Inhibition of Tyrosinase Activity by *N*,*N*-Unsubstituted Selenourea Derivatives

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Received October 2, 2004; accepted March 9, 2005; published online March 14, 2005

This study investigated inhibitory effects of *N*,*N*-unsubstituted selenourea derivatives on tyrosinase activity. Three types of *N*,*N*-unsubstituted selenoureas derivatives exhibited inhibitory effect on dopa (3,4-dihydroxyphenylalanine) oxidase activity of mushroom tyrosinase. Compound D at a concentration of 200 μM exhibited 55.5% of inhibition on dopa oxidase activity of mushroom tyrosinase. This inhibitory effect was higher than that of kojic acid (39.4%), a well known tyrosinase inhibitor. Moreover, the compound D identified as a noncompetitive inhibitor by Lineweaver–Burk plot analysis. In addition, compound D also inhibited the melanin production in melan-a cells.

Key words *N*,*N*-unsubstituted selenourea; tyrosinase inhibitor

Melanin formation is the most important determinant of mammalian skin color. Melanin is secreted by melanocyte cells, which are distributed in the basal layer of the dermis.[1] One of the roles of melanin is to protect the skin and underlying tissues from UV-induced skin injury. However, excessive melanin production in the skin has negative hyperpigmenting effects such as melasma, freckles, and senile lentigines. The synthesis of melanin starts with the conversion of the amino acid L-tyrosine to L-dopa (3,4-dihydroxyphenylalanine), the subsequent oxidation of L-dopa then yields dopaquinone. Tyrosinase is the key enzyme in the biosynthesis of melanin, and participates in the oxidation of tyrosine to L-dopa, and of dopa to dopaquinone.[2,3] Therefore, tyrosinase inhibitors are accepted as important constituents of cosmetics and as depigmenting agents in cases of hyperpigmentation.[4] In this study, we investigated inhibitory effects of *N*,*N*-unsubstituted selenourea derivatives on mushroom tyrosinase and their depigmenting effect in melan-a cells.

MATERIALS AND METHODS

General Methods Melting points were determined using a Yanagimoto micromelting point apparatus. IR spectra were obtained using a Perkin-Elmer 1600 spectrometer, and ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL-JNM-α400 (400 MHz) spectrometer. Mass spectra were obtained using a Shimadzu 9020-DF mass spectrometer, and UV spectra using a Molecular Devices E09090 microplate reader.

Materials Mushroom tyrosinase, L-dopa (3-(3,4-dihydroxyphenyl)-L-alanine), and kojic acid (5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4one) were purchased from Aldrich Chemical; Inc. (U.S.A.). Solvents used for organic syntheses were redistilled. All other chemicals and solvents were of analytical grade and used without further purification.

Typical procedure for preparation of *N*,*N*-unsubstituted selenourea derivatives, 1 M HCl (2 eq) in diethyl ether was added to *N*,*N*-dimethylcyanamide (1 eq) of THF solution. The reaction mixture became milky white suspension from colorless solution in less than 30 s and then was stirred at 0 °C for 2 h. The compound was formed *in situ*. Subsequently, LiAlHSeH (1 eq)[5] was added to the reaction mixture. The reaction mixture was further stirred at 0 °C for 3 h. After usual workup, *N*,*N*-dimethyselenourea (Compound **A**) was obtained in a 70% yield as yellow crystals

N,*N*-Dimethyselenourea: Compound **A**: mp 172.2—172.8 °C; IR (KBr) 3366, 3162, 1551 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ 3.18 (6H, br s, CH_2), 7.60 (2H, br s, NH), ^{13}C-NMR ($CDCl_3$) δ 37.9, 45.3, 177.7, ^{77}Se-NMR ($CDCl_3$) δ 230.4, MS (CI) *m/z*=153 [M^++1], HR-MS (EI) Calcd for $C_3H_8N_2Se$ 151.98522, Found 151.98346.

