Synthesis and Cosmetic Whitening Effect of Glycosides Derived from Several Phenylpropanoids

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Plant-derived phenylpropanoid compounds (4-ethyl-2-methoxyphenol, 2,6-dimethoxyphenol, 2,3-dimethoxyphenol, 3,4-dimethoxyphenol, 3,5-dimethoxyphenol, 3,4-dihydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid, and 3-hydroxy-4-methoxycinnamic acid) were glycosidated to form glycoside compounds. We evaluated the effects of these compounds on the inhibition of tyrosinase and melanin synthesis and their cytotoxicity from the viewpoint of their use as whitening agents in cosmetics. Some compounds had more potent tyrosinase-inhibiting activity than commercial arbutin, which was used as a control, and showed no cytotoxicity at low concentration ranges.

Key words——phenylpropanoid glycoside; tyrosinase inhibition; cytotoxicity

INTRODUCTION

Existing whitening cosmetics were developed to prevent spots and freckles due to sunburn. In recent years, with the nature orientation of consumers, functional cosmetics such as antiwrinkle solutions, whitening agents, and hair growth tonics have been combined with compounds from natural medicines or Chinese herbal medicines. In previous reports, we described several monoterpenoids or glycosides from compounds in Chinese herbal medicines and found that they had good whitening effects in the tyrosinase inhibition test.[1,2]

In this study, we reacted the plant-derived phenylpropanoids (4-ethyl-2-methoxyphenol (**1**), 2,6-dimethoxyphenol (**2**), 2,3-dimethoxyphenol (**3**), 3,4-dimetoxyphenol (**4**), 3,5-dimethoxyphenol (**5**), 3,4-dihydroxycinnamic acid (**6**), 4-hydroxy-3-methoxycinnamic acid (**7**) and 3-hydroxy-4-methoxycinnamic acid (**8**) with acetobromo-α-D-glucose using the Koenigs-Knorr method.[3] The results of the tyrosinase inhibition test, melanin synthesis inhibition test and cytotoxicity test in B16 melanoma cells showed that these glycosides had better whitening effects than that of commercial arbutin.

Phenylpropanoids (**1**)—(**8**) used as starting material are present plant-derived chlorogenic acid metabolites and bamboo vinegar. As an initial attempt to develop cosmetic agents that would add good permeability and hydrophilic property to the skin, we synthesized[4] condensation products (**1a**)—(**8a**) using the methods shown in Figs. 1 and 2. We focused on melanin synthesis at the cellular level, and thus subjected (**1a**)—(**8a**) to the tyrosinase inhibition test, melanin synthesis inhibition test and cytotoxicity test using B16 melanoma cells that produce melanin constantly.

EXPERIMENTAL

Materials 4-Ethyl-2-methoxyphenol (**1**), caffeic acid (3,4-dihydroxycinnamic acid) (**6**), ferulic acid (4-hydroxy-3-methoxycinnamic acid) (**7**), and isoferulic acid (3-hydroxy-4-methoxycinnamic acid) (**8**) was obtained from Tokyo Kasei Kougyou Co. 2,6-Dimethoxyphenol (**2**), and 3,5-dimethoxyphenol (**5**) were obtained from Kantou Kasei Kougyou Co. 2,3-Dimethoxyphenol (**3**), and 3,4-dimethoxyphenol (**4**) were obtained from Aldrich Co.

Analysis The structures of products (**1a**)—(**8a**) were established based on the ^{1}H-NMR and MS spectra. The ^{1}H-NMR spectra were obtained with a JAM-EX400WB (Jeol). Ms spectra were measured with a JMS-HX100 (Jeol).

