Natural Product Sciences
4(3) : 161 ~ 169 (1998)

Inhibitory Effect of Lichen Metabolites and their Synthetic Analogues on Melanin Biosynthesis in Cultured B-16 Mouse Melanoma Cells

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Abstract – The analogues of lichen components showing anti-tyrosinase activities were synthesized. 4-Alkylresorcinol derivatives showed both the inhibitory activity and cytotoxicity in B-16 melanona cells at the doses of 10 mM to 1.2 mM. Resorcinol and 4-methylresorcinol showed the inhibitory effect with a low cytotoxicity at the doses of 2.5 mM and 600 μM among 4-alkylresorcinols, respectively. Some diphenylmethane derivatives (Type A, B, and C) had strong activities with a low cytotoxicity. While xanthene derivatives had no effect. Glucosides of 4,5-alkylresorcinol and the diphenylmethane derivative (Type B) were prepared to decrese the cytotoxicity. As a result, no effect were observed. Liposome of the diphenylmethane derivative (Type B) was prepared for the same purpose, and the latter showed a remarkable effect at the dose of 15 μM with a low cytotoxicity.

Key words – Anti-tyrosinase, lichen, cultured B-16 mouse melanoma cells, liposome.

Introduction

We have already reported the anti-tyrosinase active compounds in lichen thalli of *Protousnea* spp. (Kinoshita *et al.*, 1994) and their synthetic analogues (Matsubara *et al.*, 1997). 4-Alkylresorcinols showed stronger activity than naturally occurring 5-alkyl isomrs, and the activity increases by the elongation of alkyl chain. We now report both the activity and cytotoxicity of newly synthesized diphenyl-methane derivatives (Type A-C), xanthenes (Type D), 4-alkyl-resorcinols, 5-alkyl-resorcinols, and their glucosides in cultured B-16 mouse melanoma cells.

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Experimental

Reagents – Lecitin, glucosylbromide (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), dimethyl sulfoxide (DMSO) (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) are commercial products.

Spectroscopy and chromatography – The ir spectra were measured with a JASCO A-120 ir spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded using JEOL GSX-400 (^{1}H 400 and ^{13}C 100 MHz) spectrometer in $CDCl_3$ or CD_3OD with trimethylsilane as an internal standard. The chemical shifts are expressed in ppm (δ). Column chromatography was carried out on 70-230 mesh Si gel (Merck). HPLC was performed using an SSC-3100-J pump with an Oyo-Bunko Uvilog 7

uv detector. Hrms and eims spectra were obtained using a JEOL-JMS-DX 302.

The Assay method for inhibition of melanin synthesis in cultured B16 mouse melanoma cells

Growing cells of cultured B16 mouse melanoma were implanted in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) at 2.5×10^3 cells/100 μl. The cells were incubated for 24 hr at 37°C in 5% CO_2 under high humidity of atmosphere. The culture was renewed once with fresh medium containing a sample compound, while only fresh medium as control, and each cell line was incubated for 48 hr at 37°C in a 5% CO_2. On completion of incubation, the cells were washed with phosphate-buffered saline (PBS) 100 μl, and were added to 1% Triton X 45 μl. After centrifugation at 700 rpm for 4 min, cells were added to 10 mM DOPA and centrifugation at the same condition. After incubation for 30 min at 37°C, the optical density at 490 nm of the resulting supernatant (Ab. Sample or Ab. Control) was measured by a spectrophotometer.

Tyrosinase inhibition rate (%)=
[1-(Ab. Sample/Ab. Control)]$\times$100

The Assay method for cytotoxic activity in cultured B16 mouse melanoma cells

Growing cels of cultured B16 mouse melanoma were implanted in Eagle's minimum essential medium (MEM) containing 10% FCS at 2.5×10^3 cells/100 μl. The cells were incubated for 24 hr at 37°C in 5% CO_2 under high humidity of atmosphere. The culture was renewed once with fresh medium containing a sample compound, while only fresh medium as control, and cells were incubated for 48 hr at 37°C in a 5% CO_2. On completion of incubation, the cells were washed with PBS 100 μl, and then were added to 120 μl fresh medium containing 100 μM WST-1 and 20 μM 1-methoxy PMS. After incubation for 3 hr at 37°C, the optical density at 450 nm of the resulting supernatant (Ab. Sample or Ab. Control) was measured by a spectrophotometer.

Cytotoxic activity rate (%)=
[1-(Ab. Sample/Ab. Control)]$\times$100

Preparation of liposome – Lectin from egg 100 ml and test compound 0.1 mmol were dissolved in 5 ml of chloroform. Following complete evaporation of the solvent from the surface of the aqueous mother liquid, a lipid film was formed. Then, 5 ml of pure water was added to liquid solution and emulsified with a homogenizer at 10,000 rpm for 3 min twice to form a emulsion. This emulsion was then added immediately to two volumes (10 ml) of water. Control was made using liposome without test compound.

