procedure for compound **2**-**4**, **6**-**10** was used starting from **1a**.

Physical and spectroscopic data of the synthesized compounds

8,10-diallyloxy-bergenin (1a)

White powder; yield: 84%; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.34 (1H, s H-7), 6.12-5.97 (2H, m, 2 × –CH=), 5.40 (2H, dd, *J* = 17.2, 1.8 Hz, 2 × =CHb), 5.26 (2H, dd, *J* = 10.8, 1.8 Hz, 2 × =CHa), 4.72 (1H, d, *J* = 10.4 Hz, H-10b), 4.55-4.41 (4H, m, 2 × O-CH_2), 4.01-3.83 (1H, m, H-4), 3.98 (1H, d, *J* = 9.2 Hz, H-11b), 3.91 (3H, s, OMe), 3.73-3.68 (1H, m, H-11a), 3.59-3.54 (1H, m, H-2, H-3).

11-*O*-benzoylbergenin (2)

Amorphous powder; $[\alpha]_D^{25}$ +66.8° (*c* = 0.15, MeOH); overall yield: 45%; m.p. 248.8-249.1°C; IR (KBr) ν_{max} cm^{-1}: 3474 (OH), 1722 (ester), 1610 (arom. C=C); EI-MS: *m/z* 432 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 8.01 (2H, m, H-2', 6'), 7.67 (1H, m, H-4'), 7.54 (2H, m, H-3', 5'), 7.00 (1H, s, H-7), 5.03 (1H, d, *J* = 10.8 Hz, H-10b), 4.78 (1H, dd, *J* = 12.4, 2.0 Hz, H-11b), 4.34 (1H, dd, *J* = 12.4, 7.2 Hz, H-11a), 4.03 (1H, t, *J* = 10.0 Hz, H-4a), 3.93 (1H, ddd, *J* = 9.2, 7.2, 2.0 Hz, H-2), 3.74 (3H, s, 9-OCH_3), 3.69 (1H, m, H-4), 3.41 (1H, dd, *J* = 9.2, 8.8 Hz, H-3); ^{13}C-NMR: Table I.

11-*O*-*p*-hydroxybenzoylbergenin (3)

Amorphous powder; $[\alpha]_D^{25}$ +18.3° (*c* = 0.15, MeOH);

Table I. ^{13}C-NMR spectral data for compounds **1**-**10** (δ in ppm)

Carbon number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
2	81.7	78.5	78.6	78.5	78.6	78.5	78.5	78.6	78.3	78.5
3	70.8	70.2	70.2	70.2	70.1	70.5	70.5	70.1	70.5	70.8
4	79.8	73.5	73.5	73.5	73.5	73.5	73.5	73.5	73.5	73.5
4a	79.6	79.6	79.6	79.6	79.6	79.5	79.6	79.6	79.5	79.5
6	163.2	163.4	163.4	163.4	163.4	163.3	163.3	163.3	163.3	163.2
6a	117.9	118.1	118.1	118.1	118.1	118.1	118.1	118.1	118.1	118.1
7	109.5	109.5	109.5	109.5	109.5	109.6	109.6	109.5	109.6	109.6
8	150.8	150.9	150.9	150.9	150.9	151.0	151.0	151	151.0	151.1
9	140.6	140.6	140.6	140.6	140.6	140.6	140.6	140.6	140.6	140.6
10	148.0	148.0	148.0	148.0	148.0	148.0	148.0	148	148.0	148.0
10a	115.8	115.8	115.8	115.8	115.8	115.7	115.8	115.8	115.7	115.7
10b	72.1	72.2	72.2	72.2	72.2	72.1	72.1	72.1	72.1	72.1
11	63.8	64.0	63.5	63.7	63.4	64.0	63.8	63.8	64.1	64.0
9-OCH_3	59.7	59.7	59.7	59.7	59.8	59.7	59.7	59.7	59.7	59.7
1'		129.5	120	121.7	120.2	123.4	120.2	121.6	131.5	119.0
2'		129.2	131.6	131.4	116.4	111.2	112.6	114.4	106.9	107.0
3'		128.8	115.4	114.1	145.1	148.4	147.4	146.3	160.5	147.6
4'		133.5	162.2	163.3	150.7	153.1	151.8	152.1	105.5	141.1
5'		128.8	115.4	114.1	115.3	111.7	115.2	111.5	160.5	147.7
6'		129.2	131.6	131.4	122.0	121.6	123.7	122	106.9	107.0
COO		165.6	165.5	165.4	165.6	165.3	165.4	165.5	165.2	165.3
3'-OCH_3						55.5	55.6		55.5	56.1
4'-OCH_3				55.6		55.7		55.7		
5'-OCH_3									55.5	56.1

