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Synthesis, discovery and mechanism of 2,6-dimethoxy-*N*-(4-methoxyphenyl)benzamide as potent depigmenting agent in the skin

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Abstract

In this study, a new skin-depigmenting agent, 2,6-dimethoxy-*N*-(4-methoxyphenyl)benzamide (DMPB), was synthesized using a combination of benzoic acid and aniline. DMPB exhibited significant depigmentation ability on the UV B-induced hyperpigmentation of the brown guinea pig skin. In addition, the 100 ppm treatment with this compound had a 30% inhibitory effect on melanin pigment generation in the melan-a cell line without significant cell toxicity. To search for relationship with the depigmentation, the effects of DMPB on the tyrosinase and dopachrome tautomerase were evaluated. DMPB had no effect on tyrosinase. However, it accelerated dopachrome transformation into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in the presence of dopachrome tautormerase. In addition, intracellular level of dopachrome tautomerase in melan-a cells was increased by treatment of DMPB. These results suggest that the pigment-lightening effects of DMPB might be due to biased production of DHICA-eumelanin induced by dopachrome tautormerase activation.

Keywords: Melanin; Depigment agent; Biaryl amide; Dopachrome tautomerase; Skin

Melanin is a phenolic biopolymer that is widely distributed in nature [1]. It is an important skin pigment in the animal kingdom including humans. The major determinant of skin color is the level of melanin synthesis in the melanosomes, which is a unique intracytoplasmic organelle of melanocytes [2]. Although melanin plays an important role in the absorption of free radicals generated within the cytoplasm and shields the host from UV light [3], the over production and accumulation of melanin pigment in the skin could be a serious problem resulting in a large number of skin diseases including chloasoma dermatitis, freckles, and geriatric pigment spots. Therefore, various whitening cosmetics and medicines are being developed to control melanogenesis.

Melanogenesis is started by the oxidation of tyrosine, which is oxidized to dopaquinone catalyzed by tyrosinase. Tyrosinase is believed to be the key enzyme in the melanogenesis of animal skin [4,5]. This yields dopaquinone, which is the substrate for the subsequent production of both light yellow/red colored pheomelanin and light brown/black colored eumelanin [3]. In the presence of cystein, it produces cysteinyldopa, which finally leads to the production of pheomelanin. However, in the absence of cystein, dopaquinone transforms to the non-enzymatic-generating dopachrome. Dopachrome undergoes either spontaneous decarboxylation to form 5,6-dihydroxyindole (DHI) or tautomerization to form 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The formation of DHICA from a dopachrome is controlled by dopachrome tautomerase. This dopachrome tautomerase is another major enzyme correlating with the melanogenesis pathway [6]. Dopa-

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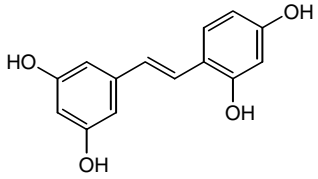
chrome tautomerase has a subcellular distribution within the melanocyte, which is similar to that of tyrosinase. It catalyzes the transformation of dopachrome into DHICA. This means that dopachrome tautomerase acts as a "dopachrome conversion" factor [7]. The production of DHICA induced by dopachrome tautomerase eventually leads to the brown DHICA-eumlanin, which is followed by further oxidation and polymerization. On the other hand, the spontaneous decarboxylation of dopachrome produces DHI, which is followed by oxidation, and the polymerization of DHI gives rise to the black DHI-eumelanin. The production ratio for the three distinct epidermal melanins is as follows in the human skin: DHI-eumelanin approximately 70%, DHICA-eumelanin approximately 25%, and pheomelanin approximately 5% [8].

Oxyresveratrol (3,5,2′,4′-tetrahydroxystilbene, Scheme 1), which is one of the ingredients of *Mori cortex* [9], has recently been reported to have a potent inhibitory effect on the dopa oxidase activity of tyrosinase, which catalyzes the rate-limiting steps of melanin biosynthesis. Its inhibitory effects are approximately 40-fold stronger than kojic acid, which is the currently used tyrosinase inhibitor in cosmetics [10,11]. However, preliminary experiments suggest that oxyresveratrol is not suitable as a whitening agent due to its high cell toxicity [12]. Furthermore, the quantity of this ingredient is limited and has many synthetic steps [13,14]. Therefore, there is a need to search for alternative materials such as the oxyresveratrol derivatives, which have a high bioactivity, lower toxicity, and can be easily obtained.

This study searched for oxyresveratrol derivatives with a pigmentation control ability and a low toxicity by modifying the chemical structure. Three groups of oxyresveratrol derivatives were synthesized and their depigmentating ability was examined using melan-a cells. During the test, several biaryl amide derivatives exhibited inhibitory effects on melanin pigment production in the melan-a cells. The biaryl amide group is modified with an amide connection chain between the two benzene rings and a lower polarity. The amide connection chain can be synthesized easily in one step [15].

In the examining of melanin pigment production and the cell viability on the melan-a cell line treated with biaryl amide compounds, the most effective agent was selected. In addition, the lightening effect on the UV B-induced hyperpigmentation on the dorsal skin of brownish guinea pigs was also investigated. Furthermore, this study investigated the effects of the biaryl amide compounds on tyrosinase and dopachrome tautomerase in order to evaluate the pathway of inhibiting melanin pigment production.



Scheme 1. Chemical structure of oxyresveratrol.

Materials and methods

Instrumentation

The ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were run using a Gemini-2000 spectrometer. The FABMS spectra were obtained from a Hewlett Packard mass spectrometer. The UV spectra were obtained using a Molecular Devices E09090 microplate reader. UV B radiation was provided using a Waldmann UV 800 (Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280–305 nm) and the degree of pigmentation was assessed as the *L*-value measured using a chromameter (CR-300, Minolta, Japan).

Reagents

All the chemicals used were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and TCI (Tokyo, Japan). The solvents used for organic synthesis were redistilled. The TLC and column chromatography were carried out on precoated silica gel F254 plates and Si gel 60 (Merck, 70–230 mesh).

Organic synthesis

*(1) Synthesis of 3,5,2′,4′-tetramethoxystilbene (**1**) and 3,5,2′,4′-tetrahydroxy stilbene (oxyresveratrol)*

3,5,2′,4′-Tetramethoxystilbene and oxyresveratrol were prepared according to a previously reported procedure [16].

*3,5,2′,4′-Tetramethoxystilbene (**1**).* White solid; yield: 30%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (d, 1H, $J = 8.4$ Hz), 7.29 (d, 1H, $J = 16.2$ Hz), 6.87 (d, 1H, $J = 16.2$ Hz), 6.60 (d, 2H, $J = 2.4$ Hz), 6.44 (m, 2H), 6.29 (t, 1H, $J = 2.1$ Hz), 3.80 (s, 3H), 3.76 (s, 3H), 3.76 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.88, 160.61, 158.10, 140.37, 127.35, 126.98, 123.85, 119.33, 107.08, 104.99, 104.38, 99.41, 98.50, 97.50, 55.51, 55.40, 55.36, 55.22; MS (EI^+) *m/z* 300 (M^+, 100), 269 (43), 254 (19), 238 (11), 211 (9), 121 (12).

