

Potent Inhibitory Effects of *N*-Aryl *S*-Alkylthiocarbamate Derivatives on the Dopa Oxidase Activity of Mushroom Tyrosinase

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This study reports the potent inhibitory effect of *N*-aryl *S*-alkylthiocarbamate derivatives on mushroom tyrosinase (MT) activity. *N*-Aryl *S*-alkylthiocarbamate derivatives were found to exhibit a potent inhibitory effect on the dopa (3,4-dihydroxyphenylalanine) oxidase activity of mushroom tyrosinase. Most of the *N*-aryl *S*-alkylthiocarbamate derivatives (compounds from A to J) exhibited higher inhibitory effects than kojic acid (IC₅₀=318 μM), a well known tyrosinase inhibitor. Tyrosinase was the most inhibited by *S*-phenetyl *N*-phenylthiocarbamate (compound E, IC₅₀=7.25 μM), and this inhibition was 44 times stronger than that of kojic acid. Compound E exhibited 95.0% of inhibition at 100 μM. A kinetic study of MT inhibition by compound E using the Lineweaver–Burk plots analysis was performed. And the kinetics profiles observed suggest that compound E competitively inhibits MT.

Key words *N*-aryl *S*-alkylthiocarbamate; mushroom tyrosinase; diethyldithiocarbamate

Tyrosinase is melanogenic copper-containing enzyme that catalyzes the transformation of tyrosine to dopaquinone.^{1,2} This enzyme is responsible for melanization in plants and animals, which leads to the undesirable browning of farm products and the coloring of an animal's skin, eyes, inner ear, and hair.^{3,4} Numerous tyrosinase inhibitors, such as kojic acid and oxyresveratrol, have been developed to remove of undesirable pigment.^{5–7} In this study, we examined the inhibitory effects of diethyldithiocarbamate derivatives on mushroom tyrosinase. Diethyldithiocarbamate (DETC) has been reported to act as a nitric oxide synthase inhibitor and as a xanthine oxidase inhibitor.^{8,9} In particular, DETC has also been reported to potentially inhibit tyrosinase,^{10,11} which lead us to investigate the inhibitory effects of diethyldithiocarbamate derivatives on tyrosinase. To identify more potent tyrosinase inhibitors, several *N*-aryl *S*-alkylthiocarbamate derivatives were synthesized by reacting isocyanates with LiAlHSH and then with alkyl halides. Thiocarbamates have been used as key intermediates for the synthesis of thioureas¹² and of isothiocyanates,¹³ and are important moieties in pesticides components.^{14,15} In this work, we investigated the structure–activity–relationships (SARs) of synthetic *N*-aryl *S*-alkylthiocarbamate derivatives on tyrosinase inhibitory activity and on these inhibition patterns.

Experimental

General Methods Melting points were determined using a Yanagimoto micromelting point apparatus. IR spectra were obtained using a Perkin-Elmer 1600 spectrometer, and ¹H- and ¹³C-NMR spectra were recorded on a JEOL-JNM-α 400 (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), Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) and DETC (diethyldithiocarbamate) were purchased from Aldrich Chemical, Inc. (U.S.A.). Oxyresveratrol (3,5,2',4'-tetrahydroxy stilbene) was synthesized in our previous work.¹⁶ Solvents for organic synthesis were redistilled. All other chemicals and solvents were of analytical grade and used without further purification. *N*-Aryl *S*-alkylthiocarbamate derivatives were prepared according to the previously reported method.^{17,18} This method give the *N*-

aryl *S*-alkylthiocarbamate derivatives under mild conditions in high yields. Briefly, the synthesis of *S*-ethyl *N*-phenylthiocarbamate (A) was conducted as follows; Phenyl isocyanate (0.22 ml, 2.0 mmol) was added to a THF solution (10 ml) of LiAlHSH (1.0 mmol). The reaction mixture was stirred at room temperature for 1 h, and ethyl iodide (0.06 ml, 1.0 mmol) was added, and stirred at room temperature for 3 h. The mixture was then extracted with dichloromethane, washed with distilled water, and the organic layer was dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography on silica gel with dichloromethane : hexane (1 : 2) to give:

Compound A; *S*-Ethyl *N*-Phenylthiocarbamate (Yield 71%): White crystals; mp 63.9–65.1 °C; IR (KBr) 1651, 3281 cm⁻¹; ¹H-NMR (CDCl₃); δ 1.33 (3H, t, *J*=7.2 Hz, CH₃), 2.98 (2H, q, *J*=7.2 Hz, CH₂), 7.09 (1H, t, *J*=7.2 Hz, Ar), 7.21 (1H, br s, NH), 7.30 (2H, t, *J*=7.2 Hz, Ar), 7.41 (2H, d, *J*=7.2 Hz, Ar); ¹³C-NMR (CDCl₃); δ 15.5, 24.7, 119.7, 124.4, 129.1 137.6 (Ar), 165.8; MS (CI): *m/z*=182 [M⁺+1].

Compound B; *S*-Propyl *N*-Phenylthiocarbamate (Yield 66%): White crystals; mp 73.2–75.6 °C; IR (KBr) 1653, 3280 cm⁻¹; ¹H-NMR (CDCl₃); δ 1.00 (3H, t, *J*=7.6 Hz, CH₃), 1.67 (2H, m, CH₂), 2.95 (2H, t, *J*=7.6 Hz, CH₂), 7.02 (1H, br s, NH), 7.10 (1H, t, *J*=7.6 Hz, Ar), 7.31 (2H, t, *J*=7.6 Hz, Ar), 7.41 (2H, d, *J*=7.6 Hz, Ar); ¹³C-NMR (CDCl₃); δ 13.2, 23.6, 32.2, 119.7–137.7 (Ar), 165.9; MS (CI): *m/z*=196 [M⁺+1].

Compound C; *S*-Isopentyl *N*-Phenylthiocarbamate (Yield 50%): White crystals; mp 66.1–69.3 °C; IR (KBr) 1655, 3290 cm⁻¹; ¹H-NMR (CDCl₃); δ 0.92 (6H, t, *J*=6.8 Hz, CH₃), 1.54 (2H, q, CH₂), 1.68 (1H, m, CH), 2.97 (2H, t, *J*=8.0 Hz, CH₂), 7.09 (1H, t, *J*=7.2 Hz, Ar), 7.19 (1H, br s, NH), 7.30 (2H, t, *J*=7.2 Hz, Ar), 7.41 (2H, d, *J*=7.2 Hz, Ar); ¹³C-NMR (CDCl₃); δ 22.1, 27.4, 28.3, 30.8, 39.1, 119.6–137.7 (Ar), 165.9; MS (CI): *m/z*=224 [M⁺+1].

Compound D; *S*-Benzyl *N*-Phenylthiocarbamate (Yield 55%): White powder; mp 92.1–94.2 °C; IR (KBr) 1653, 3250 cm⁻¹; ¹H-NMR (CDCl₃); δ 4.22 (2H, s, CH₂), 7.08–7.40 (10H, m, Ar), 7.10 (1H, br s, NH); ¹³C-NMR (CDCl₃); δ 34.4, 119.8–137.8 (Ar), 165.1; MS (CI): *m/z*=244 [M⁺+1].

Compound E; *S*-Phenetyl *N*-Phenylthiocarbamate (Yield 44%): White crystals; mp 108.1–110.3 °C; IR (KBr) 1652, 3382 cm⁻¹; ¹H-NMR (CDCl₃); δ 2.96 (2H, t, *J*=6.8 Hz, CH₂), 3.21 (2H, t, *J*=7.7 Hz, CH₂), 7.08–7.40 (10H, m, Ar), 7.43 (1H, br s, NH); ¹³C-NMR (CDCl₃); δ 31.5, 36.6, 119.7–139.9 (Ar), 165.5; MS (CI): *m/z*=258 [M⁺+1].

