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Structure–activity relationships of various amino-hydroxy-benzenesulfonic acids and sulfonamides as tyrosinase substrates

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ABSTRACT

Background: *o*-Aminophenols have been long recognised as tyrosinase substrates. However their exact mode of interaction with the enzyme's active site is unclear. Properly *vic*-substituted *o*-aminophenols could help gain some insight into tyrosinase catalytic mechanism.
Methods: Eight *vic*-substituted *o*-aminophenols belonging to two isomeric series were systematically evaluated as tyrosinase substrates and/or activators and/or inhibitors, by means of spectrophotometric techniques and HPLC-MS analysis. Some relevant kinetic parameters have also been obtained.
Results: Four *o*-aminophenolic compounds derived from 3-hydroxyorthanilic acid (2-amino-3-hydroxybenzenesulfonic acid) and their four counterparts derived from the isomeric 2-hydroxymetanilic acid (3-amino-2-hydroxybenzenesulfonic acid) were synthesised and tested as putative substrates for mushroom tyrosinase. While the hydroxyorthanilic derivatives were quite inactive as both substrates and inhibitors, the hydroxymetanilic compounds on the contrary all acted as substrates for the enzyme, which oxidised them to the corresponding phenoxazinone derivatives.
General significance: Based on the available structures of the active sites of tyrosinases, the different affinities of the four metanilic derivatives for the enzyme, and their oxidation rates, we propose a new hypothesis regarding the interaction between *o*-aminophenols and the active site of tyrosinase that is in agreement with the obtained experimental results.

1. Introduction

Tyrosinases (monophenol, *o*-diphenol:dioxygen oxidoreductases, EC 1.14.18.1) are copper-containing enzymes that catalyse the hydroxylation of monophenols to the corresponding *o*-diphenols (catechols) and the oxidation of *o*-diphenols to *o*-quinones [1]. The first activity is often referred to as cresolase or monophenolase activity and the second as diphenolase or catecholase activity. In its active site, tyrosinase binds two non-equivalent cupric ions that form a cluster of spectroscopic type III (antiferromagnetic coupling that renders the enzyme EPR-silent) and are joined by a water or hydroxide bridge in the enzyme's resting state (*met* form). The two cupric ions forming the dicopper cluster are coordinated by six conserved histidine residues belonging to the same polypeptide chain and are named $\mathbf{Cu_A}$ (the cupric ion that is coordinated by three histidines proximal to the N-terminus) and $\mathbf{Cu_B}$ (that coordinated by three histidines proximal to the C-terminus) [2]. In the enzyme's reduced (*deoxy*) form the two copper ions are in their cuprous state; in the *oxy* form, a peroxo μ–η^2:η^2 bridge between the two cupric ions is present. The active site of the enzyme is the subject of several past and ongoing studies aimed at outlining its shape and identifying the amino acid residues involved [3], studying the modes of substrate recognition and binding, and elucidating the precise mechanisms of the reactions throughout the catalytic cycle [4–8]. Mushroom tyrosinase is undoubtedly the most studied tyrosinase and has been used to generate much kinetic data. Unfortunately, it has not yet been crystallised, so its 3D structure is unknown. Comparative modelling is currently the most accurate method to predict the 3D structure of proteins when no experimental structure is available [9].

With respect to tyrosinase substrates, a wide range of phenols and catechols have been found to be *o*-hydroxylated and quinonised, respectively, by the enzyme [1]. All known tyrosinases (with rare exception [10]) show a typical lag time when acting on monophenols [11–13]. This lag time can be defined as the time required to reach the stationary state speed with respect to the diphenol concentration. The lag time can be influenced by many factors, including the presence of minute amounts of diphenols such as L-dopa. Conversely, several

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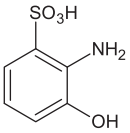
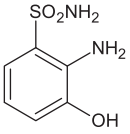
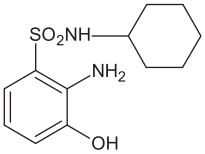
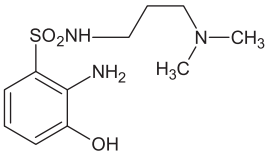
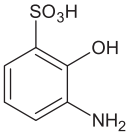
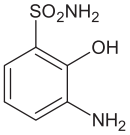
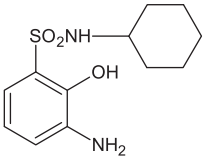
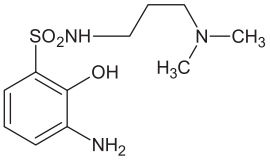
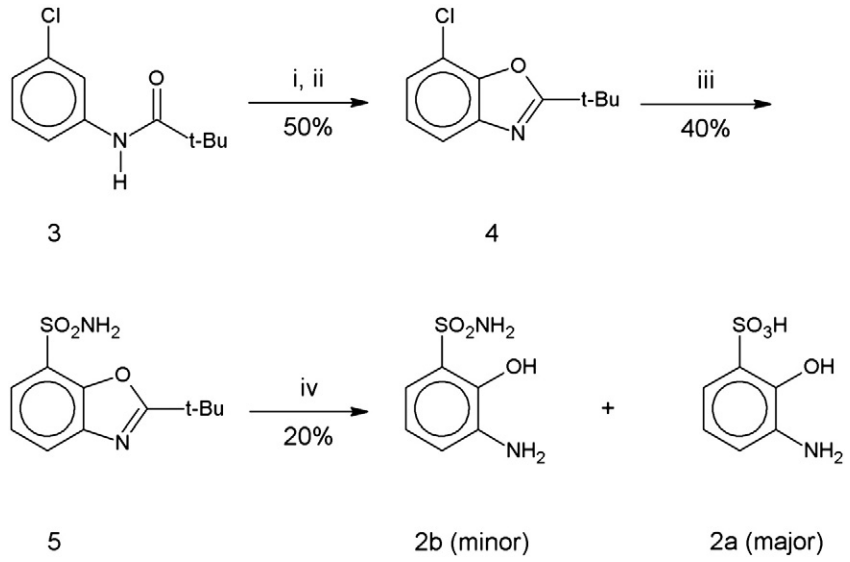
properly substituted phenols have been found to be more or less effective inhibitors of the enzyme [14].

