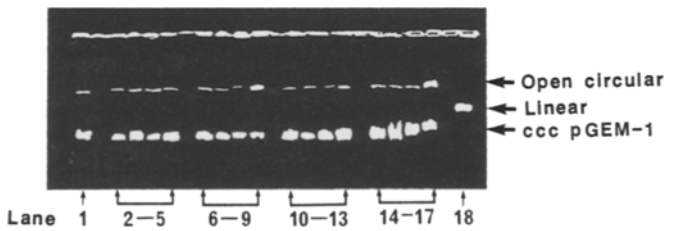

Figure 5. Interaction of **1** (lanes 2–5), **2** (lanes 6–9) and **3** (lanes 10–13) with ccc pGEM-1. Lanes 1 and 18 on the agarose gel are reference samples of ccc pGEM-1 and linear pGEM-1, respectively. Concentrations of the flavonoids: 1 μM (lanes 2, 6 and 10), 10 μM (lanes 3, 7 and 11), 100 μM (lanes 4, 8 and 12) and 1000 μM (lanes 5, 9 and 13). Effect of 0.0013, 0.013, 0.13 and 1.3 μM ethidium bromide is indicated on lanes 14–17, respectively. Experimental details are described in the text.

mycin D[8]. It has already been suggested[9] that polar flavonoids have this intercalative property. The planarity of **3** may be slightly weakened by the asymmetrically substituted B-ring (slight rotation of B-ring against the plane of the A/C ring system). Because of its nonplaner structure, **1** seems to cause no production of open circular DNA. The planarity of their structure seemed to have a correlation with the potency of flavonoids in producing open circular DNA. However, the necessary concentration (1000 μM) of the flavonoids was much higher than that (1.3 μM) of ethidium bromide.

Quite recently, (−)-epicatechin [a compound with one OH (5' position) less than **1**] has been reported to cleave the phage ϕX 174 DNA strand in the presence of Cu(II) ions[10]. It is possible that the addition of the metal ion would make the cleavage of ccc DNA by the flavonoids (**2, 3** and even **1**) possible at lower concentrations than 1000 μM.

Nose's report[11] describes the inhibitory effect of **3** on mouse RNA polymerase II activity; the transcription of naked DNA with the polymerase was strongly inhibited by mutagenic flavonoids including **3**, but weakly by non- or weakly mutagenic flavonoids. Myricetin (**2**) has also been reported to have mutagenicity[12]. In the present study, the mutagenic **2** and **3** also show a stronger inhibitory effect on the polymerase activities than the non-mutagenic **1**.

Thus, this paper is the first report on the mode of inhibitory activity of flavonoids against DNA-dependent DNA and RNA polymerases.

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Inhibition of mushroom tyrosinase by 3-amino-L-tyrosine: Molecular probing of the active site of the enzyme

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Summary. We report the ability of 3-amino-L-tyrosine to act as a fully reversible competitive inhibitor of mushroom tyrosinase. The inhibition is linked to the ortho-aminophenol structure, and a copper bridging mechanism in the active site is proposed.
Key words. Mushroom tyrosinase inhibition; 3-amino-L-tyrosine; ortho-aminophenols; di-copper active site.

Tyrosinase (EC 1.14.18.1) is a widespread copper-containing enzyme which catalyzes two oxidoreduction reactions involving molecular oxygen[1] and phenolic structures in the presence of reductants as shown in figure 1. The resulting unstable o-quinones typically polymerize to form pigments such as humin or melanin. However, in the presence of an excess of external reductant, the reaction will stop at the catechol level (reaction I, fig. 1); such a system has been shown to be an efficient source of o-dihydroxyphenols[2]. We report the competitive inhibition of reaction (I) by 3-amino-L-tyrosine and some structural analogs. On the basis of these results we suggest a mechanism for this inhibition using the model proposed earlier for the active site of this enzyme.

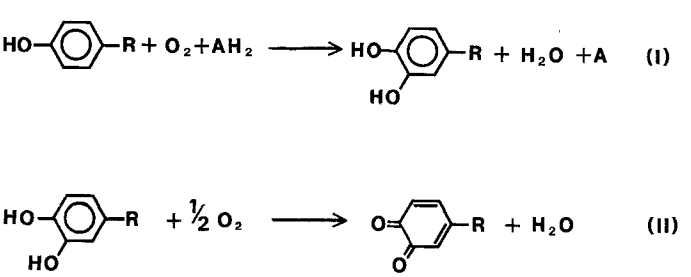


Figure 1. Mono- and di-phenoloxidase activity of tyrosinase (AH_2 = reductant).