Compound F; *S*-3-Phenyl-propyl *N*-Phenylthiocarbamate (Yield 36%): White crystals; mp 78.3–79.8 °C; IR (KBr) 1652, 3386 cm⁻¹; ¹H-NMR (CDCl₃); δ 1.99 (2H, quint, *J*=7.2 Hz, CH₂), 2.73 (2H, t, *J*=7.2 Hz, CH₂), 2.99 (2H, t, *J*=7.2 Hz, CH₂), 7.08–7.41 (10H, m, Ar), 7.17 (1H, br s, NH); ¹³C-NMR (CDCl₃); δ 29.7, 31.8, 34.7, 119.6–141.5 (Ar), 165.7; MS (CI): *m/z*=272 [M⁺+1].

Compound G; *S*-Methyl *N*-(4-Methylphenyl)thiocarbamate (Yield 59%):

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Yellow crystals; mp 102.1–103.5 °C; IR (KBr) 1654, 3242 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3); δ 2.30 (3H, s, CH_3), 2.39 (3H, s, CH_3), 7.10 (2H, d, $J=8.4$ Hz, Ar), 7.27 (1H, br s, NH), 7.28 (2H, t, $J=8.4$ Hz, Ar); $^{13}\text{C-NMR}$ (CDCl_3); δ 12.5, 20.7, 119.9–135.0, (Ar), 166.3; MS (CI): $m/z=182$ [M^++1].

Compound H; *S*-Butyl *N*-(4-Methylphenyl)thiocarbamate (Yield 46%): White crystals; mp 73.9–75.1 °C; IR (KBr) 1651, 3297 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3); δ 0.91 (3H, t, $J=7.6$ Hz, CH_3), 1.40 (2H, m, $J=7.6$ Hz, CH_2), 1.62 (2H, quint, $J=7.6$ Hz, CH_2), 2.29 (3H, s, CH_3), 2.95 (2H, t, $J=7.6$ Hz, CH_2), 7.08 (2H, d, $J=8.4$ Hz, Ar), 7.23 (1H, br s, NH), 7.28 (2H, t, $J=8.4$ Hz, Ar); $^{13}\text{C-NMR}$ (CDCl_3); δ 13.5, 20.7, 21.8, 29.9, 32.3, 119.9–135.1 (Ar), 166.0; MS (CI): $m/z=224$ [M^++1].

Compound I; *S*-Isopentyl *N*-(4-Methylphenyl)thiocarbamate (Yield 52%): White crystals; mp 33.1–35.6 °C; IR (KBr) 1657, 3318 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3); δ 0.92 (6H, t, $J=6.8$ Hz, CH_3), 1.53 (2H, q, CH_2), 1.67 (1H, m, CH), 2.30 (3H, s, CH_3), 2.96 (2H, t, $J=7.2$ Hz, CH_2), 7.06 (1H, br s, NH), 7.10 (2H, d, $J=8.4$ Hz, Ar), 7.28 (2H, t, $J=8.4$ Hz, Ar); $^{13}\text{C-NMR}$ (CDCl_3); δ 20.8, 22.1, 27.4, 28.3, 30.8, 39.1, 120.0–135.1 (Ar), 164.2; MS (CI): $m/z=238$ [M^++1].

Compound J; *S*-Ethyl *N*-(4-Chlorophenyl)thiocarbamate (Yield 47%): White crystals; mp 95.1–96.2 °C; IR (KBr) 1651, 3271 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3); δ 1.33 (3H, t, $J=7.6$ Hz, CH_3), 2.98 (2H, q, $J=7.6$ Hz, CH_2), 7.13 (1H, br s, NH), 7.26 (2H, t, $J=8.4$ Hz, Ar), 7.36 (2H, t, $J=8.4$ Hz, Ar); $^{13}\text{C-NMR}$ (CDCl_3); δ 15.4, 24.7, 120.8–136.2 (Ar), 166.0; MS (CI): $m/z=216$ [M^++1].

