# The influence of catechol structure on the suicide-inactivation of tyrosinase

**Christopher A. Ramsden,*[a] Michael R. L. Stratford[b] and Patrick A. Riley[c]**

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**3,6-Difluorocatechol, which cannot act as a monooxygenase tyrosinase substrate, is an oxidase substrate, and, in contrast to other catechols, oxidation does not lead to suicide-inactivation, providing experimental evidence for an inactivation mechanism involving reductive elimination of $Cu^0$ from the active site.**

Tyrosinase (EC 1.14.18.1) is a mixed-function oxidase that is widely distributed in nature. It is involved in several biologically significant processes including pigmentation, cuticular hardening in insects, and protection of seeds.[1] Tyrosinase is a metalloenzyme with a strongly conserved active centre containing two copper atoms that bind dioxygen to form *oxy*-tyrosinase **1**.[2,3] *Oxy*-tyrosinase is able to catalyze *ortho*-quinone formation by the oxidation of both catechols (oxidase activity) and phenols (monooxygenase activity) (Scheme 1). Phenols, including tyrosine, are oxidised to *ortho*-quinones with formation of *deoxy*-tyrosinase **2**, which then binds dioxygen to reform *oxy*-tyrosinase **1** (path a, Scheme 1). Oxidation of catechols (path b, Scheme 1) leads to an *ortho*-quinone plus *met*-tyrosinase **3**. Reduction of *met*-tyrosinase by a second molecule of catechol gives *deoxy*-tyrosinase **2** which in turn binds dioxygen to reform *oxy*-tyrosinase **1** and complete the cycle.

It has been known for more than half a century that tyrosinase is subject to reaction-inactivation (suicide-inactivation), but only when catalysing the oxidation of catechols.[4,5] Based on a structure–activity study of a variety of dihydroxy tyrosinase substrates, we recently proposed a novel mechanism to rationalise this previously unexplained feature of catechol oxidation.[6] In particular, we proposed that, in addition to acting as oxidase substrates for *oxy*-tyrosinase (path b), catechols can also act as monooxygenase substrates, and that under these circumstances the hydroxy substituent in the bound substrate can deprotonate, leading to reductive elimination of copper and irreversible formation of *inactivated* tyrosinase **4** (path c, Scheme 1). This mechanism accounts for the 50% loss of the copper from the active site reported by Dietler and Lerch.[7]

The inactivation mechanism shown in Scheme 1 leads to two predictions: (i) catechol oxidases lacking monooxygenase activity will not exhibit suicide-inactivation, and (ii) catechols having substituents that block oxygenation at positions 3 and 6 cannot act as monooxygenase substrates, and will not exhibit suicide-inactivation. Subsequently we have shown that, in agreement with prediction (i), the catechol oxidase extracted from bananas (*Musa cavendishii*), which is deficient in monooxygenase activity, is not inactivated.[8] In accord with prediction (ii), we now demonstrate that 3,6-difluorocatechol **5** is a substrate for *oxy*-tyrosinase but does not cause deactivation of the enzyme.

3,6-Difluorocatechol **5** was prepared from 5,8-difluoro-2,3-dihydro-1,4-benzodioxin using the method of Ladd and Weinstock.[9,10] Tyrosinase (ex *Agaricus bisporus*) was made up in phosphate buffer (0.1 M, pH 6.3) and catechol oxidation was monitored using combined enzyme oximetry and spectrophotometry, using the apparatus previously described.[11]

The kinetics of oxygen uptake for 3,6-difluorocatechol **5** and 4-fluorocatechol **6** are shown in Fig. 1. The enzymatic oxidation of 3,6-difluorocatechol **5** proceeds at a nearly exponential rate, dependent on the amount of enzyme added. The oxidation rate per unit of tyrosinase (2.5 nmoles $O_2$ $min^{-1}$) is considerably lower than that of 4-fluorocatechol (66 nmoles $O_2$ $min^{-1}$). However, the kinetic characteristics significantly differ from those for the oxidation of 4-fluorocatechol **6**, which demonstrates suicide-inactivation and premature termination of oxygen uptake. The $V_{max}$ for 3,6-difluorocatechol oxidation by mushroom tyrosinase was calculated as 250 nmol substrate $min^{-1}$ and the Michaelis constant as 483 μM.

