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Benzylamide derivative compound attenuates the ultraviolet B-induced hyperpigmentation in the brownish guinea pig skin

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Abstract

This study evaluated the effects of synthetic benzylamide compound I (2,6-dimethoxy-*N*-phenylbenzamide) on the ultraviolet B (UV B)-induced hyperpigmentation of the skin. UV B-induced hyperpigmentation was elicited on brownish guinea pig skin according to the method reported by Hideya *et al*. [Arch Dermatol Res 290 (1998) 375] with minor modifications. A lightening effect was observed following the topical application of compound I on UV-stimulated hyperpigmentation. The skin returned to its original color after treatment with compound I. Fontana-Masson staining indicated that melanin level in the hyperpigmented area was significantly decreased in the compound I-treated animals. However, the number of melanocytes were not changed in the compound I-treated groups using the S-100 stain, which is an immunohistochemical method. *In vitro* experiments using the cultured melanoma cells showed a 31.7% inhibition of melanin production by compound I at 100 μM. In addition, this compound had no effect on the tyrosinase enzyme function. However, it exhibited a catalyzing effect on the dopachrome transformation into 5,6-dihydroxyindole-2-carboxylic acid. Overall, the pigment-lightening effects of the compound I may due to the dopachrome tautomerase stimulation.

Keywords: Benzylamide; Melanogenesis; Whitening agents

1. Introduction

Studies on skin pigment have a history of less than 20 years. Since the 1980s, the melanin biosynthesis pathways were studied by skin cancer researchers [1,2], Which have led to the development of whitening cosmetics and medicines. Arbutin, kojic acid and its derivatives were developed in 1990s [3]. However, the clinical effect of these materials is unsatisfactory. The hydroquinone group compounds have been used as effective depigmenting agents for skin overpigmentation but they are strongly irritable and exhibits cell toxicity [4,5]. Therefore, there is a large demand for newer whitening agents.

Melanogenesis is mainly regulated by both enzymes, tyrosinase and dopachrome tautomerase. The tyrosinase catalyzes the oxidation of L-tyrosine to dopachrome. It is believed to be the most important enzyme in melanogenesis [6,7]. Another enzyme correlating with the melanogenesis pathway is dopachrome tautomerase [2]. It catalyzes the transformation of dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The early development of bleaches focused on the function of oxidizing tyrosine by tyrosinase and screening the tyrosinase inhibitor *in vitro*. However, the real application of the sorted bleaches to the human body lacks the *in vivo* biological effect with the problems of skin transmission, cell toxicity, stability, etc. Oxyresveratrol (3,5,2′,4′-tetrahydroxystilbene) has been recently reported to exert excellent inhibitory effects on mushroom tyrosinase activity than kojic acid, which is used widely as a whitening agent [8,9]. However, based on our preliminary experiments, oxyresveratrol revealed a high cell toxicity in the melan-a cell line [10]. In addition, this ingredient is not believed to be

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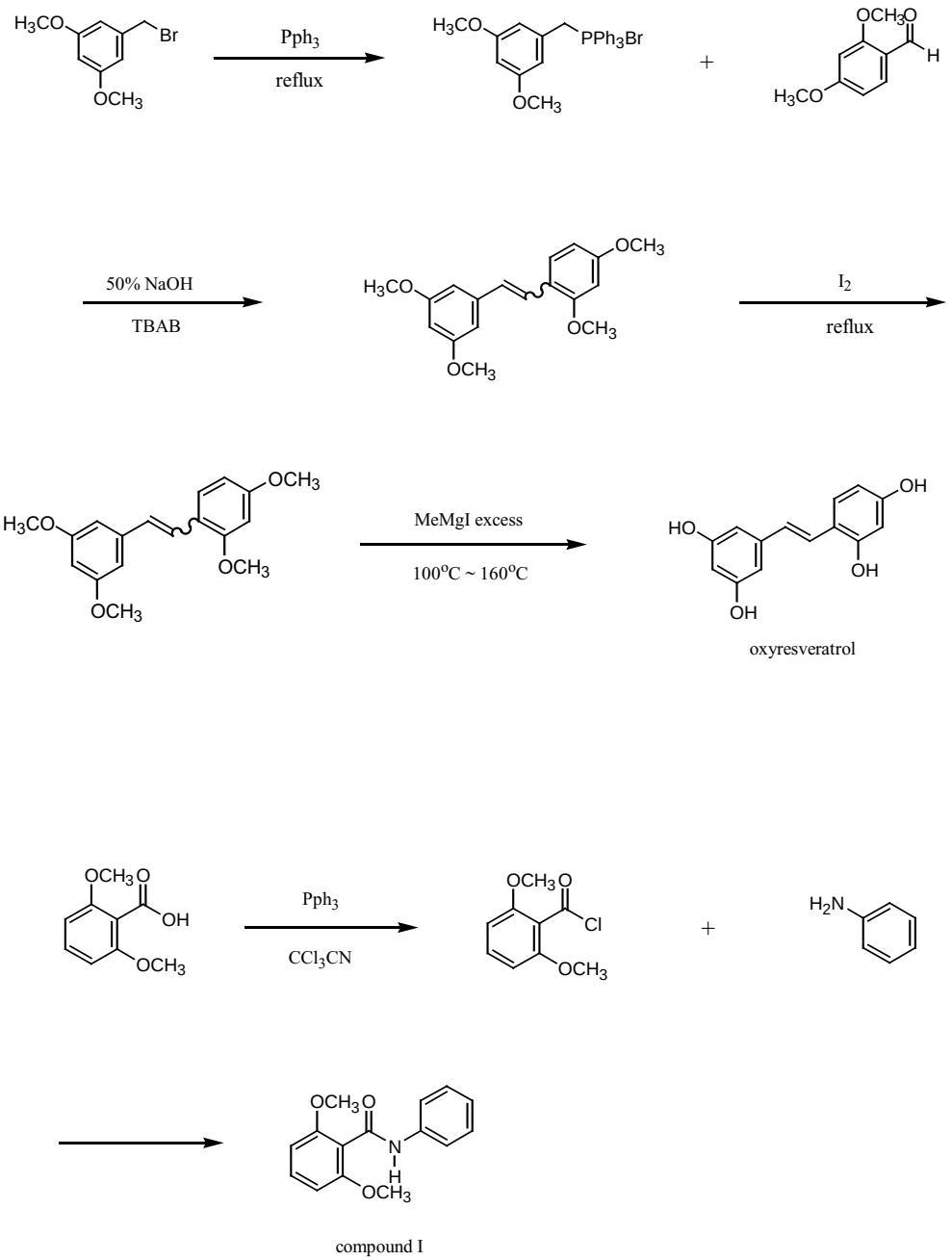

