Synthesis, Characterization, and Evaluation of Thiazolidine Derivatives of Cysteine for Suppressing Eumelanin Production

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In the pathway of melanin biosynthesis, cysteine (Cys) is utilized for the synthesis of pheomelanin. Accordingly, Cys is considered to suppress the formation of brown–black eumelanin. Although attempts have been made to utilize Cys and its derivatives as skin-whitening agents, their instability and odor hinders their application as a cosmetic agent. Herein, N-acetyl-2-methylthiazolidine-2,4-dicarboxylic acid ethyl ester (AcCp2Et) was proposed as a candidate for a stable and prolonged-release derivative of Cys to inhibit dopachrome formation after its degradation in melanocytes. It was synthesized by acetylation of 2-methylthiazolidine-2,4-dicarboxylic acid 2-ethyl ester (Cp2Et), the condensation derivative of Cys and ethyl pyruvate. AcCp2Et suppressed melanogenesis in melanocytes in vitro, was stable in phosphate buffer at 70°C for five days, and exhibited far less odor than Cp2Et. Therefore, AcCp2Et was validated to be a useful derivative of Cys for application as a skin-whitening agent. AcCp2Et comprises four stereoisomers; thus characterization of each stereoisomer was required. The stereochemistry of AcCp2Et was confirmed via a single-crystal X-ray structure analysis of N-acetyl-2-methylthiazolidine-2,4-dicarboxylic acid (AcCp) derived from AcCp2Et. In the synthesis of AcCp2Et, the acetylation of Cp2Et proceeded with epimerization at C4 to give trans-isomers when excess acetyl chloride and an organic amine was used, whereas it proceeded while retaining the original (R) configuration at C4 to give the cis- and trans-isomer when an equivalent of acetyl chloride with an inorganic base was used. These results indicate that the formation of an intermolecular mixed acid anhydride is responsible for the isomerization at the C4 asymmetric center.

Key words thiazolidine derivative; cysteine; suppressing eumelanin production; acetylation; stereoisomer

Melanin plays an important role in determining hair, eye, and skin color. It also protects the skin from UV radiation and inhibits photocarcinogenesis. However, hyperpigmentation, which is caused by melanin overproduction and accumulation, causes freckles, age spots, melasma, and melanoma. Medical cosmetics that contain skin-whitening components are used for treating hyperpigmentation. The inhibitory effect of conventional pharmaceutical skin-whitening agents such as hydroquinone, arbutin, and kojic acid on melanogenesis is attributed to the inhibition of tyrosinase. Therefore, skin-whitening agents that have a mechanism distinct from tyrosinase inhibition should be useful.

Melanin is produced in human pigment cells (melanocytes) in the skin utilizing L-tyrosine (Tyr) and L-cysteine (Cys). The melanin biosynthesis pathway comprises two main parts: the production of eumelanin and the production of pheomelanin. During eumelanogenesis, Tyr is converted to 3,4-dihydroxyphenylalanine (DOPA) by tyrosinase, which then further catalyzes the conversion of DOPA to dopaquinone. Dopaquinone is converted to 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole intermediates, eventually resulting in the formation of brown–black eumelanin. Dopaquinone also reacts with glutathione (GSH) or Cys to form glutathionyl-dopa (GSH-dopa) or cysteinyldopa, and eventually forms yellow–red pheomelanin. When Tyr, a starting material for eumelanin, is utilized for the synthesis of melanin, production of eumelanin is promoted and the skin color turns darker. Conversely, when Cys is utilized for the synthesis of pheomelanin, production of eumelanin is suppressed and the skin color turns closer to yellow. Therefore, it is considered that production of eumelanin is suppressed by supplying Cys during melanin synthesis.

Accordingly, attempts have been made to utilize thiol-containing compounds such as Cys and N-acetyl-cysteine (NAC) as cosmetics, especially as skin-whitening agents. However, Cys and NAC are easily oxidized and have problems, including poor stability and/or unpleasant odor; this makes them unsuitable for formulation as cosmetic agents or external skin preparations. To solve these problems, the development of a Cys derivative with improved stability is required. Several 2-monosubstituted thiazolidine-4-carboxylic acids, which are cyclic Cys derivatives with a masked sulfhydryl group, have been used as protective agents to liberate Cys non-enzymatically at physiological pH and temperature. Several 2-substituted phenylthiazolidine-4-carboxylic acid derivatives have been investigated as agents for inhibition of melanogenesis or as tyrosinase inhibitors. However, some of these compounds have the disadvantage of liberating toxic aldehyde upon degradation.

As an example of the application of 2,2-disubstituted derivatives (Chart 1), Wlodek and Romselspacher investigated the effect of L-2-methyl-thiazolidine-2,4-dicarboxylic acid (abbreviated as CP (I), i.e., cysteyl pyruvic acid), a condensation product of the biological substances Cys and pyruvic acid, on paracetamol-induced hepatotoxicity as a non-toxic precursor of Cys and GSH. Takizawa et al. reported that CP (I) is...
stable compared to conventional Cys derivatives, and may be used as an external skin preparation or a cosmetic.\textsuperscript{19} Suzuki \textit{et al.} reported that \textit{L}-2-methylthiazolidine-2,4-dicarboxylic acid 2-ethyl ester (abbreviated as CP2Et, \textit{i.e.}, cysteiny1 pyruvic acid 2-ethyl ester), a product of Cys and ethyl pyruvate condensation, is useful as a skin-whitening agent since it has a suppressive effect upon eumelanin production.\textsuperscript{20} However, these compounds exist in equilibrium with Cys and pyruvic acid 2-ethyl ester, respectively, through a ring-opening mechanism involving Schiff-base intermediates. Selective epimerization at C4 takes place when \textit{N}-acetylated derivatives are treated with acetic anhydride at 100°C via a mixed acid anhydride type intermediate.\textsuperscript{21} Conversely, Cremonesi \textit{et al.} reported that the acetylation of \textit{L}-2-methylthiazolidine-2,4-dicarboxylic acid 4-methyl ester with acetyl chloride in the presence of trimethylamine (Et\textsubscript{3}N) in dichloromethane afforded \textit{N}-acetyl-\textit{L}-3-acetyl-1,3-thiazolidine-4(\textit{R})-carboxylic acid, \textit{R}=ethyl (AcCP2Et) or 2,4-epimers of \textit{L}-2-methylthiazolidine-2,4-dicarboxylic acid 4-ethyl ester with acetyl chloride in the presence of trimethylamine (Et\textsubscript{3}N) in dichloromethane afforded \textit{N}-acetyl-\textit{L}-3-acetyl-1,3-thiazolidine-4(\textit{R})-carboxylic acid, \textit{R}=ethyl (AcCP2Et) as \textit{N}-acetylated derivatives are treated with acetic anhydride at 100°C via a mixed acid anhydride type intermediate.\textsuperscript{21} Conversely, Cremonesi \textit{et al.} reported that the acetylation of \textit{L}-thiazolidine-2,4-dicarboxylic acid 4-methyl ester with acetyl chloride in the presence of trimethylamine (Et\textsubscript{3}N) in dichloromethane afforded \textit{N}-acetyl-\textit{L}-3-acetyl-1,3-thiazolidine-2,4-dicarboxylic acid 4-methyl ester with retention of configuration at C4 because their substrate could not generate an acid anhydride intermediate involving the 4-carboxyl group.\textsuperscript{24}

