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Evaluation of *in vitro* and *in vivo* anti-melanogenic activity of a newly synthesized strong tyrosinase inhibitor (*E*)-3-(*2,4 dihydroxybenzylidene*)*pyrrolidine-2,5-dione* (*3-DBP*)

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ABSTRACT

Background: Tyrosinase inhibitors have become increasingly important because of their ability to inhibit the synthesis of the pigment melanin. A search for new agents with strong tyrosinase activity led to the synthesis of the tyrosinase inhibitor (*E*)-3-(2,4-dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP).
Methods: The inhibitory effect of 3-DBP on tyrosinase activity and melanin production was examined in murine melanoma B16F10 cells. Additional experiments were performed using HRM2 hairless mice to demonstrate the effects of 3-DBP *in vivo*.
Results: The novel compound, 3-DBP, showed an inhibitory effect against mushroom tyrosinase (IC_{50} = 0.53 μM), which indicated that it was more potent than the well-known tyrosinase inhibitor kojic acid (IC_{50} = 8.2 μM). When tested in B16F10 melanoma cells treated with α-melanocyte stimulating hormone (α-MSH), 3-DBP also inhibited murine tyrosinase activity, which in turn induced a decrease in melanin production in these cells. The anti-melanogenic effect of 3-DBP was further verified in HRM2 hairless mice. The skin-whitening index (L value) of HRM2 hairless mice treated with 3-DBP before irradiation with UVB was greater than that of UVB-irradiated mice that were not treated with 3-DBP.
General significance: The newly synthesized 3-DBP has a potent inhibitory effect on tyrosinase. In addition to an *in vitro* investigation of the effects of 3-DBP on tyrosinase, *in vivo* studies using an HRM2 hairless mouse model demonstrated the anti-melanogenic potency of 3-DBP. Our newly synthesized 3-DBP showed efficient tyrosinase inhibitory effect *in vivo* and *in vitro*. Our finding suggests that 3-DBP can be an effective skin-whitening agent.

1. Introduction

Tyrosinase inhibitors have become increasingly important because they inhibit synthesis of the pigment melanin [1,2]. They are important in various fields, including the cosmetic industry [3,4], as medications [5], and in the food industry [6,7] for their ability to decrease pigmentation. Although melanin provides effective protection against harmful ultraviolet radiation, abnormal melanin accumulation can cause esthetic problems such as melasma, freckles, and senile lentigines [8,9]. Although diverse tyrosinase inhibitors can be obtained from both naturally occurring and synthetic sources, safety concerns have prevented the commercialization of most of these inhibitors. There are many tyrosinase inhibitors such as hydroquinone [10], ascorbic acid derivatives [11], azeleic acid [12], retinoids [13], arbutin [14], and kojic acid [6]. However, some well-known whitening agents, such as hydroquinone and kojic acid, are considered as harmful agents because of their undesirable side effects such as cytotoxicity, skin cancer, and dermatitis. Therefore, safe and effective whitening agents are needed. Newly synthesized agents with a fully different structure moiety may address these issues and serve as a better solution for treating dermatological disorders associated with skin pigmentation.

Melanin is the primary agent responsible for skin color and plays an important role in preventing skin injury under normal physiological conditions. The photochemical properties of melanin make it an excellent photo-protectant. It absorbs harmful UV-radiation, transforming this energy into harmless heat through a process referred to as 'ultrafast internal conversion' [15]. Despite its advantages, melanin can also cause abnormal pigmentations such as freckles, age spots,

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and melanoma, which can be serious skin problems. Therefore, modulating melanogenesis is an important strategy for treating abnormal skin pigmentation.

Melanogenesis is significantly affected by tyrosinase, which catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and L-DOPA to dopaquinone [16]. Tyrosinase is a multifunctional type-3 binuclear copper-containing enzyme; the copper-containing site plays an important role in the activity of tyrosinase [17]. The best activator and substrate for human tyrosinase is L-DOPA and not L-tyrosine [18,19]. The substrate L-tyrosine and the activator L-DOPA were shown to have separate binding sites on tyrosinase [20]. Tyrosinase is a rate-limiting enzyme that catalysis steps in melanin biosynthesis and causes the abnormal accumulation of melanin pigments under unregulated conditions [21,22]. Because of its key role in melanogenesis, tyrosinase is an attractive target in the search for various types of depigmenting agents [23]. Thus, the present study focused on inhibition of tyrosinase and melanin production; to this end, a novel compound with a new type of scaffold was synthesized.

The purpose of this study was to identify and characterize a new tyrosinase inhibitor. In a search for tyrosinase inhibitors from natural sources and synthesized compounds, we synthesized the novel compound (*E*)-3-(2,4-dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP, Fig. 1a) containing a pyrrolidine-2,5-dione skeleton with powerful tyrosinase inhibitory effects. 3-DBP has not been synthesized previously or used as a potent tyrosinase inhibitor. The inhibitory effects of 3-DBP on murine tyrosinase activity and melanogenesis were evaluated using an *in vitro* model with B16F10 mouse melanoma cells. Moreover, using an HRM2 hairless *in vivo* mouse model, 3-DBP was identified to be a powerful skin-whitening agent that may be used for treating skin hyperpigmentation without perceptible cytotoxicity.

2. Materials and methods

2.1. Materials

3-DBP containing a pyrrolidine-dione moiety was synthesized using a Wittig reaction in our laboratory. Mushroom tyrosinase, L-tyrosine, α-melanocyte stimulating hormone (α-MSH), and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). Antibodies against tyrosinase, microphthalmia-associated transcription factor (MITF), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Synthesis of (E)-3-(2,4-dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP)

A suspension of 2,4-dihydroxybenzaldehyde (100 mg, 0.72 mmol) and triphenyl phosphoranylidene succinimide (260 mg, 0.72 mmol) in MeOH (5 mL) was refluxed. Before reaching the boiling point of methanol, the reaction mixture became a clear solution. After cooling the solution to room temperature, precipitates were filtered through a Buchner funnel. The filter cake was washed with methanol, methylene chloride, and water to remove the remaining starting materials, 2,4-dihydroxybenzaldehyde and triphenylphosphoranylidene succinimide, and obtain the final product.