Materials and methods. 3-Amino-L-tyrosine (3-AT), 2-aminophenol (2-AP) and o-anisidine (o-An) were purchased from Aldrich, p-aminophenylalanine (4-APhe), mushroom tyrosinase (MT, 2,480 units/mg), ascorbate oxidase (EC 1.10.3.3, 1700 units/mg) and uricase (EC 1.7.3.3, 1.0 unit/mg, in 2.0 M ammonium sulfate) were from Sigma. All other chemicals and reagents were of analytical grade. The effect of the different drugs on the enzymatic activities was measured in the range of 1 µM to 1 mM.

MT activity was measured at 24 °C in NaH_2PO_4 buffer (0.1 M, pH 7.0, final volume 550 µl) containing ascorbic acid [2] (25 mM) and variable amounts of L-tyrosine (50 µM to 400 µM). At t = 0, MT (20 µg in 100 µl of buffer) was added and air-bubbled through the solution (≃ 50 ml/min). At t = 30 s, $HClO_4$ (100 µl, 1 M) was added. An aliquot (40 µl) was removed and diluted with $HClO_4$ (1 M, 460 µl) containing dihydroxybenzylamine (DHBA, 300 ng) internal standard. Measurement of the amount of L-DOPA formed was made by HPLC (C_{18} reverse-phase column, 3 µ, 100 mm × 2 mm: mobile phase; NaH_2PO_4, 0.1 M, pH 3.4, 50 mg/l EDTA and 75 mg/l sodium octyl sulfate, flow 200 µl/min) coupled to an amperometric detector (glassy carbon working electrode, + 0.75 V, Ag/AgCl reference). The peak height obtained for L-DOPA relative to that for DHBA internal standard was compared between samples. Measurement of the amount of 3-AT in each sample could conveniently be made from the same chromatogram by comparing its peak height to that of the internal standard.

Assays for the activity of the four other enzymes were performed as described in the literature [3].

Results and discussion. 3-AT is a competitive inhibitor of tyrosine hydroxylation by MT with a K_i of 3.5×10^{-6} M (fig. 2). This inhibition is totally reversible by dialysis against 0.01 M phosphate buffer (97% activity recovered), and the low micromolar range obtained for the K_i indicates that 3-AT is among the most potent known selective MT inhibitors, comparable to methimazole, extensively studied earlier [5]. However, at tyrosine concentrations less than 200 µM, the results (fig. 2-right) have consistently indicated the presence of a secondary non-competitive inhibition of the type postulated by Christopherson in the case of L-tyrosine effect on chorismate mutase activity [4]. In the presence or absence of tyrosine plus ascorbic acid, 3-AT concentrations were unaltered following incubation with MT for up to 30 min, suggesting that 3-AT is not acting as a competitive substrate in this system.

The competitive nature of this inhibition is not surprising in view of the structural similarity between 3-AT and the substrate tyrosine. On the other hand, this finding is not consistent with the conclusion of an investigation of aromatic ring substituent effects by Passi and Nazzaro-Porro [6], who deduced that phenolic inhibitors of MT require an electron-withdrawing group on the ring. Negative σ Hammet constants show that the amino group in 3-AT is not electron-withdrawing.

The structural analogues 2-AP, 4-APhe and o-An were also investigated for their ability to inhibit MT. The results showed 2-AP is as potent as 3-AT, while o-An is only a weak inhibitor and 4-APhe had no noticable activity (table). These results suggest that the ortho-aminophenolic structure is responsible for the inhibitory effect on MT activity, and the potency of inhibition is significantly diminished when the oxygen atom is methylated.

The effect of the ortho-aminophenolic compounds was then tested on the activity of three other copper-containing enzymes. Their effects were negligible on uricase, amine oxidase and ascorbate oxidase (table). Furthermore, none of these compounds had a significant effect on tyrosine hydroxylase activity (table). In sum these observations suggest a specific structural interaction between the active site of MT and the ortho-aminophenolic structure.

The active site of hemocyanin is considered a valid model for MT [7]. The structure of oxyhemocyanin has been used to interpret the EXAFS and resonance Raman spectroscopy [8] results on oxytyrosinase from *Neurospora crassa* which have shown that the distance between the two copper atoms at the active site is 3.63 Å [9]. The calculated distance, based on standard bond lengths, between the oxygen and the nitrogen in 2-AP is about 2.8 Å. This would place the electronic doublets of both heteroatoms at a convenient distance to interact with the two copper atoms, leading to a bridged structure (fig. 3) as already proposed for L-mimosine inhibition [10]. When so positioned, the aminophenol would both block access to the site and prevent the polarization of the peroxo bridge, proposed as a possible mechanism for monophenol hydroxylation [11]. Another alternative explanation, also proposed for mimosine [10], would be displacement by 3-AT of the peroxide from oxytyrosinase, yielding the corresponding inactivated bridged deoxy-structure.