Syntheses of diphenylmethane derivatives and xanthenes

Preparation of diphenylmethanes – 94 % Paraformaldehyde was dissolved with 88% formic acid in the round bottomed flask on an oil bath at 80°C, and then 3.0 mole of resorcinol or 5-alkylresorcinols were added into the solution, respectively. The solution was refluxed at 120°C on an oil bath for 6 hr. After cooling, the reaction mixture was extracted with ether three times, and the ethereal layer was dried on anhydrous sodium sulfate. After filtering, the filtrate was evaporated to a solid. The mixture was chromatographed on a Si gel column to obtain crude compound, which was further chromatographed on a HPLC column ($CHCl_3$-MeOH, $CHCl_3$-MeOH-H_2O, MeOH-H_2O solvent system) to get pure compounds.

Bis(2,4-dihydroxyphenyl)methane – White amorphous powder, mp 181-185°C (dec.) UV λ_{max} (MeOH) nm (logε): 212 (4.16), 280 (3.69), IR ν_{max} (KBr) cm^{-1}: 3250, 1620, 1510, 1470, 1170, 830. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 3.72 (2H, s, CH_2), 6.30 (2H, d, J=2.4 Hz, H-3), 6.33 (2H, m, H-5), 6.97 (2H, d, J=8.0 Hz, H-6). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 28.9 (CH_2), 102.3 (C-5), 107.0 (C-3), 119.0 (C-1), 130.4 (C-6), 153.9 (C-4), 155.7 (C-2). EI-MS m/z: 232 (M^+), 123, 110. HR-MS m/z: 232.0733 (M^+,

calcd. for $C_{13}H_{12}O_4$, 232.0736).

Bis(2,6-dihydroxyphenyl)methane – White amorphous powder, mp 248-251°C (dec.). UV λ_{max} (MeOH) nm (logε): 208 (4.15), 270 (3.10), IR ν_{max} (KBr) cm^{-1}: 3250, 1600, 1470, 1050. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 3.91 (2H, s, CH_2), 6.45 (4H, d, J=8.0 Hz H-3 and 5), 6.94 (2H, dd, J=8.0, 8.2 Hz, H-4), 6.97 (2H, d, J=8.0 Hz, H-6). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 17.5 (CH_2), 107.9 (C-3 and 5), 113.8 (C-1), 127.6 (C-4), 154.8 (C-2 and 6). EI-MS m/z: 232 (M^+), 123, 110. HR-MS m/z: 232.0742 (M^+, calcd. for $C_{13}H_{12}O_4$, 232.0736).

2,2',4,6'-Tetrahydroxydiphenylmethane – Light brown amorphous powder, mp 209-214°C (dec.). UV λ_{max} (MeOH) nm (logε): 211 (4.16), 279 (3.52), IR ν_{max} (KBr) cm^{-1}: 3250, 1600, 1510, 1470, 1010. ^{1}H-NMR ($CDCl_3$+CD_3 OD) δ: 3.84 (2H, s, CH_2), 6.30 (2H, m, H-3 and 5), 6.37 (2H, dd, J=3.2, 8.0 Hz, H-3' and 5'), 6.85 (1H, dt, J=3.4, 8.2 Hz, H-4'), 743 (1H, dd, J=3.6, 8.6 Hz, H-6). ^{13}C-NMR ($CDCl_3$ +CD_3OD) δ: 22.5 (CH_2), 102.3 (C-5), 107.1 (C-3' and 5'), 107.2 (C-3), 114.9 (C-1'), 118.7 (C-1), 126.7 (C-4'), 132.0 (C-6), 154.0 (C-4), 154.9 (C-2' and 6'), 155.6 (C-2). EI-MS m/z: 232 (M^+), 123, 110. HR-MS m/z: 232.0738 (M^+, calcd. for $C_{13}H_{12}O_4$, 232.0736).

Bis(2,4-dihydroxy-6-methylphenyl) methane – Orange amorphus powder, mp 213-216°C (dec.). UV λ_{max} (MeOH) nm (logε): 220 (3.81), 280 (3.33), IR ν_{max} (KBr) cm^{-1}: 3220, 1610, 1470, 1280, 1140. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 2.23 (6H, CH_3), 3.81 (2H, s, CH_2), 6.20 (2H, d, J=2.5 Hz, H-3), 6.22 (2H, d, J=2.5 Hz, H-5). ^{13}C-NMR ($CDCl_3$+CD_3 OD) δ: 19.8 (CH_3), 22.0 (CH_2), 100.2 (C-3), 109.3 (C-5), 116.2 (C-1), 138.8 (C-6), 154.9 (C-4), 154.9 (C-2). EI-MS m/z: 260 (M^+), 137, 124. HR-MS m/z: 260.1045 (M^+, calcd. for $C_{15}H_{16}O_4$, 260.1049).

Bis(2,6-dihydroxy-4-methylphenyl) methane – Colorless plates, mp 244-249°C (dec.). UV λ_{max} (MeOH) nm (logε): 223 (4.15), 270 (3.23), IR ν_{max} (KBr) cm^{-1}: 3250, 1590, 1060, 820. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 2.17 (6H, CH_3), 3.84 (2H, s, CH_2), 6.28 (4H, s, H-3 and 5). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 17.0 (CH_3), 21.0 (CH_2), 108.8 (C-3 and 5), 111.0 (C-1), 137.9 (C-4), 154.2 (C-2 and 6). EI-MS m/z: 260 (M^+), 137, 124. HR-MS m/z: 260.1041 (M^+, calcd. for $C_{15}H_{16}O_4$, 260.1049).