The solid product was very pale and brown in color; the reaction time was 24 h, the yield was 82%, and the melting point was >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6) showed peaks at δ 11.18 (s, 1H, NH), 10.04 (s, 1H, OH), 9.92 (s, 1H, OH), 7.65 (s, 1H, vinyl H), 7.28 (d, 1H, J = 8.5 Hz, 6′-H), 6.37 (d, 1H, J = 2.0 Hz, 3′-H), 6.31 (dd, 1H, J = 2.0, 8.5 Hz, 5′-H), 3.50 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) showed peaks at δ 176.8 (C5), 173.2 (C2), 161.3 (C4′), 159.5 (C2′), 131.0 (benzyl C), 127.2 (C6′), 121.2 (C3), 113.5 (C1′), 108.4 (C5′), 103.0 (C3′), 35.5 (C4); low-resolution mass spectrometry-electrospray ionization (LRMS–ES) *m/z*, expected: 218 $(M–H)^-$, found: 218 $(M–H)^-$.

2.3. Cell culture system

Murine melanoma B16F10 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, NY, USA), and penicillin/streptomycin (100 IU/50 μg/mL) in a humidified atmosphere containing 5% CO_2 in air at 37 °C. B16F10 cells were cultured in 24-well plates for cell viability (MTT) assay and a 60π dish for a melanin content assay and tyrosinase activity assay. All experiments were performed at least 3 times to ensure reproducibility.

2.4. Cell viability assay

The cell viability assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Next, 5×10^4 cells were plated in each well of a 24-well plate. After the cells were treated with 3-DBP at concentrations ranging from 10 to 200 μM for 24 h, MTT solutions were added and the insoluble derivative formed by cellular dehydrogenase was solubilized in a mixture of ethanol and

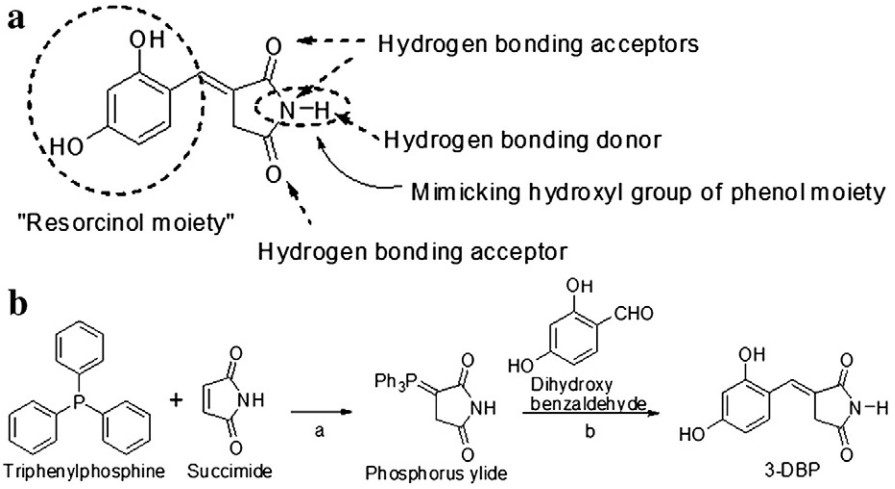


Fig. 1. Rationale for the design and synthesis of (*E*)-3-(2,4-dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP). (a) Chemical structure of 3-DBP and explanation of the rationale for design of the desired compound. (b) Brief scheme of the synthetic process of 3-DBP. Reagents and conditions were as follows: a. anhydrous acetone, reflux, 1 h, 92%; b. methanol, reflux, 24 h, 82%.

dimethylsulfoxide (EtOH–DMSO, 1:1 mixture); the absorbance of each well was determined at 560 nm using a microplate reader.

2.5. Measurement of mushroom tyrosinase activity

To evaluate the inhibitory action of 3-DBP on tyrosinase, tyrosinase isolated from mushrooms was utilized as described previously with some modifications [25]. Briefly, 20 μL of aqueous solution of mushroom tyrosinase (1000 units) was added to a 96-well microplate with 200 μL of a reaction mixture containing 1 mM L-tyrosine solution, 50 mM phosphate buffer (pH 6.5), and the test material 3-DBP (0.5 to 10 μM). The assay mixture was incubated at 25 °C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm (OD_{492}) in a microplate reader. The inhibitory concentration-50 (IC_{50}) is the concentration of a substance that inhibits a standard response by 50%. To determine the inhibitory mechanism of 3-DBP, a tyrosinase kinetic assay was carried out. Various concentrations of L-tyrosine (16, 8, 4, 2, and 1 mM) were used for the inhibition assay. After examination, the reciprocal of each value was calculated to construct Lineweaver–Burk plots. The plot shows the inverse of reaction velocity (1/V) *versus* the inverse of substrate concentration (1/[S]). On the basis of the point of convergence of lines on the plot, an inhibitory mechanism could be determined.

2.6. Determination of melanin content

The amount of melanin present was used as an index of melanogenesis in the current study. Briefly, B16 cells were plated on a 60π-dish and incubated in the presence or absence of 100 μM α-MSH. Cells were then incubated for 48 h with or without 3-DBP at concentrations ranging from 10 to 100 μM. After washing twice with PBS, samples were dissolved in 500 μL of 1 N NaOH. The samples were incubated at 60 °C for 1 h and mixed to solubilize the melanin. The absorbance at 405 nm was compared with that derived from a standard curve of synthetic melanin.

2.7. Measurements of cellular tyrosinase activity

Tyrosinase activity in B16F10 cells was examined by measuring the rate of oxidation of L-DOPA. Cells were plated in 60π-well dishes at a density of 5×10^4 cells/mL. B16 cells were incubated in the presence or absence of 100 μM α-MSH and then treated for 48 h with various concentrations (10–100 μM) of 3-DBP. The cells were lysed in 500 μL of 50 mM sodium phosphate buffer (pH 6.8) containing 25 μL of 1% Triton X-100 and 25 μL of 0.1 mM phenylmethyl-sulfonyl fluoride and then frozen at −80 °C for 30 min. After thawing and mixing, cellular extracts were clarified by centrifuging the samples at 12,000×*g* for 30 min at 4 °C. The supernatant (80 μL) and 20 μL of L-DOPA (2 mg/mL) were placed in a 96-well plate, and the absorbance at 492 nm was read every 10 min for 1 h at 37 °C using an ELISA plate reader.

2.8. Western blotting

Preparation of cell lysates was carried out as described previously, with some modifications [25]. Cell lysates (20 μg of protein each) were boiled for 5 min in gel-loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, and 0.2% bromophenol blue) at a 1:1 ratio. Total protein equivalents for each sample were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were immediately placed into blocking buffer (5% non-fat milk) in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. Blots were blocked at room temperature for 1 h. Membranes were incubated with specific primary antibodies at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz, 1:10,000), an anti-rabbit antibody (Santa Cruz, 1:10,000), or an anti-goat antibody (Santa Cruz, 1:10,000) at 25 °C for 1 h. Antibody labeling was detected using West-zol Plus and chemiluminescence FluorchemTMSP (Alpha Innotech Corporation, San Leandro, CA, USA). Pre-stained protein markers were used for molecular weight determination.

