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Modulating effects of a novel skin-lightening agent, α-lipoic acid derivative, on melanin production by the formation of DOPA conjugate products

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Abstract—Sodium zinc dihydrolipoylhistidinate (DHLHZn) is a compound of Zn^{2+}/dihydrolipoic acid derivate complex, which was developed for cosmetic/medical use. To characterize DHLHZn as a novel skin-lightening agent, inhibitory actions of DHLHZn on tyrosinase (including its reaction pathway) have been elucidated in this study. In a B16 melanoma cell system, DHLHZn was active in suppressing the synthesis of melanins as well as α-arbutin, well known as a depigmenting drug. Furthermore, in a tyrosinase assay, DHLHZn showed stronger inhibitory effect on DOPAchrome formation than other tyrosinase inhibitors such as kojic acid. Our previous report demonstrated that the sulfhydryl groups of lipoyl motif react with DOPAquinone to form lipoyl DOPA conjugates. We therefore postulated that conjugated products between DHLHZn and DOPAquinone might be formed. Upon reaction of DHLHZn with L-DOPA following tyrosinase-catalyzed oxidation, the formation of DHLH DOPA conjugated products was confirmed by HPLC-tandem mass spectrometry using reserpine as the internal standard. In addition, the inhibitory kinetics analyzed by a Lineweaver–Burk plot exhibited the reversibility of DHLHZn as a competitive inhibitor with a K_I value of 0.35 μM. These results indicate that this covalent reaction might contribute to alternating DOPAquinone, which is a tyrosinase reaction product, and result in the competitive inhibitory effect of DHLHZn on DOPAchrome formation. DHLHZn may thus serve as a potentially effective skin-lightening agent, an effectiveness that is based on the compound's covalent scavenging of DOPAquinone resulting in depigmentation.

1. Introduction

Visibly pigmented skin results from the synthesis and distribution of melanins.[1] Recently, to achieve effective control of melanin synthesis for the treatment of facial hyperpigmentation, several investigators have extensively studied the lightening of pigmented skin.[2–5] In human, melanins are predominantly synthesized within melanocytes and are classified into two general types: black and/or brown eumelanin, formed by the polymerization of dihydroxyindole precursors;[6] red and/or yellow pheomelanin, which is colored by the incorporated polymerization of cysteine conjugated precursor. Most natural melanins are known to be mixtures or copolymers of these two types.[7,8]

Melanin synthesis is mainly regulated by tyrosinase, which takes part in the initial and rate-limiting reactions (tyrosine hydroxylation to L-3,4-dihydroxyphenylalanine [L-DOPA], and L-DOPA oxidation to DOPAquinone) common to both eu- and pheomelanogenesis.[1,9] The subsequent steps after the formation of DOPAquinone are responsible for switching between these two types of melanins. The progression of these steps, including spontaneous chemical reactions, depends on the ratio of sulfhydryl compounds such as cysteine and/or glutathione (GSH) within melanocytes: in the absence of cysteine and/or GSH, DOPAquinone is oxidized to form DOPAchrome as the intermediate product of eumelanin, which results in the advance of eumelanogenesis; in the presence of these compounds, DOPAquinone is coupled with their SH groups to form cysteinyl DOPA as a precursor of sulfur-containing pigment known as pheomelanin, which corresponds to the progress of pheomelanogenesis. Thus, in addition to

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tyrosinase, thiols that capture DOPAquinone are thought to be essential for melanogenesis.

Many strategies for developing skin-lightening agents are formulated based on down-regulating melanin synthesis by inhibiting the activity of tyrosinase, including its transcription.[3,4,10–13] On the other hand, the concept behind several depigmentation agents has been based on the conjugative reactions between sulfhydryl compounds and DOPAquinone.[14] However, little chemical information is available, including data on the structure of the relevant conjugated products. We previously reported that sulfhydryl groups of dihydrolipoic acid (DHLA), the reduced form of α-lipoic acid (LA), reacted with DOPAquinone to form lipoyl DOPA conjugate products, and showed its potent depigmenting effect as an inhibitor of DOPAchrome formation.[15] However, a defect of DHLA, in terms of cosmetic/medical applications, was observed in its ease of oxidization in the presence of air and its insolubility in aqueous condition. To overcome this defect, sodium zinc dihydrolipoylhistidinate (DHLHZn) was developed;[16,17] the resultant Zn^{2+} coordination yields stability against the formation of oxides by *S*-thiolation, and the binding with histidine increases aqueous solubility (Fig. 1). In this study, we characterize the skin-lightening effects of DHLHZn. We show that DHLHZn retains the reactivity of the sulfhydryl groups toward DOPAquinone and also significantly inhibits melanin synthesis as well as α-arbutin, a known depigmentation agent.[4] Our results indicate that DHLHZn may be a strong candidate for use in skin-lightening agents.

