Ethyl-(4-hydroxyphenyl) oxamate Sodium Salt As a Strong Melanin Biosynthesis Inhibitor

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Preliminary data show that hydroxyaniline derivatives have strong inhibitory activities against mushroom tyrosinase. Twelve out of thirty-four synthesized hydroxyaniline derivatives demonstrated inhibitory activities, among which ethyl-(4-hydroxyphenyl)oxamate sodium salt (EHPONa) was found to be the most effective inhibitor. Inhibitory activities of hydroxyaniline derivatives were much higher than that of commercially available whitening agent arbutin, and comparable to that of kojic acid. Data showed that EHPONa x had a higher inhibitory activity of melanin biosynthesis than those of both kojic acid and arbutin. Based on the study of inhibition kinetics and cupric chloride effects on enzyme activity, we propose that 4-hydroxyl oxygen and oxamate oxygen moiety of EHPONa chelate the two cupric ions at the active site, and that phenol moiety interacts with the hydrophobic enzyme pocket. Accordingly, EHPONa may be a good candidate as a skin-whitening agent.

Key words: ethyl-(4-hydroxyphenyl)oxamate sodium salt, hydroxyaniline derivatives, melanin biosynthesis, skin-whitening agent, tyrosinase inhibitor

Tyrosinase (EC:1.14.18.1) is a copper-containing mono-oxygenating enzyme involved in the biosynthesis of melanin and various polyphenolic compounds. The catalytic process consists of monophenolase activity, the hydroxylation of phenols, and catecholase activity, which is responsible for the oxidation of *o*-diphenols to *o*-quinols [Mayer, 2006]. Tyrosinases exist in a wide range of plant, animal and fungi species with their respective structures and mechanisms [Jaenicke and Decker, 2003]. However, all active sites of tyrosinases contain binuclear type 3 copper ions (CuA and CuB), which bind to three histidine residues, whose copper-binding ligand shares similarity with the copper-binding sites of some arthropods and molluscan hemocyanins [Linzen, 1989; Lang and van Holde, 1991]. Proteins related to tyrosinase include polyphenol oxidase (EC:1.10.3.1) in plants, which are responsible for catalyzing the oxidation of mono- and *o*-diphenols to *o*-diquinones [Cary *et al*., 1992]. There are two mammalian tyrosinase-related proteins: TYRP1 (tyrosinase-related protein 1) [Kobayashi *et al*., 1994], which catalyzes 5,6-dihydro-xyindole-2-carboxylic acid (DHICA) to indole-5,6-quinone-2-carboxylic acid; and TYRP2 (tyrosinase-related protein 2), which is a melanogenic enzyme dopachrome tautomerase (EC:5.3.3.12) that isomerizes dopachrome to DHICA [Jackson *et al*., 1992]. It should be noted that TYRP2 binds two zinc ions instead of two copper ions [Solano *et al*., 1994].

Accumulation of a high level of melanin leads to a number of dermatologic disorders, ranging from actinic damages caused by solar UV exposure, melasma, freckles, and age spots [Curto *et al*., 1999]. A number of treatment options exist, among which hydroquinone, kojic acid, and arbutin have been widely used to inhibit tyrosinase activities [Perez-Bernal *et al*., 2000]. However, recent studies have questioned their effectiveness due to adverse side effects and health risks, which have gained much attention. For example, hydroquinone treatment has been reported to cause cytotoxicity and induce mutagenesis. Similarly, mutagenicity and contact dermatitis have put kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-on) under controversy despite being an effective bleaching

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agent and a tyrosinase inhibitor. Arbutin (hydroquinone β-D-glucopyranoside) has also been noted to produce toxicity through long-term use, because it undergoes hydrolysis in the presence of β-glycosidase to form hydroquinone. As a result, there is a growing demand for a more stable, effective, and safe tyrosinase inhibitor.

In the present study, hydroxyaniline derivatives containing an oxamate moiety were synthesized, and their anti-tyrosinase and anti-melanin biosynthesis activities were examined using mushroom tyrosinase and human melanoma cells (SK-MEL-2).

