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Synthesis and dual biological effects of hydroxycinnamoyl phenylalanyl/prolyl hydroxamic acid derivatives as tyrosinase inhibitor and antioxidant

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ABSTRACT

We previously reported that caffeoyl-amino acidyl-hydroxamic acid (CA-Xaa-NHOH) acted as both a good antioxidant and tyrosinase inhibitor, in particular when caffeic acid was conjugated with proline or amino acids having aromatic ring like phenylalanine. Here, various hydroxycinnamic acid (HCA) derivatives were further conjugated with phenylalanyl hydroxamic acid and prolyl hydroxamic acid (HCA-Phe-NHOH and HCA-Pro-NHOH) to study the structure and activity relationship as both antioxidants and tyrosinase inhibitors. When their biological activities were evaluated, all HCA-Phe-NHOH and HCA-Pro-NHOH exhibited enhanced antioxidant activity compared to HCA alone. Moreover, derivatives of caffeic acid, ferulic acid, and sinapic acid inhibited lipid peroxidation more efficiently than vitamin E analogue (Trolox). In addition, derivatives of caffeic acid and sinapic acid efficiently inhibited tyrosinase activity and reduced melanin content in melanocytes Mel-Ab cell.

Reactive oxygen species (ROS) are generated by the reduction of oxygen or oxidation of water molecules resulting in superoxide ($O_2^{\cdot-}$, ·OOH), hydrogen peroxide (H_2O_2), peroxyl (ROOH·) and hydroxyl (·OH) radicals. When ROS exists in moderate concentration, it participates in physiological processes for desired cellular responses. In healthy bodies, the amount of ROS can be controlled by antioxidant-mediated self-defense systems. For example, antioxidants such as vitamin C, vitamin E and glutathione get rid of free radical intermediates and terminate free radical-mediated chain reactions to prevent cellular damage. However, overproduced ROS leads to oxidative stress by disrupting the balance of the cellular oxidation state. This results in a number of cellular damage by oxidation of lipids, protein, DNA and RNA, which causes the deconstruction of cell membranes, deactivation of enzymes, acceleration of the aging process, and mediation of severe disease states such as chronic lung diseases, diabetes, and neurogenerative disorders.[1] For these reasons, antioxidants that efficiently suppress ROS activity have been intensively studied, not only for the prevention of diseases but also for use in other applications such as cosmetic ingredients to delay skin aging process and food preservatives to prevent food discoloration and deterioration.

Hydroxycinnamic acids (HCAs) such as caffeic acid (CA; 3,4-dihydroxy-cinnamic acid), *p*-coumaric acid (pCoA; 4-hydroxy-cinnamic acid), ferulic acid (FA; 3-methoxy-4-hydroxy-cinnamic acid) and sinapic acid (SA; 3,5-dimethoxy-4-hydroxy-cinnamic acid) are well-known antioxidants, which are distributed in many plants and found in fruits, tea, coffee, and wine.[2,3] They exhibit strong antioxidant activity because they can have resonance stabilized phenoxy radicals after the hydrogen radical is abstracted due to the phenolic nucleus and extended side chain conjugation.[4] In particular, CA, pCoA and dihydrocaffeic acid (DHCA) possessing phenol or catechol moiety have drawn attention due to their multiple biological and pharmaceutical activities.[5] Various natural and synthetic hydroxycinnamic acids analogues were reported and their potential activities have been intensively investigated.[6–10]

Melanocytes produce the dark pigment melanin through a complex pathway in which tyrosinase (EC 1.14.18.1) plays a crucial role. Tyrosinase is a metalloenzyme, which contains dinuclear copper in the active site. It catalyzes both *ortho*-hydroxylation of L-tyrosine and the subsequent conversion of 3,4-dihydroxyphenylalanine (L-DOPA) to DOPA-quinone in the early stage of melanogenesis, which is responsible for skin and hair color.[11,12] The DOPA-quinone yields melanin, which is a polymer comprising