2,2',4,6'-Tetrahydroxy-4',6-dimethylphenylmethane – Orange amorphous powder, mp 300-302°C. UV λ_{max} (MeOH) nm (logε): 227 (3.88), 277 (3.63), IR ν_{max} (KBr) cm^{-1}: 3250, 1590, 1140, 1060, 820. ^{1}H-NMR ($CDCl_3$ +CD_3OD) δ: 2.16 (6H, CH_3), 2.37 (3H, s, CH_3), 3.86 (2H, s, CH_2), 6.21 (4H, m, H-3, 3', 5 and 5'). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 18.8 (CH_2), 19.8 (CH_3), 20.5 (CH_3), 99.3 (C-3), 107.8 (C-3' and 5'), 109.6 (C-5), 110.5 (C-1'), 116.7 (C-1), 136.7 (C-4') 140.0 (C-6), 154.3 (C-4), 154.8 (C-2), 155.1 (C-2' and 6'). EI-MS m/z: 260 (M^+), 137, 124. HR-MS m/z: 260.1039 (M^+ calcd. for $C_{15}H_{16}O_4$, 260.1049).

1,8-Dihydroxy-3,6-dimethylxanthene – White amorphous powder, mp 205°C (dec.). ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 2.24 (6H, s, CH_3), 3.73 (2H, s, CH_2), 6.33 (2H, s, H-2,7), 6.38 (2H, s, H-4,5). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 17.3 (CH_2), 2.10 (CH_3), 104.7 (C-1a and 8a), 108.3 (C-2 and 7), 109.6 (C-4 and 5), 137.4 (C-3 and 6), 152.0 (C-1 and 8), 154.5 (C-4a and 5a). EI-MS m/z: 242 (M^+), 137, 124. HR-MS m/z: 242.0945 (M^+, calcd. for $C_{15}H_{14}O_3$, 242.0943).

Bis(2,6-dihydroxy-4-propylphenyl) methane – Colorless plates, mp 182-184°C. UV λ_{max} (MeOH) nm (logε): 215 (4.28), 280 (3.10), IR ν_{max} (KBr) cm^{-1}: 3300, 2950, 1590, 1440, 1050, 820. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.90 (6H, t, J=7.4 Hz, H-3'), 1.55 (4H, m, H-2'), 2.41 (4H, t, J=7.6 Hz, H-1'), 3.84 (2H, s, CH_2), 6.29 (4H, s, H-3,5) ^{13}C-NMR ($CDCl_3$+ CD_3OD) δ: 13.5 (C-3'), 17.1 (CH_2), 24.0 (C-2'), 37.5 (C-1'), 107.9 (C-3 and 5), 111.2 (C-1), 142.6 (C-4), 154.2 (C-2 and 6) EI-MS m/z: 316 (M^+), 165, 152. HR-MS m/z: 316.1670 (M^+, calcd. for $C_{19}H_{24}O_4$, 316.1675).

1,8-Dihydroxy-3,6-dipropylxanthene – White amorphous, mp 159-162°C. UV λ_{max}

(MeOH) nm (logε): 229 (4.62), 246 (4.24), 273 (4.16), IR ν_{max} (KBr) cm^{-1}: 3450, 1630, 1425, 1040. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.94 (6H, t, J=7.3 Hz, H-3'), 1.62 (4H, m, H-2'), 2.47 (4H, t, J=7.3 Hz, H-1'), 3.74 (2H, s, CH_2), 6.35 (2H, d, J=1.3 Hz, H-2 and 7), 6.39 (2H, d, J= 1.1 Hz, H-3 and 4). ^{13}C-NNR ($CDCl_3$+CD_3OD) δ: 13.5 (C-3'), 17.4 (CH_2), 24.0 (C-2'), 37.6 (C-1'), 105.0 (C-1a and 8a), 107.4 (C-2 and 7), 108.9 (C-4 and 5), 142.2 (C-3 and 6), 151.0 (C-1 and 8), 154.6 (C4a and 5a). FAB-MS m/z: 299 $[M+H]^+$.

Bis(2,6-dihydroxy-4-pentylphenyl) methane – Orange plates, mp 154-156°C. UV λ_{max} (MeOH) nm (logε): 217 (4.39), 270 (3.40), IR ν_{max} (KBr) cm^{-1}: 3300, 2920, 1590, 1050, 830. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.87 (6H, t, J=6.8 Hz, H-5'), 1.28 (8H, m, H-3' and 4'), 1.53 (4H, m, H-2'), 2.42 (4H, t, J=7.6 Hz, H-1'), 3.83 (2H, s, CH_2), 6.29 (4H, s, H-3 and 5). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 13.8 (2H, s, CH_2), 17.2 (CH_2), 22.4 (C-4'), 30.7 (C-2'), 31.3 (C-3'), 35.5 (C-1'), 108.0 (C-3 and 5), 111.3 (C-1), 142.9 (C-4), 154.3 (C-2 and 6). EI-MS m/z: 372 (M^+), 193, 180. HR-MS m/z: 372.2298 (M^+, calcd. for $C_{23}H_{32}O_4$, 372.2302).