3,5,2,4-Tetrahydroxystilbene (oxyresveratrol). Yellowish oil; yield: 19.3%; ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 8.53 (s, 1H), 8.34 (s, 1H), 8.13 (s, 2H), 7.41 (d, 1H, $J = 8.7$ Hz), 7.33 (d, 1H, $J = 16.2$ Hz), 6.89 (d, 1H, $J = 16.5$ Hz), 6.52 (d, 2H, $J = 2.1$ Hz), 6.40 (m, 2H), 6.24 (t, 1H, $J = 2.1$ Hz); ^{13}C NMR (75 MHz, $(CD_3)_2CO$): δ 159.52, 159.07, 156.86, 141.64, 128.23, 126.28, 124.32, 117.26, 108.43, 105.45, 103.56, 102.26; MS (EI^+) *m/z* 244 (M^+, 100), 226 (39), 198 (31), 181 (11), 137 (61), 123 (39).

*(2) Synthesis of (3,5-dimethoxybenzylidene)-(2,4-dimethoxyphenyl)-amine (**2**)*

3,5-Dimethoxybenzaldehyde (1 g, 6 mmol) and 2,4-dimethoxyaniline (858 μl, 6 mmol) were dissolved in toluene (12 ml). The reaction mixture was equipped with a dean-stark apparatus and refluxed for 12 h. The toluene was evaporated and the products were recrystallized in 30 ml of methanol at 20 °C. The yield of the compound was 440.6 mg (27%).

*(3,5-Dimethoxybenzylidene)-(2,4-dimethoxyphenyl)amine (**2**).* Brownish solid; yield: 27.0%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.42 (s, 1H), 7.05 (d, 2H, $J = 2.4$ Hz), 7.01 (d, 1H, $J = 8.4$ Hz), 6.55 (m, 2H), 6.49 (dd, 1H, $J = 2.7$ Hz; 2.7 Hz), 3.87 (s, 3H), 3.84 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.47, 138.63, 134.76, 120.70, 115.13, 107.10, 107.04, 106.24, 104.31, 104.15, 103.84, 99.48, 99.31, 55.82, 55.69, 55.50, 55.44; MS (EI^+) *m/z* 301 (M^+, 100), 286 (19), 271 (8), 242 (8), 228 (11), 164 (40), 150 (21), 135 (18).

*(3) General procedure for preparing the biaryl amides using trichloroacetonitrile and triphenyl phosphine (**3a–3c**)*

To a mixture of selected benzoic acid (1.0 mmol) and trichloroacetonitrile (200 μl, 2.0 mmol) in CH_2Cl_2 (3.2 ml), Ph_3P (530 mg, 2 mmol) in CH_2Cl_2 (1.8 ml) was added under argon at room temperature. After

stirring for 3–6 h, the reaction mixture was treated with the selected aniline (123 mg, 1.0 mmol) and stirred for 6–12 h. The reaction mixture was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash column chromatography on a silica gel to give the desired biaryl amides.

*N-(2,4-Dimethoxyphenyl)-3,5-dimethoxybenzamide (**3a**).* White solid; yield: 43.0%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.32 (d, 1H, $J = 9.3$ Hz), 8.21 (s, 1H), 6.94 (d, 2H, $J = 2.1$ Hz), 6.54 (t, 1H, $J = 2.1$ Hz), 6.46 (m, 1H), 6.44 (s, 1H) 3.82 (s, 3H), 3.79 (s, 6H), 3.75 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.76, 160.96, 156.55, 149.50, 137.71, 121.33, 120.67, 105.02, 103.85, 103.41, 99.66, 55.82, 55.61, 55.67; MS (EI$^+$) m/z 317 (M^+, 75), 165 (100), 137 (37), 122 (22), 107 (11).

*3,5-Dimethoxy-N-(4-methoxyphenyl)benzamide (**3b**).* White solid; yield: 56.2%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11 (s, 1H), 7.51 (d, 2H, $J = 8.7$ Hz), 6.94 (d, 2H, $J = 2.4$ Hz), 6.84 (m, 2H), 6.55 (t, 1H, $J = 2.4$ Hz), 3.78 (s, 3H), 3.76 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.39, 161.01, 156.63, 137.30, 130.95, 121.99, 114.23, 104.93, 103.66, 55.60, 55.49; MS (EI$^+$) m/z 287 (M^+, 74), 165 (100), 137 (50), 107 (20).

*2,6-Dimethoxy-N-(4-methoxyphenyl)benzamide (DMPB, **3c**).* White solid; yield: 35.7%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.52–7.47 (m, 2H), 7.28 (s, 1H), 7.24 (t, 1H, $J = 8.4$ Hz), 6.81 (m, 2H), 6.52 (d, 2H, $J = 8.4$ Hz), 3.76 (s, 6H), 3.73 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.45, 157.61, 156.29, 131.60, 130.96, 128.39, 121.35, 114.11, 104.12, 56.02, 55.52; MS (EI$^+$) m/z 287 (M^+, 51), 165 (100), 122 (20), 107 (21).

*(4) General procedure for preparing the biaryl amides using carbonyldimidazole (**3d**)*

The selected benzoic acid (1.0 mmol) was added to a solution of carbonyldiimidazole (1.0 mmol) in 5.0 ml acetonitrile. After stirring for 1–2 h, aniline (1.0 mmol) was added to the mixture and stirred for 10–12 h. The reaction mixture was poured into water and extracted with ethyl acetate. The extract was then washed with brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash column chromatography on a silica gel to give the desired biaryl amides.

*2-Methoxy-N-(2-methoxyphenyl)benzamide (**3d**).* White solid; yield: 13.4%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.60 (s, 1H), 8.65 (dd, 1H, $J = 2.1$ Hz; 2.1 Hz), 8.31 (dd, 1H, $J = 2.1$ Hz; 2.1 Hz), 7.49 (m, 1H), 7.16–6.91 (m, 6H), 7.07 (m, 3H), 4.07 (s, 3H), 3.96 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.94, 157.32, 148.31, 132.98, 132.40, 128.60, 123.39, 122.20, 121.47, 121.28, 120.30, 111.49, 109.96, 56.02, 55.97; MS (EI$^+$) m/z 257 (M^+, 49), 226 (9), 135 (100), 92 (21), 77 (30).

*(5) General procedure for preparing the biaryl amides using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (**3e**–**3h**)*

The selected benzoic acid (1.0 mmol) was added to a stirred solution of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (1.0 mmol) in anhydrous THF (10 ml). The selected anisidine (1.0 mmol) was then added. After stirring for 4–12 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was washed with brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was chromatographed on a silica gel to afford the desired biaryl amides.

*2,5-Dimethoxyl-N-(4-methoxyphenyl)benzamide (**3e**).* White solid; yield: 30.0%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.85 (d, 1H, $J = 2.7$ Hz), 7.61–7.56 (m, 3H), 7.03 (dd, 1H, $J = 2.7$ Hz; 2.7 Hz), 6.97 (d, 1H, $J = 9.0$ Hz), 6.91 (d, 2H, $J = 9.0$ Hz), 4.01 (s, 3H), 3.85 (s, 3H), 3.82 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.69, 156.25, 154.08, 151.42, 131.55, 122.37, 121.93, 119.59, 115.57, 114.07, 113.19, 56.76, 55.76, 55.43; MS (EI$^+$) m/z 287 (M^+, 31), 165 (100), 149 (9), 136 (8), 122 (22), 107 (25), 95 (19), 81 (33), 69 (63).