Conversely, little information is available about the stereoselectivity of \textit{CP} derivatives in \textit{N}-acetylation, \textit{i.e.}, 2,2-disubstituted thiazolidine-4-carboxylic acid. The CP derivative also comprises four stereoisomers. In addition, it has a tetra-substituted asymmetric carbon center at C2. Schubert obtained AcCP (3) and its acid anhydride by the reaction of \textit{CP} (1) and acetic anhydride/pyridine. However, the stereochemistry of the reaction product was not disclosed.\textsuperscript{25}

In this report, we present a detailed study of the stereo-specificity in the \textit{N}-acetylation of \textit{CP} (1) and CP2Et (2), and explore the stability of AcCP2Et (4) and its suppression of eumelanin production \textit{in vitro}.

**Results and Discussion**

The synthesis of \textit{N}-acetyl-2-methylthiazolidine-2,4-dicarboxylic acid (AcCP) derivatives is outlined in Chart 1. Derivatives of \textit{2-methyl-thiazolidine-2,4-dicarboxylic acid (CP; 1a, b, CP2Et; 2a, b) were prepared by condensation of pyruvic acid or ethyl pyruvate with \textit{L}-(\textit{R})-Cys. When these compounds are reacted in ethanol or water at room temperature (r.t.), a

| Chart 1. Synthesis of \textit{N}-Acetyl-2-methyl-2,4-thiazolidinedicarboxylic Acid and Its 2-Ethyl Ester |
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| **1** | 2-Methyl-2,4-thiazolidinedicarboxylic acid (cysteinyl pyruvic acid, CP); **2** | 2-Methyl-2,4-thiazolidinedicarboxylic acid 2-ethyl ester (cysteinyl pyruvic acid 2-ethyl ester, CP2Et); **3** | \textit{N}-Acetyl-2-methyl-2,4-thiazolidinedicarboxylic acid (AcCP); **4** | \textit{N}-Acetyl-2-methyl-2,4-thiazolidinedicarboxylic acid 2-ethyl ester (AcCP2Et); **5** | \textit{N}-Acetyl-2-methyl-2,4-thiazolidinedicarboxylic acid anhydride (AcCP anhydride). |
| Synthesis of \textit{N}-Acetyl-2-methyl-2,4-thiazolidinedicarboxylic Acid and Its 2-Ethyl Ester |
| 1a: \textit{R}=H, \textit{R}’=H’ |
| 1b: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=H’ |
| 1c: \textit{R}=H’, \textit{R}’=\textit{CH}_3\text{CH}_2 |
| 1d: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=\textit{CH}_3\text{CH}_2 |
| 2a: \textit{R}=H, \textit{R}’=H’ |
| 2b: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=H’ |
| 2c: \textit{R}=H’, \textit{R}’=\textit{CH}_3\text{CH}_2 |
| 2d: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=\textit{CH}_3\text{CH}_2 |
| 3: \textit{R}=H, \textit{R}’=H’ |
| 4: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=H’ |
| 5a: \textit{R}=H, \textit{R}’=H’ |
| 5b: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=H’ |
| 5c: \textit{R}=H’, \textit{R}’=\textit{CH}_3\text{CH}_2 |
| 5d: \textit{R}=\textit{CH}_3\text{CH}_2, \textit{R}’=\textit{CH}_3\text{CH}_2 |
spontaneous condensation–cyclization reaction leads to the formation of a mixture of the two diastereoisomers of the CP derivatives (1a, b or 2a, b) in a ratio of ca. 1:1, as determined by 1H-NMR spectroscopy. These derivatives were converted to N-acetyl-2-methylthiazolidine-2,4-dicarboxylic acid derivatives (AcCP: 3, AcCP2Et: 4) under various reaction conditions as shown in Table 1. Since AcCP2Et (4) exists as four stereoisomers due to its two asymmetric centers at C2 and C4, characterization of each stereoisomer is complicated. Therefore, their stereochemistry was assigned on the basis of their analytical and spectral data. A reversed-phase HPLC (RP-HPLC) analysis and a chiral HPLC analysis were performed to evaluate the stereoisomer ratios of AcCP2Et (4a–d), and typical chromatograms are shown in Supplementary Fig. S1.

Acetylation of 2-Methylthiazolidine-2,4-dicarboxylic Acid (CP: 1a, b) with Acetic Anhydride  Entry 1 in Table 1 shows the reaction of a ca. 1:1 mixture of CP (1a, b) and acetic anhydride. When CP (1a, b) and acetic anhydride was heated at 100°C according to Schubert’s method,25 a dark brown homogeneous solution formed in 2h. The acid anhydride of AcCP (5) was found to be stable to silica gel column chromatography and isolated in modest yield. Acidic hydrolysis of 5 gave a single stereoisomer, i.e., the cis-isomer of AcCP (3), as indicated by 1H-NMR spectroscopy. In addition, this compound proved to be a mixture of cis-enantiomers of AcCP (3a, e) based on chiral HPLC analysis and the single crystal X-ray structure analysis described below (Fig. 1A). As reported by Szilágyi and Györgydeák,21 the N-acetylation of cis-(2S,4R)-CP (1a) proceeded with retention of configuration at C2 and C4 to give intramolecular acid anhydride 5a, whereas N-acetylation of trans-(2S,4R)-CP (1b) proceeded with epimerization at C4 via intermolecular mixed acid anhydride such as 6b and c followed by formation of intramolecular acid anhydride 5c as shown in (A) in Chart 2.