Materials and Methods

Strains and reagents. Human melanoma cells (SK-MEL-2) were purchased from KCLB (Korean Cell Line Bank). Cell culture media and reagents, fetal bovine serum (FBS), trypsin, and antibiotics were purchased from Gibco BRL (New York, USA). Reagents used in tyrosinase inhibition, cytotoxicity, and the synthesis of hydroxyaniline derivatives, including mushroom tyrosinase, L-DOPA, sodium cyanide, dimethylsulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and α-melanocyte stimulating hormone (a-MSH) were of the highest purity (Sigma Co., St. Louis, USA).

Synthesis of ethyl *N*-(4-hydroxyphenyl)oxamate. To a solution of 4-aminophenol (60 g, 550 mmol) in methylene chloride (150 mL), K_2CO_3 (83.3 g, 570 mmol) was added. The mixture was heated to 120-130°C and stirred at the same temperature for 5 h. TLC ($EtOAc/CH_2Cl_2$, 1/10, v/v) showed that 4-aminophenol was consumed completely. After the addition of 2:1 mixture of ethanol and water (v/v; total volume 600 mL) to the above mixture, the crude product was precipitated, washed with water (5×100 mL), and recrystallized employing a mixture of methylene chloride and ethyl acetate (10:1, v/v) to give the title product as a white solid (100 g, 87%).

Synthesis of ethyl *N*-(4-hydroxyphenyl)oxamate mono- and di-sodium salt (11, 12). To a solution of ethyl *N*-(4-hydroxyphenyl)oxamate (20 mmol) in methanol (30 mL), sodium hydroxide (19.5 mmol for mono-salt and 39 mmol for di-salt) was added. The resulting mixture was refluxed for 2 h. The removal of the solvent resulted in a crude product, which was recrystallized employing ethyl acetate (50 mL) to give the title product as a white solid in quantitative yield: EHPONa, white amorphous powder, ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.74 (m, 2H, aromatic), 7.52 (m, 3H, aromatic), 9.39 (broad s, 1H, OH), 10.53 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$) 115.10, 122.12, 129.00, 154.96, 1598.80, 160.95.

Synthesis of 2-[4-(ethoxyoxalylamino)-phenoxy]-2-methyl-propionic acid ethyl ester. To a solution of ethyl *N*-(4-hydroxyphenyl)oxamate (2.09 g, 10 mmol) in methylene chloride (150 mL), ethyl hydrozyisobutyrate (1.45 g, 11 mmol) and PPh_3 (3.15 g, 12 mmol) were added, followed by a slow addition of diethylazodicarboxylate (DEAD; 1.92 g, 11 mmol) while maintaining the temperature of the reaction mixture to about 20°C. After the addition, the resulting solution was stirred at room temperature for 11 h. TLC showed that ethyl *N*-(4-hydroxyphenyl)oxamate was consumed completely. Subsequently, H_2O (30 mL) was added to the resulting mixture. The mixture was then stirred for additional 30 min. The mixture was then separated, and the organic phase was dried over $MgSO_4$. Removal of the solvent resulted in a crude product, which was purified by silica gel column chromatography employing a mixture of methylene chloride and ethyl acetate (10:1, v:v) as a solvent to give the title product as a white solid (2.10 g, 65%).

Synthesis of 2-[4-(ethoxyoxalylamino)-phenoxy]-2-methyl-propionic acid ethyl ester sodium salt. To a solution of 2-[4-(ethoxyoxalylamino)-phenoxy]-2-methyl-propionic acid ethyl ester (20 mmol) in MeOH (30 mL), NaOH (0.78 g, 19.5 mmol) was added. The resulting mixture was refluxed for 2 h. Removal of the solvent resulted in the crude product, which was recrystallized employing ethyl acetate (50 mL) to give the title product as a white solid.

Synthesis of 2-[4-(ethoxyoxalylamino)-phenoxy]-propionic acid ethyl ester. To a solution of ethyl *N*-(4-hydroxyphenyl)oxamate (2.09 g, 10 mmol) in methylene chloride (150 mL), (S)-(-)-ethyl lactate (1.28 g, 11 mmol) and PPh_3 (3.15 g, 12 mmol) were added. DEAD (1.92 g, 11 mmol) was then slowly added into the mixture, while maintaining the temperature of the reaction mixture to about 20°C, and stirred at room temperature for 11 h. TLC showed that ethyl-*N*-(4-hydroxyphenyl)oxamate was consumed completely. Subsequently, H_2O (30 mL) was added into the resulting mixture. The mixture was stirred for additional 30 min. The mixture was then separated, and the organic phase was dried over $MgSO_4$. Removal of the solvent resulted in the crude product, which was purified by silica gel column chromatography employing a mixture of methylene chloride and ethyl acetate (10:1, v:v) as solvent to give the title product as a white solid (1.79 g, 58%).

