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INHIBITION OF MUSHROOM TYROSINASE BY TROPOLONE*

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Key Word Index—Tropolone, mushroom tyrosinase, inhibition

Abstract—Tropolone inhibits both mono- and *o*-dihydroxyphenolase activity of mushroom tyrosinase Most of the inhibition exerted by tropolone was reversed by dialysis or by excess Cu^{2+} The data indicate that tropolone and *o*-dihydroxyphenols compete for binding to the copper at the active site of the enzyme Comparison between the effectiveness of various copper chelators showed that tropolone is one of the most potent inhibitors of mushroom tyrosinase, 50% inhibition was observed with $0 4 \times 10^{-6}$ M tropolone

INTRODUCTION†

Tropolone (2,4,6-cycloheptatriene-1-one, 2-hydroxy-2,4,6-cycloheptatrienone) can complex with metals The complex formation of ferric tropolone [1], cupric tropolone [1, 2] and magnesium tropolone [3] is well documented

Goldstein *et al* [4] showed that 4-isopropyl tropolone is a potent inhibitor of dopamine-β-hydroxylase of beef adrenal glands At 1×10^{-6} M it gave 40% inhibition Dopamine-β-hydroxylase and tyrosinase [monophenol mono-oxygenase, phenolase (EC 1 10 3 1)] are both copper-containing enzymes Visser proposed recently [5] that the mechanism of action of dopamine-β-hydroxylase and that of tyrosinase are analogous, except that in tyrosinase, *o*-diphenol replaces ascorbate as a reductant (AH_2) In view of the above it was of interest to test if tropolone is also an effective inhibitor of tyrosinase

RESULTS AND DISCUSSION

Effect of tropolone on the monohydroxyphenolase activity of mushroom tyrosinase

The hydroxylation of a monohydroxyphenol by mushroom tyrosinase is characterized by an initial lag period The data in Fig 1 show that tropolone extended the lag period of tyrosine hydroxylation (for details, see Fig 1, inset) Moreover, tropolone also slowed the rate of dopachrome formation (*A* at 475 nm) following the lag period (Fig 1)

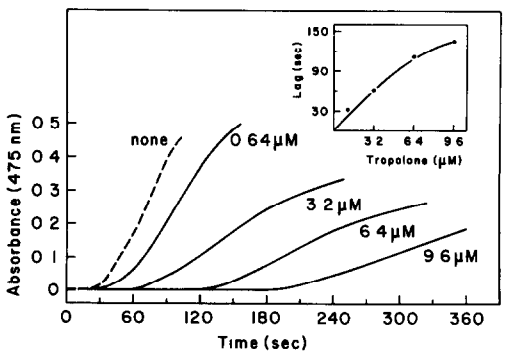

Fig 1 Effect of tropolone on the rate of tyrosine hydroxylation by mushroom tyrosinase The reaction mixture included, in a total volume of 3 ml, 2 mM L-tyrosine, 47 mM sodium phosphate buffer (pH 6 5), 100 μg mushroom tyrosinase (added last) and tropolone as indicated The lag periods were calculated from the curves obtained and are shown in the inset as a function of tropolone concentration

Effect of tropolone on o-dihydroxyphenolase activity of mushroom tyrosinase

Tropolone inhibited the *o*-dihydroxyphenolase activity of mushroom tyrosinase when different *o*-hydroxyphenols were used as the substrates In the range tested (0 3–200 μM), the higher the tropolone concentration used, the greater the extent of inhibition of the rate of formation of pigmented oxidation products obtained from each of the *o*-dihydroxyphenols tested The effect of preincubating mushroom tyrosinase with tropolone at either 1 or 5 μM for a period of up to 20 min was tested Samples were withdrawn from the preincubation mixture at different times and *o*-dihydroxyphenolase activity was assayed immediately It was found that the same extent of inhibition is seen after exposing mushroom tyrosinase to 5 μM tropolone for 1 or 20 min, suggesting that tropolone is an inhibitor rather than an inactivator of the enzyme, although the possibility that a rapid inactivation (complete within 1 min) is responsible for the loss of activity cannot, at present, be excluded

The inhibition exerted by tropolone on the oxidation of DL-DOPA (3,4-dihydroxyphenylalanine), dopamine and 4-methyl catechol by mushroom tyrosinase is summarized

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†For abbreviations see Table 1.

