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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 681–683

2-Hydroxy-4-isopropylbenzaldehyde, a potent partial tyrosinase inhibitor

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Received 12 September 2003; revised 11 November 2003; accepted 16 November 2003

In honor of Professor Andrew S. Kende's 70th birthday.

Abstract—Chamaecin (2-hydroxy-4-isopropylbenzaldehyde) was synthesized and tested for its tyrosinase inhibitory activity. It partially inhibits the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase with an IC_{50} of 2.3 μM. The inhibition kinetics analyzed by Dixon plots found that chamaecin is a mixed type inhibitor. This inhibition may come in part from its ability to form a Schiff base with a primary amino group in the enzyme.

Tyrosinase (EC 1.14.18.1), a copper-containing enzyme, catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and conversion of an *o*-diphenol to the corresponding *o*-quinone.[1] The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is also the initial step in catecholamine synthesis. The enzymatic oxidation of L-tyrosine to melanin is of considerable importance since melanin has many functions. Alterations in melanin synthesis occur in many disease states. Melanin pigments are also found in the mammalian brain. Tyrosinase may play a role in neuromelanin formation in the human brain and could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease.[2] Melanoma specific anticarcinogenic activity is also known to be linked with tyrosinase activity.[3]

In our continuing search for tyrosinase inhibitors from plants, 2-hydroxy-4-methoxybenzaldehyde (**1**), was recently isolated as a potent tyrosinase inhibitor from African medicinal plants. It inhibits the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase with an IC_{50} of 30 μM.[4] The inhibition kinetics analyzed by Lineweaver–Burk plots found 2-hydroxy-4-methoxybenzaldehyde to be a mixed type inhibitor. This inhibition comes from its ability to form a Schiff base with a primary amino group and to chelate copper in the enzyme.[4] Simultaneously, cuminaldehyde (4-isopropylbenzaldehyde) (**2**) was characterized as a tyrosinase inhibitor from the seeds of *Cuminum cyminum*, known as cumin and widely used as a food spice.[5] Subsequently, various benzaldehyde derivatives were tested for their tyrosinase inhibitory activity for comparison. Notably, cuminaldehyde is approximately 16-fold more potent compared to benzaldehyde (**3**) as far as their IC_{50}s are compared, though anisaldehyde (**4**) is only slightly more potent than benzaldehyde. In contrast, salicylaldehyde (**5**) is about 4-fold less potent than benzaldehyde. Interestingly, 2-hydroxy-4-methoxybenzaldehyde is 11-times more potent than anisaldehyde. Hence, introducing an isopropyl group at the 4-position in salicylaldehyde can be expected to enhance the inhibitory activity (Fig. 1). 2-Hydroxy-4-isopropylbenzaldehyde (**6**)[6] was previously isolated from various plants. It was first characterized from *Eucalyptus cneralifolia*[7] and *Chamaecyparis taiwanensis*, and named as chamaecin.[8]

In a previous report,[9] Reimer–Tiemann reaction was described as a convenient method for the preparation of various salicylaldehyde derivatives from the corresponding phenols as a one step synthesis.[10] This reaction was first applied to commercially available 3-isopropylphenol (**7**). However, the complex mixture was

Keywords: Mushroom tyrosinase; Benzaldehydes; Chamaecin; Vilsmeier reaction; Partial mixed type inhibitor.
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doi:10.1016/j.bmcl.2003.11.033

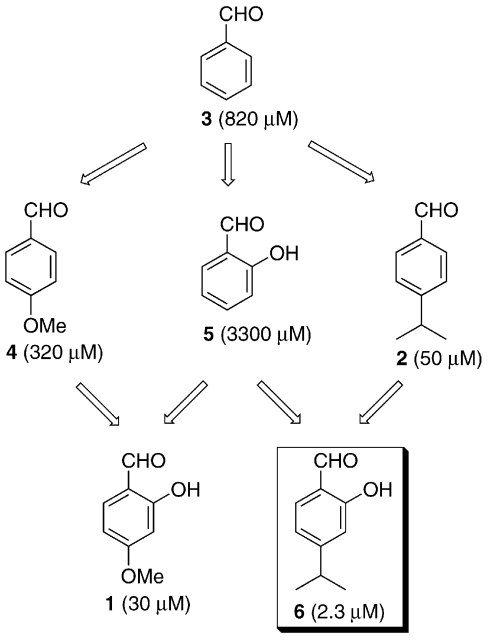
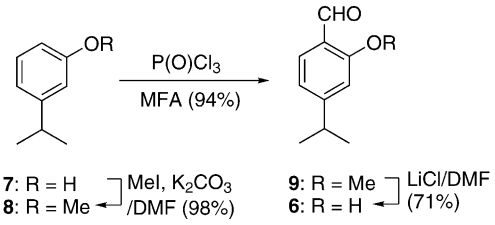