Tyrosinase substrates are not limited to phenols and catechols; many *o*-aminophenols and aromatic *o*-diamines have been found to be quinonised by tyrosinase, and even monoamines have been reported to be *o*-hydroxylated by the enzyme [15–18]. While aromatic *o*-diamines are unnatural compounds, *o*-aminophenols are commonly found in living systems; the most studied is perhaps 2-amino-3-hydroxybenzoic acid (3-HAA, 3-hydroxyanthranilic acid), which arises from tryptophan metabolism [19]. 3-HAA is not only a good substrate for tyrosinase but also an activator of its monophenolase activity; treatment of tyrosinase with 3-HAA substantially shortens the lag time [20]. The oxidation product of 3-HAA arises from an oxidative coupling of an intermediate quinoneimine derivative; it has a peculiar structure, based on a tricyclic phenoxazinone nucleus, and is known as cinnabarinic acid (2-amino-3-H-phenoxazin-3-one-1,9-dicarboxylic acid, CA) after the white-rot fungus *Picnoporus cinnabarinus*, from which it is isolated [21]. Other phenoxazinone derivatives closely resembling CA are well known to exist in nature. These include 2-amino-3-H-4,6-dimethyl-3-phenoxazin-3-one-1,9-dicarboxylic acid (4,6-dimethylcinnabarinic acid, also known as actinocin), which is the non-amino acidic component of the dicyclopeptidic antitumor and antibiotic drug actinomycin D [22]. In general, phenoxazinone derivatives are expected to arise whenever an *o*-aminophenol with free 4- and 5-positions is properly oxidised [23].

Very recently, some studies have explored the synthesis of a number of phenoxazinone derivatives whose outstanding feature is the presence of sulfonate or sulfonamide functionalities directly bound to the tricyclic cores of those molecules [24,25]. In the present study, some *o*-aminophenols bearing the sulfonate or sulfonamide functionalities (Table 1) were synthesised and studied as putative

Table 1
The structures of the studied compounds, series 1(a–d) and 2(a–d).

Compound	IUPAC name	Chemical structure
1a	2-Amino-3-hydroxy-benzenesulfonic acid (3-hydroxy-orthanilic acid, 3-HOA)	
1b	2-Amino-3-hydroxy-benzenesulfonamide	
1c	2-Amino-3-hydroxy-benzene-*N*-cyclohexyl sulfonamide	
1d	2-Amino-3-hydroxy-benzene-*N*-(3-dimethylamino-propyl)sulfonamide	
2a	3-Amino-2-hydroxy-benzenesulfonic acid (2-Hydroxymetanilic acid, 2-HMA)	
2b	3-Amino-2-hydroxy-benzenesulfonamide	
2c	3-Amino-2-hydroxy -benzene-*N*-cyclohexyl sulfonamide	
2d	3-Amino-2-hydroxy- benzene-*N*-(3-dimethylamino-propyl)sulfonamide	



Scheme 1. Synthesis of 3-amino-2-hydroxy-benzenesulfonamide (Method A: [24,25]). Reagents and conditions: (i) *n*-BuLi (2.1 equiv.), −78 °C to −45 °C, 2 h; (ii) SO_2Cl_2 (5 equiv.), −78 °C to 25 °C, overnight; (iii) 25% aq. NH_3, DCM, 25 °C, overnight; (iv) 5 N HCl, 100 °C, overnight.

tyrosinase substrates with the aims of shedding light on the structural features required for a good fit in the enzyme active site and of gaining more information about the mode of action of tyrosinase.

2. Experimental

2.1. Preparation of aminophenols

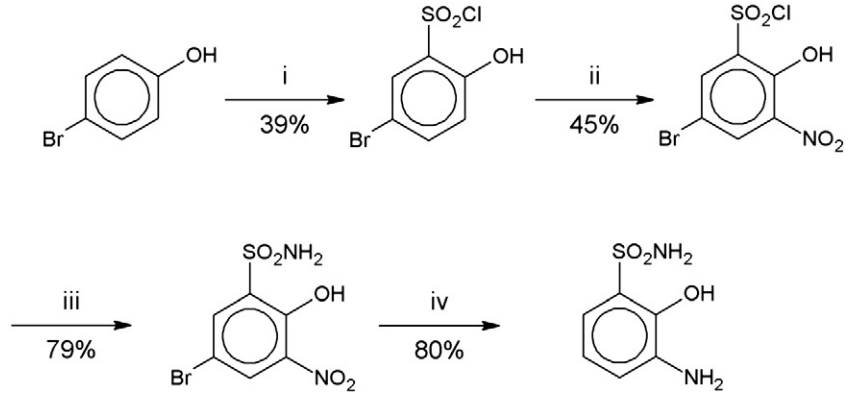
The tyrosinase substrates were prepared using the standard equipment in the Laboratory of Organic and Medicinal Chemistry at UCL (Louvain-la-Neuve, Belgium).