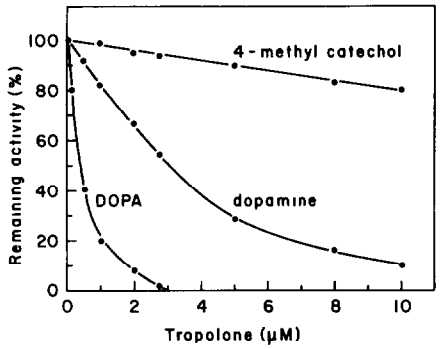


Fig 2 Effect of tropolone on the *o*-dihydroxyphenolase activity of mushroom tyrosinase The reaction mixture included, in a total volume of 3 ml, 6 7 mM *o*-dihydroxyphenol (4-methylcatechol, DL-DOPA or dopamine), 47 mM sodium phosphate buffer (pH 6 5), 100 μg mushroom tyrosinase (added last) and tropolone as indicated Activity in the absence of tropolone was taken as 100%

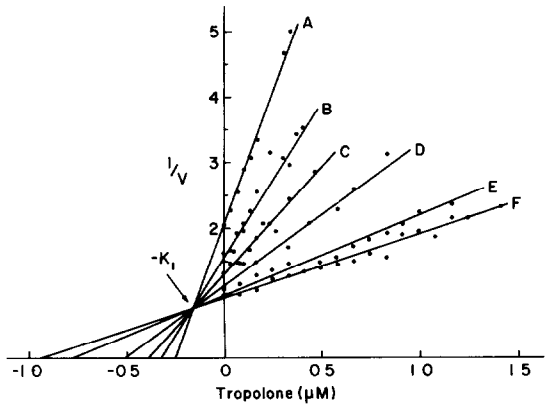


Fig 3 Inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase by different concentrations of tropolone The reaction mixture included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6 5), 100 μg mushroom tyrosinase (added last), different concentrations of tropolone and 0 33, 0 66, 1 33, 2 66, 5 00 and 6 66 mM of DL-DOPA in A, B, C, D, E and F, respectively Velocity (V) refers to initial *o*-dihydroxyphenolase activity (A at 475 nm/min) The data were plotted as $1/V$ vs tropolone concentration and the K_i estimated from the graph obtained, as described by Webb [6]

in Fig 2 It is clear that the effectiveness of tropolone as an inhibitor of the enzyme depends on the substrate inhibition is highest with DL-DOPA, intermediate with dopamine and lowest with 4-methyl catechol We have shown (unpublished data) that the K_m values of mushroom tyrosinase for DL-DOPA and dopamine are 4 8 $\times 10^{-4}$ M and 3 6 $\times 10^{-4}$ M, respectively, while that for 4-methyl catechol is *ca* 0 8 $\times 10^{-4}$ M Thus, it appears that the higher the K_m of the substrate, the lower the concentration of tropolone required to inhibit the enzyme to the same extent These data are consistent with the hypothesis that tropolone and substrate compete for binding to the copper at the active site of the enzyme

To obtain further information on the type of inhibition exerted by tropolone on mushroom tyrosinase, *o*-dihydroxyphenolase activities (V), were measured as a function of DL-DOPA concentration for several concentrations of tropolone A plot of $1/V$ vs tropolone concentration (Fig 3) shows that tropolone produced a mixed inhibition [6] The K_m value for DL-DOPA is estimated from these data to be 4 8 $\times 10^{-4}$ M, in agreement with the estimate of K_m mentioned above, while the K_i value for tropolone is 1 5 $\times 10^{-5}$ M The mixed inhibition exerted by tropolone may come from its ability to chelate copper at the active site and its ability to bind to that portion of the enzyme that normally binds the substrate, and/or due to the possibility that tropolone might inhibit the isoenzymes of mushroom tyrosinase differently

