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Inhibitory effects of novel synthetic methimazole derivatives on mushroom tyrosinase and melanogenesis

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ABSTRACT

In this study, we synthesized 4 methimazole (2-mercapto-1-methylimidazole, MMI) derivatives. The kinetics of inhibition on mushroom tyrosinase by methimazole and its derivatives were investigated. The results indicated that *tert*-butyl 3-methyl-2-sulfanylidene-2,3-dihydro-1*H*-imidazole-1-carboxylate (compound 3; **3**), 2-mercaptoimidazole (MI; compound 1; **1**) and MMI (compound 2; **2**) significantly inhibited tyrosinase activity in a dose-dependent manner, exhibiting an IC_{50} value of 1.50 mM, 4.11 mM, and 1.43 mM. However, compound 4 (**4**), compound 5 (**5**), and compound 6 (**6**) exerted no inhibitory effect on mushroom tyrosinase activity. Kinetic analysis indicated that **3** was a noncompetitive tyrosinase inhibitor, whereas both **1** and **2** were exhibited as mixed-type tyrosinase inhibitors. Furthermore, **3** exerted a potent inhibitory effect on intracellular melanin formation in the B16/F10 murine melanoma cells and did not cause cytotoxicity, as **1** and **2** did.

1. Introduction

Melanin is synthesized by melanocytes distributed in the innermost layer of the epidermis.[1] It plays a crucial role in protecting the skin from the harmful effects of ultraviolet (UV) irradiation from the sun.[2] Although melanin can serve as a photoprotector, excessive accumulation of melanin can cause hyperpigmentation in food, which reduces food quality, and in skin, which induces age spots and melasma spots.[3,4] It has been reported that effective tyrosinase inhibitors could potentially improve food quality and cancer remedies, and ameliorate skin hyperpigmentation.[5]

Tyrosinase (EC1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis: the hydroxylation of tyrosine by monophenolase action and the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) to *o*-dopaquinone.[6] Tyrosinase consists of two copper ions bound to six histidines at the active site. Three types of tyrosinase (met-, oxy-, and deoxytyrosinase) exhibiting different binuclear copper structures of the active site are involved in the formation of melanin pigments.[7] Based on the interaction between the inhibitor and the enzyme, tyrosinase inhibitors can be classified into four types, namely competitive, uncompetitive, noncompetitive, and mixed.[8]

According to a previous study, thio-containing compounds act as *o*-dopaquinone scavengers, which are well-known melanogenesis inhibitors that react with dopaquinone to form colorless products and reduce melanin production.[8,9] In our previous study, a natural amino acid ergothioneine, which contains a methimazole 2-mercapto-1-methylimidazole (MMI)-like structure, exerts a potent inhibitory effect on tyrosinase activity.[10] In this study, we conducted experiments to synthesize methimazole derivatives, including a methimazole amino acid, to evaluate their tyrosinase inhibitory effects. Methimazole is a thiourea derivative and is used as oral antithyroid medication.[11] In previous studies, the topical application of 5% MMI on the skin of brown guinea pigs for 6 weeks caused a significant reduction in the amount of epidermal melanin, resulting in visually recognizable cutaneous depigmentation.[12] In murine melanoma cells B16/F10, MMI acted as a potent melanin production inhibitor.[13] The results indicated that MMI can serve as a suitable lead compound for developing novel tyrosinase inhibitors.

This study investigated the inhibitory effects of 2-mercaptoimidazole (MI; compound 1; **1**), MMI (compound 2; **2**), and four other synthetic MMI derivatives (Fig. 1) on tyrosinase activity and intracellular melanin formation in B16/F10 murine melanoma cells. We also executed kinetic analysis to evaluate the enzyme kinetic parameters and inhibition types.

2. Materials and methods

2.1. Chemicals

Mushroom tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), kojic acid, 2-mercapto-1-methylimidazole, potassium carbonate,

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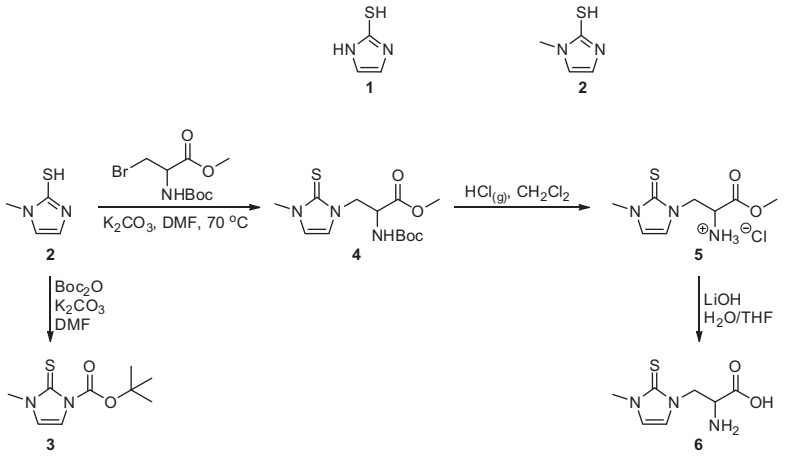

Figure 1. The structures and synthetic pathway of methimazole derivatives.

