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ARTICLE

Picolyl Alkyl Amines As Novel Tyrosinase Inhibitors: Influence of Hydrophobicity and Substitution

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Several novel picolyl alkyl amine derivatives (**A**−**L**) were synthesized, and the influence of hydrophobicity and substitution on the inhibition of mushroom tyrosinase toward both monophenolase and diphenolase activities are described. α-, β-, and γ-picolyl amines are neither the substrates nor the inhibitors; however, the inhibition is induced by the incorporation of an alkyl chain. The inhibition was strongly dependent on the substitution on a pyridine ring, and the inhibition follows the trend of α-picolyl alkyl amines (**A**, **D**, **G**) < β-picolyl alkyl amines (**B**, **E**, **H**) < γ -picolyl alkyl amines (**C**, **F**, **I**). The inhibition kinetics have been investigated, and γ-substituted derivatives were found to be a mixed type of inhibitor, whereas β-substituted derivatives were found to exhibit uncompetitive inhibition toward the oxidation of L-DOPA.

KEYWORDS: Mushroom tyrosinase; picolyl amines; L-DOPA; enzyme-inhibition; mixed inhibitors; uncompetitive inhibitors

INTRODUCTION

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase, is a copper-containing multifunctional oxidase that catalyzes two different reactions involving molecular oxygen, the hydroxylation of monophenolic compounds to diphenolic compounds and the oxidation of *o*-diphenols to *o*-quinones (*1*, *2*). Tyrosinase widely exists in plants and animals and is involved in the formation of melanin pigments. In the food industry, tyrosinase is a very important enzyme in controlling the quality and economics of fruit and vegetable storage and processing including fruit pulp manufacturing (*3*, *4*). Tyrosinase catalyzes the oxidation of phenolic compounds to the corresponding quinone and is responsible for the enzymatic browning of fruits and vegetables. In addition to the undesirable color and flavor, the quinones produced are highly reactive compounds and can polymerize spontaneously to form high molecular weight compounds or brown pigments, and in the browning reaction, they may irreversibly react with the amino and sulfhydryl groups of proteins that enhance the brown color produced (*5*, *6*). Browning can cause deleterious changes in the appearance and organoleptic properties of the food product, resulting into shorter shelf life, loss of quality, and decreased commercial value. The quinone−protein reaction results in decreased digestibility of the protein and therefore low bioavailability of essential amino acids, including lysine and cysteine. Tyrosinase inhibition may be a potential approach to prevent and control the enzymatic browning reactions and improve the quality and nutritional value of food products (*7*, *8*).

Mammalian tyrosinase is the key enzyme in melanin biosynthesis, catalyzing the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and its subsequent oxidation to dopaquinone, and further reactions lead to melanin formation; however, overproduction of melanin leads to hyperpigmentation. It is well recognized that tyrosinase inhibitors are important for their potential application in medical and cosmetic products that may be used to prevent or treat hyperpigmentation. Tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation (*9*) and are also cosmetically important for skin lightening effects (*10*, *11*); therefore, there is a need to identify the compounds that inhibit mushroom tyrosinase activity. A number of tyrosinase inhibitors from both synthetic (*12*) and natural sources that inhibit monophenolase, diphenolase, or both of these activities have been identified (*13*, *14*).

It has been reported that gallic acid, which is a substrate of mushroom tyrosinase, can become an inhibitor as an ester by the incorporation of alkyl chains (*7*). There are reports where p-substituted benzaldehyde derivatives (*14*), *p*-alkoxybenzoic acid (*15*), alkylbenzoic acids (*16*), alkyl xanthates (*17*), and synthetic *n*-alkyl dithiocarbamates with different alkyl chains (*18*) have been investigated for inhibition toward mushroom tyrosinase. In all these reports, the effect of hydrophobicity or alkyl chain length has been studied toward inhibition of mushroom tyrosinase activities. It is very important to understand the role of hydrophobicity and substitution toward the inhibition of mushroom tyrosinase, which can lead to the designing of new potent inhibitors of mushroom tyrosinase.

In the literature, several copper chelators are known for tyrosinase inhibition (*19*); although picolyl amines and derivatives are known copper chelators (*20*−*22*), their tyrosinase inhibition studies with structural modifications are scarce. In this article, we have reported the tyrosinase inhibition efficiency of picolyl amines, synthesized several picolyl alkyl amine derivatives, and studied their inhibitory effect toward monophenolase and diphenolase activities of mushroom tyrosinase. Furthermore,

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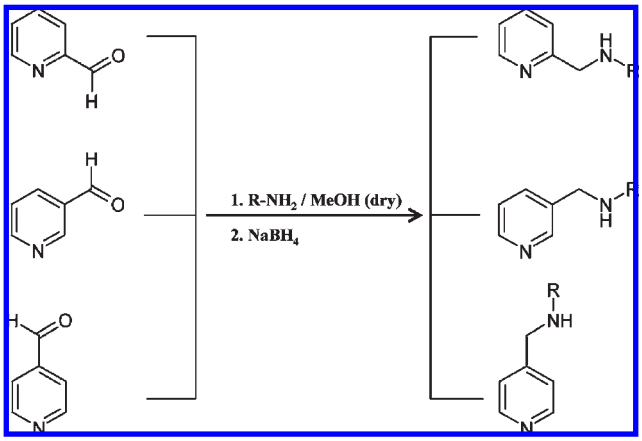
Scheme 1. Synthesis of α-, *β*-, and *γ*-Picolyl Alkyl Amines



the aim was also to carry out a kinetic study of the inhibition of the diphenolase and monophenolase activity of tyrosinase by these derivatives to understand the inhibition mechanism as a function of substitution and to evaluate the kinetic parameters and inhibition constants.