The multistep syntheses of compounds **1a** (3-HOA) and compounds **1b**, **1c**, **1d**, **2a** (2-MA), **2c** and **2d** fully described in references [24] and [25] respectively, were performed according to the literature. The novel compound **2b** was similarly prepared, as depicted in Scheme 1 (Method A). Briefly, 2-*tert*-butylbenzoxazole-7-sulfonyl chloride (**4**), prepared in two steps from *N*-(3-chlorophenyl)-2,2-dimethylpropionamide (**3**), was dissolved in CH_2Cl_2 (5 mL/mmol) and treated at 0 °C with 25% NH_4OH aqueous solution (5 mL/mmol). The mixture was stirred for 17 h at 20 °C. The organic phase was separated, washed with 1 N HCl (3×5 mL/mmol), dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography (hexane/ethyl acetate, 7:3) to produce 2-*tert*-butylbenzoxazole-7-sulfonamide (**5**) as a white solid (yield = 40%). Hydrolysis with 6 N HCl (10 mL/mmol) at 100 °C for 17 h produced a mixture of **2b** (quantitatively determined to be minor) and **2a** (2-HMA; quantitatively determined to be major). Pure 3-amino-2-hydroxy-benzenesulfonamide (**2b**) was obtained by concentration *in vacuo* and flash chromatography (ether/ethyl acetate, 7:3) as a brown powder (yield = 20%): ^{1}H NMR (300 MHz, CD_3OD) δ = 7.08 (d, J = 7.8 Hz, 1 H), 6.93 (d, J = 7.2 Hz, 1 H), 6.77 (dd, J = 7.8, 7.2 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, CD_3OD) δ = 143.7, 138.8, 128.5, 121.5, 120.7, 117.8 ppm. MS (ESI) m/z = 187.1 [M–H$^+$] for $C_6H_8N_2O_3S$. The compound synthesised here was identical to a reference sample obtained according to Method B (Scheme 2) from Binggeli et al. (Hoffman-La Roche Patent US 2007093521, WO 2007025897) [26], which was practical on the multigram scale.

All other chemicals used were obtained from Sigma-Fluka-Aldrich (Milan, Italy) and were of reagent grade and used without further purification.

2.2. Enzyme and assays

Mushroom tyrosinase was purified as previously described [27]. Enzyme activity was estimated in the presence of a given amount of enzyme, 100 mM potassium phosphate buffer (pH 6.5), and 0.33 mM L-tyrosine. We consider the amount of enzyme that increases the absorbance at 305 nm by 0.001 per min in the assay conditions to be one enzyme unit [28]. Because tyrosinase catalyses a reaction between two



Scheme 2. Synthesis of 3-amino-2-hydroxy-benzenesulfonamide (Method B: US Patent 2007093521). Reagents and conditions: (i) HO_3SCl (7 equiv.), 0 °C to 20 °C, 16 h; (ii) HNO_3-H_2SO_4 (1:1, 1.5 equiv.), DCM, 5 °C to 20 °C, 2 h; (iii) 25% aq. NH_3, (1.5 equiv.), THF, Et_3N (1.5 equiv.), 0°C to 20 °C, 16 h; (iv) H_2 (1 atm), Pd-C (10%), MeOH, 4 h, 20 °C.

substrates, molecular oxygen and a mono- or diphenol, the assays were carried out in air-saturated solutions.

Stock solutions of aminophenolic derivatives to be tested as putative tyrosinase substrates were prepared at 1 mM in 10% (v/v) aqueous ethanol. The effect of tyrosinase on each of the aminophenolic compounds was measured in a final volume of 1 mL 0.1 M potassium phosphate buffer at pH 6.5. The reaction mixture also included 200 μM aminophenol and 130 tyrosinase units. The reaction mixtures contained 1% (v/v) ethanol. This concentration of ethanol had no measurable effect on enzyme activity.

In some experiments monophenolase activity was also measured in the presence of *tert*-butylphenol (BP), while diphenolase activity was tested with 4-*tert*-butylcatechol (BC) as the substrate. Indeed, the catalysed oxidation of these substrates gives rise to 4-*tert*-butyl-*o*-benzoquinone (BQ), which is more stable than the product derived from oxidation of L-tyrosine or L-dopa. Moreover, this *o*-quinone can be coupled to an aromatic amine to yield a blue adduct [29,30]. This adduct exhibits spectral features different from those of the parent phenol and its *o*-quinone counterpart and is therefore a better readout of the effect of some of the experimental compounds on enzyme activity. Furthermore, the higher sensitivity of this test allowed for the use of lower enzyme amounts (13 U) when measuring diphenolase activity. K_M values for compounds **2(a–d)** were measured using 20 mM stock solutions in 20% (v/v) ethanol. All other conditions were the same.

All the experiments were performed in triplicate and the given data are the corresponding mean values.

Characterization of reaction products was carried out using a LC–MS system composed by a Varian 212-LC HPLC (Varian Inc, CA, USA) and a Varian 310-MS triple quadrupole mass spectrometer (Varian Inc, CA, USA) with positive electrospray ionisation mode using full scan monitoring (*m/z*: 330.0→380.0). HPLC was equipped with a Pursuit® C18 column (100 mm×2.0 mm i.d., particle size 5 μm). An isocratic mobile phase (100% methanol) at flow rate of 0.2 mL/min was used. Settings used were as follows: needle voltage 5.7 kV; nebulising gas (N_2) 45.0 psi; shield voltage 0.6 kV; drying gas 200 °C; 20.0 psi; capillary voltage 40 V. The LC–MS system was controlled by Varian MS Workstation software, and data were collected with the same software.

