Synthesis and Biological Evaluation of Kojic acid Derivatives as Tyrosinase Inhibitors

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Tyrosinase is a copper-containing enzyme which is widely distributed in nature. This enzyme is associated with the production of melanin for the protection of the skin from solar irradiation. However, overproduction of melanin in the skin is of particular concern to woman, for example, in the conditions melasma and lentigo. Tyrosinase catalyzes the first two steps of melanin biosynthesis, namely the hydroxylation of L-tyrosine to L-dopa and the subsequent oxidation of L-dopa to dopaquinone. Tyrosinase is contained in vegetables and fruits and is responsible for the undesirable enzymatic browning that occurs upon long term storage of these.

Therefore, tyrosinase inhibitors have attracted attention as important depigmenting agents for the treatment of hyperpigmentation and as anti-browning agents in vegetables and fruits. Kojic acid (1) is a well known tyrosinase inhibitor. However, its inhibitory activity is not potent enough for the above purposes. To overcome this drawback, many semi-synthetic kocialic acid derivatives have been synthesized, usually by modification of the C-2 hydroxyl group. Recently, we synthesized kocialic acid derivatives containing thioether and ester linkers and evaluated their tyrosinase inhibitory activities. Among them, 2-((cyclohexylthio)methyl)-5-hydroxy-4H-pyran-4-one (2b), showed the most potent inhibitory activity. Compound 2b is composed of three parts, including the kocialic acid moiety, a thioether linker, and a hydrophobic cyclohexane group. We synthesized and evaluated additional compounds to get more structural insight into the tyrosinase inhibitory activity of the kocialic acid derivatives. The crystallographic structure of tyrosinase has recently been established, enabling closer examination of its three-dimensional structure, which revealed the presence of a hydrophobic protein pocket adjoining the binuclear copper active site. We also conducted a molecular docking study to elucidate the structural importance of the kocialic acid derivatives in binding with the active site of tyrosinase.

The synthetic pathways of the kocialic acid derivatives are shown in Scheme 1. Thioether derivatives (2a-2e) were synthesized by the condensation of koyal chloride with the potassium salts of thiols. Ester derivatives (3a-3e) were synthesized by the condensation of koyal chloride with the potassium salts of acids.

The inhibitory activities of the kocialic acid derivatives on mushroom tyrosinase were investigated using koyalic acid as a positive control. The results are summarized in Table 1.

Figure 1. Structures of the kocialic acid derivatives.

Scheme 1. Reaction conditions: (a) thionyl chloride (1.5 equiv.), dimethylformamide, room temperature, 0.5 h, 72% yield; (b) the potassium salts of thiols (1.0 equiv.), dimethylformamide, 110 °C-120 °C, 1 h, 65%-81% yields; (c) the potassium salts of acids (1.0 equiv.), dimethylformamide, 110 °C-120 °C, 1 h, 70%-84% yields.
Kojic acid (1) inhibited mushroom tyrosinase with an IC\textsubscript{50} value of 50.07 µM. Dramatically enhanced activities were observed with the thioether compounds (2a-2e). Compound 2a, which contains a hexyl group, showed a potent inhibitory activity (IC\textsubscript{50} = 0.87 µM). Compound 2b, 2-((cyclohexylthio)methyl)-5-hydroxy-4H-pyran-4-one, showed the most potent inhibitory activity (IC\textsubscript{50} = 0.25 µM). However, compounds with a greater hydrophobic character, for example, those with the adamantine or planar aromatic groups such as phenyl and naphthyl, had a negative influence on inhibitory activity (2c, 2d, and 2e). The activities of compounds containing an ester linker were lower than those of the thioether compounds. Compound 3b, (5-hydroxy-4-oxo-4H-pyran-2-yl)methyl cyclohexanecarboxylate, exhibited decreased activity (IC\textsubscript{50} = 2.74 µM). The ester derivatives (3a-3e) were found to show a similar activity trend to the thioether derivatives. The adamantanyl compound (3e) and phenyl compound (3d) showed decreased inhibitory activities (IC\textsubscript{50} = 3.26 µM and 5.90 µM, respectively). In the naphthyl compound 3e, a greater decrease in activity was observed (IC\textsubscript{50} = 21.01 µM). In the tyrosinase assay, the thioether linkage, and flexible and bulky naphthyl group caused a steric hindrance. The planar and bulky naphthyl group caused a steric hindrance that changed its binding to the active site of tyrosinase (Fig. 2(c)). For this reason, ligand 3e also lost important interactions, such as the H-bond with peroxide and π–π interaction with His 194 in the active site. After evaluating the tyrosinase inhibitory activity, we selected compound 2b as a candidate for an effective depigmenting agent and evaluated its inhibitory potency against melanin formation. The results are summarized in Table 3.