Fig. 1. Synthetic pathway of oxyresveratrol and compound I.

adequate in cell transmission because of its high polarity. Therefore, an attempt to search for substitute materials as oxyresveratrol derivatives, which have a lower toxicity and a lower polarity, is warranted.

Synthetic compound I is an oxyresveratrol derivative with an amide connection chain between the two benzene rings and a lower polarity. The compound can be synthesized simply in one flask (Fig. 1) [11]. This study suggests that melanin production in the melan-a cell line treated with compound I decreases in a dose-dependent manner without cell toxicity. In addition, compound I had a lightening effect on UV-B-induced hyperpigmentation on the dorsal skin of brownish guinea pigs.

2. Materials and methods

2.1. Instrumentation and general techniques

The UV spectra were obtained using a Molecular Devices E09090 microplate reader. The ^{1}H NMR (300 MHz) and ^{13}C (75 MHz) NMR spectra were run on a Gemini-2000 spectrometer. The FABMS spectra were measured on a Hewlett Packard GC-mass spectrometer. The TLC and column chromatography were carried out on Merck precoated silica gel F254 plates and Si gel 60 (Merck, 70–230 mesh). All other chemicals and solvents were of analytical grade and used without further purification.

2.2. Synthesis of oxyresveratrol

1. After dissolving 3,5-dimethoxybenzyl bromide (3 g, 13.2 mmol) in anhydrous benzene (44 mL), triphenylphosphine (8.38 g, 19.8 mmol) was added and refluxed for 2 hr. A precipitate was generated after cooling. 6.58 g (99%) of 3,5-dimethoxybenzyl triphenylphosphonium bromide was obtained by filtration.
2. In 8.2 mL of methylene chloride, 3,5-dimethoxybenzyl triphenylphosphonium bromide (200 mg, 0.407 mmol) and 2,4-dimethoxy benzaldehyde (68 mg, 0.447 mmol) were dissolved along with a catalyst, tetrabutylammonium bromide (10 mg). Subsequently, a 50% aqueous solution (1.2 mL) was added slowly. After stirring for 12 hr, 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 *in vacuo*. The residue was purified by flash column chromatography on a silica gel (eluent: CH_2Cl_2) gave 91.3 mg (75%) of 3,5,2′,4′-tetramethoxystilbene as a white solid.
3. Magnesium (285 mg, 11.7 mmol) and methyl iodide (2.73 mL, 43.9 mmol) in dry ether (12 mL) were stirred for 10 min. After the exothermic reaction was completed, 3,5,2′,4′-tetramethoxystilbene (150 mg, 0.5 mmol) in dry ether (12 mL) was added drop wise. The solution was concentrated using an aspirator and heated to 100° under vacuum. The reaction mixture was then heated up to 160° for 15 min, under nitrogen. After cooling, a 10% ammonium chloride solution was slowly added. The product was extracted with ethyl acetate and washed with brine. It was dried over sodium sulfate and evaporated. The residue was purified by chromatography over a silica gel (eluent: hexane/EtOAc = 1/2), which afforded 31.4 mg (26%) into oxyresveratrol as a yellowish oil.

2.3. Synthesis of compound I

1. To a mixture of 2,6-dimethoxybenzoic acid (100 mg, 0.55 mmol) and trichloroacetonitrile (158 mg, 1.1 mmol) in CH_2Cl_2 (2 mL), Ph_3P (288 mg, 1.1 mmol) in CH_2Cl_2 (1 mL) was added under argon at room temperature. After stirring for 1 hr, the reaction mixture was treated with aniline (51.2 mg, 0.55 mmol) followed by triethylamine (0.28 mL, 2 mmol), and the mixture was stirred for 1 hr. The reaction mixture was poured into water and extracted with ethylacetate. 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 (eluent: CH_2Cl_2/MeOH = 60/1) to give 42.3 mg (30%) of compound I as a white solid.

Oxyresveratrol; yellowish oil; ^{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), 6.44 (d, 1H, J = 2.4 Hz), 6.38 (dd, 1H, J = 2.4 Hz), 6.24 (t, 1H, J = 2.1 Hz); ^{13}C NMR ($(CD_3)_2CO$): 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).