Synthesis

Synthesis of Glycosides (1a)—(5a) from Bamboo Vinegar Components The synthesis of 4-ethyl-2-methoxyphenyl glucopyranoside (**1a**) is described as an example. A solution of (**1**) (0.500 g, 1.5×10^{-3} mol) and 1,1,3,3-tetramethylurea (0.200 g, 1.7×10^{-3} mol) in CH_2Cl_2 (dehydrated, 5 ml) was stirred
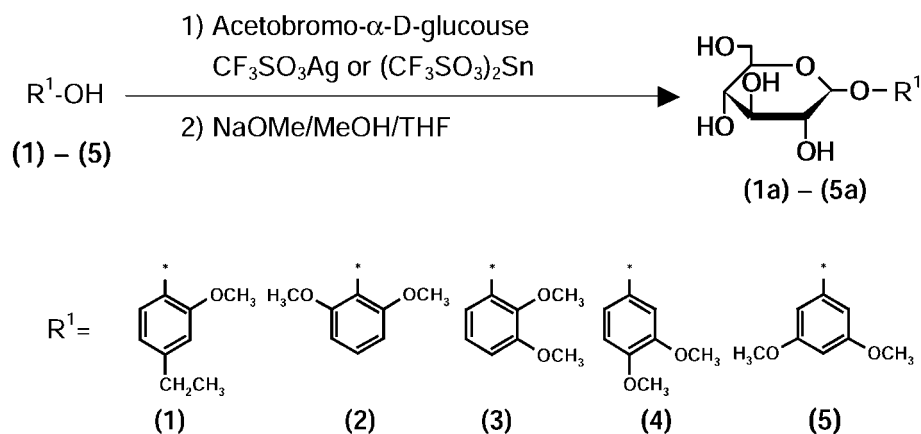
*e-mail: nomura@hiro.kindai.ac.jp

Fig. 1.   Synthesis of Glycosides (1a)—(5a) from Bamboo Vinegar Components
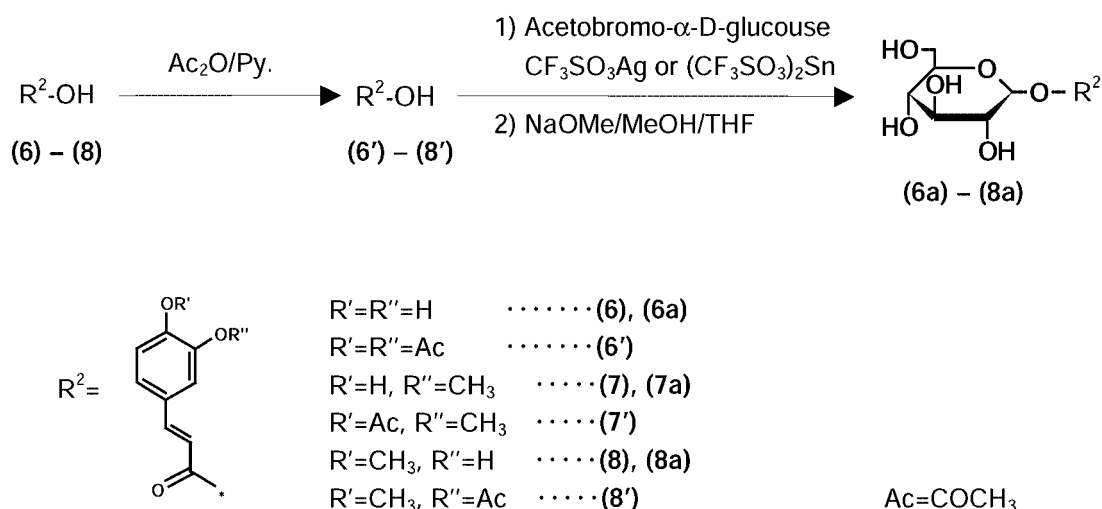

Fig. 2.   Synthesis of Glycosides (6a)—(8a) from Cinnamic Acids

with acetobromo-α-D-glucose (0.575 g, 1.4×10⁻³ mol) and stannous triflate (0.625 g, 1.5×10⁻³ mol) in CH₂Cl₂ (dehydrated, 5 ml) at room temperature (20±2°C). After 24 h, aqueous NaHCO₃ solution (100 ml) and EtOAc (100 ml) were added to the mixture. Then EtOAc was washed twice with saturated aqueous NaCl and dried over anhydrous Na₂SO₄. The EtOAc extract was evaporated under reduced pressure and purified on column chromatography over silica gel with hexane-EtOAc (1 : 1) to afford the acetylglycoside derivative. Then CH₃ONa was added to the solution of the acetylglycoside derivative in CH₃OH-THF (5 : 4) and neutralized with ion-exchange chromatography (DOWEX-50W). After evaporation under reduced pressure, the residue was refined on silica gel chromatography with CHCl₃-CH₃OH (5 : 1) to afford 4-ethyl-2-methoxyphenyl gluco-

pyranoside (1a) 0.350 g (1.1×10⁻³ mol, 35.5%).