MATERIALS AND METHODS

Reagents. α-Pyridine carboxaldehyde, *β*-pyridine carboxaldehyde, *γ*-pyridine carboxaldehyde, α-picolyl amine, *β*-picolyl amine, *γ*-picolyl amine, *n*-propyl amine, *n*-pentyl amine, *n*-heptyl amine, *n*-nonyl amine, L-tyrosine, and 3,4-dihydroxy-L-phenyl alanine (L-DOPA) were purchased from Aldrich and $CuSO_4 \cdot 5H_2O$ from SD Fine Chemicals, India. Methanol and dimethyl sulfoxide (DMSO) were of high performance liquid chromatography (HPLC) grade obtained from Merck, India.

Mushroom tyrosinase (polyphenol oxidase (EC 1.14.18.1)) was purchased from Worthington Biochemical Corporation, New Jersey, USA.

Stock solutions: 0.1 M phosphate buffer (KH_2PO_4, pH 6.5), 3.5 mM L-tyrosine in phosphate buffer, 3.5 mM L-DOPA in phosphate buffer, 1 mg/mL mushroom tyrosinase enzyme (100 kU) in phosphate buffer, and 10 mM $CuSO_4$ in water. Also, 1000 mM inhibitor solutions were prepared in DMSO and further diluted in phosphate buffer to give 10 mM and 20 mM inhibitor stock solutions; thus, the amount of DMSO in test assays were almost negligible.

Dopachrome formation was studied by reading the absorbance at 450 nm using a TECAN spectrophotometer.

Synthesis of α-, *β*-, and *γ*-Picolyl Alkyl Amine. *n*-Alkyl amine (1.0 mmol) was taken in dry methanol (10 mL) and to this stirring solution, (α/*β*/*γ*-pyridine carboxaldehyde (1.1 mmol) was added over a period of 1 h (**Scheme 1**). The solution was left stirring for 3 h under N_2 atmosphere. Progress of reaction was monitored by thin layer chromatography (TLC). After the completion of the reaction (as noted by TLC), the reduction of corresponding imine was done by adding sodium borohydride (1.5 mmol). After stirring for 2 h, the methanol was evaporated under vacuum, and the solid was washed with saturated brine solution (5 × 10 mL) and the residue extracted in chloroform.

The residue was purified by column chromatography to obtain pure picolyl alkyl amine derivatives as a yellowish liquid (**Table 1**). All of the derivatives were characterized by mass and NMR spectroscopy.

α-*Picolyl Propyl Amine* (***A***). The molecular formula is $C_9H_{14}N_2$, and the calculated MW = 150. Mass spectra: M^+ peak observed at 149.6 along with $M^+ + 23$ peak at 171.6 corresponding to $[C_9H_{13}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.95 (t, 3H), 1.55 (m, 2H), 2.65 (t, 2H), 3.9 (s, 2H), 7.16 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.3 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.65 (ddd, 1H, *J* = 8.1, 8.1, 2.5 Hz), 8.55 (dd, 1H, *J* = 9 Hz).

β-*Picolyl Propyl Amine* (***B***). The molecular formula is $C_9H_{14}N_2$, and the calculated MW = 150. Mass spectra: M^+ peak observed at 149.6 along with $M^+ + 23$ peak at 171.6 corresponding to $[C_9H_{13}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.94 (t, 3H), 1.51 (m, 2H), 2.52 (t, 2H), 3.56 (s, 2H), 7.27 (m, 1H), 7.68 (d, 1H, *J* = 9.0 Hz), 8.48 (dd, 1H, *J* = 9, 2.1 Hz), 8.55 (d, 1H, *J* = 2.1 Hz).

γ-*Picolyl Propyl Amine* (***C***). The molecular formula is $C_9H_{14}N_2$, and the calculated MW = 150. Mass spectra: M^+ peak observed at 149.6 along with $M^+ + 23$ peak at 171.6 corresponding to $[C_9H_{13}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.95 (t, 3H), 1.55 (m, 2H), 2.6 (t, 2H), 3.62 (s, 2H), 7.27 (d, 2H, *J* = 6.5 Hz), 8.53 (d, 2H, *J* = 6.5 Hz).