Acetylation of 2-Methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl Ester (CP2Et: 2a, b) with Acetyl Chloride and Organic Amine  Entry 2 in Table 1 shows the reaction of a ca. 1:1 mixture of CP2Et (2a, b) and ethyl pyruvate, with acetyl chloride and Et3N. Contrary to CP (1a, b), CP2Et (2a, b) is highly soluble in ethyl acetate (AcOEt), and was acetylated by acetyl chloride in the presence of Et3N at r.t. When 1.5 equiv. of acetyl chloride and 2.0 equiv. of Et3N were used, acetylation completed to give AcCP2Et (4) as a single stereoisomer, as indicated by 1H-NMR spectroscopy, in satisfactory isolated yield. However, the existence of two stereoisomers was indicated by chiral HPLC. Similar results were obtained when other organic amines such as pyridine, N-methylmorpholine, or diisopropylethylamine were used instead of Et3N.

Then, AcCP2Et (4) was subjected to alkaline saponification...
acetate also gave an enantiomeric mixture of mixed acid anhydrides (7a,4b). The trans relative stereochemistry of the obtained AcCP (3) was estimated by comparing its 1H-NMR signals with those of cis-enantiomers of AcCP (3a, c). In addition, the single crystal X-ray structure analysis described below revealed that this compound is a mixture of enantiomers of trans-isomers, i.e., trans-(2S,4R)-AcCP (3b) and trans-(2R,4S)-AcCP (3d) (Fig. 1B). It is concluded that the trans-enantiomers of AcCP2Et (4b, d) were obtained under these acetylation conditions. Therefore, it is proposed that the configuration at C4 of cis-(2R,4R)-AcCP (4a) is inverted to give the thermodynamically stable trans-(2R,4S)-AcCP2Et (4d) via an intermolecular mixed acid anhydrides (7a, d) (Chart 2B).

In addition, acetylation of CP2Et (2a, b) with more than 2 equiv acetic anhydride without amine in refluxing ethyl acetate also gave an enantiomeric mixture of trans-enantiomers of AcCP2Et (4b, d) (Table 1, entry 3). The presence of their anhydrides with acetic acid such as 7d (Chart 2B) were confirmed by 1H-NMR spectroscopy of the crude reaction mixture. This observation strongly suggests the involvement of a mixed acid anhydride in the epimerization. Such a mechanism is consistent with that proposed in a study by Szilágyi and Györgydeák, in which selective epimerization at C4 of a mixed acid anhydride in the epimerization. Such a mechanism is consistent with that proposed in a study by Szilágyi and Györgydeák, in which selective epimerization at C4 of thiazolidine-4-carboxylic acid (Fig. 1B) was induced using CP (1a, b, c, d, e, f, g, h). The stereo-selectivity of N-acetylation with a long-chain fatty acid chloride yielded surfactant-like prodrugs such as N-hexadecanoyl derivatives (11 and 12). The stereo-selectivity of N-acetylation with long-chain fatty acid chloride is similar to that of N-acetylation with acetyl chloride, and depends on the amount of acylation reagent and the kind of base used.

Acetylation of 2-Methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl Ester (CP2Et: 2a, b) with Acetyl Chloride and Inorganic Base Entry 4 in Table 1 shows the reaction of a ca. 1:1 mixture of CP2Et (2a, b) with acetyl chloride in the presence of potassium carbonate (K2CO3) in ethyl acetate at r.t. Under these reaction conditions, more than 1.2 equiv. of acetyl chloride and 1.2 equiv. of K2CO3 were required to complete the reaction in satisfactory yield. Tetrahydrofuran (THF) or CH2Cl2 were found to be suitable solvents for this reaction, and potassium hydrogen carbonate, sodium carbonate, and sodium hydrogen carbonate were assessed as the inorganic base. Following these reaction conditions, a ca. 1:1 mixture of the cis- and trans-isomers of AcCP2Et (4) was indicated by 1H-NMR. Chiral HPLC analysis revealed that they were the cis-(2R,4R)-AcCP2Et (4a) and trans-(2S,4R)-AcCP2Et (4b). Therefore, it was concluded that the formation of a mixed acid anhydride and the epimerization at C4 are avoided under these conditions using an inorganic base as in the case of the 4-methyl ester derivative reported by Cremonesi et al.,24 and that N-acetylation proceeded with retention of the C4 configuration. Similarly, another set of stereoisomers of CP2Et (2c, d) obtained by the reaction of D-(S)-Cys and ethyl pyruvate gave a mixture of cis-(2S,4S)- and trans-(2R,4S)-AcCP2Et (4e, d) as shown in entry 5 in Table 1.

Hydrolysis of cis-(2R,4R)- and trans-(2S,4R)-AcCP2Et (4a, b) under alkaline conditions at 60°C for 16 h gave cis-(2R,4R)- and trans-(2S,4R)-AcCP (3a, b). In addition, formation of ca. 5% of the trans-(2R,4S)-AcCP (3d) was observed (Table 1, entry 4). Therefore, the C4 asymmetric center of cis-(2R,4R)-AcCP2Et (4a) and/or cis-(2R,4S)-AcCP (3a) are liable to isomerize under basic conditions to generate the stable trans-(2R,4S)-AcCP (3d).