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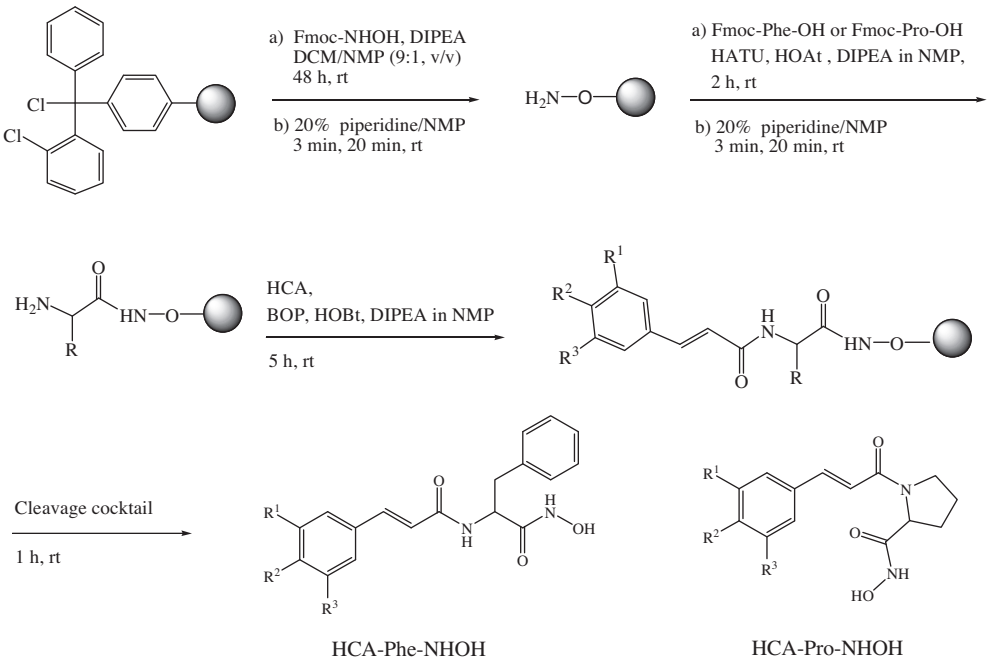

Scheme 1. Solid phase synthesis of HCA-Phe-NHOH and HCA-Pro-NHOH. CA: $R^1 = R^2 = OH$, $R^3 = H$; DHCA: $R^1 = R^2 = OH$, $R^3 = H$, without the 2,3-double bond; pCoA: $R^1 = R^3 = H$, $R^2 = OH$; FA: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = H$; SA: $R^1 = R^3 = OCH_3$, $R^2 = OH$.

numerous smaller component molecules such as 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), through a series of enzymatic reactions, sequential oxidation and oxidative cyclization.[13] It participates in the enzymatic browning of fruits and vegetables,[14] and pigmentary skin diseases.[15] Therefore, studies on tyrosinase inhibitors have attracted a lot of interest because tyrosinase inhibitors have broad applications in medicinal, food and cosmetic industries.[16–18] Numerous naturally existing tyrosinase inhibitors have been discovered in plants, but have mostly shown poor[19] to moderate activity.[20] Various copper chelators including kojic acid (KA)[21] and *N*-phenylthiourea (PTU)[22] have also been reported as tyrosinase inhibitors. Of these, we chose hydroxamic acid moiety for tyrosinase inhibitory activity because hydroxamic acid group had strong metal chelating ability with a wide range of metal ions such as Fe(III), Zn(II) and Cu(II). Hydroxamic acids supposedly inhibit the metalloenzyme by metal chelation and hydrogen bonding, or through the formation of a charge transfer complex in the enzyme.[23] Recently, several reports showed that hydroxamate derivatives had tyrosinase inhibitory activity.[24,25]

In our previous work, we showed that caffeoyl-amino acidyl-hydroxamic acid (CA-Xaa-NHOH) acted as both a good antioxidant and tyrosinase inhibitor, in particular, caffeoyl-prolyl-hydroxamic acid (CA-Pro-NHOH) was capable of inhibiting tyrosinase activity as well enhancing antioxidant activity, and caffeoyl aromatic amino acid hydroxamic acid such as caffeoyl-phenylalanyl-hydroxamic acid (CA-Phe-NHOH) exhibited good tyrosinase inhibitory activity.[26] Here, we further studied on these bioactive compounds by conjugating phenylalanyl-hydroxamic acid and prolyl-hydroxamic acid with various hydroxycinnamic acids (HCA-Phe-NHOH and HCA-Pro-NHOH). In addition, we demonstrated that our compounds were effective in the cell system.

All HCA-Pro-NHOH and most of HCA-Phe-NHOH were obtained with high purity (93∼99%) (Scheme 1). Some HCA-Phe-NHOH

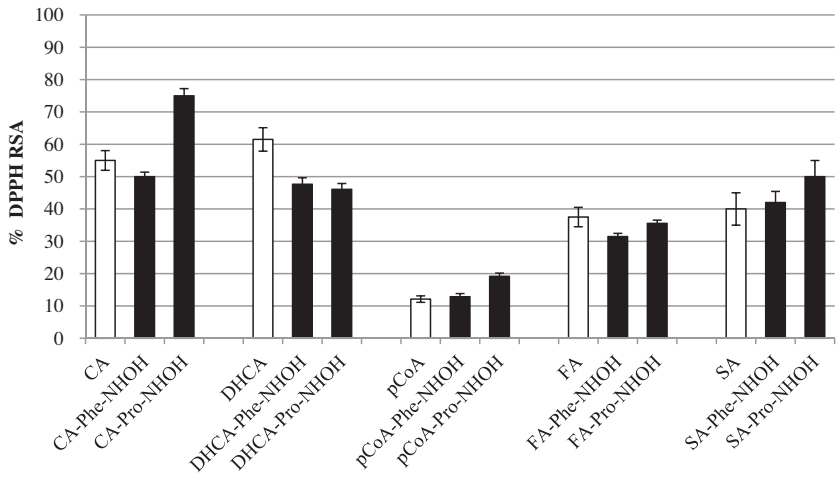

Figure 1. DPPH radical scavenging activity of HCA-Phe-NHOH, HCA-Pro-NHOH (black solid bars) and HCA (white solid bars). Conditions: [Antioxidant]/[DPPH] (mol/mol) = 0.25 for CA, DHCA, and their derivatives; 0.50 for pCoA, FA, SA, and their derivatives. The reaction was performed for 10 min at 25 °C. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error.