α-*Picolyl Pentyl Amine* (***D***). The molecular formula is $C_{11}H_{18}N_2$, and the calculated MW = 178. Mass spectra: M^+ peak observed at 177.7.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.88 (t, 3H), 1.32 (m, 4H), 1.55 (m, 2H), 2.65 (t, 2H), 3.9 (s, 2H), 7.15 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.3 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.65 (ddd, 1H, *J* = 8.1, 8.1, 2.5 Hz), 8.55 (d, 1H, *J* = 9.0 Hz).

β-*Picolyl Pentyl Amine* (***E***). The molecular formula is $C_{11}H_{18}N_2$, and the calculated MW = 178. Mass spectra: M^+ peak observed at 177.7.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.9 (t, 3H), 1.3 (m, 4H), 1.5 (m, 2H), 2.65 (t, 2H), 3.8 (s, 2H), 7.26 (m, 1H), 7.68 (d, 1H, *J* = 9 Hz), 8.49 (dd, 1H, *J* = 9, 2.1 Hz), 8.55 (d, 1H, *J* = 2.1 Hz).

γ-*Picolyl Pentyl Amine* (***F***). The molecular formula is $C_{11}H_{18}N_2$, and the calculated MW = 178. Mass spectra: M^+ peak observed at 177.7.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.9 (t, 3H), 1.35 (m, 4H), 1.52 (m, 2H), 2.65 (t, 2H), 3.82 (s, 2H), 7.26 (d, 2H, *J* = 6.5 Hz), 8.53 (d, 2H, *J* = 6.5 Hz).

α-*Picolyl Heptyl Amine* (***G***). The molecular formula is $C_{13}H_{22}N_2$, and the calculated MW = 206. Mass spectra: $M^+ + 23$ peak observed at 227.8 corresponding to $[C_{13}H_{21}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.88 (t, 3H), 1.3 (m, 8H), 1.55 (m, 2H), 2.65 (t, 2H), 3.9 (s, 2H), 7.15 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.3 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.65 (ddd, 1H, *J* = 8.1, 8.1, 2.5 Hz), 8.55 (d, 1H, *J* = 9.0 Hz).

β-*Picolyl Heptyl Amine* (***H***). The molecular formula is $C_{13}H_{22}N_2$, and the calculated MW = 206. Mass spectra: $M^+ + 23$ peak observed at 227.8 corresponding to $[C_{13}H_{21}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.85 (t, 3H), 1.25 (m, 8H), 1.5 (m, 2H), 2.65 (t, 2H), 3.8 (s, 2H), 7.25 (m, 1H), 7.68 (d, 1H, *J* = 9.0 Hz), 8.49 (dd, 1H, *J* = 9, 2.1 Hz), 8.55 (d, 1H, *J* = 2.1 Hz).

γ-*Picolyl Heptyl Amine* (***I***). The molecular formula is $C_{13}H_{22}N_2$, and the calculated MW = 206. Mass spectra: $M^+ + 23$ peak observed at 227.8 corresponding to $[C_{13}H_{21}N_2Na]$.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.88 (t, 3H), 1.28 (m, 8H), 1.52 (m, 2H), 2.65 (t, 2H), 3.82 (s, 2H), 7.26 (d, 2H, *J* = 6.5 Hz), 8.53 (d, 2H, *J* = 6.5 Hz).

α-*Picolyl Nonyl Amine* (***J***). The molecular formula is $C_{15}H_{26}N_2$, and the calculated MW = 234. Mass spectra: M^+ peak observed at 233.9.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.87 (t, 3H), 1.24 (m, 12H), 1.52 (m, 2H), 2.65 (t, 2H), 3.92 (s, 2H), 7.15 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.28 (dd, 1H, *J* = 8.1, 2.5 Hz), 7.65 (ddd, 1H, *J* = 8.1, 8.1, 2.5 Hz), 8.55 (d, 1H, *J* = 9.0 Hz).

β-*Picolyl Nonyl Amine* (***K***). The molecular formula is $C_{15}H_{26}N_2$, and the calculated MW = 234. Mass spectra: M^+ peak observed at 233.8.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.86 (t, 3H), 1.25 (m, 12H), 1.5 (m, 2H), 2.65 (t, 2H), 3.8 (s, 2H), 7.26 (m, 1H), 7.68 (d, 1H, *J* = 9.0 Hz), 8.49 (dd, 1H, *J* = 9, 2.1 Hz), 8.56 (d, 1H, *J* = 2.1 Hz).

γ-*Picolyl Nonyl Amine* (***L***). The molecular formula is $C_{15}H_{26}N_2$, and the calculated MW = 234. Mass spectra: M^+ peak observed at 233.9.

^{1}H NMR in $CDCl_3$ (300 MHz) δ ppm: 0.88 (t, 3H), 1.28 (m, 12H), 1.5 (m, 2H), 2.62 (t, 2H), 3.82 (s,2H), 7.26 (d, 2H, *J* = 6.5 Hz), 8.54 (d, 2H, *J* = 6.5 Hz).

Enzyme Activity Assay. *o*-Monophenolase and *o*-diphenolase activities of mushroom tyrosinase were determined with L-tyrosine and L-DOPA, respectively as the substrate by measuring the rate of dopachrome formation at 450 nm (ε = 3700 M^{-1} cm^{-1}) (*14*).