*N-(2,4-Dimethoxyphenyl)-2,4-dimethoxybenzamide (**3f**).* White solid; yield: 21.0%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.28 (s, 1H), 8.52 (d, 1H, $J = 9.6$ Hz), 8.26 (d, 1H, $J = 8.7$ Hz), 6.64 (dd, 1H, $J = 2.4$ Hz; 2.4 Hz), 6.54–6.50 (m, 4H), 4.03 (s, 3H), 3.92 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.40, 162.45, 158.55, 155.92, 149.46, 133.85, 122.44, 120.76, 115.23, 105.44, 103.76, 98.62, 55.94, 55.49; MS (EI$^+$) m/z 317 (M^+, 38), 165 (100), 150 (7), 122 (13), 107 (11), 92 (8), 79 (10).

*2,4-Dimethoxy-N-(4-methoxyphenyl)benzamide (**3g**).* White solid; yield: 48.9%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.56 (s, 1H), 8.26 (d, 1H, $J = 8.7$ Hz), 7.57 (m, 2H), 6.89 (m, 2H), 6.65 (dd, 1H, $J = 2.4$ Hz; 2.4 Hz), 6.53 (d, 1H, $J = 2.4$ Hz), 4.02 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.57, 162.91, 158.47, 156.11, 134.09, 131.76, 122.02, 114.76, 114.06, 105.59, 98.71, 56.13, 55.53, 55.46; MS (EI$^+$) m/z 287 (M^+, 37), 165 (100), 150 (6), 122 (14), 107 (10), 79 (8).

*2-(2-Methoxybenzoylamino)benzoic acid methyl ester (**3h**).* White solid; yield: 30.0%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.18 (s, 1H), 8.94 (dd, 1H, $J = 0.9$ Hz; 1.2 Hz), 8.20 (dd, 1H, $J = 1.8$ Hz; 1.8 Hz), 8.03 (dd, 1H, $J = 1.8$ Hz; 1.8 Hz), 7.57 (m, 1H), 7.47 (m, 1H), 7.09 (m, 2H), 7.02 (m, 1H), 4.07 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.76, 164.51, 157.53, 141.18, 134.04, 133.11, 132.34, 130.71, 122.71, 122.52, 121.98, 120.93, 116.79, 111.32, 55.51, 52.00; MS (EI$^+$) m/z 285 (M^+, 26), 151 (24), 135 (100), 120 (5), 92 (25), 77 (29).

*(6) General procedure for preparing the biaryl amides using 1,3-dicyclohexylcarbodiimide (**3i**–**3k**)*

The selected benzoic acid (1.0 mmol) was added to a stirred solution of 1,3-dicyclohexylcarbodiimide (1.0 mmol) in anhydrous THF (10 ml). The selected aniline (0.9 mmol) was then added and mixed with the reaction solution. After 12 h, the product was filtered and concentrated under vacuum. The residue was chromatographed (silica gel) and recrystallized (ethyl acetate/hexane) to afford the desired biaryl amides.

*2,6-Dimethyl-N-p-tolylbenzamide (**3i**).* White solid; yield: 36.3%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27 (m, 1H), 7.24 (m, 1H), 7.22 (s, 1H), 7.08 (m, 3H), 7.05 (m, 2H), 2.43 (s, 6H), 2.43 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.17, 135.84, 131.76, 130.45, 127.96, 19.93, 19.92; MS (EI$^+$) m/z 239 (M^+, 4), 223 (7), 195 (4), 133 (100), 105 (26), 91 (6), 77 (18).

*N-(3,4-Dimethoxyphenyl)-2,4,6-trimethoxybenzamide (**3j**).* White solid; yield: 40.3%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.64 (d, 1H, $J = 2.4$ Hz), 7.41 (s, 1H), 6.91–6.80 (m, 2H), 6.15 (s, 2H), 3.92 (s, 3H), 3.87 (s, 3H), 3.85 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.42, 162.31, 158.70, 148.94, 145.43, 132.38, 111.22, 111.04, 108.92, 104.52, 90.62, 56.09, 55.96, 55.87, 55.39; MS (EI$^+$) m/z 347 (M^+, 24), 195 (100), 180 (10), 152 (13), 137 (12).

*2,6-Dimethoxy-N-(4-butoxyphenyl)benzamide (**3k**).* White solid; yield: 40.3%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55 (m, 2H), 7.34–7.28 (m, 2H), 6.88 (m, 2H), 6.61 (s, 1H), 6.58 (s, 1H), 3.96 (t, 2H, $J = 6.6$ Hz), 3.84 (s, 6H), 1.75 (m, 2H), 1.50 (m, 2H), 0.98 (t, 3H, $J = 7.2$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.44, 157.54, 155.81, 131.39, 130.93, 121.27, 115.98, 114.73, 104.05, 67.97, 56.00, 31.29, 19.19, 13.82; MS (EI$^+$) m/z 329 (M^+, 29), 165 (100), 150 (16), 107 (15), 77 (4).

Cell culture

The melan-a cells and HM3KO cells were a kind gift from Dr. Byeong Gon Lee at the skin research institute, Amore-Pacific Co. Kyunggi, Korea. The melan-a cells were cultured in RPMI1640 medium with 10% FBS, 1% PS, and 200 nM TPA (phobol 12-myristate 13-acetate) conditions. Similarly, the HM3KO cells were maintained in MEM with 10% FBS (Sigma, St. Louis). Both cell types in the 25–35th passage were used.

Melanin pigment content and cell viability determination in cultured melan-a cells

The cells were grown to confluence after four days at 37 °C and under a 5% CO_2 atmosphere. They were then seeded with 10^5 cells/well in the 24-well culture plate and incubated for 24 h. Each well was changed with 990 μl of the medium each day and treated with 10 μl of 10,000 ppm, 1000 ppm, and 100 ppm of the test sample for three days. The test samples were dissolved in propylene glycol/EtOH/H_2O = 5:3:2.

(1) Determination of cell viability. The percentage of viable cells was determined by staining the cells with crystal violet. The cells were washed

with PBS after removing the media from each well. Two hundred microliters of the staining solution (0.1% crystal violet, 10% EtOH, and the remainder is PBS) was added to the well. The well was incubated at room temperature for 5 min and washed twice with water. After adding 1 ml EtOH, it was shaken at room temperature for 10 min. The crystal violet absorption was measured at 590 nm.

(2) Determination of the melanin pigment level. The melanin pigment content was measured using a slight modification of the method reported by Wright et al. [17] and Hosoi et al. [18]. The wells were washed with PBS after removing the media from each well. This was followed by adding 1 ml of 1 N NaOH and shaking them to dissolve the melanin. The absorbance was measured at 400 nm, and the melanin pigment content per well was calculated, and is expressed as a percentage of the control. Phenylthiourea (PTU), which is a melanogenesis inhibitor acting on tyrosinase, was used as the positive control [19,20].

Tyrosinase extraction

The melan-a and HM3KO cells (human melanoma cell line) were disrupted by resuspending them in a tyrosinase buffer (80 mM PO_4 buffer + 1% Triton X-100 + 100 μg/ml PMSF), which was followed by sonication in an ice bath. After centrifugation at 12,500 rpm for 15 min, the supernatant was used for the enzyme assay. One hundred and fifty grams of the proteins was required for each reaction [21].