Reversal of the inhibition exerted by tropolone on mushroom tyrosinase

Goldstein *et al* [4] attributed the inhibition of dopamine-β-hydroxylase by tropolone to the copper-chelating properties of tropolone, although the inhibition by tropolone could not be reversed by Cu^{2+} and could only be reversed by a 24 hr dialysis against sodium phosphate buffer (pH 6 4)

The effect of dialysis on tropolone-inhibited mushroom tyrosinase was tested as follows Mushroom tyrosinase (1 5 mg) in 86 mM sodium phosphate buffer (pH 6 5) was incubated with 0, 1, 2 or 3 mM tropolone (samples A, B, C and D, respectively) for 10 min, yielding a completely inhibited enzyme Each sample was then dialysed for 48 hr (against several changes of 0 05 M sodium phosphate buffer, pH 6 5) and its *o*-dihydroxyphenolase activity was tested with 6 7 mM DL-DOPA (in 47 mM sodium phosphate buffer, pH 6 5) as the substrate It was found that 90% of the inhibition caused by tropolone (in samples B–D) was reversed by dialysis (i e 90% activity was recovered), indicating that the tyrosinase–tropolone complex is dissociable

Figure 4 shows that, in the absence of Cu^{2+}, but in the presence of 1 66 μM tropolone, *o*-dihydroxyphenolase activity showed a ΔA of 0 07 at 475 nm/min (see *x*-axis at zero copper sulphate concentration) compared with a ΔA of 0 44 at 475 nm/min in the absence of tropolone (indicated by an arrow on the *x*-axis at zero copper sulphate concentration) Upon addition of copper sulphate at various concentrations, the *o*-dihydroxyphenolase activity of the tropolone-inhibited enzyme was recovered, with maximum observed recovery achieved at *ca* 0 1 mM copper sulphate A large molar excess of copper sulphate–tropolone was required to obtain maximum reversal of the inhibitory effect of 1 66 μM tropolone indicating that, perhaps, tropolone has a higher binding constant to the enzyme relative to the binding of tropolone to Cu^{2+} The observation that only *ca* 70% of the original activity was recovered by addition of copper sulphate is due to the fact that added copper sulphate alone inhibits *o*-dihydroxyphenolase activity of the enzyme, as seen in the inset to Fig 4 (inset) Further experiments showed that brief preincubation of 1 66 μM tropolone with a 200- or 400-fold excess copper sulphate prior to the addition of mushroom tyrosinase, significantly reduced the inhibitory effect of tropolone on the enzyme

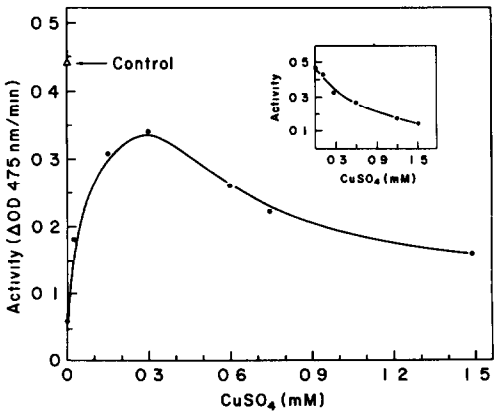


Fig 4 Effect of copper sulphate on tropolone inhibition of mushroom tyrosinase and the inhibiting effect of copper sulphate alone on mushroom tyrosinase (inset) The reaction mixture included, in a total volume of 3 ml, 6 7 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6 5), 1 66 μM tropolone, 50 μg mushroom tyrosinase (added last) and copper sulphate as indicated (Inset) The reaction mixture was as above, but without tropolone *o*-Dihydroxyphenolase activity of control (without tropolone or copper sulphate) showed a ΔA of 0 44 at 475 nm/min (shown on the *x*-axis of the inset and indicated by an arrow on the *x*-axis of the main graph)

