Synthesis and Biological Evaluation of Kojic acid Derivatives as Tyrosinase Inhibitors

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Tyrosinase[1] 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.[2] 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.[3] 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[4] (**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 kojic acid derivatives have been synthesized, usually by modification of the C-2 hydroxyl group.[5] Recently, we synthesized kojic acid derivatives containing thioether[6] and ester[7] linkers and evaluated their tyrosinase inhibitory activities. Among them, 2-((cyclohexylthio)methyl)-5-hydroxy-4*H*-pyran-4-one (**2b**), showed the most potent inhibitory activity. Compound **2b** is composed of three parts, including the kojic 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 kojic 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.[8] We also conducted a molecular docking study to elucidate the structural importance of the kojic acid derivatives in binding with the active site of tyrosinase.

The synthetic pathways of the kojic acid derivatives are shown in Scheme 1. Thioether derivatives (**2a-2e**) were synthesized by the condensation of kojyl chloride **4** with the potassium salts of thiols. Ester derivatives (**3a-3e**) were synthesized by the condensation of kojyl chloride **4** with the potassium salts of acids.

The inhibitory activities of the kojic acid derivatives on mushroom tyrosinase were investigated using kojic acid as a positive control. The results are summarized in Table 1.
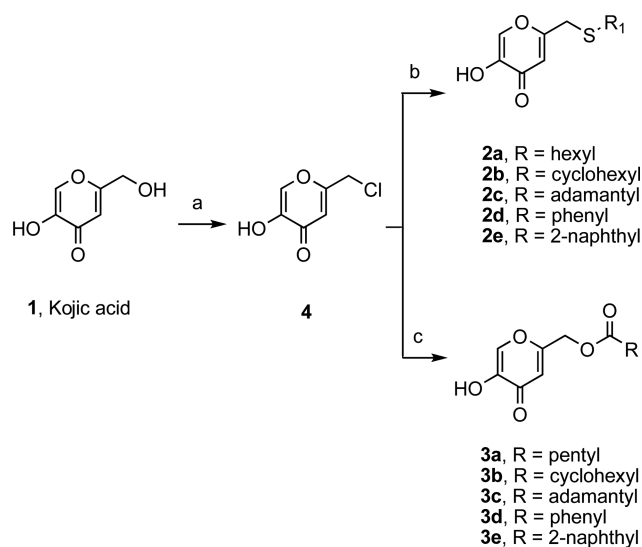
Scheme 1. Reaction conditions: (a) thionyl chloride (1.5 equiv.), dimethylformamide, room temperature, 0.5 h, 72% yield; (b) the potassium salts of thiols (1.0 equiv.), dimethylformamide, 110 °C-120 °C, 1 h, 65%-81% yields; (c) the potassium salts of acids (1.0 equiv.), dimethylformamide, 110 °C-120 °C, 1 h, 70%-84% yields.
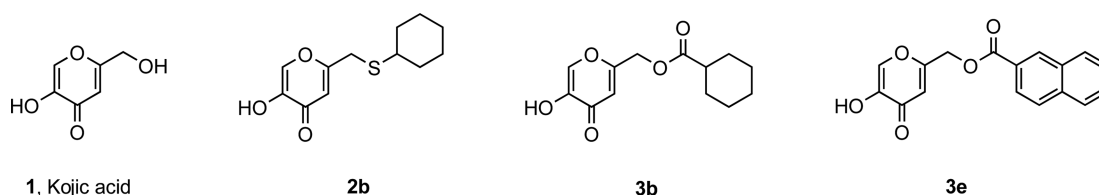

Figure 1. Structures of the kojic acid derivatives.

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

Compounds	$IC_{50}{}^a$	Compounds	$IC_{50}{}^a$
2a	0.87 μM	**3a**	2.55 μM
2b	0.25 μM	**3b**	2.74 μM
2c	1.39 μM	**3c**	3.26 μM
2d	1.59 μM	**3d**	5.90 μM
2e	1.10 μM	**3e**	21.01 μM
Kojic acid	50.07 μM		

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

Table 2. Docked scores of the kojic acid derivatives (**2a-2e**) and (**3a-3e**)

Compounds	Docked score^a	Compounds	Docked score^a
2a	5.43	**3a**	4.45
2b	5.51	**3b**	4.50
2c	5.18	**3c**	4.39
2d	4.89	**3d**	3.84
2e	5.22	**3e**	3.98
Kojic acid	3.69		

^{a}Molecular modeling study was carried out using SYBYL-X version 1.2

Kojic acid (**1**) inhibited mushroom tyrosinase with an IC_{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 ($IC_{50} = 0.87$ μM). Compound **2b**, 2-((cyclohexyl-thio)methyl)-5-hydroxy-4*H*-pyran-4-one, showed the most potent inhibitory activity ($IC_{50} = 0.25$ 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-4*H*-pyran-2-yl)methyl cyclohexanecarboxylate, exhibited decreased activity ($IC_{50} = 2.74$ μM). The ester derivatives (**3a-3e**) were found to show a similar activity trend to the thioether derivatives. The adamantanyl compound (**3c**) and phenyl compound (**3d**) showed decreased inhibitory activities ($IC_{50} = 3.26$ μM and 5.90 μM, respectively). In the naththyl compound **3e**, a greater decrease in activity was observed ($IC_{50} = 21.01$ μM). In the tyrosinase assay, the thioether linkage, and flexible normal alkyl and cycloalkyl groups were critical factors for the inhibitory activity.