2.3. Molecular modelling

The primary structure of *Agaricus bisporus* tyrosinase was obtained from the Swiss-Prot database (accession number O42713). The identification of homologues of tyrosinase was performed using the protein–protein BLAST algorithm (Basic Local Alignment Search Tool) [31] on the Protein Data Bank (BLOSUM62 matrix). The programme compares protein sequences to sequence databases and calculates the statistical significance of matches (*E* value). *Vitis vinifera* polyphenol oxidase [32] (PDB code 2P3XA) was selected as the most appropriate template (sequence identity 23%; amino acid positives 38%). An automated homology modelling programme, ESyPred3D [9] was used to build the three-dimensional (3D) structure of tyrosinase from *Agaricus bisporus*. The amino acid sequence of *A. bisporus* tyrosinase and the sequence of the polyphenol oxidase template were submitted to the programme. This programme used a new alignment methodology: by comparing the results from various multiple alignment algorithms, it derived a "consensus" alignment of the target sequence and the template. The 3D models were visualised using the Mercury view programme (v. 2.3, http://www.ccdc.cam.ac.uk./free_services/mercury/).

3. Results

The synthesised aminophenols represented in Table 1 were tested as tyrosinase substrates. All compounds **1(a–d)** showed a negligible ability to behave as enzyme substrates, and therefore their absorption spectra remained almost unchanged.

On the contrary, compounds **2(a–d)** were all enzyme substrates, although enzymatic oxidation of compound **2a** was comparatively slow. Fig. 1 shows the spectral variations caused by tyrosinase action towards compounds **2(a–d)**. Fig. 2 shows a quantitative comparison of tyrosinase action on the four different substrates.

It is worth noting that sulfonyl moieties in position 1 should render the corresponding quinoneimines particularly reactive as electrophiles due to the strong electron-withdrawing effect of these substituents. Hence, the formation rates for the corresponding phenoxazinone derivatives should be very high, with absorption maximum centred around 440 nm (at pH 7) [16,25]. In fact, recent studies demonstrated that the laccase-mediated oxidation of *o*-aminophenols brings to the formation of phenoxazinone derivatives [25,33]. Here, mushroom tyrosinase allowed to obtain the same kind of oxidised products. Indeed, Fig. 3 shows the mass spectrum (positive electrospray ionisation mode) of the main products obtained by the oxidation of compound **2b**.

The peaks at 372 and 373 *m/z* have quite high abundances and are compatible with phenoxazinone derivatives. This is also confirmed by the intense peaks at 356 and 357 *m/z*, due to the formation of stable ions likely obtained by the loss of a NH_2 group (16 *m/z*), and by those at 340 and 341 due to the loss of a further NH_2 group. As the four phenoxazinone derivatives have similar structures, the respective ε values should also be similar, allowing for quantitative comparison. In

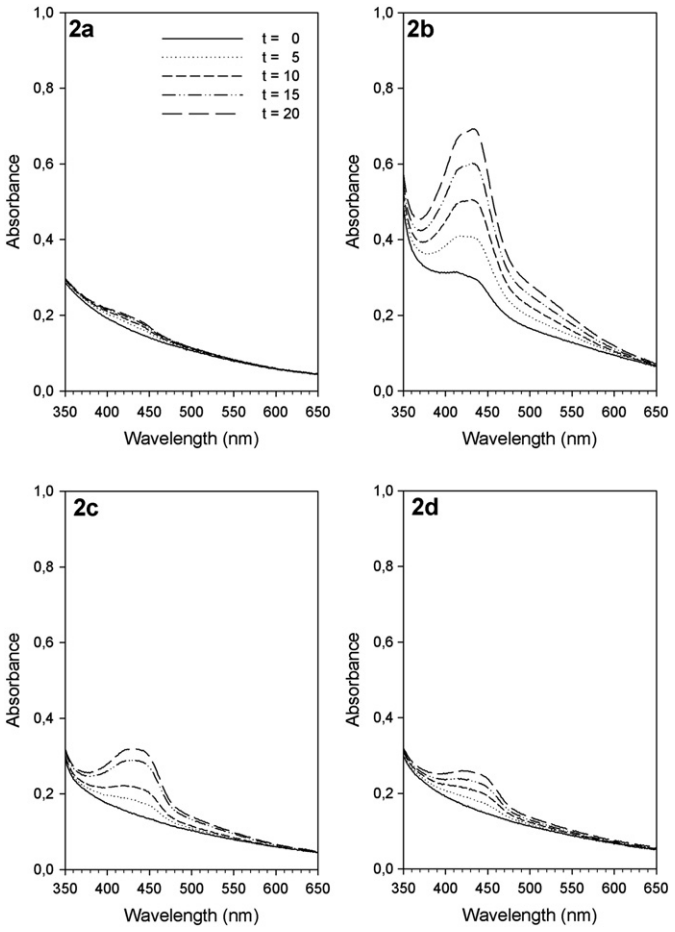


Fig. 1. Absorption spectra of aminophenolic compounds **2(a–d)** with tyrosinase. Reaction media contained the aminophenolic compound (200 μM), tyrosinase (130 U), and potassium phosphate buffer (100 mM) in a final volume of 1 mL. Scans were taken every 5 min over a total time of 20 min. In all cases, possible autoxidation was ruled out before scanning.

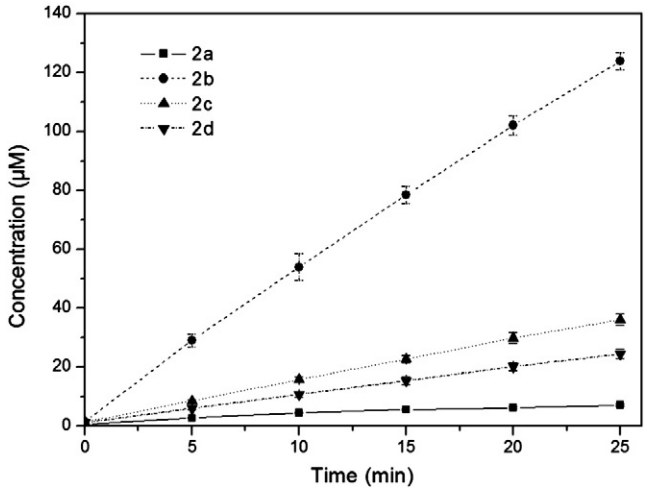


Fig. 2. Quantitative comparison of compounds **2(a–d)** as tyrosinase substrates. Activity was given as μMol phenoxazinone derivatives versus reaction time. All other reaction conditions as described in Fig. 1.