Synthesis of Various Derivatives of 2-Methylthiazolidine-2,4-dicarboxylic Acid Chart 3 shows examples of the derivatives of 2-methylthiazolidine-2,4-dicarboxylic acid produced using CP (1a, b, c, d, e, f, g, h) and 2-methylthiazolidine-2,4-dicarboxylic acid 2-methyl ester (CP2Me, h). These derivatives were expected to possess different lipophilicities depending on the introduced functional groups. Reaction of CP2Et (2a, b) with a long-chain fatty acid chloride yielded surfactant-like prodrugs such as N-hexadecanoyl derivatives (11 and 12). The stereo-selectivity of N-acetylation with long-chain fatty acid chloride is similar to that of N-acetylation with acetyl chloride, and depends on the amount of acylation reagent and the kind of base used. Esterification of the 2-carboxylic acid group in thiazolidine-2,4-dicarboxylic acid 2-methyl ester (CP2Mt, h). These derivatives were expected to possess different lipophilicities depending on the introduced functional groups. Reaction of CP2Et (2a, b) with a long-chain fatty acid chloride yielded surfactant-like prodrugs such as N-hexadecanoyl derivatives (11 and 12).
(3a, c) (Table 1, entry 1) and the mixture of trans-enantiomers of AcCP (3b, d) (Table 1, entry 3) were determined by single crystal X-ray structure analysis. A summary of the crystallographic analyses is shown in Supplementary Table S1.

Figure 1A illustrates the crystal packing in the racemic crystal of cis-(2R,4R)- and cis-(2S,4S)-AcCP (3a, c) as an ORTEP drawing. This crystal belongs to the space group P-1 and its unit cell contains one molecule of cis-(2R,4R)-AcCP (3a), one molecule of cis-(2S,4S)-AcCP (3c), and two water molecules. The cis-enantiomers of AcCP (3a, c) molecule forms an intramolecular hydrogen bond between oxygen atoms O2 and O3, and adopts a relatively globular conformation. The hydrogen bonding network between a water molecule and oxygen atoms O1, O4, and O5 of neighboring cis-enantiomers of AcCP (3a, c) molecules plays an important role in the crystal packing.

Figure 1B demonstrates the crystal packing in the racemic crystal of trans-(2S,4R)- and trans-(2R,4S)-AcCP (3b, d) as an ORTEP drawing. This crystal belongs to the space group P21/c and its unit cell contains two molecules of trans-(2S,4R)-AcCP (3b) and two molecules of trans-(2R,4S)-AcCP (3d). Contrary to the cis-enantiomers of AcCP (3a, c) structure, the trans-enantiomers of AcCP (3b, d) molecule adopts a relatively extended conformation. Two kinds of intermolecular hydrogen bond, i.e., the hydrogen bond between O1 atom and O2 atom of neighboring enantiomer, and the hydrogen bond between O3 atom and O5 atom of neighboring enantiomer, play an important role in the crystal packing.

Suppression of Eumelanin Production Figure 2 shows the cell viabilities and the melanogenesis inhibition activity of the examined compounds.

We treated B16 melanoma cells with L-Cys, CP2Et (2a, b), and trans-enantiomers of AcCP2Et (4b, d) (Table 1, entry 2) to determine whether the compounds have cytotoxic effects. Cell viabilities were determined using Neutral Red (NR) assays. The results show that Cys is not cytotoxic at 2 mM, and that CP2Et (2a, b) and AcCP2Et (4b, d) are not cytotoxic at concentrations of 2, 4, 8, and 10 mM to B16 melanoma over 3 d.

B16 melanoma cells were incubated with various concentrations of kojic acid (a positive control), Cys, CP2Et (2a, b), and AcCP2Et (4b, d) under the same conditions for 3 d, and the melanin contents were measured to determine the melanogenesis inhibition of the test molecules. Cys, CP2Et (2a, b), and AcCP2Et (4b, d) were found to reduce the melanin content in a concentration-dependent manner with IC50 values of 1108, 3120, and 4544 µM, respectively. The activity of AcCP2Et (4b, d) is estimated to be approximately one fourth of that of Cys under these experimental conditions. As the stability of the compounds increases, their inhibition activity decreases. Cys is considered to be a key compound in the control of melanogenesis, as it regulates tyrosinase activity as well as the ability of l-dopaquinone to generate eumelanin or pheomelanin.7,14 We believe that the Cys liberated by degradation of CP2Et (2a, b) or AcCP2Et (4b, d) is responsible for the melanogenesis inhibition observed. In the case of CP2Et (2a, b), it is thought that Cys released by extracellular non-enzymatic degradation is taken up into cells. Conversely, it is expected that AcCP2Et (4b, d) is first taken up into the cells; Cys is then liberated inside the cells by enzymatic cleavage of its amide bond and/or ester bond and followed by non-enzymatic ring opening. Since AcCP2Et (4b, d) is more active than trans-enantiomers of AcCP (3b, d) (data not shown), AcCP2Et (4b, d) may be more easily taken up into cells, which may be responsible for its higher activity relative to AcCP (3b, d). These data suggest that AcCP2Et (4b, d) may have the potential as a stable derivative of Cys for skin whitening. Research on the detection of Cys released from AcCP2Et (4b, d) in a skin model incorporating melanocytes is currently under investigation in our group, and the results will be published in due course.

Stability of N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl Ester (AcCP2Et, 4a, c) As mentioned above, alkaline hydrolysis of the ethyl ester group at the quaternary C2 in AcCP2Et (4) is slow. Therefore, it was assumed that AcCP2Et (4) would be stable in neutral or weakly acidic conditions. The stability of a ca. 1 : 1 mixture (20 mg/20 mL) of the cis-(2R,4R)- and trans-(2S,4R)-isomers of AcCP2Et (4a, b) (Table 1, entry 4) over a pH range of 4 to 7 was investigated, and the results are shown in Table 2. No degradation was observed at 70°C after 5 d for the AcCP2Et (4b) at pH 5 to 7 or for the AcCP2Et (4a) at pH 7. Three to 7% degradation was observed for the AcCP2Et (4b) at pH 4 and for the AcCP2Et
A considerable degree of hydrolysis of the ethyl ester functionality in the AcCP2Et (4a) was observed at pH 4. Therefore, the AcCP2Et (4b) is quite stable to prolonged storage at pH 5 to 7. It is noteworthy that the degradation products are cis-(2R,4R)- and trans-(2S,4R)-isomers of AcCP (3a, b), which is also expected to function as a prodrug of Cys. Stability to enzymatic hydrolysis is currently under investigation in our group, and the results will be reported in due course.