Compound I; white solid; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58 (d, 2H, J = 7.5 Hz), 7.36 (br s, 1H), 7.26 (dd, 3H, J = 16.5, 8.4 Hz), 7.05 (t, 1H, J = 7.4 Hz), 6.53 (d, 2H, J = 8.4 Hz), 3.77 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 162.67, 156.62, 137.36, 133.75, 130.09, 127.95, 123.10, 118.61, 103.12, 55.05; MS (EI^+) m/z 257 (M^+, 13), 165 (100), 150 (13), 122 (8), 107 (11).

2.4. Cell line and culture procedures

The melan-a cells and HM3KO cells were kindly donated by Dr. Byeong Gon Lee at the Skin Research Institute, Amore-Pacific Corporation. The melan-a cells were cultured in RPMI1640 medium with 10% FBS and 200 nm TPA (phorbol 12-myristate-13-acetate) conditions. Ten milliliters of the medium was added to a 100 mm culture dish, and seeded with approximately 5×10^5 cells. Similarly, the HM3KO cells were maintained in the MEM medium with 10% FBS [12].

2.5. Human tyrosinase extraction

The HM3KO cells (human melanoma cell line) were disrupted by resuspending them in an tyrosinase buffer (80 mM PO_4 buffer + 1% Triton-X 100 + 100 μg/mL PMSF) followed by sonication in an ice bath. After centrifugation at 16,600 *g* for 15 min, the supernatant was used for the enzyme assay. 150 μg of proteins was required for each reaction [13].

2.6. Dopachrome tautomerase extraction

If the melan-a cells grew to confluently in the 100 mm culture dish, the media was removed and the cells were washed with PBS. 300 μL of a hypotonic medium (10 mM phosphate buffer, pH 6.8, containing 1% Brij 35) was added in to the cells. The resuspended cells were sonicated for 5 min in an ice bath. After centrifugation at 17,950 *g* for 10 min, the supernatant was used for enzyme activity measurements.

2.7. Inhibition of tyrosinase activity

Tyrosinase activity was measured by its dopa oxidase activity using a modification of the method reported by Shono *et al.* [14]. Each concentration (1 mM, 500, 100, 10 μM) of the test substance was dissolved in MeOH. 40 μL of L-dopa (15 mM), 80 μL of 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

then 40 μL of human tyrosinase were added. The amount of dopachrome in the reaction mixture was determined after incubation at 37° for 30 min. Based on the optical density at 490 nm (OD_{490}), the inhibitory activity of the sample was expressed as a concentration that inhibits 50% of the enzyme activity (IC_{50}). Kojic acid was used as a reference.

2.8. Measurement of 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. The dopachrome prepared was used immediately. The assay reagents consisted of 125 μL of crude dopachrome tautomerase (7 mg total protein), 250 μL of the dopachrome solution, 525 μL of the 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, leaded to the enzyme-catalyzed formation of DHICA from dopachrome [15].

2.9. Melanin and cell viability determination in cultured melan-a cell

The cells were grown to confluence after 4 days under 37° and a 5% CO_2 atmosphere. They were seeded with 10^5 cells/well in the 24-well plate and incubated for 24 hr. Each well was changed with 990 μL of medium everyday and treated with 10 μL of 10,000, 1000 and 100 ppm test sample for 3 days (solvent system: propylene glycol/EtOH/H_2O = 5/3/2). It was then incubated for 1 day.

2.9.1. Cell viability

The percentages of viable cells was determined by staining the cell population with crystal violet. After removing the media on each well, the cells were washed with PBS. The 200 μL of crystal violet (CV 0.1%, 10% EtOH, the rest is PBS) was added. It was incubated at room temperature for 5 min, and washed twice with water. After adding 1 mL of EtOH, it was shaken at room temperature for 10 min. The crystal violet absorption was measured at 590 nm.

2.9.2. Measurement of the melanin level

The melanin content was measured using a modification of the methods reported Wright [16] and Hosoi *et al.* [17]. After removing the media from each well, it was washed with PBS. This was followed by adding 1 mL of 1 N NaOH to dissolve the melanin. The absorption maxima was measured at 400 nm, and the melanin content per well was calculated, and is expressed as a percentage of the control. Phenylthiourea (PTU), which is an inhibitor of melanogenesis acting on tyrosinase, was used as a positive standard control [18,19].

2.10. UV B-induced hyperpigmentation in brown guinea pigs

UV B-induced hyperpigmentation was induced on the backs of brownish guinea pigs weighing approximately 500 g (Jungang Animal Co) using a modification of the methods reported by Hideya *et al.* [20] and Imokawa *et al.* [21]. The guinea pigs were anesthetized with pentobarbital (30 mg/kg), and separate areas (1 cm diametrical circle) on the back of each animal were exposed to UV B radiation (Waldmann UV 800, Herbert Waldmann GmbH, Philis TL/12 lamp emitting 280–305 nm). The total energy dose of UV B was 500 mJ/cm^2 per exposure. Groups of four