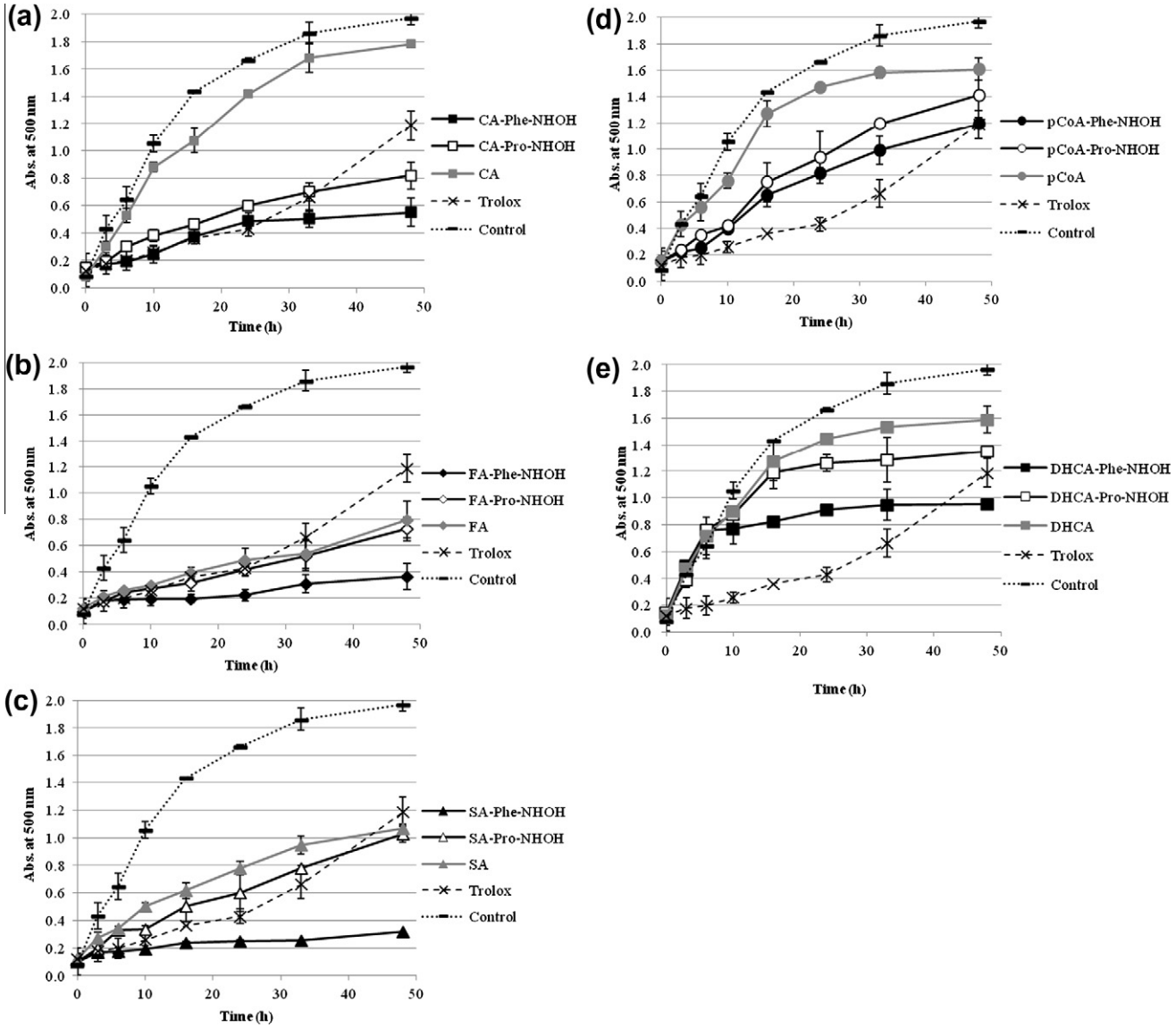

Figure 2. Antioxidant activity of HCA, HCA-Phe-NHOH, HCA-Pro-NHOH and Trolox (positive control) on lipid peroxidation for 48 h. The absorbance was measured at 500 nm by ferric thiocyanate method. Conditions: lipid peroxidation proceeded in emulsified system. The final concentration of each antioxidant was 90 μM; reaction was carried out at 50 °C under the dark. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error.

obtained with moderate purity (~80%) were further purified by semi-preparative RP-HPLC. They were identified by mass spectrometry.[27]

