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Discovery of a new type of scaffold for the creation of novel tyrosinase inhibitors

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ABSTRACT

Tyrosinase is known as the key enzyme for melanin biosynthesis, which is effective in preventing skin injury by ultra violet (UV). In past decades, tyrosinase has been well studied in the field of cosmetics, medicine, agriculture and environmental sciences, and a lot of tyrosinase inhibitors have been developed for their needs. Here, we searched for new types of tyrosinase inhibitors and found phenylbenzoic acid (PBA) as a unique scaffold. Among three isomers of PBA, 3-phenylbenzoic acid (3-PBA) was revealed to be the most potent inhibitor against mushroom tyrosinase (IC_{50} = 6.97 μM, monophenolase activity; IC_{50} = 36.3 μM, diphenolase activity). The kinetic studies suggested that the apparent inhibition modes for the monophenolase and diphenolase activities were noncompetitive and mixed type inhibition, respectively. Analyses by in silico docking studies using the crystallographic structure of mushroom tyrosinase indicated that the carboxylic acid group of the 3-PBA could adequately bind to two cupric ions in the tyrosinase. To prove this hypothesis, we examined the effect of modification of the carboxylic acid group of the 3-PBA on its inhibitory activity. As expected, the esterification abrogated the inhibitory activity. These observations suggest that 3-PBA is a useful lead compound for the generation of novel tyrosinase inhibitors and provides a new insight into the molecular basis of tyrosinase catalytic mechanisms.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a type 3 copper protein, which is widely distributed in nature. This enzyme catalyzes both the hydroxylation of monophenols, such as L-tyrosine, into *ortho*-diphenols (*o*-diphenols) (monophenolase activity) and the oxidation of *o*-diphenols, such as L-DOPA, into *ortho*-quinones (*o*-quinones) (diphenolase activity).[1,2] Two cupric ions in tyrosinases are individually coordinated with six histidine residues at the active sites, and are involved in the oxidation processes.[3,4] The *o*-quinone products are reactive precursors for the synthesis of melanin pigments.[5] Mushroom tyrosinase has been suggested to be present in three forms: *deoxy*-tyrosinase (E*deoxy*) binds to oxygen to form *oxy*-tyrosinase (E*oxy*); E*oxy* can bind to both L-tyrosine and L-DOPA. In the monophenolase activity, L-tyrosine is converted to L-DOPA. In this process, the enzyme is finally recycled as E*deoxy* form. In the diphenolase activity, L-DOPA is oxidized to dopaquinone (DQ) and the enzyme loses only one oxygen atom to form *met*-tyrosinase (E*met*). E*met* is considered to have higher affinity to L-DOPA as compared to other forms.[5] After catalyzation of L-DOPA, E*met* loses the oxygen atom remaining in its active pocket to regenerate E*deoxy* form.

In mammals, tyrosinase is responsible for skin, hair and eye pigmentation. Furthermore, pigmentation disorders have been suggested to be involved in hyperpigmentation status such as melasma, freckles, and melanoma, etc., and to cause serious medicinal and esthetic problems.[6] On the other hand, plant and fungal tyrosinases cause browning in vegetables and fruits when their tissues are injured.[7] For several decades, tyrosinase inhibitors have been widely discovered and several inhibitors have been used as cosmetics, medicines and agrichemicals.

Abbreviations: DQ, dopaquinone; EE, ethyl ester; L-DOPA, L-3,4-dihydroxyphenylalanine; MM-GB/SA, molecular mechanics-generalized born/surface area; PBA, phenylbenzoic acid; SASA, solvent-accessible surface area; UV, ultra violet; VDW, van der Waals.

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Previously, Cheng classified tyrosinase inhibitors into five classes, (1) Polyphenols; (2) Benzaldehyde and Benzoate Derivatives; (3) Long-chain Lipids and Steroid; (4) Others; (5) Irreversible; based on either the chemical structures or the inhibitory mechanisms.[8] Lee reported that benzoic acid is a mushroom tyrosinase inhibitor against L-DOPA oxidation activity (IC_{50} = 710 μM).[9] Recently, we showed that γ- and β- but not α-thujaplicins inhibit mushroom and human tyrosinases.[10] The IC_{50} values of γ- and β-thujaplicins against mushroom/human tyrosinases were calculated to be 0.07/1.15 μM and 0.09/8.93 μM, respectively. Furthermore, in silico studies suggested that the chelating effects of the α-hydroxycarbonyl (O=C—C—OH) group in the tropolone structures of γ- and β-thujaplicins on cupric ions in the tyrosinases are critical for their specific binding.[11] These data indicate that simple aromatic rings possessing a free carboxylic acid group in adequate positions, which could chelate cupric ions, may become a new type of tyrosinase inhibitors. To our knowledge, only few benzoic acid inhibitors, such as *p*-methoxybenzoic acid and 4-aminobenzoic acid (IC_{50} values against mushroom tyrosinase = 420 and 3.8 μM, respectively) have been reported.[12,13]

In this study, we examined a new type of scaffold, phenylbenzoic acid (PBA) as a unique lead structure for tyrosinase inhibitors (Table 1). Among the PBA isomers, 3-phenylbenzoic acid (3-PBA) was found to be the most potent inhibitor of mushroom tyrosinase. The kinetic studies suggested the complicated catalytic mechanisms for mushroom tyrosinase. Analyses by the esterification of the carboxylic acid group in 3-PBA and the in silico docking simulations against mushroom tyrosinase revealed that the specific interaction of the carboxylic acid group in 3-PBA with cupric ions in the active site of the tyrosinase is involved in the inhibitory activity. These results provide a new insight into the catalytic mechanisms of tyrosinases and the active pockets for the creation of novel tyrosinase inhibitors.