^{13}C-NMR spectral data were recorded at 100 MHz in DMSO-d_6 using tetramethylsilane (TMS) as internal standard.

overall yield: 48%; m.p. 214.6-215.5°C; IR (KBr) ν_{max} cm^{-1}: 3380 (OH), 1712 (ester), 1608 (arom. C=C), 1238 (C-O); EI-MS: *m/z* 448 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.85 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.00 (1H, s, H-7), 6.86 (2H, d, *J* = 8.8 Hz, H-3', 5'), 5.02 (1H, d, *J* = 10.0 Hz, H-10b), 4.71 (1H, dd, *J* = 12.0, 1.6 Hz, H-11b), 4.26 (1H, dd, *J* = 12.0, 6.4 Hz, H-11a), 4.03 (1H, t, *J* = 9.8 Hz, H-4a), 3.87 (1H, ddd, *J* = 9.2, 6.4, 1.6 Hz, H-2), 3.73 (3H, s, 9-OCH_3), 3.69 (1H, t, *J* = 9.2 Hz, H-4), 3.40 (1H, m, H-3); ^{13}C-NMR: Table I.

11-*O*-*p*-methoxybenzoylbergenin (4)

Amorphous powder; $[\alpha]_D^{25}$ +44.4° (*c* = 0.15, MeOH); overall yield: 44%; m.p. 258.9-260.4°C; IR (KBr) ν_{max} cm^{-1}: 3373 (OH), 2959 (C-H), 1720 (ester), 1609 (arom. C=C), 1237 (C-O); EI-MS: *m/z* 462 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.95 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.05 (2H, d, *J* = 8.8 Hz, H-3', 5'), 7.00 (1H, s, H-7), 5.03 (1H, d, *J* = 10.0 Hz, H-10b), 4.74 (1H, dd, *J* = 12.0, 2.1 Hz, H-11b), 4.29 (1H, dd, *J* = 12.0, 6.8 Hz, H-11a), 4.04 (1H, t, *J* = 10.0 Hz, H-4a), 3.89 (1H, m, H-2), 3.83 (3H, s, 4'-OCH_3), 3.74 (3H, s, 9-OCH_3), 3.69 (1H, t, *J* = 8.8 Hz, H-4), 3.40 (1H, t, *J* = 8.8 Hz, H-3); ^{13}C-NMR: Table I.

11-*O*-protocatechuoylbergenin (5)

Amorphous powder; $[\alpha]_D^{25}$ +25.2° (*c* = 0.25, MeOH); overall yield: 26%; m.p. 167.2-173.5°C; IR (KBr) ν_{max} cm^{-1}: 3364 (OH), 1709 (ester), 1610 (arom. C=C), 1227 (C-O); EI-MS, *m/z* 464 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.38 (1H, d, *J* = 1.6 Hz, H-2'), 7.35 (1H, d, *J* = 8.4 Hz, H-6'), 7.00 (1H, s, H-7), 6.81 (1H, d, *J* = 8.4 Hz, H-5'), 5.05 (1H, d, *J* = 10.4 Hz, H-10b), 4.67 (1H, brd, *J* = 11.6 Hz, H-11b), 4.26 (1H, dd, *J* = 11.6, 6.0 Hz, H-11a), 4.01 (1H, t, *J* = 10.0 Hz, H-4a), 3.86 (1H, m, H-2), 3.74 (3H, s, 9-OCH_3), 3.69 (1H, t, *J* = 9.2 Hz, H-4), 3.39 (1H, t, *J* = 9.2 Hz, H-3); ^{13}C-NMR: Table I.