2. Results

2.1. DHLHZn suppresses melanin biosynthesis in murine melanoma B-16 as well as well-known depigmenting agent, α-arbutin

DHLHZn is a compound of Zn^{2+}/DHLA derivate complex (Fig. 1), which was developed for cosmetic/medical use.[16,17] To assess the depigmenting activity of DHLHZn against melanin synthesis, we measured the levels of melanins in B16 melanoma cells cultured with DHLHZn for 3 days, and compared the results with α-arbutin and DHLA. Treatment with DHLHZn at 75 μM for 3 days showed a strong inhibitory effect with 41.5% inhibition (Fig. 2a). In this density, the remarkable toxicity of DHLHZn was not observed. DHLA and α-arbutin at the same concentration showed almost the same inhibitory effect, at 36.3% and 38.3%, respectively. This result indicates that DHLHZn suppresses the synthesis of melanins as effectively as α-arbutin, a well-known depigmenting drug.[4]

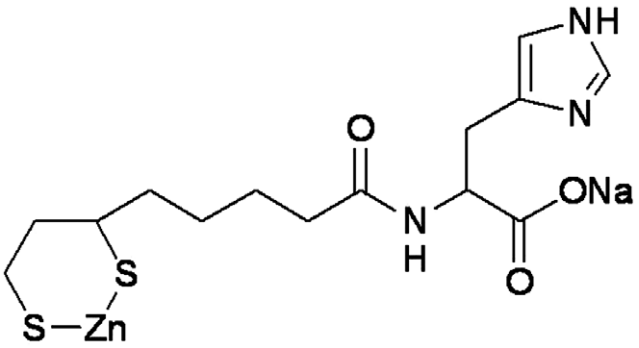


Figure 1. Structure of sodium zinc dihydrolipoylhistidinate (DHLHZn).

2.2. DHLHZn specifically inhibits DOPAchrome formation upon tyrosinase-catalyzed reaction

DHLA, but not LA, has been reported to effectively inhibit the formation of melanin precursors such as DOPAchrome and cysteinyl DOPA.[15,18] Therefore, we evaluated the inhibitory activity of DHLHZn on DOPAchrome formation via the tyrosinase-catalyzed oxidation of L-DOPA. As shown in Figure 2b, DHLHZn and DHLA inhibited DOPAchrome formation to a similar extent. In comparison to kojic acid and α-arbutin, well-known tyrosinase inhibitors,[4,19,20] both DHLA and DHLHZn seem to act as more specific inhibitors of tyrosinase-catalyzed reactions.

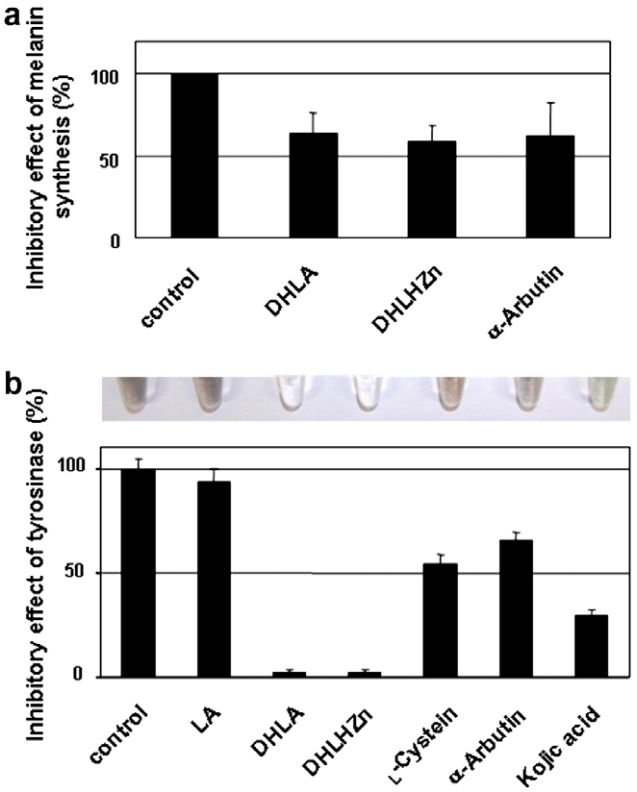


Figure 2. (a) Effects of DHLHZn on melanin synthesis. The amount of total melanins is represented as a percentage of the control. B16 melanoma cells were cultured with or without skin-lightening agents (DHLA, DHLHZn, and α-arbutin, respectively; 75 μM). Values are expressed as means ± SD of four independent experiments. Inhibitory effects of DHLAZn on DOPAchrome formation. (b) L-DOPA (2.5 mM) was mixed with or without each agent (LA, DHLA, DHLHZn, cysteine, α-arbutin, or kojic acid; 120 μM) and then incubated with melanocyte extracts as enzyme sources of the cell-free tyrosinase for 1 h at 37 °C. Values are represented as inhibition (%).

2.3. DHLHZn covalently reacts with DOPAquinone to form DHLH DOPA conjugates

In our previous report, we demonstrated that the thiol groups of DHLA covalently react with DOPAquinone to form lipoyl DOPA conjugated products.[15] Based on this evidences, we expected that DHLHZn might also react with DOPAquinone following tyrosinase-catalyzed oxidation to form the conjugated products between DHLHZn and DOPAquinone.