Synthesis of 2-[4-(ethoxyoxalylamino)-phenoxy]-propionic acid ethyl ester sodium salt. To a solution of 2-[4-(ethoxyoxalylamino)-phenoxy]-propionic acid ethyl ester (6.2 g, 20 mmol) in MeOH (30 mL), NaOH (0.78 g, 19.5 mmol) was added. The resulting mixture was refluxed for 2 h. Removal of the solvent gave the crude product, which was recrystallized employing ethyl acetate (50

mL) to give the title product as a white solid.

Synthesis of *N*-(4-ethoxycarbonylmethoxyphenyl)-oxalamic acid ethyl ester. To a solution of ethyl *N*-(4-hydroxyphenyl)oxamate (2.09 g, 10 mmol) in CH_2Cl_2 (150 mL), ethyl glycolate (1.14 g, 11 mmol) and PPh_3 (3.15 g, 12 mmol) were added. DEAD (1.92 g, 11 mmol) was then added into the mixture slowly while maintaining the temperature of the reaction mixture to about 20°C. After the addition, the resulting solution was stirred at room temperature for 11 h. TLC showed that ethyl *N*-(4-hydroxyphenyl)oxamate was consumed completely. H_2O (30 mL) was then added into the resulting mixture. The mixture was stirred for additional 30 min. The mixture was separated, and the organic phase was dried over $MgSO_4$. Removal of the solvent resulted in the crude product, which was purified by silica gel column chromatography employing a mixture of methylene chloride and ethyl acetate (10:1, v/v) as a solvent to give the title product as a white solid (1.95 g, 66%).

Synthesis of *N*-(4-ethoxycarbonylmethoxy-phenyl)-oxalamic acid ethyl ester sodium salt. To a solution of *N*-(4-ethoxycarbonylmethoxyphenyl)-oxalamic acid ethyl ester (5.90 g, 20 mmol) in MeOH (30 mL), NaOH (0.78 g, 19.5 mmol) was added. The resulting mixture was refluxed for 2 h. The removal of the solvent resulted in the crude product, which was recrystallized employing ethyl acetate (50 mL) to give the title product as a white solid.

Synthesis of fluoro-substituted phenyloxalamic acid ethyl esters. To a solution of ethyl *N*-(4-hydroxyphenyl) oxamate (2.09 g, 10 mmol) in CH_3CN (30 mL), *p*-fluorobenzylbromide (102 mg, 10.2 mmol) and K_2CO_3 (1.52 g, 11 mmol) were added. The resulting solution was refluxed for 6 h. TLC (EtOAc/CH_2Cl_2, 1/10, v/v) showed that ethyl *N*-(4-hydroxyphenyl)oxamate was consumed completely. Methylene chloride (100 mL) and water (300 mL) were added into the resulting mixture. The mixture was stirred for additional 10 min. The mixture was filtered to remove the insoluble solid. The filtrate was separated, and the organic phase was dried over $MgSO_4$. Removal of the solvent resulted in the crude product, which was purified by silica gel column chromatography employing a mixture of methylene chloride and ethyl acetate as the solvent (5:1, v:v). The solid obtained was recrystallized employing a mixture of *n*-hexane and ethyl acetate (2:1, v:v) to give the title product as a white solid.

Tyrosinase assay. The reaction mixture (1 mL) contained 50 mM phosphate (pH 6.8), 1.5 mM L-tyrosine, and 20 mU tyrosinase. Enzyme activity was determined by measuring the increase in absorbance at 475 nm with a UV-VIS spectrophotometer (Perkin Elmer Bio30, Foster City, USA). The reaction mixture was preincubated at 25°C, and the enzyme reaction was initiated by adding tyrosinase. Enzyme inhibition was carried out by adding compounds at variable concentrations. IC_{50} indicates the concentration of inhibitor, at which half of the enzymes are inactivated. All tests have been carried out at least three times.