11-*O*-(3',4'-dimethoxybenzoyl)-bergenin (6)

Amorphous powder; $[\alpha]_D^{25}$ +32.6° (*c* = 0.025, MeOH); overall yield: 48%; m.p. 260.6-262.7°C; IR (KBr) ν_{max} cm^{-1}: 3395 (OH), 1716 (ester), 1598 (arom. C=C), 1225 (C-O); EI-MS: *m/z* 492 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.63 (1H, dd, *J* = 8.4, 1.6 Hz, H-6'), 7.48 (1H, d, *J* = 1.6 Hz, H-2'), 7.09 (1H, d, *J* = 8.4 Hz, H-5'), 7.00 (1H, s, H-7), 5.05 (1H, d, *J* = 10.0 Hz, H-10b), 4.83 (1H, brd, *J* = 12.0 Hz, H-11b), 4.21 (1H, dd, *J* = 12.0, 7.2 Hz, H-11a), 4.04 (1H, dd, *J* = 10.0, 9.6 Hz, H-4a), 3.91 (1H, m, H-2), 3.84 (3H, s, 4'-OCH_3), 3.83 (3H, s, 3'-OCH_3), 3.73 (3H, s, 9-OCH_3), 3.70 (1H, t, *J* = 9.6 Hz, H-4), 3.38 (1H, m, H-3); ^{13}C-NMR: Table I.

11-*O*-vanilloylbergenin (7)

Amorphous powder; $[\alpha]_D^{25}$ +56.4° (*c* = 0.1, MeOH); overall yield: 40%; m.p. 268.5-270.5°C; IR (KBr) ν_{max} cm^{-1}: 3365 (OH), 1717 (ester), 1599 (arom. C=C), 1222 (C-O); EI-MS: *m/z* 478 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.51 (1H, dd, *J* = 8.4, 2.0 Hz, H-6'), 7.48 (1H, d, *J* = 2.0 Hz, H-2'), 7.00 (1H, s, H-7), 6.88 (1H, d, *J* = 8.4 Hz, H-5'), 5.05 (1H, d, *J* = 10.8 Hz, H-10b), 4.81 (1H, dd, *J* = 11.6, 2.0 Hz, H-11b), 4.19 (1H, dd, *J* = 11.6, 8.0 Hz, H-11a), 4.04 (1H, dd, *J* = 10.4, 9.6 Hz, H-4a), 3.89 (1H, ddd, *J* = 8.0, 7.6, 2.0 Hz, H-2), 3.85 (3H, s, 3'-OCH_3), 3.73 (3H, s, 9-OCH_3), 3.69 (1H, dd, *J* = 9.6, 8.8 Hz, H-4), 3.39 (1H, m, H-3); ^{13}C-NMR: Table I.

11-*O*-isovanilloylbergenin (8)

Amorphous powder; $[\alpha]_D^{25}$ +42.4° (*c* = 0.1, MeOH); overall yield: 36%; m.p. 226.6-227.1°C; IR (KBr) ν_{max} cm^{-1}: 3376 (OH), 1714 (ester), 1608 (arom. C=C), 1237 (C-O); EI-MS: *m/z* 478 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.47 (1H, dd, *J* = 8.4, 2.0 Hz, H-6'), 7.40 (1H, d, *J* = 2.0 Hz, H-2'), 7.02 (1H, d, *J* = 8.4 Hz, H-5'), 7.00 (1H, s, H-7), 5.03 (1H, d, *J* = 10.4 Hz, H-10b), 4.70 (1H, dd, *J* = 12.0, 2.0 Hz, H-11b), 4.28 (1H, dd, *J* = 12.0, 6.4 Hz, H-11a), 4.02 (1H, dd, *J* = 10.4, 9.2 Hz, H-4a), 3.89 (1H, ddd, *J* = 7.6, 6.4, 2.0 Hz, H-2), 3.82 (3H, s, 4'-OCH_3), 3.73 (3H, s, 9-OCH_3), 3.69 (1H, dd, *J* = 9.2, 8.8 Hz, H-4), 3.39 (1H, m, H-3); ^{13}C NMR: Table I.