First, we evaluated antioxidant activity by DPPH radical scavenging test[28,29] and lipid peroxidation inhibition test.[30,31] CA and DHCA has strong DPPH radical scavenging activity (RSA) because the *ortho*-dihydroxy group can generate stabilized phenoxy radical by hydrogen bond after donating hydrogen radical.[32] Since pCoA, FA and SA had relatively weak DPPH RSA compared to CA, we performed DPPH RSA test under two different assay conditions to compare the conjugation effect of amino acidyl-hydroxamic acid. The ratio of CA and DHCA derivatives to DPPH was 0.25, and the ratio of pCoA, FA, SA and their derivatives to DPPH was 0.5. As shown in Figure 1, only CA-Pro-NHOH showed significantly enhanced DPPH RSA, whereas SA-Pro-NHOH exhibited slightly enhanced DPPH RSA, and FA derivatives just maintained the activity of FA itself. In the case of DHCA, its strong activity for releasing hydrogen radical seemed to be a little hindered by conjugation with amino acidyl-hydroxamic acid. pCoA and its derivatives did not show any sufficient DPPH RSA.

The results of lipid peroxidation inhibition were quite different when compared to those of DPPH RSA in hydrophilic environment. All of HCA-Phe-NHOH derivatives showed better lipid peroxidation inhibition activity than HCA-Pro-NHOH derivatives (Fig. 2). CA-Phe-NHOH and CA-Pro-NHOH significantly enhanced antioxidant activity compared to CA. FA-Pro-NHOH and SA-Pro-NHOH also exhibited enhanced antioxidant activity, and FA-Phe-NHOH and SA-Phe-NHOH showed excellent antioxidant activity for 48 h.

The order of lipid peroxidation inhibition activity was FA-Phe-NHOH ≈ SA-Phe-NHOH > FA-Pro-NHOH ≈ CA-Phe-NHOH ≈ CA-Pro-NHOH > SA-Pro-NHOH > Trolox (Fig. 2a, b, and c). DHCA and pCoA derivatives showed inferior lipid peroxidation inhibition activity to Trolox, even though they had enhanced activity compared to DHCA or pCoA alone (Fig. 2d and e).

Next, we evaluated tyrosinase inhibition activity of HCA-Phe-NHOH and HCA-Pro-NHOH derivatives.[33] They revealed from moderate to good tyrosinase inhibitory activity, although none of HCAs showed tyrosinase inhibitory activity when L-DOPA was used as a substrate. We hypothesized that amino acidyl-hydroxamic acid had tyrosinase inhibitory activity and the antioxidant group HCA

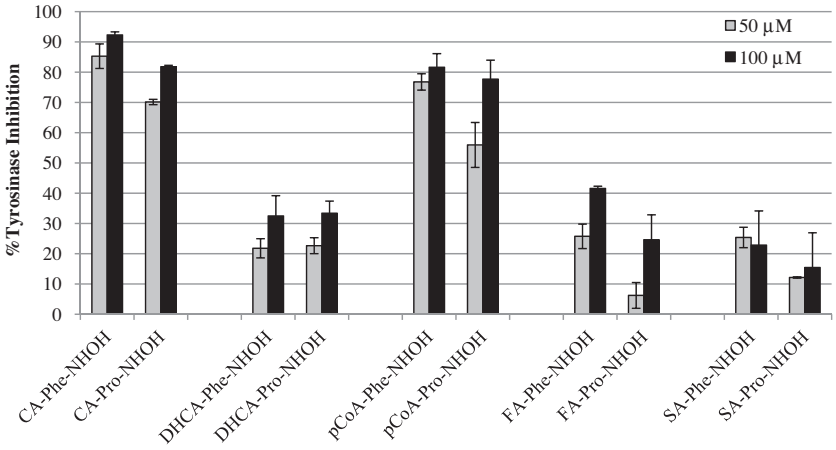

Figure 3. Mushroom tyrosinase inhibitory activity of HCA-Phe-NHOH and HCA-Pro-NHOH at 50 μM (gray solid bars) and 100 μM (black solid bars). Conditions: % Tyrosinase inhibition activity was determined after treating 50 μM (gray solid bars) or 100 μM (black solid bars) of each sample with L-DOPA (2.5 mM) and mushroom tyrosinase (100 μg/ml), and incubating at 25 °C for 10 min. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error.

