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Mushroom Tyrosinase Inhibition by Two Potent Uncompetitive Inhibitors

F. KARBASSI[a], A.A. SABOURY[a,*], M.T. HASSAN KHAN[b], M. IQBAL CHOUDHARY[b] and Z.S. SAIFI[b]

[a]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran;* [b]*HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan*

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Two new bi-pyridine compounds, [1,4′] Bipiperidinyl-1′-yl-naphthan-2-yl-methanone (I) and [1,4′] Bipiperidinyl-1′-yl-4-methylphenyl-methane (II) were synthesized and examined for inhibition of the catecholase activity of mushroom tyrosinase in 10 mM phosphate buffer pH 6.8, at 293 K using UV spectrophotometry. Inhibition kinetics indicated that they were uncompetitive inhibitors and the value of the inhibition constants were 5.87 and 1.31 μM for I and II, respectively, which showed high potency. Fluorescent studies confirmed the uncompetitive type of inhibition for these two inhibitors. The inhibition mechanism presumably comes from the presence of a particular hydrophobe site which can accommodate these inhibitors. This site could be formed due to a probable conformational change that was induced by binding of substrate with the enzyme.

Keywords: Mushroom tyrosinase; Uncompetitive inhibition; Fluorescence

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase, which is widely distributed in microorganisms, animals, and plants.[1–4] In the presence of molecular oxygen it catalyses the *o*-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity), and the oxidation of catechols to the corresponding *o*-quinones (diphenolase or catecholase activity).[2,5–8] Quinones are highly reactive compounds and can polymerise spontaneously (nonenzymatically) to form high-molecular-weight compounds or brown pigments (melanins), or react with amino acids and proteins that enhance the brown color produced.[9] This phenomenon, which is called "browning", can cause deleterious changes in the quality of some fruits and vegetables and decreases their nutritional and market values significantly.[4,10,11] This unfavorable darkening from the enzymatic oxidation of phenols has therefore been of great concern.[12]

Tyrosinase is responsible not only for browning but also melanization in animals.[13–16] So, to reduce the undesired browning in vegetables and fruits, tyrosinase inhibitors have also become increasingly important.[17–25] Furthermore, tyrosinase inhibitors should be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and are also important in cosmetics for whitening and depigmentation after sunburn.[23,24]

As tyrosinase is the major enzyme responsible in the enzymatic browning of mushrooms, causing severe economical losses to mushroom growers, its inhibition has been of great concern. It must be noted that although there are some alternatives like using ascorbic acid, sulfites, etc.[25] to control browning, the widely used method is the use of tyrosinase inhibitors.

Many naturally occurring tyrosinase inhibitors have been described.[17,26–29] One of them, kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone), is an antibiotic produced by species of *Aspergillus* and *Penicillum* in the aerobic process[30] and is widely used as a cosmetic whitening agent.[31–33] It has been shown to be a potent, "slow-binding", competitive inhibitor of tyrosinase.[28,34–37] Some of the natural inhibitors have been extracted from plants[38–40] including flavonoids that have generally

*Corresponding author. Tel.: +98-21-6956984. Fax: +98-21-6404680. E-mail: saboury@chamran.ut.ac.ir

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a competitive type of inhibition.[17,18,21,41,42] Finally the inhibitors have been obtained by synthesis,[43] and these show different types of inhibition.[22,44] In the present work, two synthetic bi-pyridine inhibitors are described and their kinetic analysis and type of inhibition has been elucidated.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase (MT; EC 1.14.18.1), specific activity 3400 units/mg, and dimethyl sulfoxide (DMSO) were purchased from Sigma. 4-[(4-Methylbenzo)azo]-1,2-benzendiol (MeBACat) (Figure 1) was prepared as already described.[45] [1,4′] Bipiperidinyl-1′-yl-naphthan-2-yl-methanone (**I**) and [1,4′] Bipiperidinyl-1′-yl-4-methylphenyl-methane (**II**) (Figure 2) were synthesized at the University of Karachi, Pakistan, and their synthesis and characterization is being published elsewhere. The buffer used in the assay was 10 mM phosphate buffer, pH = 6.8, and its salts were obtained from Merck. All experiments were carried out in 293 K.