Dopachrome tautomerase extraction

If the melan-a cells grew to confluence in the 100-mm culture dish, the media were removed and the cells were washed with PBS. Three hundred mictoliters of a hypotonic medium (10 mM phosphate buffer, pH 6.8, containing 1% Brij 35) was then added to the cells. The resuspended cells were sonicated for 5 min in an ice bath. After centrifuging at 13,000 rpm for 10 min, the supernatant was used to measure the enzyme activity.

Measurement of the tyrosinase activity

The tyrosinase activity was measured by its dopa oxidase activity using a slight modification of the method reported by Shono et al. [22]. Each concentration (1 mM, 500 μM, 100 μM, and 10 μM) of the test substance was dissolved in MeOH. 120 μl of L-dopa (5 mM, dissolved in a 67 mM phosphate buffer, pH 6.8) and 40 μl of either the same buffer or the test sample were added to a 96-well microplate, and 40 μl of tyrosinase was then added. The amount of dopachrome in the reaction mixture was measured after incubation at 37 °C for 30 min. Based on the optical density at 490 nm (OD_{490}), the inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}). Kojic acid was used as the reference.

Measurement of the dopachrome tautomerase activity

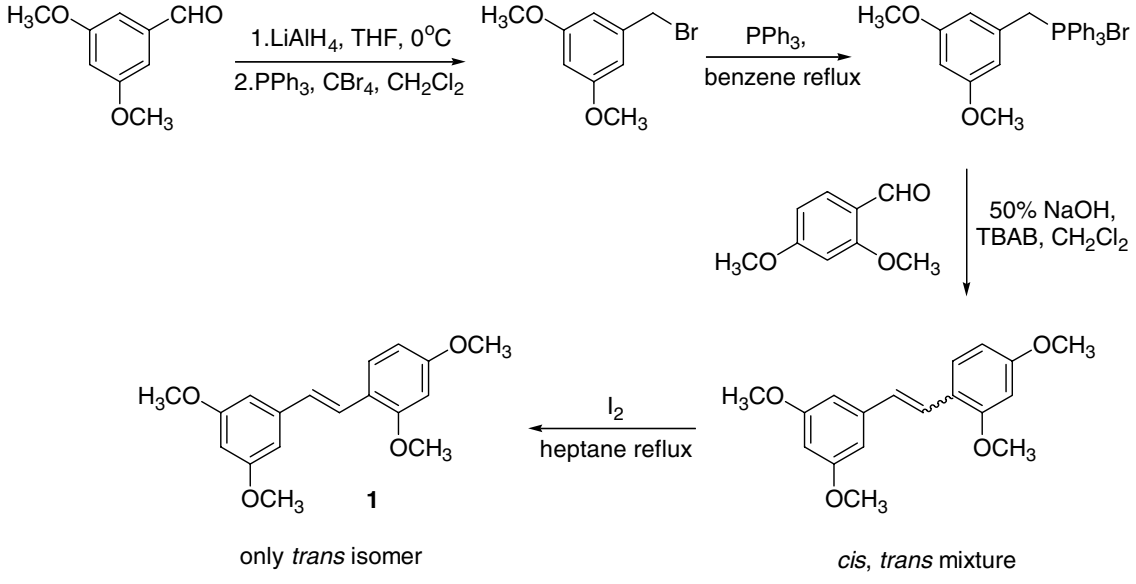
Dopachrome was produced by mixing cold dopa (0.5 mg L-dopa/ml in 0.05 M sodium phosphate, pH 6.8) with silver oxide (6 mg Ag_2O/mg dopa) for 3 min. After filtering through a 0.22 μm Millipore filter, the supernatant was treated with Chelex 100 to remove all traces of sliver. This procedure resulted in the 77% conversion of dopa to dopachrome. The dopachrome was prepared immediately before use owing to its instability. The assay reagents consisted of 125 μl of crude dopachrome tautomerase (0.9 mg total protein), 250 μl of the dopachrome solution, 525 μl of a 0.05 M sodium phosphate buffer, pH 6.8, and 100 μl of either MeOH or 1 mM of the test sample dissolved in MeOH. The dopachrome tautomerase activity was determined by measuring the increase in absorbance at 308 nm, indicating the enzyme-catalyzed formation of DHICA from dopachrome [7].

Western immunoblotting analysis

The melan-a cells were harvested and extracted in a triple-detergent lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml PMSF, and 1 μg/ml aprotinin). The protein content was measured using a protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of the protein was separated on 8% SDS–polyacrylamide gels and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 5% skim milk and incubated with tyrosinase (Santa cruz Biotech, 1/250 dilution) and TRP-2 (Santa cruz Biotech, 1/300 dilution) primary antibodies and anti-goat secondary antibodies. The following detection was performed using ECL (Amersham Pharmacia Biotech).

UV B-induced hyperpigmentation in brown guinea pigs

UV B-induced hyperpigmentation was induced on the backs of the brownish guinea pigs weighing approximately 500 g (SLC, Shizuoka,



Scheme 2. Synthesis of tetramethoxy analogue of oxyresveratrol (**1**).

Japan) using a slight modification of the methods reported by Hideya et al. [23] and Imokawa et al. [24]. The guinea pigs were anesthetized with pentobarbital (30 mg/kg), and separate areas (1 cm diametrical circle) of the back of each animal were exposed to the UV B radiation (Waldmann UV 800, Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280–305 nm). The total UV B dose was 500 mJ/cm^2 per exposure. Groups of four animals were used in the experiments. The animals were exposed to the UV B radiation once a week for three consecutive weeks. The candidate for the whitening agent was given the material topically to the hyperpigmented areas (1% in propyleneglycol/EtOH/H_2O = 5:3:2, 5 μl/circle) twice a day for 8 weeks from the next day of the last tanning. The degree of pigmentation was assessed by the *L*-value measured using a chromameter (CR-300, Minolta, Japan). Eight weeks later, skin biopsies were taken and processed for Fontana-masson staining [25].

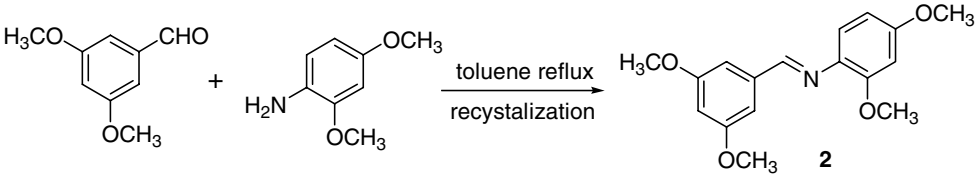
Statistical analysis

The data are presented as means ± SE. The statistical comparisons between the different treatments were performed using Student's *t*-test.

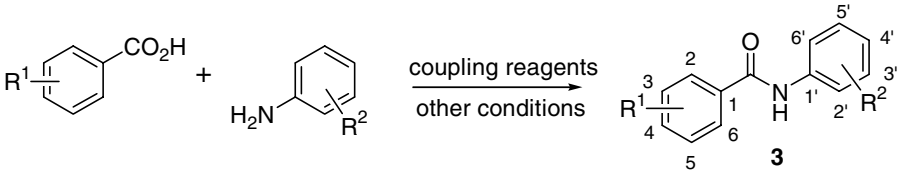
Results

Chemistry

The preparation of oxyresveratrol has many synthetic steps and this compound has a high cell toxicity [16]. Therefore, the new depigmenting agents related to oxyresveratrol are needed. Based on the oxyresveratrol chemical



Scheme 3. Synthesis of tetramethoxy imine analogue of oxyresveratrol (**2**).