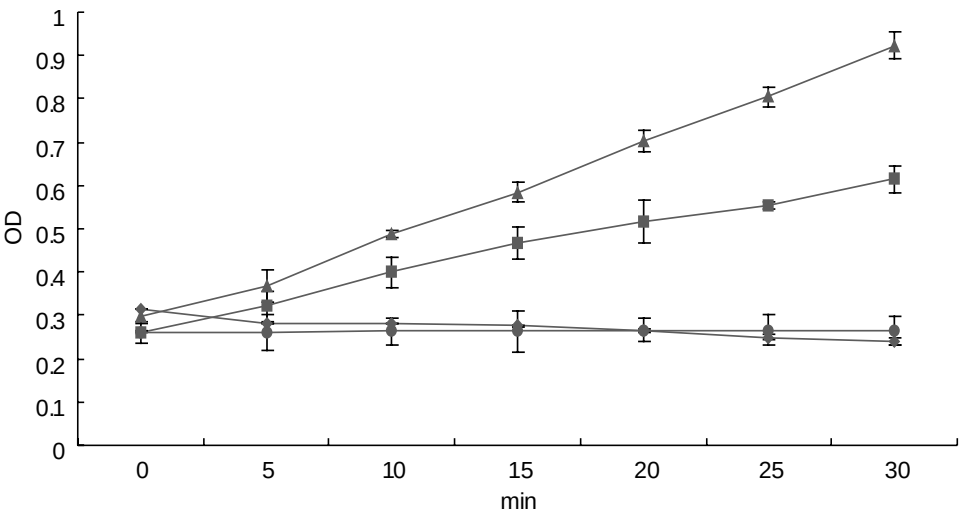

Fig. 2. Changes in the UV absorbance at 308 nm. Vehicle + dopachrome (diamond), 100 μM compound I + dopachrome (circle), vehicle + dopachrome + enzyme (rectangle), dopachrome + enzyme + 100 μM compound I (triangle).

animals were used in the experiments. The animals were exposed to UV B radiation once a week for 3 consecutive weeks. The candidates for the whitening agent was then topically applied to the hyperpigmented areas (1% in propyleneglycol:EtOH:H_2O = 5:3:2, 5 μL/circle) twice a day for 8 weeks from next day of last tanning. The degree of pigmentation was assessed as the *L*-value measured using a chromameter (CR-300, Minolta). Eight weeks later, skin biopsies were taken and processed for Fontana-Masson and S-100 staining [22].