Sixty microliters of 10 mM inhibitor solution was taken to have the resultant inhibitor concentration of 3 mM, 8 μL of mushroom tyrosinase enzyme, and 3.5 mM L-tyrosine (57 μL, 1 mM) for monophenolase activity, and 3.5 mM L-DOPA (57 μL, 1 mM) for diphenolase activity. The reaction volume was adjusted (q.s.) to 200 μL with KH_2PO_4 buffer, and the progress of dopachrome formation was observed by measuring the absorbance at 450 nm. Assays were performed in a flat bottom 96 well microlitre plate.

The extent of inhibition by the compounds is expressed as the inhibitor concentration leading to 50% decrease in enzyme activity (IC_{50}).

Mode of Enzyme Inhibition Assay. In order to determine the mode of enzyme inhibition, detailed kinetic studies were done. The assay was

Table 1. Chemical Yields, log $P_{o/w}$, IC_{50}, Inhibition Type, Inhibition Constants for Diphenolase

compound	R-	substitution on pyridine ring	log $P_{o/w}$ (predicted)	yield (%)	IC_{50} (mM)	inhibition type (monophenolase)	inhibition type (diphenolase)	iinhibition constants (mM) K_I	K_{IS}
A[a]	nC_3H_7	α	1.09	70	>12				
B	nC_3H_7	β	1.09	77	12	uncompetitive	uncompetitive		31
C	nC_3H_7	γ	1.09	74	5.8	uncompetitive	mixed	23	37
D[a]	nC_5H_{11}	α	2.152	75	>12				
E	nC_5H_{11}	β	2.152	80	12	uncompetitive	uncompetitive		29
F	nC_5H_{11}	γ	2.152	76	4.2	uncompetitive	mixed	20	42
G[a]	nC_7H_{15}	α	3.215	79	>12				
H	nC_7H_{15}	β	3.215	81	9.5	uncompetitive	uncompetitive		24
I	nC_7H_{15}	γ	3.215	80	6.8	uncompetitive	mixed	18	32
J[b]	nC_9H_{19}	α	4.278	77					
K[b]	nC_9H_{19}	β	4.278	74					
L[b]	nC_9H_{19}	γ	4.278	80					

[a] Inhibition type could not determined as the inhibition was very low. [b] Inhibitory effects of **J**, **K**, and **L** could not be tested because of the low solubility in the assay media.

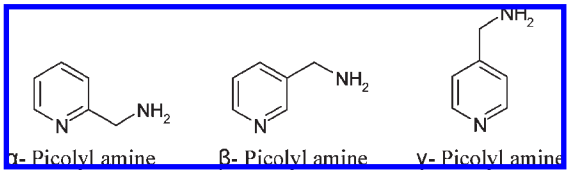


Figure 1. Chemical structures of α-picolyl amine, β-picolyl amine, and γ-picolyl amine.

performed by varying the enzyme concentration at different inhibitor concentrations at the same substrate (L-DOPA) concentration. To the KH_2PO_4 buffer (q.s. to 200 μL) in a flat bottom 96 well plate was added 20, 30, 40, and 50 μL of 20 mM inhibitor solutions to obtain desired inhibitor concentrations of 2, 3, 4, and 5 mM in assay medium. Then to each set of inhibitor concentration, 2, 4, 6, 8, and 10 μL of mushroom tyrosinase enzyme was added, which ultimately resulted in the enzyme units 2, 4, 6, 8, and 10, respectively. Finally, 3.5 mM L-DOPA (57 μL, 1 mM) was added to the resultant solution, and dopachrome formation was observed by measuring the absorbance at 450 nm. The mode of inhibition was determined from the nature of the graph when the enzyme concentration was plotted against the reaction velocity (absorbance/min).

Enzyme Kinetic Studies. To the KH_2PO_4 buffer (q.s. to 200 μL) in a flat bottom 96 well plate was added 20−120 μL of 20 mM inhibitor solution in order to have the resultant inhibitor concentration in the range of 2−12 mM. Then 8 μL of enzyme was added to each well. Finally, 29, 40, 57, and 100 μL of 3.5 mM L-DOPA was added to each set to yield 0.5, 0.7, 1.0, and 1.75 mM final concentration of L- DOPA, respectively, and dopachrome formation was observed for 10 min by measuring the absorbance at 450 nm. The inhibition type was assessed by Lineweaver−Burke plots using initial velocities for the dopachrome formation, and the inhibition constants were determined by the secondary plots of the kinetic parameters versus the inhibitor concentrations (*23*).

