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Synthesis and tyrosinase inhibitory activity of novel *N*-hydroxybenzyl-*N*-nitrosohydroxylamines

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Abstract

Several novel *N*-substituted *N*-nitrosohydroxylamines were synthesized. They all inhibited mushroom tyrosinase, but the type of inhibition was different depending on the substituent. Some *N*-(mono- or dihydroxybenzyl)-*N*-nitrosohydroxylamines exhibited uncompetitive inhibition with respect to L-dopa. Among them, compound **6** was also a competitive inhibitor with respect to oxygen. This observation suggests that another interaction by the *meta*- or *para*-hydroxyl group might stabilize the binding of the inhibitor to the enzyme through the oxygen binding site.

Keywords: Nitrosohydroxylamine; Tyrosinase; L-dopa; Enzyme inhibition

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing enzyme that catalyzes two different reactions, the hydroxylation of monophenolic compounds to *o*-diphenols and the oxidation of the *o*-diphenols to *o*-quinones. The mammalian tyrosinase is the key

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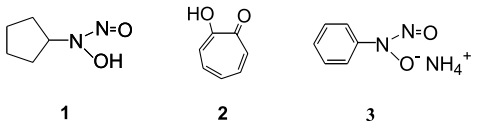


1 2 3

Fig. 1. Chemical structures of *N*-cyclopentyl-*N*-nitrosohydroxylamine (**1**), tropolone (**2**), and cupferron (**3**).

enzyme in melanin biosynthesis [1], catalyzing the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-dopa) and its subsequent oxidation to dopaquinone. Therefore, inhibitors of tyrosinase should be useful as therapeutic agents for the treatment of melanin hyperpigmentation [2] and cosmetic materials for whitening after sunburn [3]. Moreover, tyrosinase, also known as polyphenol oxidase, is known to cause a browning reaction. Thus, in the food industry, tyrosinase inhibitors are becoming important to prevent enzymatic browning [4]. In the previous paper, we reported that *N*-substituted *N*-nitrosohydroxylamines strongly inhibit mushroom tyrosinase [5]. Among the compounds examined, *N*-cyclopentyl-*N*-nitrosohydroxylamine (**1**, Fig. 1) exhibited the most potent activity, having an IC_{50} value of 0.6 μM, which is as potent as tropolone (**2**) [6], the most potent known inhibitor of tyrosinase. It was suggested that the inhibitory activity was caused by the chelation of the copper in the active site of the enzyme by the *N*-nitrosohydroxylamino moiety. The kinetic analysis showed that cupferron (**3**) was a competitive inhibitor with respect to L-dopa, but compound 1 was not a competitive one. Thus, the mechanism of inhibition by *N*-substituted *N*-nitrosohydroxylamines might be different depending on the *N*-substituent. In order to further investigate the effect of the *N*-substituent on the mechanism, we synthesized several *N*-hydroxybenzyl-*N*-nitrosohydroxylamines and related compounds to examine their inhibitory activities. The results are reported herein.

2. Materials and methods

2.1. Materials

Tyosinase was obtained from Sigma and used without further purification. Sodium cyanoborohydride was purchased from Aldrich. All other chemical reagents were supplied by Tokyo Kasei.

2.2. Syntheses

Three *N*-monohydroxybenzyl derivatives (**4**, **5**, and **6**), two *N*-dihydroxybenzyl derivatives (**7** and **8**), 4′-chlorobenzyl derivatives (**9**), 4′-methoxybenzyl derivatives (**10**), and 4′-*tert*-butylbenzyl derivatives (**11**) were synthesized by the same method as described previously [5], starting from the corresponding benzaldehydes, through the reduction of oximes. All *N*-substituted *N*-nitrosohydroxylamines synthesized

were isolated as ammonium salts, which were characterized by chemical and spectral methods. ^{1}H NMR spectra were recorded on a Hitachi R-24 60 MHz spectrometer. FTIR spectra were recorded on a Bio-Rad FTS-60A spectrometer. HRMS data were obtained on a JEOL JMS-700 ms station spectrometer. The general procedure for the preparation of *N*-hydroxybenzyl-*N*-nitrosohydroxylamines is described for compound **4**.