Methods

Enzyme activity was assayed according to a recently described method[46,47] using a Shimadzu spectrophotometer, UV-3100 model, with jacketed cell holders. All the enzymatic reactions were run in phosphate buffer (10 mM) at pH 6.8 and 293 K in a conventional quartz cell. Freshly prepared mushroom tyrosinase at a constant concentrations of 11.6 μg/ml, 39.5 units/ml, was used in each catecholase reaction. The rates of the enzymatic reactions were monitored spectrophotometrically through the depletion of MeBACat at its λ_{max} (364 nm). The temperature during the assays was regulated by

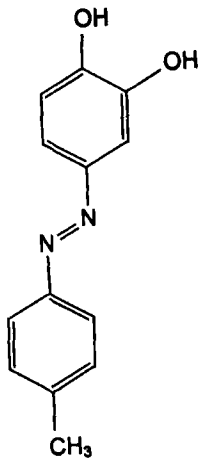
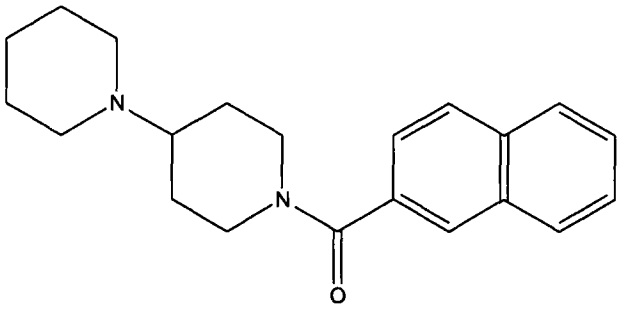
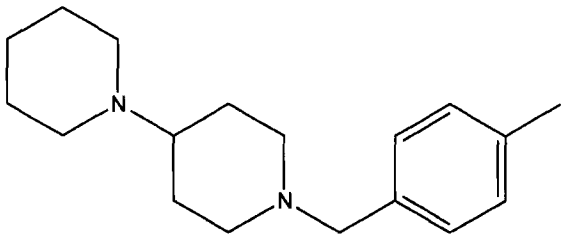


FIGURE 1 Structure of substrate: 4-[(4-methylbenzo)azo]-1,2-benzendiol (MeBACat).



Compound I



Compound II

FIGURE 2 Structure of inhibitors: [1,4′] Bipiperidinyl-1′-yl-naphthan-2-yl-methanone (**I**) and [1,4′] Bipiperidinyl-1′-yl-4-methylphenyl-methane (**II**).

an external thermostated water circulator within ±0.05°C. All the inhibitors were dissolved in DMSO and used for the experiments after diluting. Activities were measured over 5 different fixed concentrations of substrate (29.2–68.1 μM) in different concentrations of the inhibitor (2, 6.5, 8 and 12 μM for **I** and 0.25, 1.72, 2.15 and 3 μM for **II**) and the assays were repeated at least three times. The concentration of substrate was calculated using $\varepsilon = 15400\,M^{-1}cm^{-1}$.

Fluorescenence intensity measurements were carried out on a Hitachi spectrofluorimeter, MPF-4 model, equipped with a thermostatically controlled cuvette compartment. The intrinsic emission of protein, 0.15 mg/ml was seen at the excitation wavelength of 280 nm. Since the protein concentration in this experiment is 13 times higher than the concentration used in the enzyme assays, the inhibitor concentrations were increased 13-fold higher than the maximum concentration of inhibitors used to obtain the Lineweaver-Burk plots. The maximum concentrations of inhibitors used in the enzyme assays were 12 and 3 μM for **I** and **II**, respectively, so the fluorescent experiments were done in the presence of 156 and 39 μM of **I** and **II**, respectively. Hence, the stoichiometry remains constant.

RESULTS AND DISCUSSION

The oxidation of MeBACat catalyzed by mushroom tyrosinase was inhibited by **I** in a dose—dependent