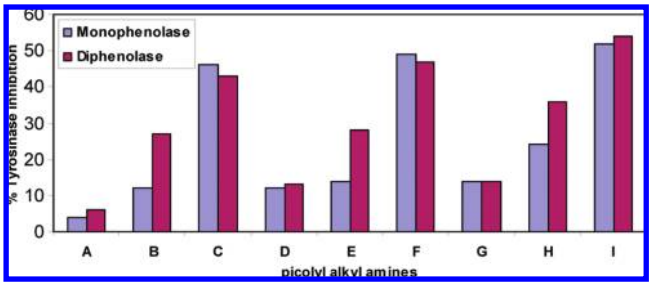
Effect on Lag Phase during Monophenolase Activity. The monophenolase activity with L-tyrosine as the substrate was performed in way similar to that described for the enzyme activity assay and the progress of dopachrome formation was immediately monitored (*23*).

UV Absorption Studies. Copper chelation efficiency of picolyl alkyl amines was studied by UV absorption spectroscopy (220−350 nm) using $CuSO_4$ (in molar ratio, 1:2). To get more insight into the mechanism of inhibition, mushroom tyrosinase enzyme (10 U) was incubated with the inhibitor solutions (10 mM) for 30 min at room temperature, the solutions were diluted to 1 mL with 0.1 M phosphate buffer (KH_2PO_4, pH 6.5) to study the absorbance spectra (220−350 nm) (*24*).

RESULTS AND DISCUSSION

Series of α-, β-, and γ-picolyl alkyl amines (C_3, C_5, C_7, and C_9) were synthesized using Schiff-base condensation reaction between pyridine carboxaldehyde and *n*-alkyl amines. These derivatives were tested for their effect toward monophenolase and diphenolase activities of mushroom tyrosinase.

Chart 1. Inhibition Efficacy of Different Picolyl Alkyl Amines toward the Monophenolase and Diphenolase Activity of Mushroom Tyrosinase at 3 mM Picolyl Alkyl Amines



α-, β-, and γ-picolyl amines (**Figure 1**) are known copper chelators, when tested for their action toward monophenolase and diphenolase activities of mushroom tyrosinase, they did not inhibit any of the two activities. However, introduction of alkyl chain (C_3, C_5, and C_7) into the picolyl amines influenced the inhibitory action toward both monophenolase and diphenolase activities of mushroom tyrosinase. C_9 derivatives could not be studied because of the low solubility in assay media.

The inhibition was not greatly induced with α-substitution, and the inhibition toward monophenolase and diphenolase activities were estimated to be in the range of 4−15% for C_3, C_5, and C_7 picolyl alkyl amines.

Alkyl chain introduction at the β-position influenced the inhibition activity to a greater extent as compared to that at the α-position. The inhibition for C_3, C_5, and C_7 was estimated to be 12%, 12%, and 24% for monophenolase activity and 27%, 28%, and 36% for diphenolase activity.

It was further observed that γ-picolyl alkyl amine led to the maximum inhibition toward both the monophenolase and diphenolase activities of the mushroom tyrosinase. The inhibitions for C_3, C_5, and C_7 were estimated to be in the range of 40−55% toward both monophenolase and diphenolase activities. Comparison of the percentage of inhibition of monophenolase and diphenolase activities of mushroom tyrosinase by these derivatives at 3 mM are shown in **Chart 1**.

Enzyme activity was found to decrease with increasing concentration of inhibitors. The plot of remaining enzyme activity versus the concentration of enzyme at various concentrations of **H** and **I** gave a family of straight lines which passed through the origin, as shown in **Figures 2** and **3**, respectively. It was found that

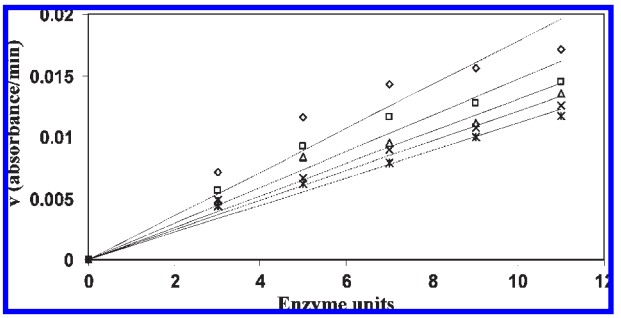


Figure 2. Relationship of enzyme activity and the enzyme concentration at different concentrations of **H** where (◇) 0 mM, (□) 3 mM, (△) 5 mM, (×) 7 mM, and (∗) 10 mM.

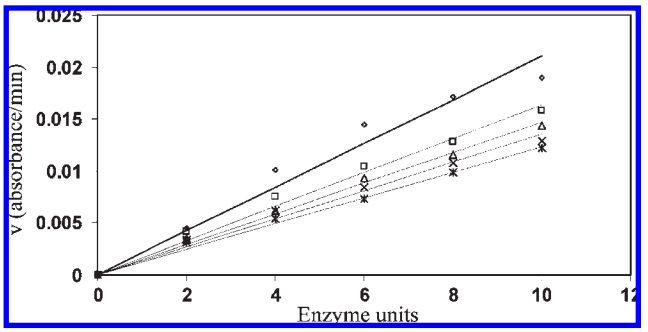


Figure 3. Relationship of enzyme activity to the enzyme concentration at the different concentrations of inhibitor **I** where (◇) 0 mM, (□) 2 mM, (△) 3 mM, (×) 4 mM, and (∗) 5 mM.

increasing the inhibitor concentration resulted in a descending slope of the line, which is indicative of the reversible effect of inhibitor on the enzyme (*16*). At higher concentrations of enzyme, a plateau was obtained, indicating that the reaction may be limited by the substrate concentration (L-DOPA). All of the derivatives of β-picolyl alkyl amines and γ-picolyl alkyl amines were found to be the reversible inhibitors of mushroom tyrosinase for the oxidation of L-DOPA.