To validate our prediction, we next performed the reaction of DHLHZn with L-DOPA in the presence of mushroom tyrosinase and then estimated the amount of DHLH DOPA conjugates formed. Thus, the reaction mixture was analyzed by HPLC with UV detection and electrospray-mass spectrometry in positive ion mode (ESI^+-MS). The three molecular ion peaks of the expected monoconjugate product, named peak I, II (a shoulder peak of peak III), and III, were detected at *m/z* 541; the substances coeluted at the time characteristic of the major reaction products (Fig. 3a and b). These peaks were not detected when incubating DHLHZn or L-DOPA alone followed by tyrosinase-catalyzed oxidation (data not shown). Moreover, spectrographic analysis showed peaks at 256 and 291 nm, in agreement with the previously defined characteristic of DHLA DOPA conjugated products (supplementary data). These results indicate that these three products are isomers arising through the addition of the sulfhydryl groups of DHLHZn to the 5, 2, and 6 positions of the DOPAquinone, since three similar product peaks were also detected in DHLA DOPA conjugated reaction.[15]

Among these expected monoconjugate peaks, peak III was prominent enough to isolate as a mixture including peak II by HPLC. The fraction of this peak area was collected and processed without further purification (see Section 4). The structure was confirmed by a collision-induced MS/MS experiment (Fig. 3c). The formulas of the fragment ions were assigned on the basis of the fragment patterns of DHLHZn and DHLA by collision-induced tandem mass spectrometry (MS/MS) experiment; the fragment patterns in the figure are speculative but reasonable assignments. The fragments of *m/z* 155.8 and 110.0 confirm the presence of a histidine moiety, since these fragments overlapped with those of DHLHZn, but not of DHLA.

To further confirm the formation of DHLH DOPA (the conjugates in the reaction of DHLHZn), DHLHZn was incubated with L-DOPA in the presence of B16 cell

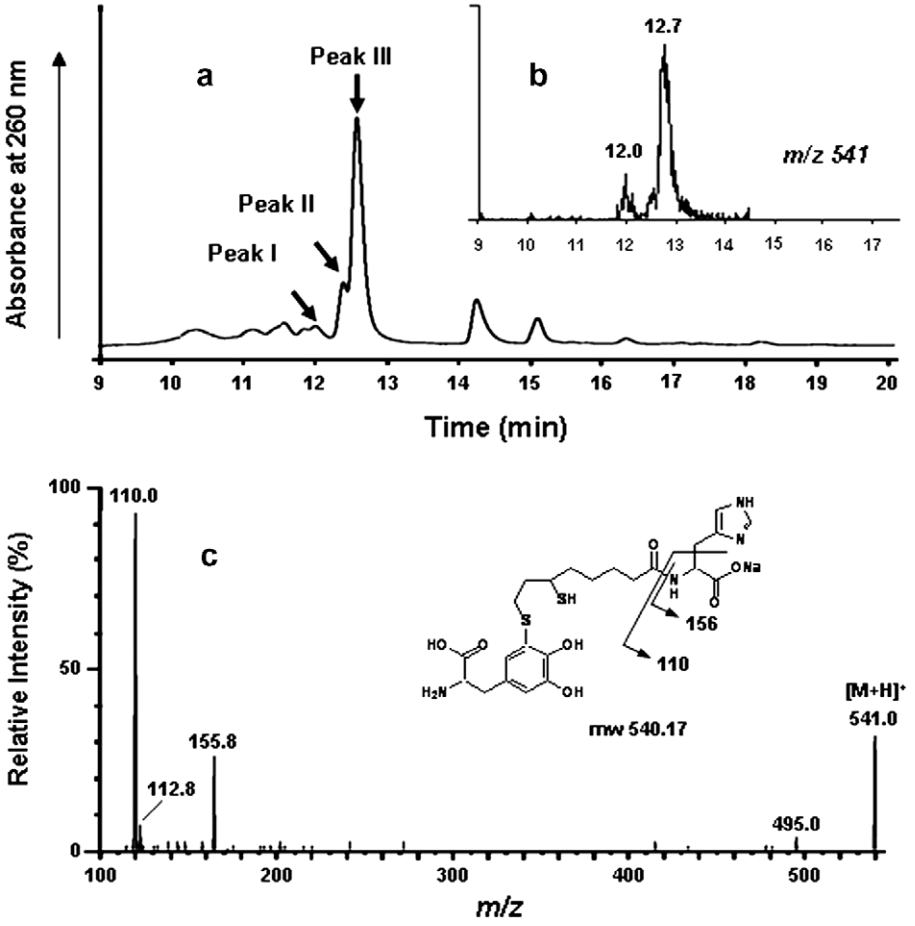


Figure 3. (a) HPLC analysis of adducts from the incubation of DHLHZn with L-DOPA following tyrosinase-catalyzed oxidation. (b) HPLC/ESI^+-MS analysis of the reaction mixture under the same chromatographic conditions. The overlapping chromatographic peak at 12.7 min, representing the elution of DHLH DOPA conjugate product of *m/z* 541. (c) Tandem mass spectrum of DHLH DOPA conjugate product of *m/z* 541.

extracts, and the formation of the conjugated products was investigated. After the reaction, the mixture was applied to the HPLC/MS/MS experiment (*m/z* 541.0 $[M+H]^+ \rightarrow$ 155.8 and 110.0). The retention times of the resultant ions were in total agreement with those of mushroom tyrosinase-catalyzed reaction, suggesting that DHLH DOPA conjugates were formed upon reaction of DHLHZn with B16 cell-extract-treated DOPA as well as mushroom-tyrosinase-treated DOPA (Fig. 4a–c). These results show that the sulfhydryl groups of DHLHZn also react with DOPAquinone in a sulfhydryl-compound-specific fashion.