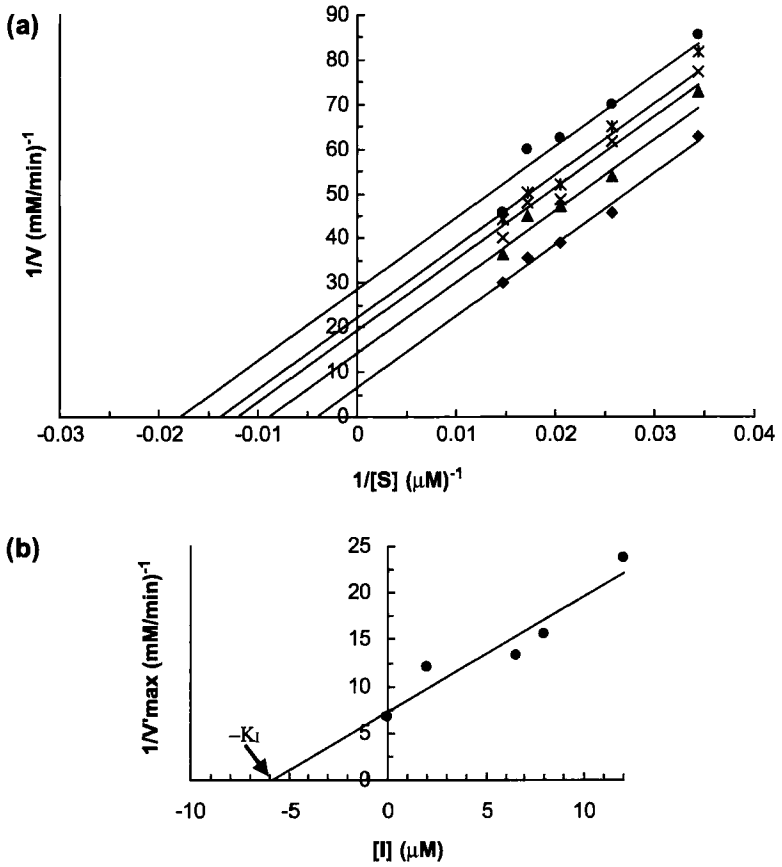


FIGURE 3 a) Double reciprocal Lineweaver-Burk plots for MT kinetics in phosphate buffer, pH = 6.8 and T = 293 K, in the presence of different fixed concentrations of compound I: 0 μM (◆), 2 μM (▲), 6.5 μM (×), 8 μM (*), 12 μM (•). The concentration of the enzyme is 11.6 μg/ml. b) The secondary plot of 1/V-axis intercept versus [I] is shown, [I] is inhibitor.

manner and double reciprocal Lineweaver-Burk plots are shown in Figure 3a. This plot was obtained with different concentrations of compound I at pH = 6.8 and T = 293 K. The plots show a family of parallel straight lines with the same slope so that compound I decreases the apparent values of V_{max} with no effect on K_m/V_{max} values, which confirms uncompetitive inhibition of the enzyme with I. The values of the reciprocal apparent maximum velocity ($1/V'_{max}$) at any concentration of I were obtained from Figure 3a and plotted versus the concentration of inhibitor I, (a secondary plot) from which the inhibition constant (K_I) was obtained with a value of 5.87 μM (Figure 3b).

Figure 4a depicts the double reciprocal Lineweaver-Burk plot for the catecholase activity of mushroom tyrosinase with different concentrations of compound II at pH = 6.8 and T = 293 K. The plots again show parallel straight lines and an uncompetitive type of inhibition. The inhibition constant, K_I = 1.31 μM, was calculated from the relevant secondary plot (Figure 4b).

If the type of inhibition is uncompetitive, the inhibitors should bind to the enzyme-substrate complex but not the free enzyme. Figure 5 depicts the intrinsic fluorescence emission of both free MT and MT incubated for 15 minutes with each of these inhibitors. It can be seen that the intrinsic emission spectra do not notably change. It would seem that substrate binding to the enzyme causes inhibitor binding to MT in agreement with the uncompetitive inhibition by compounds I and II.

The inhibition constant in an uncompetitive type of inhibition is the dissociation constant for binding of the inhibitor to the ES complex and is equal in value to IC_{50}: the inhibitor concentration leading to 50% activity loss. The comparison of these values (5.87 and 1.31 μM) with common inhibitors of MT[18–21,48] reveals that these two inhibitors can be classified as potent inhibitors of MT. These inhibitors are about 7 and 31-fold stronger in their inhibitory effect than kojic acid, a depigmentation agent used as a cosmic material for skin whitening effect and a medical agent for hyperpigmentation. Among the known tyrosinase inhibitors only a few of them, like cuminaldehyde and oxyresveratrole, are comparable with these two inhibitors in respect to their inhibition potency.[27,39,49] A number of MT inhibitors from both natural and synthetic sources are listed in Table I.[50]

