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# Structural insight into the active site of mushroom tyrosinase using phenylbenzoic acid derivatives

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ABSTRACT

So far, many inhibitors of tyrosinase have been discovered for cosmetic and clinical agents. However, the molecular mechanisms underlying the inhibition in the active site of tyrosinase have not been well understood. To explore this problem, we examined here the inhibitory effects of 4′-hydroxylation and methoxylation of phenylbenzoic acid (PBA) isomers, which have a unique scaffold to inhibit mushroom tyrosinase. The inhibitory effect of 3-PBA, which has the most potent inhibitory activity among the isomers, was slightly decreased by 4′-hydroxylation and further decreased by 4′-methoxylation against mushroom tyrosinase. Surprisingly, 4′-hydroxylation but not methoxylation of 2-PBA appeared inhibitory activity. On the other hand, both 4′-hydroxylation and methoxylation of 4-PBA increased the inhibitory activity against mushroom tyrosinase. *In silico* docking analyses using the crystallographic structure of mushroom tyrosinase indicated that the carboxylic acid or 4′-hydroxyl group of PBA derivatives could chelate with cupric ions in the active site of mushroom tyrosinase, and that the interactions of Asn260 and Phe264 in the active site with the adequate-angled biphenyl group are involved in the inhibitory activities of the modified PBAs, by parallel and T-shaped π-π interactions, respectively. Furthermore, Arg268 could fix the angle of the aromatic ring of Phe264, and Val248 is supposed to interact with the inhibitors as a hydrophobic manner. These results may enhance the structural insight into mushroom tyrosinase for the creation of novel tyrosinase inhibitors.



Tyrosinases (EC 1.14.18.1), which is a type 3 copper protein, are widely distributed in nature from microorganism to human to produce melanin pigments. They function to defense their bodies from various circumstances, such as skin injury by UV irradiation.[1] However, overproduction of melanin pigments become problems in the cosmetic and clinical points of views, such as melasma, freckles and melanosis.[2,3]

Tyrosinase catalyzes the oxidation of monophenols, such as L-tyrosine, into *ortho*-quinones (*o*-quinones) (monophenolase activity) and the oxidation of *ortho*-diphenols (*o*-diphenols) such as L-DOPA into *o*-quinones (diphenolase activity).[4,5] The active pocket of the enzyme contains two cupric ions and one oxygen ($O_2$) molecule. Until now, a large number of studies about tyrosinase inhibitors have been reported.[6–8] These compounds has been mainly used as skin whitening and depigmenting agents. Phenolic compounds are studied as tyrosinase inhibitors because of the analogy for true substrate, L-tyrosine. There are some phenylphenol compounds reported as tyrosinase inhibitors such as 4,4′-dihydroxybiphenyl, fortuneanoside E and honokiol with $IC_{50}$ values of 1.91, 140 and 67.9 μM, respectively.[9–11]

Our previous data suggested that 3-phenylbenzoic acid (PBA) (Compound 2) is a new type of tyrosinase inhibitor (Table 1).[12] However, 2- and 4-PBA (Compounds 1 and 3, respectively) had weaker inhibitory activities. These results suggested that the carboxylic acid group could chelate with cupric ions, and that the substituted phenyl groups are involved in their inhibitory activities against mushroom tyrosinase.[12] Furthermore, the molecular

*Abbreviations:* DMSO, dimethyl sulfoxide; MM-GB/SA, molecular mechanics-generalized born/surface area; PBA, phenylbenzoic acid; UV, ultra violet.

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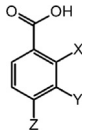
**Table 1**
The chemical structures of PBA derivatives.



| Compound No. | X | Y | Z |
|---|---|---|---|
| 1 | $-C_6H_5$ | – | – |
| 2 | – | $-C_6H_5$ | – |
| 3 | – | – | $-C_6H_5$ |
| 4 | $-C_6H_4OH$ | – | – |
| 5 | – | $-C_6H_4OH$ | – |
| 6 | – | – | $-C_6H_4OH$ |
| 7 | $-C_6H_4OCH_3$ | – | – |
| 8 | – | $-C_6H_4OCH_3$ | – |
| 9 | – | – | $-C_6H_4OCH_3$ |

docking studies revealed that Asn260 in the active site of mushroom tyrosinase is an important residue for the interaction with 3-PBA.[12]

In this study, we investigated the molecular basis of the tyrosinase inhibition occurred in the active site of mushroom tyrosinase, using PBA derivatives. Tested compounds were purchased from Tokyo Chemical Industry Co., Ltd. (Compounds 1 and 3) (Tokyo, Japan), Combi-Blocks, Inc. (Compounds 2 and 4–6) (San Diego, USA), Key Organics Ltd. (Compound 7) (Cornwall, UK) and Vitas-M (Compounds 8 and 9) (Kowloon, Hong Kong) (Table 1). To assess the inhibitory activity of these compounds against mushroom tyrosinase, monophenolase activity was measured using L-tyrosine as substrate.[12,13] Mushroom tyrosinase and L-tyrosine were purchased from Sigma (St. Louis, USA). Briefly, 10 μL of mushroom tyrosinase solution (400 units/mL) was added to a 96-well microplate in a total volume of 100 μL assay mixture containing 0.1 mM L-tyrosine with 50 mM potassium phosphate buffer (pH 6.5). The enzyme reaction was monitored by measuring changes in absorbance at 475 nm at 30 °C with every 60 s for 25 times by Envision (Perkin Elmer Co., Ltd., Waltham, USA).[12,13]