Compounds	R^1	R^2	Coupling reagents	Other conditions
3a	3,5-OCH_3	2',4'-OCH_3	TCA/PPh_3	CH_2Cl_2
3b	3,5-OCH_3	4'-OCH_3	TCA/PPh_3	CH_2Cl_2
3c (DMPB)	2,6-OCH_3	4'-OCH_3	TCA/PPh_3	CH_2Cl_2
3d	2-OCH_3	2'-OCH_3	CDI	CH_3CN
3e	2,5-OCH_3	4'-OCH_3	EDCI	THF
3f	2,4-OCH_3	2',4'-OCH_3	EDCI	THF
3g	2,4-OCH_3	4'-OCH_3	EDCI	THF
3h	2-OCH_3	2'-CO_2CH_3	EDCI	THF
3i	2,6-CH_3	4'-CH_3	DCC	THF
3j	2,4,6-OCH_3	3',5'-OCH_3	DCC	THF
3k	2,6-OCH_3	4'-$OCH_2CH_2CH_2CH_3$	DCC	THF

Scheme 4. Synthetic scheme of biaryl amide analogues (**3a**–**3k**) Four kinds of coupling reagents were used in this study. Abbreviations of the coupling regents are as follows; TCA, Trichloroacetonitrile; DCC, Dicyclohexylcarbodiimide; CDI, Carbonyl diimidazole; EDCI, 1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide.

skeleton, three groups of derivatives (the stilbene group, nitrogen group and biaryl amide group) were designed by structural modifications. The structural modifications were performed to introduce better structural stability and a lower polarity for better skin transmission. Also, a simpler synthetic pathway for mass production and possibility of having a higher bioactivity and lower toxicity were considered.

A Wittig reaction between a selected benzaldehyde and a selected aromatic phosphonium was used to synthesize the stilbene derivatives with a carbon double bond (Scheme 2) [14,16,26–28]. The nitrogen derivative was synthesized using the reductive amination of the selected aniline and selected benzaldehyde (Scheme 3) [29]. In addition, the biaryl amide derivatives were made by treating the selected amines with a selected aromatic acid in the presence of a coupling reagent (Scheme 4) [15,30]. The chemical structure of the products was identified by ^{1}H NMR, ^{13}C NMR, and MS spectroscopy.

Effects on melanin pigment production and cell viability in cultured melan-a cells

Melan-a cells were used in this study to investigate whether or not the oxyresveratrol derivative compounds inhibited melanin pigment production in the melanocytes. Melan-a cells are syngeneic associated with the B16 melanoma and its sublines, and provide an excellent parallel non-tumorigenic line for examining the melanoma malignancy [31]. As shown in Table 1, treatment with DMPB for 3 days resulted in a significant reduction in the melanin pigment content with no cell toxicity. The morphological changes are shown in Fig. 1. However, treatment with positive controls, oxyresveratrol and phenylthiourea was toxic to the cells at over 100 ppm and 10 ppm, respectively. In addition, kojic acid did not have an inhibitory effect on melanin pigment production at any concentrations. The structure–activity relationship between the synthetic oxyresveratrol derivatives and the depigmenting activity is shown in Fig. 2. In the class of functional groups, the methoxy group had a higher depigmenting activity than the hydroxy or methyl groups. This tendency was confirmed by comparing the activity between oxyresveratrol and compound **1**, and DMPB and compound **3i**. In the same functional group, the amide connection chain (**3a**) had a higher activity than the nitrile connection chain (**2**) and a C–C double bond connection chain (**1**). Furthermore, the biaryl amide derivatives with methoxy groups at positions 2 and 6 were found to be more potent than positions 3 and 5 in the depigmenting ability by comparing the activity of compound **3b** and DMPB.

Table 1
Effects of each compound on cell growths and melanin pigment production of melan-a cells

Samples	Concentrations (ppm)	Melanin pigment production(%)	Cell viability (%)
1	1	95.0 ± 7.1	97.4 ± 2.9
	10	49.3 ± 0.5	51.7 ± 3.0
	100	35.0 ± 1.7	44.7 ± 1.3
2	1	93.8 ± 5.6	102.0 ± 7.0
	10	100.8 ± 4.7	94.6 ± 2.8
	100	57.8 ± 5.5	71.3 ± 4.2
3a	1	100.0 ± 4.3	97.4 ± 3.9
	10	88.7 ± 2.7	103.0 ± 1.9
	100	36.0 ± 7.2	55.8 ± 6.1
3b	1	96.7 ± 10.6	101.8 ± 7.8
	10	52.9 ± 4.1	75.2 ± 0.4
	100	10.5 ± 8.8	20.0 ± 4.0
DMPB (**3c**)	1	97.2 ± 2.8	105.7 ± 4.7
	10	88.7 ± 1.3	99.7 ± 6.3
	100	67.6 ± 4.4	95.3 ± 4.9
3d	1	97.4 ± 3.9	99.4 ± 9.2
	10	65.7 ± 11.3	89.0 ± 8.2
	100	50.0 ± 9.0	74.1 ± 10.6
3e	1	97.9 ± 2.5	98.2 ± 1.2
	10	90.5 ± 9.1	98.3 ± 7.4
	100	66.4 ± 7.5	82.2 ± 7.0
3f	1	96.4 ± 2.7	98.1 ± 1.9
	10	66.5 ± 12.4	72.4 ± 7.9
	100	—[a]	—
3g	1	100.0 ± 1.3	98.5 ± 4.0
	10	83.1 ± 2.0	98.7 ± 4.3
	100	66.8 ± 9.7	64.4 ± 7.6
3h	1	97.8 ± 1.9	97.7 ± 2.2
	10	62.9 ± 7.2	88.2 ± 7.1
	100	9.9 ± 8.2	11.1 ± 2.9
3i	1	100.4 ± 8.0	97.1 ± 4.0
	10	93.1 ± 11.0	94.3 ± 9.9
	100	73.9 ± 9.2	86.9 ± 6.1
3j	1	94.3 ± 0.3	96.0 ± 2.8
	10	97.2 ± 1.9	87.4 ± 2.2
	100	—	—
3k	1	99.6 ± 2.4	96.3 ± 5.2
	10	90.7 ± 6.8	97.3 ± 2.7
	100	65.2 ± 12.9	84.1 ± 12.0
Phenylthiourea	1	88.9 ± 7.6	97.3 ± 1.3
	10	41.4 ± 9.3	80.1 ± 9.3
	100	25.3 ± 8.4	72.3 ± 9.4
Kojic acid	1	107.1 ± 9.9	96.6 ± 7.5
	10	96.0 ± 2.6	98.6 ± 6.4
	100	91.9 ± 4.0	84.3 ± 5.8
Oxyresveratrol	1	94.9 ± 8.6	104.5 ± 6.5
	10	88.1 ± 7.3	94.4 ± 8.4
	100	20.1 ± 8.4	16.8 ± 5.0

Viability and melanin pigment content of the solvent (vehicle) treated cells was set to 100%. Each value represents the mean ± SE of three experiments.

