Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1233–1240

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis of *N*-substituted *N*-nitrosohydroxylamines as Inhibitors of Mushroom Tyrosinase

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Received 10 August 2000; accepted 13 December 2000

Abstract—A series of *N*-substituted *N*-nitrosohydroxylamines including six new compounds were synthesized and examined for inhibition of mushroom tyrosinase. Corresponding hydroxylamines were reacted with *n*-butyl nitrite to give substituted nitrosohydroxylamines as their ammonium salt. The *N*-substituted hydroxylamines were prepared from the primary amines via the oxaziridine, or from the carbonyl compounds via the oxime. Most of the nitrosohydroxylamines tested inhibited mushroom tyrosinase. Among them, *N*-cyclopentyl-*N*-nitrosohydroxylamine exhibited the most potent activity ($IC_{50} = 0.6\,\mu M$), as powerful as that of tropolone, one of the most powerful inhibitors. As removal of nitroso or hydroxyl moiety, the enzyme inhibitory activity was completely diminished. Both *N*-nitroso group and *N*-hydroxy group were suggested to be essential for the activity, probably by interacting with the copper ion at the active site of the enzyme. Lineweaver–Burk plotting showed that cupferron was a competitive inhibitor but that *N*-cyclopentyl-*N*-nitrosohydroxylamine was not.

Introduction

Tyrosinase (E. C.1.14.8.1) is a copper-containing enzyme widely distributed in plants and animals. It catalyses the *o*-hydroxylation of monophenols and also the oxidation of *o*-diphenols to *o*-quinones. Tyrosinase is known to be a key enzyme for melanin biosynthesis in plants and animals. Therefore, tyrosinase inhibitors should be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation[1] and also important in cosmetics for whitening and depigmentation after sunburn.[2,3] In addition, tyrosinase is known to be involved in the molting process of insects[4] and adhesion of marine organisms.[5]

Several *N*-substituted *N*-nitrosohydroxylamines are known to inhibit various types of enzymes; e.g., dopastin (**1**) is an inhibitor of dopamine-β-monohydroxylase (E. C.1.14.2.1),[6] nitrosoxacins inhibit 5-lipoxygenase (E. C.1.13.11.12),[7] and cupferron (**2**) and neocupferron (**3**), which are metal-chelating agents, inhibit superoxide dismutase (E. C.1.15.1.1)[8] (Fig. 1). In the present paper, we found that dopastin (**1**) also inhibited mushroom tyrosinase effectively. The inhibitory activity of dopastin (**1**) is likely to be derived from the *N*-nitroso-*N*-hydroxylamino group, which can interact with the copper ion at the active site of the enzyme. Therefore, we synthesized several *N*-nitroso-*N*-hydroxylamines and examined their inhibitory activity on mushroom tyrosinase to study the structure–activity relationship.

Results and Discussion

Synthesis of *N*-substituted *N*-nitrosohydroxylamines

Eleven *N*-substituted *N*-nitrosohydroxylamines (**4**–**14**) including six new compounds (**5**–**8, 12** and **13**) were synthesized. The procedure for the synthesis of the *N*-substituted *N*-nitrosohydroxylamines is outlined in Scheme 1. The *N*-nitrosohydroxylamines were prepared by nitrosation of the corresponding hydroxylamine, which were obtained by procedures A or B in Scheme 1. Procedure A included condensation of the primary amine with benzaldehyde and oxidation of the resulting imine with *m*-chloroperbenzoic acid followed by hydrolysis of the oxaziridine.[9,10] Procedure B included condensation of the aldehyde or ketone with hydroxylamine and reduction of the resulting oxime.[11] Nitrosation of all of

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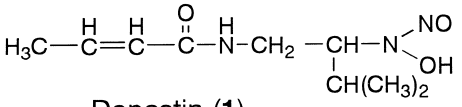
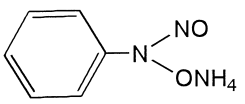
PII: S0968-0896(01)00003-7





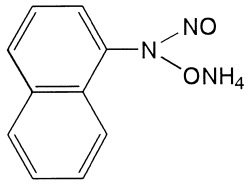


Figure 1. Structure of dopastin and related *N*-nitrosohydroxylamines.

the hydroxylamines obtained was achieved by use of *n*-butyl nitrite in etherial solution saturated with ammonia, and *N*-nitroso-*N*-hydroxylamines were isolated as ammonium salts. All compounds synthesized were characterized by chemical and spectral methods.

Inhibition of tyrosinase

Most of the *N*-nitroso-*N*-hydroxylamines tested inhibited the mushroom tyrosinase, and the IC_{50} values ranged widely from 0.6 to 273 μM, as summarized in Table 1.