Figure 1. Structure of chamaecin and related compounds. Number in parenthesis is IC_{50} value.



Scheme 1. Synthesis of chamaecin.

observed on TLC and their separation was not easily performed. Hence, to introduce a carbonyl group at the *ortho* position of the phenolic group in **7**, Vilsmeier reaction[11,12] was selected. The efficient total synthesis of chamaecin was carried out as follows (Scheme 1). 3-Isopropylphenol was converted to 3-isopropylanisole (**8**) using iodomethane and K_2CO_3. Applying Vilsmeier reaction to **8** with *N*-methylformanilide (MFA) and $P(O)Cl_3$ afforded **9** in excellent yield.[12] In this step, 2-alkylbenzaldehyde could not be furnished, at least not as a major product because of the steric hindrance of the adjacent isopropyl group. Finally the removal of the methyl group from **9** by reflux with LiCl in DMF was achieved and chamaecin was obtained in high yield.[13,14] The overall yield from **7** was 65%.

Dose–response curve of tyrosinase inhibition by chamaecin was shown in Figure 2.[15] The IC_{50} was estimated to be 2.3 μM which is about 350-fold more potent than that of benzaldehyde. It should be noted that as the concentrations of chamaecin increased, the remaining enzyme activity was decreased but not completely suppressed. The remaining enzyme activity was about 6.7% when the concentration of chamaecin reached to 1.0 mM. In order to get more diagnostic signature, dose-response plots obtained were transformed to the plots of $1/v$ versus [I] (inset in Fig. 2).[16] It displays an apparent hyperbolic nature so that

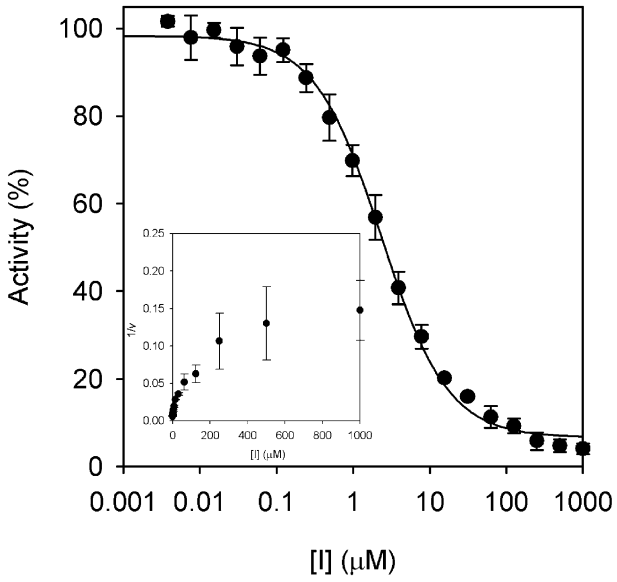


Figure 2. Dose–response plots by chamaecin on tyrosinase oxidation of L-DOPA. Plots in inset was converted from dose–response plots.

chamaecin acts as a partial inhibitor on diphenolase activity by tyrosinase.

The kinetic behavior of the oxidation of L-DOPA catalyzed by tyrosinase at different concentrations of chamaecin was studied. The inhibition kinetics analyzed by Dixon plots showed that the plots of $1/v$ versus [I] give a family of hyperbolic curves,[17] but intersected one another near the x-axis, indicating that chamaecin is a mixed (competitive and noncompetitive mixed) type inhibitor as shown in Figure 3. This observed behavior indicates that inhibitor can bind not only with free enzyme, but also with the enzyme–substrate complex, and their equilibrium constants are different. The mixed inhibition exerted by chamaecin comes from its ability to form a Schiff base with a primary amino group and to chelate copper in the active site, although its direct effect to the active site contributes more to the inhibition since the dissociation equilibrium constant, K_i (1.1

