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Novel synthetic kojic acid-methimazole derivatives inhibit mushroom tyrosinase and melanogenesis

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In this study, two kojic acid-methimazole (2-mercapto-1-methylimidazole, MMI, 1) derivatives, 5-hydroxy-2-{[(1-methyl-1H-imidazol-2-yl)thio]methyl}-4H-pyran-4-one (compound 4) and 5-methoxy-2-{[(1-methyl-1H-imidazol-2-yl) thio]methyl}-4H-pyran-4-one (compound 5), were synthesized to examine their inhibitory kinetics on mushroom tyrosinase. Compound 4 exhibited a potent inhibitory effect on monophenolase activity in a dose-dependent manner, with an IC_{50} value of 0.03 mM. On diphenolase activity, compound 4 exhibited a less inhibitory effect (IC_{50} = 1.29 mM) but was stronger than kojic acid (IC_{50} = 1.80 mM). Kinetic analysis indicated that compound 4 was both as a noncompetitive monophenolase and diphenolase inhibitor. By contrast, compound 5 exhibited no inhibitory effects on mushroom tyrosinase activity. The IC_{50} value of compound 4 for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was 4.09 mM, being much higher than the IC_{50} of compound 4 for inhibiting the tyrosinase activity. The results indicated that the antioxidant activity of compound 4 may be partly related to the potent inhibitory effect on mushroom tyrosinase. Compound 4 also exerted a potent inhibitory effect on intracellular melanin formation in B16/F10 murine melanoma cells, and caused no cytotoxicity. Furthermore, compound 4 induced no adverse effects on the Hen's egg test-chorioallantoic membrane (HET-CAM).

[**Key words:** Kojic acid; Methimazole; Mushroom tyrosinase; Kinetics; Melanin]

Tyrosinase (EC 1.14.18.1), a copper-containing enzyme, is widely distributed in fungi, plants, and animals (1). Tyrosinase is ubiquitously distributed in organisms, and has multicatalytic functions namely the hydroxylation of tyrosine to generate L-dopa (monophenolase activity), and oxidation of L-dopa to generate dopaquinone (diphenolase activity) (2). Tyrosinase expression or activity is responsible for pigmentation disorders (3). Therefore, tyrosinase is a crucial target for treating pigmentation disorders. Moreover, tyrosinase has also been reported to be related to Parkinson's disease and other neurodegenerative diseases (4,5). Three types of tyrosinase (oxy-, met-, and deoxytyrosinase), exhibiting different binuclear copper structures of the active sites, participate in the formation of melanin pigments (6,7). The two copper ions of tyrosinase, located in the active center of the enzyme, are individually connected to three histidine residues (8). Tyrosinase inhibitors can be classified into the following four types: competitive inhibitors, uncompetitive inhibitors, mixed-type inhibitors, and noncompetitive inhibitors (9).

In humans, the primary determinant of skin color is melanin. The melanin in the skin is produced by melanocytes, which are distributed in the basal layer of the epidermis (10). Melanin plays a crucial role in the function of the skin as a protective barrier by absorbing UV radiation, and thus protecting skin cells against UV radiation-induced damage (11). Therefore, tyrosinase inhibitors could be useful for maintaining food quality and developing cancer remedies, as well as for treating skin hyperpigmentation (12).

Kojic acid is a well-known antityrosinase agent, and is also a scavenger of free radicals (13,14). Kojic acid shows a competitive inhibitory effect on monophenolase activity, and a mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase (15). However, kojic acid has been reported with cytotoxicity and chemical instability during storage which may result in skin irritation. (16). Therefore, synthesized novel kojic acid derivatives with low toxicity and high effectiveness still have great potential in reducing melanogenesis.