2,2',4,6'-tetrahydroxy-4',6-dipentylphenylmethane – Orange amorphous powder, mp 68-70°C. UV λ_{max} (MeOH) nm (logε): 220 (4.37), 280 (3.68), IR ν_{max} (KBr) cm^{-1}: 3300, 2850, 1610, 1580, 1450, 1040. ^{1}H-NMR ($CDCl_3$ +CD_3OD) δ: 0.86 (6H, m, CH_2), 1.27 (8H, m, CH_2), 1.42 (2H, m, CH_2), 1.52 (2H, m, CH_2), 2.40 (2H, t, J=7.7 Hz, CH_3), 2.75 (2H, t, J=7.6 Hz, CH_2), 3.89 (2H, s, CH_2), 6.19 (3H, m, H-3', 5 and 5'), 6.25 (1H, d, J=0.8 Hz H-3). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 13.6 (CH_3), 13.7 (CH_3), 18.4 (CH_2), 22.3 (CH_3), 22.4 (CH_3), 30.6 (CH_2), 31.2 (CH_2), 31.7 (CH_2), 32.8 (CH_2), 35.3 (CH_2), 99.7 (C-3), 107.1 (C-3'and 5'), 108.8 (C-5), 111.1 (C-1'), 116.2 (C-1), 142.0 (C-4'), 145.2 (C-6), 154.3 (C-4), 155.0 (C-2), 155.0 (C-2' and 6'). EI-MS m/z: 372 (M^+), 193, 180. HR-MS m/z: 3772.2299 (M^+, calcd. for $C_{23}H_{32}O_4$, 372.2302).

1,8-Dihydroxy-3,6-dipentylxanthene – Colorless needles, mp 157-160°C. UV λ_{max} (MeOH) nm (logε): 217 (4.32), 250 (3.48), 275 (3.40), IR ν_{max} (KBr) cm^{-1}: 3400, 2900, 1620, 1420, 1040. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.88 (6H, t, J=7.0 Hz, H-5'), 1.32 (8H, m, H-3' and 4'), 1.59 (4H, m, H-2'), 2.48 (4H, t, J=7.7 Hz, H-1'), 3.74 (2H, s, CH_2), 6.34 (2H, d, J=1.4 Hz, H-2 and 7), 6.40 (2H, d, J=1.1 Hz, H-4 and 5). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 13.7 (C-5'), 17.3 (CH_2), 22.3 (C-4'), 30.0 (C-2'), 31.2 (C-3'), 35.4 (C-1'), 104.9 (C-ia and 8a), 107.3 (C-2 and 7), 108.8 (C-4 and 5), 142.4 (C-3 and 6), 151.9 (C-1 and 8), 154.6 (C-4a and 5a). FAB-MS m/z: 354 $[M+H]^+$. HR-MS m/z: 354.2194 (M^+, calcd. for $C_{23}H_{30}O_4$ 354.2196).

Synthesis of monoglucosides of 5-pentylresorcinol, 4-pentylresorcinol and bis (2,6-dihydroxy-4-pentylphenyl)methane – Acetobromoglucose (1 mmol), aglycone (1 mmol) and powdered molecular sieves 4A were put in a round bottomed flask. 20 ml of methy lene chloride and perchloric acid (1 mmol) were added into the mixture. The mixture was standing for 24 hr at room temperature. After filtering, the filtrate was evaporated to a solid. The crude material was chromatographed on a Sephadex LH20 to remove aglycone fraction, and to obtain crude glucoside. It was chromatographed on an HPLC column ($CHCl_3$-MeOH solvent system) to obtain a pure compound.

5-Pentylresorcinol-2,3,4,6-tetra-O-β-glucoside (yield 19.0%) – Colorless oil. UV λ_{max} ($CHCl_3$) nm (logε): 238 (2.76), 271 (3.05), IR ν_{max} (NaCl) cm^{-1}: 3460, 2940, 1750, 1600, 1220, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J =6.7 Hz, C-5'), 1.31 (4H, m, C-3' and 4'), 1.57 (2H, m, H-2'), 2.04 (3H, s, AcO), 2.05 (3H, s, AcO), 2.06 (3H, s, AcO), 2.09 (3H, s, AcO), 2.50 (2H, t, J=7.8 Hz, H-1'), 3.86 (1H, ddd, J=2.4, 5.4, 10.0 Hz, H-5'), 4.18 (1H, dd, J=2.2, 12.4 Hz, H-6"), 4.28 (1H, dd, J=5.2, 12.2 Hz, H-6"), 5.03 (1H, d, J=7.3 Hz, H-1"), 5.15 (1H, t, J=9.4 Hz, H-4"), 5.24 (1H, t, J=8.5 Hz, H-2"), 5.31 (1H, q, J=9.1 Hz, H-3"), 5.77 (1H, OH), 6.3-6.4 (3H, m, H-2, 4 and 6). ^{13}C-NMR ($CDCl_3$)

δ: 14.0 (C-5'), 20.6 (AcO), 22.5 (C-4'), 30.7 (C-2'), 31.4 (C-3'), 35.9 (C-1'), 62.0 (C-6"), 68.3 (C-4"), 71.1 (C-2"), 71.9 (C-5"), 72.7 (C-3"), 98.9 (C-1"), 101.7 (C-2), 109.2 (C-4), 110.4 (C-6), 145.9 (C-5), 150.7 (C-3), 157.8 (C-1), 169.5 (COO), 170.4 (COO), 170.9 (COO). FAB-MS *m/z*: 511 $[M+H]^+$.