Compound K was synthesized by following method; to a mixture of benzoic acid (300 mg, 2.5 mm) and trichloro acetonitrile (490 μl , 4.9 mm) in CH_2Cl_2 (7 ml), Ph_3P (1.3 g, 4.9 mm) in CH_2Cl_2 (5 ml) was added under nitrogen at room temperature. After stirring for 4 h, the reaction mixture was treated with aniline (225 ml, 5 mm) and mixture was stirring for 12 h.

The reaction mixture was poured into water and extracted with ethylacetate. The extract was washed with brine, and the organic layer was dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography on silica gel with dichloromethane:methanol (60:1) to give:

Compound K; *N*-Phenyl-benzamide (Yield 40%): White crystals; $^1\text{H-NMR}$ (CDCl_3); δ 7.08 (1H, m), 7.30 (2H, m), 7.38–7.51 (4H, m), 7.56 (1H, t, $J=1.2$ Hz, Ar), 7.59 (1H, t, $J=1.2$ Hz, Ar), 7.79 (1H, t, $J=1.5$ Hz, Ar), 7.81 (1H, t, $J=1.5$ Hz, Ar); $^{13}\text{C-NMR}$ (CDCl_3); δ 120.2, 124.6, 127.0, 128.8, 129.1, 131.8, 135.0, 137.9, 165.7; MS (CI): $m/z=197$ [M^++1].

Assay of Tyrosinase Activity The test compounds were dissolved in methanol at various concentrations (500 μM , 250 μM , 50 μM , 5 μM). 120 μl of L-dopa (8 mM, dissolved in 67 mM phosphate buffer, pH 6.8) and 40 μl of each *N*-aryl *S*-alkylthiocarbamate compound solution was added to a 96-well microplate, and 40 μl of mushroom tyrosinase (125 U) was added. After incubation at 37 °C for 20 min, the amount of dopachrome in the reaction mixture was determined. Based on the optical density at 490 nm, the inhibitory activity was expressed as a concentration, *i.e.*, the concentration required to inhibit the enzyme activity by 50% (IC_{50}). Kojic acid was used as a positive control. The pattern of inhibition of the test compound was determined by Lineweaver–Burk's plot at various L-dopa concentrations.

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

Results and Discussion

Inhibitory Effects of Compounds on Tyrosinase Activity Tyrosinase inhibitory effects by the *N*-aryl *S*-alkylthiocarbamate derivatives are presented in Table 1. The majority of these *N*-aryl *S*-alkylthiocarbamate compounds inhibited tyrosinase more strongly than kojic acid. Compound **E** had the highest inhibitory effects with an IC_{50} of 7.25 μM , and the level of inhibition increased dose dependently over the concentration range 1–100 μM . At 50 μM , the inhibitory effect of compound **E** exceeded 90% (Fig. 1).

SARs of *N*-Aryl *S*-Alkylthiocarbamate Derivatives Compounds **D**, **E** and **F** with aromatic ring containing substituents at R_2 showed higher tyrosinase inhibition than the other *N*-aryl *S*-alkylthiocarbamate derivatives or DETC. Among these compounds, compound **E** (R_2 =phenetyl) had the greatest potency *versus* compound **D** (R_2 =*S*-benzyl) or

Table 1. Inhibitory Effects of *N*-Aryl *S*-Alkylthiocarbamate Derivatives, DETC, Oxysresveratrol and Kojic Acid on Mushroom Tyrosinase