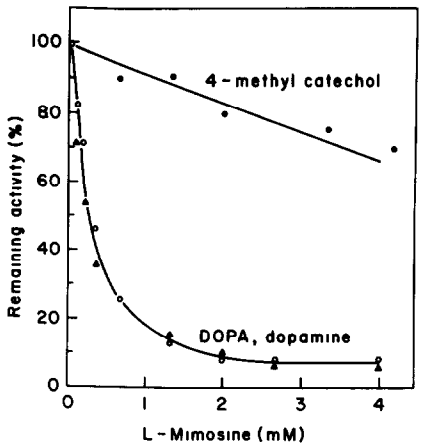
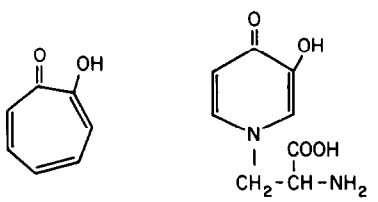


Fig 5 Effect of L-mimosine on *o*-dihydroxyphenolase activity of mushroom tyrosinase The reaction mixture included, in a total volume of 3 ml, 6 7 mM *o*-dihydroxyphenol [(4-methylcatechol) (●), DOPA (▲) or dopamine (○)], 47 mM sodium phosphate buffer (pH 6 5), 100 μg mushroom tyrosinase (added last) and L-mimosine as indicated

Comparison between the effectiveness of tropolone, L-mimosine and other copper chelators in inhibiting mushroom tyrosinase

L-Mimosine [β-(*N*-3-hydroxypyridone-4)-α-aminopropionic acid] is the pyridone analogue of DOPA, which can chelate metals such as Cu^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+} [7] The stability constants of these complexes were evaluated by Stunzi *et al* [7] The ability of tropolone and mimosine to chelate metals is attributed in both cases to the presence of a hydroxyl and an oxygen adjacent to each other (see formulae),



TROPOLONE L-MIMOSINE

It was interesting to compare the effect of L-mimosine on mushroom tyrosinase with that of tropolone The data in Fig 5 show that L-mimosine, like tropolone, is a more effective inhibitor of mushroom tyrosinase when DL-DOPA or dopamine are the substrates compared with 4-methyl catechol as the substrate A comparison between the effectiveness of tropolone and L-mimosine to inhibit the oxidation of DL-DOPA by mushroom tyrosinase showed that, under the same conditions, 50% inhibition was achieved with $0\,5 \times 10^{-6}$ M tropolone or 1×10^{-4} M L-mimosine Therefore, tropolone is 200-fold more effective as inhibitor of mushroom tyrosinase than L-mimosine

Hashiguchi and Takahashi [8] studied the effect of L-mimosine on mouse melanoma tyrosinase and on dopamine-β-hydroxylase of bovine adrenal medula They found that L-mimosine, at 10 μM or more, inhibited tyrosinase activity when L-DOPA was the substrate The inhibition of tyrosinase by L-mimosine was reported to be competitive with L-DOPA, with a K_i of 54 μM, while that of dopamine-β-hydroxylase with tyramine as the substrate was uncompetitive, with a K_i of 180 μM [8] Hashiguchi and Takahashi attributed the inhibition of tyrosinase by L-mimosine mainly to the structural similarity between L-mimosine and L-DOPA and only partially to the copper-chelating property of L-mimosine, while the inhibition of dopamine-β-hydroxylase by L-mimosine was attributed mainly to the copper-chelating capacity of the latter [8]