N-(2′-hydroxybenzyl)-N-nitrosohydroxylamine, ammonium salt ***(4)***. To a mixture of salicylaldehyde (3.0 g, 25 mM) in methanol (150 mL) and hydroxylamine hydrochloride (2.6 g, 37 mM) in water (5 mL) was added aqueous solution of 6 M NaOH (7.5 mL). After 2 h of stirring, the mixture was concentrated in vacuo. The residue was extracted with ethyl acetate. The extract was dried over Na_2SO_4 and evaporated to give the crude oxime. To a solution of the crude oxime in methanol (50 mL), containing a trace of methyl orange, $NaBH_3$ CN (1.6 g, 25 mM) was added. Subsequently, 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 cacuo*. The residue was suspended in water and brought to pH 10 using aqueous solutions of 6 M NaOH and saturated $NaHCO_3$, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in cacuo* to dryness. Recrystallization of the crude product from ethyl acetate gave 1.4 g of *N*-(2′-hydroxybenzyl)hydroxylamine with a yield of 37% from salicylaldehyde. *N*-(2′-hydroxybenzyl)hydroxylamine (100 mg, 0.6 mM) was dissolved in 1 mL of ethyl acetate and added to 8 mL of ether. Subsequently, *n*-butyl nitrite (0.3 mL) was added and then ammonia gas was bubbled through the mixture at 0 °C for 10 min. After 30 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 followed by *n*-hexane to give white crystals, which were dried using nitrogen gas. The yield of *N*-(2′-hydroxybenzyl)-*N*-nitrosohydroxylamine, ammonium salt was 80 mg (72%). mp 137.5–142.2 °C; IR (KBr): 3423 (broad), 1604, 1463, 927, 749, 698 cm^{-1}; ^{1}H NMR (D_2O)δ: 5.02 (2H, s), 6.68–7.15 (4H, m); HRMS (FAB) calcd. for $C_7H_7N_2O_3$ $[M–H]^-$ 167.046, found 167.046. Both Liebermann [7] and Griess' [8] reactions were positive indicating the presence of the nitroso group.

N-(3′-hydroxybenzyl)-N-nitrosohydroxylamine, ammonium salt ***(5)***. The overall yield from 3-hydroxybenzaldehyde was 30.6%. mp 126.6–130.1 °C; IR (KBr): 3422 (broad), 1605, 1466, 929, 740, 694 cm^{-1}; ^{1}H NMR (D_2O)δ: 4.90 (2H, s), 6.65–7.10 (4H, m); HRMS(FAB) calcd. for $C_7H_7N_2O_3$ $[M–H]^-$ 167.046, found 167.046. Both Liebermann and Griess' [7,8] reactions were positive indicating the presence of the nitroso group.

N-(4′-hydroxybenzyl)-N-nitrosohydroxylamine, ammonium salt ***(6)***. The overall yield from 4-hydroxybenzaldehyde was 22.4%. mp 101.4–103.4 °C; IR (KBr): 3427 (broad), 1602, 1436, 945, 748, 696 cm^{-1}; ^{1}H NMR (D_2O)δ: 4.85 (2H, s), 6.65 (2H, d, $J = 8.0$ Hz), 7.05 (2H, d, $J = 8.0$ Hz); HRMS(FAB) calcd. for $C_7H_7N_2O_3$ $[M–H]^-$ 167.046, found 167.045. Both Liebermann and Griess' reactions were positive indicating the presence of the nitroso group [7,8].

2.3. Kinetics

Tyrosinase activity was measured by a spectrophotometric method using a Shimadzu UV-160A spectrophotometer or by a polarographic method using a Central Oxygraph-9 equipped with a Clark-type oxygen electrode [5].

The IC_{50} values were obtained from the spectrophotometric assay. Thus, 3 mL of 0.5 mM L-dopa in 67 mM phosphate buffer (pH 6.8) and 0.1 mL of the test sample solution were mixed at 30 °C. Subsequently, 0.1 mL of the aqueous solution of mushroom tyrosinase (80 units) was added to the mixture to measure immediately, the initial rate of linear increase in optical density at 475 nm, corresponding to the formation of dopachrome. The inhibitory effect of the sample was represented as percentage of inhibition compared with the control.

The effect of copper sulfate on the inhibition of mushroom tyrosinase by compound **6** was performed by the spectrophotometric assay. Accordingly, the mixture contained, in a total volume of 3.2 mL, 67 mM phosphate buffer (pH 6.8), 0.5 mM L-dopa, 13.7 μM of compound **6** (equivalent amount of IC_{50}), and 50 μM copper sulfate. The mixture was incubated at 30 °C for 3 min. Subsequently, 0.1 mL of the aqueous solution of mushroom tyrosinase (80 units) was added to the mixture. The reaction was monitored at 475 nm.