Compounds	Substituent		Inhibition at 100 μM (%) ^{a)}	IC_{50} ^{b)} (μM)
	R_1	R_2		
A	H	CH_2CH_3	3.4 \pm 5.9	>100
B	H	$(\text{CH}_2)_2\text{CH}_3$	36.8 \pm 12.5	>100
C	H	$(\text{CH}_2)_2\text{CH}(\text{CH}_3)_2$	82.2 \pm 7.3	38.1
D	H	$\text{CH}_2\text{C}_6\text{H}_5$	93.2 \pm 4.9	18.4
E	H	$(\text{CH}_2)_2\text{C}_6\text{H}_5$	95.0 \pm 4.0	7.3
F	H	$(\text{CH}_2)_3\text{C}_6\text{H}_5$	59.9 \pm 7.6	46.6
G	CH_3	CH_3	74.3 \pm 1.7	48.5
H	CH_3	$(\text{CH}_2)_3\text{CH}_3$	55.1 \pm 7.3	87.5
I	CH_3	$(\text{CH}_2)_2\text{CH}(\text{CH}_3)_2$	70.9 \pm 5.2	47.4
J	Cl	CH_2CH_3	20.2 \pm 4.5	>100
K			13.5 \pm 3.5	>100
DETC		—	61.9 \pm 0.5	61.2
Oxysresveratrol		—	73.4 \pm 1.8	53.7
Kojic acid		—	19.0 \pm 2.6	318.0

a) Each value represents the mean \pm S.E. of three experiments. b) 50% inhibitory concentration (IC_{50}).

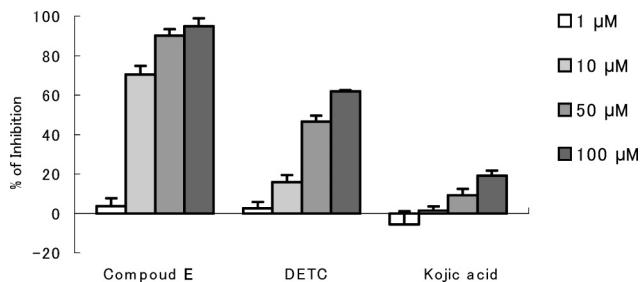
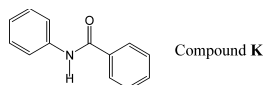


Fig. 1. Inhibitory Effects of Compound **E**, DETC, and Kojic Acid on Mushroom Tyrosinase at Several Concentrations

Each value represents the mean \pm standard error of experiments performed in triplicate.

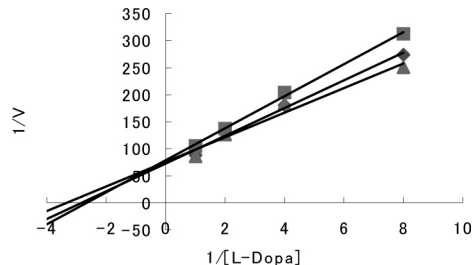


Fig. 2. Lineweaver–Burk Plots of Mushroom Tyrosinase Activity Changes Caused by Compound **E**

10 μM (rectangle), 5 μM (diamond) and blank (triangle).

compound **F** (R_2 =*S*-3-phenyl-propyl). However, introduction of the aliphatic chain at R_2 (compounds **A**, **B**, **C**) reduced inhibitory activity. This tendency was confirmed by comparing the inhibitory abilities of compounds **A** and **D**, with those of

compounds **B** and **E**. In terms of the R₁ positions, no functional group (compound **C**) was better than the methyl group (compound **I**) for tyrosinase inhibition on the same basic chemical skeleton.

In addition, the presence of sulfur may play a very important role in tyrosinase inhibitory activities. In the case of compound **K** including no sulfur atom, the inhibitory activity of the compound was very low.

Inhibition Pattern of Compound E Kinetic analysis showed that the compound **E** is a competitive inhibitor of mushroom tyrosinase. Compound **E** had the same V_{\max} value at several concentrations, but the K_m value reduced with increasing concentration. Therefore, compound **E** was identified as competitive inhibitor of mushroom tyrosinase. As competitive inhibitor, kojic acid is well known.

In this study, compound **E** exhibited 44-fold higher tyrosinase inhibition than kojic acid, which also exceeds the inhibitory effect of oxyresveratrol, a recently reported potent tyrosinase inhibitor. The present study identified a useful candidate for potent tyrosinase inhibitor. Furthermore, our results suggest that compound **E** may act as a potent depigmenting agent.

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