[a] The compound did not dissolve in the media at concentration of 100 ppm.

Inhibitory effects on tyrosinase activity

Three kinds of tyrosinase, which were extracted from the melan-a cells (mouse originated), HM3KO cells (human originated), and mushroom, were used in this study. The melan-a extracted tyrosinase inhibitory activities of PTU and kojic acid at 200 μM were 90.8% and 83.7%, respectively. Similarly, the inhibitory effect on mushroom tyrosinase at a concentration of 200 μM was 58.0% and 38.4% for PTU and kojic acid, respectively. The HM3KO extracted tyrosi-

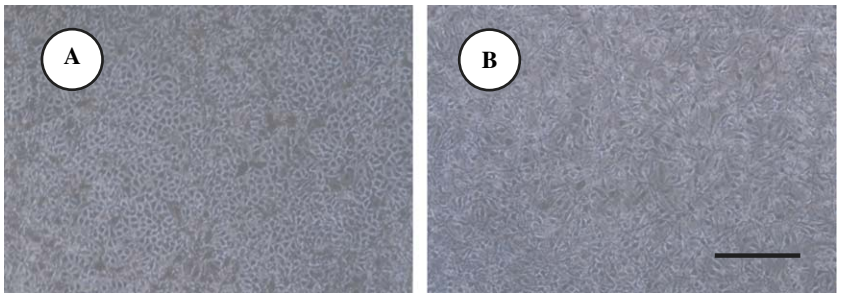


Fig. 1. The melan-a cells in 100 ppm concentration of DMPB. Three days after the treatment. (A) Vehicle, (B) DMPB (magnification 200×, scale bar 100 μm).

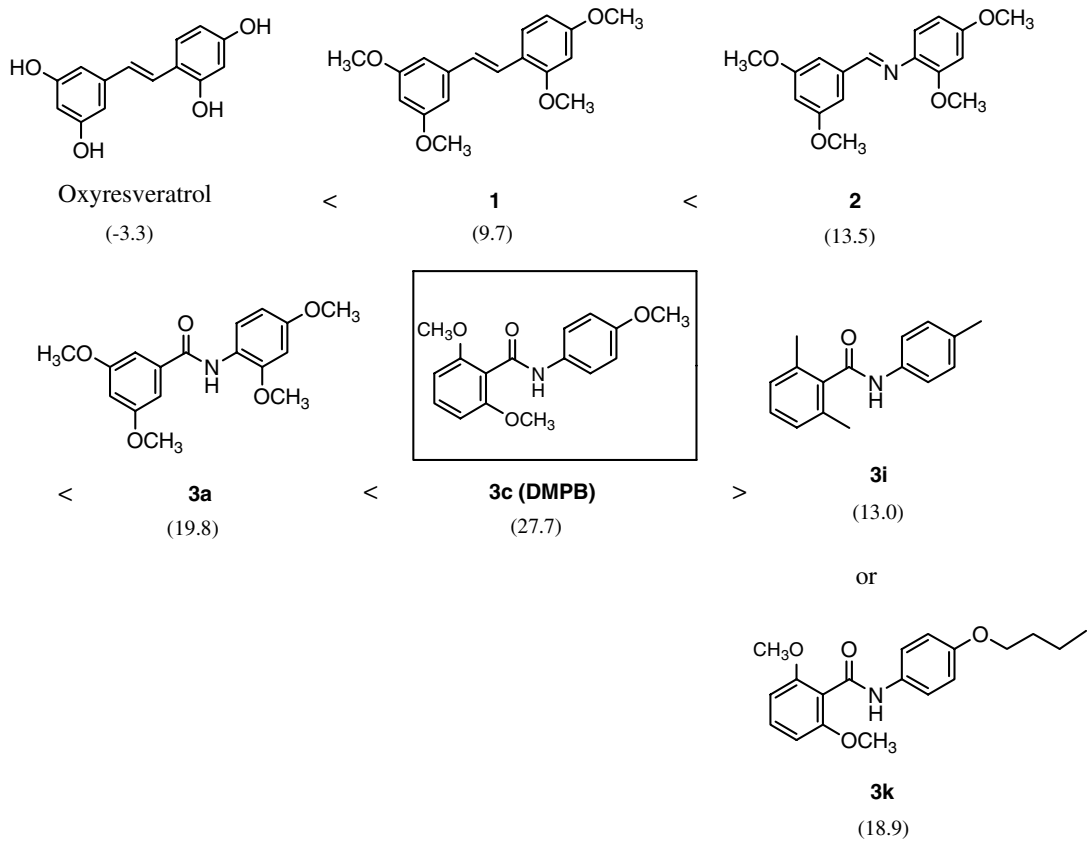


Fig. 2. The structure–activity relationships of the oxyresveratrol derivatives on the inhibition of melanogenesis in the melan-a cells. Measurement of the depigmenting activity was performed as described in Materials and methods. The activity of each compound was determined by comparing the level of melanin pigment production with the cell toxicity at a concentration of 100 ppm.

nase inhibitory activity of kojic acid, as a positive control, was 57.9% at the same concentration. In addition, oxyresveratrol exhibited a stronger inhibitory activity against the HM3KO extracted and mushroom tyrosinase than kojic acid. However, interestingly, DMPB has no inhibitory effect on any kind of tyrosinase (Table 2).

Effects on the dopachrome tautomerase activity

Dopachrome tautomerase catalyzes the transformation of dopachrome into DHICA. The formation of DHICA led to an increase in the absorbance at 308 nm [32]. As shown in Fig. 3, the reaction of DMPB (star) treatment showed the highest increase in absorbance at 308 nm when the dopachrome tautomerase was present. This increase in level of absorbance was much higher than that of dopachrome tautomerase alone. However, DMPB could not stimulate enzymatic activity in the absence of dopachrome tautomerase. Therefore, DMPB can be described as a dopachrome tautomerase stimulator.

Effects on the intracellular level of the enzyme in melan-a cells

In order to examine the regulation of the melanin generation related proteins at the translational level follow-

Table 2
Inhibitory effects of each compound on tyrosinase activity

Samples	Concentrations (μM)	Melan-a tyrosinase inhibition (%)	IC_{50}[a](μM)	HM3KO tyrosinase inhibition (%)	IC_{50} (μM)	Mushroom tyrosinase inhibition[b] (%)	IC_{50} (μM)
DMPB	2	3.7 ± 5.5	—	4.4 ± 5.5	—	9.2 ± 4.0	—
	20	2.7 ± 3.2		6.0 ± 6.8		2.2 ± 1.5	
	100	3.3 ± 1.5		1.3 ± 4.5		1.6 ± 8.5	
	200	5.3 ± 3.2		7.0 ± 6.3		6.4 ± 2.9	
Kojic acid	2	12.8 ± 6.8	105.2	0.2 ± 6.2	161.7	8.3 ± 6.2	275.6
	20	13.3 ± 3.1		15.8 ± 5.2		10.9 ± 2.3	
	100	58.3 ± 10.1		37.5 ± 6.6		23.4 ± 1.5	
	200	83.7 ± 2.7		57.9 ± 3.7		38.4 ± 1.5	
Oxyres	2	28.4 ± 6.0	36.5	3.8 ± 2.0	148.1	17.8 ± 5.1	73.2
-veratrol	20	48.9 ± 5.1		19.6 ± 2.9		39.3 ± 4.3	
	100	84.3 ± 4.8		41.8 ± 1.3		73.4 ± 1.8	
	200	91.7 ± 5.4		61.3 ± 1.0		77.8 ± 1.1	

Percentage of tyrosinase inhibition is the result as compared with the MeOH treated (Vehicle) group. Each value represents the mean ± S.E. of three experiments.
[a] 50% inhibitory concentration.
[b] 125 U/ml concentration of the mushroom tyrosinase was used in this study.