11-*O*-(3',5'-dimethoxybenzoyl)-bergenin (9)

Amorphous powder; $[\alpha]_D^{25}$ +157.9° (*c* = 0.025, MeOH); overall yield: 46%; m.p. 185.9-194.2°C; IR (KBr) ν_{max} cm^{-1}: 3355 (OH), 1717 (ester), 1602 (arom. C=C), 1237 (C-O); EI-MS: *m/z* 492 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.19 (2H, d, *J* = 2.4 Hz, H-2', 6'), 7.00 (1H, s, H-7), 6.79 (1H, t, *J* = 2.4 Hz, H-4'), 5.05 (1H, d, *J* = 10.4 Hz, H-10b), 4.90 (1H, dd, *J* = 12.0, 1.6 Hz, H-11b), 4.23 (1H, dd, *J* = 12.0, 7.6 Hz, H-11a), 4.04 (1H, dd, *J* = 10.4, 9.6 Hz, H-4a), 3.91 (1H, ddd, *J* = 9.2, 7.6, 1.6 Hz, H-2), 3.81 (6H, s, 3', 5'-OCH_3), 3.73 (3H, s, 9-OCH_3), 3.69 (1H, m, H-4), 3.38 (1H, m, H-3); ^{13}C-NMR: Table I.

11-*O*-syringylbergenin (10)

Amorphous powder; $[\alpha]_D^{25}$ +24.5° (*c* = 0.1, MeOH); overall yield: 40%; m.p. 240.7-241.3°C; IR (KBr) ν_{max} cm^{-1}: 3371 (OH), 1718 (ester), 1610 (arom. C=C), 1235 (C-O); EI-MS: *m/z* 508 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.26 (2H, s, H-2', 6'), 7.00 (1H, s, H-7), 5.07 (1H, d, *J* = 10.8 Hz, H-10b), 4.89 (1H, dd, *J* = 11.6, 2.4 Hz, H-11b), 4.12 (1H, dd, *J* = 11.6, 8.8 Hz, H-11a), 4.06 (1H, t, *J* = 10.0 Hz, H-4a), 3.92 (1H, ddd, *J* = 10.0, 8.1, 2.4 Hz, H-2), 3.85 (6H, s, 3', 5'-OCH_3), 3.72 (3H, s 9-OCH_3), 3.69 (1H, m, H-4), 3.38 (1H, m, H-3); ^{13}C-NMR: Table I.

Oxygen radical absorbance (ORAC) assay

The ORAC method used with fluorescein (FL) as the "fluorescent probe" was described in the literature method (Zulueta et al., 2009). The automated ORAC assay was carried out on a MTP-800AFC (Corona Electric Co., Ltd.) with fluorescence filters for an excitation wavelength of 480 nm and emission wavelength of 540 nm. The measurements were made in a plate with 96 black flat-bottom wells (Greiner Bio-One). The reaction was performed at 37°C as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.4) because of the sensitivity of FL to pH. Fig. 2 is a diagram of the reaction of the fluorescein during the ORAC assay. The fluorescein stock solution was made in 75 mM phosphate buffer (pH 7.4) and stored under dark conditions at 4°C. AAPH and trolox solutions in 75 mM phosphate buffer (pH 7.4) were prepared daily. The samples were dissolved in 50% acetone. The mixture was incubated for 1 h at room temperature while being continuously mixed. The reaction was performed in 75 mM phosphate buffer (pH 7.4) and