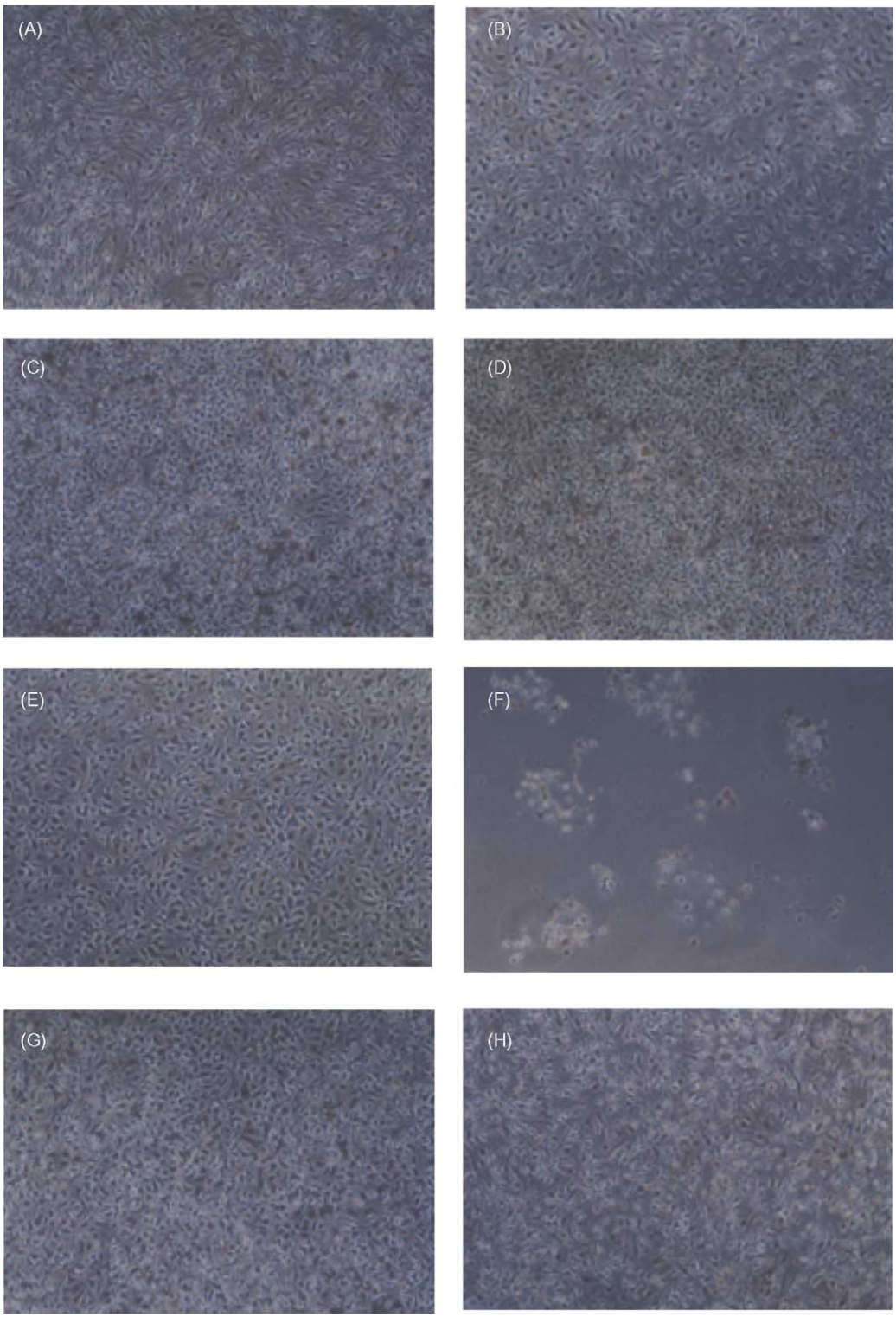

Fig. 3. Photograph of melan-a cells in various concentrations of each compounds. (A) PTU 10 ppm, (B) PTU 100 ppm, (C) kojic acid 10 ppm, (D) kojic acid 100 ppm, (E) oxyresveratrol 10 ppm, (F) oxyresveratrol 100 ppm, (G) compound I 10 ppm, (H) compound I 100 ppm.

2.11. Statistical analysis

The data are presented as a mean ± SE. The statistical comparisons between the different treatments were done by Student's *t* test.

3. Results and discussion

3.1. Organic synthesis of compounds

This study was undertaken to search for a candidate for use as new raw material for a skin whitening cosmetic and whitening medicine. The synthesis of the compound I was started from a preparation of 2,6-dimethoxybenzoic acid and trichloroacetonitrile. Compound I was synthesized in one step with a 30% yield. This compound designed to have a lower polarity for cell transmission and a greater structural stability. In contrast, oxyresveratrol has many synthetic steps [23,24], a higher polarity and an unstable structure at room temperature.

3.2. Tyrosinase inhibitory effects

Compound I, oxyresveratrol and kojic acid as a positive control were examined for their tyrosinase inhibitory activity. The human tyrosinase inhibitory activities of oxyresveratrol and kojic acid at a concentration of 20 μM were 19.6 and 15.8% for oxyresveratrol and kojic acid, respectively. Similarly, at a concentration of 100 μM, inhibitory effect on mushroom tyrosinase was 41.8 and 37.5%, respectively. Oxyresveratrol (IC_{50} = 148.1 μM) exhibited stronger inhibitory activity against human tyrosinase than kojic acid (Table 1). However, compound I had no inhibitory effect on tyrosinase at any dose tested.

Table 1
Inhibitory effects of compound I, oxyresveratrol and kojic acid on human tyrosinase activity.

Samples	Concentrations (μM)	Human tyrosinase inhibition[a] (%)	IC_{50}[b] (μM)
Kojic acid	2	0.2 ± 6.2	161.7
	20	15.8 ± 5.2	
	100	37.5 ± 6.6	
	200	57.9 ± 3.7	
Oxyresveratrol	2	3.8 ± 2.0	148.1
	20	19.6 ± 2.9	
	100	41.8 ± 1.3	
	200	61.3 ± 1.0	
Compound I	2	4.6 ± 2.8	–
	20	0.2 ± 7.5	
	100	3.4 ± 1.7	
	200	8.5 ± 5.3	

Percentage of tyrosinase inhibition was result from compared with MeOH-treated (vehicle) group.
[a]Each value represents the mean ± SE of three experiments.
[b]50% inhibitory concentration.

Table 2
Effects of PTU, kojic acid, oxyresveratrol and compound I on cell growths and melanin production of melan-a cells

Samples	Concentrations (ppm)	Melanin production[a] (%)	Cell viability[a] (%)
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
Compound I	1	96.8 ± 1.0	93.6 ± 4.5
	10	90.5 ± 1.6	101.1 ± 9.3
	100	68.3 ± 4.0	99.2 ± 6.2

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

3.3. Effects on dopachrome tautormerase activity

Dopachrome tautomerase is an important regulatory factor in the melanogenesis pathway. This enzyme is a dopachrome conversion factor that catalyzes the transformation of dopachrome into DHICA [15]. As shown in Fig. 2, the reaction of treatment with compound I (triangle) showed highest increase in absorbance at 308 nm due to the enzyme-specific tautomerization of dopachrome to DHICA. The formation of DHICA led to an increase in absorbance at 308 nm [25]. Therefore, compound I can be described as a dopachrome tautormerase stimulator.

3.4. Melanin production in cultured melan-a cells

Melan-a cells are syngeneic with the B16 melanoma and its sublines, and provide an excellent parallel non-tumorigenic line for studying the melanoma malignancy [26]. This study measured the cell toxicity and melanin production for each compound by use of the melan-a cell line. The results and morphological changes were represented at Table 2 and Fig. 3. Treatment with a positive control, PTU, at concentrations of 1, 10 and 100 ppm for

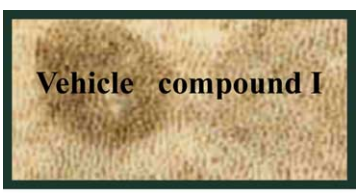

Fig. 4. Representative photographs showing the lightening effects on UV B-induced hyperpigmentation in the guinea pig skin (8 week after). The vehicle was not affected at the skin color compare to the control (data not shown). (A) Vehicle, (B) 1% compound I.