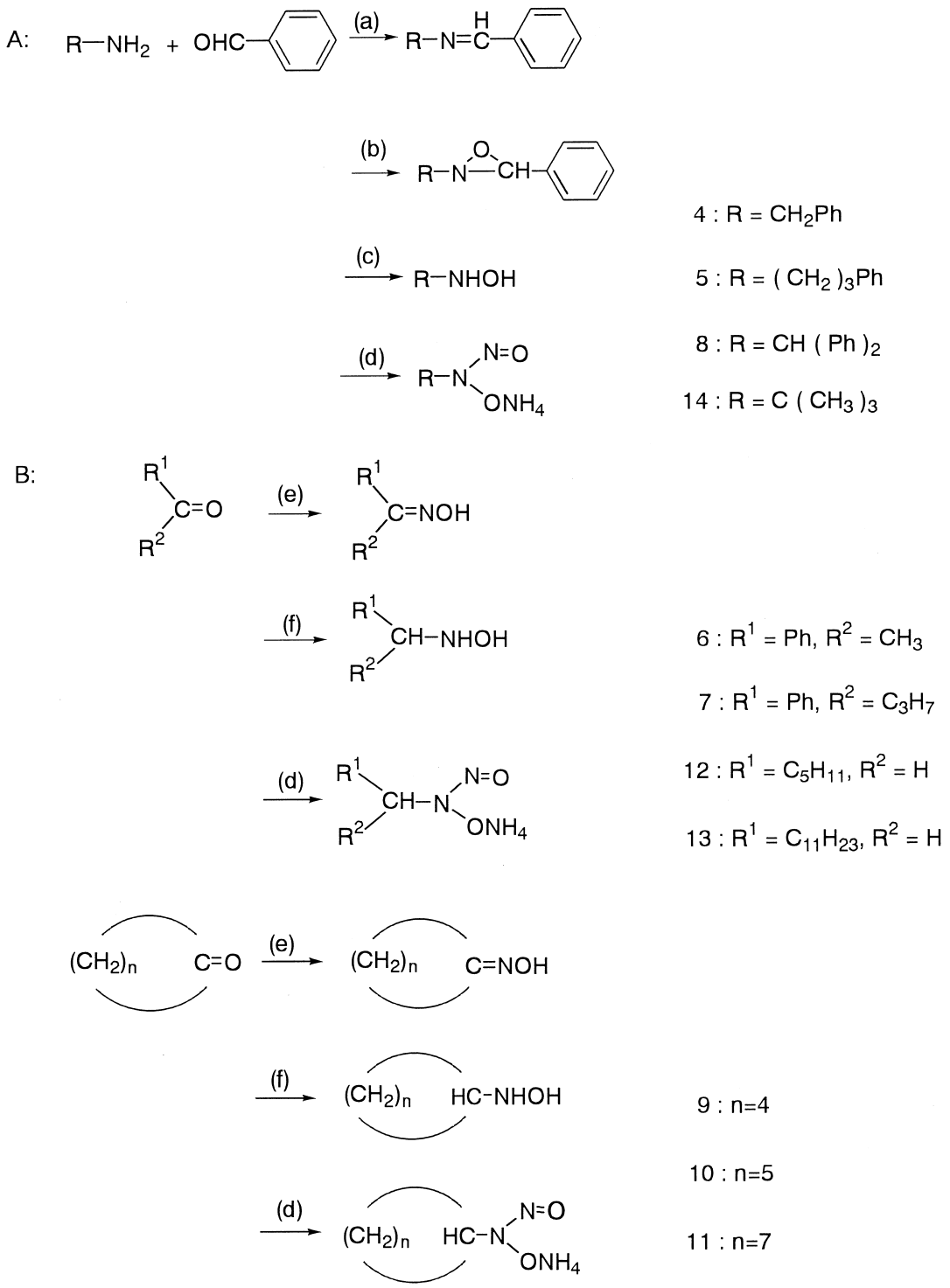
Tropolone,[12,13] kojic acid[14–16] and mimosine,[12,17,18] are known to be the most powerful inhibitors of tyrosinase. Among the compounds tested, cupferron (**2**; $IC_{50} = 1.1$ μM), *N*-cycloalkyl derivatives (**9**–**11**; 0.6–1.2 μM), and *N*-*n*-alkyl derivatives (**12** and **13**; 1.5 and 2.2 μM, respectively) exhibited the inhibitory activity of almost the same order with the reported inhibitors. Interestingly, activities of *N*-*n*-alkyl derivatives were not affected by the length of their alkyl chain (**12** and **13**). On the other hand, the inhibitory activity dramatically decreased with an increase in the length of the alkyl chain (**4**, **6** and **7**; 3.0–273 μM) in the compounds having the phenyl substituent at the α-carbon of the *N*-nitroso-*N*-hydroxylamino group. Compound **8,** having two phenyl groups at the α-carbon, exhibited the activity two orders lower than that of the corresponding monophenyl derivative (**4**). The activity of the *N*-*tert*-butyl derivative (**14**; 19.3 μM) was one order lower than that of *N*-*n*-alkyl derivatives (**12** and **13**). Therefore, the inhibitory activity was affected significantly with either the phenyl group or alkyl group as a substituent on the α-carbon of the *N*-nitroso-*N*-hydroxylamino group, but not by subsituents at other positions. These observations suggest that the phenyl or alkyl substituent at the α-carbon of the *N*-nitroso-*N*-hydroxylamino group may cause steric hindrance for the approach of the inhibitors to the active site of the enzyme.