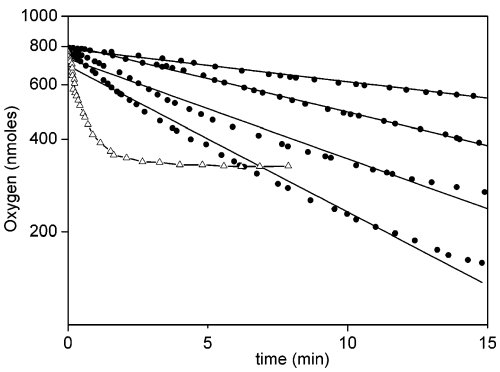
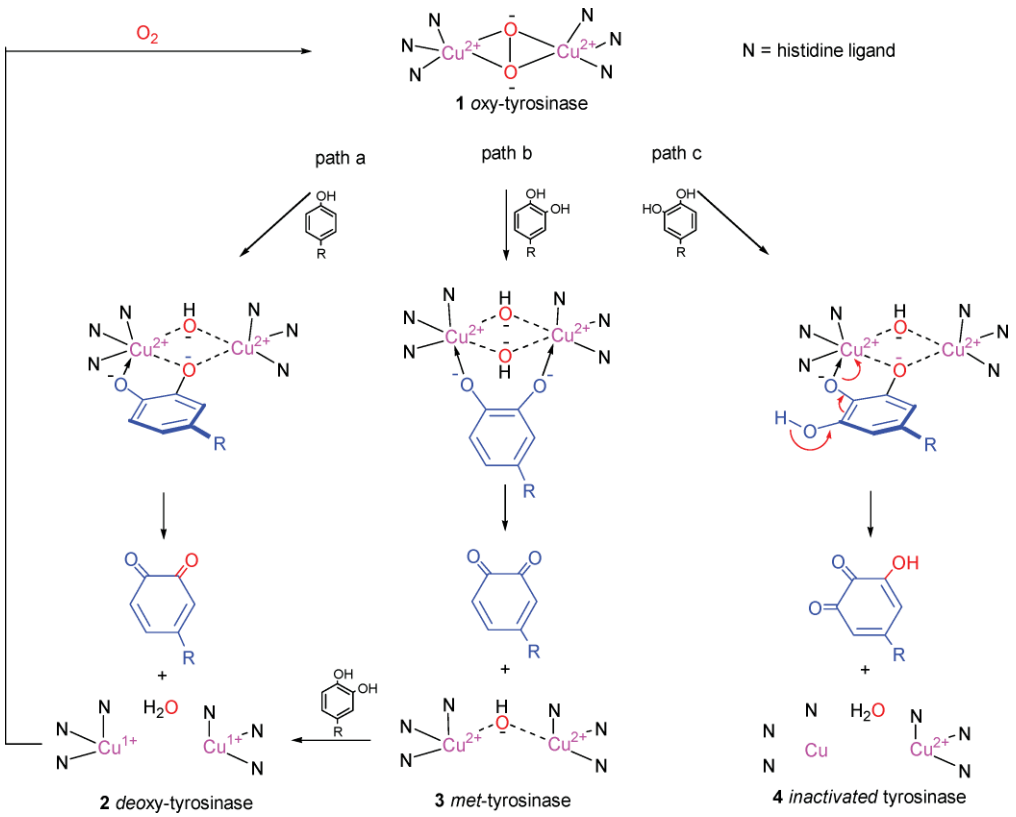



**Fig. 1** Oximetric data for tyrosinase-catalysed oxidation of 3,6-difluorocatechol **5** compared to 4-fluorocatechol **6** at 30 °C. Oxidation of **5** (960 μM) (●), shown for additions of 7.5, 10, 15 and 30 units enzyme (in 3.65 mL), fits a set of exponential curves (mean correlation coefficient 0.987), in contrast to the kinetics of oxidation of equimolar **6** (△), in which tyrosinase (15 units) is subject to suicide-inactivation. The data are shown as a semi-logarithmic plot of the residual oxygen as a function of incubation time.