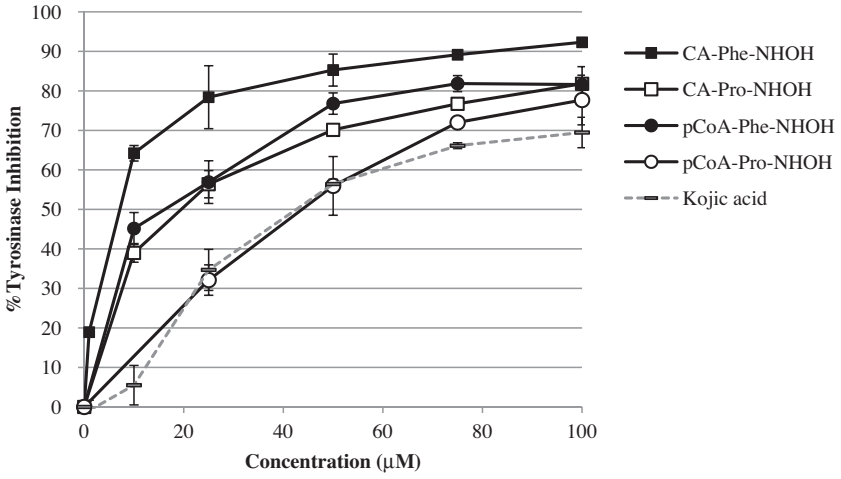

Figure 4. Tyrosinase inhibition activity of CA derivatives, pCoA derivatives, and kojic acid. Conditions: % Tyrosinase inhibition activity was determined after treating each sample with L-DOPA (2.5 mM) and mushroom tyrosinase (100 μg/ml), and incubating at 25 °C for 10 min. Each enzyme reaction was performed for 10 min at 25 °C. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error.

delayed L-DOPA oxidation. However, tyrosinase inhibitory activity of Ac-Phe-NHOH and Ac-Pro-NHOH was only 6% and 5%, respectively, even at a relatively high concentration of 100 μM. In addition, as shown in Figure 3, tyrosinase inhibition activity of HCA-Phe-NHOH and HCA-Pro-NHOH was more dependent on the kinds of antioxidant group, even though HCA-Phe-NHOH seemed to exhibit 5–20% higher tyrosinase inhibitory activity than HCA-Pro-NHOH. This means that HCA plays an important role in binding to tyrosinase as well as in reducing substrate oxidation. CA moiety may act as a substrate analogue because the 3,4-dihydroxyphenyl ring is similar to the natural substrate L-DOPA. FA and SA derivatives exhibited poor tyrosinase inhibition activity because the methoxy group next to *para*-hydroxyl group may have introduced steric hindrance when approaching to the active site of tyrosinase. In addition, FA and SA moiety have less structural similarity to the natural substrate and exhibited less reducing activity than CA in hydrophilic environment.

We also measured tyrosinase inhibition activity of DHCA and pCoA derivatives to prove the effect of CA moiety on tyrosinase inhibition. pCoA-Phe-NHOH and pCoA-Pro-NHOH showed good tyrosinase inhibition activity probably because pCoA had similar

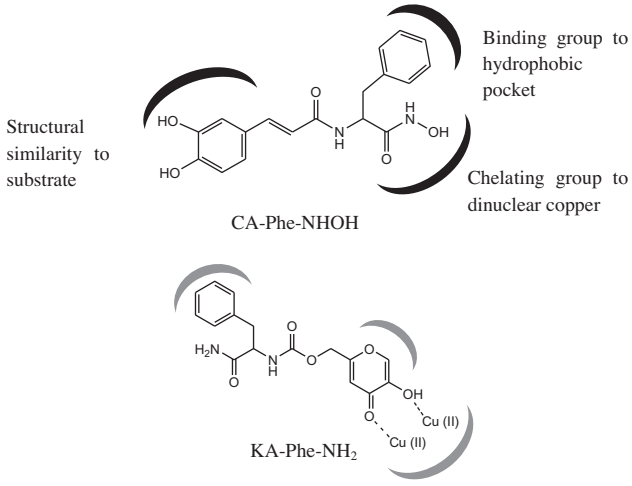

Figure 5. Comparison of possible tyrosinase inhibition mechanism between CA-Phe-NHOH and KA-Phe-NH_2.

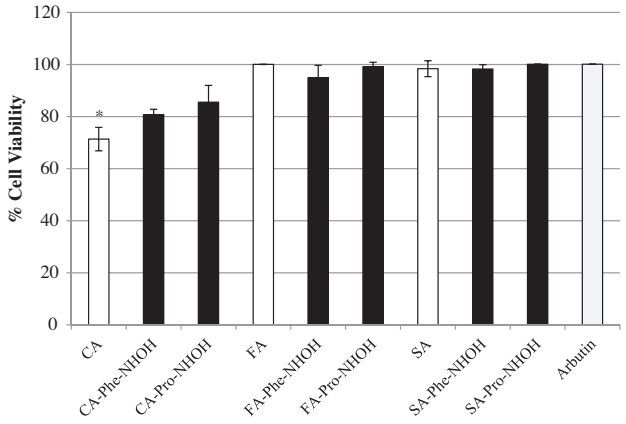

Figure 6. Effect of HCA-Phe-NHOH, HCA-Pro-NHOH, HCA and arbutin on cell viability in Mel-Ab cells. The cells were treated with samples (100 μM) containing new serum-free media and incubated for 24 h. CCK-8 solution was added and cells were incubated for another 2 h at 37 °C. The amount of water-soluble formazan generated by the activity of dehydrogenase in cells was measured by optical density at 450 nm. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error. $^{*}P$ <0.01

structure to the natural substrate L-tyrosine. However, their tyrosinase inhibition activity was inferior to CA derivatives because CA had stronger antioxidant activity than pCoA in hydrophilic environment (Figs. 1 and 4). Interestingly, DHCA derivatives did not sufficiently inhibit tyrosinase activity, even though DHCA had 3,4-dihydroxyphenyl ring like CA and exerted higher antioxidant activity than CA in hydrophilic environment. This is most likely due to the fact that presence of the conjugated double bond makes the molecule less flexible and leads to form a stable Schiff base with a primary amino group of tyrosinase.[34,35]