The percentage of tyrosinase activity was calculated as follows: Relative activity (%) = (A/B) × 100 (%), where A is the initial slope of absorbance versus time with the tested sample and B is the initial slope of absorbance versus time with dimethyl sulfoxide (DMSO) instead of the sample.[12,13] The $IC_{50}$ values were determined when Y-axis revealed 50% of inhibition.

Previously, 2-PBA (Compound 1) had little inhibitory activity against mushroom tyrosinase. This suggested little availability of the carboxylic acid group in 2-PBA to chelate with cupric ions in the active site.[12] Here, surprisingly, 4′-hydroxylation of 2-PBA (Compound 4) resulted in the appearance of inhibitory activity ($IC_{50}$ = 100.18 μM: Fig. 1A). However, this inhibitory effect is abolished by the subsequent methoxylation of the hydroxyl group (Compound 7). These observations indicate that the hydroxyl group of Compound 4 works as a chelating unit.

Our previous data showed that 3-PBA (Compound 2) is the most potent tyrosinase inhibitor ($IC_{50}$ = 6.97 μM) among the PBA isomers.[12] As described above, the substitution of hydrogen atom on 4′-position to hydroxyl group in 2-PBA (Compound 1) improved its inhibition activity (Compound 4). So, we tried to examine the effects of 4′-hydroxylation of 3-PBA (Compound 5) on the inhibitory activity. Unfortunately, this substitution resulted in the decrease of the inhibitory activity ($IC_{50}$ = 10.59 μM: Fig. 1B). Furthermore, the 4′-methoxylation (Compound 8) reduced the inhibitory activity more than 4′-hydroxylation ($IC_{50}$ = 15.30 μM: Fig. 1B). The newly introduced substitution groups are considered to change the electron densities of the neighboring phenyl rings. These changes may cause alterations of the binding affinities of Compounds 5 and 8 in the active pocket of mushroom tyrosinase.

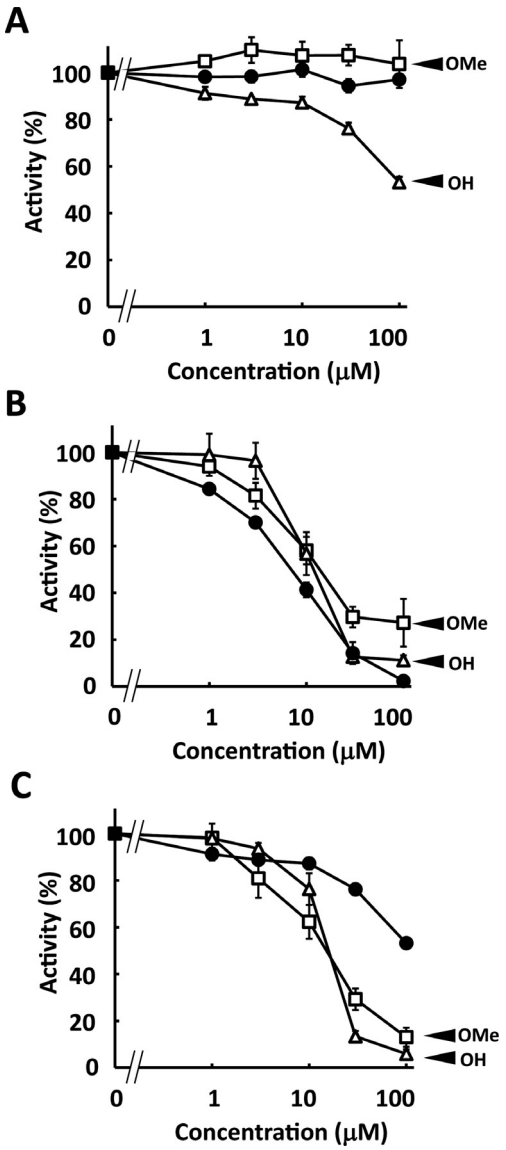



**Fig. 1.** Dose-dependent effects of phenylbenzoic acid derivatives on mushroom tyrosinase. The mushroom tyrosinase activity was measured using L-tyrosine as substrate. The data are shown as the percentages of activities in the presence of various concentrations of (A) Compounds 1 (*closed circle*), 4 (*open triangle*) and 7 (*open square*). (B) Compounds 2 (*closed circle*), 5 (*open triangle*) and 8 (*open square*). (C) Compounds 3 (*closed circle*), 6 (*open triangle*) and 9 (*open square*). The abbreviations of OH and OMe indicate 4′-hydroxylated and methoxylated derivatives, respectively. The data are the averages of three independent experiments and bars show the SE values.