2.9. Determinations of depigmenting activity in HRM2 hairless mouse

The *in vivo* depigmenting efficacy of 3-DBP was assessed through animal experiments that were performed in accordance with the guidelines for animal experimentation of Pusan National University. Six-week-old male HRM-2 melanin-possessing hairless mice were obtained from Hoshino Laboratory Animals (Yashino, Saitama, Japan) and housed in a controlled room (23 °C ± 1 °C, 55 ± 5% humidity, 12-h light/dark cycle) with *ad libitum* access to water and standard laboratory diet. After an acclimation period (2 weeks), mice were randomly divided into 5 groups of 6 animals. 3-DBP was prepared at 3 concentrations (0.4, 2, and 10 μM) in a solution of propylene glycol and ethanol (3:7). Control solution and 3-DBP-dissolved solution (200 μL) were topically applied to a designated site (3 cm × 3 cm) on the dorsal skin of the animal once per day. Animals were exposed to UVB in a CROSSLINKER (BEX-800, Ultra-Lum, Inc., Claremont, CA, USA) at 150 mJ/cm^2. Colors of skin sites were measured using a CR-10 spectrophotometer (Konica Minolta Sensing, Inc., Sakai, Osaka, Japan) in which the colors are described by L*, a*, and b* values according to the Commission Internationale de l'Eclairage color system.

2.10. Fontana–Masson staining

Skins were fixed in 4% paraformaldehyde overnight at room temperature and stained for melanin by using a Fontana–Masson staining kit from American Mastertech, Inc. (Lodi, CA, USA) according to the manufacturer's instructions. Briefly, sliced skins were stained with ammoniacal silver solution for 60 min at 60 °C followed by incubation in 0.1% gold chloride and then in 5% sodium thiosulfate.

2.11. Statistical analysis

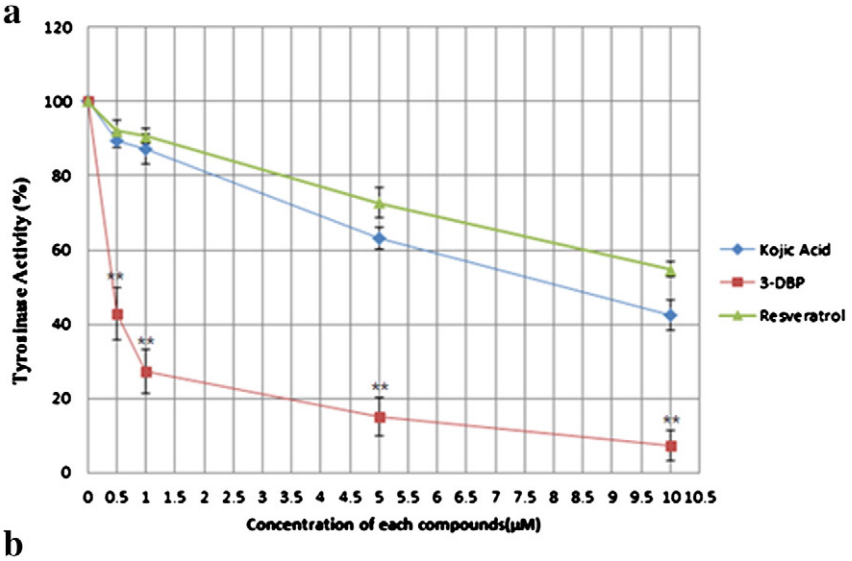
Tyrosinase activity is expressed as a percentage of activity on the basis of the formula [(A · 100)/B], where A = OD_{492} with a test sample and B = OD_{492} without a test sample. Values are presented as means ± SEM (Fig. 2a, n = 6; Fig. 2b, n = 6; Fig. 2c, n = 5; Fig. 3a, n = 6; Fig. 3b, n = 5; Fig. 3c, n = 5, and Fig. 4b and c, n = 6 samples per group). Analysis of variance (ANOVA) was used to analyze differences among all groups. Differences in the means of individual groups were assessed using the Fischer's protected least significant difference post hoc test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Design and synthesis of 3-DBP

3-DBP was designed by combining the structure characteristics of resorcinol and pyrrolidine-2,5-dione into 1 compound. 3-DBP (Fig. 1a) was prepared *via* the Wittig reaction between 2,4-dihydroxybenzaldehyde and triphenylphosphorylidenyl succinimide, which was synthesized from a Michael addition reaction of triphenylphosphine to maleimide (Fig. 1b). The Wittig reaction proceeded smoothly under reflux conditions in methanol and yielded 3-DBP, a very pale, brownish solid, with an 82% yield. The isomer with the (*E*)-configuration was exclusively obtained from filtration of the precipitates generated from the Wittig reaction. The structure of 3-DBP was determined using ^{1}H and ^{13}C NMR

a



b

Compound	IC50 (μM)
3-DBP	0.53 ± 0.11
Kojic acid	8.2 ± 1.3
Resveratrol	13.48 ± 0.96