In order to compare the inhibitory potencies, the IC_{50} values (the concentration leading to 50% activity lost) of all of the tested inhibitors were determined under the same experimental conditions. The IC_{50} values of picolyl alkyl amine derivatives ranged from 4 mM to 10 mM comparable to that of arbutin (6 mM), a well-known tyrosinase inhibitor (*11*) not as potent as kojic acid (0.07 mM) another well-known tyrosinase inhibitor (*25*). Plots to obtain the IC_{50} values for derivatives **H** and **I** are shown in **Figure 4a** and **b**, respectively, and **Table 1** summarizes the IC_{50} of tested compounds for comparison.

To obtain further information on the mechanism of action of these derivatives on mushroom tyrosinase, kinetics of the reaction at various concentrations of L-DOPA and inhibitors was studied.

Under the conditions employed in the present investigation with L-DOPA as the substrate to study the diphenolase activity of mushroom tyrosinase in the presence of synthesized derivatives (**I**), the oxidation reaction followed the Michaelis−Menten equation by the Lineweaver−Burke plot (**Figure 5**). The plot resulted in a family of straight lines with different slopes and intercepts, but they intersect one another in the second quadrant. This indicates that **I** exhibits a mixed type of inhibition, which means that it can bind to the free enzyme as well as the enzyme−substrate complex; likewise, other γ-substituted derivatives also showed a mixed type of inhibition. The inhibition constants for inhibitor (**I**) binding with the free enzyme, K_I, was determined

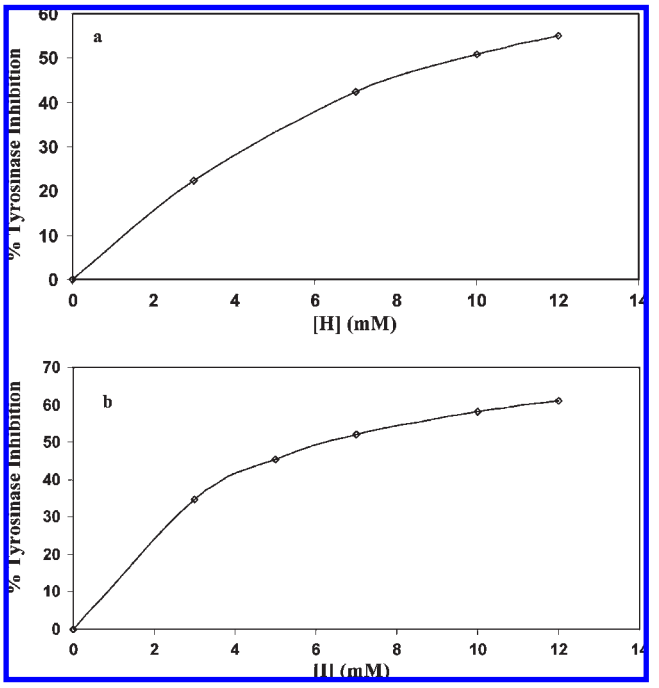


Figure 4. Ability of picolyl alkyl amine derivatives to inhibit the enzymatic activity of mushroom tyrosinase was measured using L-DOPA as substrate. (**a**) IC_{50} for **H** and (**b**) IC_{50} for **I**.

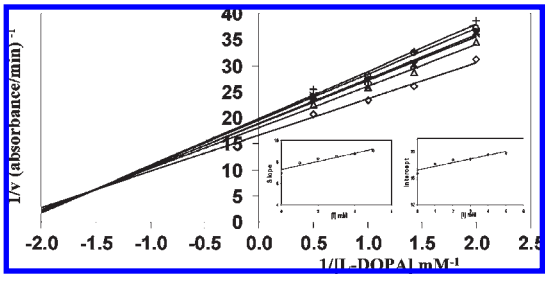


Figure 5. Lineweaver−Burke Plots for the inhibition of mushroom tyrosinase in the presence of **I** where (◇) 0 mM, (△) 1 mM, (×) 2 mM, (∗) 3 mM, (○) 4 mM, and (+) 5 mM.

from a plot of the slopes (K_m/V_m) versus the inhibitor concentration, and for the enzyme−substrate complex, K_{IS} was determined from a plot of the intercept on the Y-axis versus the inhibitor concentration, as shown in the inset of **Figure 5**. K_I and K_{IS} for **I** are found to be 18 mM and 30 mM, respectively.