TABLE 1. Time-Course Stability Test of AcCP2Et (4a, b) at 70°C

| pH | Residual values (%) |
|----|---------------------|
|    | 4a (cis-Isomer)     | 4b (trans-Isomer) |
| 4  | 69.6                | 95.8               |
| 5  | 93.5                | 99.8               |
| 6  | 96.9                | >99.9              |
| 7  | >99.9               | >99.9              |

The residual values (%) after five days are shown. Sample concentration: 20 mg sample in 20 mL of 25 mM phosphate buffer.

Odor Test Aqueous solutions (1%) at pH 4 and 7 of trans-enantiomers of AcCP2Et (4b, d) (Table 1, entry 2) and CP2Et (2a, b) in sealed vials were kept in a thermostatic bath at 70°C for 6 d. The odor of each sample was evaluated by six panelists, and the evaluation scores of the six panelists were totaled (see Experimental). CP2Et (2a, b) is in equilibrium with Cys and ethyl pyruvate in aqueous solution via non-enzymatic ring opening and hydrolysis; therefore odor originating for Cys may be detected. Indeed, a strong sulfur odor was detected in both samples of CP2Et (2a, b), while a slight sulfur odor was detected in AcCP2Et (4b, d) at pH 4 and almost no odor was detected at pH 7. It was concluded that AcCP2Et (4b, d) shows less sulfur odor due to decomposition, and exhibits superior preservation stability to CP2Et (2a, b) on storage. Therefore, AcCP2Et is a useful compound with potential as a cosmetic ingredient.

Conclusion AcCP2Et (4) was designed as a stable and prolonged-release derivative of Cys for the suppression of eumelanin production, and was synthesized by acetylation of CP2Et (2a, b), a simple condensation product of Cys and ethyl pyruvate. trans-Enantiomers of AcCP2Et (4b, d) exhibited eumelanin suppression in vitro, indicating that AcCP2Et (4b, d) is cleaved enzymatically followed by non-enzymatic ring opening to liberate the parent Cys. AcCP2Et (4b, d) was highly stable in 25 mM phosphate buffer at pH 4 to 7 at 70°C for 5 d. In addition, AcCP2Et (4b, d) exhibited far less odor than the original CP2Et (2a, b). These results indicate that AcCP2Et (4b, d) may have the potential as a stable derivative of Cys for skin-whitening.

AcCP2Et (4) comprises four stereoisomers because of its two asymmetric centers at C2 and C4, making it necessary to characterize each stereoisomer obtained by acetylation. N-Acetylation of cis-(2R,4R)-isomer of CP2Et (2a) proceeded with epimerization at C4 via an intermolecular mixed acid anhydride such as 7a and d (Chart 2B), producing stable trans-(2R,4S)-AcCP2Et (4d) (Table 1, entry 2), when large excess acetyl chloride and an organic amine was used, while it proceeded with retention of the (R) configuration at C4 when an equivalent of acetyl chloride and an inorganic base was used, to produce cis-(2R,4R)-isomers of AcCP2Et (4a) (Table 1, entry 4). HPLC analysis of AcCP2Et (4) stereoisomers, and single crystal X-ray structure analysis of trans-enantiomers...
of AcCP (3b, d) derived from trans-enantiomers of AcCP2Et (4b, d), confirmed that N-acetylation by both methods leaves the original configuration at C2 unchanged. These results suggest that the formation of an intermolecular mixed acid anhydride is responsible for the isomerization at the C4 asymmetric center.

Experimental

1H-NMR spectra were recorded using a Brucker Avance 400 (400 MHz) and electron spray ionization (ESI)-MS spectra were measured using a Thermo Quest TSQ 700. The melting point (mp) of the obtained crystal was measured using an IA9100 digital melting point measuring apparatus manufactured by Electrothermal (U.K.)

**RP-HPLC Conditions** Column: Inertsil ODS-3 (high-pressure type, GL Sciences, Tokyo, Japan, particle size 3 µm, inner diameter 4.6 mm, length 250 mm). Detector: UV absorption spectrophotometer (measurement wavelength: 210 nm).

**Chiral HPLC Conditions** Column: CHIRALPACK ON-AQ (0.46 cm × 15 cm). Buffer: MeOH–AcOH–AcONH4 = 98: 2: 0.5 (v/v/v). Flow rate: 1.0 mL/min. Temperature: 40°C. Detection: UV 250 nm. Sample concentration: 1% in MeOH. Injection: 10 µL. Retention time of stereoisomers of N-AcCP2Et (4): 4a, c (cis-isomers): 30.5 min; 4b, d (trans-isomers): 32.4 min.

**2-Methylthiazolidine-2,4-dicarboxylic Acid (CP, 1a, b)** Under an argon atmosphere, l-Cys (15.0 g, 124 mmol) was dissolved in dry ethanol (35 mL), pyruvic acid (18.6 mL) was added at r.t., and the mixture was stirred for 3 h. The resulting precipitate was collected by filtration, washed with ice-cooled ethanol, and dried under reduced pressure to give 1a and b (ca. 1:1 diastereomer mixture, 23.0 g, 120 mmol, 97% yield).

1H-NMR (dimethyl sulfoxide (DMSO)-d6): δ: 1.59 (1.5H, s), 1.62 (3H, s), 2.81 (1H, dd, J=8.6, 10.0 Hz), 3.15 (0.5H, dd, J=8.4, 9.9 Hz), 3.26 (0.5H, dd, J=6.5, 10.1 Hz), 4.09 (2H, dd, J=7.1, 13.9 Hz). Diastereomer 2: 1.19 (3H, t, J=7.1 Hz), 1.75 (3H, s), 2.97 (1H, dd, J=8.6, 10.0 Hz), 3.29 (1H, m), 4.09 (2H, m), 4.16 (1H, m). ESI-MS m/z: 220.0 (M+H)+.

**N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid (cis-Isomer of AcCP, 3a, e)** (Table 1, Entry 1) Compounds 1a and b (2.0 g, 105 mmol) and acetic anhydride (40 mL, 418 mmol) were heated at 100°C for 2 h. The reaction mixture was concentrated under reduced pressure, aqueous citric acid (5%, 200 mL) was added thereto, and the mixture stirred overnight at r.t. To the mixture was added concentrated hydrochloric acid (2.0 mL) and the mixture was stirred at 70°C for 1 h. The reaction mixture was filtered to remove tar material, and the filtrate was concentrated under reduced pressure to give 3a and c (cis-isomers, 5.3 g, 22.7 mmol, 21.7%).