4-Pentylresorcinol-2,3,4,6-tetra-O-acetyl-β-glucoside (yield 6.9%) – Colourless oil. UV λ_{max} ($CHCl_3$) nm (logε): 239 (2.68), 275 (3.15), IR ν_{max} (NaCl) cm^{-1}: 3460, 2940, 1750, 1220, 1020. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, *J*=6.8 Hz, C-5'), 1.32 (4H, m, C-3' and 4'), 1.57 (2H, m, H-2'), 2.04 (6H, s, AcO), 2.06 (3H, s, AcO), 2.09 (3H, s, AcO), 2.53 (2H, t, *J*=7.7 Hz, H-1'), 3.85 (1H, ddd, *J*=2.4, 5.4, 10.0 Hz, H-5"), 4.17 (1H, dd, *J*=2.6, 12.1 Hz, H-6"), 4.30 (1H, dd, *J*=5.2, 12.3 Hz, H-6") 5.03 (1H, d, *J*=7.5 Hz, H-1"), 5.16 (1H, t, *J*=9.5 Hz, H-4"), 5.24 (1H, t, *J*=8.5 Hz, H-2"), 5.28 (1H, q, *J*=9.5 Hz, H-3"), 5.60 (1H, s, OH), 6.49 (1H, d, *J*=2.4 Hz, H-2), 6.50 (1H, d, *J*=2.4 Hz, H-6), 7.00 (1H, d, *J*=9.1 Hz, H-5). ^{13}C-NMR ($CDCl_3$) δ: 14.0 (C-5'), 20.5 (AcO), 20.6 (AcO), 20.7 (AcO), 22.5 (C-4'), 29.3 (C-2') 29.5 (C-1'), 31.6 (C-3'), 62.0 (C-6"), 68.3' (C-4"), 71.1 (C-2"), 71.9 (C-5"),72.7 (C-3"), 99.2 (C-1"), 104.8 (C-2), 108.5 (C-6), 123.9 (C-4), 130.4 (C-5), 154.4 (C-3), 155.8 (C-1), 169.5 (COO), 170.4 (COO), 170.9 (COO). FAB-MS *m/z*: 511 $[M+H]^+$.

Deacetylation of acetylglucosides – Acetylglucoside (0.06 mmol) was dissolved into 15 ml of methanol, and then sodium methoxide (5 drops) was added into the reaction mixture. The mixture was stirred for 4 hr, and then purified water (5 ml) was poured into the mixture. The reaction mixture was stirred for another 1 hr. the mixture was evaporated to a solid. The crude material was chromatographed on an ODS HPLC column (MeOH solvent system) to obtain pure compound.

5-Pentylresorcinol-β-glucoside (yield 89.7 %) – Colorless granulate. mp 118-120°C. UV λ_{max} (MeOH) nm (logε): 220 (3.44), 278 (2.80), IR ν_{max} (KBr) cm^{-1}: 3440, 2950, 1590, 1085. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, *J*=7.0 Hz, C-5'), 1.31 (4H, m, C-3' and 4'), 1.58 (2H, m, H-2'), 2.48 (2H, t, *J*=7.8 Hz, H-1'), 3.86 (1H, ddd, *J*=2.4, 5.4, 10.0 Hz, H-5'), 4.8 (1H, dd, *J*=2.2, 12.4 Hz, H-6"), 4.28 (1H, dd, *J*=5.2, 12.2 Hz, H-6"), 5.03 (1H, d, *J*=7.3 Hz, H-1"), 5.15 (1H, t, *J*=9.4 Hz, H-4"), 5.24 (1H, t, *J*=8.5 Hz, H-2"), 5.31 (1H, q, *J*=9.1 Hz, H-3"), 5.77 (1H, OH), 6.3-6.4 (3H, m, H-2, 4 and 6) ^{13}C-NMR ($CDCl_3$) δ: 14.4 (C-5'), 23.6 (C-4'), 32.1 (C-2'), 32.6 (C-3'), 37.0 (C-1'), 62.5 (C-6"), 71.4 (C-4"), 74.9 (C-2"), 78.0 (C-5"), 78.0 (C-5"), 78.1 (C-3"), 102.2 (C-1"), 102.4 (C-2), 109.2 (C-4), 110.6 (C-6), 146.4 (C-5), 159.3 (C-3), 160.1 (C-1). FAB-MS *m/z*:343 $[M+H]^+$.