c

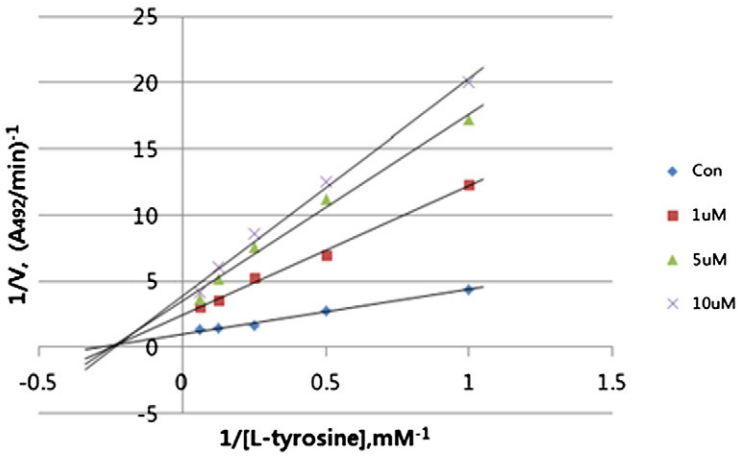


Fig. 2. Effects of 3-DBP on mushroom tyrosinase activity and determination of the inhibitory mechanism *in vitro*. (a) Various concentrations of 3-DBP (0.5–10 μM) were used to treat mushroom tyrosinase. Kojic acid and resveratrol were used as positive controls at the same concentrations of 3-DBP. Data are shown as the percent of tyrosinase activity. (3-DBP: rectangle shape, Kojic acid: diamond shape, resveratrol: triangle shape). A double asterisk (**) denotes the statistical significance between 3-DBP *vs.* kojic acid, $P<0.001$ in a Student's *t* test. (b) IC_{50} values of 3-DBP, resveratrol, and kojic acid for mushroom tyrosinase activity. The IC_{50} value of 3-DBP was 0.53 μM, which was significantly smaller than that of kojic acid (8.2 μM) and resveratrol (13.48 μM). (c) Determination of the inhibitory mechanism of 3-DBP. The inhibitory mechanism of 3-DBP was determined on the basis of a Lineweaver–Burk plot. Data were obtained as mean values of 1/V, the inverse of the absorbance increase at a wavelength 492 nm per min, of 3 independent tests at different concentrations of L-tyrosine as a substrate. Four different enzyme inhibitor concentrations were used to determine the inhibitory mechanism (10 μM = cross shape, 5 μM = triangle shape, 1 μM = rectangle shape, and 0 μM = diamond shape). The modified Michaelis–Menten equation used was $1/V_{max} = 1/K_m\,(1 + [S]/K_i)$. V denotes the velocity of the reaction, S is the L-tyrosine concentration, and K_i is the inhibitor constant.

and mass spectroscopic analyses. The brief synthetic scheme is shown in Fig. 1b.

3.2. Determination of anti-melanogenic effects on mushroom tyrosinase

Inhibitory activities of the synthesized compound and control compounds were examined using mushroom tyrosinase as described previously with minor modifications [26,27]. As shown in Fig. 2a, 3-DBP inhibited tyrosinase activity in a concentration-dependent manner. IC_{50} values of 3-DBP and reference compounds (resveratrol and kojic acid) are shown in Fig. 2b. The low IC_{50} value of 3-DBP ($IC_{50} = 0.53 \pm 0.11$ μM) indicates that the potency is significantly stronger than that of resveratrol ($IC_{50} = 13.48 \pm 0.96$ μM) and kojic acid ($IC_{50} = 8.2 \pm 1.3$ μM), which were used as positive controls.

The inhibitory mechanism of 3-DBP on mushroom tyrosinase for oxidizing L-DOPA was determined from Lineweaver–Burk double-reciprocal plots. Fig. 2c showed double-reciprocal plots of enzyme inhibition by 3-DBP. The 1/V *vs.* 1/[S] plot showed 4 different lines with different slopes, which intersected on the same horizontal axis. Accompanying the increase in the concentration of the compound,