1H-NMR (DMSO-d6): δ: 1.80 (3H, s), 2.02 (3H, s), 3.49 (1H, dd, J=1.0, 12.0 Hz), 3.61 (1H, dd, J=6.3, 12.0 Hz), 5.22 (1H, dd, J=1.0, 6.2 Hz). 1H-NMR (D2O): δ: 1.80 (3H, s), 2.03 (3H, s), 3.41 (1H, dd, J=12.4 Hz), 3.72 (1H, dd, J=6.4, 12.4 Hz), 5.05 (1H, dd, J=6.4 Hz). 13C-NMR (D2O): δ: 22.8, 24.2, 34.1, 67.1, 73.7, 172.4, 174.7, 176.7. ESI-MS m/z: 234.0 (M+H)+, 232.0 (M−H−). FAB-MS m/z: 234.0451 (M+H+). (Calcd for C7H11NO3S, 234.0436).

5a, e Compounds 1a and b (2.0 g, 10.5 mmol) and acetic anhydride (40 mL, 42.0 mmol) were heated at 100°C for 2 h. The reaction mixture was then concentrated under reduced pressure. To the residue was added water (10 mL) and the residue was extracted with ethyl acetate (15 mL, twice). The extract was washed with saturated brine (100 mL), dried over anhydrous MgSO4, and concentrated under reduced pressure to give 5a and e (cis-isomers, 5.3 g, 22.7 mmol, 21.7%).

1H-NMR (DMSO-d6): δ: 1.80 (3H, s), 2.02 (3H, s), 3.49 (1H, dd, J=1.0, 12.0 Hz), 3.61 (1H, dd, J=6.3, 12.0 Hz), 5.22 (1H, dd, J=1.0, 6.2 Hz). 1H-NMR (D2O): δ: 1.80 (3H, s), 2.03 (3H, s), 3.41 (1H, dd, J=12.4 Hz), 3.72 (1H, dd, J=6.4, 12.4 Hz), 5.05 (1H, dd, J=6.4 Hz). 13C-NMR (D2O): δ: 22.8, 24.2, 34.1, 67.1, 73.7, 172.4, 174.7, 176.7. ESI-MS m/z: 234.0 (M+H)+, 232.0 (M−H−). FAB-MS m/z: 234.0451 (M+H+). (Calcd for C7H11NO3S, 234.0436).
layer was dried over anhydrous MgSO₄, and concentrated. The residue (trans–cis=95:5) was crystallized from ethyl acetate and n-heptane to give 4b and d (trans-isomer: 99%) as pale yellow crystals (7.0 g, 26.8 mmol, 58.8%).

1H-NMR (CDCl₃) δ: 1.27 (3H, t, J=7.1 Hz), 1.94 (3H, s), 2.18 (3H, s), 3.40 (1H, d, J=11.6 Hz), 3.56 (1H, dd, J=5.5, 11.0 Hz), 4.20 (2H, qd, J=7.1, 7.2 Hz), 5.00 (1H, d, J=5.9 Hz), 9.10 (1H, brs). 1H-NMR (DMSO-d₆) δ: 1.15 (3H, t, J=7.1 Hz), 1.77 (3H, s), 2.03 (3H, s), 3.36 (1H, dd, J=10.6, 11.7 Hz), 4.04 (2H, qd, J=7.1, 9.0 Hz), 5.34 (1H, d, J=5.2 Hz). 13C-NMR (DMSO-d₆, 100 MHz) δ: 14.3, 23.2, 23.9, 33.6, 61.4, 65.4, 70.7, 168.9, 171.4, 171.9. FAB-MS m/z: 262.0753 (M+H) (Calcd for C₈H₁₈NO₅S: 262.0749). mp: 138–141°C.

N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomers of AcCP, 3b and d, Table 1, Entry 2) Compounds 4b and d (20.0 g, 91.3 mmol) obtained from compounds 2a and b by an operation similar to that described above was dissolved in ethanol (40 mL), and sodium hydroxide solution (4N, 57.0 mL) was added to the solution. The mixture was stirred with heating at 60°C under an argon atmosphere overnight. The mixture was allowed to cool to r.t., and the pH of the solution was adjusted to 1 with hydrochloric acid solution (6N). The solution was kept at r.t. overnight, and the resulting white crystals were collected by filtration and washed with a small amount of cold water. The crystals were dried under reduced pressure to give 3b and d (trans-isomers, 14.2 g, 61.0 mmol, 66.8%).

1H-NMR (DMSO-d₆) δ: 1.73 (3H, s), 2.01 (3H, s), 3.36 (2H, d, J=3.6 Hz), 5.28 (1H, t, J=3.6 Hz). 1H-NMR (D₂O) δ: 1.77 (3H, s), 2.10 (3H, s), 3.43 (1H, d, J=12.0 Hz), 3.50 (1H, dd, J=6.0, 12.0 Hz), 5.24 (1H, d, J=5.6 Hz). 13C-NMR (D₂O) δ: 22.1, 23.0, 32.9, 66.1, 70.8, 172.6, 173.6, 175.4. ESI-MS m/z: 234.0 (M+H)⁺, 232.0 (M−H)⁻. FAB-MS m/z: 234.0451 (M+H) (Calcd for C₇H₁₄NO₄S: 234.0436).

N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl Ester 4-Gly Methyl Ester Amide (cis- and trans-Isomers of AcCP2Et, 4a and b, Table 1, Entry 4) Compounds 2a and b (20.0 g, 91.3 mmol) was dissolved in ethyl acetate (190 mL), and the solution was kept at 0°C. To the solution was added potassium carbonate (15.2 g, 0.77 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI·HCl, 0.89 g, 4.64 mmol), and 4-dimethylaminopyridine (DMAP; 95 mg, 0.77 mmol). To the solution were added ethanol (0.27 mL, 4.60 mmol), water (15 mL), and the reaction mixture was kept at 0°C. The mixture was allowed to gradually rise to r.t., and the mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure, and water (10 mL) was added. The mixture was then extracted with ethyl acetate (10 mL, three times). The combined organic layer was washed with aqueous citric acid (5%, 10 mL, twice), aqueous sodium hydrogen carbonate (5%, 10 mL, twice) and saturated sodium chloride (10 mL), dried over anhydrous MgSO₄, and concentrated to give 10a as a colorless oil (cis–trans=45:55, 1.0 g, 3.48 mmol, 91.0%).