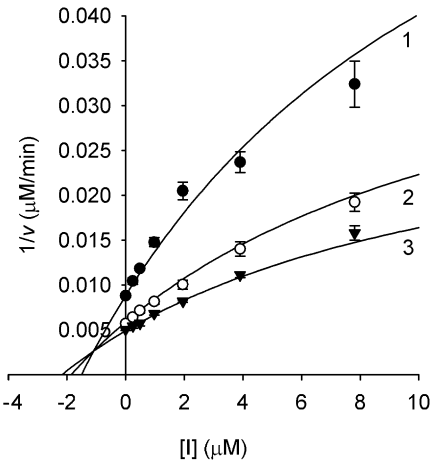


Figure 3. Dixon plots for inhibition of chamaecin on mushroom tyrosinase for the catalysis of L-DOPA. The inhibitor concentrations used were 0.24, 0.49, 0.98, 1.95, 3.91, and 7.81 μM. The concentration of L-DOPA for curves 1–3 was 0.25, 0.50, and 0.75 mM, respectively.

μM) is lower than αK_i (6.4 μM). On the other hand, the kinetics experiments by chamaecin provided burst amplitude (β-value) (0.43) that is between 0 and 1. Hence, this molecule dose not completely block the ability of the enzyme to turnover when bounded to the inhibitor.

Although the precise explanation how chamaecin interacts with the enzyme on a molecular basis is still unknown, the ability to form a Schiff base with a primary amino group in the enzyme is more likely. The 2-hydroxy-4-methoxybenzaldehyde that form more stable Shiff base adducts with a primary amino group showed more potent activity.[4] The Schiff base is expected to be largely governed by those factors affecting the stability of the carbon–nitrogen double bond. Since 2-methoxy-4-isopropylbenzaldehyde (**9**) did not show any inhibitory activity up to 0.5 mM, the salicylaldehyde moiety was requisite to interact with enzyme. In addition, the partial inhibition kinetics of chamaecin was probably explained by the fact that the Schiff base adduct generated is too small to prevent complete adsorption of the substrate in the active pocket.[18]

The primary amino group very likely plays an important role in the tertiary structure of tyrosinase. For instance, the amino group in the enzyme may be involved with hydrogen bonding which is essential to maintain the tertiary structure of the enzyme. This may be supported by the previous report that the hydrogen-bonding interactions are known to stabilize the *oxy*-form of *Streptomyces glaucescens* tyrosinase.[19] Chamaecin, a relatively nonpolar molecule, may form a Schiff base with an amino group possibly located nearby hydrophobic region of the enzyme. However, native proteins form a sort of intramolecular micelle in which the nonpolar portion is likely to be out of contact in the water based-test solution. Hence, some substrates first approach the binuclear active site and form the enzyme-substrate complex, and then chamaecin forms a Schiff base with a free primary amino group of the enzyme. The low conformational stabilities of native proteins make them easily susceptible to denaturation by altering the balance of the weak nonbonding forces that maintain the native conformation. It appears that chamaecin indirectly disrupts the tertiary structure of the enzyme. However, the conclusive interpretation remains to be clarified since the structure of tyrosinase used for this study has not yet been established.

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14. Chamaecin; IR (film) 3140, 2950, 1650, 1620, 1565, 1500, 1320, 1195, 940 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz, δ): 1.26 (d, $J=7.0$ Hz, 6H), 2.92 (sep, $J=7.0=$Hz, 1H), 6.87 (d, $J=1.5$ Hz, 1H), 6.90 (dd, $J=1.5$, 7.5 Hz, 1H), 7.45 (d, $J=7.5$ Hz, 1H), 9.84 (s, 1H), 11.05 (s, 1H). HRMS-EI $[M]^+$ calcd for $C_{10}H_{12}O_2$ 164.0837, found 164.0824. IR and ^1H NMR spectra are consisted with those reported by Lin, Y. T et al.[8]
15. The assay was performed as previously reported[4] with slight modifications.[20] The commercial mushroom tyrosinase purchased from Sigma was purified by the procedure as previously reported.[21] All data show that the mean of three separate experiments and were reproducible to within 15% of average. The IC_{50}s were obtained by fitting experimental data to the logistic curve by Sigma Plot (SPSS Inc, Chicago, IL).
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