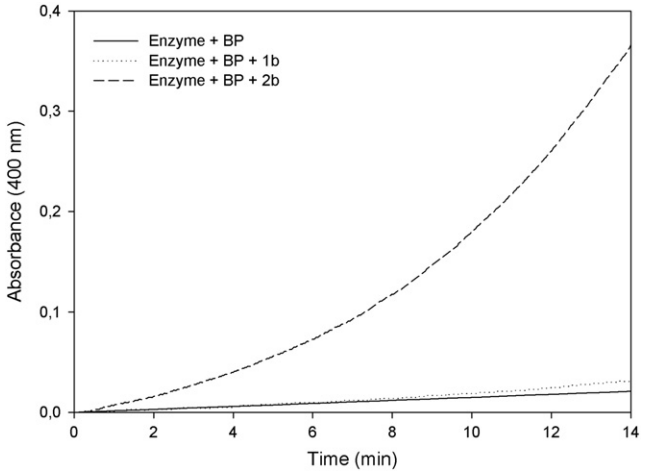


Fig. 4. Spectrophotometric measurement at 400 nm of the effect of compounds **1b** and **2b** (200 μM) on monophenolase activity of tyrosinase in the presence of 500 μM *tert*-butylphenol (BP) as substrate. The reaction media contained enzyme (130 units) and potassium phosphate buffer (100 mM) in a final volume of 1 mL.

fact, these ε values ranged from 3950 (**2b**) and 5100 (**2a**) M^{-1} cm^{-1} [24,34]. The corresponding K_M values are 17.31, 0.17, 0.81, and 1.76 mM for the four compounds **2(a–d)** respectively. The k_{cat} are 0.6, 12.5, 3.6, and 3.4 s^{-1} respectively. These latter values show that compounds **2(a–d)** are on the whole very poor substrates in comparison to *o*-diphenols.

Compounds **1(a–d)** were essentially inert as tyrosinase substrates as confirmed by experiments aimed at more thoroughly understanding their action on monophenolase activity. Therefore, their ability to influence the lag time shown by the enzyme when acting on monophenols was tested.

Compound **2b** was chosen as a representative of the 2-HMA series because it is the best substrate and was compared to its analogue, compound **1b** (which was inactive as a substrate).

Fig. 4 shows that **2b** exerted a marked effect on *tert*-butylphenol oxidation whereas **1b** was almost ineffective.

Compound **1b** was also tested for effects, particularly inhibitory ones, on the catecholase activity of the enzyme. In this case, to minimise cross-reactions, *tert*-butylcatechol was chosen as the substrate, and the production of 4-*tert*-butyl-*o*-benzoquinone was monitored as described above. Inspection of Fig. 5 shows that 200 μM **1b** did not have any inhibitory effect on the speed of oxidation of *tert*-butylcatechol. In the presence of **2b**, however, a green adduct quickly formed. A deeper investigation of the reaction has shown that the blue adduct would arise anyway, but it non-enzymatically reacted with **2b** leading to the observed green compound.

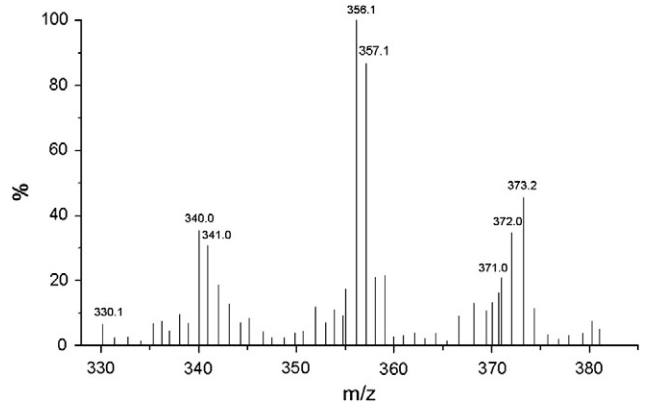


Fig. 3. Mass spectrum (positive electrospray ionisation mode) of the reaction mixture obtained by the tyrosinase mediated oxidation of compound **2b**.

4. Discussion

In general, aromatic amines, *o*-aminophenols, and even *o*-diamines enter and interact with tyrosinase active site and behave as enzyme substrates; amines are hydroxylated to the corresponding *o*-aminophenols, and *o*-aminophenols are oxidised to the corresponding *o*-quinoneimines [15,18]. A very similar affinity pattern is also observed when comparing *o*-diamines, *o*-aminophenols, and *o*-diphenols. Very different effects are observed when it comes to reaction rates: substituting nitrogen for oxygen (e.g., converting monophenols to monoamines, diphenols to aminophenols, or aminophenols to diamines) causes a dramatic drop in reaction speed. This has been ascribed to the need for phenol deprotonation [18] as a *condicio sine qua non* for reaction; while such a deprotonation is favourable for phenols (pKa ≈ 10), it is quite unfavourable for aromatic amines (pKa > 25). Also, the significant influence of *meta* and *para* substitutions in *o*-aminophenols on tyrosinase activity has been stressed, in particular for some amino-hydroxybenzoic acids [15].