Of interest in this regard is the common geometric pattern of the nucleophilic centers of 3-AT, L-mimosine and fusaric acid, another inhibitor of tyrosinase [12] (fig. 3). This feature

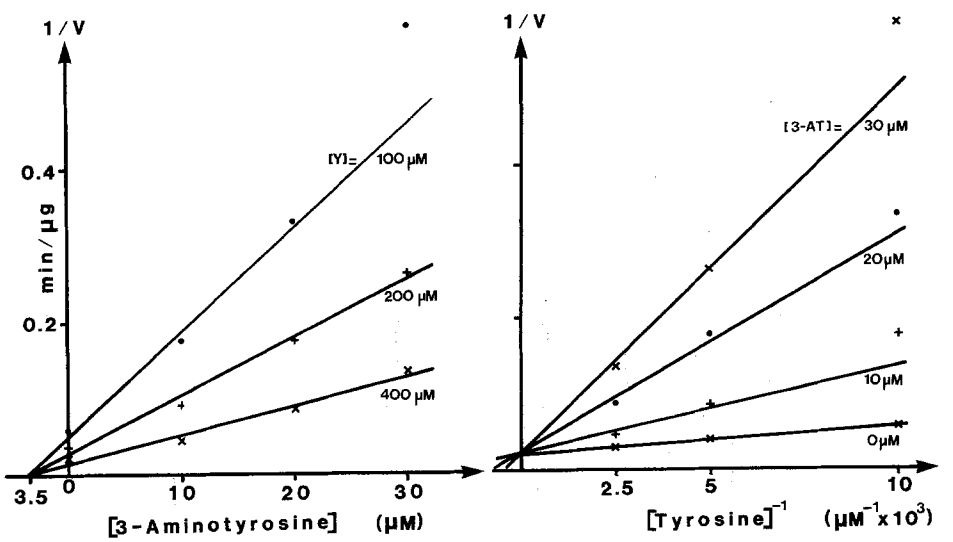
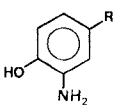
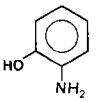
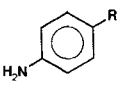
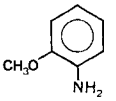


Figure 2. Dixon (left) and Lineweaver-Burk (right) plots of mushroom tyrosinase kinetics in the presence of 3-amino-L-tyrosine.

Concentrations of 3-amino-L-tyrosine, 2-aminophenol, 4-amino-L-phenylalanine and o-anisidine necessary for 50% inhibition of enzyme activities (I_{50}).

Enzyme (origin)	3-AT 	2-AP 	4-APhe 	o-An 
Tyrosinase (mushroom)	14 μM	2 μM	> 1 mM	≃ 200 μM
Uricase (porcine liver)	> 1 mM	> 1 mM	> 1 mM	–
Amine oxidase (human plasma)	> 1 mM	≃ 250 μM	> 1 mM	–
Ascorbate oxidase (curcubita species)	> 1 mM	–	> 1 mM	–
Tyrosine hydroxylase (canine basal ganglia)	≃ 300 μM	> 1 mM	> 1 mM	–

R = CH_2–$CH(NH_2)COOH$

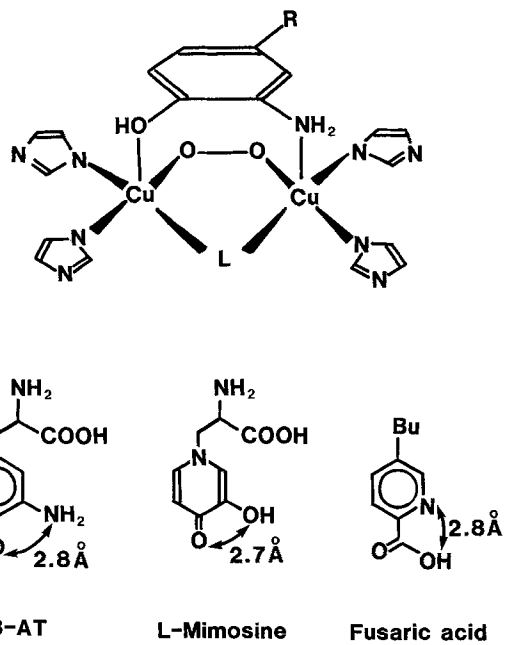


Figure 3. Proposed structure for the active site of oxytyrosinase complexed with 3-aminotyrosine (R = $CH_2CH(NH_2)COOH$, L = endogenous ligand).

suggests these three compounds could act through this same bridging mechanism. The observation that o-An is less potent than 3-AT can be explained in this context by the decreased nucleophilicity and/or the increased steric hindrance of the oxygen atom, both factors reducing its bridging ability.

We are currently working on a theoretical calculation of the relative energy states of different conformations of a simplified model of this coupled binuclear copper system in an attempt to get a mechanistic description of the very first steps of the hydroxylation process.

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