**N-(3,5-Dihydroxybenzoyl)-6-hydroxytryptamine as a Novel Human Tyrosinase Inhibitor That Inactivates the Enzyme in Cooperation with L-3,4-Dihydroxyphenylalanine**

Yoshimitsu Yamazaki* and Yasuhiro Kawano

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology; 1–1–1 Higashi, Tsukuba, Ibaraki 305–8566, Japan.

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Inhibitors of tyrosinase (EC 1.14.18.1) are potentially applicable to skin lightening agents. A major class of tyrosinase inhibitors is phenolic compounds structurally analogous to the substrates, L-tyrosine and L-3,4-dihydroxyphenylalanine (DOPA). Recently, we found that introduction of a dihydroxyphenyl group into serotonin (that is, a 5-hydroxyindole derivative) improved its tyrosinase inhibitory activity. The succeeding study with positional isomers of hydroxyindoles revealed that 6-hydroxyindole was more potent than 5-hydroxyindole as an inhibitor of human HMV-II melanoma tyrosinase. The IC₅₀ values for 2 and three reference compounds, N-(3,5-dihydroxybenzoyl)serotonin, 6-hydroxyindole, and kojic acid, were 9.1, 842, 22, and 310 μM, respectively, indicating that the 6-hydroxyindole moiety was more effective than 5-hydroxyindole as the pharmacophore of polyphenolic tyrosinase inhibitors and that the inhibitory activity of 6-hydroxyindole was strengthened by the link with a resorcinol group. Furthermore, compound 2 exhibited a unique property of inactivating the human tyrosinase in the presence of low concentrations of DOPA. This inactivation was attenuated by high concentrations of DOPA and for the most part was irreversible as confirmed by activity stain in native polyacrylamide gel electrophoresis and by removal of 2 and DOPA using gel permeation chromatography. Tyrosinase is the enzyme that oxidizes tyrosine to DOPA and further oxidizes DOPA to the melanin precursor dopaquinone. A compound such as 2 that inactivates the enzyme in the presence of a small amount of DOPA is therefore attractive as a new type of tyrosinase inhibitor. Unfortunately, 2 hardly suppressed the melanogenesis in melanoma cell culture. However, the above strong inhibitory activity and the unique property in the combination with DOPA suggest that this compound is a useful lead in designing new antimelanogenic agents.

**Key words** tyrosinase; inhibitor; human; L-3,4-dihydroxyphenylalanine; hydroxyindole; phenolic

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**Results and Discussion**

The compound 2 was synthesized by coupling of 6-benzyl-oxytryptamine hemisulfate with 3,5-dihydroxybenzoic acid using dicyclohexylicarbodiimide (DCC) followed by catalytic hydrogenation to remove the benzyl group. Although the intrinsic substrate, DOPA, contains an ortho-diphenol (catechol) group, meta-diphenolic acid was selected because the catechol group was not necessarily superior to the meta-diphenol in inhibiting tyrosinase as judged from the facts that 3,5,2-tetrahydroxybenzamide was the strongest tyrosinase inhibitor among many hydroxy-substituted N-benzylbenzamides, and N-(2,4-dihydroxybenzoyl)serotonin was a stronger inhibitor than N-(3,4-dihydroxybenzoyl)serotonin, and oxyresveratrol was a much stronger inhibitor than piceatannol (Table 1). N-(3,5-Dihydroxybenzoyl)serotonin was also synthesized by DCC-mediated coupling of serotonin hydrochloride with 11 for comparison between 6- and 5-hydroxyindole moieties. The inhibitory activities of these compounds together with five related and reference compounds (6-hydroxyindole, N-(3,4-dihydroxybenzoyl)serotonin, 6-hydroxytryptamine creatinine sulfate salt, serotonin hydrochloride, and kojic acid) were tested for the catecholase activity of HMV-II melanoma tyrosinase. The results are summarized in Table 1. The IC₅₀ value for compound 2 against HMV-II tyrosinase was less than one half of the value for 6-hydroxyindole (1), showing that the introduction of the dihydroxyphenyl moiety enhanced the inhibitory activity of 1. The compound 2 was the second strongest inhibitor of HMV-II tyrosinase in Table 1, but the IC₅₀ was only 1.5 μM higher than that for the strongest inhibitor, oxyresveratrol (7). The IC₅₀ for 2 against HMV-II tyrosinase was markedly smaller (1/90—1/40) than
those for the corresponding 5-hydroxyindole derivatives 3
and 4. 6-Hydroxytryptamine creatinine salt (5) also showed a
very small IC₅₀ as compared to serotonin hydrochloride (6).
These facts confirm that the 6-hydroxy group in indole is more
effective than the 5-hydroxy group for the tyrosinase
inhibition. Many kinds of tyrosinase inhibitors have been
developed, but their inhibitory activities were mostly assayed
with mushroom and mouse B16 melanoma tyrosinase.1)
However, the activity of inhibitors against mushroom tyrosi-