In separate experiments we have shown that prior exposure (10 min) of tyrosinase (20 units) to 3,6-difluorocatechol **5** (225 μM) does not diminish the oxidation of 4-methylcatechol (790 μM) relative to a control experiment using fresh tyrosinase.

[a]*Lennard-Jones Laboratories, School of Physical and Geographical Sciences, Keele University, Staffordshire, ST5 5BG, UK*
[b]*Gray Institute for Radiation Oncology & Biology, University of Oxford, Old Road Campus Research Building, Oxford, OX3 7DQ, UK*
[c]*Totteridge Institute for Advanced Studies, The Grange, Grange Avenue, London, N20 8AB, UK*




**Scheme 1**

The spectrophotometric data show a similar kinetic distinction between 3,6-difluorocatechol **5** and 4-fluorocatechol **6** oxidation. 3,6-Difluorocatechol **5** has a strong absorbance peak at 220 nm and a shoulder at 260 nm. On oxidation by tyrosinase there is an initial rise in absorbance at 400 nm, consistent with the formation of the corresponding *ortho*-quinone **7**, and a rapidly increasing absorbance in the 260–320 nm region (Fig. 2). The final spectrum is very broad, extending to above 600 nm suggesting the formation of melanoid polymers.

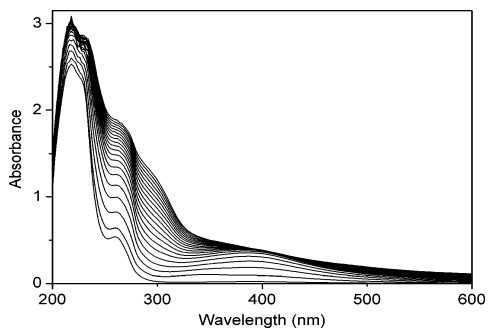



**Fig. 2** Successive spectral scans at 30-second intervals during oxidation of 960 μM 3,6-difluorocatechol **5** by tyrosinase (30 units).

Data from LC-MS examination of an oxidation mixture in phosphate buffer (0.1 M, pH 6.3) (1 mL) containing 3,6-difluorocatechol **5** (700 μM) and tyrosinase (3 units) indicated the formation of several products of which four major components were clearly identified by their molecular ions at *m*/*z* (M-H) 289 (RT 6.2 min), 159 (RT 3.7), 305 (RT 6.4), and 429 (RT 6.8). None of the products identified exhibited a quinone absorbance, indicating that any quinones formed (*e.g.* **7**–**9**) are highly reactive. Fluoroquinones are known to undergo substitution with release of fluoride,[12] and we examined the kinetics of fluoride release by ion chromatography. Fig. 3 shows the time-dependent yield of fluoride and Fig. 4 shows the change of the products identified by LC-MS with time.[13,14]

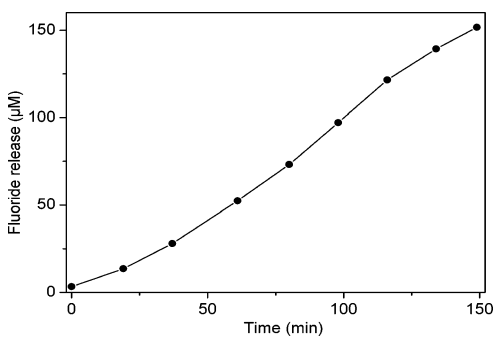



**Fig. 3** Time course of fluoride release from 3,6-difluorocatechol **5** (700 μM) and tyrosinase (3 units in 0.1 M phosphate buffer, pH 6.3) (1 mL).