The related hydroxylamines and *N*-nitrosoamines did not inhibit the enzyme, as shown in Table 2. Therefore, both *N*-nitroso group and *N*-hydroxyl group are essential for inhibition of the enzyme.

It has been reported that the inhibitory activities of cupferron (**2**) on superoxide dismutase and dopastin (**1**) on dopamine-β-monohydroxylase were due to the chelation between the nitrosohydroxylamino group and the copper ion in the enzyme.[6,8] It has also been reported that some *N*-substituted *N*-nitrosohydroxylamines were copper chelating compounds.[19,20] Therefore, we also examined the chelating activity of *N*-substituted *N*-nitrosohydroxylamines. The addition of each *N*-substituted *N*-nitrosohydroxylamine to a copper carbonate solution gave the corresponding chelate compound as the precipitate, indicating the ability of each compound to chelate Cu^{2+}. Besides, the addition of copper sulfate to the reaction medium in the inhibitory assay recovered the enzyme activity inhibited by *N*-cyclopentyl-*N*-nitrosohydroxylamine (**9**), as shown in Figure 2. Based on these results, we propose that the synthesized or desigaed inhibitors might bind to the copper ion at the active site of the enzyme.

Kinetic analysis for *o*-dihydroxyphenolase inhibition with respect to L-dopa and oxygen was carried out with cupferron (**2**) and compound **9**, as shown in Figure 3. The *o*-dihydroxyphenolase reaction is an orderded bi-bi reaction. Thus, cupferron was competitive with L-dopa ($K_i = 0.3$ μM) and noncompetitive with oxygen. On the other hand, compound **9** did not show competitive inhibition. Therefore, these two compounds affect the enzyme in different ways. To look into the mechanism of inhibition, we tested the effect of pre-incubation of the enzyme with the inhibitor prior to the addition of L-dopa. Pre-incubation of the enzyme in the presence of cupferron or compound **9** and in the absence of the substrate decreased the enzyme activity significantly. The inhibitory activity at an equivalent amount of IC_{50} of either compound was increased 1.6 times by the pre-incubation. As the enzyme pre-incubated is mostly *met* tyrosinase, known as the resting form of the enzyme,[21,22] the above results indicate that both cupferron and compound **9** can react with the *met*-form of tyrosinase before oxygen binds.

Since the most potent inhibitors such as kojic acid, mimosine, and toropolone resemble the structure of the substrate in part (Fig. 4A),[12,23,24] this structural similarity may explain the inhibitory activity. Though *N*-nitrosohydroxylamines seem to have no structural similarity, some of them exhibited inhibitory activity of the same order. Recently, *N*-nitrosohydroxylamines were reported to exist mainly as a tautomeric hydroxydiazenium *N*-oxide form, being a planar structure (Fig. 4B).[25] As a planar structure, the distance between the two oxygens of the nitrosohydroxylamino group can be estimated from a molecular model, and it was nearly the same as that of the two hydroxy groups of catechols. Therefore, the *N*-nitrosohydroxylamino group may interact with binuclear copper at the active site of the enzyme, as a bridge structure similar to the substrate (Fig. 4C). There are two separate substrate-binding sites in the binuclear copper active center of the *oxy*-form of tyrosinase, one for catechol and the other for oxygen.[26] Being a competitive inhibitor, cupferron only interacts with the catechol-binding site. On the other hand, compound **9** may interact not only with the



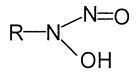
Scheme 1. Routes for synthesis of *N*-nitrosohydroxylamines: (a) CH_2Cl_2, $MgSO_4$, rt, 2 h; (b) *m*-chloroperbenzoic acid, CH_2Cl_2, 4 °C, 4 h; (c) H_2SO_4/MeOH/H_2O, 4 °C, 18 h; (d) *n*-butyl nitrite, ether, NH_3, 0 °C, 15 min; (e) NH_2OH·HCl, NaOH, MeOH, rt, 2 h; (f) $NaBH_3CN$, HCl, MeOH, rt, 16 h.

catechol-binding site but also with the oxygen-binding site in the *oxy*-form of tyrosinase.