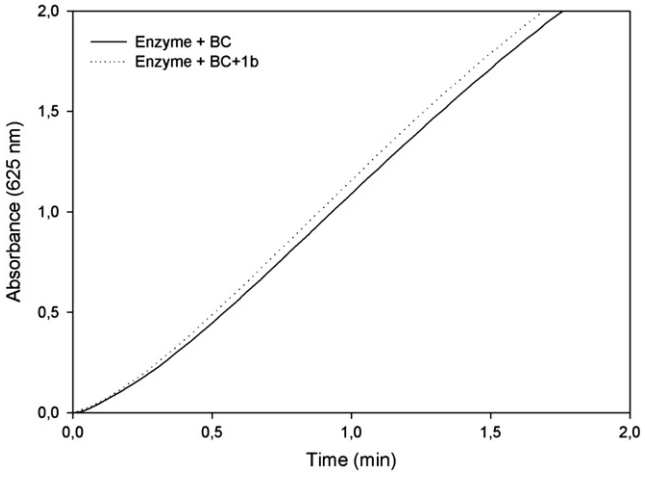
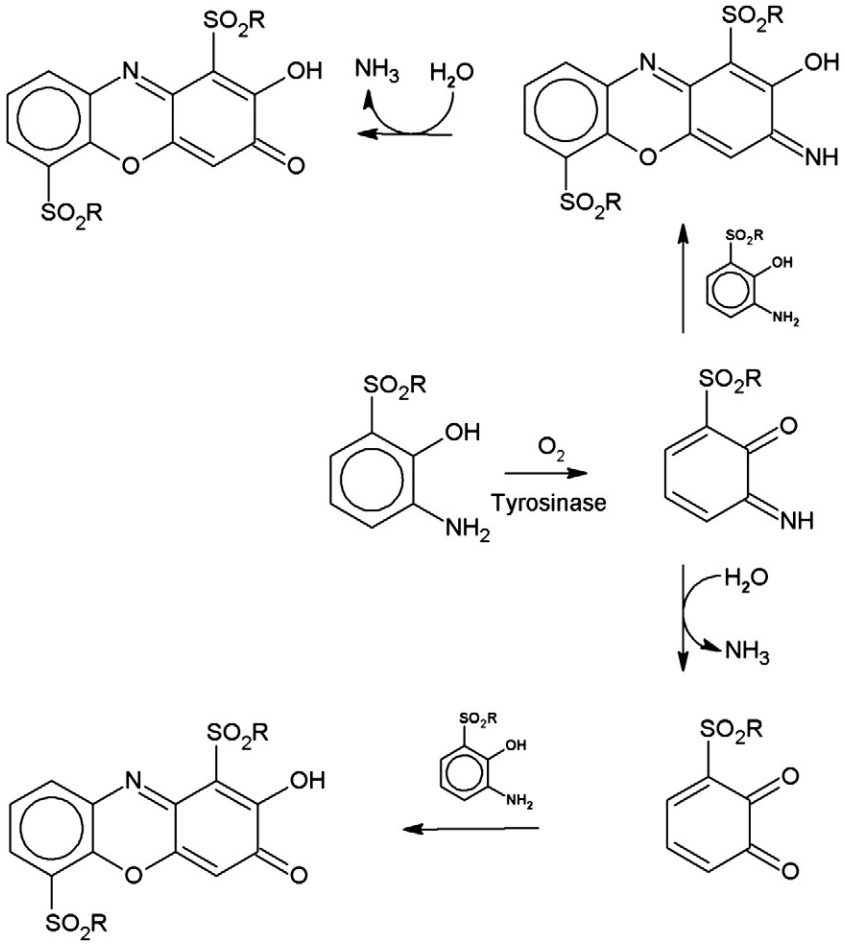


Fig. 5. Spectrophotometric measurement at 625 nm of the effect of compound **1b** (200 μM) on catecholase activity of tyrosinase in the presence of 5 mM *tert*-butylcatechol (BC) as substrate. The reaction medium contained enzyme (13 U) and potassium phosphate buffer (100 mM) in a final volume of 1 mL.

Previous interest in the transformation of *o*-aminophenols by tyrosinase [19] and the fact that some synthetic *vic*-aminohydroxybenzenesulfonic derivatives have recently become available [24,25] prompted this study on possible interactions between these compounds and mushroom tyrosinase. Two series of such molecules are available, deriving from 3-hydroxy-orthanilic acid (2-amino-3-hydroxy-benzenesulfonic acid, 3-HOA) and 2-hydroxy-metanilic acid (3-amino-2-hydroxy-benzenesulfonic acid, 2-HMA). The structures of the studied substances are reported in Table 1. It is worth noting that **1a** is the sulphur-containing analogue of 3-hydroxyanthranilic acid (3-HAA), which is well known as both a substrate for and an activator of mushroom tyrosinase [16]. We were surprised, therefore, to find that **1a** was not a substrate for the enzyme and that an almost complete lack of activity was also found for its derivatives **1(b–d)**. Whatever the case, the possible lack of activity towards phenols, catechols, and *o*-aminophenols bearing electron-withdrawing substituents does not exclude the possibility that these compounds could interact with the dicupric cluster at the active site of tyrosinase [33–35]. In such a case, competitive inhibition of the enzyme would be observed, as in the case of 4-nitrophenol [40]. In contrast, neither the monophenolase nor the diphenolase activity of tyrosinase is influenced by compounds **1(a–d)**; in particular, no shortening of the lag time was observed. The following important conclusion can therefore be drawn: compounds **1(a–d)** likely cannot enter the active site. 3-HAA has been previously recognised as both a substrate and as an activator for mushroom tyrosinase [16]; so why can it enter the active site while its sulphur analogue **1a** cannot? The major difference between the two compounds is that the tetrahedral geometry induced by the presence of sulphur could form an obstacle that prevents the compound from entering the active site. In contrast, the carboxyl moiety of 3-HAA can easily become coplanar with the aromatic ring, allowing 3-HAA to gain access to the enzyme cleft containing the dicopper cluster. This consideration cannot be split from an insight on the proper interaction mode between the dicopper cluster and the *o*-aminophenol functionality of the studied compounds. As shown below, Cu_A and Cu_B are **not** equivalent in their ability to bind with phenol or amine moieties (*vide infra*).