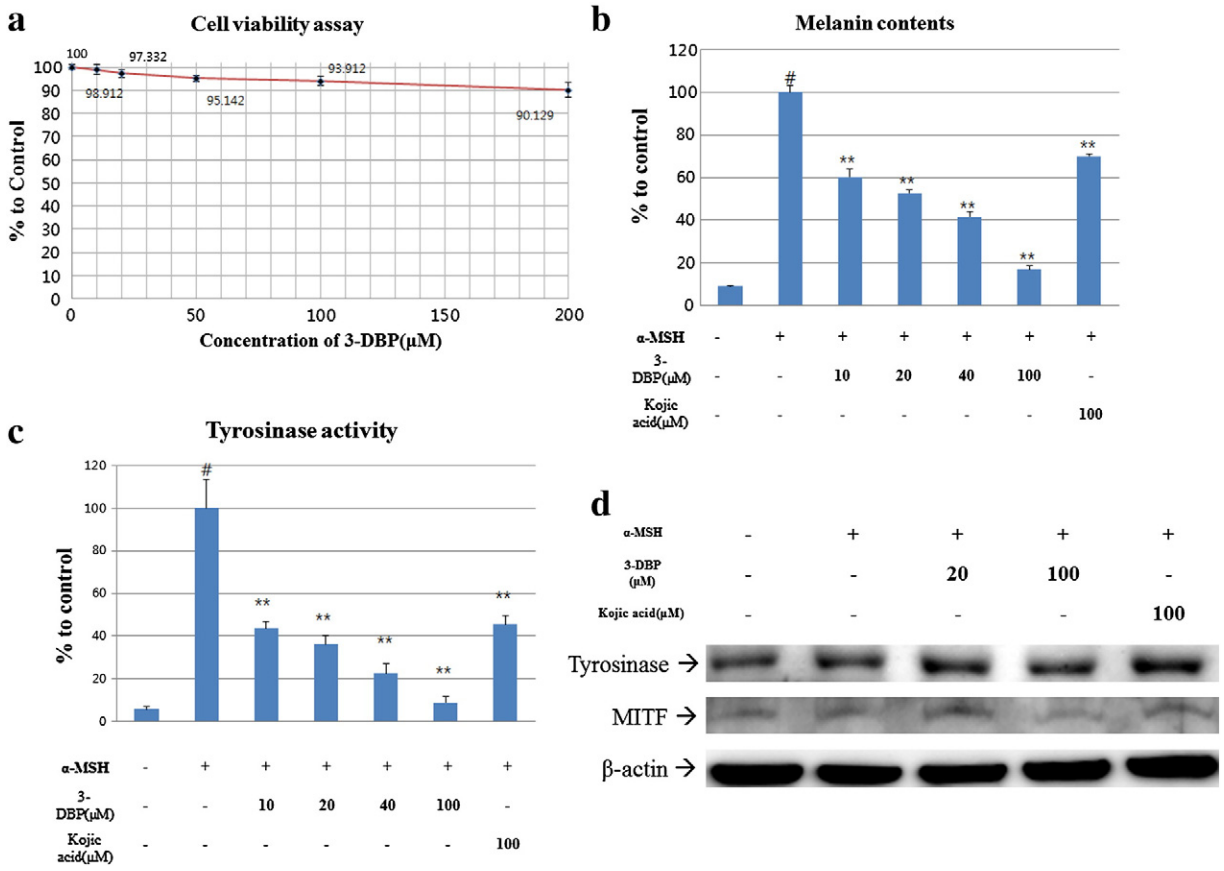


Fig. 3. Effects of 3-DBP on B16F10 melanoma cells. (a) The effect of 3-DBP on the viability of B16F10 cells. Cells were treated with various concentrations of 3-DBP (10–200 μM) and were examined using the MTT assay. Data are expressed as the percent of cell viability. (b) Effect of 3-DBP on melanin content. B16F10 cells were treated with vehicle or 3-DBP (10–100 μM) and incubated in the absence or presence of 100 μmol of α-MSH for 48 h. Intracellular melanin extracted using 0.1 M NaOH was quantified spectrophotometrically and normalized for protein content. A sharp (#) denotes the statistical significance between non-treated cells and α-MSH-treated cells; $P<0.001$ in the Student's *t* test. A double asterisk (**) denotes the statistical significance between α-MSH-treated cells and compound (3-DBP or kojic acid)-treated cells; $P<0.001$ in the Student's *t* test. (c) Tyrosinase activities were examined in B16F10 cells. B16F10 cells were treated with vehicle or 3-DBP (10–100 μM) and incubated in the absence or presence of 100 μmol of α-MSH for 48 h. Treated cells were lysed to obtain cell-free extracts, and tyrosinase activity was assayed using L-DOPA as the substrate. Results are expressed as percentages of the control (only in the α-MSH-treated group). Kojic acid (100 μM) was used as a positive control. A # denotes statistical significance between non-treated cells and α-MSH-treated cells; $P<0.001$ in the Student's *t* test. A double asterisk (**) denotes statistical significance between α-MSH-treated cells and compound (3-DBP or kojic acid)-treated cells; $P<0.001$ in the Student's *t* test. (d) Analysis of total tyrosinase protein and nuclear MITF expression change after α-MSH and/or 3-DBP treatment. β-actin was measured as a control.

V_{max} decreased, but K_m remained the same, suggesting that 3-DBP is a non-competitive inhibitor of tyrosinase.

3.3. Evaluating depigmenting activity of 3-DBP in cell culture system

We used a B16F10 melanoma cell system to evaluate the depigmenting activity of 3-DBP. The results for *in vitro* treatment of B16F10 cells with 3-DBP for cell survival, cellular tyrosinase activity, and melanin contents are shown in Fig. 3. Results from the cell viability assay using MTT for B16F10 cells (Fig. 3a) indicate that 3-DBP is relatively non-cytotoxic to the cells under the experimental conditions used.

The inhibitory effect of 3-DBP on cellular melanogenesis was compared to that of kojic acid, which is widely used in cosmetics as a depigmenting agent [28]. To assess the effect of 3-DBP on the melanin contents of B16F10 cells, 3-DBP was used to treat B16F10 cells in the presence of 100 μM α-MSH at various concentrations; no significant cell cytotoxicity was observed. As shown in Fig. 3b, 3-DBP inhibited cellular melanogenesis, which was augmented by α-MSH in a dose-dependent manner. The inhibitory effect of 3-DBP was stronger than that of kojic acid. To analyze the inhibition mechanism of melanin by 3-DBP, we examined the inhibitory effect of 3-DBP on cellular tyrosinase activity by using murine-derived tyrosinase as depicted in Fig. 3c. Cellular tyrosinase activity exhibited a similar profile to that of the specific melanin content. On the basis of the potent tyrosinase inhibitory activity of 3-DBP, melanogenesis inhibition by 3-DBP may be attributed to its ability to suppress tyrosinase.

To investigate whether 3-DBP-mediated depigmenting activity is involved in modulating tyrosinase gene expression, we further evaluated tyrosinase gene expression and MITF gene expression. As shown in Fig. 3d, no changes in expression levels of tyrosinase and MIFT were observed, indicating that the inhibitory effect of 3-DBP is limited to inhibition of tyrosinase activity.

3.4. Effects of 3-DBP on in vivo skin pigmentation

The inhibitory effects of 3-DBP on *in vivo* skin pigmentation were examined in melanin-possessing hairless mice that were treated according to the schedule shown in Fig. 4a. Animals treated with control vehicle or 3-DBP-condddtaining solution for 3 days prior to UVB exposure did not show any skin irritation. Repeated UVB exposure led to visible pigmentation in mice (Fig. 5a). The colors of the skin sites were measured using a spectrophotometer. UVB exposure led to a decrease in L^* values, which represent pigmentation (Fig. 4b). The UVB-induced decreases in L^* values were significantly and dose-dependently recovered in the 3-DBP treated animals than in the control animals treated with vehicle (Fig. 4b and c), demonstrating the potent depigmenting effect of 3-DBP. UVB exposure of mice