CA-Phe-NHOH and CA-Pro-NHOH showed significantly higher tyrosinase inhibition activity than FA and SA derivatives. In particular, CA-Phe-NHOH showed excellent tyrosinase inhibition activity (IC_{50} = 4.9 μM), which was comparable to KA-Phe-NH_2 (IC_{50} = 4.2 μM), the strongest tyrosinase inhibitor reported in our lab to date[36] (Fig. 5).

When we performed in vitro tube tests to evaluate tyrosinase inhibition activity and antioxidant activity of HCA-Phe-NHOH and HCA-Pro-NHOH, the most active compound was found to be different in each assay system. We could only comprehensively measure the activity of these compounds in the melanocytes because the cell system contained both hydrophilic and hydrophobic environments and biological melanin synthetic pathway was affected by both tyrosinase and antioxidant.

HCA-Phe-NHOH, HCA-Pro-NHOH and HCA did not affect on cell viability, while only CA showed cytotoxicity at 100 μM in Mel-Ab cells[37] (Fig. 6).

As shown in Figure 7, CA-Pro-NHOH, SA-Phe-NHOH and SA-Pro-NHOH inhibited melanogenesis as much as arbutin, which is used as a hypo-pigmenting agent in the cosmetic industry. HCAs and FA-Phe-NHOH hardly reduced melanin contents, and FA-Pro-NHOH did not sufficiently inhibit melanogenesis.[38] In cell system, HCA-Pro-NHOH showed similar or 10–30% higher anti-melanogenesis activity than HCA-Phe-NHOH. This is clrealy shown in FA-Phe-NHOH and FA-Pro-NHOH.

Interestingly, SA-Phe-NHOH and SA-Pro-NHOH showed hypo-pigmenting effect in melanocyte, even though sufficient tyrosinase inhibition activity was not observed. This is probably due to different cell permeability of each molecule and the different tyrosinase structures in the assay system because mushroom tyrosinase was used in vitro tube test and mouse melanocyte was used for cell assay system. Moreover, there is a possibility that SA-Phe-NHOH and SA-Pro-NHOH can affect not only tyrosinase inhibition and retardation of substrate oxidation, but also other pathways related to receptor-mediated melanogenesis in melanocytes. To understand the role of SA derivatives in melanocytes more clearly, further study is needed.

In summary, various HCA-Phe-NHOH and HCA-Pro-NHOH derivatives were prepared and their antioxidant and tyrosinase inhibition activity was compared to those of CA-Phe-NHOH and CA-Pro-NHOH. All of HCA-Phe-NHOH and HCA-Pro-NHOH showed enhanced antioxidant activity in lipid peroxidation test, in particular, CA derivatives, FA derivatives and SA derivatives surpassed trolox. In the structure-tyrosinase inhibition activity study, HCA-Phe-NHOH showed 5–20% higher tyrosinase inhibition activity than HCA-Pro-NHOH, and HCA moiety played an important role in both binding to tyrosinase and reducing substrate. Among them, CA derivatives exhibited good tyrosinase inhibition activity due to substrate similarity and strong reducing activity.

CA-Pro-NHOH, SA-Pro-NHOH and SA-Phe-NHOH reduced melanin contents in Mel-Ab cells by 30%, on the other hand,

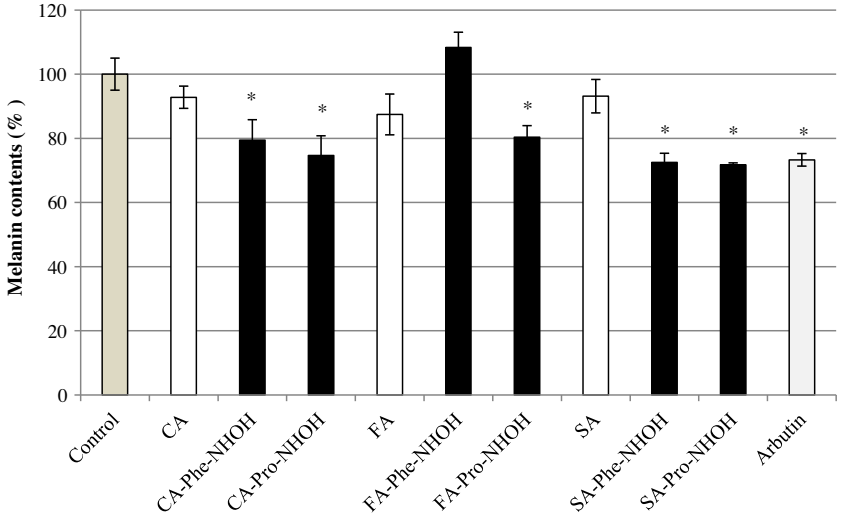

Figure 7. Effect of HCA-Phe-NHOH, HCA-Pro-NHOH, HCA and arbutin on melanogenesis in Mel-Ab cells. The inhibitors were treated at the concentrations of 100 μM for 4 days. The inhibitor-treated cells were dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min, and centrifuged for 20 min at 16,000*g*, after which the optical densities of the supernatants were measured at 400 nm. Each experiment was performed in triplicate and averaged. The values are given as the mean ± standard error. $^{*}P$ <0.01.