This procedure was repeated for compounds (2)—(5): (2) was synthesized to 2,6-dimethoxyphenyl glucopyranoside (2a) (yield 52.9%), (3) to 2,3-dimethoxyphenyl glucopyranoside (3a) (yield 40.1%), (4) to 3,4-dimethoxyphenyl glucopyranoside (4a) (yield 9.7%), and (5) to 3,5-dimethoxyphenyl glucopyranoside (5a) (yield 8.1%).

(1a): ¹H-NMR (CD₃OD) δ: 1.19 (3H, t, J=7.6 Hz, −CH₂CH₃), 2.58 (2H, q, J=7.6 Hz, −CH₂CH₃), 3.36—3.38 (2H, m, Glc-4H, 5H), 3.44—3.48 (2H, m, Glc-2H, 3H), 3.68 (1H, dd, J=5.2 Hz, 12.1 Hz, Glc-6aH), 3.84 (3H, s, −OCH₃), 3.85 (1H, dd, J=2.7 Hz, 15.6 Hz, Glc-6bH), 4.83 (1H, d, J=7.6 Hz, Glc-1H), 6.72 (1H, d, J=8.4 Hz, ph-H), 6.83 (1H, s, ph-H), 7.06 (1H, d, J=8.4

Hz, ph-H). MS m/z (relative abundance %): 315 ([M+H]$^+$, 24), 255 (22), 153 (100), 93 (67), 85 (48).

(2a): ^{1}H-NMR (CD$_3$OD) δ: 3.14—3.19 (1H, m, Glc-4H), 3.35—3.48 (3H, m, Glc-2H, 3H, 5H), 3.44 (1H, ddd, J=2.7 Hz, 8.3 Hz, 15.1 Hz, Glc-5H), 3.62 (1H, dd, J=5.1 Hz, 12.0 Hz, Glc-6aH), 3.73 (1H, dd, J=2.2 Hz, 12.1 Hz, Glc-6bH), 3.79 (6H, s, −OCH$_3$× 2), 4.81 (1H, d, J=7.6 Hz, Glc-1H), 6.63 (2H, d, J=8.3 Hz, ph-H), 6.99 (1H, t, J= 8.3 Hz, ph-H). MS m/z (relative abundance %): 317 ([M+H]$^+$, 4), 277 (62), 155 (27), 93 (67), 85 (48).

(3a): ^{1}H-NMR (CD$_3$OD) δ: 3.38—3.57 (4H, m, Glc-2H, 3H, 4H, 5H), 3.68 (1H, dd, J=5.1 Hz, 12.0 Hz, Glc-6aH), 3.81 (3H, s, −OCH$_3$), 3.83 (3H, s, −OCH$_3$), 3.86 (1H, d, J=12.5 Hz, Glc-6bH), 4.90 (1H, d, J= 7.3 Hz, Glc-1H), 6.71 (1H, d, J=8.3 Hz, ph-H), 6.82 (1H, d, J=8.5 Hz, ph-H), 6.99 (1H, t, J=8.4 Hz, ph-H). MS m/z (relative abundance %): 317 ([M+H]$^+$, 4), 277 (62), 155 (27), 93 (67), 85 (48).

(4a): ^{1}H-NMR (CD$_3$OD) δ: 3.25—3.27 (1H, m, Glc-4H), 3.28—3.36 (3H, m, Glc-2H, 3H, 5H), 3.59 (1H, dd, J=5.9 Hz, 12.0 Hz, Glc-6aH), 3.69 (3H, s, −OCH$_3$), 3.72 (1H, s, −OCH$_3$), 3.81 (1H, dd, J=2.2 Hz, 12.0 Hz, Glc-6bH), 4.69 (1H, d, J=7.6 Hz, Glc-1H), 6.58 (1H, dd, J=2.8 Hz, 5.9 Hz, ph-H), 6.73 (1H, d, J=2.8 Hz, ph-H), 6.76 (1H, d, J=8.8 Hz, ph-H). MS m/z (relative abundance %): 317 ([M+H]$^+$, 5), 277 (75), 155 (10), 93 (100), 75 (99), 57 (53).