2.4. The formation of DHLH DOPA conjugates proceeds at an extremely rapid rate, but their yields decay at late phase

To clarify the formation of DHLH DOPA conjugates in a cell-free system, the reaction mixture of DHLHZn with B16 cell-extract-treated DOPA was analyzed by a HPLC/MS/MS quantitative system using reserpine quantitative behavior of (*m/z* 609.0 $[M+H]^+ \rightarrow$ 195.0) as the internal standard (Fig. 4d). The formation of DHLH DOPA conjugates was almost completed in a very short time period of 10 min (Fig. 5a). The maximum yield of those peaks was attained at about 4 h, but decreased at a late phase and was almost undetectable 24 h later. In addition, we detected these new peaks from reaction in the equal lower density consisting of DOPA and DHLHZn clearly (supplementary data). This quantitative validation was common to all three conjugated products.

Figure 5b shows the time course of the inhibitory effect of DHLHZn on DOPAchrome formation. The inhibitory effect of DHLHZn was active until 24 h of incubation, but decreased 48 h after that. These results indicate that the quantitative variance of DHLH DOPA

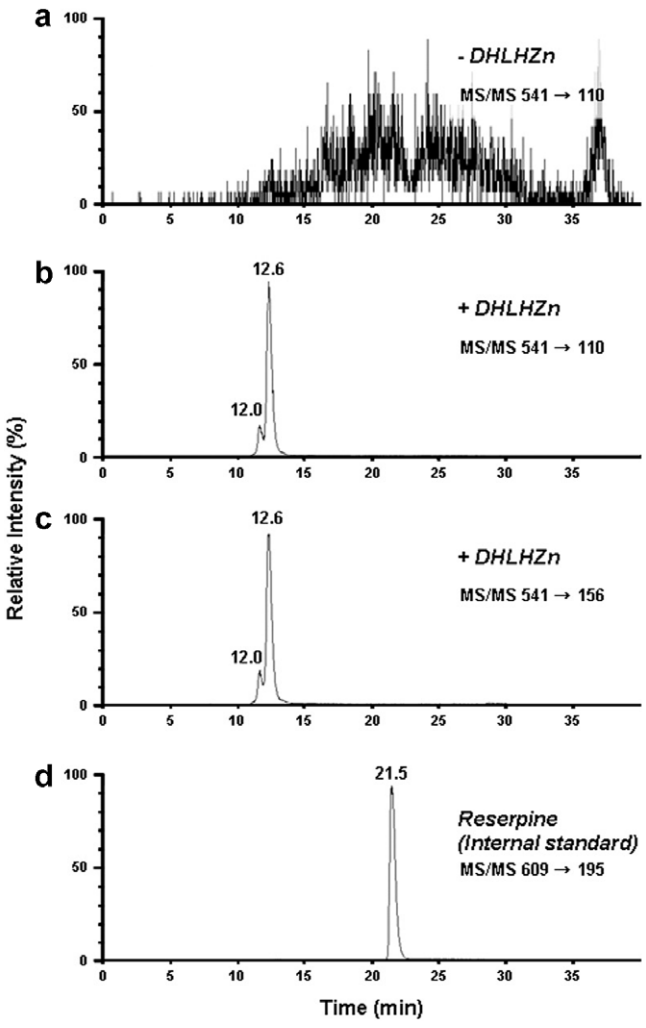


Figure 4. HPLC/MS/MS profile for reaction of L-DOPA without (a) or with DHLHZn (b and c) in the presence of melanocyte extracts as enzyme sources of the cell-free tyrosinase at 37 °C. The reaction mixtures were monitored in MRM mode for *m/z* = 541 → 110 and 156 (DHLH DOPA conjugates) and *m/z* = 609 → 195 (d; reserpine) using a semi-micro column (2.0 × 50 mm).