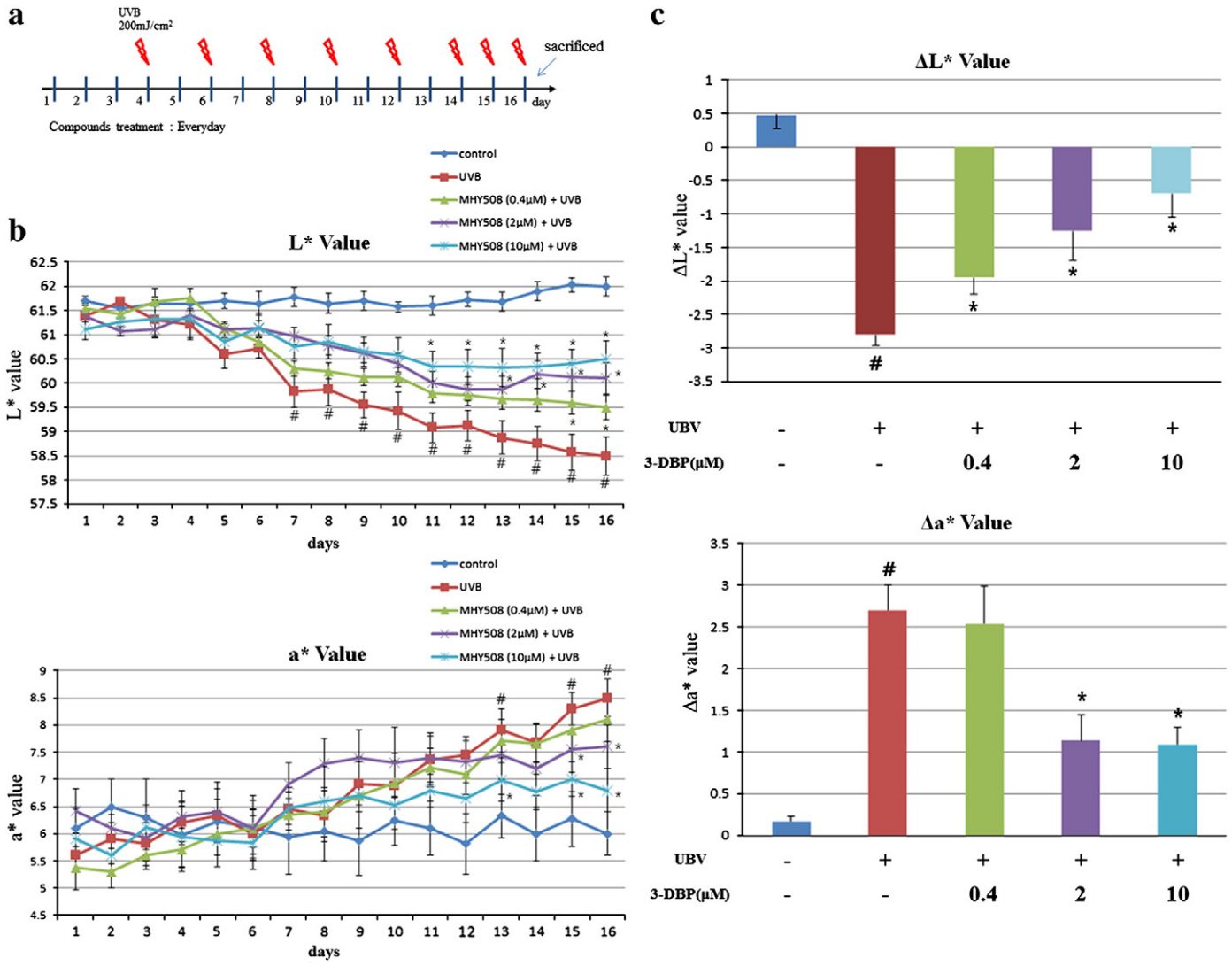


Fig. 4. Effects of 3-DBP on pigmentation in animal skins exposed to UVB. (a) HRM2 melanin-possessing hairless mice were treated with vehicle or 3-DBP on a designated site on the dorsal skins according to the indicated schedules. UVB irradiation was conducted 8 times at the indicated doses. (b) The upper panel shows the lightness (L* value) of the designated skin sites, which was measured prior to 3-DBP or vehicle application on each day. Graph shows changes in lightness (L* value). The lower panel shows the redness (a* value) of the designated skin sites, which was measured prior to 3-DBP or vehicle application on each day. A # denotes statistical significance between control mice and UVB-treated mice; $P<0.05$ in the Student's *t* test. An asterisk (*) denotes statistical significance between UVB-treated mice *vs.* 3-DBP-treated mice; $P<0.05$ in the Student's *t* test. (c) The upper panel shows ΔL* values calculated as the average values after the final UVB exposure (day 16) minus the average baseline values before treatment (day 1). The lower panel shows Δa* values, which were calculated as the average values after final UVB exposure (day 16) minus the average baseline values before treatment (day 1).

led to an increase in a* values, which represent sunburn induction. The UVB-induced increase in the a* value was significantly recovered when the mice were treated with more than 2 μM 3-DBP. Fontana–Masson staining, which highlights melanin [29], confirmed the effects of 3-DBP. As shown in Fig. 5b, UVB-irradiated animals showed increased melanin spots when stained with collected skin. Consistent with the numerical data collected using the spectrophotometer, 3-DBP-treated animals showed decreased stained melanin spots compared to UVB-irradiated controls.

4. Discussion

Tyrosinase, which mediates skin pigmentation, is a copper-containing enzyme; its substrates are L-tyrosine and L-DOPA [16,17]. Therefore, most inhibitors of tyrosinase are Cu^{2+} chelators or phenolic compounds that are structurally analogous to L-tyrosine and L-DOPA. Recently, however, there have been reports stating that resorcinol-containing derivatives exhibited better tyrosinase inhibition and melanin product inhibition in murine B16 melanoma cells without cell toxicity and showed better mushroom tyrosinase inhibition than catechol-containing derivatives [30,31]. Phenolic hydroxyl groups can act as both hydrogen-bonding donors and acceptors. Therefore, compounds with a resorcinol moiety on a side of a double bond and a structure capable of playing a role as both a hydrogen-bonding donor and acceptor on opposite sides of the double bond can be regarded as potential tyrosinase inhibitors. The amino group of the imide in pyrrolidine-2,5-dione can serve as both a hydrogen-bonding donor and acceptor, similar to the hydroxyl group of phenolic compounds. Additionally, pyrrolidine-2,5-dione may provide additional interactions with several amino acid residues of tyrosinase through hydrogen-bonding interactions of its 2 carbonyl groups, implying that these additional interactions with tyrosinase may induce tyrosinase inhibitory activity.

On the basis of these findings, we designed and synthesized (*E*)-3-(2,4-dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP) by combining the structural characteristics of resorcinol and pyrrolidine-2,5-