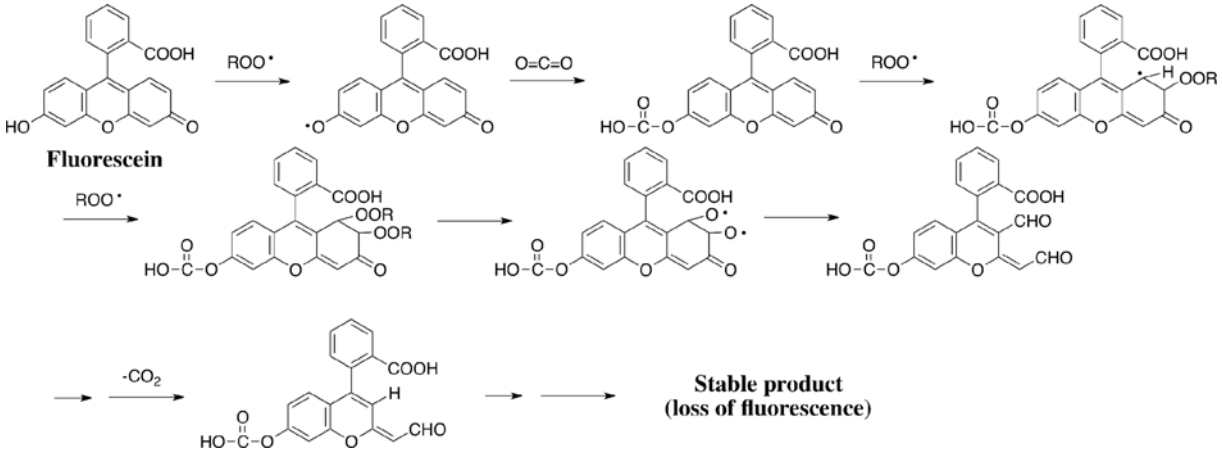

Fig. 2. The proposed Fluorescein pathway in the presence of AAPH.

the final assay mixture (200 μL) contains fluorescein (160 μL, 63 nM final concentration) as an oxidizable substrate, AAPH (20 μL, 12.8 mM final concentration) as an oxygen radical generator, and trolox (20 μL, 5-20 μM final concentration) or samples (20 μL, 5-20 μM final concentration). The reaction was performed at 37°C, and fluorescence was recorded every minute for 120 min. A blank (control) using phosphate buffer instead of the antioxidant was carried out in each experiment. All reaction mixtures were prepared in duplicate, and at least three independent runs were performed for each sample. BHT was used as a reference standard for antioxidant. Fluorescent measurements were normalized to the curve of the blank (no antioxidant). The ORAC values were expressed as Trolox equivalents (mean ± S.D.) by applying the following formula:

$$\text{ORAC value} = \frac{C_{Trolox} \times (AUC_{Sample} - AUC_{Blank})}{C_{Sample} \times (AUC_{Trolox} - AUC_{Blank})}$$

where C_{Trolox} is the concentration (μM) of trolox, C_{Sample} is the concentration of the sample, and AUC is the area below the fluorescence decay curve of the sample, blank and trolox, respectively, calculated by applying the following formula:

$$\text{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 \ldots + f_n/f_0$$

where f_0 is the initial fluorescence and f_n is the fluorescence at time n.

Tyrosinase inhibitory assay

The tyrosinase assay was performed by the method of Baek et al. (2008) with slight modifications, using L-tyrosine as the substrate. 100 μL of 0.1 M phosphate buffer (pH 7.0), 36 μL of 1.5 mM L-tyrosine, and 10 μL of sample solution containing DMSO + 0.05% Tween® 20 to dissolve the sample were added to each well of a 96-well plate and then incubated at 37°C for 10 min. Then 16 μL of mushroom tyrosinase (500 units/mL, 0.1 M phosphate buffer at pH 7.0) was added, and the assay mixture was incubated at 37°C for 20 min. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 492 nm in a microplate reader (Corona Electric Co., Ltd). Arbutin and kojic acid were used as a positive control. The extent of tyrosinase inhibition by the different compounds added was calculated and expressed as the percentage necessary for 50% inhibition concentration (IC_{50}).

The percentage of tyrosinase activity was calculated as follows:

$$\text{Tyrosinase activity (\%)} = [(C - D)/(A - B)] \times 100,$$

where A is the absorbance at 492 nm without test sample, B is the absorbance at 492 nm without test sample and substrate, C is the absorbance at 492 nm with test sample, D is the absorbance at 492 nm with test sample, but without substrate. All data are the mean of three experiments.