In the presence of **H**, the Lineweaver−Burke plot (**Figure 6**) gave a family of parallel straight lines with the same slopes. Values of both K_m and V_{max} were enhanced, but the ratios of K_m/V_{max} remained constant with increasing concentrations of **H**. The slopes were found to be independent of the concentration of **H**, which indicates that it is an uncompetitive type of inhibition with respect to L-DOPA oxidation. This means that the inhibitor binds at a site distinct from the substrate and combines with the enzyme−substrate complex and not with the free enzyme. The inhibition constant, K_{IS}, for binding with the enzyme−substrate complex was obtained from a plot of the vertical intercept ($1/V_m$) versus the inhibitor concentration, which is linear and is shown in the inset of **Figure 6**. The K_{IS} value was estimated to be 24 mM. The inhibitory activities of the synthesized picolyl alkyl amines on mushroom tyrosinase, the type of inhibition obtained from the Lineweaver−Burke plot, and the inhibitor constants are listed in **Table 1**. The mode of inhibition could not be studied with α-substituted derivatives because of low inhibition.

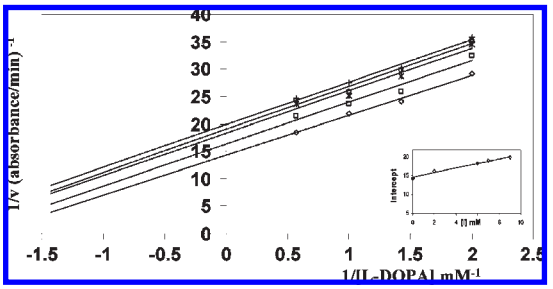


Figure 6. Lineweaver−Burke Plots for the inhibition of mushroom tyrosinase in the presence of **H** where (◇) 0 mM, (□) 2 mM, (∗) 6 mM, (○) 7 mM, and (+) 9 mM.

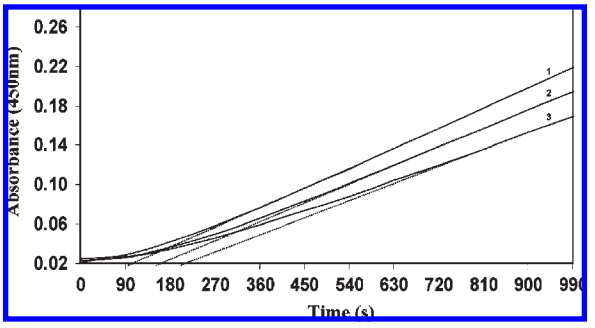


Figure 7. Inhibition by **F** on monophenolase activity of mushroom tyrosinase showing that the lag time had been lengthened by **F** where 1, 2, and 3 represent 0, 3, and 6 mM **F**, respectively.

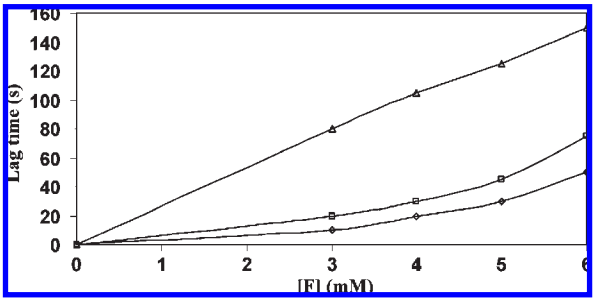


Figure 8. Effect of monophenol concentration on lengthening the lag time of mushroom tyrosinase by **F** where (◇) 0.5 mM, (□) 0.75 mM, and (△) 1 mM of L-tyrosine.

All the derivatives of β- and γ-picolyl alkyl amines showed uncompetitive inhibition toward the monophenolase activity of tyrosinase (**Table 1**).

These derivatives were also tested for their effect on lag period during monophenolase activity. When the enzymatic reaction was started by the action of tyrosinase on L-tyrosine, a marked lag period, characteristic of monophenolase activity was observed, and the system reached a steady state after the lag period, which was obtained by the extrapolation of the linear portion of the curve (*26*). The lag period is known to be dependent on both enzyme and substrate concentrations in the reaction medium, and many monophenolase inhibitors are known for extending the lag time such as tropolone (*27*) or cumic acid (*13*). There are some inhibitors such as kaempferol (*24*) or α,β-unsaturated aldehydes (*28*), which do not lengthen the lag phase.

α- and β-picolyl alkyl amines did not show any effect on lag time; however, steady state was influenced, whereas γ-substituted derivatives were found to influence both the lag time and the steady state. The lag time was estimated to be 100 s in the absence

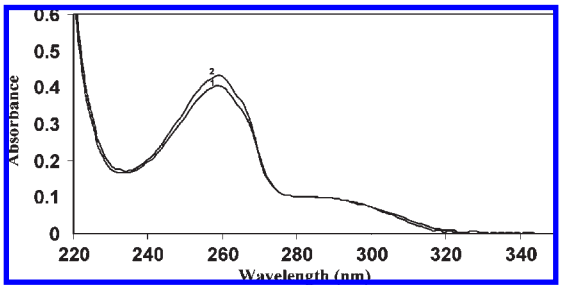


Figure 9. UV Spectrum of **I** (1) without and (2) with tyrosinase.

of **F**, extended to 150 s in the presence of 3 mM of **F**, and to 200 s at 6 mM of **F** (**Figure 7**). Hence, lag time extended up to 50% with 3 mM and to 100% in the presence of 6 mM of **F**.