Conclusion

We found that *N*-substituted *N*-nitrosohydroxylamines inhibited mushroom tyrosinase. Their potency was affected by the structure of *N*-substitutent. *N*-Substitution might cause steric hindrance for the approach of inhibitors to the active site of the enzyme. As a mechanism of inhibition, we suggested that both *N*-nitroso and *N*-hydroxyl groups were essential to inhibit the enzyme, interacting with the copper ion at the active site of the enzyme. Furthermore, kinetic analysis of the enzyme indicated that *N*-cyclopentyl-*N*-nitrosohydroxylamine might interact to the copper ion at the active site of the enzyme not only from the substrate-binding site but also from the oxygen-binding site.

Table 1. Inhibitory activity of *N*-substituted *N*-nitrosohydroxylamines on mushroom tyrosinase



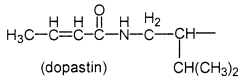
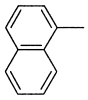
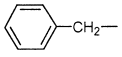
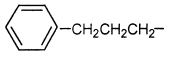
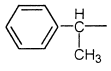
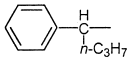
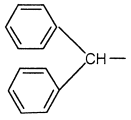
Compd	R	IC_{50} (μM)
1	 (dopastin)	20
2	 (cupferron)	1.1
3	 (neocupferron)	46.0
4		3.0
5		1.3
6		23.5
7		273
8		139
9		0.6
10		1.2
11		1.1
12	$H_3C-(CH_2)_4-CH_2-$	1.5
13	$H_3C-(CH_2)_{10}-CH_2-$	2.2
14		19.3

Table 2. Effect of *N*-hydroxyl and *N*-nitroso amines on tyrosinase

R	IC_{50} (mM)	
	R-NHOH	R-N(NO)-R′
Phenyl (**2**)[a]	N.T.	12.8 (R′=CH_3)
Benzyl (**4**)[a]	4.4	1.0 (R′=CH_3)
Cyclohexyl (**10**)[a]	> 0.28	N.T.
Cyclooctyl (**11**)[a]	0.22	N.T.
tert-butyl (**14**)[a]	5.5	> 78 (R′=C_2H_5)

[a]Compound number of related chemical in Table 1.
N.T.: not tested.

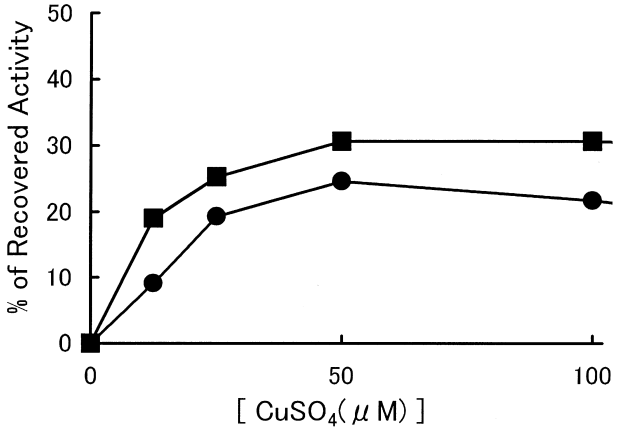


Figure 2. Effect of Cu^{2+} ion on the inhibitory activity of *N*-cyclopentyl-*N*-Nitrosohydroxylamine. The reaction was carried out with 0.5 mM L-dopa, 67 mM phosphate buffer (pH 6.8), 80 units of mushroom tyrosinase, $CuSO_4$ and 1.0 μM (●) or 2.0 μM (■) of *N*-cyclopentyl-*N*-nitrosohysroxylamine. Percentage of recovered activity was calculated in reference to the control value.

Experimental

Materials

Cupferron (**2**) and neocupferron (**3**) were purchased from Tokyo Kashei. Dopastine (**1**) was kindly supplied by Dr. H. Iinuma, Institute of Microbial Chemistry, Tokyo. Tyrosinase was obtained from Sigma.

Syntheses

The structures were determined by 60 MHz ^1H NMR (Hitachi R-24), IR (Jasco A-100, Bio-Rad FTS-60A (FTIR)), HRMS (JEOL JMS-700 MSstaition) and the elemental analysis. The typical procedure for the preparation of *N*-nitrosohydroxylamines **5** and **8** (procedure A) is described for **5**.