1H-NMR (CDCl₃) δ: cis-isomer; 1.28 (3H, t, J=7.1 Hz), 1.34 (3H, t, J=7.1 Hz), 1.96 (3H, s), 2.17 (3H, s), 3.50 (1H, dd, J=6.1, 11.8 Hz), 3.61 (1H, dd, J=1.8, 11.9 Hz), 4.34–4.27 (2H, m), 4.34–4.25 (2H, m), 4.85 (1H, dd, J=1.8, 6.0 Hz). trans-Isomer; 1.27 (3H, t, J=7.1 Hz), 1.33 (3H, t, J=7.1 Hz), 1.93 (3H, s), 2.12 (3H, s), 3.36 (1H, d, J=11.7 Hz), 3.54 (1H, dd, J=6.2, 11.6 Hz), 4.12–4.25 (2H, m), 4.25–4.33 (2H, m), 4.95 (1H, d, J=5.8 Hz). FAB-MS m/z: 290.1063 (M+H) (Calcd for C₁₀H₁₄NO₅S: 290.1062).

N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl 4-Isopropyl Diester (10b, Chart 3) An operation similar to the one described for 10a was performed using isopropanol, and 10b was obtained in an overall yield of 86% as a colorless oil (cis–trans=27:73).

1H-NMR (CDCl₃) δ: cis-isomer; 1.28 (3H, t, J=7.1 Hz), 1.33 (3H, t, J=6.2 Hz), 1.96 (3H, s), 2.16 (3H, s), 3.49 (1H, dd, J=6.1, 11.9 Hz), 3.59 (1H, dd, J=2.0, 1.9 Hz), 4.81 (1H, dd, J=2.0, 6.1 Hz), 5.19–5.12 (1H, m). trans-Isomer; 1.28 (3H, t, J=7.1 Hz), 1.31 (6H, d, J=6.2 Hz), 1.93 (3H, s), 2.12 (3H, s), 3.36 (1H, dd, J=0.7, 11.6 Hz), 3.55 (1H, dd, J=6.2, 11.6 Hz), 4.17–4.28 (2H, m), 4.91 (1H, d, J=5.6 Hz), 5.12–5.19 (1H, m). FAB-MS m/z: 304.1208 (M+H) (Calcd for C₁₈H₂₆NO₅S: 304.1219).

N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 4-Gly Methyl Ester Amide (13a, Chart 3) Compounds 4a and b (cis–trans=48:52, 2.0 g, 7.68 mmol)
was dissolved in dichloromethane (20 mL), and the reaction mixture was kept at 0°C. To the solution were added 1.62 g of 2-trans methyl ester hydrochloride (0.963 g, 7.67 mmol), EDCI·HCl (1.62 g, 8.47 mmol) and 1-hydroxy-1H-benzotriazole hydrate (HOBT·H₂O, 1.14 g, 8.45 mmol). The reaction temperature was allowed to gradually rise to r.t., and the mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure, and water (20 mL) was added. The mixture was then extracted with ethyl acetate (80 mL, three times). The combined organic layer was washed with aqueous citric acid solution (5%, 20 mL, twice) and saturated sodium chloride (20 mL), dried over anhydrous MgSO₄, and concentrated. The residue was purified by silica gel chromatography eluted using compounds methanol (50 : 50, 10.0 g, 45.6 mmol) was dissolved in 20.0° steps at 0.0°. A second sweep was performed using CuKα radiation (λ=1.54187 Å) at a temperature of 1.54187 Å at a temperature of 20±1°C. The crystal-to-detector distance was 127.40 mm. Readout was performed in the 0.100 mm pixel mode. The ex-

### N-Hexadecanoyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomer, 12, Chart 3) To the solution of compound 11 (trans-isomer, 5.12 g, 11.2 mmol) in EtOH (10 mL) was added aqueous NaOH solution (4N, 6.97 mL, 27.88 mmol), and the solution was stirred overnight at 60°C. The pH of the solution was adjusted to 1 by addition of aqueous HCl (6N). The solution was then extracted with ethyl acetate (30 mL, twice) and the combined organic layer was washed with water (30 mL, twice) and saturated sodium chloride (30 mL, twice), dried over anhydrous MgSO₄, and concentrated. The precipitate was recrystallized from mixed solvent of ethyl acetate and n-hexane to give 12 as a white solid (trans-isomer, 4.16 g, 9.68 mmol, 86.4%).

### N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Ethyl Ester 4-α-Ala Methyl Ester Amide (13b, Chart 3) An operation similar to the one described for 13a and b was performed using methyl pyruvate, and 8 was obtained in an overall yield of 71.5% as a white solid (ca. 50 : 50 diastereomer mixture).

### N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Methyl Ester (AcCP2Me, 9, Chart 3) An operation similar to the one described for compounds 2a and b was performed using methyl pyruvate, and 8 was obtained in an overall yield of 71.5% as a white solid (ca. 50 : 50 diastereomer mixture).

### N-Hexadecanoyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomer, 11, Chart 3) Compounds 2a and b (trans-cis=50 : 50, 10.0g, 45.6mmol) was dissolved in ethyl acetate (90 mL), and the solution was added Et₃N (12.7 mL, 91.2 mmol), and the solution was stirred overnight. The reaction mixture was kept at 0°C. To the solution were added Gly (HOBt·H₂O, 1.14 g, 8.45 mmol). The reaction temperature was allowed to gradually rise to r.t., and the mixture was stirred overnight. Citric acid solution (5%, 20 mL, twice) and saturated sodium chloride (20 mL), dried over anhydrous MgSO₄, and concentrated. The residue was purified by silica gel chromatography eluted using compounds methanol (50 : 50, 10.0 g, 45.6 mmol) was dissolved in 20.0° steps at 0.0°. A second sweep was performed using CuKα radiation (λ=1.54187 Å) at a temperature of 20±1°C. The crystal-to-detector distance was 127.40 mm. Readout was performed in the 0.100 mm pixel mode. The ex-

### N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Methyl Ester (AcCP2Me, 9, Chart 3) An operation similar to the one described for compounds 2a and b was performed using methyl pyruvate, and 8 was obtained in an overall yield of 71.5% as a white solid (ca. 50 : 50 diastereomer mixture).