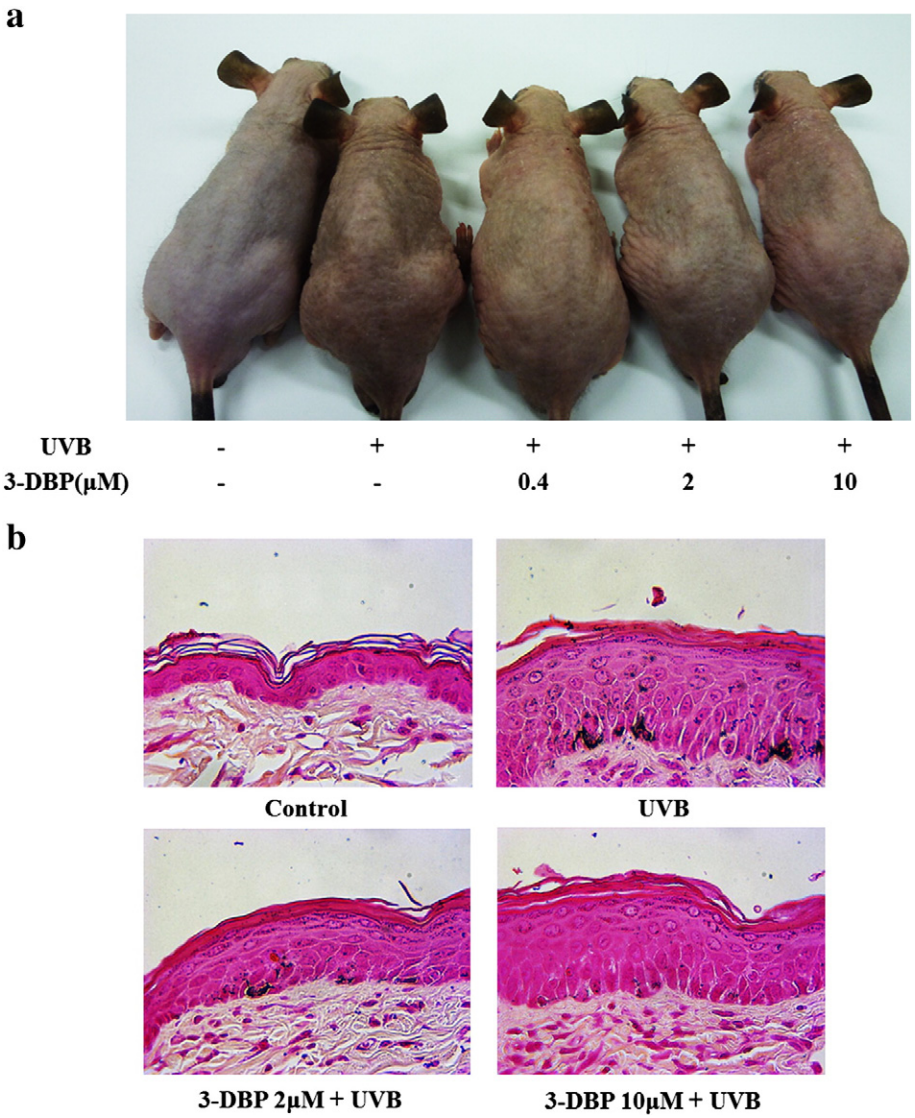


Fig. 5. Effects of 3-DBP on pigmentation in animal skins exposed to UVB. (a) The photos indicate pigmentation differences between the dorsal skins of tested animals. UVB-irradiated vehicle-treated mice (day 16) tanned markedly in contrast to control mice that were not irradiated. Changes were observable with the naked eye or with a normal camera. As shown in the photos, 3-DBP-treated mice showed a significant increase in lightness compared to vehicle-treated mice. Optical data were coincident with numerical data. (b) Fontana–Masson staining of dorsal skin sections from the mice shown in (a) reveals differences in melanin content.

dione into one compound (Fig. 1a) and evaluated its inhibitory activity against tyrosinase and melanin production.

Our recent experiments to synthesize and evaluate new compounds with potent inhibitory effects on tyrosinase revealed several possibilities for developing new types of tyrosinase inhibitors [24,32,33]. These studies, involving a computational docking system between tyrosinase and other compounds, showed efficacy for developing new and potent tyrosinase inhibitors. Although these studies clearly demonstrated the anti-melanogenic effect of newly synthesized compounds in melanoma cells, important questions remained regarding its *in vivo* efficacy.

Through continuous design and synthesis of potent tyrosinase inhibitors, the novel 3-DBP was found to be an effective tyrosinase inhibitor by using *in vitro* and *in vivo* models. Although it was difficult to demonstrate docking of 3-DBP with tyrosinase due to its non-competitive inhibitory mechanism, significant efficacy was demonstrated *in vitro*. The IC_{50} value of 3-DBP *in vitro* was 16-fold lower than that of kojic acid, and the molecule showed no adverse effects on cell viability.

An HRM2 melanin-possessing hairless mouse model was used as an *in vivo* model. Although many other melanogenesis inhibitors identified through *in vitro* studies failed to show *in vivo* efficacy, likely due to the inability to enter the stratum corneum barrier [34], 3-DBP showed significant efficacy in modulating UVB-induced *in vivo* melanogenesis (Fig. 4). We are currently conducting studies to examine how 3-DBP works as a non-competitive inhibitor by using a computational docking system and to demonstrate the mechanism of 3-DBP efficacy by using an *in vivo* model.

Recent studies have focused on the mechanism of tyrosinase inhibition in different pathways [35]. A suitable compound may be more potent if it contains 2 mechanisms for reducing melanogenesis, including direct tyrosinase activity inhibition and the reduction of tyrosinase expression. However, it may be safer to target a minimum number of molecules. The inhibitory effect of 3-DBP on melanogenesis is attributed to the direct inhibition of tyrosinase activity rather than suppression of tyrosinase gene expression.

In summary, we identified 3-DBP as a novel anti-melanogenic agent in both *in vitro* and *in vivo*. The compound appears to decrease

melanogenesis stimulated by α-MSH through non-competitive inhibition of tyrosinase *in vitro*. 3-DBP also showed great efficacy against UVB-induced melanogenesis in an animal model.