nase is often very different from that against mammalian ty-

rosinase, so if the inhibitor is to be applied to human skin
agents, it is beneficial to test them with human tyrosinase.
Though few data are presently available concerning human
tyrosinase, the IC₅₀ for 2 against HMV-II tyrosinase belongs
to the smallest group among the reported IC₅₀ for arbutin,9,10)
aloesin,10) (+)-imperanone,11) and kinoben.12) Also in Table
1, the IC₅₀ for 2 is one thirty-fourth that for kojic acid (9),
a standard tyrosinase inhibitor, and as small as that for
oxyresveratrol (7), the active ingredient in the whitening cos-
metics from Morus alba.13) Thus, the inhibitory activity of 2
against the human tyrosinase is higher than or comparable to
those of the known inhibitors.

The Lineweaver–Burk plots obtained with different con-
centrations of 2 for HMV-II tyrosinase activity cross at a
point slightly to the left of the Y-axis, indicating that 2 is a
mixed type inhibitor with respect to DOPA (Fig. 2). The ap-
parent Michaelis constant (Kₘ) was 1.1 mM and replots of the
slope and Y-intercept of the lines in Fig. 2 vs. concentration
of 2 gave the inhibitor constants Kᵢ=1.7 μM for Enzyme-
Inhibitor complex and Kᵢ′=53 μM for Enzyme-Substrate-
Inhibitor complex, respectively.14) Although the simple
Michaelis–Menten kinetics is not properly applied here be-
cause of the enzyme inactivation induced by 2 and DOPA
(see the later section), it is most likely that compound 2 pri-
marily competes for the enzyme active site with DOPA.
Tyrosinase contains a binuclear copper complex in the active
center for the substrate binding and oxidation. Since the two
resorcinol derivatives 2 and 3 showed the very different in-
hibitory activities, 2 is bound to the copper center for the in-
hibition probably through 6-hydroxyindole moiety rather
than the resorcinol moiety. In addition, HPLC analysis
showed that the peak of 2 did not change after incubation
with HMV-II tyrosinase for 1 h, while DOPA diminished by
71% under the same condition (Fig. 3). This result confirms
that 2 is not a substrate of the tyrosinase under this experi-
mental condition.

To study the inhibition mechanism in more detail, HMV-II
tyrosinase was pretreated with compound 2 before assay with
DOPA. The pretreatment mixtures were prepared without or
with a small amount of DOPA to investigate the effect of 2
on the enzyme turning over in catalysis. Thus, HMV-II tyrosi-

nase solutions containing 2 and/or DOPA, or none of them
were incubated at 37 °C for 1 h and then DOPA was added
to the solutions to 2.5 mM (i.e., the concentration used
in the standard assay). After standing at room temperature
for 24 h, the mixtures pretreated with 2 plus 10 μM DOPA
showed melamin formation as low as the control level (autox-
idation of DOPA), while all mixtures pretreated with 2 alone
became dark with much melamin (Fig. 4A). This pretreatment
effect of an inhibitor plus DOPA was not found with compound
3 though its concentration was ten times that used for
2. In addition, 6-hydroxyindole (1) and oxyresveratrol (7)
showed a similar combination effect with DOPA on the
melanin suppression (data not shown). The tyrosinase activity
itself decreased by the pretreatment with 2 plus DOPA
and the activity loss increased with the preincubation time.
When treated only with 2 or DOPA, the tyrosinase activity