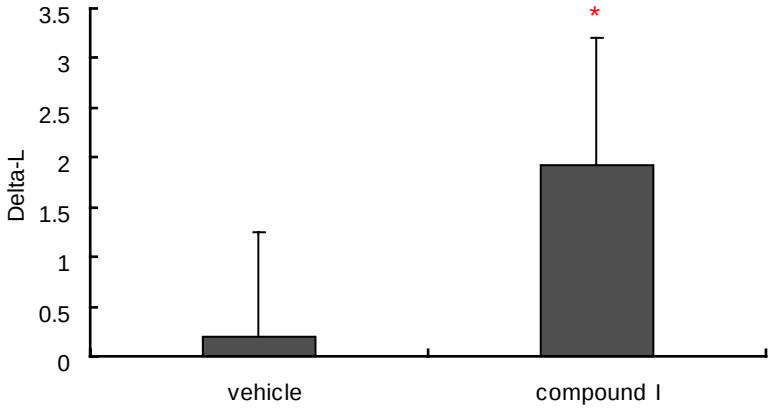

Fig. 5. The degree of pigmentation decrease (ΔL-value) before and 8 weeks after daily topical applications of the vehicle and compound I. The data are expressed as a mean ΔL-value ± SEM, t test was used for the statistical analysis of the data ($^{*}P < 0.05$ vs. control). Groups of four animals were used in this experiment.

3 days resulted in a significantly reduction (74.7% at 100 ppm) in melanin formation in a melan-a cells (Table 2). However, PTU had a cell toxicity at over a 10 ppm concentration. In addition, 100 ppm oxyresveratrol exhibited cell toxicity, and this cell toxicity also had effects on decreasing melanin production (Fig. 3). Treatment with compound I at a concentration of 100 ppm for 3 days resulted in a significant reduction in the melanin content with no cell toxicity. As shown in Table 2, Kojic acid did not show an inhibitory effect of melanin production at any concentrations.

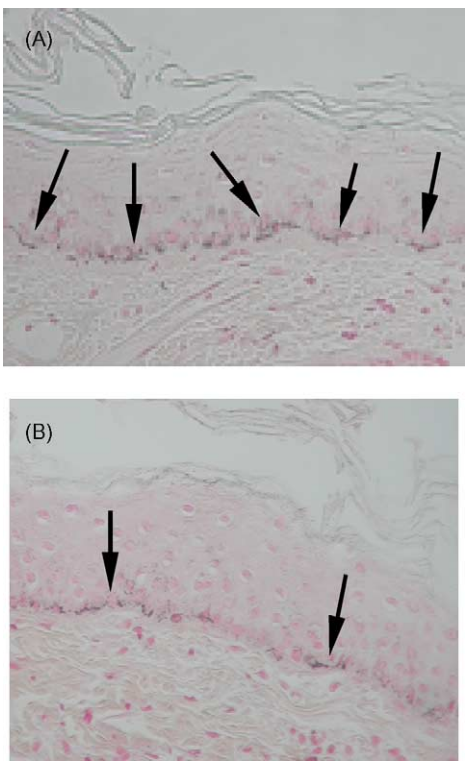

Fig. 6. Effects of compound I in UV-stimulated melanin production of guinea pig skin. Fontana-Masson staining was performed on 5 μm sections embedded in paraffin. There was decrease in the melanin content at the treated site. When the photos were analyzed directly in the optima image analysis program (6.5, Media Cybernetics), 1% of compound I decrease the level of melanin content by 40.0% compared to vehicle. (A) Vehicle, (B) compound I (original magnification: 400×).

3.5. Depigmenting effects on brown guinea pig skin

The whitening effect of compound I was examined under a UV B-induced hyperpigmentation model in brown guinea pigs. UV B-induced hyperpigmentation was elicited on the dorsal skin of the brownish guinea pigs according to a slight modification of the method reported

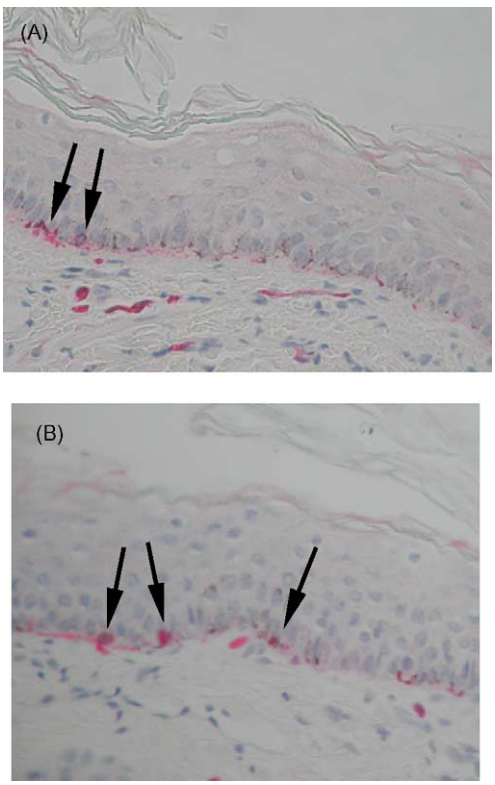

Fig. 7. Effects of compound I on the melanocytes of guinea pig skin. S-100 immunostaining was performed on 5 μm sections embedded in paraffin. (A) vehicle, (B) compound I (original magnification: 400×).

by Hideya *et al*. [20]. Figure 4 shows a photograph of the whitening effects on the guinea pig dorsal skin. Compound I was topically applied 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 2 weeks after the treatment with compound I, when compared to the vehicle group. In addition, a dramatically visible decrease in hyperpigmentation was observed at 8 weeks after treatment with compound I compared to the control vehicle group (Fig. 5). Furthermore, visible edema was not observed at any sites where the dorsal skin was treated with compound I during all experimental days.