***N*-(3-Phenylpropyl)hydroxylamine.** *N*-(3-Phenylpropyl) amine (6.76 g, 0.05 M) in dichloromethane (75 mL), benzaldehyde (10 g, 0.05 M) in dichloromethane (25 mL), and anhydrous $MgSO_4$ (8.7 g, 0.05 M) were mixed, and stirred for 2 h at room temperature. After removal of $MgSO_4$ by filtration, the filtrate was evaporated in vacuo. The residue was dissolved in 100 mL of dichloromethane and added dropwise by 12.1 g of *m*-chloroperbenzoic acid in 150 mL of dichloromethane, and the mixture was stirred at 4 °C for 4 h. After filtration, the filtrate was washed with 10% K_2CO_3 aq solution, dried, and concentrated in vacuo. The residue was dissolved in 50 mL of methanol and then H_2SO_4:CH_3OH:H_2O (32:75:93 mL) was added dropwise at 4 °C, and then the mixture was stirred for 18 h. After concentration, the acidic solution was neutralized with 6 M NaOH aq solution, and the pH of the solution was adjusted to above 10 with saturated $NaHCO_3$ aq solution. The alkaline solution was extracted with 300 mL of ethyl acetate, and the extract was dried over Na_2SO_4 and evaporated to dryness. The residue was crystallized from *n*-hexane and benzene to give 0.85 g of *N*-(3-phenylpropyl)hydroxylamine with a total yield of 11.2%: mp 41.8–43.4 °; IR (KBr): 3245, 3140, 3025, 2930, 2850, 1600, 1490, 1450, 1060, 740, 685 cm^{-1};

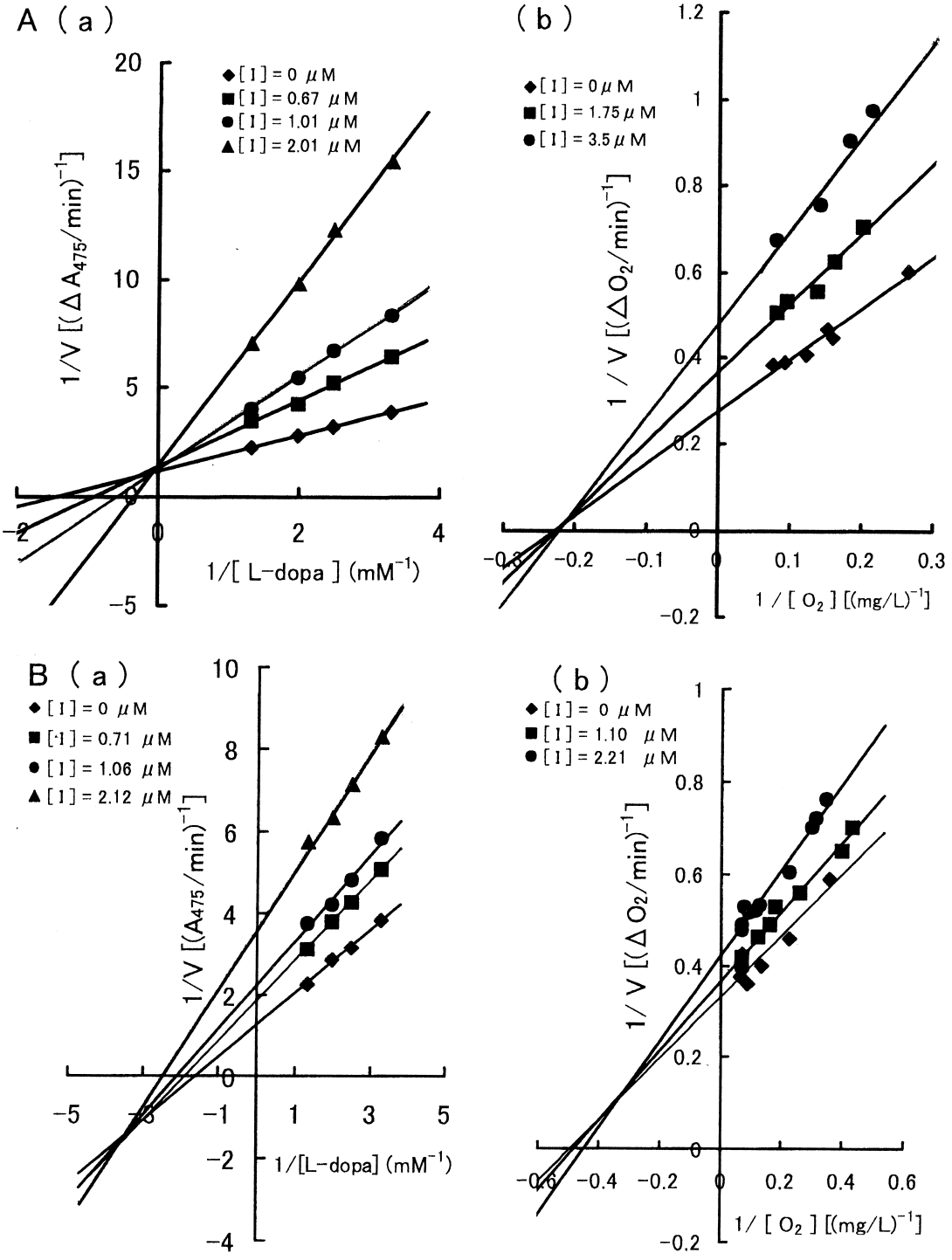


Figure 3. Lineweaver–Burk plots of the mushroom tyrosinase reaction with (A) cupferron and (B) *N*-cyclopentyl-*N*-nitrosohydroxylamine: (a) respect to L-dopa; (b) respect to oxygen.