### Table 1. Inhibitory Activity of the Compounds for Tyrosinase from Human HMV-II Melanoma Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>% inhibition at 100 μM</th>
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<tbody>
<tr>
<td>Serotonin hydrochloride (6)</td>
<td>&gt;1000</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>6-Hydroxytryptamine creatinine sulfate (5)</td>
<td>7.6 ± 0.3</td>
<td>98</td>
</tr>
<tr>
<td>Piceatannol (8)</td>
<td>&gt;300</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Kojic acid (9)</td>
<td>310 ± 40</td>
<td>27 ± 1</td>
</tr>
</tbody>
</table>

a) Values are means ± S.D. (standard deviation), n=3. b) 5 was dissolved in water as the sample solution (0.1—10 mM) and water was used as the control. Creatinine (100 μM) was confirmed to be inactive in this inhibition assay. c) Calculated by subtracting the relative activity (% of control) from 100. d) n=2. e) At 30 μM.
slightly changed in the experimental period (Fig. 4B). The loss of tyrosinase activity was dependent on the dose of 2 and DOPA (Fig. 4C). The plots of the residual activities determined after preincubation for 10 min show that DOPA itself caused the decrease in tyrosinase activity at the high concentrations, but that the effect of DOPA was increased by the presence of 2. The maximum effect of DOPA was found at about 100 \( \mu \text{M} \) in the combination with 10 \( \mu \text{M} \) 2. The entity of tyrosinase treated with 2 and DOPA was investigated by the method of activity stain in native polyacrylamide gel electrophoresis (PAGE).\textsuperscript{15} The untreated tyrosinase gave the main activity band (colored red) at molecular weight of 67 kDa (lit.\textsuperscript{16} 62610 Da), whose migration was consistent with that of the immunologically stained tyrosinase band (Figs. 4D, F; see also the color print in Graphical Abstract). The activity band for the samples treated with 10 \( \mu \text{M} \) 2 plus 10 or 2500 \( \mu \text{M} \) DOPA was faint and it did not appear for the samples treated with 10 \( \mu \text{M} \) 2 plus 100 or 1000 \( \mu \text{M} \) DOPA. The activity band was also lighter for the samples treated with 1000 or 2500 \( \mu \text{M} \) DOPA alone than those for the samples treated with 0—100 \( \mu \text{M} \) DOPA. This coloration is nearly parallel to the enzyme activity shown in Fig. 4C, suggesting that the activity decrease by 2 and DOPA is due to disappearance of the main tyrosinase activity.

The combination effect of 2 with DOPA was further studied by separating the pretreated enzyme from 2 and DOPA by gel permeation chromatography (GPC). Samples containing tyrosinase without or with 10 \( \mu \text{M} \) 2 and/or 10 \( \mu \text{M} \) DOPA were incubated at 37°C for 1 h and then directly assayed with 2.5 mM DOPA (Fig. 5A) or assayed with the protein fractions after passing through a Sephadex\textsuperscript{©} G-50 column (Fig. 5B). The initial velocity (% of the control) for the sample pretreated only with 2 recovered from 42 to 78% by the GPC treatment (cf. plots c and c'), but that for the sample pretreated with 2 plus DOPA recovered from 0% (plot d) to only 9% (plot d'). This enzyme activity did not significantly increase when the same sample (the column eluate) was left at room temperature for 6 h before initiating the assay (plot d').

The most interesting property of compound 2 is that the inhibition of HMV-II tyrosinase by 2 is greatly strengthened by the presence of low concentrations of DOPA. Since this inhibition remains long after the condition is altered by the addition of high concentrations of DOPA, it might be reasonable to conclude that the enzyme is inactivated by the treatment with 2 plus DOPA. The disappearance of activity stain in PAGE (Fig. 4D) and the limited recovery of activity after GPC separation (Fig. 5B) imply that this inactivation is mostly irreversible. Tyrosinase is known to be inactivated during the oxidation of DOPA\textsuperscript{17,18} and several phenolic substrates.\textsuperscript{19—21} This type of inactivation is called suicide inactivation, whose mechanism has been discussed for many years.\textsuperscript{17—23} For example, a two-electron transfer from the substrate to the active center Cu\textsuperscript{2+} ion leading to its liberation as a Cu\textsuperscript{0} atom was proposed as the mechanism for inactivation of tyrosinase in the enzymatic oxidation of resorcinol.\textsuperscript{21} Compound 2 has a resorcinol moiety, but this is probably not the cause of the present inactivation, since another resorcinol derivative 3 did not suppress the melanin formation (Fig. 4A). The above-mentioned electron transfer might be possible from the 6-hydroxyindole moiety with pro-
duction of a conjugated iminoketone, but presently we have no evidence for it. Although DOPA may act as the suicide substrate in the present phenomenon, its effective concentration (around 100 μM) is considerably low as compared to those (0.25—10 mM) used for suicide inactivation under aerobic conditions. The effectiveness of DOPA at such a low concentration implies a possibility that 2 enhances the suicide inactivation by DOPA, for instance, through conformational change of the enzyme protein by binding. However, this mechanism does not explain the fact that there was the inactivation of the enzyme protein by binding. Nevertheless, compound 2 was rather inactive in suppressing the melanogenesis in cultured HMV-II cells. The melanin formation in these cells was not significantly suppressed by 3—20 μM 2, while 10—30 μM 2 decreased the cell viability (data not shown). These problems must be solved by further modification of the chemical structure. However, the above strong inhibitory activity and the unique property in the combination with DOPA suggest that this compound is a useful lead in designing new antimelanogenic agents. The mechanism of the present inactivation is to be studied through detailed kinetic analysis using purified HMV-II tyrosinase in future.