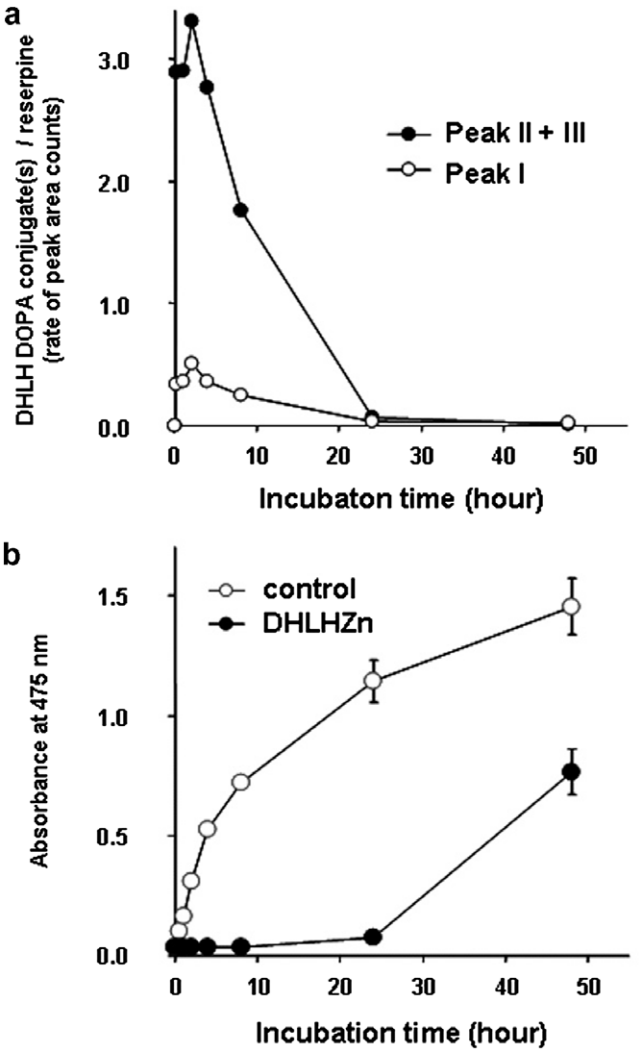


Figure 5. (a) Formation of DHLH DOPA conjugates (-○-, peak I; -●-, peak II and III) by tyrosinase-catalyzed oxidation of L-DOPA in the presence of DHLHZn. (b) Time course experiment results of effects of DHLHZn (-○-) and vehicle (-●-) on DOPAchrome formation are exhibited in a temporal pattern. L-DOPA (2.5 mM) was mixed with or without DHLHZn (120 μM) and then incubated with melanocyte extracts as enzyme sources of the cell-free tyrosinase for 1 h at 37 °C. Values are means ± SE from three separate experiments.

conjugate formation correlated with a decline in the inhibitory effect on DOPAchrome formation, suggesting that these conjugated products might not be stable end products but rather contribute to production of a pigment ingredient following further chain reactions. On the other hand, cysteine is a well-known sulfhydryl compound that forms cysteinyl DOPAs, but its inhibitory effect at the same concentration is exerted for a period of only 30 min (data not shown).

2.5. DHLHZn shows a competitive inhibition toward tyrosinase-catalyzed reaction without inactivating tyrosinase

To determine the inhibitory mode of DHLHZn, inhibitory effects of DHLHZn n mushroom tyrosinase activity have been evaluated. Effects on the mushroom tyrosinase activity were 82.9% inhibition at 4.0 μM and an IC_{50} value of 1.3 μM by DHLHZn (Fig. 6). A kinetic study of L-DOPA oxidation catalyzed by mushroom tyrosinase was accomplished in the presence of DHLHZn. DHLHZn increased the K_m value of mushroom tyrosinase activity but did not change the V_{max} value. The V_{max} value (ΔA_{492}/min) of mushroom tyrosinase activity was 1.3×10^{-4}, and the K_m value was 0.86 mM L-DOPA. Mushroom tyrosinase activity in the presence of DHLHZn at 1.3 μM exhibited the same V_{max} value of 1.3×10^{-4} and K_m value of 4.1×10^{-3}, respectively. Therefore, DHLHZn inhibited DOPAchrome formation following tyrosinase-catalyzed reaction competitively without the inactivation of tyrosinase. This suggests that the formation of conjugated products between DHLHZn and DOPAquinone, which is a tyrosinase reaction product, might result in the competitive inhibitory effect of DHLHZn.

3. Discussion and conclusions

In the current study, we demonstrated that DHLHZn, which was developed for cosmetic/medical use,[16,17] takes part in a skin-lightening effect to modulate melanin synthesis. DHLHZn suppressed melanin biosynthesis in murine melanoma B-16 as well as well-known depigmenting agent, α-arbutin.[4] In addition, DHLHZn showed a strong inhibitory effect on L-DOPA oxidation by tyrosinases from mushroom and murine melanoma B-16, suggesting that DHLHZn is a more specific inhibitor of tyrosinase activity than the other depigmenting agents.