Enzyme Inhibition (%)=[1−enzyme activity in the presence of inhibitor enzyme activity in the absence of inhibitor]×100(%)

Cell culture. A human melanoma cell line (SK-MEL-2) was maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/mL theophylline. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Melanin inhibition assay. Human melanoma cells were seeded in 200 μL medium at a density of 1×10^4 cells per well in 96-well micro test tissue culture plates. After 24 h seeding, serial dilutions of MSH peptides were added to the wells, each containing 50 μL medium. All concentration points were assayed in triplicates. The cells were incubated for 3 days at 37°C and then equilibrated to ambient temperature and atmosphere. The absorbance of each well was measured in a reader at 405 nm. The absorbance values were compared with a standard curve obtained using synthetic melanin (Sigma Co., St. Louis, USA), which was dissolved in 0.85 N KOH, diluted in culture medium, and distributed in 96-well plates. The standard curve was linear within the range of experimental values.

MTT assay. MTT was dissolved in isotonic phosphate buffer (pH 7.4) solution at 5 mg/mL and filtered to sterilize and remove insoluble residues. Human melanoma cells were cultured in 96-well plates and incubated for 4 h in Dulbecco's Modified Eagle's Medium. Cell survival was assayed by measuring the conversion of yellow, water-soluble tetrazolium MTT into blue, water-insoluble formazan. The optical density at 570 nm (proportional to the number of surviving cells) relative to that of 630 nm was assessed with a microplate reader (Bio Rad 550).

Results and Discussion

Synthesis of hydroxyaniline derivatives. Thirty-four hydroxyaniline derivatives were synthesized, of which twelve compounds showed melanin biosynthesis-inhibitory activities (Fig. 1).

Comparison of tyrosinase inhibitory activities by hydroxyaniline derivatives. Synthesized hydroxyaniline derivatives were dissolved in a solvent and tested for their inhibitory activity on tyrosinase. Based on inhibition

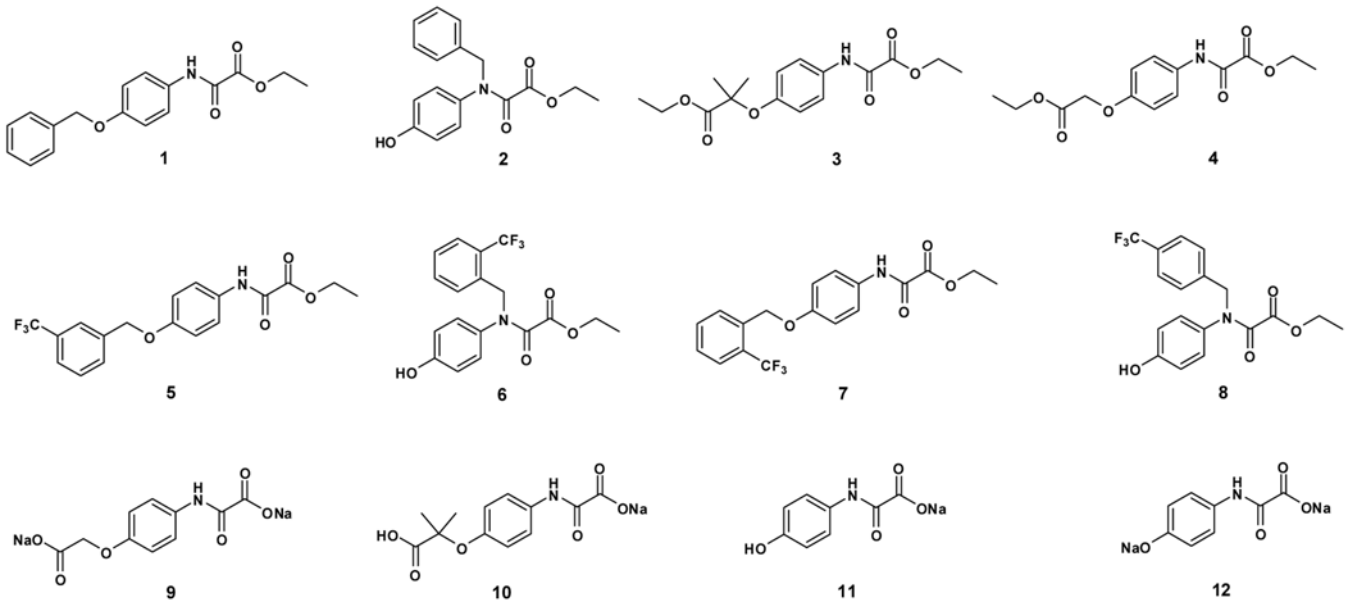


Fig. 1. The chemical structure of hydroxyaniline derivatives synthesized in this work.