^{1}H NMR ($CDCl_3$)δ1.92 (2H, m), 2.68 (2H, t, J=7 Hz), 2.94 (2H, t, J=7 Hz), 6.12 (2H, s), 7.18 (5H, s). Anal. calcd for $C_9H_{13}NO$: C, 71.49; H, 8.67; N, 9.26. Found: C, 71.41; H, 8.60; N, 9.18.

***N*-Nitroso-*N*-(3-phenylpropyl)hydroxylamine ammonium salt (5).** (3-Phenylpropyl)hydroxylamine (150 mg, 1 mM) dissolved in ether (8 mL) saturated ammonia at 0 °C. After 15 min of stirring, the reaction mixture was centrifuged at 500 *g* for 10 min at 4 °C. After decantation of the supernatant, the precipitate was washed three times sequentially with dried ether and *n*-hexane to give the white crystal, which was dried with nitrogen gas. Yield: 181 mg (0.92 mM, 92%); mp 86.8 °C; IR (KBr): 3150 (broad), 3028, 2932, 1500, 1450, 1402, 1075, 937, 742, 698 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 2.04 (2H, m), 2.61 (2H, t, J=7 Hz), 3.88 (2H, t, J=6 Hz), 6.92 (4H, s, broad), 7.20 (5H, s); HRMS(FAB) calcd for $C_9H_{11}N_2O_2$ $(M-NH_4)^-$ 179.0820, found 179.0835. Anal. calcd for $C_9H_{15}N_3O_2$: C, 54.80; H, 7.67; N, 21.31. Found: C,

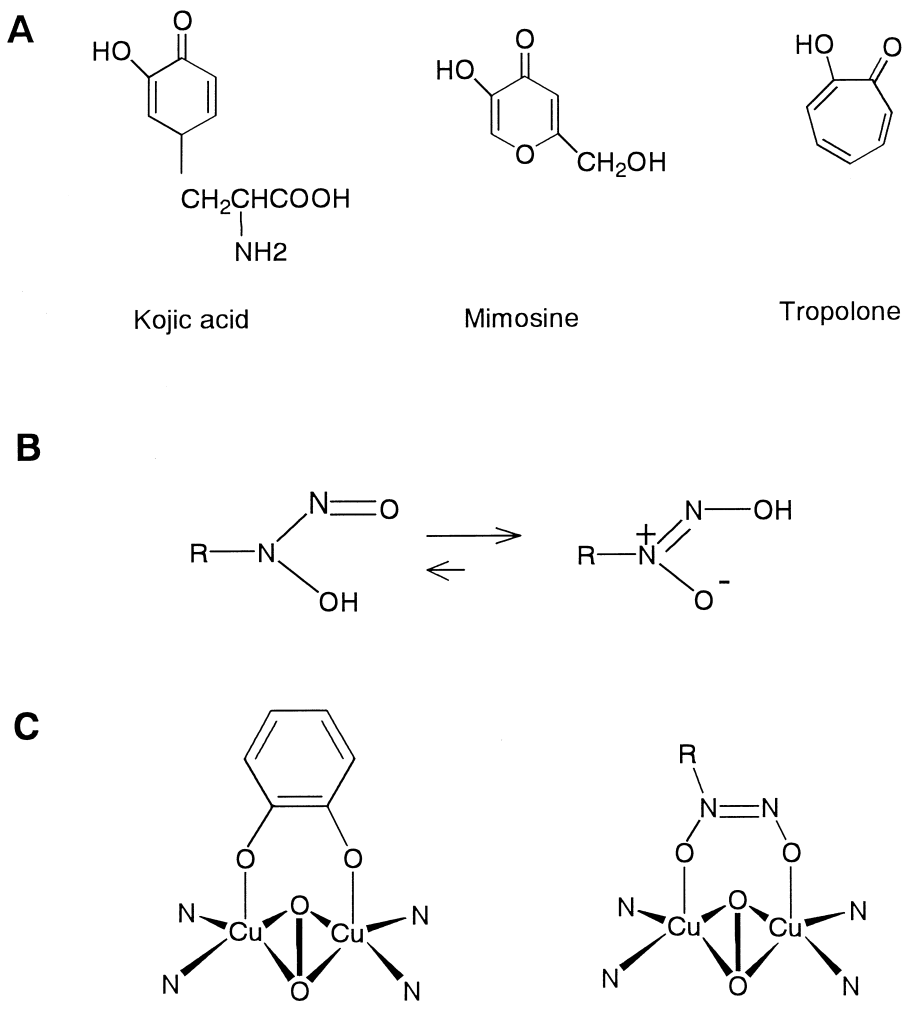


Figure 4. (A) The known potent inhibitors. (B) A possible planar structure of the tautomeric hydroxydiazenium *N*-oxide. (C) Binding models to the binuclear copper active center in the *oxy*-form of tyrosinase. *N*-nitrosohydroxylamines (right) may also interact with the binuclear copper as shown for catechol (left, ref 22).