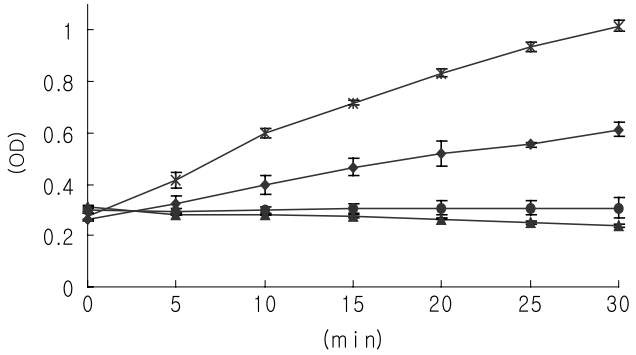


Fig. 3. The effect of DMPB on dopachrome tautomerization. The enzyme-specific tautomerization of dopachrome to DHICA led to an increase in the absorbance at 308 nm. Vehicle + dopachrome (triangle), 100 μM DMPB + dopachrome (circle), Vehicle + dopachrome + enzyme (diamond), Dopachrome + enzyme + 100 μM DMPB (star).

Fig. 4. Western immunoblotting of tyrosinase and dopachrome tautomerase in melan-a cells. (A) tyrosinase, (B) dopachrome tautomerase. Melan-a cells were cultured with or without 100 ppm of DMPB for 3 days.

ing the DMPB treatment, the level of this enzyme in the control and in DMPB-treated cells was determined by metabolic labeling using the enzyme-specific antibodies. Fig. 4 shows the effects of DMPB on the tyrosinase protein level and the TRP-2 (tyrosinase related protein-2, dopachrome tautomerase) level. The result demonstrated that there were no changes on the tyrosinase level in the melan-a cells after the DMPB treatment for 3 days. On the other hand, TRP-2 level was increased by DMPB treatment.

Depigmenting effects on guinea pig skin

Brownish guinea pigs were used in this study as a model for determining the depigmenting effects of DMPB because they have functional melanocytes in their epidermis, which respond well to several stimuli including UV B light. UV B-induced hyperpigmentation was elicited on the dorsal skin of the brownish guinea pigs using a slight modification of the method reported by Hideya et al. [23]. Ten microliters of DMPB (10 mg/ml) was applied topically to the UV-stimulated hyperpigmented dorsal skin areas twice a day for 8 weeks from the day after the last tanning. A visible decrease in hyperpigmentation was observed 4 weeks after the DMPB treatment, when compared with the vehicle group. Fig. 5 shows the depigmenting effects of 1% DMPB on the guinea pig dorsal skin after 8 weeks. The degree of pigmentation decreased (ΔL-value) before and 8 weeks after the applications of the DMPB was 4.3. There was no visible edema at any of the sites where the dorsal skin was treated with the DMPB. In addition, this study used histological methods such as Fontana-masson stain for melanin pigment. In the DMPB-treated groups' skin, histological staining using the Fontana-massons method showed a decrease in the amount of melanin pigment (Fig. 6).

Discussion

Oxyresveratrol has been reported to have 40 times the inhibitory effects on tyrosinase than kojic acid. However, our preliminary experiments found that oxyresveratrol was unsuitable as a raw material for whitening cosmetics and medicines due to its high cell toxicity. Therefore, this study modified the oxyresveratrol structure, and synthesis, and selected DMPB performed in order to overcome the disadvantage of oxyresveratrol. DMPB has an advantage over oxyresveratrol in some respects. (1) DMPB was syn-

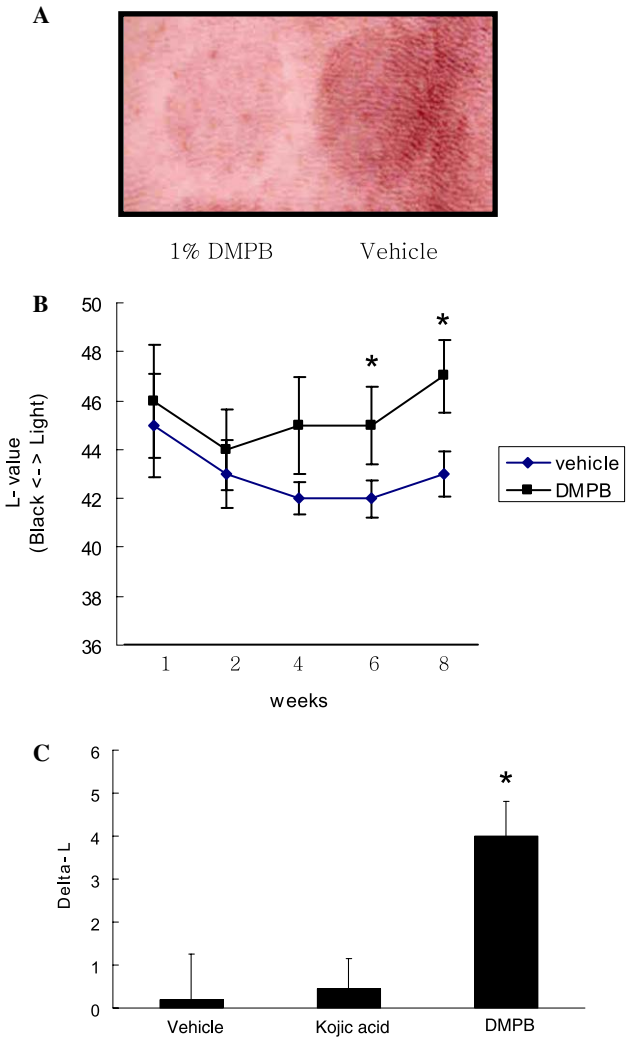


Fig. 5. Effect of DMPB on UV B-induced hyperpigmentation in the guinea pig skin. (A) Representative photographs showing the lightening effects (8 weeks after). The vehicle did not affect the skin color compared with the control. (B) Changes of *L*-value after the daily topical applications of the vehicle and DMPB. (C) The degree of pigmentation decreased (ΔL-value) before and 8 weeks after. The data are expressed as a mean ΔL-value + SEM. The ΔL-value was measured using a chromameter and a *t*-test was used for the statistical analysis of the data ($^{*}P < 0.05$ vs control). Groups of four animals were used in this experiment.

thesized using a single-step process. However, oxyresveratrol needs to pass many synthetic steps and only a very small quantity of oxyresveratrol was found in the natural products. Therefore, DMPB can be more easily obtained than oxyresveratrol. (2) DMPB has a lower cell toxicity than oxyresveratrol. As the result, DMPB had no significant cell toxicity at 100 ppm on mouse-originated melan-a cells. In contrast, the same concentration of oxyresveratrol exhibited only 16.8% of the cell viability.