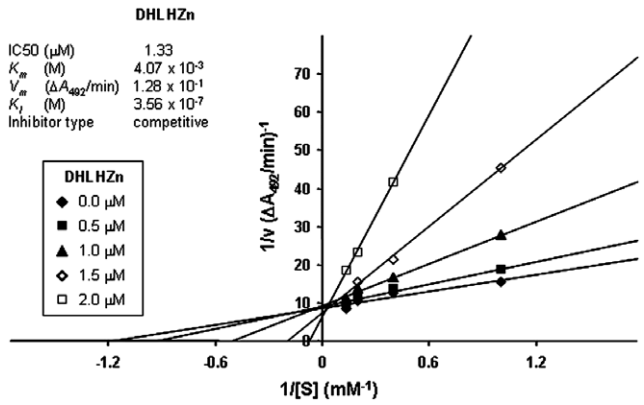
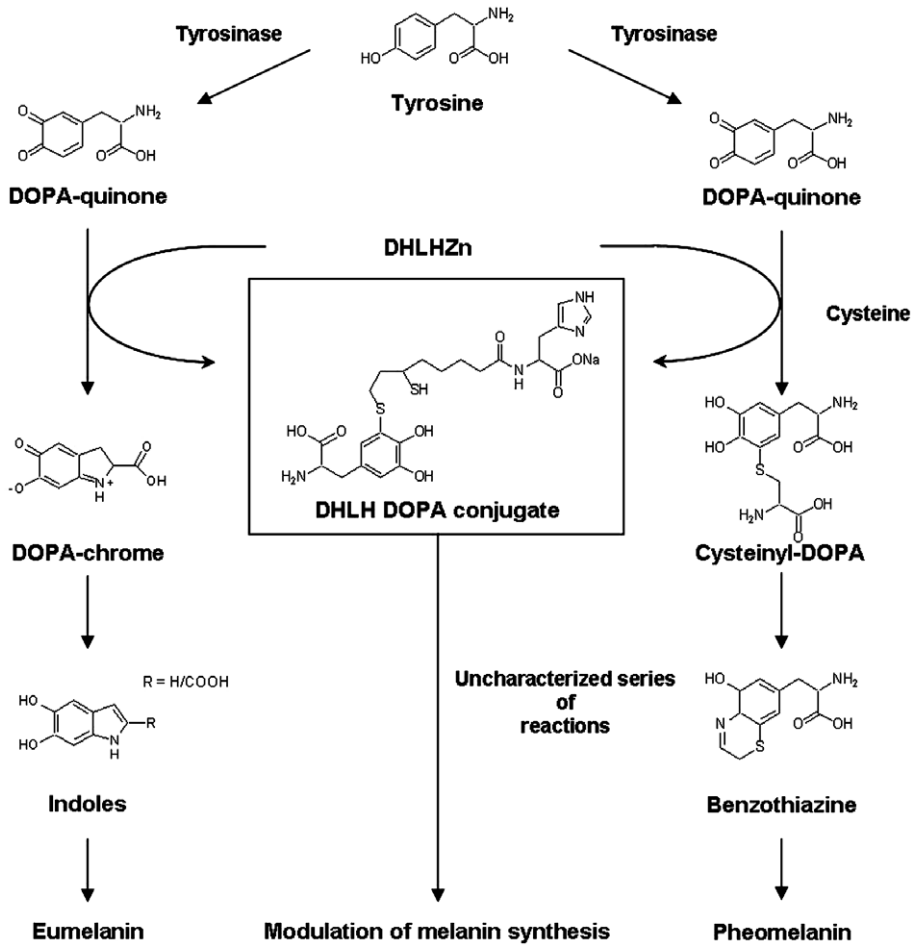


Figure 6. Lineweaver–Burk plot for the inhibition of the mushroom tyrosinase by DHLHZn. Data were obtained as mean values of $1/V$, the inverse of the increase of absorbance at a wavelength 92 nm per min ($\Delta A492$/min), of three independent tests with different concentrations of L-DOPA as a substrate. The reaction was performed in the presence of DHLHZn [final concentration of 0, 0.5, 1.0, 1.5 and 2.0 μM].

The regulation mechanism of DHLHZn is based on the formation of DHLH DOPA conjugates, which is formed upon reaction of the sulfhydryl groups of DHLHZn with DOPAquinone (Scheme 1). Sulfhydryl compounds, such as cysteine and GSH, are known to combine with DOPAquinone in a reaction that proceeds much faster than the other important steps in the early phase of melanogenesis.[21–25] In the presence of cysteine, DOPAquinone following tyrosinase-catalyzed oxidation is preferentially consumed by the generation of cysteinyl DOPAs, as these reactions are regulated by the intrinsic chemical reactivity of DOPAquinone. Our kinetics study of mushroom tyrosinase shows that the inhibitory effect of DHLHZn on DOPAchrome formation should not result from the inactivation of tyrosinase, and that DHLHZn inhibits tyrosinase activity in a competitive manner. In general, a competitive inhibitor is regarded as a substance that combines with a free enzyme in a manner that prevents substrate binding. However, DHLHZn including DHLA seems not to be a nonmetabolizable analogue or derivative of L-DOPA. Therefore, we suggest that the reaction pathway of DOPAquinone, which is a tyrosinase-reaction product, might switch to form DHLH DOPA conjugates as an alternative product to DOPAchrome. In brief, the formation of DHLH DOPA conjugates would compete with DOPAchrome formation.

The presence of sulfhydryl compounds in the pigment cell leads to modulating eumelanogenesis and an increased ratio of pheomalanin/eumalanin due to its quenching of DOPAquinone.[23–25] Our chemical proof may also provide information about the chemistry of pheomelanogenesis, since little evidence was previously supported from a chemical viewpoint, with the exception of the analysis of cysteine and GSH. Although DHLH DOPA conjugates were formed very rapidly in a tyrosinase-catalyzed reaction, their peaks completely disappeared at a final stage (Fig. 5). These results indicate that these conjugated products might not be stable end products, but contribute to the production of a pigment ingredient following further chain reactions. The results also raise the possibility that DHLH DOPA conjugates might have further reactions that promote pigment formation, including pheomelanin and/or trichochrome. In addition, DHLHZn effectively inhibited DOPAchrome formation for a longer period than cysteine (data not shown). This prominent difference between DHLHZn and cysteine might be attributed to the difference in behavior between each DOPA conjugated product in the stages of polymerization. We speculate that subsequent polymerization of DHLH DOPA conjugates might progress more slowly than that of



Scheme 1. Proposed mechanism of melanogenesis in melanocytes showing the formation of DHLHZn products.

indoles or cysteinyl DOPAs. These results indicate that the formation of DHLH DOPA conjugates might contribute to delaying the progress of cyclization and/or polymerization, which in turn depresses melanin synthesis. These results are also in accordance with observations by Jara et al.[23]