54.31; H, 7.92; N, 20.85. Both Liebermann and Griess' reactions for the nitroso group were positive. Compound **8** was obtained in a similar way from the appropriate amine.

***N*-Nitroso-*N*-(diphenylmethyl)hydroxylamine ammonium salt (8).** Overall yield 12.3%; mp 114.2–117.6 °C; IR (KBr): 3211 (broad), 1485, 1457, 1385, 1072, 934, 750, 698 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.48 (1H, s), 6.65 (4H,broad), 7.12 (10H, s); HRMS(FAB) calcd for $C_{13}H_{11}N_2O_2$ $(M-NH_4)^-$ 227.0821, found 227.0838; Both Liebermann and Griess' reactions were positive.

The typical procedure for the preparation of *N*-nitrosohydroxylamines **6**, **7**, **12** and **13** (procedure B) is described for **12**.

***N*-Hexylhydroxylamine.** To a mixture of hexylaldehyde (4.0 g, 0.04 M) in methanol (100 mL) and hydroxylamine hydrochloride (4.1 g, 0.06 M) in water (10 mL), 6 M NaOH aq solution (18 mL) was added. After 2 h of stirring, the mixture was concentrated; and the residue was then extracted with ethyl acetate. The extract was dried over Na_2SO_4 and evaporated to give crude oxime. To a solution of the crude oxime in methanol (60 mL) containing a trace of methyl orange, $NaBH_3CN$ (1.5 g, 0.024 M) was added; and then 5 M HCl-MeOH was added dropwise with stirring to maintain the red color of the solution for 30 min. The reaction mixture was stirred overnight, and methanol was removed in vacuo. The residue was suspended in water and brought to pH 10 using 6 M NaOHaq solution and saturated $NaHCO_3$ aq solution, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated in vacuo to dryness. Recrystallization of the crude product from *n*-hexane gave 1.3 g of *N*-hexylhydroxylamine with a yield of 29% from *n*-hexylaldehyde: mp 65.0–65.8 °C; IR (KBr): 3266, 3162, 2924, 2857, 1460, 1378 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.83 (3H, t, $J=4$ Hz), 1.05–1.55 (8H, m), 2.88 (2H, t, $J=6$ Hz), 6.25 (2H, s); HRMS(FAB) calcd for $C_6H_{16}NO$ $(M+H)^+$ 118.1232, found 118.1253. Anal. calcd for $C_6H_{15}NO$: C, 61.49; H, 12.90; N, 11.95. Found: C, 61.78; H, 13.09; N, 11.88.

***N*-Nitroso-*N*-hexylhydroxylamine ammonium salt (12).** Nitrosation of *N*-hexylhydroxylamine was accomplished by the same method described above. Yield of 82%; IR (KBr): 3181(broad), 2930, 2860, 1460, 1066, 941 cm^{-1}; ^1H NMR (D_2O)δ 0.68 (3H, t, $J=4$ Hz), 1.08

(6H, m), 1.61 (2H, m), 3.78 (2H, t, $J=4$ Hz); HRMS(FAB) calcd for $C_6H_{13}N_2O_2$ $(M-NH_4)^-$ 145.0977, found 145.0969. Anal. calcd for $C_6H_{17}N_3O_2$: C, 44.15; H, 10.50; N, 25.75. Found: C, 43.36; H, 10.51; N, 25.02. Both Liebermann and Griess' reactions were positive. Compounds **6**, **7** and **13** were obtained in a similar way from the appropriate aldehyde or ketone.

***N*-Nitroso-*N*-(α-metylbenzyl)hydroxylamine ammonium salt (6).** Overall yield 20.6%; mp 71.3–78.0 °C; IR (KBr): 3176 (broad), 1447, 1063, 931, 703 cm^{-1}; ^1H NMR (D_2O)δ 1.65 (3H, d, $J=7.0$ Hz), 5.36 (1H, q, $J=7.0$ Hz), 7.25 (5H, s); HRMS(FAB) calcd for $C_8H_9N_2O_2$ $(M-NH_4)^-$ 165.0664, found 165.0659. Anal. calcd for $C_8H_{13}N_3O_2$: C, 52.44; H, 7.15; N, 22.94. Found: C, 51.93; H, 7.42; N, 22.40; Both Liebermann and Griess' reactions were positive.