activity, IC_{50} were obtained for the top 12 inhibitory derivatives (Table 1), among which, compounds **1**, **5**, **11**, and **12** showed the highest inhibitory activity, with IC_{50} of 92.5, 82.7, 12.9, and 12.3 μM, respectively. Therefore, compounds **11** (EHPONa) and **12** (EHPONa$_2$) were chosen for further study. For comparison with known commercial inhibitors, IC_{50} were obtained under identical conditions. Table 2 shows that inhibitory activity are in the order of kojic acid, compound **11,** compound **12,** ethyl-4-nitrobenzoylacetate, 3-hydroxy-4-methoxycinnamic acid, 4-hydroxyacetophenone, 4-hydroxycinnamic acid, 2,5-dihydroxybenzaldehyde, *trans*-4-hydroxycinnamic acid, 4-hydroxybenzoic acid, arbutin, 2,5-dihydroxybenzoic acid, ethyl-4-nitrophenylglyoxylate, and 2,4-dihydroxybenzoic acid. Results showed **12** could replace kojic acid, the use of which is prohibited in most countries due to its susceptibility to carcinogenesis. The inhibitory activities of **11** and **12** were more potent than that of arbutin, showing tyrosinase activity with IC_{50} values of 1.17 mM. From the structure-activity point of view, we have found that a *para*-configuration of hydroxyl group with an oxamate moiety could contribute to the inhibitory activity against tyrosinase enzyme. However, hydroxiniline derivatives with an alkyl substituent were less inhibitive against tyrosinase activity, indicating that bulky hydroxyniline derivatives do not fit into the active site of tyrosinase. Based on the reported $IC_{50,}$ **11** and **12** are more potent inhibitors than flavonoids, which have been recently spotlighted. The reported IC_{50} of flavonoids are

Table 1. IC_{50} of hydroxyaniline derivatives

Compound	IC_{50} (μM)	Solvent
1	92.5	ethanol
2	609.1	methanol
3	203.8	ethanol
4	195.5	ethanol
5	82.7	DMSO*
6	311.7	DMSO
7	301.1	DMSO
8	160.0	DMSO
9	143.2	H_2O
10	319.9	H_2O
11	12.9	H_2O
12	12.3	H_2O

*DMSO represents dimethylsulfoxide

Table 2. IC_{50} of various tyrosinase inhibitors

Compound	IC_{50}	Solvent
Compound **11**	12.9 μM	H_2O
Compound **12**	12.3 μM	H_2O
Kojic acid	12.5 μM	H_2O
Ethyl-4-nitrobenzoylacetate	16.3 μM	methanol
3-Hydroxy-4-methoxyacetophemone	51.2 μM	methanol
4-Hydroxybenzaldehyde	88.6 μM	H_2O
4-Hydroxycinnamic acid	89.5 μM	ethanol
2,5-Dihydroxybenzaldehyde	107.6 μM	ethanol
trans-4-Hydroxycinnamic acid	145.2 μM	ethanol
4-Hydroxybenzoic acid	201.4 mM	H_2O
Arbutin	221.7 mM	H_2O
2,5-Dihydroxybenzoic acid	560.6 mM	H_2O
Ethyl-4-nitrophenylglyoxylate	625.3 mM	ethanol
2,4-Dihydroxybenzoic acid	752.9 mM	H_2O