RESULTS AND DISCUSSION

Antioxidant activities of bergenin (1) and its derivatives (2-10)

Bergenin (**1**) and all bergenin derivatives (**2**-**10**) were tested that showed antioxidative activity Trolox equivalents (Table II). The antioxidative activities against peroxyl radicals were in the order of bergenin derivatives > Trolox > BHT, indicating that bergenin and all bergenin derivatives (**2**-**10**) were more active than the reference antioxidants. 11-*O*-(3',4'-Dimethoxybenzoyl)-bergenin (**6**) showed the lowest activity in the assay (1.27 ± 0.07 μmol of Trolox equiv. per μmol of **6**). 11-*O*-Protocatechuoylbergenin (**5**), on the other hand, presented the highest antioxidant activity (3.75 ± 0.20

Table II. Antioxidant activity of the bergenin derivatives by ORAC assay

Compound	Trolox-equivs.	Compound	Trolox-equivs.
bergenin (**1**)	1.79 ± 0.11	benzoic acid (**2a**)	0.05 ± 0.02
11-*O*-benzoylbergenin (**2**)	1.56 ± 0.12	*p*-hydroxy benzoic acid (**3a**)	1.84 ± 0.88
11-*O*-*p*-hydroxybenzoylbergenin (**3**)	2.49 ± 0.39	*p*-anisc acid (**4a**)	0.02 ± 0.002
11-*O*-*p*-methoxybenzoylbergenin (**4**)	1.86 ± 0.10	protocatechuic acid (**5a**)	2.26 ± 0.33
11-*O*-protocatechuoylbergenin (**5**)	3.75 ± 0.20	3,4-dimethoxy benzoic acid (**6a**)	0.02 ± 0.001
11-*O*-(3',4'-dimethoxybenzoyl)-bergenin (**6**)	1.27 ± 0.07	vanillic acid (**7a**)	1.17 ± 0.09
11-*O*-vanilloylbergenin (**7**)	2.94 ± 0.08	isovanillic acid (**8a**)	1.16 ± 0.08
11-*O*-isovanilloylbergenin (**8**)	1.69 ± 0.33	3,5-dimethoxy benzoic acid (**9a**)	0.10 ± 0.01
11-*O*-(3',5'-dimethoxybenzoyl)-bergenin (**9**)	1.50 ± 0.05	syringic acid (**10a**)	1.25 ± 0.14
11-*O*-syringylbergenin (**10**)	1.54 ± 0.06	BHT	0.12 ± 0.06
		trolox	1.00

Results are presented as the mean ± S.D., n=3.

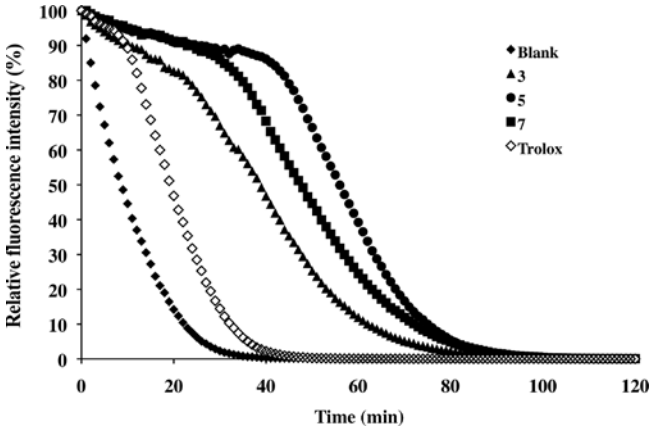

Fig. 3. Fluorescence intensity decay curve of fluorescein in the presence of compound **3**, **5**, **7** and trolox. Concentrations for curves were 20 μM, respectively.

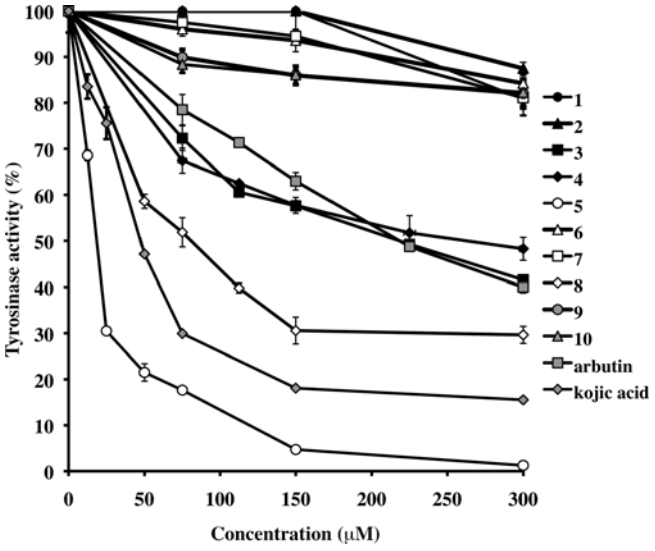

Fig. 4. The dose-dependent inhibitory effects of bergenin (**1**) and bergenin derivatives (**2-10**) on tyrosinase activity. Tyrosinase activity was measured using L-tyrosine as the substrate. Values are means ± S.E. of n = 3 determination. Error bars show S.E. of triplicates.