N,*N*-Diethyselenourea: Compound **B**: mp 121.8—122.7 °C; IR (KBr) 3340, 3176, 1534 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ 1.27 (6H, t, *J*=6.8 Hz, CH_3), 3.49 (2H, br s, CH_2), 3.97 (2H, br s, CH_2), 6.48 (2H, br s, NH), ^{13}C-NMR ($CDCl_3$) δ 12.2, 42.9, 51.1, 176.6, ^{77}Se-NMR ($CDCl_3$) δ 209.8, MS (CI) *m/z*=181 [M^++1], HR-MS (EI) Calcd for $C_5H_{12}N_2Se$ 180.0165, Found 180.0147.

1-Selenocarbamoylpyrrolidine: Compound **C**: mp 215.1—215.9 °C; IR (KBr) 3292, 3159, 1523 cm^{-1}, ^{1}H-NMR (DMSO-d_6) δ 1.80 (2H, m, CH_2), 1.98 (2H, m, CH_2), 3.26 (2H, t, *J*=6.8 Hz, CH_2), 3.62 (2H, t, *J*=6.8 Hz, CH_2), 7.73 (2H, br s, NH), ^{13}C-NMR (DMSO-d_6) δ 24.4, 25.9, 47.5, 54.2, 173.6, ^{77}Se-NMR (DMSO-d_6) δ 245.1, MS (CI) *m/z*=179 [M^++1], HR-MS (EI) Calcd for $C_5H_{10}NSe$ 163.1011, Found 163.1014.

1-Selenocarbamoylpiperidine: Compound **D**: mp 145.0—146.2 °C; IR (KBr) 3303, 3169, 1522 cm^{-1}, ^{1}H-NMR (DMSO-d_6) δ 1.47 (4H, m, CH_2), 1.59 (2H, m, CH_2), 3.76 (4H, br s, CH_2), 7.75 (2H, br s, NH), ^{13}C-NMR (DMSO-d_6) δ 23.6, 25.3, 176.0, ^{77}Se-NMR (DMSO-d_6) δ 218.5, MS (CI) *m/z*=193 [M^++1], HR-MS (EI) Calcd for $C_6H_{12}N_2Se$ 192.0165, Found 192.01499.

Assay of Tyrosinase Activity Tyrosinase activity was measured by determining its dopa oxidase activity using a modification of the method reported by Shono *et al*.[6] Test substances were dissolved in MeOH to 1 mM, 500 μM, 100 μM, or 10 μM. 120 μl of L-dopa (8 mM, dissolved in 67 mM phosphate buffer, pH 6.8) and 40 μl of either the same buffer or test sample were added to each well of a 96-well microplate, and then 40 μl of mushroom tyrosinase (125 U)

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was added.

The amount of dopachrome in the reaction mixture was determined after 30 min at 37 °C. Inhibitory activity was expressed as the concentration that inhibited 50% of tyrosinase activity (IC_{50}), as determined by optical density at 490 nm (OD_{490}). Kojic acid was used as a positive control. The test compound inhibition patterns were determined using Lineweaver–Burk's plot at various L-dopa concentrations.

Cell Line and Culture Procedures Melan-a cells were kindly donated by Dr. B. Lee at the Skin Research Institute at the Pacific Co., Korea. The cells were cultured in RPMI1640 medium containing 10% FBS and 200 nM phorbol 12-myristate 13-acetate (TPA). Cells were then seeded in a 100 mm diameter tissue culture dish (5×10^5 cells/well). Cells grew to confluency after 3 to 4 d at 37 °C in 5% CO_2, and were seeded at 10^5 cells/well in a 24 well plate, and then incubated for a further 24 h. Each well was renewed with 990 μl of RPMI1640 medium daily and treated with 10 μl of 10000, 1000 or 100 μM of test sample for 3 d (solvent system: propylene glycol/EtOH/H_2O=5/3/2). And then it was incubated for 1 d in CO_2 incubator.