CA-Phe-NHOH, FA-Pro-NHOH, and FA-Phe-NHOH did not sufficiently reduce melanin synthesis. Overall, CA-Pro-NHOH was the best promising bioactive compound acting as an excellent antioxidant in both hydrophilic and hydrophobic environments, and a good tyrosinase inhibitor at the same time. This study could be a good starting point for further refinement of potentially useful bioactive compounds in the fields of medicine, agriculture, and cosmetic industry.

Acknowledgments

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27. *Solid phase synthesis of HCA-Phe-NHOH and HCA-Pro-NHOH:* Hydroxylamine hydrochloride (834 mg, 12 mmol) was dissolved in 40 mL of aqueous sodium hydrogen carbonate (2.2 g, 26 mmol), and cooled to 5 °C. *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu, 4.0 g, 12 mmol) dissolved in 40 mL ethyl acetate was added drop wise to the rapidly stirred hydroxylamine solution in an ice-bath and stirred for 4 h at room temperature. The reaction was monitored by TLC (ethyl acetate/hexane = 1:1, R_f = 0.4). After the water layer was removed, the organic layer was washed with saturated aqueous potassium hydrogen sulfate and brine. This organic extract was concentrated in high vacuum, and then *N*-Fmoc protected hydroxylamine (Fmoc-NHOH) was obtained as a white crystalline solid after trituration in hexane and stored overnight (80% yield). Its structure was identified by ^{1}H NMR (JNM-LA300 spectrometer, JEOL Ltd, Tokyo, Japan): (δ_H, $CDCl_3$) 4.21 (1H, t, Fmoc CH), 4.32 (2H, d, Fmoc CH_2), 7.28–7.43, 7.68, 7.86 (8H, m, Fmoc Ar. CH), 8.77 (1H, s, NH), 9.75 (1H, br s, OH). Fmoc-NHOH (2 equiv) was coupled to 2-chlorotrityl chloride (CTC) resin (1.43 mmol/g) with *N,N'*-diisopropylethylamine (DIPEA; 4 equiv) in dichloromethane (DCM) for 48 h. Fmoc-NHOH loaded CTC resin was treated with 10% DIPEA/methanol (v/v) to block the remaining chloride groups. The resulting resin was filtered, and its loading level was 1.0 mmol/g, which was determined by Fmoc titration. After treating with 20% piperidine/*N*-methyl-2-pyrrolidone (NMP) for 30 min to remove Fmoc groups, Fmoc-L-Pro-OH or Fmoc-L-Phe-OH (2 equiv) was coupled to the resin with 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyl uronium hexafluorophosphate methanaminium (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and DIPEA (4 equiv) for 1.5 h at room temperature. After removing Fmoc groups by 20% piperidine/NMP, HCA (2 equiv) was coupled to the amino acid anchored resin with benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP; 2 equiv), hydroxybenzotriazole (HOBt; 2 equiv) and DIPEA (3 equiv) for 5 h. The final product was cleaved from the resin by 30% trifluoroacetic acid (TFA)/DCM (v/v) for 1 h. The resin was filtered, and the filtrate was concentrated in high vacuum, followed by precipitation with cold diethyl ether. The resulting HCA-Phe-NHOH and HCA-Pro-NHOH were identified by QUATTRO Triple Quardrupole Tandem mass spectrometer (Micromass & Waters, Milford, MA, USA) at National Instrumentation Center for Environmental Management (NICEM): CA-Phe-NHOH (*m*/*z* calcd: 343.1 $[M+H]^+$; found: 343.0), CA-Pro-NHOH (*m*/*z* calcd: 293.1 $[M+H]^+$; found: 293.1), DHCA-Phe-NHOH (*m*/*z* calcd: 345.1 $[M+H]^+$; found: 345.1), DHCA-Pro-NHOH (*m*/*z* calcd: 295.1 $[M+H]^+$; found: 295.1), pCoA-Phe-NHOH (*m*/*z* calcd: 327.1 $[M+H]^+$; found: 327.1), pCoA-Pro-NHOH (*m*/*z* calcd: 277.1 $[M+H]^+$; found: 277.0), FA-Phe-NHOH (*m*/*z* calcd: 357.1 $[M+H]^+$; found: 357.1), FA-Pro-NHOH (*m*/*z* calcd: 307.1 $[M+H]^+$; found: 307.0), SA-Phe-NHOH (*m*/*z* calcd: 387.1 $[M+H]^+$; found: 387.0), SA-Pro-NHOH (*m*/*z* calcd: 337.1 $[M+H]^+$; found: 337.1). Their purities were analyzed by RP-HPLC (Thermo Scientific Spectra System AS300; Thermo-Fisher, Waltham, MA, USA) using C18 reverse phase column (120 Å, 5 μm, 4.6 × 250 mm; AAPPTec, Louisville, KY, USA) using the following conditions: gradient elution with A: 0.1% TFA/water, B: 0.1% TFA/acetonitrile; from 10% to 90% over 30 min, a flow rate: 1.0 mL/min; detection: UV, 280 or 326 nm. HCA-Phe-NHOH were purified by a semi-preparative RP-HPLC column using an A to B gradient (A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile; from 10% to 90% B over 30 min, at a flow rate of 4.0 mL/min) and freeze-dried.