The major product (*m*/*z* 289), which rapidly forms, is the diaryl ether **10**, formed from the *ortho*-quinone **7** and its precursor **5** (Scheme 2). The second initial product (*m*/*z* 159) is a tetrahydroxy product **11** and/or **12**, formed with fluoride release from the initial *ortho*-quinone **7** and water. The secondary products are formed by further oxidation, substitution and addition reactions of the initial

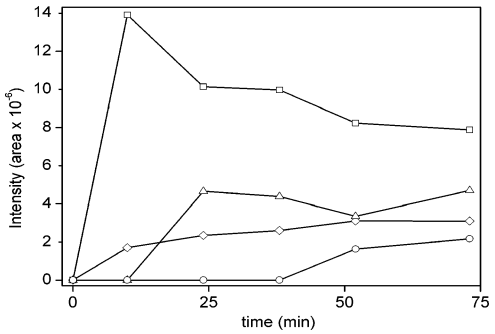
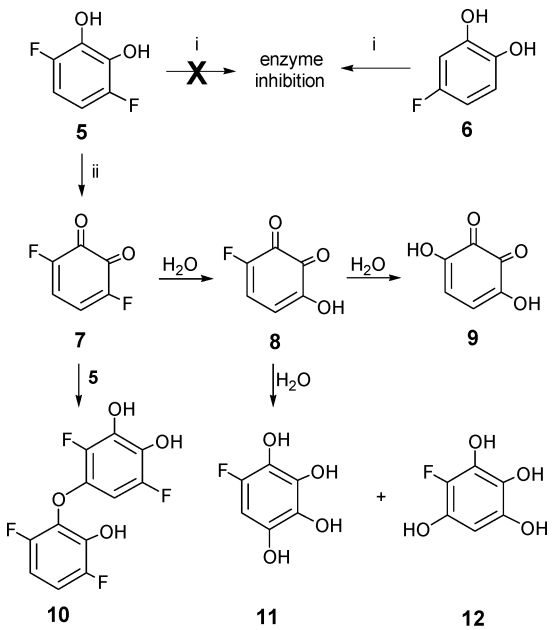



**Fig. 4** Time-dependent changes of major oxidation products identified by LC-MS. The products illustrated are those with $m/z$ 289 (□), 159 (◇), 305 (△), and 429 (○).




Reagent: i, monooxygenase activity; ii, oxidase activity

**Scheme 2**

product **10**. Fluoride release is slower than formation of the ether **10** and concomitant with the rate of formation of the monofluoro products **11** and **12**.

The data show that 3,6-difluorocatechol **5** is a substrate for tyrosinase and forms 3,6-difluoro-*ortho*-quinone **7** *via* the oxidase mechanism (path b, Scheme 1). In contrast to other catechols, there is no inactivation of tyrosinase. The rate of oxidation is slow in comparison with 4-fluorocatechol and 3-fluorocatechol,[6] but the kinetics of oxygen utilization differ significantly from these substrates. Both the oximetric and spectrophotometric data show that the oxidation kinetics of 3,6-difluorocatechol **5** fit a pseudo-first-order function and resemble those found using catechol oxidase extracted from bananas.[8] They differ from the usual kinetics of catechol oxidation by tyrosinase, which exhibit suicide-inactivation.[6] We attribute this result to the fluoro substituents flanking the catecholic group which prevent the difluorocatechol **5** from being processed by the monooxygenase route (path c, Scheme 1). This provides further evidence for the inactivation mechanism that we have proposed to account for the suicide-inactivation of tyrosinase by catecholic substrates.

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13 LC-MS studies used a similar system to that previously described,[8] but using an Atlantis dC18 column, 150 × 3 mm (Waters), the organic modifier was MeCN, and the gradient was 15–60% (flow rate of 0.5 mL min$^{-1}$). Electrospray ionisation was carried out in negative mode, capillary voltage 2.1 kV, cone voltage 21 V.
14 Separation of fluoride was carried out on an AS12 column (Dionex) using water and 37.5 mM NaOH, 50 mM sodium tetraborate as eluents. Initial conditions were 15% buffer, with a linear gradient to 75% from 1–8 min (flow rate 1.5 mL min$^{-1}$). Detection was by conductivity using a Dionex ED40 with external water suppression.

 