Cell Viability Percentages of viable melan-a cells were determined by staining the cell population with crystal violet. After removing the medium from each well, the cells were washed with PBS, and 200 μl of crystal violet (CV 0.1%, EtOH 10%, PBS 89.9%) was added. The cells were then incubated at room temperature for 5 min and washed twice with distilled water. After adding 1 ml of EtOH to the cells, they were shaken at room temperature for 10 min. Crystal violet absorption was measured at 590 nm.

Determination of Melanin Level Melanin contents were measured using a modification of the methods described by Hosoi *et al.*[7] After removing the media from each wall, it was washed with PBS. 1 ml of 1 M NaOH was then added to the well to dissolve the melanin. The absorption maxima of melanin was measured at 400 nm, and the melanin content per well calculated, and expressed as a percentage to the vehicle. Phenylthiourea was used as a positive control.[8,9]

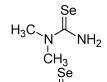
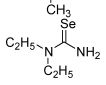
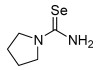
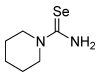
Statistical Analysis Data are presented as means±S.E. of three independent experiments. Different treatments were compared using the Student's *t*-test.

RESULTS

Inhibitory Effects of Compounds against Tyrosinase Activity The tyrosinase inhibitory effects of *N*,*N*-unsubstituted selenourea derivatives, compounds **A**, **B**, **C**, and **D**, and of kojic acid are detailed in Table 1. Among *N*,*N*-unsubstituted selenourea derivatives, 1-selenocarbamoylpiperidine (compound **D**) showed the highest inhibitory effect on mushroom tyrosinase. Compound **D** at 2, 20, 100, and 200 μM inhibited tyrosinase by 3.8%, 15.1%, 37.0%, and 55.5%, respectively (Fig. 1). The tyrosinase inhibitory effect of compound **D** was greater than kojic acid (39.4% at 200 μM); the IC_{50}s of compound **D** and kojic acid were 170 and 277 μM, respectively.

Inhibition Pattern of Mushroom Tyrosinase by Compound D The kinetics behavior of compound **D** during the oxidation of L-DOPA was studied. When various concentrations of L-DOPA was used as a substrate, compound **D** decreased the V_{max} value of tyrosinase in a dose-dependent manner but did not change the K_m value. Therefore, compound **D** was identified as a noncompetitive tyrosinase inhibitor by Lineweaver–Burk plot analysis (Fig. 2).

Table 1. Inhibitory Effects of *N*,*N*-Unsubstituted Selenourea Derivatives and Kojic Acid on Mushroom Tyrosinase.

	Compounds	Inhibition at 200 μM[a] (%)	IC_{50}[b] (μM)
A		36.7±9.8	>200
B		10.6±6.1	>200
C		43.3±5.3	232
D		55.5±4.9	170
	Kojic acid	39.4±7.9	277

a) Values represent means±S.E. of three experiments. *b*) 50% inhibitory concentration (IC_{50}).

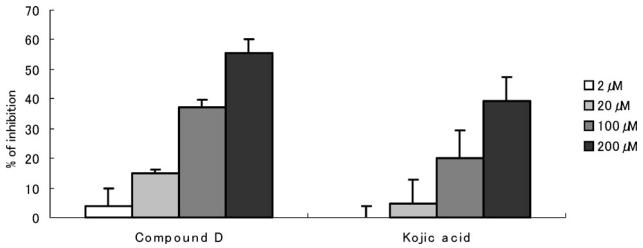


Fig. 1. Inhibitory Effects of Compound **D** and of Kojic Acid on Mushroom Tyrosinase at Several Concentrations

Values represent the means±standard errors of three independent experiments performed in triplicate.

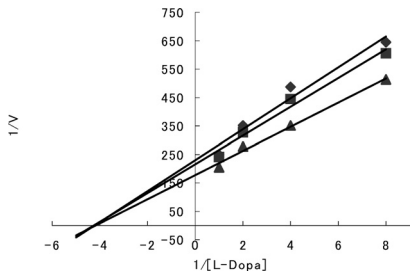


Fig. 2. Lineweaver–Burk Plots of Mushroom Tyrosinase Activity Changes Caused by Compound **D**

100 μM (rectangle), 50 μM (diamond), and blank (triangle).