N,*N*-dimethylformamide, di-*tert*-butyl dicarbonate, ethyl acetate, hexane, MI, MMI, methyl 3-bromo-2-[(*tert*-butoxycarbonyl)-amino]propanoate, dichloromethane, tetrahydrofuran, lithium hydroxide monohydrate, 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 (FBS), phosphate buffer solution (PBS), 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 (Chemi Co. Ltd, Japan). B16/F10 mouse melanoma cell lines were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan).

2.2. Synthetic methimazole derivatives

Figure 1 shows the structures and synthetic pathways of methimazole derivatives. Briefly, compound 3 (**3**) was prepared through the reaction of an MMI (compound 2; **2**) (400 mg, 3.5 mmol) and potassium carbonate (968 mg, 7 mmol) in 7 mL of *N*,*N*-dimethylformamide and the subsequently di-*tert*-butyl dicarbonate (1.1 mL, 5.2 mmol) was added. The reaction mixture was stirred at 60 °C for 30 min in an N_2 atmosphere. The resulting mixture was partitioned between ethyl acetate (40 mL) and H_2O (20 mL). The organic layer was washed with brine (20 mL), dried over $MgSO_4$, and then concentrated in vacuo. The residue was separated by chromatography over silica gel and eluted with hexane/ethyl acetate (1:1) to afford 665 mg (89% yield) of **3** (Fig. 1).

Compound 4 (**4**) was prepared through the reaction of a mixture of **2** (1.12 g, 9.8 mmol) and methyl 3-bromo-2-[(*tert*-butoxycarbonyl)-amino]propanoate (2.79 g, 9.8 mmol) in 35 mL of *N*,*N*-dimethylformamide and the subsequently potassium carbonate (2.71 g, 19.6 mmol) was added. The reaction mixture was stirred at 70 °C for 1 h in an N_2 atmosphere. The resulting mixture was filtered and concentrated in vacuo. The residue was separated with chromatography over silica gel and eluted with hexane/ethyl acetate (3:7) to afford 2.60 g (87% yield) of **4**.

Compound 5 (**5**) was prepared through the reaction of a mixture of a solution of **4** (1 g, 3.2 mmol) in 16 mL of dichloromethane and subsequently purged with hydrogen chloride gas for 30 min. The mixture was partitioned in ether to afford 685 mg (86% yield) of **5** as a yellow solid.

Compound 6 (**6**) was prepared through the reaction of **5** (616 mg, 2.4 mmol) in 9.6 mL of tetrahydrofuran and subsequently lithium hydroxide monohydrate (302 mg, 7.2 mmol) and H_2O were added. The mixture was stirred at room temperature for 1.5 h and the pH value was then adjusted to 4–5 with 1 N $HCl_{(aq)}$. The resulting mixture was concentrated in vacuo and purified by preparative high-performance liquid chromatography (HPLC) with a gradient MeOH/H_2O system to afford 192 mg (39% yield) of **6**.

The products were purified through recrystallization from ethanol and were identified using nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS) analyses. The NMR data were acquired on a Mercury 400 MHz NMR spectrometer from Varian (Agilent Technologies, Inc.).

2.3. Inhibitory effect of methimazole derivative on mushroom tyrosinase

The reaction media, containing 20 μL of 0.1, 1, 2.5, and 10 mM MMI and its derivatives, were mixed with 160 μL of 1 mM L-tyrosine and 20 μL of 212.64 μg/mL mushroom tyrosinase and dissolved in 5 mM dipotassium hydrogen phosphate in a 96-well plate. A dipotassium hydrogen phosphate buffer served as a vehicle control. All experiments were repeated in triplicate. The mixture was incubated at 25 °C for 30 min before measuring absorbance at 475 nm, using an ELISA reader (Tecan, Austria). The tyrosinase inhibition rate (%) was calculated as $(1 - ABS_{sample}/ABS_{control}) * 100\%$. The IC_{50} value was determined through regression of a constructing dose–response curve at which 50% target activity was lost.

2.4. Determining inhibition type and inhibition constant

In a 96-well plate, 20 μL of 0.1 (or 0.5), 1, 2, and 5 mM MMI and its derivatives, were mixed with 160 μL of L-tyrosine (0.25, 0.5, 1, 2, and 4 mM). Subsequently, 20 μL of varying concentrations of mushroom tyrosinase were added and the formation of dopachrome in the solution was monitored for 10 min by measuring absorbance at 475 nm. The reaction was executed at a constant temperature of 25 °C. All experiments were conducted in triplicate. The inhibition mechanism was assessed using Lineweaver–Burk plots, and the inhibition constants were obtained from the second plots of the apparent $1/V_{max}$ and apparent K_m/V_{max} against the inhibitor concentration, as described in Liao et al.[10]

2.5. Cell viability assay

B16/F10 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin G, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin, and then incubated at 37 °C with 5% CO_2. The viability of cells treated with 1 mM and