DHLHZn overcomes two drawbacks of DHLA by both forming a stable six-membered ring by chelation of Zn^{2+} and by binding with histidine as an aqueous soluble product (Fig. 1), and is further a highly stable molecule under physiologic conditions (data not shown). Our previous report mentioned the potential for DHLA to be used as a depigmenting agent,[15] but the material difficulties involved (considering that DHLA is easily oxidized and insoluble in aqueous conditions) have prevented such application. Furthermore, DHLHZn suppressed both DOPAchrome formation and melanin synthesis as strongly as DHLA (Fig. 2), although we initially expected that the chelation of Zn^{2+} might affect its inhibitory effect. Our chemical studies using HPLC/MS/MS system also indicate that the thiol groups of DHLHZn might have a higher affinity for DOPAquinone than a Zn^{2+} ion, and that DHLHZn yields stability against *S*-thiolation without loss of its inhibitory effect on DOPAchrome formation. Accordingly, the Zn^{2+} addition toward a DHLA derivate would provide stability without losing its essential skin-lightening effect.

Several recent reports have showed that the ability to chelate copper at the active site of tyrosinase and quench reactive oxygen species (ROS) might also contribute to inhibiting tyrosinase-catalyzed reactions;[19,20,26,27] kojic acid is known as a good chelator of copper, and vitamin B_6 compounds quench singlet oxygen well. Both of these compounds have been shown to inhibit tyrosinase-catalyzed reactions.[19,20,27] DHLA is known to show chelation on copper and also to quench ROS as well;[28,29] therefore, DHLHZn may also have the mechanism employed by either or both of the abilities. To understand the potential of DHLHZn as a depigmenting agent in detail, we must also evaluate the focal point of the structure–activity relationships between tyrosinase and DHLHZn including DHLA.

In the current study, we demonstrated that DHLHZn engages in a skin-lightening effect to modulate melanin synthesis without sacrificing the inhibitory activities of DHLA toward melanin biosynthesis and DOPAchrome formation. The regulation mechanism of DHLHZn is based on the formation of DHLH DOPA conjugates, which are formed upon reaction of the sulfhydryl groups of DHLHZn with DOPAquinone (Scheme 1). DHLHZn has a dual advantage for cosmetic/medical applications: aqueous solubility and stability against oxidation via *S*-thiolation. The results in this study raise

the possibility that DHLHZn may serve as a useful agent for the treatment of hyperpigmentation.

4. Materials and methods

4.1. Materials

L-Cysteine, L-DOPA, DHLA, kojic acid, and tyrosinase from mushroom (EC 1.14.18.1) were purchased from Sigma Chemical Corp. (St. Lous, MO). Reserpine was purchased from Wako Pure Chemicals Industries (Osaka, Japan). LA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). α-Arbutin was purchased from Ezaki Glico Co. Ltd (Osaka, Japan). DHLHZn (*N*-(6,8-dimercaptooctanoyl)-L-histidine zinc complex sodium salt) was obtained from Iwaki Co., Ltd (Tokyo, Japan). All other chemicals were of the highest commercially available grade.

4.2. Cell culture

Mouse melanoma B16F10 cells (Cell Resource Center for Biomedical Research, Tohoku University) were maintained as monolayer cultures in Eagle's minimal essential medium (Sigma Chemical Corp.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

4.3. Measurement of melanin content

B16F10 cells were treated with test substance for 3 days. Cells were collected by trypsinization and washed twice with phosphate-buffered saline (PBS). To determine spectrophotometrically the amount of total melanins, cell pellets were washed 3 times with 5% trichloroacetic acid, ethanol–ether (3/1; v/v), and ether, respectively. The dried precipitates were solubilized with 1 N NaOH/10% dimethylsulfoxide by heating at 80 °C for 30 min and then monitored at 475 nm using a Perkin-Elmer Japan Corp., Ltd (Kanagawa, Japan) 1420 ARVO series multilabel counter.

4.4. Tyrosinase assay

Tyrosinase activity was evaluated as DOPA oxidase activity using a method slightly modified from that previously described.[30] Briefly, approximately 2.5×10^6 cells were pelleted and then washed twice with PBS. After centrifugation, the supernatant was discarded. The precipitated cells were dissolved in 1.0 ml of phosphate buffer (pH 6.8) containing 0.1% *t*-octylphenoxypolyethoxyethanol and protease inhibitor cocktail, then sonicated (TOMY Sonicator UD201; Tomy, Tokyo, Japan; 3 × 10-s burst; output power = 2) and centrifuged at 11,000*g* for 10 min. After centrifugation, supernatants were used as tyrosinase sources. A reaction mixture consisting of 200 μl of L-DOPA (2.5 mM) and each agent (120 μM as final concentration) in a 0.1 M phosphate buffer (pH 6.8) was mixed with 200 μl of the cell lysate. Assays were performed at 37 °C. The absorbance was then monitored at 475 nm by a 1420 ARVO series multilabel counter. Corrections for autooxidation of L-DOPA in controls were made. For analytical experiments, the reaction mixtures were further analyzed using HPLC/MS/MS as described below.