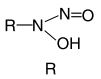
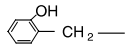
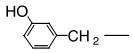
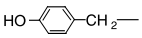
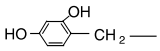
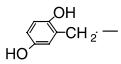
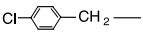
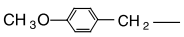
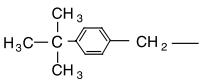
3. Results and discussion

The inhibitory activity of the newly synthesized compounds on mushroom tyrosinase and the type of inhibition obtained from Lineweaver–Burk plots are listed in Table 1. The synthesized compounds show activities with IC_{50} values ranging from 10 to 24 μM, which are nearly comparable with that of the well-known potent inhibitor, kojic acid (**12**, Fig. 2) [9]. Since these compounds have IC_{50} values less than the parent compound, *N*-benzyl-*N*-nitrosohydroxylamine ($IC_{50} = 3.0\,\mu M$), the substituents on the benzene ring slightly decrease the inhibition activities, regardless of the nature of the substituent or its position. Compound **6** showed an IC_{50} value that is two orders of magnitude greater than that of the corresponding hydroxylamine ($IC_{50} = 1.1$ mM), confirming that the activity is caused by the *N*-nitrosohydroxylamino group as reported in our previous paper [5].

The inhibition data obtained at pH 6.8, were plotted using Lineweaver–Burk plots (Fig. 3) and analyzed. Compounds **4**, **9**, **10**, and **11** showed mixed type of inhibition with respect to L-dopa. On the other hand, compounds **5**–**8**, which have a phenolic hydroxyl group at the *meta*- or *para*-position, inhibited uncompetitively with respect to L-dopa. Moreover, compound **6** exhibited competitive inhibition with respect to oxygen (Fig. 3).

To confirm the mechanism of inhibition by compound **6**, the effect of copper sulfate on the inhibitory activity was examined. The results showed that the activity of compound **6** decreased from 50% of inhibition to 41% by the addition of copper sulfate, similar to that observed with cupferron in the presence of copper sulfate (38%). These observations suggest that the mechanism of inhibition by compound **6** may be

Table 1
Inhibition of tyrosinase by *N*-substituted *N*-nitrosohydroxylamines

Compound		IC_{50} (μM)	Type of inhibition with respect to L-dopa
4		19.2	Mixed
5		20.3	Unccompetitive
6		13.7	Unccompetitive
7		11.6	Unccompetitive
8		23.8	Unccompetitive
9		13.1	Mixed
10		9.7	Mixed
11		15.8	Mixed

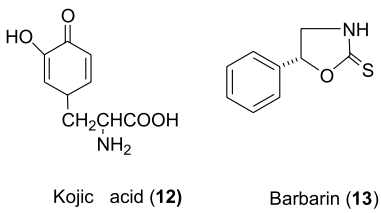


Fig. 2. Chemical structures of kojic acid (**12**) and barbarin (**13**).

due to its chelation of copper ion in the active site of the enzyme as has been shown for cupferron, even though their type of inhibition is different. In addition, when the enzyme was pre-incubated with cupferron without L-dopa, the enzyme activity was decreased [5]. However, pre-incubation of the enzyme with compound **6** did not change activity. Since the resting form of the enzyme is mostly *met*-tyrosinase [10], it appears that compound **6** interacts with only *oxy*-tyrosinase, but not with *met*-tyrosinase, in contrast to cupferron.

Among the *N*-monohydroxybenzyl derivatives (**4**–**6**), the *ortho*-hydroxyl derivative (4) showed mixed type of inhibition while both *N*-dihydroxybenzyl derivatives