In this study, B16/F1 melanoma cells were used without α-melanocyte stimulating hormone treatment as a stimulant of melanogenesis. We treated B16/F1 melanoma cells with compound 2b at various concentrations (2.5 µM-20 µM). A higher docked score (5.51); whereas compound 3e, showed decreased tyrosinase inhibitory activity (IC\textsubscript{50} = 21.01 µM) and exhibited a lower docked score (3.98). The selected docked conformations of compounds 2b, 3b, and 3e in the tyrosinase binding site are shown in Figure 2.

The major factors for a tight binding mode are an H-bonding interaction between the oxygen atoms of the ligands and a π–π interaction with His 194 in the active site. Compound 2b had a stronger interaction with peroxide, with a hydrogen bond distance of 2.80 Å, than that of compound 3b (3.34 Å). Moreover, compound 2b also formed H-bonding interactions with residue Ser 206 in the binding pocket. In addition, a π–π interaction between compound 2b and His 194 was found, and the position of the pyranone ring of kojic acid was nearly identical to that of superimposed L-tyrosinase (Fig. 2(a)). However, compound 3e (3.98) exhibited a different binding pattern due to the naphthyl moiety. The planar and bulky naphthyl group caused a steric hindrance that changed its binding to the active site of tyrosinase (Fig. 2(c)). For this reason, ligand 3e also lost important interactions, such as the H-bond with peroxide and π–π interaction with His 194 in the active site.

To understand the binding mode of the kojic acid derivatives in the active site of tyrosinase, we conducted a modeling study. The results are summarized in Table 2.

Table 1. Inhibitory activities of the kojic acid derivatives (2a-2e) and (3a-3e) on mushroom tyrosinase

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} (µM)</th>
<th>Compounds</th>
<th>IC\textsubscript{50} (µM)</th>
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<tbody>
<tr>
<td>2a</td>
<td>0.87</td>
<td>3a</td>
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</tr>
<tr>
<td>2b</td>
<td>0.25</td>
<td>3b</td>
<td>2.74</td>
</tr>
<tr>
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<td>2d</td>
<td>1.59</td>
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<tr>
<td>2e</td>
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<td>Kojic acid</td>
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Table 2. Docked scores of the kojic acid derivatives (2a-2e) and (3a-3e)

<table>
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<th>Compounds</th>
<th>Docked score*</th>
<th>Compounds</th>
<th>Docked score*</th>
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<td>2b</td>
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<tr>
<td>Kojic acid</td>
<td>3.69</td>
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</table>

*Values were determined from the logarithmic concentration-inhibition curves and are given as the mean values of the results of three experiments.

Notes

*aMolecular modeling study was carried out using SYBYL-X version 1.2.