4-Pentylresorcinol-β-glucoside (yield 63.9 %) – White amorphous. mp 179-183°C. UV λ_{max} (MeOH) nm (logε): 220 (3.51), 276 (3.44), IR ν_{max} (NaCl) cm^{-1}: 3400, 2950, 1080. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, *J*=6.5 Hz, H-5'), 1.31 (4H, m, H-3', 4'), 1.54 (2H, m, H-2'), 2.50 (2H, t, *J*=7.6 Hz, H-1'), 3.39 94H, m, H-2", 3", 4" and 6"), 3.70 (1H, dd, *J*=5.1, 12.0 Hz, H-6"), 3.89 (1H, dd, *J*=1.9, 11.1 Hz, H-6"), 4.85 (1H, d, *J*=7.4, H-1"), 6.50 (1H, dd, *J*=2.4, 8.3 Hz, H-6), 6.54 (1H, d, *J*=2.2 Hz, H-2), 6.90 (1H, d, *J*=8.3 Hz, H-5) ^{13}C-NMR ($CDCl_3$) δ: 14.5 (C-5'), 23.7 (C-4'), 30.6 (C-2'), 30.9 (C-3'), 32.8 (C-1'), 62.6 (C-6"), 71.4 (C-4"), 74.9 (C-2"), 78.1 (C-5"), 78.1 (C-3"), 102.5 (C-1"), 104.9 (C-2), 108.5 (C-6), 124.5 (C-4), 131.2 (C-5), 156.9 (C-3), 158.1 (C-1), FAB-MS *m/z*: 343 $[M+H]^+$.

Results and Discussion

Anti-tyrosinase activity represents the inhibition of L-dopaquinone production from L-DOPA in cultured B-16 mouse melanoma cells (Saeki *et al.*, 1978). WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium-salt] was a good substrate for dehydrogenase in mitochondria, and it was converted into WST formozan in the presence of 1-methoxy PMS (1-methoxy-5-methyl-phenazinium methylsulfate) by the enzyme in cultured B-16 mouse melanoma

cells (Ishiyama *et al.*, 1993). WST formozan production is proportional to the number of living cell, so that the decrease of the production means its toxicity for the cell.

The syntheses of 4-alkyl and 5-alkylresorcinols were described in our earier paper (Matsubara *et al.*, 1997).

Diphenylmethane derivatives were prepared by the reaction of 5-alkylresorcinols and paraformaldehyde as shown in Fig. 1. All tested compound were listed in Fig. 2 and Fig. 3.

5-alkylresorcinols showed strong inhibitory activity against tyrosinase like as arbution (**1**) and kojic acid (**2**), which have been used as cosmetics for whitening the facial skin. However, cytotoxicity was observed in 5-alkylresorcinols in parallel with their activity. The elongation of alkyl chain showed the increase of the cytotoxicity (Table 1).

4-Alkylresorcinol showed the activity and the cytotoxicity in parallel with the concentration of 10 mM to 1.2 mM. However, those compounds at the concentration of 600 μM showed stronger activity decreasing cytotoxicity. Especially 4-methylresorcinol (**7**) showed stronger activity than arbution (**1**) and kojic acid (**2**) at the concentration of 600 μM to 80 μM without cytotoxicity (Table 2).

5-Alkylresolcinol dimer (**10**) in Type A, 5-propylresorcinol (**14**) in type B, and 5-pentylresorcinol (**17**) in Type C showed strong inhibitory activity with less toxicity. The compounds in Type D did not show any remarkable activity and toxicity (Table 3). In order to determine the stable conformation of three types of compounds, dimer type A, B and C, MM2 were applied, and then lower energy conformers were determined by Monte Carlo conformational search. After several thousand runs were made, we found 6-8 conformers within the region of 2.4 kcal/mol. No obvious difference was observed in type A, B and C conformers giving overlapped patterns in MM2 and Monte Carlo conformational search. Their anti-tyrosinase activity may be affected by the difference of the connecting position of the dimer and the