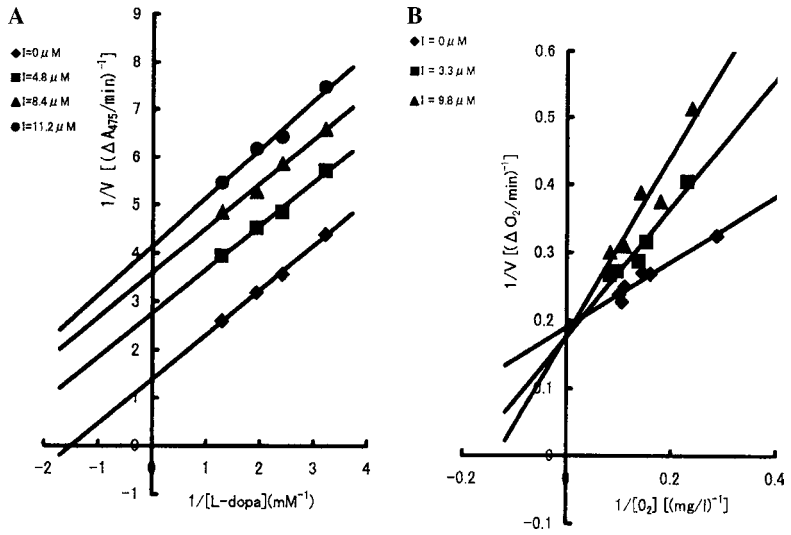


Fig. 3. Lineweaver–Burk plots of the mushroom tyrosinase reaction with *N*-(4′-hydroxybenzyl)-*N*-nitrosohydroxylamine (**6**) at pH 6.8: (A) with respect to L-dopa and (B) with respect to oxygen.

(**7** and **8**) exhibited uncompetitive inhibition, as noted above. Therefore, the *meta*- or *para*-hydroxyl group on the benzyl residue would play a role in this mechanism. One possibility is that the *meta*- or *para*-hydroxyl group strengthens the interaction between the inhibitors and the copper in the active site of the *oxy*-tyrosinase at the oxygen binding site, by forming a hydrogen bond to the amino nitrogen near to the active site of the enzyme (Fig. 4). This idea was supported by the results of inhibition under weakly acidic conditions. At pH 5.8, compound **6** exhibited mixed type of inhibition with respect to both L-dopa and oxygen, in contrast to uncompetitive inhi-

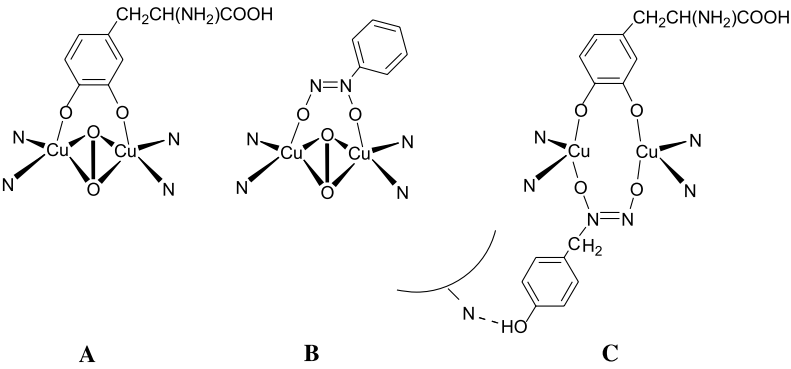


Fig. 4. Proposed binding models for the active site of the *oxy*-tyrosinase. (A) A model of L-dopa as a substrate [13]; (B) a model of cupferron as a competitive inhibitor [5]; (C) a model of compound **6** as an uncompetitive inhibitor with L-dopa as a substrate.

bition with respect to L-dopa and competitive inhibition with respect to oxygen at pH 6.8. Under acidic conditions, hydrogen bonding between phenolic hydroxyl group of compound **6** and the nitrogen might weaken so that the interaction at the oxygen binding site might decrease. In case of compound **4**, hydrogen bonding of the *ortho*-hydroxyl group may be weakened by steric hindrance, making it difficult to show uncompetitive inhibition.

Most tyrosinase inhibitors that chelate the copper in the active site of the enzyme show competitive inhibition such as tropolone (**2**) and kojic acid (**12**) (Figs. 1 and 2). Here, we have shown that *N*-substituted *N*-nitrosohydroxylamine derivatives, which have the ability to chelate the copper, exhibit a different type of inhibition depending on the substituent. The different type of inhibition might be due to the differences in the binding ability of the inhibitor to the copper in the active site of enzyme as well as the differences in the interaction of the *N*-substituent with the enzyme. Among tyrosinase inhibitors, uncompetitive inhibitor with respect to L-dopa is rare. Barbarin (**13**, Fig. 2) has been known as an uncompetitve tyrosinase inhibitor with L-dopa, but its mechanism of inhibition has not been elucidated yet [11]. Although cyanide is a competitive inhibitor of oxygen, it exhibited noncompetitive inhibition with respect to L-dopa [12]. In this paper, we showed that *N*-(4′-hydroxybenzyl)-*N*-nitrosohydroxylamine (**6**) is a tyrosinase inhibitor, which is uncompetitive with L-dopa and competitive with oxygen, and acts through a chelation mechanism.

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