Histological methods, such as Fontana-Masson stain for melanin pigment and immunohistochemical stain for melanocytes, were used in this study. We evaluated the biopsy specimens from the dorsal skin of guinea pigs. In the compound I-treated group's skin, histological staining using Fontana-Masson's method resulted in a decrease in melanin pigment (Fig. 6). Immunoreactivity was found to be patchy in some areas with present staining of the mature melanocytes. S-100 was the most sensitive melanocyte marker. Significant amounts of the S-100 protein were demonstrated in both the vehicle groups and the compound I-treated groups (Fig. 7). The S-100 protein-positive cells lay close together, and many areas were stained strongly. This indicates that compound I has no toxicity on guinea pig melanocytes.

This study was conducted to evaluate the action of a benzamide derivative, compound I, on the ultraviolet B-induced hyperpigmentation of the skin. According to the result, It can be speculated that compound I inhibits pigmentation due to the stimulation of dopachrome tautomerase. In the melanization pathway (Fig. 8), the conversion of dopachrome is a branch road to determine whether 5,6-dihydroxyindole (DHI)-eumelanin or DHICA-eumelanin will be produced. Of these, the fast and main pathway is the production of DHI-eumelanin, which resulted from the conversion of dopachrome into DHI [27]. Dopachrome tautomerase converts dopachrome into DHICA [15,28], thereby finally producing DHICA-eumelanin and inhibiting DHI-eumelanin production through the fast pathway. The pathway for producing DHICA-eumelanin is slower than that for producing

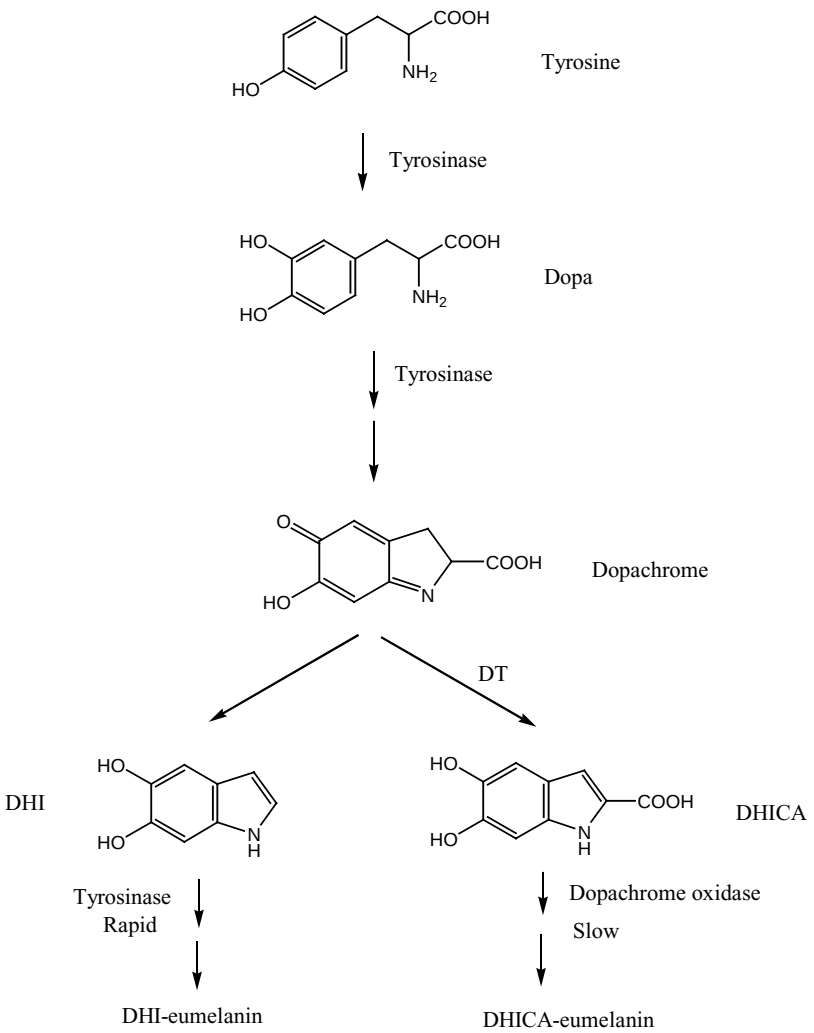

Fig. 8. Melanization pathway in vertebrate animals. DHI: dihydroxyindole, DHICA: dihydroxyindole-2-carboxylic acid, DT: dopachrome tautomerase.

DHI-eumelanin, which is due to the higher stability of DHICA than DHI [28,29]. Thus, the production of DHICA by the stimulation of dopachrome tautomerase is consequently described to have delayed effects in melanogenesis. Moreover, 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 [27]. This is also considered to result in the lightening effect.

However, our suggestion remains to be seen that all pigment producing melanocytes have DHICA polymerizing factor otherwise DHICA-melanin would not results. Hence, future studies are necessary to determine whether compound I may inhibit DHICA polymerization to find more direct evidence for depigment activity of compound I.