**Experimental**

**Materials** 6-Hydroxytryptamine creatinine sulfate (5) and 2-(6-benzyl-oxyindolyl)ethylamine hemisulfate (10) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), 6-hydroxyindole (1), piceatannol (8), and serotonin hydrochloride (6) were from Wake Pure Chemicals Inc. (Osaka, Japan), kojic acid (9) and 3,5-dihydroxybenzoic acid (11) were from Tokyo Chemical Industries Ltd. (Tokyo, Japan), and oximeseratol (7) was from Hangzhou Great-Forest Biomedical Ltd. (Hangzhou, P. R. China).

**Synthesis** N-(3,5-Dihydroxybenzoyl)-6-hydroxytryptamine (2) was synthesized by the DCC method. The amine salt 10 (100 mg) was suspended in 5 ml dimethylformamide (DMF) containing 2 ml pyridine and mixed with a solution of 100 mg 3,5-dihydroxybenzoic acid (11) and 100 mg DCC in 0.5 ml DMF. The mixture was stirred at room temperature for 1 h. The insoluble material was collected by centrifugation, suspended in 1 ml dimethylsulfoxide containing 0.2 ml pyridine, and supplemented with 200 mg 11 and 200 mg DCC. To the supernatant from the above centrifugation, 200 mg DCC was added. After stirring at room temperature overnight, the two mixtures were filtered and the filtrates were concentrated by a rotary evaporator. The residues were respectively dissolved in 30 ml ethyl acetate, and washed successively with 10% citric acid, 10% NaHCO3, and saturated NaCl in water. Finally, the ethyl acetate solutions were combined, dried over Na2SO4, concentrated by an evaporator, and applied to a silica gel column. The eluate with ethyl acetate was dissolved in 4 ml ethanol containing 0.2 ml acetic acid, and hydrogenated over 30 mg Pd/C at an atmospheric pressure for 4 h. The catalyst was filtered off and the filtrate was concentrated to give a residue, which was purified by silica gel column chromatography. The main product (2) was eluted with ethyl acetate and crystallized from ethyl acetate and benzene as fine needles (34 mg, 34% yield), dec. 213—217°C, Anal. Calcd for C17H16N2O4·1/2H2O: C, 63.54; H, 5.33; N, 8.72%. Found: C, 63.37; H, 5.31; N, 8.46%; FAB-MS m/z 313.1197 ([M+H]+). (Caled C17H17N2O4·1/2H2O: 313.1187; and 'H-NMR (270 MHz, acetone-d6): δ: 2.99 (2H, t, J=7 Hz, CH3), 3.64 (2H, m, CH2N), 6.47 (1H, t, J=2 Hz, H-4), 6.64 (1H, J=2 Hz, H-3)).