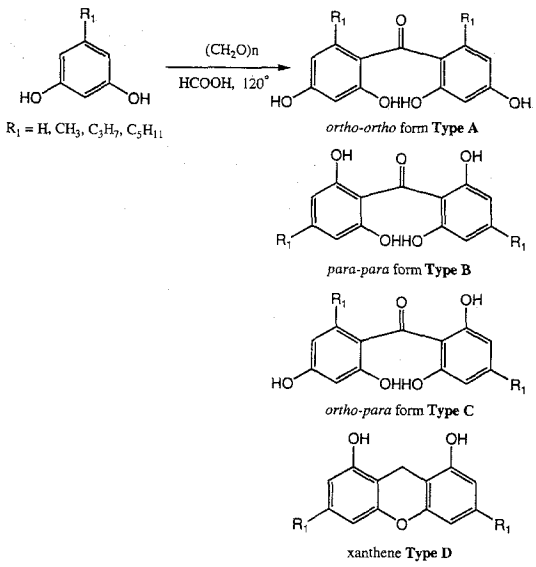

Fig. 1. Synthesis of Diphenylmethane and xanthene derivatives

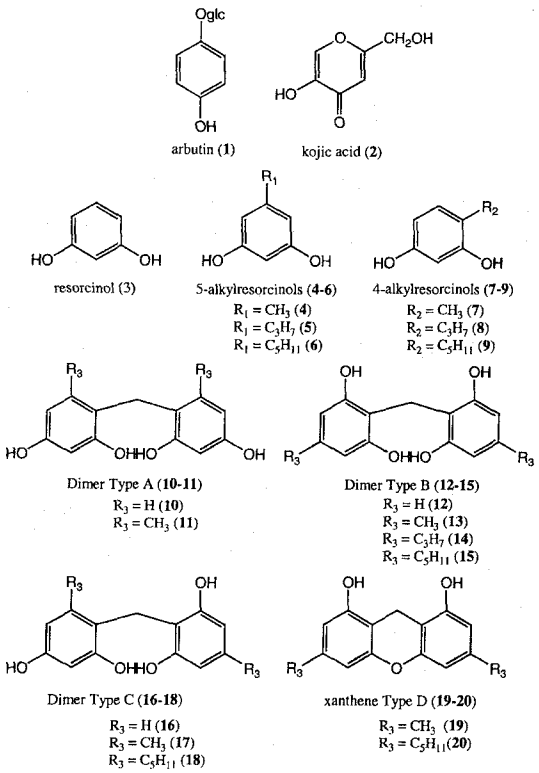

Fig. 2. Tested compounds.

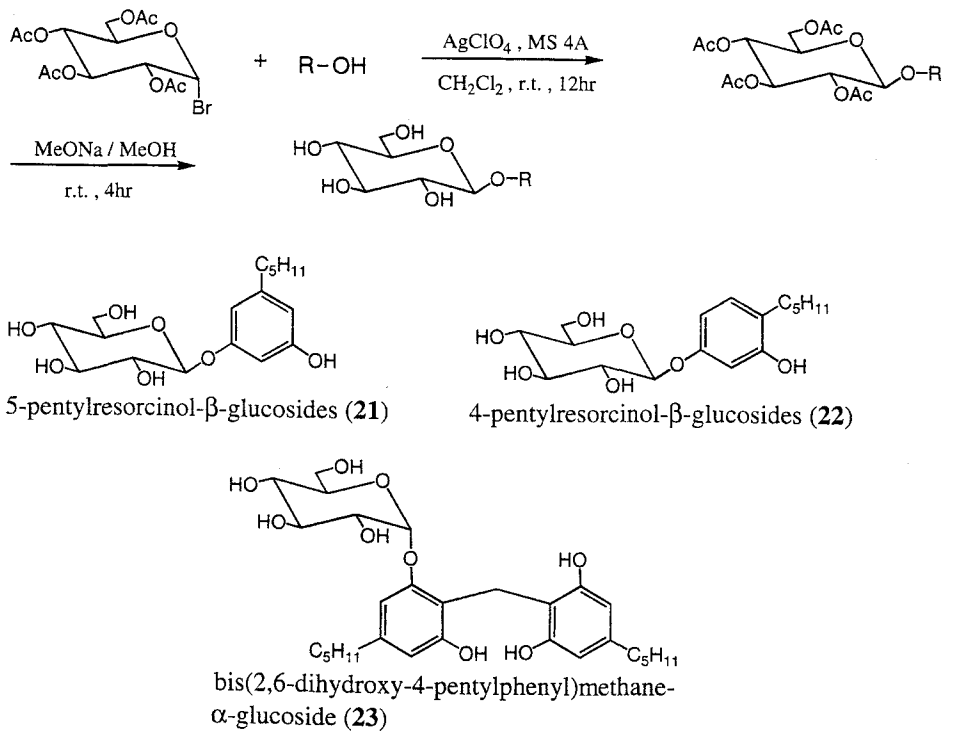

Fig. 3. Synthesis of glucosides ad their structures.

Table 1. The inhibitory effects of 5-alkylresorcinol derivatives on melanin synthesis in B-16 mouse melanoma cells

Compounds	Tyrosinase inhibition rate (%)/Cytotoxicity rate (%) Concentration (mM)			
	10	50	2.5	1.2
Arbutin (**1**)	67.6/ 22.1	70.2/ 4.2	62.2/ -11.7	45.9/ -46.6
Kojic acid (**2**)	83.3/ 43.0	67.6/ 17.6	43.2/ 17.6	27.0/ 17.9
3	92.1/ 57.6	78.3/ 61.8	75.0/ 66.2	31.9/ 62.7
4	100.0/ 99.2	73.6/ 82.5	44.4/ 78.2	23.6/ 71.3
5	94.7/ 99.8	100.0/ 99.8	100.0/ 99.4	70.8/ 89.5
6	89.2/ 97.7	83.8/ 99.0	91.9/ 99.8	94.6/ 99.8

Table 2. Tyrosinase inhibition rate and cytotoxicity rate of 4-alkylresorcinols

Compounds	Tyrosinase inhibition rate (%)/Cytotoxicity rate (%) Concentration (mM)							
	10 mM	5	2.5	1.2	600 μM	300	150	80
1	67.6/ 22.1	70.2/ 4.2	62.2/ -11.7	45.9/46.6	40.5/42.9	29.7/51.9	39.3/49.6	8.7/27.3
2	83.3/ 43.0	67.6/ 17.6	43.2/ 17.6	27.0/17.9	24.3/15.7	16.2/20.1	10.7/25.8	4.3/10.7
3	96.3/ 43.7	**81.5/ 18.4**	48.7/ -3.2	21.9/ 4.9	12.5/ -7.9	17.1/ -3.9	14.3/ -8.6	14.3/ -1.3
7	**96.3/101.6**	96.3/ 94.3	**96.3/ 65.7**	**90.6/38.4**	**78.1/ -0.5**	**68.6/ 3.4**	**51.4/ -9.9**	**48.6/ -3.4**
8	100.0/102.4	100.0/102.4	100.0/101.2	96.9/91.0	96.9/61.7	94.3/46.8	91.4/51.1	82.3/44.2
9	96.3/ 97.8	92.1/ 94.8	94.7/ 97.0	94.7/97.8	94.7/96.3	92.1/76.3	84.2/74.4	71.1/45.0

position of alkyl groups at the resorcinol position.