(5a): ^{1}H-NMR (CD$_3$OD) δ: 3.35—3.50 (3H, m, Glc-2H, 3H, 4H), 3.44 (1H, ddd, J=2.2 Hz, 7.3 Hz, 18.9 Hz, Glc-5H), 3.68 (1H, dd, J=5.9 Hz, 12.2 Hz, Glc-6aH), 3.74 (6H, s, −OCH$_3$×2), 3.88 (1H, dd, J=2.2 Hz, 12.1 Hz, Glc-6bH), 4.85 (1H, d, J=7.6 Hz, Glc-1H), 6.15 (1H, t, J=2.2 Hz, ph-H), 6.30 (2H, d, J=2.2 Hz, ph-H). MS m/z (relative abundance %): 317 ([M+ H]$^+$, 10), 277 (65), 93 (100), 75 (76), 57 (40).

Synthesis of Glycosides (6a)—(8a) from Cinnamic Acids 3,4-Dihydroxycinnamic acid (6) (1.0 g, 5.5×10^{-3} mol) stirred with pyridine (7 ml) at speci-

fied temperature (0—5°C), and acetic anhydride (1.1 ml) was added dropwise to the mixture for 30 min. After 48 h, 2 N HCl solution was added and extracted with diethylether. The organic layer was washed twice with saturated aqueous NaCl and dried over anhydrous Na$_2$SO$_4$. The diethylether extract was evaporated under reduced pressure and purified on column chromatography with silica gel with EtOAc to afford 3,4-diacetylcinnamic acid (6').

To the solution of (6') (0.500 g, 1.5×10^{-3} mol) 1,1,3,3-tetramethylurea (0.200 g, 1.7×10^{-3} mol) in CH$_2$Cl$_2$ (dehydrated, 5 ml) was added, and the mixture was stirred with acetobromo-α-D-glucose (0.575 g, 1.4×10^{-3} mol) and stannous triflate (0.625 g, 1.5 ×10^{-3} mol) in CH$_2$Cl$_2$ (dehydrated, 5 ml) at room temperature (20±2°C). After 24 h, aqueous NaHCO$_3$ solution (100 ml) and EtOAc (100 ml) were added to the mixture for extraction. Then EtOAc was washed twice with saturated aqueous NaCl and dried over anhydrous Na$_2$SO$_4$. The EtOAc extract was evaporated under reduced pressure and purified on column chromatography with silica gel with hexane-EtOAc (1 : 1) to afford the acetylglycoside derivative. Then CH$_3$ONa was added to the acetylglycoside derivative solution in CH$_3$OH-THF (5 : 4) and neutralized with ion-exchange chromatography (DOWEX-50 W). After evaporation under reduced pressure, the residue was refined on silica gel chromatography with CHCl$_3$-CH$_3$OH (5 : 1) to afford 3,4-dihydroxycinnamic acid glucopyranoside (6a) (yield 25.3%).

This procedure was repeated for compounds (7) and (8): (7) was synthesized to 4-hydroxy-3-methoxycinnamic acid glucopyranoside (7a) (yield 25.3%), and (8) to 3-hydroxy-4-methoxycinnamic acid glucopyranoside (8a) (yield 36.2%).

(6a): ^{1}H-NMR (CD$_3$OD) δ: 3.33—3.49 (4H, m Glc-2H, 3H, 4H, 5H), 3.69 (1H, dd, J=4.6 Hz, 12.2 Hz, Glc-6aH), 3.85 (1H, dd, J= 2.0 Hz, 12.1 Hz, Glc-6bH), 5.57 (1H, d, J= 7.8 Hz, Glc-1H), 6.31 (1H, d, J=15.9 Hz, −CH=CHCOO-), 6.79 (1H, d, J=8.3 Hz, ph-H), 6.97 (1H, dd, J=2.0 Hz, 8.2 Hz, ph-H), 7.07 (1H, d, J=2.0 Hz, ph-H), 7.66 (1H, d, J=15.9 Hz, −CH=CHCOO-). MS m/z (relative abundance %): 343 ([M+ H]$^+$, 15), 341 (70), 325 (74), 311 (68), 275 (73), 183 (100), 179 (38), 91 (97), 89 (71), 59 (71).