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.

The docking results agreed with the observed *in vitro* data, which showed that compound **2b** had the most potent tyrosinase inhibitory activity ($IC_{50} = 0.25$ μM), exhibiting a higher docked score (5.51); whereas compound **3e**, showed decreased tyrosinase inhibitory activity ($IC_{50} = 21.01$ 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 planer 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
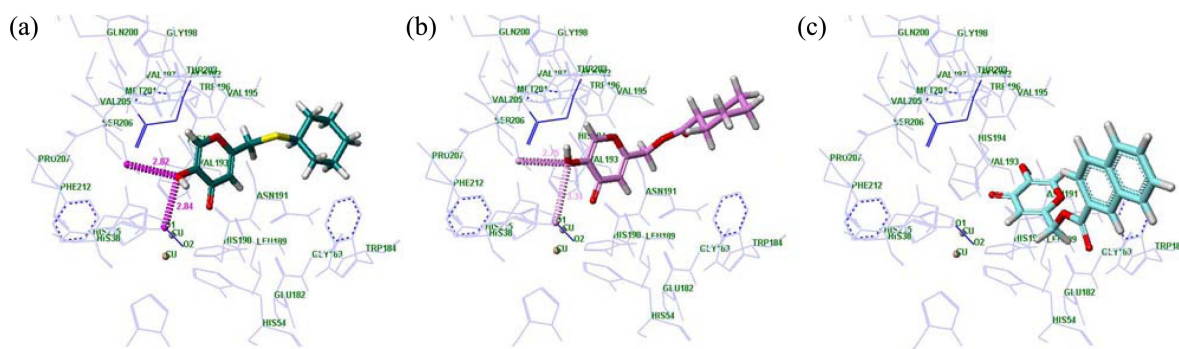

Figure 2. Docked conformation of compounds **2b**, **3b**, and **3e** in the binding site of tyrosinase: (a) compound **2b**, (b) compound **3b**, (c) compound **3e**.

Table 3. Depigmenting activities of compound **2b** and kojic acid

Compound	Concentration	Melanin production (%)[a]	Cell viability (%)[a]
2b	2.5 µM	92.11	100.87
	5 µM	74.41	96.62
	10 µM	48.38	94.88
	20 µM	18.30	76.30
Kojic acid	0.25 mM	59.57	99.96
	0.5 mM	41.25	96.19
	1 mM	33.12	93.55
	2 mM	28.32	90.47

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

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 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)-4*H*-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)-4*H*-pyran-4-one (2d). To a stirred solution of kojyl chloride **4** (4.80 g, 30.0 mmol) in DMF (100 mL) under N$_2$ was added potassium benzenethiolate (4.43 g, 30.0 mmol). The reaction mixture was stirred for 1 h at 110 ºC-120 ºC, after which DMF was evaporated *in vacuo*. The residue was extracted with ethyl acetate (500 mL), washed with water. The organic layer was dried with anhydrous MgSO$_4$ 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.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (s,1H), 7.97 (s, 1H), 7.26-7.35 (m, 5H), 6.21 (s, 1H), 4.10 (s, 2H). ^{13}C-NMR

(125 MHz, DMSO-d_6) δ 174.0, 164.2, 146.1, 140.26, 140.21, 134.3, 130.2, 129.6, 127.4, 113.1, 112.9, 34.8. FABMS: (*m/e*) 235 [M+H]$^+$.

(5-Hydroxy-4-oxo-4*H*-pyran-2-yl)methyl benzoate (3d). To a stirred solution of kojyl chloride **4** (4.80 g, 30.0 mmol) in DMF (100 mL) under N$_2$ was added potassium benzoate (4.79 g, 30.0 mmol) with benzoic acid (2.74 g, 22.5 mmol). The reaction mixture was stirred for 1 h at 110 ºC-120 ºC, after which DMF was evaporated *in vacuo*. The residue was extracted with ethyl acetate (500 mL), washed with water. The organic layer was dried with anhydrous MgSO$_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give **3d** (5.00 g) in 68 % yields.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.11 (s, 1H), 8.02 (m, 2H), 7.71 (m, 1H), 7.58 (m, 2H), 6.55 (s, 1H), 5.22 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.5, 164.9, 161.4, 146.0, 139.9, 133.7, 129.3, 128.8, 128.7, 112.6, 61.9. FABMS, *m/e* 245.1 [M-H]$^+$.

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 ºC. The inhibitory activity of each sample is expressed as the concentration that inhibits enzyme activity by 50% (IC$_{50}$).

Molecular Modeling. Molecular modeling study was carried out on linux system using SYBYL-X version 1.2. To prepare the tyrosinase structure, the crystal structure of the oxy form of S. castaneoglobisporus tyrosinase was taken from the Protein Data Bank (PDB cod 1 wx2) because there is no crystal structure of mushroom tyrosinase published yet. The caddie protein and water molecules were removed. Hydrogen atoms were added to the enzyme using the SYBYL. For the molecular docking method, Surflex-Dock version 2.5 was used using standard parameters and allowing the hydrogen of protein movement.

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 ºC in a humidified atmosphere of 5% CO$_2$.

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 ºC 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 a (Molecular Devices Co., Sunnyvale, CA, USA).

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).

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