μmol of Trolox equiv. per μmol of **5**). The other active compounds, compound **3** and **7**, also exhibited antioxidant activities (2.49-2.94 μmol of Trolox equiv. per μmol of **3** and **7**). As presented in Fig. 3, the protective effect of an antioxidant was measured by assessing the fluorescence decay curve (AUC) of the sample compared to a blank, in which no antioxidant was presented. Furthermore, compound **2**, **6**, **8**, **9** and **10** showed moderate antioxidant activities. Whereas, all benzoic acid derivatives (**2a**-**10a**) had lower activities than bergenin derivatives. These results indicate that the bergenin moiety affects the antioxidant activity, and the presence of the hydroxy group at 4'-position on bergenin derivative was a potent factor of antioxidant activity. When the antioxidant activities of compound **5** and **6** were compared, it was found that the potency increased in 5 which was greater than 6. Therefore, we considered that the bergenin moiety and catechol moiety have a great influence on antioxidant activity.

Tyrosinase inhibitory activities of bergenin (1) and its derivatives (2-10)

We investigated the tyrosinase inhibitory activity of bergenin derivatives. Bergenin and its derivatives were used as the effectors. Tyrosinase is a copper-containing enzyme that catalyzes two reactions: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the conversion of DOPA to DOPA quinones (Hearing and Jimenz, 1987). The tested compounds were assayed for tyrosinase inhibitory activity, which demands L-tyrosine as substrate. All of them have inhibitory effects on enzyme activity with dependence on the concentrations as shown in Fig. 4. Four compounds (**3**, **4**, **5**, **8**) showed tyrosinase inhibition with IC_{50} varying from 17.5 to 259.6 μM. Among the compounds, compound **5** exhibited potent tyrosinase inhibitory effect with IC_{50} value of 17.5 ± 0.04 μM as compared to positive control, arbutin (IC_{50} value = 217 ± 2.0 μM) and kojic acid (IC_{50} value = 46.6 ± 3.8 μM).

Among the bergenin derivatives **2**-**10**, introduction of substituents, the addition of the OH or OMe functional group at the 4'-position makes compound **3** (IC_{50} value = 216.9 ± 3.5 μM) and **4** (IC_{50} value = 259.6 ± 2.8 μM) about five times more active than compound **2** which has no substituents of benzoic acid moiety (12.5 ± 1.3% at 300 μM), which were much the same effect as a potent inhibition of arbutin (IC_{50} value = 217.0 ± 2.0 μM). When an OH group was located at the 3' position, and the *para*-hydrogen was substituted by an OH group or OMe group (compound **5** and **8**), inhibitory activities were enhanced, (IC_{50} value = 17.5 ± 0.04 and 79.8 ± 3.3 μM). In the case of compound **5**, the introduction of another OH group in an *ortho*-position to an OH group led to a dramatic increase in inhibitory activity relative to compound **3** and **7**.

On the other hand, when an OMe group was located at the 4' position, and the *meta*-hydrogen was substituted by an OMe group or OH group (compound **6** and **8**), the inhibitory activity of compound 8 was much more potent than that of compound **6**. Other compounds (**9** and **10**) had shown a weak inhibition of mushroom tyrosinase.