Melanin Production and Cell Viability of Melan-a Cells Melan-a cells are synergistic with the B16 melanoma cell line and its sublines, and provide an excellent parallel non-tumorigenic line for studying melanoma malignancy.[10] In this study, the melan-a cell line was used for testing the melanin production and cell toxicity. Compound **D**, kojic acid and phenylthiourea were treated in the cultured melan-a cells for 3 d. The testing results, cell viability and melanin contents, were represented at Table 2. As the results, compound **D** slightly attenuates the melanin content at above 50 μM (Fig. 3). The difference between cell viability and melanin produc-

Table 2. Effects of PTU, Kojic Acid and Compound **D** on Cell Growth and the Melanin Production in Melan-a Cells

Samples	Concentrations (μM)	Melanin production (%)	Cell viability (%)
Phenylthiourea	10	76.3±6.5	102.8±5.2
	100	33.5±5.0	99.0±5.4
Kojic acid	10	98.1±3.2	97.8±2.9
	100	96.9±4.8	95.3±5.6
Compound **D**	10	95.7±3.4	103.3±5.6
	100	49.5±5.9	60.6±4.8

Test sample and medium were renewed daily. The cell viabilities and the melanin contents of melan-a cells were determined after 3 d. The data shown represents the means±S.E. of four independent experiments performed in duplicate.

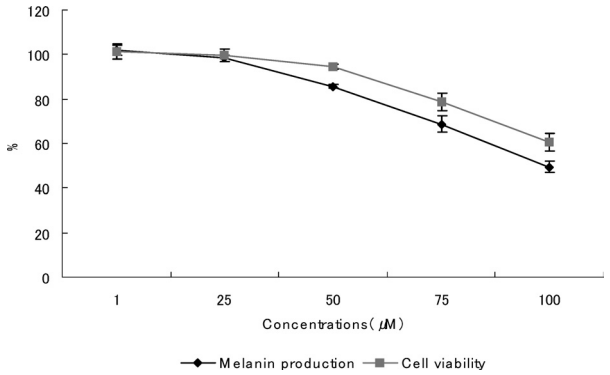


Fig. 3. Effect of Compound **D** on Cell Viability and Melanin Production in Melan-a Cells in Various Concentrations

Values represent the means±standard errors of four independent experiments in duplicate.

tion is about 10—20%. Phenylthiourea was used as a positive control.

DISCUSSION

This study investigated inhibitory effects of *N*,*N*-unsubstituted selenourea derivatives on tyrosinase activity. Among *N*,*N*-unsubstituted selenourea derivatives, 1-selenocarbamoylpiperidine (compound **D**) showed the highest inhibitory effect on mushroom tyrosinase. Moreover, this inhibitory effect was higher than that of kojic acid (39.4%), a well known tyrosinase inhibitor.

In terms of chemical structure, 5-membered ring piperidine (compound **D**) had a greater inhibitory effect than 6-membered ring pyrrolidine (compound **C**). *N*,*N*-Dimethyselenourea (compound **A**) and *N*,*N*-diethyselenourea (compound **B**) did not show strong inhibitory effects.

In kinetics study, compound **D** identified as a noncompetitive inhibitor of the mushroom tyrosinase with same K_m value. Therefore, compound **D** might indicate the enzyme inhibitory activity by binding at other sites except cupper active site of mushroom tyrosinase.

Furthermore, compound **D** was tested with respect to its anti-melanogenic effect in cultured melan-a cells at various concentrations (1, 25, 50, 75, 100 μM). Compound **D** showed melanin inhibitory effect in range from 50 to 100 μM (Fig. 3). Compound **D** was found to slightly attenuate the melanin content. Based on these results, we can suggest that compound **D** may act as a depigmenting agent.

Acknowledgments This work was supported by the grants from the 2002 Good Health Research and Development Project (Ministry of Health and Welfare, Korea, 02-PJ1-PG1-CH11-0001) and Brain Korea 21 Projects (Ministry of Education, Korea).

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