Moreover, the 100 ppm DMPB treatment had a more than 30% inhibitory effect on melanin pigment generation in the melan-a cells without significant cell toxicity. In addition, DMPB has a depigmentation ability on UV B-induced hyperpigmentation of brown guinea pig skin. The skin returned to its original color after the DMPB treatment. Fontana-masson staining (Fig. 6) indicated that the melanin pigment level in the hyperpigmented area was significantly lower in the DMPB-treated animals.

Furthermore, this study was carried out to evaluate the action of a biaryl amide derivative, DMPB, on the melanogenesis of melanocytes. DMPB had no effect on the tyrosinase activity and intracellular level. However, DMPB catalyzes the action of dopachrome tautomerase by transformation of dopachrome into DHICA and increases intracellular level of dopachrome tautomerase. According to the result, it can be speculated that DMPB inhibits pigmentation due to the stimulation of dopachrome tautomerase. In the melanization pathway, the conversion of dopachrome is a turning point that determines if DHI-eumelanin or DHICA-eumelanin will be produced. Of these, the main pathway is the production of DHI-eumelanin [3,33]. Dopachrome tautomerase converts dopachrome into DHICA [34], which finally produces DHICA-eumelanin, and relatively decreases the level of DHI-eumelanin production. The DHICA-eumelanin pigment produced by dopachrome tautomerase stimulation has a yellow to light brown color, which is milder than the light brown to black of DHI-eumelanin [3]. Therefore, the production of DHICA-eumelanin by the stimulation of dopachrome tautomerase is consequently described to have lightening effects (Fig. 7). Indeed, recent comparative analyses of

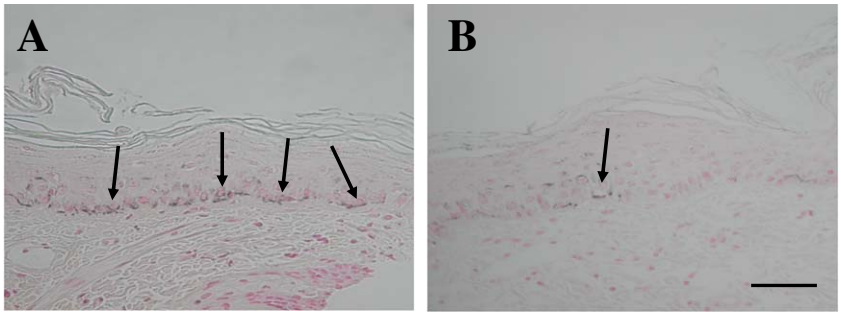


Fig. 6. Fontana-masson staining of the effect of the UV-stimulated guinea pig skin treated with DMPB. Fontana-masson staining was performed on 5 μm sections embedded in paraffin. There was a decrease in the melanin pigment content at the treated site. When the photographs were analyzed directly in the optimas image analysis program (6.5, Media Cybernetics, USA), 1% of DMPB decreased the level of melanin pigment content by 71.3%, as compared with the vehicle. (A) Vehicle, (B) 1% DMPB (original magnification: 400×, scale bar 50 μm).

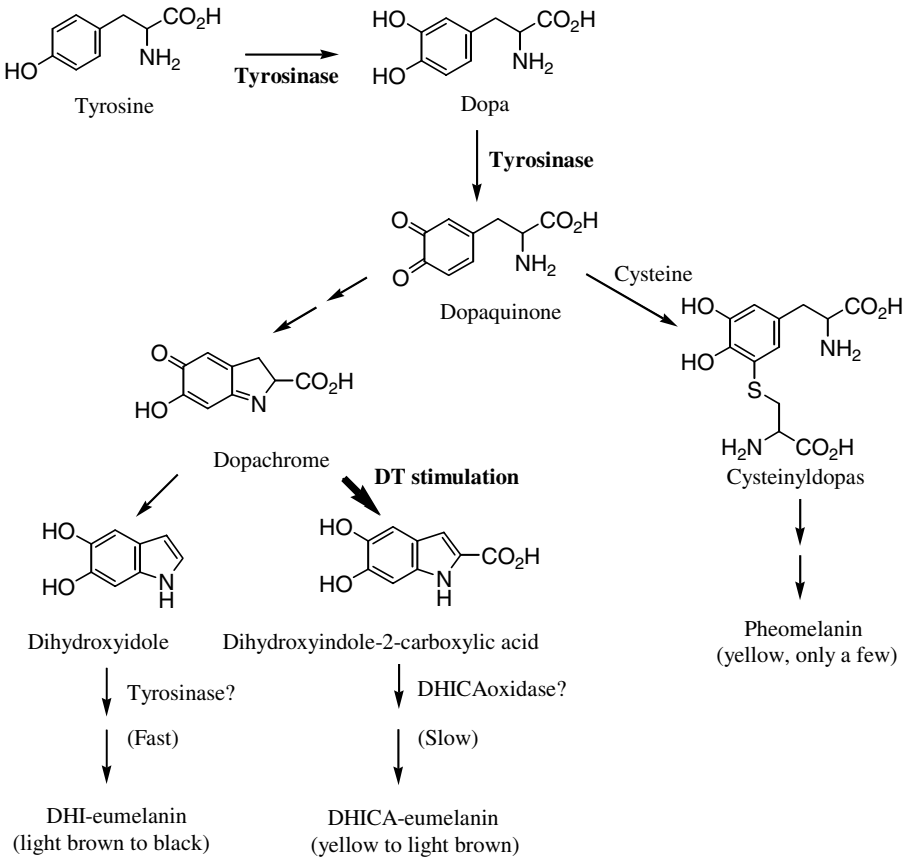


Fig. 7. Depigmenting mechanism of DMPB. The pigment-lightening effect of the DMPB may be due to the dopachrome tautomerase stimulation. DHI, dihydroxyindole; DHICA, dihydroxyindole-2-carboxylic acid; DT, dopachrome tautomerase.

the melanin composition at the photoexposed and photoprotected human skin sites demonstrated that there is a higher DHICA-eumelanin level in the photoprotected skin, whereas the relative abundance of the darker DHI-eumelanin is reduced at these sites [8].

On the other hand, our suggestion remains to be seen in terms of mouse mutation at the dopachrome tautomerase gene. It was shown that the presence of dopachrome tautomerase makes the skin black and its absence gives it light color [35,36]. However, based on the evidences from several current studies, it is possible that it is not the dopachrome tautomerase action but the high cytotoxicity of DHI and the corresponding decarboxylated indolic melanogenic intermediates. DHI has a much higher cytotoxicity to melanoma cells than DHICA [37]. In the absence of a dopachrome tautomerase function, most of the dopachrome is converted to DHI with decarboxylation, and the over-produced DHI causes a high cytotoxicity. This cytotoxicity can affect the survival of melanocytes in the skin. Therefore, changes to a lighter skin color, which is induced by a mutation at the dopachrome tautomerase gene, may be due to the over production of cytotoxic DHI and the corresponding decarboxylated indolic cytotoxic intermediates. Studies on detoxification by dopachrome tautomerase [36–38] support the results of this study. To the best of our knowledge, a new dopachrome tautomerase activator was introduced and this acts as depigmenting agent in melanocytes. Based on this result and other experimental evidences, increase of dopachrome tautomerase activity might be resulting in skin depigmentation. DMPB is a new conceptual depigmenting agent as compared with tyrosinase inhibitor, thereby laying the important foundation for development of the optimum skin-depigmenting agent.

Acknowledgments

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