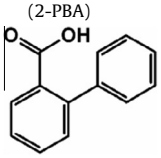
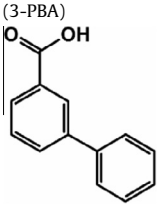
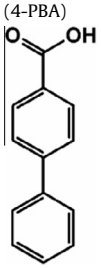
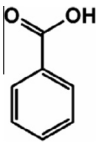
2. Results

2.1. Inhibitory activities of the PBA isomers against tyrosine hydroxylation (monophenolase) activity

The effects of 2-, 3- and 4-PBA isomers on mushroom tyrosinase activity were examined by measuring the hydroxylation of L-tyrosine (monophenolase activity). Benzoic acid was selected as a control compound, since the inhibitory activity had been already reported (IC_{50} = 710 μM).[9] As shown in Figure 1A, 3- and 4-PBA were found to inhibit the monophenolase activity in dose-dependent manners. The concentrations to inhibit 50% of the monophenolase activity (IC_{50}) are summarized in Table 2. 3-PBA inhibited mushroom tyrosinase most potently (IC_{50} = 6.97 μM) among the PBA isomers. The IC_{50} value of 3-PBA was approximately 30 times lower than that of benzoic acid. 4-PBA had a weak inhibitory activity (IC_{50} = 63.24 μM). However, 2-PBA showed little inhibitory activity.

These observations indicate that the substitution of the hydrogen atom by the phenyl group in benzoic acid (3- or 4-position) results in the stronger inhibitory effect on mushroom tyrosinase activity than that of benzoic acid. Thus, the position of phenyl group is suggested to be the main concern to the different inhibitory activities between the three isomers of PBA.

Table 1
The chemical structures of benzoic acid and phenylbenzoic acid (PBA) isomers

Compounds			
2-Phenylbenzoic acid (2-PBA)	3-Phenylbenzoic acid (3-PBA)	4-Phenylbenzoic acid (4-PBA)	Benzoic acid
			

PBA: Phenylbenzoic acid.

2.2. Inhibitory effects of the PBA isomers on L-DOPA oxidation (diphenolase) activity

We next examined whether the PBA isomers inhibit the diphenolase activity of mushroom tyrosinase using L-DOPA as substrate. As shown in Figure 1B, 3- and 4-PBA inhibited the synthesis of dopachrome from L-DOPA. 3-PBA was found to be the most potent inhibitor (IC_{50} = 36.32 μM) among the isomers as was the case in the tyrosine hydroxylation experiments (Fig. 1A). Reflecting the reaction velocities of the diphenolase activity, the IC_{50} value of 4-PBA increased to 216.05 μM. These IC_{50} values of 3- and 4-PBA against the diphenolase activity measured with L-DOPA were about 3 to 5-times higher than those against the monophenolase activity with L-tyrosine (Table 2).

It is noteworthy that the inhibitory effects of 3- and 4-PBA on the diphenolase activity are weaker than those in the monophenolase activity, suggesting the subtle different interactions of these inhibitors with the active pocket of mushroom tyrosinase in the presence of the different substrates, L-tyrosine and L-DOPA. Nevertheless, the phenyl substituted benzoic acid at the 3- and 4-positions could inhibit the diphenolase activity of mushroom tyrosinase.

2.3. Kinetic analyses of the inhibition on mushroom tyrosinase by 3-PBA

The kinetics of mushroom tyrosinase during the hydroxylation (monophenolase activity) and the oxidation (diphenolase activity) were examined by using L-tyrosine and L-DOPA as substrates, respectively. The concentrations of L-tyrosine and L-DOPA were changed to decide the inhibition modes of 3-PBA by Lineweaver–Burk plot analysis. Changes of the apparent constants, K_m^{app} and V_{max}^{app} of Michaelis–Menten equations could be determined through Lineweaver–Burk plots (Fig. 2). Interestingly, in the case that L-tyrosine was used as substrate, K_m^{app} was constant and V_{max}^{app} decreased (Fig. 2A). On the other hand, in the case that L-DOPA was used as substrate, K_m^{app} increased and V_{max}^{app} slightly decreased (Fig. 2B). These results suggest that the inhibition modes of 3-PBA are different with substrates, L-tyrosine and L-DOPA. 3-PBA was considered to act as the noncompetitive inhibitor of the monophenolase activity and the mixed-type inhibitor of the diphenolase activity.

2.4. Binding modes of the PBA isomers with mushroom tyrosinase

In order to understand the binding modes of the three isomers of PBA with mushroom tyrosinase, they were docked at the active site by Autodock program[14] as described in Section 4. The *met*-form of mushroom tyrosinase (E*met*) is considered to have higher affinity to L-DOPA as compared to other *oxy*- and *deoxy*-forms. From our kinetic analyses, we considered that 3-PBA may bind to the E*met*. Favre et al. suggested E*met* is the main target for the discovery of tyrosinase inhibitors.[15] So, we used here the E*met* model for docking simulation. The lowest scoring poses were chosen in