28. *DPPH radical scavenging test:* We adopted the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay[29] to measure free radical scavenging activity of HCA-Phe-NHOH and HCA-Pro-NHOH. Methanolic DPPH solution (0.1 mM, 1480 μL) was mixed with 20 μL of 1.85 mM or 3.70 mM sample dissolved in methanol. The absorbance was measured at 516 nm after 10 min by UV/visible spectrophotometer (Optizen 2120 UV, Mecasys Co. Ltd, Korea). The percentage of DPPH radical scavenging activity (RSA) was calculated as % RSA = (1−A/B) × 100, where A represented the absorbance of reaction mixture containing antioxidant sample, and B represented the absorbance of reference solution containing methanol instead of sample.
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30. *Lipid peroxidation inhibition test:* Linoleic acid (0.284 g) and 0.284 g of Tween 20 were mixed together in 0.1 M sodium phosphate buffer (pH 7.0) to prepare 50 mM linoleic acid emulsion. The linoleic acid emulsion (2.5 mL), 2.0 mL of the phosphate buffer, 0.5 mL of water, and 0.5 mL of test samples dissolved in methanol or methanol alone were mixed in a glass vial (10 mL-volume). The total reaction volume was fixed at 5.5 mL, and the final concentration of test samples was 90 μM. These glass vials containing reaction mixture were tightly capped by a rubber septum, and then kept at 50 °C under the dark for 48 h. Ferric thiocyanate assay[31] was used with a slight modification to evaluate antioxidant activity of test samples. Aliquots of the reaction mixture (25 μL) were taken out at specific intervals, and mixed with 1.175 mL of 75% ethanol, 25 μL of 20 mM ferrous chloride in 3.5% HCl, and 25 μL of 30% ammonium thiocyanate. The absorbance was measured at 500 nm after 3 min, when the color development by ferric thiocyanate complex reached maximum. Each experiment was performed in triplicate and averaged.
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33. *Tyrosinase inhibition test:* Tyrosinase inhibition activity was carried out with L-DOPA as a substrate. Two hundred fifty microliters of phosphate buffer (0.1 M, pH 6.8), 250 μL of 2.5 mM L-DOPA, 200 μL of water, and 25 μL of inhibitor dissolved in DMSO or DMSO alone were mixed together in an Eppendorf tube (1.5 mL-volume). The reaction mixture was incubated at 25 °C for 10 min after treating with 25 μL of aqueous mushroom tyrosinase solution (100 μg/mL), after which the UV absorbance was measured at 475 nm. The percentage of tyrosinase inhibition activity was calculated as% inhibition = (1−A/B) × 100, where A represents the absorbance of reaction mixture containing inhibitor, and B represents the absorbance of reference reaction mixture containing DMSO instead of inhibitor. Each experiment was performed in triplicate. The values are given as the mean ± standard error.
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37. *Cell viability test:* The cell viability was tested using Cell Counting Kit-8 (CCK-8; CK04, Dojindo, Kumamoto, Japan). Mel-Ab cells (2×10^3 per well) were seeded into 96-well plates. Culture media were replaced with serum-free DMEM after 24 h, and incubated for another 24 h. Then, cells were treated with samples (100 μM) containing new serum-free media and incubated for 24 h. CCK-8 solution was added and cells were incubated for another 2 h at 37 °C. The

amount of water-soluble formazan generated by the activity of dehydrogenase in cells was measured by optical density at 450 nm using SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

38. *Melanogenesis inhibition assay:* Mel-Ab cells were cultured in DMEM with 10% FBS, 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 nM cholera toxin (CT), 50 μg/mL of streptomycin, and 50 U/mL penicillin at 37 °C in 5% CO_2. Effects of the inhibitors on melanin formation in Mel-Ab cell line were estimated. When the samples were treated at the concentrations of 100 μM for 4 days, the inhibitors-treated cells produced less amount of melanin than the cells without inhibitors. The treated cells were then dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min, and centrifuged for 20 min at 16,000*g*, after which the optical densities of the supernatants were measured at 400 nm. Each experiment was performed in triplicate and averaged.