At various concentrations of L-tyrosine, the lag period of the reaction was lengthened by the γ-substituted derivatives to different extents. **Figure 8** shows that with increase in L-tyrosine concentration, the lag time increases at a particular concentration of **F**.

It is well known that tyrosinase exists in three different forms, met-, oxy-, and deoxy-tyrosinase. During the monophenolase reaction, the oxy-form of the enzyme participates in the hydroxylation of L-tyrosine, whereas during the diphenolase reaction, both the oxy- and met- forms participate in L-DOPA oxidation. No effect on lag period during the monophenolase reaction in the presence of α- and β-picolyl alkyl amines indicates that they may preferentially be interacting with met-tyrosinase, whereas with γ-picolyl alkyl amines, the substantial enhancement in lag time is indicative of an interaction with both the oxy- and met-forms of tyrosinase (*29*).

The copper chelation efficiency of these inhibitors was studied through change in their UV absorption in presence of $CuSO_4$. α-Picolyl alkyl amines and γ-picolyl alkyl amines were found to form complexes with copper, whereas β-picolyl alkyl amines did not form complexes with copper. The length of the alkyl chain length did not affect the copper chelation efficiency of these derivatives.

To get more insight into the interaction of picolyl alkyl amines with tyrosinase, the UV absorption spectrum (220−350 nm) of these derivatives were studied (*24*). It was found that when **I** was incubated with tyrosinase there was increase in the absorption (λ max = 260 nm) of the **I** (**Figure 9**), indicating a different local environment (may be copper chelation), whereas incubation of tyrosinase with β-substituted derivatives (**H**) did not result in any change in its absorption behavior, indicating no interaction with the free enzyme. Although α-substituted derivatives were found to be copper chelators, they did not show any change in the absorption pattern, indicating inaccessibility to the enzyme. Inhibition of tyrosinase activity by β-substituted derivatives indicates that hydrophobicity plays an important role in the inhibition of tyrosinase activity.

The γ-substituted derivatives have been found to be the most potent inhibitors as compared to α- and β-substituted derivatives, which indicates that γ-substituted derivatives can be well positioned and could be easily incorporated in the hydrophobic pocket of the enzyme (*16*). The other reason for the ineffectiveness of α-substituted derivatives and the low inhibition activities of β-substituted derivatives could be steric crowding, which can cause spatial blocks, making the inhibitors unable to interact with the free enzyme whereas, the γ-substituted derivatives which are least sterically crowded showed considerable inhibition. The marginal increase in inhibition with increase in alkyl chain length indicates that the longer the chain length, the higher the hydrophobic interaction with the hydrophobic pocket of the enzyme, although

derivatives with higher chain length were synthesized but could not be tested because of low solubility.

Since the structure of mushroom tyrosinase is largely unknown, the precise explanation for how these derivatives are interacting with the enzyme on a molecular basis is still unknown, but possibly, the hydrophobicity enhances the van der Waal's interaction with the protein residue, and the tertiary structures of the enzyme can also get disrupted because of intermolecular hydrogen bonding. Low activities of α-picolyl alkyl amine could also be attributed to the fact that in α-substituted derivatives the chances of intramolecular hydrogen bonding are higher, thus reducing the chances of interaction with the enzyme via intermolecular hydrogen bonding interactions (*30*).

Because of the planar structure of γ-picolyl alkyl amines, the chances of intermolecular bonding is higher, and the known 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. Therefore, it can be concluded that γ-picolyl alkyl amine interacts strongly with enzymes as compared to that of α- and β-picolyl alkyl amine. In addition, we have also studied the preincubation of the enzyme with inhibitors in the absence of the substrate L-DOPA, where the enzyme activity was found to remain unchanged, suggesting that these picolyl alkyl derivatives are inhibitors rather than inactivators of enzymes (*31*).

In summary, a series of picolyl alkyl amines has been reported as novel tyrosinase inhibitors. Detailed investigation on the inhibition of monophenolase and diphenolase activities of mushroom tyrosinase indicates that hydrophobicity and substitution play an important role in enzyme inhibition. The results indicate that hydrophobicity and steric crowding are critical for the interaction of inhibitors with enzymes. In addition, the predicted log $P_{o/w}$ (**Table 1**) of these derivatives indicates that these compounds will have easier cellular permeability (*32*) and therefore are potential candidates for reducing melanogenesis in humans and enzymatic browning in foods. The basic structure of these picolyl alkyl derivatives is similar to that of a naturally occurring class of alkaloids which have a large variety in their biochemical and botanical origin (*33*). On the basis of the understanding discussed in the present work, this class of molecules and their derivatives can be used to control enzymatic browning.

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Supporting Information Available: Spectroscopic data for the picolyl alkyl amines. This material is available free of charge via the Internet at http://pubs.acs.org.

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