### N-Hexadecanoyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomer, 12, Chart 3) To the solution of compound 11 (trans-isomer, 5.12 g, 11.2 mmol) in EtOH (10 mL) was added aqueous NaOH solution (4N, 6.97 mL, 27.88 mmol), and the solution was stirred overnight at 60°C. The pH of the solution was adjusted to 1 by addition of aqueous HCl (6N). The solution was then extracted with ethyl acetate (30 mL, twice) and the combined organic layer was washed with water (30 mL, twice) and saturated sodium chloride (30 mL, twice), dried over anhydrous MgSO₄, and concentrated. The residue was recrystallized from mixed solvent of ethyl acetate and n-hexane to give 12 as a white solid (trans-isomer, 4.16 g, 9.68 mmol, 86.4%).

### N-Acetyl-2-methylthiazolidine-2,4-dicarboxylic Acid 2-Methyl Ester (AcCP2Me, 9, Chart 3) An operation similar to the one described for compounds 2a and b was performed using methyl pyruvate, and 8 was obtained in an overall yield of 71.5% as a white solid (ca. 50 : 50 diastereomer mixture).

### N-Hexadecanoyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomer, 11, Chart 3) Compounds 2a and b (trans-cis=50 : 50, 10.0g, 45.6mmol) was dissolved in ethyl acetate (90 mL), and the solution was kept at 0°C. To the solution was added Et₃N (12.7 mL, 91.2mmol), and n-hexadecanoyl chloride (20.7 mL, 68.4mmol) was added dropwise over 30 min. The reaction temperature was allowed to gradually rise to r.t., and the mixture was stirred overnight. Citric acid solution (5%, 30 mL) was then added to the reaction mixture, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (50 mL, twice) and saturated sodium chloride (50 mL, twice), dried over anhydrous MgSO₄, and concentrated. The residue was purified by silica gel chromatography eluted using n-hexane–ethyl acetate=9 : 1 to 1 : 1, 1% AcOH) to give a pale yellow solid. The obtained solid was washed with n-hexane to give 11 as a pale yellow wax (trans-isomer, 13.2g, 28.8mmol, 63.2%).

### N-Hexadecanoyl-2-methylthiazolidine-2,4-dicarboxylic Acid (trans-Isomer, 13, Chart 3) Compounds 2a and b (trans-cis=50 : 50, 10.0g, 45.6mmol) was dissolved in ethyl acetate (90 mL), and the solution was kept at 0°C. To the solution was added Et₃N (12.7 mL, 91.2mmol), and n-hexadecanoyl chloride (20.7 mL, 68.4mmol) was added dropwise over 30 min. The reaction temperature was allowed to gradually rise to r.t., and the mixture was stirred overnight. Citric acid solution (5%, 30 mL) was then added to the reaction mixture, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (50 mL, twice) and saturated sodium chloride (50 mL, twice), dried over anhydrous MgSO₄, and concentrated. The residue was purified by silica gel chromatography eluted using n-hexane–ethyl acetate=9 : 1 to 1 : 1, 1% AcOH) to give a pale yellow solid. The obtained solid was washed with n-hexane to give 11 as a pale yellow wax (trans-isomer, 13.2g, 28.8mmol, 63.2%).

### Single Crystal X-Ray Study Single crystals of the mixture of cis-(2R,4R)- and cis-(2S,4S)-isomers of AcCP (3a, e) (Table 1, entry 1) and the mixture of trans-(2S,4R)- and trans-(2R,4S)-isomers of AcCP (3b, d) (Table 1, entry 2) suitable for X-ray analysis were obtained from MeOH via slow evaporation.

X-Ray diffraction (XRD) data were collected using a Rigaku RAXIS RAPID imaging plate area detector with filtered CuKa radiation (λ=0.154187 Å) at a temperature of 20±1°C. The crystal-to-detector distance was 127.40 mm. Readout was performed in the 0.100 mm pixel mode. The exposure was set to 2.05s. The XRD data of each crystal were recorded to a total of 45 oscillation images. A first sweep of data was performed using ω scans from 80.0 to 260.0° in 20.0° steps at χ=0.0° and φ=0.0°. A second sweep was performed using ω scans from 80.0 to 260.0° in 20.0° steps at χ=54.0° and φ=0.0°. A third sweep was performed using ω scans from 80.0 to 260.0° in 20.0° steps at χ=54.0° and
absorbance at 450 nm after 3 d was measured with a predetermined concentration of each sample for 3 d. Cells were treated and subsequently fixed with 1% formaldehyde and 1% CaCl$_2$. Cells were incubated with neutral red dye (50 µg/mL) solution. Cells were incubated in a humidified 5% CO$_2$ atmosphere at 37°C.

B16 melanoma cells were plated in 96-well microplates at 8.0×10$^3$ cells per well in 200 µL of DMEM containing 10% FBS without phenol red. After overnight incubation, the medium was replaced with 200 µL of DMEM containing each sample at a predetermined concentration. Cells were treated with a predetermined concentration of each sample for 3 d. The absorbance of the medium at 450 nm after 3 d was measured by a microplate reader.

The concentration necessary for suppressing eumelanin production in each sample by 50% was calculated relative to the absorbance at 540 nm in a microplate reader. The absorbance after 3 d following the addition of a predetermined concentration of each sample was divided by the absorbance at all, 2 points: a slight sulfur odor was detected, 1 point: sulfur odor was detected, and 0 point: strong sulfur odor was detected. The results of the 6 panelists were totaled as follows; AcCP2Et (4b, d) at pH 4: 8 points, AcCP2Et (4b, d) at pH 7: 17 points, CP2Et (2a, b) at pH 4: 1 point, CP2Et (2a, b) at pH 7: 1 point.

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Conflict of Interest All authors were employees of Ajinomoto Co., Inc. when this study was conducted and have no further conflicts of interest to declare.

Supplementary Materials The online version of this article contains supplementary materials.

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