Thiol containing compounds are crucial melanogenesis inhibitors, which react with dopaquinone to form colorless products and decrease melanin production (17,18). In our previous study, both 2-mercapto-1-methylimidazole (MMI, **1**) and ergothioneine (a natural amino acid that contains an MMI-like structure) exerted potent inhibitory effects on tyrosinase activity (19,20). Therefore, we synthesized kojic acid-methimazole derivatives 5-hydroxy-2-{[(1-methyl-1H-imidazol-2-yl)thio]methyl}-4H-pyran-4-one (compound **4**) and 5-methoxy-2-{[(1-methyl-1H-imidazol-2-yl) thio]methyl}-4H-pyran-4-one (compound **5**) (Fig. 1), and evaluated their inhibitory effects against tyrosinase, enzyme kinetic

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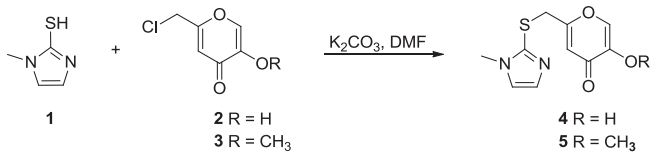

FIG. 1. Structures and synthetic pathway of kojic acid-methimazole derivatives.

parameters, intracellular melanin formation in B16/F10 murine melanoma cells and the Hen's egg test-chorioallantoic membrane (HET-CAM) for mucosa-irritating effects.

MATERIALS AND METHODS

Reagents Compound **4**/**5** were synthesized by our team. Mushroom tyrosinase, 3-(4-hydroxyphenyl)-L-alanine (L-tyrosine), 3,4-dihydroxy-L-phenylalanine (L-dopa), kojic acid, vitamin C, 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphate buffer solution dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), alpha-melanocyte-stimulating hormone (α-MSH), hydrogen peroxide, and magnesium sulfate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, phosphate buffer solution, penicillin G, streptomycin, and amphotericin were purchased from Gibco Life Technologies Inc. (Carlsbad, CA, USA). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Showa Chemical Industry Co. Ltd. (Japan). A B16/F10 mouse melanoma cell line was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan).

Synthetic methimazole derivatives The structures and synthetic pathways of kojic acid-methimazole derivatives are shown in Fig. 1. In brief, compound **4** was prepared by mixing compound **1** (0.92 g, 8.0 mmol) with potassium carbonate (1.11 g, 8.0 mmol) in 20 mL of *N*,*N*-dimethylformamide, followed by adding kojyl chloride (compound **2**; 1.29 g, 8.0 mmol). The mixture was stirred at room temperature for 20 h, and passed through a pad of Celite afterward. The residue was separated using chromatography over silica gel, and eluted with methanol/ethyl acetate (1/9) to afford 1.02 g compound **4** with a 54% yield (Fig. 1).

Compound **5** was prepared by mixing compound **1** (0.23 g, 2.0 mmol) with potassium carbonate (0.28 g, 2 mmol) in 6 mL of *N*,*N*-dimethylformamide, followed by adding 2-(chloromethyl)-5-methoxy-4*H*-pyran-4-one (compound **3**; 0.35 g, 2 mmol). The mixture was stirred at room temperature for 3 h, and passed through a pad of Celite afterward. The residue was separated using chromatography over silica gel, and eluted with methanol/ethyl acetate (1/9) to afford 456 mg of compound **5** with a 90% yield.

The products were purified through recrystallization from ethanol, and analyzed using nuclear magnetic resonance (NMR) and electron ionization mass spectrometry (EI-MS) for identification.

5-Hydroxy-2-{[(1-methyl-1H-imidazol-2-yl)thio]methyl}-4H-pyran-4-one(compound 4) Mp 166–167°C; ^{1}H NMR (CD_3OD, 400 MHz) δ 3.62 (s, 3 H), 3.96 (s, 2 H), 6.06 (s, 1 H), 7.04 (d, J = 1.0 Hz, 1 H), 7.20 (d, J = 1.0 Hz, 1 H), 7.90 (s, 1 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 34.1, 37.8, 113.4, 125.4, 130.3, 139.5, 141.4, 147.5, 166.1, 176.4; HRMS calcd for $C_{10}H_{10}N_2O_3S$ 238.0412, found 238.0413.

5-Methoxy-2-{[(1-methyl-1H-imidazol-2-yl)thio]methyl}-4H-pyran-4-one(compound 5) Mp 114–115°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.52 (s, 3 H), 3.71 (s, 3 H), 3.99 (s, 2 H), 6.15 (s, 1 H), 6.89 (d, J = 0.8 Hz, 1 H), 7.05 (d, J = 0.8 Hz, 1 H), 7.48 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 33.0, 35.8, 56.0, 113.4, 122.8, 129.7, 137.4, 138.4, 148.2, 163.5, 173.5; HRMS calcd for $C_{11}H_{12}N_2O_3S$ 252.0569, found 252.0563.