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References

[1] Q.X. Chen, I. Kubo, Kinetics of mushroom tyrosinase inhibition by quercetin, J. Agric. Food Chem. 50 (2002) 4108–4112.
[2] K. Maeda, M. Fukuda, Arbutin: mechanism of its depigmenting action in human melanocyte culture, J. Pharmacol. Exp. Ther. 276 (1996) 765–769.
[3] K. Nihei, I. Kubo, Identification of oxidation product of arbutin in mushroom tyrosinase assay system, Bioorg. Med. Chem. Lett. 13 (2003) 2409–2412.
[4] R. Matsuura, H. Ukeda, M. Sawamura, Tyrosinase inhibitory activity of citrus essential oil, J. Agric. Food Chem. 54 (2006) 2309–2313.
[5] L. Maeda, K. Yoshizaki, Intestinal term pregnancy without rupture, Nippon Sanka Fujinka Gakkai Zasshi 43 (1991) 361–363.
[6] S.Y. Seo, V.K. Sharma, N. Sharma, Mushroom tyrosinase: recent prospects, J. Agric. Food Chem. 51 (2003) 2837–2853.
[7] K. Tsuji-Naito, T. Hatani, T. Okada, T. Tehara, Modulating effects of a novel skin-lightening agent, alpha-lipoic acid derivative, on melanin production by the formation of DOPA conjugate products, Bioorg. Med. Chem. 15 (2007) 1967–1975.
[8] A. Slominski, D.J. Tobin, S. Shibahara, J. Worteman, Melanin pigmentation in mammalian skin and its hormonal regulation, Physiol. Rev. 84 (2004) 1155–1228.
[9] C.R. Goding, Melanocytes: the new Black, Int. J. Biochem. Cell Biol. 39 (2007) 275–279.
[10] A. Garcia, J.E. Fulton Jr., The combination of glycolic acid and hydroxyquinone or kojic acid for the treatment of melasma and related conditions, Dermatol. Surg. 22 (1996) 443–447.
[11] K. Kameyama, C. Sakai, S. Kondoh, K. Yonemoto, S. Nishiyama, M. Tagawa, T. Murata, T. Ohnuma, J. Quigley, A. Dorsky, D. Bucks, K. Blanock, Inhibitory effect of magnesium L-ascorbyl-2-phosphate (VC-PMG) on melanogenesis *in vitro* and *in vivo*, J. Am. Acad. Dermatol. 34 (1996) 29–33.
[12] J.F. Hermanns, L. Petit, C. Pierard-Franchimont, P. Paquet, G.E. Pierard, Assessment of topical hypopigmenting agents on solar lentigines of Asian women, Dermatology 204 (2002) 281–286.
[13] K. Yoshimura, K. Tsukamoto, M. Okazaki, V.M. Virador, T.C. Lei, Y. Suzuki, G. Uchida, Y. Kitano, K. Harii, Effects of all-*trans* retinoic acid on melanogenesis in pigmented skin equivalents and monolayer culture of melanocytes, J. Dermatol. Sci. 27 (2001) 68–75.
[14] M. Nakajima, I. Shinoda, Y. Fukuwatari, H. Hayasawa, Arbutin increases the pigmentation of cultured human melanocytes through mechanisms other than the induction of tyrosinase activity, Pigment Cell Res. 11 (1998) 12–17.
[15] M. Paul, R. Jennifer, Radiative relaxation quantum yields for synthetic eumelanin, Photochem. Photobiol. 79 (2007) 211–216.
[16] C.J. Cooksey, P.J. Garratt, E.J. Land, S. Pavel, C.A. Ramsden, P.A. Riley, N.P. Smit, Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase, J. Biol. Chem. 272 (1997) 29226–29235.
[17] I. Kubo, I. Kinst-Hori, S.K. Chaudhuri, Y. Kubo, Y. Sanchez, T. Ogura, Flavonols from *Heterotheca inuloides*: tyrosinase inhibitory activity and structural criteria, Bioorg. Med. Chem. 8 (2000) 1749–1755.
[18] T.B. Fitzpatrick, A.B. Lerner, Biochemical basis of human melanin pigmentation, AMA Arch Derm. Syphilol. 69 (1954) 133–149.
[19] J.M. Wood, K.U. Schallreuter, Studies on the reaction between human tyrosinase, superoxide anion, hydrogen peroxide and thiols, Biochim. Biophys. Acta 1074 (1991) 378–385.
[20] C. Olivares, J.C. Garcia-Borron, F. Solano, Identification of active site residues involved in metal cofactor binding and stereospecific substrate recognition in mammalian tyrosinase, Biochemistry 41 (2002) 679–686.
[21] V.J. Hearing, M. Jimenez, Analysis of mammalian pigmentation at the molecular level, Pigment Cell Res. 2 (1989) 75–85.
[22] M. Fairhead, L. Thony-Meyer, Bacterial tyrosinase: old enzymes with new relevance to biotechnology, N. Biotechnol. 29 (2012) 183–191.
[23] S. Briganti, E. Camera, M. Picardo, Chemical and instrumental approaches to treat hyperpigmentation, Pigment Cell Res. 16 (2003) 101–110.
[24] Y.M. Ha, Y.J. Park, J.Y. Lee, D. Park, Y.J. Choi, E.K. Lee, J.M. Kim, J.A. Kim, J.Y. Park, H.J. Lee, H.R. Moon, H.Y. Chung, Design, synthesis and biological evaluation of 2-(substituted phenyl)thiazolidine-4-carboxylic acid derivatives as novel tyrosinase inhibitors, Biochimie 94 (2012) 533–540.
[25] J.M. Kim, H.S. Heo, Y.M. Ha, B.H. Ye, E.K. Lee, Y.J. Choi, B.P. Yu, H.Y. Chung, Mechanism of AngII involvement in activation of NF-κB through phosphorylation of p54 during aging, Age (Dordr.) 34 (2012) 11–25.
[26] V.J. Hearing, K. Tsukamoto, Enzymatic control of pigmentation in mammals, FASEB J. 5 (1991) 2902–2909.
[27] J.K. No, D.Y. Soung, Y.J. Kim, K.H. Shim, Y.S. Jun, S.H. Rhee, T. Yokozawa, H.Y. Chung, Inhibition of tyrosinase by green tea components, Life Sci. 65 (1999) 241–246.
[28] G.A. Burdock, M.G. Soni, I.G. Carabin, Evaluation of health aspects of kojic acid in food, Regul. Toxicol. Pharmacol. 33 (2001) 80–101.
[29] P.G. Josh, N. Nair, G. Begum, Melanocyte–keratinocyte interaction induces calcium signaling and melanin transfer to keratinocytes, Pigment Cell Res. 20 (2007) 380–384.
[30] Y. Yamazaki, Y. Kawano, A. Yamanaka, S. Maruyama, N-[(Dihydroxyphenyl)acyl] serotonins as potent inhibitors from mouse and human melanoma cells, Bioorg. Med. Chem. Lett. 19 (2009) 4178–4182.
[31] L.T. Ng, H.H. Ko, T.M. Lu, Potential antioxidants and tyrosinase inhibitors from synthetic polyphenolic deoxybenzoins, Bioorg. Med. Chem. 17 (2009) 4360–4366.
[32] Y.M. Ha, J.A. Kim, Y.J. Park, D. Park, J.M. Kim, K.W. Chung, E.K. Lee, Y.J. Park, J.Y. Lee, H.J. Lee, H.Y. Moon, H.Y. Chung, Analogs of 5-(substituted benzylidene) hydantoin as inhibitors of tyrosinase and melanin formation, Biochim. Biophys. Acta 6 (2011) 612–619.
[33] Y.M. Ha, J.Y. Park, Y.J. Park, D. Park, Y.J. Choi, J.M. Kim, E.K. Lee, Y.K. Han, J.A. Kim, J.Y. Lee, H.R. Moon, H.Y. Chung, Synthesis and biological activity of hydroxy substituted phenyl-benzo[d]thiazole analogues for antityrosinase activity in B16 cells, Bioorg. Med. Chem. Lett. 8 (2011) 2445–2449.
[34] F. Solano, S. Briganti, M. Picardo, Hypopigmenting agents: an updated review on biological and clinical aspects, Pigment Cell Res. 19 (2006) 550–571.
[35] T.S. Chang, An updated review of tyrosinase inhibitors, Int. J. Mol. Sci. 10 (2009) 2440–2475.