(**7a**): ^{1}H-NMR (CD$_3$OD) δ: 3.37—3.47 (4H, m, Glc-2H, 3H, 4H, 5H), 3.69 (1H, dd, J=4.6 Hz, 12.2 Hz, Glc-6aH), 3.85 (1H, dd, J=2.0 Hz, 12.5 Hz, Glc-6bH), 3.89 (3H, s, $-$OCH$_3$), 5.58 (1H, d, J=7.1 Hz, Glc-1H), 6.41 (1H, d, J=15.9 Hz, $-$CH$=$CHCOO-), 7.11 (1H, d, J=2.0 Hz, ph-H), 7.17 (1H, dd, J=2.0 Hz, 8.3 Hz, ph-H), 7.20 (1H, d, J=2.0 Hz, ph-H), 7.73 (1H, d, J=15.9 Hz, $-$CH$=$CHCOO-). MS m/z (relative abundance %): 317 ([M+H]$^+$, 5), 277 (75), 155 (10), 93 (100), 75 (99), 57 (53).

(**8a**): ^{1}H-NMR (CD$_3$OD) δ: 3.31—3.43 (3H, m, Glc-2H, 3H, 5H), 3.38 (1H, t, J=6.6 Hz, Glc-4H), 3.52 (1H, dd, J=4.6 Hz, 12.2 Hz, Glc-6aH), 3.83 (1H, dd, J=2.2 Hz, 12.1 Hz, Glc-6bH), 3.87 (3H, s, $-$OCH$_3$), 4.51 (1H, d, J=7.8 Hz, Glc-1H), 6.33 (1H, d, J=16.1 Hz, $-$CH$=$CHCOO-), 6.91 (1H, d, J=7.8 Hz, ph-H), 6.98 (1H, d, J=8.3 Hz, ph-H), 7.04 (1H, dd, J=2.0 Hz, 8.3 Hz, ph-H), 7.30 (1H, d, J=15.9 Hz, $-$CH$=$CHCOO-). MS m/z (relative abundance %): 317 ([M+H]$^+$, 10), 277 (65), 93 (100), 75 (76), 57 (40).

Biological Activity

Tyrosinase Inhibition Test in B16 Mouse Melanoma Cells

1) Preparation of Crude Tyrosinase Mixture Cells were collected in a 96-well plate at a concentration of 5.0×10^4 cells/well and 50 μl of (Dulbecco's phosphate-buffered saline, Sigma) with 1.0% Triton-X was added and mixed well. After centrifugation supernatant was used as the crude enzyme mixture.

2) Tyrosinase Inhibition Test A specific quantity of sample was dissolved in 50 μl of DMSO mixture add 50 μl of (final concentration, 2.5 mM) DOPA solution 7.6 mM and 50 μl of crude tyrosinase mixture prepared in step 1 were added). After 5 min of mixing, the solution was incubated for 3 h at 37°C. Then the optical density (A) at 475 nm was measured with a spectrophotometer. As a blank test, DMSO was used instead of samples and as a color control test, phosphate buffer was used instead of crude enzyme buffer. The results of the blank test and color control test were referred to as B and C respectively. The percentage of tyrosinase inhibition was calculated as follows:

Tyrosinase inhibition (%)

$$= \{B-(A-C)\}/C \times 100$$

Melanin Synthesis Inhibition Test Cells were seeded into 24-well plates at a density of 3.0×10^4 cells/ml and incubated for 24 h. The medium was changed to 90.0 μg/ml of theophylline and 1.0% DMSO mixture with a specific quantity of sample and incubated for 3 days in a CO$_2$ incubator. The medium was removed and the melanin produced was dissolved with 3 N NaOH solution. Optical density (A) at 405 nm was measured with a spectrophotometer. As a blank test, DMSO was used instead of the sample mixture and its result was referred to as B. The percentage of melanin synthesis inhibition was calculated as follows:

Melanin synthesis inhibition (%)

$$= (B-A)/B \times 100$$

Cytotoxicity (MTT Assay) Cells were seeded into 96-well plates at a density of 1.0×10^4 cells/90 μl and incubated for 24 h in a CO$_2$ incubator. Then microliters 10 μl of DMSO mixture including a specific quantity of samples was added and incubated for 48 h in a CO$_2$ incubator. Then 10 μl of phosphate-buffered MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide) mixture (MTT 5 mg/ml) was added and incubated for 6 h. After removal of the MTT mixture by suction, 200 μl of acidic isopropanol (alcohol including 1.0% by volume of 4 N HCl solution) was added to produce MTT formazan, and mixed for 10 min with microplate mixer. Optical density (A) at 560 nm was measured with a spectrophotometer. As a blank test, DMSO was added instead of samples and optical density (B) was measured. The ratio of cell damage (cytotoxicity) was calculated as follows:

Cell damage (%) $= (B-A)/B \times 100$

RESULTS AND DISCUSSION

(**1a**)—(**8a**) did not showed the expected significant tyrosinase-inhibitory activity in the tyrosinase inhibition test with crude tyrosinase mixture from B16 melanoma cells, but (**6a**) and (**7a**) showed more potent tyrosinase inhibitory activity than commercial arbutin (Table 1). Hydrophilicity improved with glycosidation, but tyrosinase-inhibitory activity decreased compared with that of precursor compounds (**1**)—(**5**) (i.e., (**1**)→(**1a**), 46.3%→7.9%; (**2**)→(**2a**), 72.3%→9.6%). These results suggest that hydroxyl in the benzene ring is required for improved tyrosinase-inhibitory activity.

Table 1.　Inhibition of Tyrosinase Activity by (1a)—(8a)

Compound	Concentration (mM)[a]					
	2.0	1.0	0.5	0.25	0.13	0.06
(1a)	7.9	1.1	2.0	14.0	3.9	1.8
(2a)	0.2	9.2	6.6	8.9	16.6	15.4
(3a)	4.0	16.4	11.1	11.2	5.3	11.9
(4a)	9.6	6.5	10.6	13.0	7.6	15.7
(5a)	1.8	7.2	15.0	6.5	5.8	3.5
(6a)	27.3	27.2	11.4	11.4	4.5	1.8
(7a)	55.5	39.5	25.6	12.5	8.3	10.2
(8a)	21.4	18.5	11.1	6.4	16.0	9.2
Arbutin	33.0	16.0	6.2	7.6	—	—

a) Inhibition rate (%).

Table 2.　Inhibition of Melanin Formation by (1a)—(8a)

Compound	Concentration (mM)[a]				
	2.0	1.0	0.5	0.25	0.05
(1a)	46.9	37.1	28.3	20.5	—
(2a)	10.3	—	—	—	—
(3a)	47.6	37.1	17.2	12.3	—
(4a)	2.9	—	—	—	—
(5a)	71.1	36.5	12.8	16.8	—
(6a)	31.0	—	—	—	—
(7a)	41.1	31.5	15.3	28.9	—
(8a)	41.9	38.2	10.0	16.8	—
Arbutin	45.4	47.9	37.4	28.5	5.7

a) Inhibition rate (%).

Table 3.　Cytotoxic Effects of (1a), (3a), (5a), (7a), and (8a)

Compound	Concentration (mM)[a]				
	20.0	10.0	5.0	2.5	1.0
(1a)	48.1	24.0	10.1	0.0	0.0
(3a)	22.3	16.8	7.2	5.5	0.0
(5a)	32.9	29.2	17.4	10.2	0.0
(7a)	0.0	0.0	0.0	0.0	0.0
(8a)	41.1	19.4	0.0	0.0	0.0
Arbutin	39.9	16.2	9.6	0.0	0.0

a) Inhibition rate (%).

The results of the melanin synthesis inhibition test showed that (1a), (3a), (5a), (7a), and (8a) have significant inhibitory activity at final concentrations of 2.0 mM—0.25 mM (Table 2). In particular, (1a) and (5a) showed similar or more potent inhibition to that of commercial arbutin at some concentration. These results suggest that these compounds do not inhibit tyrosinase directly.

Melanin has protective effects against ultraviolet radiation. Inhibition of melanin synthesis with whitening cosmetics may cause progression to permanent vitiligo due to the lack of melanin production. We therefore examined the concentrations at which compounds (1a), (3a), (5a), (7a), and (8a) showed potent melanin synthesis inhibitory activity without cytotoxicity. The cytotoxicity of all five compounds was less than that of their precursors. We also examined cytotoxicity at the low concentration of 1.0 mM and found that these compounds expressed melanin synthesis-inhibitory activity without cytotoxicity (Table 3).

This study showed that glycosides of several phenylpropanoids have hydrophilic properties. Particular, (7a) has significant whitening effects and has the potential for practical application as a whitening cosmetic.

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