Tyrosinase activity assay In a 96-well plate, 20 μL of various concentrations of mushroom tyrosinase were mixed with 20 μL of various concentrations of compound **4** (21). After 160 μL of 0.5 mM L-tyrosine or L-dopa was added to each solution, we monitored the formation of dopachrome for 10 min by measuring the linear increase in optical density at 475 nm. The reaction was conducted at a

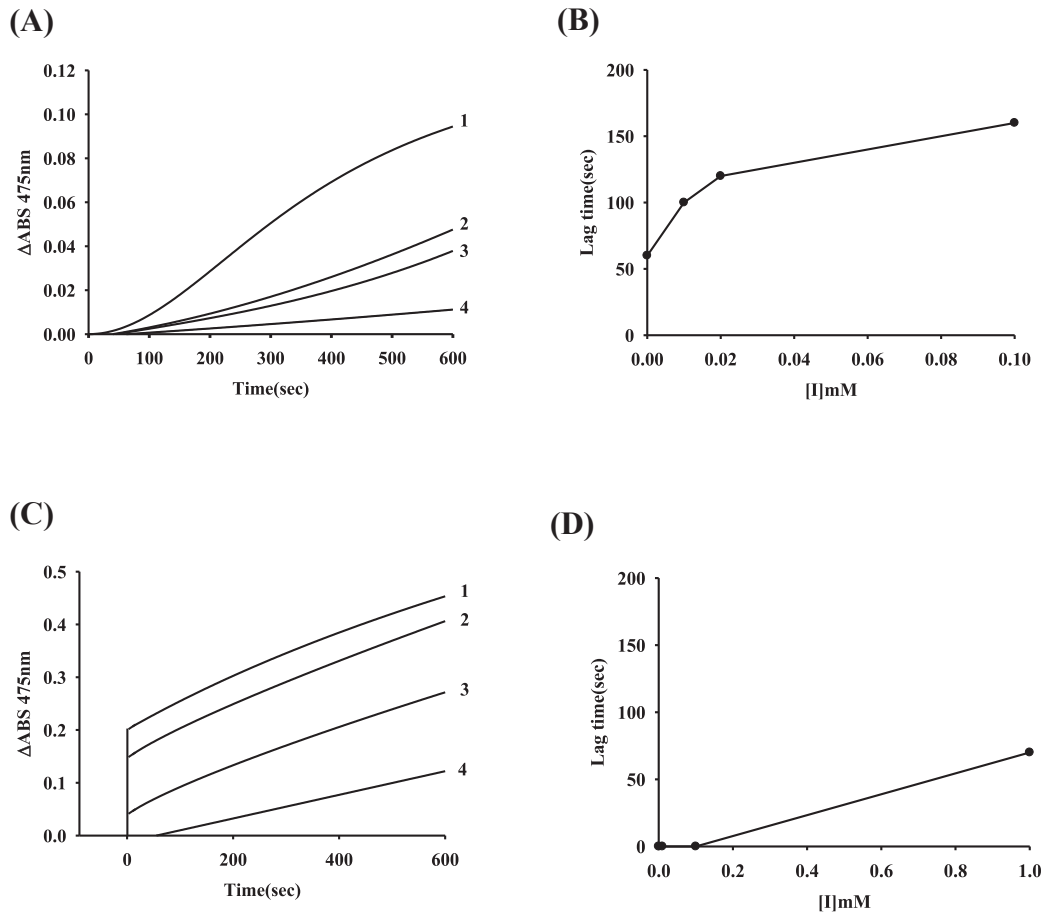

FIG. 2. Effects of the monophenolase activity and diphenolase of compound **4** on mushroom tyrosinase. (A) Time-course of L-tyrosine oxidation reaction. The concentrations of compound **4** for curves 1, 2, 3, and 4 were 0, 0.01, 0.02, and 0.1 mM, respectively. (B) Effects of compound **4** on the lag time of monophenolase activity of mushroom tyrosinase. (C) Time-course of L-dopa oxidation reaction. The concentrations of compound **4** for curves 1, 2, 3, and 4 were 0, 0.01, 0.1, and 1 mM, respectively. (D) Effects of compound **4** on the lag time of diphenolase activity of mushroom tyrosinase.