4.5. Isolation and analysis of the DHLH DOPA conjugate

For isolation of the DHLH DOPA conjugate, DHLHZn (5 mM), L-DOPA (5 mM), and mushroom tyrosinase (25 U) were dissolved in 5 ml of 10 mM phosphate buffer (pH 6.5). The reaction mixture was incubated for 2 h at 37 °C. After incubation, the crude mixture was purified using HPLC method I as described below. The solution eluted under the chromatographic peak corresponding to the product was collected and immediately frozen at −80 °C (dry ice bath). The resulting eluents were freeze-dried to give a solid, dry product. To obtain the tandem mass spectrum, the purified product was further analyzed using HPLC/MS/MS in direct injection mode as described below.

In an analytical experiment, DHLHZn (120 μM), L-DOPA (2.5 mM), and mushroom tyrosinase (2.5 U) were dissolved in 10 mM phosphate buffer (pH 6.5). The reaction mixture was incubated for 1 h at 37 °C. The reaction mixtures were analyzed using HPLC-method II and HPLC/MS, as described below.

4.6. HPLC

HPLC was performed employing a JASCO Corp. gradient system equipped with dual Model PU-2089 plus pumps (10 ml pump heads), a Rheodyne (Cotati, CA) Model 7725i equipped with a 5.0 ml sample loop, and a JASCO Corp. Model MD-2010 plus multi-UV detector. Two mobile phase solvents were employed. Solvent A was prepared by adding concentrated acetic acid (0.01%) to deionized water. Solvent B was prepared by adding acetic acid (0.01%) to HPLC grade acetonitrile. Under Method I for isolation, HPLC was performed employing a reverse phase column (Develosil C_{18}, 5 μm, 8.0 × 250 mm, Nomura Chemical Corp., Ltd, Aichi, Japan) and the following mobile gradient: 0–30 min, linear gradient from 100% solvent A to 75% solvent B; 30–40 min, linear gradient to 100% solvent B; 40–45 min, 100% solvent B. The flow rate was constant at 2.0 ml min^{-1}. As the analytical method, HPLC method II was performed employing a reverse phase column (Develosil C_{18}, 3 μm, 2.0 × 50 mm, Nomura Chemical Corp., Ltd) and the following mobile gradient: 0–30 min, linear gradient from 100% solvent A to 75% solvent B; 30–40 min, linear gradient to 100% solvent B. The flow rate was constant at 200 μl min^{-1}.

4.7. HPLC/MS/MS

HPLC/MS/MS analyses were performed on an Applied Biosystems (Forster, CA) API 2000 triple-quadrupole mass spectrometer equipped with an ESI source operating in positive ion mode. The heated capillary of the ESI source was kept at 450 °C. Nitrogen was used as the sheath, nebulization, and auxiliary gas, and maintained

at 25, 30, and 40 arbitrary units, respectively. The spray voltage was 5.5 kV.

The samples (10 μl), including 500 nM of the internal standard reserpine,[31] were analyzed by on-line HPLC/MS (*m*/*z* 541) and HPLC/MS/MS (*m*/*z* 110 and 156, as product ion of *m*/*z* 541; *m*/*z* 195, as product ion of *m*/*z* 609 $[\text{reserpine} + \text{H}]^+$). Online HPLC separations were conducted using Agilent Technologies, Inc. (Palo Alto, CA) model 1100 HPLC series, including a binary gradient pump, a vacuum degasser, an auto sampler, and a diode array detector, and carried out as described above under analytical HPLC conditions. HPLC/MS was used in Q1 positive ion scan mode, and HPLC/MS/MS was used in multiple reaction monitoring (MRM) positive ion scan mode. The column used was a Develosil C_{18}, 3 μm, 2.0 × 50 mm, using a flow rate of 200 μl min^{-1}. To prevent contamination in mass spectrometry, a switching valve was used, and then the elution for a period between 8.0 and 30 min was introduced into the spectrometer.

Direct injection of the HPLC-purified adduct was performed by introducing 30 μl of sample dissolved in 30% acetonitrile in 0.01% acetic acid into the mass spectrometer in order to obtain the daughter ion spectra for the adduct. The MS/MS collision energy was 67 V.

4.8. Kinetic analysis

L-DOPA oxidation by tyrosinase was spectrophotometrically determined as described previously with minor modifications.[32] The reaction mixture consisted of four different concentrations of L-DOPA (0.5–2 mM) as a substrate and mushroom tyrosinase in a 100 mM sodium phosphate buffer. DHLHZn of several concentrations was added to the reaction mixture, respectively. The initial rate of DOPAchrome formation from the reaction mixture was determined as the increase of absorbance at wavelength 492 nm per min (ΔA_{492}/min) by using a 420 ARVO series multilabel counter. Michaelis constant (K_m) and maximal velocity (V_{max}) of mushroom tyrosinase were determined by Lineweaver–Burk plots. The values were calculated using the following equations: $1/V = K_m/V_{max}\ (1 + [I]/K_I)\ \ 1/[S] + 1/V_{max}$. Inhibition constants ($K_i$) of the competitive inhibitors were calculated by the following equation: $K_{mapp} = K_m [1 + ([I]/K_I)]$ where K_{mapp} is the apparent K_m in the presence of DHLHZn concentration.

4.9. Statistics

Effects on tyrosinase by test samples were represented as a control % of ((sample ΔA_{492}/min)/(control ΔA_{492}/min)) × 100. Statistical analyses were performed by ANOVA with Dunnett's multiple comparison of means test.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.12.042.

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