Furthermore, we evaluated the inhibitory activities of benzoic acid derivatives and bergenin (**1**) to clarify whether the inhibitory activities of compounds **3**, **4**, **5**,

Table III. Inhibition effects of bergenin (**1**), bergenin derivatives (**2**-**10**), and benzoic acid derivatives (**3a**, **4a**, **5a**, and **8a**) against tyrosinase

Compound	Concentration (μM)	Inhibition (%)	IC_{50} (μM)[a]	Compound	Concentration (μM)	Inhibition (%)	IC_{50} (μM)[a]
1	75.0	0.0 ± 0.5		**8**	50.0	41.3 ± 1.5	
	150.0	0.0 ± 0.7			75.0	48.1 ± 3.1	
	300.0	19.1 ± 3.7	> 300		112.5	60.2 ± 1.0	79.8 ± 3.3
2	75.0	0.0 ± 2.6		**9**	75.0	10.1 ± 1.7	
	150.0	0.0 ± 4.2			150.0	14.0 ± 1.9	
	300.0	12.5 ± 1.3	> 300		300.0	17.9 ± 3.1	> 300
3	150.0	42.3 ± 1.1		**10**	75.0	11.6 ± 1.7	
	112.5	50.7 ± 0.3			150.0	13.9 ± 0.9	
	300.0	58.4 ± 0.4	216.9 ± 3.5		300.0	17.7 ± 2.9	> 300
4	150.0	42.3 ± 1.7		**3a**	75.0	6.1 ± 1.5	
	225.0	48.2 ± 3.7			150.0	9.2 ± 1.5	
	300.0	51.7 ± 2.4	259.6 ± 2.8		300.0	18.6 ± 3.5	> 300
5	12.5	31.4 ± 1.4		**4a**	75.0	0.0 ± 3.5	
	25.0	52.9 ± 1.1			150.0	4.49 ± 4.0	
	50.0	78.5 ± 0.7	17.5 ± 0.04		300.0	13.0 ± 3.5	> 300
6	75.0	3.94 ± 0.3		**5a**	75.0	4.6 ± 0.24	
	150.0	6.4 ± 2.4			150.0	9.5 ± 2.6	
	300.0	15.7 ± 1.7	> 300		300.0	12.0 ± 1.7	> 300
7	75.0	2.4 ± 2.1		**8a**	75.0	0.0 ± 0.3	
	150.0	5.4 ± 0.49			150.0	0.7 ± 0.3	
	300.0	18.9 ± 3.7	> 300		300.0	1.1 ± 1.7	> 300
arbutin[b]			217.0 ± 2.0	**kojic acid**[b]			46.6 ± 3.8

[a]The results are the means ± S.E. of three experiments; [b]Standard inhibitors of the enzyme tyrosinase

and **8** are due to benzoic acid moiety or bergenin moiety. *p*-Hydroxybenzoic acid (**3a**) (inhibition: 18.6 ± 3.5% at 300 μM), anisic acid (**4a**) (inhibition: 13.0 ± 3.5% at 300 μM), protocatechuic acid (**5a**) (inhibition: 12.0 ± 1.7% at 300 μM), isovanillic acid (**8a**) (inhibition: 1.1 ± 1.7% at 300 μM) and bergenin (**1**) (inhibition: 19.1 ± 3.7% at 300 μM) exhibited low inhibitory activity. Each of the benzoic acid derivatives exhibited a relatively poor inhibitory activity as compared with these bergenin derivatives. The results are summarized in Table III. Due to the inhibitory activities of these benzoic acid derivatives, we assumed that the linkage of "benzoic acid" and "bergenin" as well as benzoic acid moiety and bergenin moiety are important factors of tyrosinase inhibitory activity. In addition to these reports, the observation of the inhibitory activities of compound **5** and **8** indicated that 3' position OMe group is also a potent factor of tyrosinase inhibitory activity.

In conclusion, we have undertaken synthesis a series of bergenin analogues. Compound **5** and **8** showed the most potent inhibition of mushroom tyrosinase (IC_{50} value = 17.5 μM and 79.8 μM), but antioxidant activity of compound **8** is less than compound **7**. The inhibition data indicated that the two activities are not directly correlated. However, the substituent groups of bergenin derivatives affected both oxygen radical absorbance capacity and tyrosinase inhibitory activity. Furthermore, in the biologic activities (antioxidant activity and tyrosinase inhibitory activity), the linkage of "bergenin moiety" and "benzoic acid moiety" affected them. In this study, we reported the correlation between tyrosinase inhibition and structures of bergenin derivatives for the first time. Some bergenin derivatives (**2**, **6**, **7**, **9** and **10**) might not be drug candidates themselves since their inhibitory activity on tyrosinase is not strong. However, these results suggest that bergenin derivatives may serve as the designed development of novel tyrosinase inhibitors.

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