L-Mimosine was found to be a competitive inhibitor of *Neurospora* tyrosinase, with a K_i of 10 μM [9] Polacheck *et al* [10] showed that L-mimosine at 1 mM was a very effective inhibitor of PPO of *Cryptococcus neoformas*, while PPO of *Mycobacterium leprae* was not inhibited but, rather, used mimosine as a substrate

Further comparison between the effectiveness of tropolone and other metal-complexing agents showed that, among the agents tested, tropolone and 2-mercaptobenzothiazole (MBT) are the most potent inhibitors of mushroom tyrosinase (Table 1)

MBT has been reported to be a potent inhibitor of PPO from banana, potatoes and tobacco leaves ([11] and references therein) To the best of our knowledge, however, this is the first report of tropolone as a potent inhibitor of tyrosinase

EXPERIMENTAL

Materials Mushroom tyrosinase (grade III), tropolone (*ca* 98%, mp 51–54°), L-mimosine (*ca* 99%, mp 228°, $[\alpha]_D^{22} + 21°$), L-ascorbic acid, *p*-toluic acid, methimazole, DL-DOPA, L-

Table 1 Comparison between the effectiveness of various copper chelators to inhibit the oxidation of DOPA by mushroom tyrosinase

Copper chelator	i_{50}
Sodium salicylate	4.1×10^{-3}
Glycine	1.2×10^{-3}
Sodium thiosulphate	1.0×10^{-3}
Sodium azide	0.8×10^{-3}
Sodium ascorbate	0.4×10^{-3}
Cysteine	0.3×10^{-3}
p-Toluic acid (4-methyl benzoic acid)	0.3×10^{-3}
L-Mimosine	0.1×10^{-3}
Methimazole (1-methyl imidazole-2-thiol)	0.1×10^{-3}
MBI (2-mercaptobenzimidazole)	0.8×10^{-6}
DETC (diethyldithiocarbamate)	0.8×10^{-6}
Tropolone	0.4×10^{-6}
MBT (2-mercaptobenzothiazole)	0.13×10^{-6}

The reaction mixture included, in a total volume of 3 ml, 6.7 mM DL-DOPA 47 mM sodium phosphate buffer (pH 6.5), 100 μg mushroom tyrosinase (added last) and various concentrations of each of the copper chelators indicated. The *o*-dihydroxyphenolase activity (ΔA at 475 nm/min) was computed from the curves obtained and a graph of relative remaining activity vs inhibitor concentration was plotted in each case. The i_{50} value was obtained from each graph and represents the molar concentration of the copper chelator needed to inhibit *o*-dihydroxyphenolase activity by 50%.

tyrosine and DL-cysteine were obtained from Sigma, glycine and Na thiosulphate were from BDH, NaN_3 was from I.T. Baker, and 2-mercaptobenzimidazole was from Aldrich. All other chemicals were reagent grade.

Monohydroxyphenolase activity of mushroom tyrosinase was assayed in a reaction mixture of 3 ml that included 2 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5) and mushroom tyrosinase (added last) as indicated. The rate of tyrosine hydroxylation was followed by measuring dopachrome formation at 475 nm. The lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the *x*-axis as suggested in ref. [12].

o-Dihydroxyphenolase activity of mushroom tyrosinase was assayed in a reaction mixture of 3 ml that included 6.7 mM *o*-dihydroxyphenol (DL-DOPA, dopamine or 4-methyl catechol), 47 mM NaPi buffer (pH 6.5) and mushroom tyrosinase (added last) as indicated.

The rate of oxidation of DL-DOPA and of dopamine was followed at 475 nm and that of 4-methyl catechol at 410 nm. *o*-Dihydroxyphenolase activity was estimated from the initial linear portion of each curve and is expressed as ΔA at 475 nm/min when DL-DOPA or dopamine served as the substrates or as ΔA at 410 nm/min when 4-methyl catechol served as the substrate.

Spectroscopic data were obtained using a Varian 635 spectrophotometer equipped with a recorder.

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