Acknowledgments

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References

[1] Korner A, Pawelek J. Mammalian tyrosinase catalases three reactions in the biosynthesis of melanin. Science 1982;217:1163–5.
[2] Pawelek JM. After dopachrome? Pigment Cell Res 1991;4:53–62.
[3] Chen JS, Wei C, Marshall MR. Inhibition mechanism of kojic acid on polyphenol oxidase. J Agric Food Chem 1991;39:1897–901.
[4] Maeda K, Fukuda M. *In vivo* effectiveness of several whitening cosmetic components in human melanocytes. J Soc Cosmet Chem 1991;42:361–8.
[5] Ernest VC, Cecil K, Heino H, Hansruedi G, Chie S, Victoria V, Vincent JHJ, Thomas PD. Inhibitors of mammalian melanocyte tyrosinase: *in vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochem Pharmacol 1999;57:663–72.
[6] Iozumi K, Hoganson GE, Pemella R, Everett MA, Fuller BB. Role of tyrosinase as the determinant of pigmentation in cultured human melanocytes. J Invest Dermatol 1993;100:806–11.
[7] Aroca P, Urabe K, Kobayashi T, Tsukamoto K, Hearing VJ. Melanin biosynthesis patterns following hormonal stimulation. J Biol Chem 1993;268:25650–5.
[8] Shin NH, Ryu SY, Choi EJ, Kang SH, Chang IM, Min KR, Kim YS. Oxyresveratrol as potent inhibitor on dopa oxidase activity of mushroom tyrosinase. Biochem Biophys Res Commun 1998;243:801–3.
[9] Kim YM, Yun J, Lee CK, Lee H, Min KR, Kim YS. Oxyresveratrol and hydrostilbene compounds: inhibitory effect on tyrosinase and mechanism of action. J Biol Chem 2002;277:16340–4.
[10] Gomez-Cordoves C, Bartolome B, Vieira W, Virador VM. Effects of wine phenolics and sorghum tannins on tyrosinase activity and growth of melanoma cells. J Agric Food Chem 2001;49:1620–4.
[11] Borger D, Jiang W, Goldberg J. Convergent solution-phase combinatorial synthesis with multiplication of diversity though rigid biaryl and diarylacetylene couplings. J Org Chem 1999;64:7094–100.
[12] Oka M, Ogita K, Ando H, Horikawa T, Hayashibe K, Saito N, Kikkawa U, Ichihashi M. Deletion of specific protein kinase C subspecies in human melanoma cells. J Cell Physiol 1996;167:406–12.
[13] Fuller BB, Drake MA, Spaulding DT, Chaudhry F. Downregulation of tyrosinase activity in human melanocyte cell culture by yohimbine. J Invest Dermatol 2000;114:268–76.
[14] Shono S, Toda K. The effect of fatty acids on tyrosinase activity. In: Seiji M, editor. Pigment cell. Tokyo: University of Tokyo Press; 1981. p. 263–8.
[15] Barber J, Townsend D, David P, Olds MS, King RA. Dopachrome oxidoreductase: a new enzyme in the pigment pathway. J Invest Dermatol 1984;83:145–9.
[16] Wright S. Dietary supplementation with $n-6$ essential fatty acids in atopic eczema. J Dermatol Treat 1990;1:47–9.
[17] Hosoi J, Abe E, Suda T, Kuroki T. Regulation of melanin synthesis of B16 mouse melanoma cells by 1,2,5-dihydroxy vitamin D and retinoic acid. Cancer Res 1985;45:1474–8.
[18] Poma A, Bianchini S, Miranoa M. Inhibition of L-tyrosine-induced micronuclei production by phenylthiourea in human melanoma cells. Mutat Res 1999;446:143–8.
[19] Nordlund, Boissy, Hearing, King, Ortonne. The pigmentary system. Oxford University Press; 1998. p. 406.
[20] Hideya A, Atsuko R, Akira H, Masahiro O, Masamitsu I. Linoleic acid and α-linoleic acid lightens ultraviolet-induced hyperpigmentation of the skin. Arch Dermatol Res 1998;290:375–81.
[21] Imokawa G, Kawai M, Mishima Y, Motegi I. Differential analysis of experimental hypermelanogenesis induced by UVB, PUVA and allergic dermatitis using a brownish guinea pig model. Arch Dermatol Res 1986;278:352–62.
[22] Ludger K, Albert MK, Tracy S. The sodium hydroxide erosion assay: a revision of alkali resistance test. Arch Dermatol Res 1998;290:382–7.
[23] Alonso E, Ramon D, Yus M. Simple synthesis of 5-substituted resorcinols. J Org Chem 1997;62:417–21.
[24] Zang J, Doi W, Ronald G. Synthesis of higher oxidized metabolites of dibenz[*a*,*j*]anthracene implicated in the mechanism of carcinogenesis. J Org Chem 1998;63:8125–32.
[25] Aroca P, Solano F, Jose C, Borron G, Lozano JA. A new spectrophotometric assay for dopachrome tautomerase. J Biochem Biophys Methods 1990;21:35–46.
[26] Bennett D, Cooper P, Hart I. A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. Int J Cancer 1987;39:414–8.
[27] Sugumaran M. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 2002;15:2–9.
[28] Jose H, Solano F, Jose C, Borron G, Jose R, Jose A. α-MSH and other melanogenic activators mediate opposite effects on tyrosinase and dopachrome tautomerase in B16/F10 mouse melanoma cells. J Invest Dermatol 1992;99:435–9.
[29] Fang J, Han Q, Johnson JK, Christensen BM, Li J. Functional expression and characterization of *Aedes aegypi* dopachrome conversion enzyme. Biochem Biophys Res Commun 2002;290:287–93.