Testing compounds **2(a–d)** as tyrosinase substrates proved that the sulfonyl substituents are not capable of preventing the interaction with tyrosinase *per se*; in fact, all of these compounds were converted to the corresponding quinoneimines and in turn to phenoxazinone derivatives according to the reaction depicted in Scheme 3. Most of the expected products have been isolated and unambiguously characterised by means of NMR, MS, UV, HPLC techniques [25]. More recently, other aminophenols have been studied and their attitude to produce phenoxazinones upon enzymatic oxidation has been assessed and exploited [34].

The affinities of the four substrates for the active site and their oxidation speeds have been compared. Inspection of K_M values (see Results) shows that affinities increase in the order **2a**≪**2d**≤**2c**<**2b**. Somewhat surprisingly, **2a**—which bears the smallest substituent—shows the lowest affinity, whereas **2d** and **2c** are much more efficiently recognised by the enzyme in spite of their bulky alkylsulfonamide substituents; **2b** is the most favoured. This behaviour is clearly related to the fact that only **2a** bears a net negative charge which is independent on pH (because —SO_3H corresponds to a strong acid). Therefore, an electrostatic repulsion between **2a** and an



Scheme 3. The biotransformation of compounds **2(a–d)** mediated by tyrosinase. Spontaneous hydrolysis of the iminogroup (before or after condensation) leads to 2-hydroxyphenoxazin-3-one (the product detected by HPLC-MS).

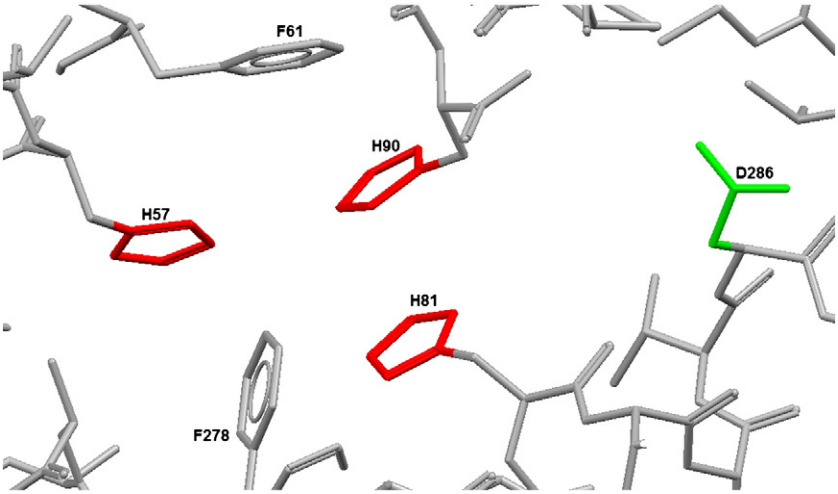


Fig. 6. Modelling of *Agaricus bisporus* tyrosinase CuA domain. The figure illustrates tyrosinase CuA domain with the three copper-binding histidine residue (H57, H81, H90) showed in red. The D 286 residue is showed in green. The theoretical 3D structure of the CuA domain from *A. bisporus* was calculated with the homology-modelling programme ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/), using as the template the structure of *Vitis vinifera* polyphenol oxidase (PDB code 2P3XA).

anionic residue at the active site is most probably responsible for the comparatively low affinity.

A conserved aspartate residue (D 286) in the vicinity of $\mathbf{Cu_A}$ might exert this electrostatic repulsion (Fig. 6). The distances between the carboxylate moiety and the nitrogen atoms (of the three histidines) engaged in the bonding to $\mathbf{Cu_A}$ have been estimated to fall between 5 and 10 Å.

This residue is close enough to the substrate cleft to exert its electrostatic repulsion towards the sulfonate anion, thus substantially lowering the affinity for the substrate. On the other hand, it is far enough away from the active site that it does not completely forbid **2a** access to the cleft. The finding also helps to exclude the residue as the acceptor of the proton derived from the dissociation of the phenolic hydroxyl from the substrate [35,36]. With respect to the E 247 residue, the computational model used shows that it is too far away to exert a noticeable electrostatic effect; moreover, it is in an orientation opposite to that of the active site. Consequently, the fact that **2b** is the best substrate is not surprising if one considers that its unsubstituted sulfonamide group is both small and uncharged.

For the sake of completeness, 3-amino-2-hydroxybenzoic acid (3-aminosalicylic acid) was also tested as a tyrosinase substrate. It was a somewhat less efficient substrate than the isomeric 3-HAA, most likely because its —OH group is part of a *quasi*-aromatic ring with the adjacent carboxylate ion, as is well documented for *o*-hydroxybenzoic acids.