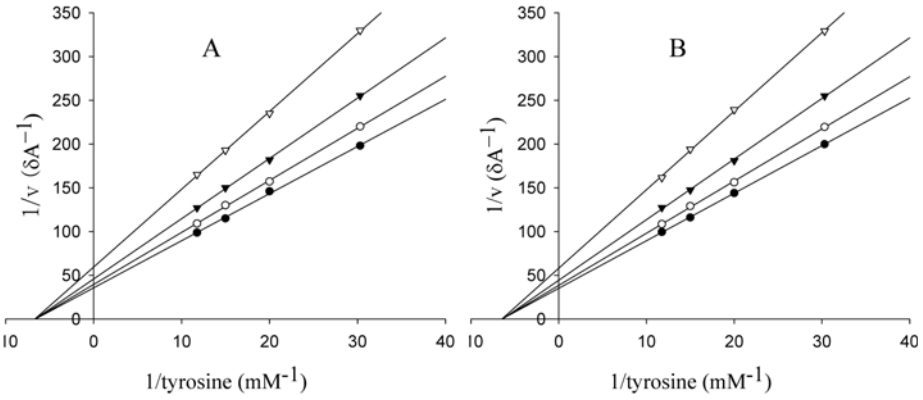


Fig. 2. Lineweaver-Burk plots for inhibition of compounds 33 (A) and 34 (B) on monophenolase activity of mushroom tyrosinase. Concentrations of the inhibitor for curves were ●-●: 0 μM, ○-○: 6.7 μM, ▼-▼: 16.7 μM, ▽-▽: 32 μM.

quercetin 0.07 mM [Kubo *et al*., 2000], kaempferol 0.23 mM [Kubo *et al*., 2000], luteolin 0.19 mM [Kubo *et al*., 2000], morin 2.32 mM [Kubo *et al*., 2000], aloesin 10 mM [Fujita *et al*., 1995], cuminaldehyde 0.05 mM [Kubo and Kinst-Hori, 1988], cumic acid 0.26 mM [Kubo and Kinst-Hori, 1988], 3,4-dihydroxycinnamic acid 0.97 mM [Lee, 2002], 4-hydroxy-3-methoxycinnamic acid 0.33 mM [Lee, 2002], anisaldehyde 0.38 mM [Lee, 2002].

Inhibition pattern of compounds 11 and 12. To determine the type of inhibition of compounds **11** and **12**, tyrosinase was assayed at varying concentrations of L-tyrosine and inhibitors. Fig. 2 shows that double-reciprocal plots of compounds **11** and **12** yielded a family of straight lines with a common intercept on the x-axis, but with different slopes, indicating that they are noncompetitive inhibitors of monophenolase activity of mushroom tyrosinase.

Kojic acid showed a competitive inhibitory effect on the monophenolase activity and a mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase [Battaini *et al*., 2005]. Kanade *et al*., [2007] reported that quercetin and kaempferol competitively inhibited tyrosinase by their ability to chelate cupric ion at the active site, leading to the inactivation of tyrosinase. Earlier studies also indicated that, with respect to both monophenolase [Jin *et al*., 1999] and diphenolase [Yagi *et al*., 1987] activities of mushroom tyrosinase, oxyresveratrol is a noncompetitive inhibitor, whereas arbutin is a competitive inhibitor. It should be noted that monophenol reacts with oxytyrosinase instead of mettyrosinase to generate *o*-quinone. As mentioned above, compounds **11** and **12** each showed noncompetitive inhibitions, whereas both kojic acid and arbutin demonstrated competitive inhibitions for monophenolase activity.

Inhibition of melanin biosynthesis by compounds 11 and 12. Compounds **11** and **12** showed 54.1 and 55.3% inhibition at 0.15 mM, and 69.2 and 70.2% inhibition at 0.3 mM in melanin biosynthesis inhibition using human melanocyte (SK-MEL-2), respectively (Table 3). Accordingly, this is higher inhibition, compared with kojic acid 28.7% and arbutin 8.2% at 0.15 mM and 41.6 and 12.1% inhibition at 0.3 mM. Here, we observed that inhibition activities for mushroom tyrosinase are not proportional to inhibition activities for melanin biosynthesis, as the source of each enzyme differs in its respective inhibition activities. However, the data on the inhibition of tyrosinase and melanin biosynthesis imply that compounds **11** and **12**, including kojic acid and arbutin, inhibit melanin biosynthesis at an enzyme level rather than nucleic acid or post melanin biosynthesis level. On the other hand, compounds **11** and **12**, together with arbutin and kojic acid did not show cytotoxic effects at concentrations up to 0.5 mM in the cultured human melanoma cells. These results indicate **11** and **12** can be utilized for the development of a new candidate for the treatment of tyrosinase-related disorders.