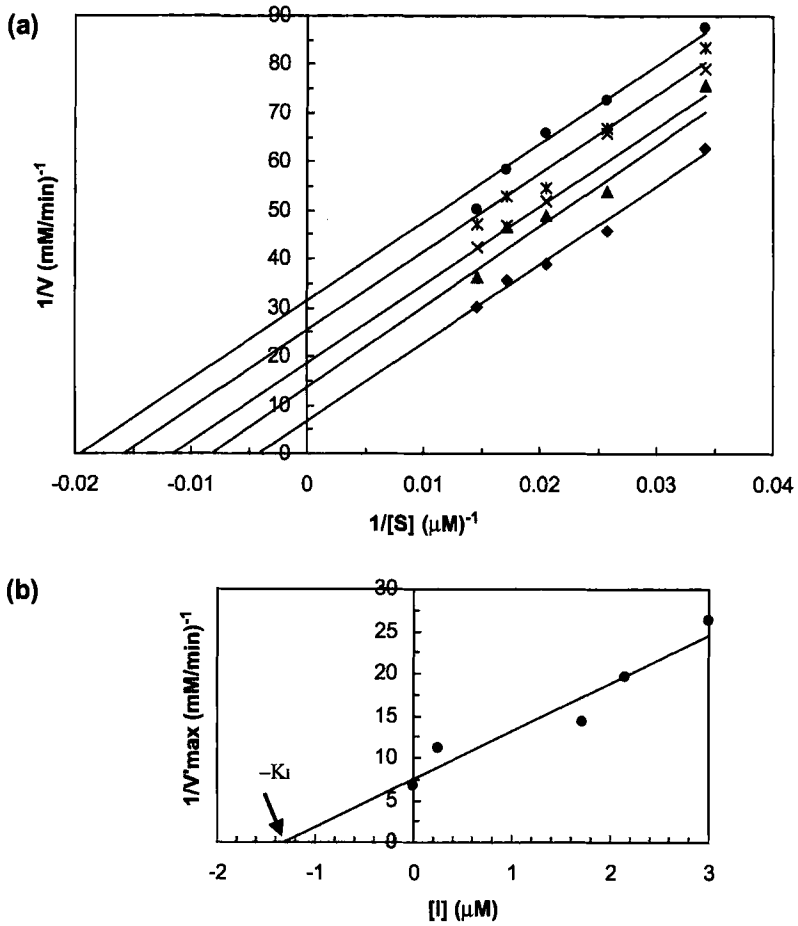


FIGURE 4 a) Double reciprocal Lineweaver-Burk plots for MT kinetics in phosphate buffer, pH = 6.8 and T = 293 K, in the presence of different fixed concentrations of compound **II**: 0 μM (◆), 0.25 μM (▲), 1.72 μM (×), 2.15 μM (*), 3 μM (•). The concentration of the enzyme is 11.6 μg/ml. b) The secondary plot of 1/V-axis intercept versus [I] is shown, [I] is inhibitor.

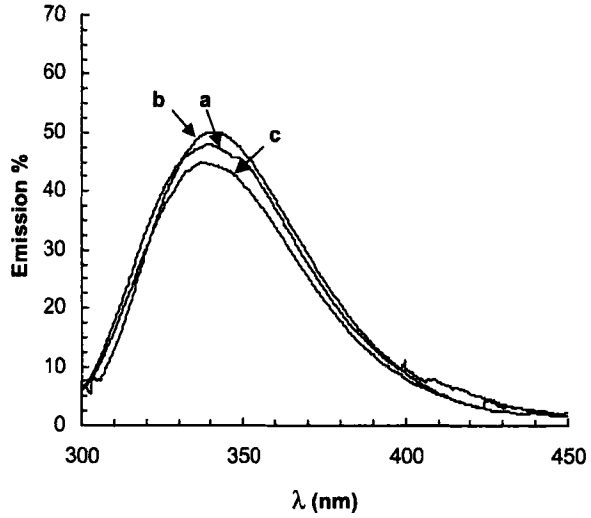


FIGURE 5 Intrinsic fluorescence emission spectra of MT (a), MT in the presence of compound **I**, 156 μM, (b) MT in the presence of compound **II**, 39 μM, (c) MT in phosphate buffer, pH = 6.8 and T = 293 K. The excitation wavelength was 280 nm. The concentration of the enzyme is 0.15 mg/ml.

The uncompetitive type of inhibition indicates that these inhibitors bind at a site distinct from the active site and bind only with the enzyme-substrate (ES) complex and not with the free enzyme (E). Presumably due to binding of the substrate to the enzyme, a conformational change is induced in the enzyme and this establishes a proper site for binding of these two bi-pyridine inhibitors. This site must be formed in a hydrophobic region which is sufficiently spacious to accommodate these bulky hydrophobic compounds. This proper environment at the inhibitor binding site with high affinity exist to justify the low value of the inhibition binding constants for these two inhibitors.

Due of the inhibitory potency of these two compounds and, as mentioned earlier, the great importance of tyrosinase inhibition in medicine and agriculture, it is proposed to study the effect of other bi-pyridine compounds on tyrosinase inhibition.

TABLE I Some mushroom tyrosinase inhibitors

Inhibitor	Source	Type of inhibition	IC_{50} (μM)	Reference
quercetin	*Heterotheca inuloides*	competitive	70	51
Aloesin	*Aloe vera*	non-competitive	100	52
metallothionein	*Aspergillus niger*	mixed	220	53
oxyreveratrole	*Morus alba*	non-competitive	1	27
cuminaldehyde	*Cumin seed*	non-competitive	50	39
Arbutin	*Gvae grsi*	competitive	40	52
kojic acid	*Penicillium*	competitive	40	28
benzaldehyde	synthetic	non-competitive	820	54
p-hydroxy-benzaldehyde	synthetic	competitive	1200	55
compound **I**	synthetic	uncompetitive	5.87	
compound **II**	synthetic	uncompetitive	1.31	

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