Comparison of the behaviour of the two sets **1(a–d)** and **2(a–d)**, together with the information available about the structures of related proteins containing the same type of dicopper cluster (haemocyanins, catechol oxidases, and—recently—tyrosinases [2,37,38]), could suggest some hypotheses about the structure of tyrosinase's active site and its chemistry throughout the catalytic cycle. Although similar in their coordinative spheres, the two cupric ions do not necessarily behave identically. It is still debatable whether a mono- or di-phenolic substrate must bind to $\mathbf{Cu_A}$ or to $\mathbf{Cu_B}$, even if a growing consent on $\mathbf{Cu_B}$ as the primary binding site exists [2,3,7,37,39–42]. Recently, structural studies on some tyrosinases [2] have shown that $\mathbf{Cu_A}$ is more sterically restricted than $\mathbf{Cu_B}$. Although it has been suggested that $\mathbf{Cu_B}$ would be the primary site for monophenol interaction with the enzyme prior to its *o*-hydroxylation by the oxytyrosinase, the exact bonding mode of *o*-diphenols remains to be ascertained. Of particular interest is the question of whether these molecules form a mono-cupric (most probably, with $\mathbf{Cu_B}$) or a dicupric catechol/enzyme adduct. It is worth noting that both *met* and *oxy* forms can perform the two-electron redox reaction leading from catechols to their *o*-quinone counterparts, albeit with different reaction rates [3], and this piece of evidence would lend some support to the idea that an adduct involving both the copper ions together with the two phenolic hydroxyls is formed, at least for the *oxy* form [43].

With respect to the *oxy* form, the hypothetical substrate adduct with one phenolic oxygen bridging the two copper ions must be indeed ruled out. Obviously, no peroxide bridge is present in the *met* form, where the redox reaction results in reduction of the two cupric ions to their cuprous, *deoxy* form. Therefore, such an adduct is most likely formed when *met* tyrosinase acts on *o*-diphenols, as suggested by growing experimental and computational evidence [34,38].

With concern to *o*-aminophenols, the amino group—owing to its electronic structure—simply cannot act as a chemical bridge between the two copper ions, not even in the *met* form. This is another indirect proof that the enzyme recognises firstly the amino group of *o*-aminophenols, and later the *o*-phenoxide ion could exert its bridging role in the *met* form of the enzyme (Fig. 7).

Experimental evidence also shows that the same reaction pathway observed for catechols must take place for *o*-aminophenols; this invariably generates the expected *o*-quinoneimines as (transient) oxidation products. It is also worth noting that—at least for *Neurospora* tyrosinase—amines show affinities for the enzyme that are comparable to or even higher than those of phenols. Apart from the substantially lower k_{cat} values [18], the most obvious difference between *vic*-substituted *o*-aminophenols and catechols is the presence of the amino

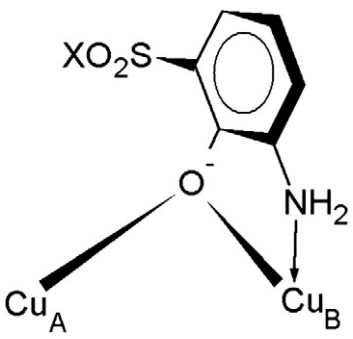


Fig. 7. The proposed mode of interaction of the compounds **2(a–d)** with the dicupric cluster at the active site of *met*-tyrosinase, showing the specific bond of the amino group with $\mathbf{Cu_B}$, and the phenolate ion bridging the two cupric ions.

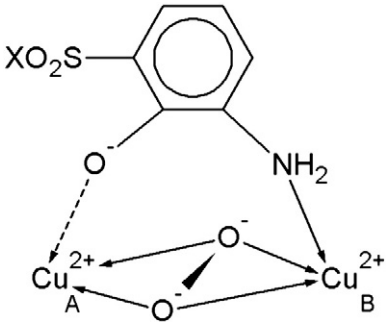


Fig. 8. The proposed mode of interaction of the compounds **2(a–d)** with the dicupric cluster at the active site of *oxy*-tyrosinase, showing the specific bond of the amino group with $\mathbf{Cu_B}$, and the hypothetical linkage between the phenolate ion and $\mathbf{Cu_A}$.

group, which allows two *vic*-isomers to exist, as in the case of the two series **1(a–d)** and **2(a–d)**. An important conclusion of our study is therefore that although it is wide, the substrate-binding cleft of tyrosinase is capable of clearly discriminating between the two isomeric series. Even if the data presented here are not enough to unequivocally decide which of the two cupric ions is the primary site for substrate binding in tyrosinase, they are quite compatible with the hypothesis of a preferential interaction between the amino groups of the compounds **2(a–d)** and the $\mathbf{Cu_B}$, as reported above.

Such an interaction could allow another linkage, at least in the case of the *oxy* form of the enzyme, that of the phenoxide with $\mathbf{Cu_A}$ (Fig. 8).

In conclusion, the mode of interaction proposed here (—NH_2 with $\mathbf{Cu_B}$, —O^- as a bridging ligand between the two cupric ions, or perhaps with $\mathbf{Cu_A}$ in the *oxy* form of the enzyme) is compatible with the observed affinities. It also takes into account the fact that the major obstacle blocking the approach of substrates **2(a–d)** to the dicupric cluster depends on the substituent *ortho* to the hydroxyl. This obstacle is not principally caused by steric hindrance but by an electrostatic repulsion due to the vicinity of the D 286 residue. As a matter of fact, the most active substrate, compound **2b**, features a very small sulfonylated substituent and is not (negatively) charged under the testing conditions. Therefore, a substrate interaction with the dicopper cluster, in the manner depicted here, that causes leaning of the sulfonyl substituents toward $\mathbf{Cu_A}$, is in full agreement with the electrostatic effect hypothesised for the substrate series **2(a–d)**. On the other hand, this hypothesis explains why the compounds belonging to the series **1(a–d)** behave as nonsubstrates: this is due to the presence of the "axial" histidine 260 hindering the approach of the sulfonyl substituents to the $\mathbf{Cu_B}$.

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