Figure 2. Docked conformation of compounds 2b, 3b, and 3e in the binding site of tyrosinase: (a) compound 2b, (b) compound 3b, (c) compound 3e.
slight decrease in cell viability was observed at 20 μM concentration. After confirming cell viability, we evaluated the depigmenting activity of compound 2b at the same concentrations (2.5 μM-20 μM). Lower melanin content (%) equated to a higher inhibition of melanin synthesis. Compound 2b significantly inhibited melanogenesis in a dose-dependent manner. At concentrations of 5 μM and 10 μM, the melanin content of compound 2b was 74.41% and 48.38%, respectively. However, kojic acid (1) inhibited melanin production at higher concentrations (0.25 mM-2 mM). Thus, compound 2b, containing a thioether linkage and hydrophobic cyclohexane group, is more effective than kojic acid and kojic acid derivatives (2a-3e) in inhibiting melanin production.

In conclusion, we synthesized a series of kojic acid thioether derivatives (2a-2e) and kojic acid ester derivatives (3a-3e). The tyrosinase inhibitory activities for the ten synthesized compounds were evaluated to investigate the structure-activity relationships (SAR). The molecular docking results closely agreed with the observed in vitro data and clearly explained the SAR pattern. Among them, 5-hydroxy-2-(cyclohexylthio)methyl-4H-pyran-4-one (2b) exhibited the most potent tyrosinase inhibitory activity (IC_{50} = 0.25 μM). In the cell-based assay, compound 2b decreased the melanin content in a dose-dependent manner without cytotoxicity. Overall, these results suggest that compound 2b could be used as an effective depigmenting agent.

### Experimental Section

**5-Hydroxy-2-((phenylthio)methyl)-4H-pyran-4-one (2d).** To a stirred solution of kojyl chloride 4 (4.80 g, 30.0 mmol) in DMF (100 mL) under N₂, was added potassium benzenethiolate (4.43 g, 30.0 mmol). The reaction mixture was stirred for 1 h at 110 °C-120 °C, after which DMF was evaporated in vacuo. To the residue, was added 100 mL water. The organic layer was dried with anhydrous MgSO₄ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give 2d (5.18 g) in 74% yields.

**Mushroom Tyrosinase Assay.** Mushroom tyrosinase and l-tyrosine were purchased from Sigma Chemical (Saint Louis, Missouri, USA). The reaction mixture for mushroom tyrosinase activity consisted of 150 μL of 0.1 M phosphate buffer (pH 6.5), 3 μL of sample solution, 8 μL of mushroom tyrosinase (2,100 unit/mL, 0.05 M phosphate buffer at pH 6.5), and 36 μL of 1.5 mM l-tyrosine. Tyrosinase activity was determined by reading the optical density at 490 nm using a microplate reader (Bio-Rad 3550, Richmond, CA, USA) after incubation for 20 min at 37 °C. The inhibitor activity of each sample is expressed as the concentration that inhibits enzyme activity by 50% (IC$_{50}$).

**Cell Culture.** The B16/F1 melanoma cells line was obtained from the Korean cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco’s Modified Eagle medium (DMEM) containing Fetal Bovine Serum (FBS, 10%), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) at 37 °C in a humidified atmosphere of 5% CO₂.

**Measurements of Cell Viability.** Cell viability was measured using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were plated in 96-well plates and cultured for 24 h. After treatment with kojic acid and kojic acid derivatives (2a-2e) and (3a-3e), 100 μL MTT (5 mg/mL in PBS) was added to each well. Cells were incubated at 37 °C for 30 min, dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and the absorbance was measured at 560 nm using a microplate reader (Bio-Rad 3550, Richmond, CA, USA). The inhibitory activity of each sample is expressed as the concentration that inhibits enzyme activity by 50% (IC$_{50}$).
Measurements of Melanin Content. Cells (2 × 10^4 cells/mL) were seeded into 24-well plates and dicinnamoylamide derivatives were added in triplicate. The medium was changed daily and after 4 d of culture, the cells were lysed with 0.1 mL of 1 N NaOH. Then 100 μL of each crude cell extract was transferred to a 96-well plate. Relative melanin content was measured at 400 nm with a microplate reader (Molecular Devices).

References