***N*-(α-Propylbenzyl)hydroxylamine.** Mp 65.3–66.7 °C; IR (KBr): 3261, 3148, 1635, 1496, 1027, 752, 694 cm^{-1}; ^1H NMR($CDCl_3$) δ 0.83 (3H, t, $J=5.0$ Hz), 1.12 (2H, m), 1.65 (2H, m), 3.85 (1H, dd, $J=8.0$ Hz), 5.58 (2H, s), 7.21 (5H, s); HRMS(FAB) calcd for $C_{10}H_{16}NO$ $(M+H)^+$ 166.1232, found 166.1215. Anal. calcd for $C_{10}H_{15}NO$: C, 72.69; H, 9.15; N, 8.48. Found: C, 72.88; H, 9.00; N, 8.62.

***N*-Nitroso-*N*-(α-propylbenzyl)hydroxylamine ammonium salt (7).** Overall yield 47.1%; mp 103.9–108.9 °C; IR (KBr): 3150 (broad), 1456, 1061, 943, 748, 700 cm^{-1}; ^1H NMR (D_2O) δ 0.98 (3H, t, $J=5.0$ Hz), 1.35 (2H, m), 2.10 (2H, m), 5.35 (1H, m), 7.40 (5H, s). HRMS(FAB) calcd for $C_{10}H_{13}N_2O_2$ $(M-NH_4)^-$ 193.0977, found 193.0977; Both Liebermann and Griess' reactions were positive.

***N*-Dodecylhydroxylamine.** Mp 88.6–90.7 °C; IR (KBr): 3263, 3156, 2918, 2851, 1462, 1378–1061 cm^{-1}; ^1H NMR ($CDCl_3$)δ 0.90 (3H, t, $J=5.0$ Hz), 1.28 (20H, s), 2.94 (2H, t, $J=5.0$ Hz), 5.10 (2H, broad); HRMS(FAB) calcd for $C_{12}H_{28}NO$ $(M+H)^+$ 202.2171, found 202.2165. Anal. calcd for $C_{12}H_{27}NO$: C, 71.58; H, 13.52; N, 6.96. Found: C, 71.61; H, 13.82; N, 6.95.

***N*-Nitroso-*N*-dodecylhydroxylamine ammonium salt (13).** Overall yield 31.8%; mp 62.3–64.2 °C; IR (KBr): 3157 (broad), 2920, 2850, 1462, 949 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.63 (3H, s), 1.02 (20H, s), 2.96 (4H, s), 3.70 (2H, t, $J=7.0$ Hz). HRMS(FAB) calcd for $C_{12}H_{25}N_2O_2$ $(M-NH_4)^-$ 229.1916, found 229.1922; Both Liebermann and Griess' reactions were positive.

The other *N*-substituted *N*-nitrosohydroxylamines were synthesized in the same way. Compound **4**[27] and **14**[28] were obtained by the procedure A with total yield of 12.8 and 9.9%, respectively. Compound **9**,[29] **10**[28] and **11**[30] were obtained by the procedure B with total yield of 26.3%, 21.9% and 25.1%, respectively.

Tyrosinase assay by spectrophotometry

The enzyme activity was measured by a modified spectrophotometric method[20] with L-dopa as the substrate. The reaction mixture (3.2 mL of final volume) containing 0.54 mM L-dopa, 67 mM phosphate buffer (pH 6.8), 80 units of mushroom tyrosinase, and the test sample was incubated at 30 °C for 2 min. The formation of dopachrome was monitored spectrophotometrically by absorption at 475 nm with a Shimadzu UV-160A spectrophotometer.

Tyrosinase assay by oxygen monitor

The enzyme activity was measured for oxygen consumption by a modified method.[31] The reaction mixture (0.92 mL of final volume) containing 0.5 mM L-dopa, 67 mM phosphate butter (pH 6.8), 80 units of mushroom tyrosinase, and the test sample was incubated at 30 °C for 5 min. Before addition of the enzyme, the initial oxygen concentration was controlled by passing nitrogen into the reaction mixture. Oxygen consumption rates were determined with a Central Oxygraph-9, a Clark-type oxygen electrode.

Effect of pre-incubation of mushroom tyrosinase with inhibitor

Pre-incubation mixtures consisted of 0.1 mL of the sample solution (equivalent amount of IC_{50}) and 0.1 mL of the aqueous solution of the tyrosinase (80 units). The mixture was pre-incubated at 0 °C for 5 min. Then, 3.0 mL of 0.54 mM L-dopa was added, and the reaction monitored at 475 nm for 15 min.

Acknowledgements

The authors wish to thank Dr. H. Iinuma, Institute of Microbial Chemistry, for the gift of dopasitin and valuable suggestions.

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