**Fig. 5. Tyrosinase Activity Pretreated with 2 and/or DOPA and That Recovered from the Mixtures by GPC**

(A) Monitor of DOPA oxidation by tyrosinase 1 h preincubated with 10 μM 2 and 10 μM DOPA (plot d), 10 μM 2 alone (c), 10 μM DOPA alone (b), or none of them (a). (B) Monitor of DOPA oxidation by tyrosinase in the protein fractions from GPC of the same preincubated samples as above. Plot a’, b’, c’, d’, and d” correspond to plot a, b, c, d, and d, respectively. Plot d’ was recorded after the column eluate was left at room temperature for 6 h. The total activities indicated by plot a and plot a’ were almost equal as calculated with the volumes of assay mixture and sample solution.
dd, J=8 and 2 Hz, H-5’), 6.82 (1H, d, J=2 Hz, H-7’), 6.84 (2H, d, J=2 Hz, H-2,5’). 6.99 (1H, m, H-2’), 7.43 (1H, d, J=8 Hz, H-4’), 7.56 (1H, brs, NHCO), 7.82 (1H, br, H-6), 8.41 (2H, brs, OH×2), and 9.64 (1H, br, H-1’).

N-(3,5-Dihydroxybenzoyl)serotonin (3) was similarly prepared from 6 and 11, crystalline solid, 94 mg (64% yield), mp 113—117°C and dec. 215—218 °C (with multiple phase transitions), FAB-MS m/z 313 (M+H+). (Calded C18H14N2O3S=312). 1H-NMR (270 MHz, acetone-d6) δ: 2.98 (2H, t, J=7 Hz, CH2), 3.66 (2H, m, CHN), 6.51 (1H, t, J=7 Hz, H-4), 6.71 (1H, dd, J=8 and 2 Hz, H-6’), 6.84 (2H, d, J=2 Hz, H-2,5’), 7.06 (1H, J=2 Hz, H-4’), 7.10 (1H, brs, H-2’), 7.46 (1H, d, J=8 Hz, H-7’), 7.72 (1H, brs, NHCO), 7.98 (1H, br, H-6), 8.33 (1H, brs, OH), and 9.75 (1H, br, H-1’). N-(3,4-Dihydroxybenzoyl)serotonin (4) was synthesized in the previous work.

Tyrosinase Assay

Human HMV-II melanoma cells (Dai-Nippon-Sumitomo Pharmaceuticals Inc., Osaka, Japan) were grown in RPMI-1640 medium containing 15% fetal bovine serum. Tyrosinase was extracted from cells with a lysis buffer containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% polyoxyethylene(9) octylphenyl ether (NP-40), 0.1% Na dodecylsulfate, and 0.1% Na deoxycholate in 10 mM Tris/HCl buffer (pH 7.4) by sonication at 4 °C. After removing the cell debris by centrifugation (15000 rpm, 10 min), the supernatant was used as the tyrosinase source. Protein was determined by the bicinchoninic acid method with bovine serum albumin as standard. The tyrosinase activity was assayed with DOP A as the substrate. Briefly, assay solutions were prepared in 50 mM phosphate buffer (pH 6.8). Compound concentrations below 2%, unless otherwise stated. (1) Samples (134 μl each) containing 5.4 mU/ml tyrosinase and 10 μM DOP A were preincubated at 37 °C for 10 min and then mixed with 10 μM DOP A or none of them were preincubated at 37 °C for 0, 3, 10, 30, or 60 min and then the enzyme was assayed by adding 0.5 ml prewarmed (37 °C) solutions of 5 mM DOP A. (2) Samples (0.5 μl each) containing 5.4 mM tyrosinase and 10 μM DOP A were preincubated at 37 °C for 0, 3, 10, 30, or 60 min and then the enzyme was assayed by adding 0.5 ml prewarmed (37 °C) solutions of 5 mM DOP A. (3) Samples (0.5 μl each) containing 5.4 mM tyrosinase and 0—10 μM DOP A and/or 0—100 μM dopamine were preincubated at 37 °C for 1 h and then mixed with 10 μl loading buffer (4% sodium dodecyl sulfate, 20% glycerol and 0.02% Bromophenol blue in 0.1% Tris/acetate buffer, pH 6.8). The mixtures (10 μl each) were immediately applied to a 1.5% 20% denaturing gradient polyacrylamide gel and electrophoresed under the condition of Laemmli26 at 4 °C for 80 min. The gel was immersed in 30 ml 50 mM phosphate buffer (pH 6.8) containing 2.6 mM DOP A and 10 μM dopamine for 1 h, and then mixed with 2.6 mM DOP A to assay tyrosinase activity. The same experiment was repeated with the sample for 2 plus DOP A, but the eluate from the column was left at room temperature for 6 h before initiating the assay as above.

References