Arbution (**1**), a glucoside of hydroquinone, showed lower toxicity than its aglycone,

Table 3. Tyrosinase inhibition rate and cytotoxicity rate of 5-alkylresorcinol dimers and xanthenes

Compounds		Tyrosinase inhibition rate (%)/Cytotoxicity rate (%) Concentration (μM)			
		100	50	25	12
Type A (**10**)	**R_1=H**	**55.9/ -40.0**	**11.8/ -25.4**	**12.5/ -3.2**	**0.0/ -8.3**
(**11**)	$R_1=CH_3$	90.0/ 68.4	56.7/ 40.2	23.5/ 17.9	-2.9/ 4.9
Type B (**12**)	R_1=H	11.8/ -1.9	-17.6/ -5.7	-7.5/ -5.5	-10.0/ -4.6
(**13**)	$R_1=CH_3$	16.7/ 9.8	-23.3/ 3.0	-20.6/ 0	-14.7/ 0
(**14**)	**$R_1=C_3H_7$**	**90.0/ 67.9**	**72.1/ -4.7**	**18.6/ -3.3**	**0.0/ 18.6**
(**15**)	$R_1=C_5H_{11}$	91.7/100.0	86.1/ 98.8	77.3/ 11.1	47.7/ 13.1
Type C (**16**)	R_1=H	17.6/ 8.1	-29.4/ -1.4	-2.5/ -7.3	-2.5/ -8.7
(**17**)	$R_1=CH_3$	36.7/ -1.3	-20.0/ -6.4	-23.5/ -13.7	-32.4/ -4.9
(**18**)	**$R_1=C_5H_{11}$**	**94.4/100.4**	**83.3/ 98.4**	**56.8/ 3.7**	**11.4/ -57.4**
Type D (**19**)	$R_1=C_3H_7$	96.7/100.3	95.3/100.0	86.0/ 82.2	27.9/ 36.4
(**20**)	$R_1=C_5H_{11}$	94.4/100.4	80.6/ 99.2	56.8/ 98.0	0.0/ -13.1

Table 4. Tyrosinase inhibition rate and cytotoxicity of glucosides

Compounds	Tyrosinase inhibition rate (%)/Cytotoxicity rate (%) Concentration							
	1,000 μM	500	250	125	60	30	15	8
21	64.3/ 71.5	16.4/ 57.7	-3.2/ 55.4	-4.8/ 38.2	-1.5/ 7.0	4.5/ 2.1	-1.5/ -4.8	3.0/ -11.9
22	95.2/100.2	93.4/ 75.7	85.5/ 73.2	66.1/ 72.9	34.8/ 55.7	31.8/ 61.0	22.7/ 39.8	15.0/ -0.8
23	88.1/100.0	90.2/100.0	93.5/100.6	95.2/ 95.2	95.5/ 80.3	93.9/ 82.0	83.3/ 48.8	9.1/ 53.0

Table 5. Tyrosinase inhibition rate and cytotoxicity rate of liposome preparations

Compounds	Tyrosinase inhibition rate (%)/Cytotoxicity rate (%) Concentration							
	1 mM	500 μM	250	125	60	30	15	8
5	-11.7/18.2	-13.6/21.7	39.4/18.7	-19.7/12.2	-33.3/13.0	-13.6/ 9.2	0.0/11.4	6.1/ 4.0
8	0 /37.7	-2.6/39.9	-25.6/41.9	16.9/46.8	8.5/48.3	25.6/31.7	16.7/23.4	21.2/22.0
15	91.2/96.2	87.2/90.1	87.2/74.8	88.1/37.8	81.4/46.5	72.7/53.2	40.9/ -1.1	28.8/ -1.9

Therefore, some glucosides of 5-pentylresorcinol (**21**), 4-pentylresorcinol (**22**) and 5-pentylresocinol dimer Type B (**23**) were prepared. The activity and cytotoxicity of 4-pentylresorcinol-β-glucoside (**22**) decreased dose-dependently, whereas other compounds gave no remarkable decrease of toxicities (Table 4).

Liposome was prepared to decrease the cytotoxicity of the compounds in keeping their activity. Bis(2,6-dihydroxy-4-pentylphenyl) methane (**15**) showed strong activity without any toxicity at the concentration of 15 μM (Table 5).

Acknowledgement

The authors wish to thank Emeritus Professor of Tokyo University, Shoji Shibata, for his encouragement and suggestion, and Professor of Nagoya University, Yasushi Tomita, for his gift of cultured B-16 mouse melanoma cells.

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(Accepted July 20, 1998)