Table 3. Effects of tyrosinase inhibitors on melanin biosynthesis

Compound	Concentration (mM)	Inhibition of melanin biosynthesis (%)
Control	-	-
Kojic acid	0.15	28.7
	0.30	41.6
Arbutin	0.15	8.2
	0.30	12.1
Compound **11**	0.15	54.1
	0.30	69.2
Compound **12**	0.15	55.3
	0.30	70.2

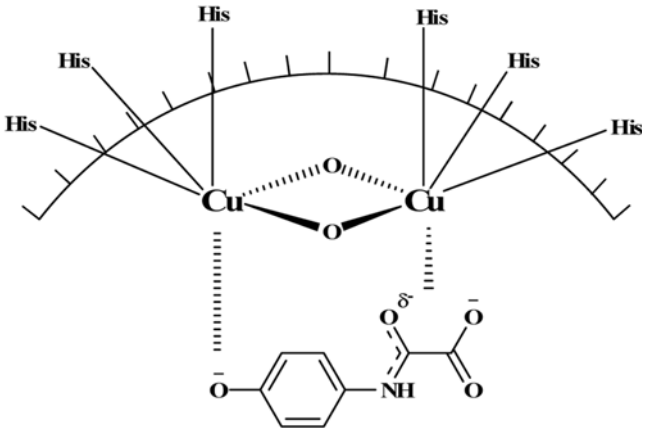
Table 4. Effect of $CuCl_2$ on tyrosinase inhibition by compound 12*

$CuCl_2$ (μM)	Relative Activity (%)
Control	100
Control (+compound **12**)	20.8
17 (+compound **12**)	51.6
33 (+compound **12**)	90.5
60 (+compound **12**)	98.5

*The reaction mixture (1 mL) contained 50 mM phosphate (pH 6.8), 1.5 mM L-tyrosine and 20 mU tyrosinase
*Final concentration of compound **12** was 40 μM

Chelation of copper ion by compounds 11 and 12. Tyrosinase has a pair of copper ions in the active site and is classified into three types depending on the function of copper ions and the presence of oxygen. Oxy-tyrosinase (E_{oxy}, Cu^{2+}-O_2-Cu^{2+}) is active and contains a pair of divalent copper ion and O_2. Each cupric ion is coordinated by three amino groups of histidines, with a weak axial and two strong equatorial bondings. O_2 binds as a peroxide to the site, forming a bridge between the two cupric ions. Met-tyrosinase (E_{met}, Cu^{2+}-O-Cu^{2+}) is active and contains a pair of divalent copper ion and atomic oxygen forming a tetragonal bicupric structure with an oxygen bridge. On the other hand, inactive deoxy-tyrosinase (E_{deoxy}, Cu^{+}-Cu^{+}) contains monovalent copper ion but no oxygen. Thus, an oxygen bridge is not observed in this structure [Kim and Uyama, 2005]. A study focusing on the catalytic mechanism for monophenolase and diphenolase activities of tyrosinase [Likhitwitayawuid, 2008] indicated a characteristic lag phase in monophenolase activity, as tyrosinase contains 15% oxy sites in its resting form [Solomon *et al*., 1996]. To determine the effects of copper ion on the inhibitory activities of **11** and **12**, copper chloride was added to the reaction mixture. Table 4 shows copper ion reduces the inhibitory activities of **11** and **12**, indicating that **11** and **12** inhibit tyrosinase by chelating copper ion at the active site. Lerch [1988] reported that six histidines in the active site are similar to six histidines in hemocynin, two of which chelate with two copper ions.

A recent study by Matoba *et al*. [2006] on the crystallographic structure of *Streptomyces castaneo-globisporus* tyrosinase examined its three-dimensional structure and catalytic mechanism. Their experiments focused on two sites of combination: the binuclear copper ion active center and the adjoining hydrophobic enzyme active site, composed of functional amino acids such as Ile 42, Arg 55, Glu 182, Trp 184, His 190, and Ala 202. Our data suggest that **11** and **12** interact with binuclear copper ions. In conclusion, 4-hydroxyl oxygen and oxamate oxygen moiety of **11** and **12** are proposed to chelate the two cupric ions at the active site as shown in Scheme 1.



Scheme 1. Proposed model of ethyl-(4-hydroxyphenyl) oxamate (di-sodium salt) binding to the cupric ion in the active site of tyrosinase.

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