Molecular Catalysis

Considerations about the kinetic mechanism of tyrosinase in its action on monophenols: a review

--Manuscript Draft--

Manuscript Number:	MOLCAA-D-21-01244R1
Article Type:	Review Paper
Section/Category:	Enzymatic Catalysis
Keywords:	tyrosinase; polyphenol oxidase; monophenols; kinetic mechanism L-tyrosine.
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Abstract:	The mechanism of action of tyrosinase on monophenols is complex, several processes overlap in time, such as the hydroxylation of monophenols to o- diphenols, the oxidation of these to o- quinones and the evolution of the latter towards melanin. The enzyme's mechanism of action is unique but depending on the chemical nature of the substrate it may show different exceptions. In this review we want to dissect the kinetic mechanism for the action of the enzyme on: a) Its physiological substrate L-tyrosine and related compounds, whose o- quinones in their chemical evolution accumulate o- diphenol in the medium (Type A). b) Substrates that cannot accumulate o- diphenol in the medium because it is easily oxidized and they need the presence of hydrogen peroxide for the enzyme to show activity, such as hydroquinone and related compounds (Type B). c) Substrates that release o- diphenol into the medium and the enzyme oxidizes it generating a stable o- quinone and therefore does not generate more o- diphenol in the medium, as is the case of 4-tert-butylphenol and related compounds (Type C). d) Substrates that do not release or generate o- diphenol in the medium, as is the case with deoxyarbutin, which produces a stable o- quinone (Type D). The different mechanisms that explain the enzymatic activity are proposed, a kinetic analysis is established for each mechanism and by means of numerical integration results are obtained that are discussed and compared with experimental data. To help and support the results and discussion, molecular docking for substrates (L-tyrosine, hydroquinone, 4-tert-butylphenol, and deoxyarbutin) to both the oxy and met forms of tyrosinase was carried out.

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Newcastle upon Tyne, 20th November 2021

Prof Frank Hollmann,
Delft University of Technology, Delft, Netherlands

Dear Prof. Hollman,

Please find enclosed the revised version of the review manuscript entitled "*Considerations about the kinetic mechanism of tyrosinase in its action on monophenols: a review*" (reference MOLCAA_S_21_01480). We would like to submit it for consideration and possible publication as a review article in "*Molecular Catalysis*".

We have addressed all Reviewers' suggestions and change them into the revised version. Also, we attached the answers, point-by-point, to them.

Thank you, in advance, for your attention and courtesy. I look forward to hearing from you.

Sincerely yours

Dr. Jose Luis Muñoz-Muñoz

Reviewer #1

We want to acknowledge Reviewer 2's comments about our work, which encourages us to continue our work on the enzyme tyrosinase. Next, we go on to answer to each of the clarifications that he/she suggest. We believe that all his/her comments contribute to increase the quality of the work.

In the present manuscript you bring together the results from decades of work on the kinetic foundations of the enzymatic hydroxylation and oxidation of monophenols (and, as an aside, also of diphenols) by tyrosinase. The classification according to whether or not the action of the enzyme on a given substrate is accompanied by the accumulation of diphenol in the reaction media is useful, especially with respect to the discrimination between alternative substrates and true inhibitors. The writing style is clear and easy to follow, although one must study the abbreviation list before venturing on with the manuscript.

I request a small number of clarifications.

1) **Considering the presentation of the docking poses: It is quite unusual not to display at least all six copper-coordinating histidines. Besides these details there is one bigger point: The absence of any mention of suicide inactivation. This is a topic which imposes itself for some of the substrates you mention in the manuscript and as the list of authors of the present manuscript brings together (most of?) the creators of the "Murcia model" of tyrosinase suicide inactivation, there is ample expertise for a description of this phenomenon in this review. In this context a comparison to the Quintox model (authored primarily by Ramsden, Land, Stratford and Riley) should not be omitted, even if the predictions are quite similar from a practical point of view. I believe this addition to be necessary for the review to be useful for the high number of researchers who use tyrosinase but have not read (or are even aware of) the many specialized reports concerning the pitfalls to be avoided when characterizing one tyrosinase kinetically or also just using its activity to drive an application.**

As suggested by the Reviewer, the docking poses now display the six copper-coordinating histidines.

Following the suggestions of the Reviewer, a section regarding the suicidal inactivation of the enzyme has been introduced (*3.5. Influence of monophenols on the kinetics of suicide inactivation of tyrosinase*) and Figure 13 has also been added to make this process clearer.

Specific comments:

2) Abstract:

There is evidence for L-tyrosine as the physiological substrate only for mammalian tyrosinase. Most of your work is based on mushroom tyrosinase for which the natural substrate is not known.

We totally agree with the Reviewer since the work has been done with tyrosinase from *Agaricus bisporus*. We have removed the word physiological throughout the manuscript but we have kept it in the abstract saying that it is the physiological substrate of mammalian tyrosinase. In addition, L-tyrosine leads to the formation of *o*-dopaquinone, which regenerates *o*-diphenol in the medium.

3) Highlights:

3rd point: In formal texts colloquial abbreviations are frowned upon. => doesn't -> does not

Following the directions of the Reviewer, “doesn’t” has been changed to does not in the 3rd point and in the ghapical abstract.

4)Abbreviations:

(Page 3) EmHQ, met-tyrosinase/hidroquinone complex -> its "hydroquinone"

K1TBF, Constante de disociacion del complejo EmTBF -> dissociation constant of the complex EmTBF

michaelis constant for (O2), in the presence of TBF: Names of persons should be capitalised. -> Michaelis constant.

3-HBA, 3-hydroxy alcohol benzylic: its "3-hydroxybenzyl alcohol".

All changes have been addressed as suggested by the Reviewer.

5) 1. Introduction:

(Page 5) Besides the forms found in the catalytic cycle there is also the enzymatically inactive form where at least one copper has been lost.

Following Reviewers' suggestions, the inactive form with Cu^0Cu^{+2} has been added.

(Page 5) Why did you formulate the "four kinetic approaches" as hypothetical statements?

Four groups have been made because all the substrates with which we have worked are included in four groups (A, B, C and D).

6) Scheme 1:

"QH" is not in the list of abbreviations.

QH has been added to the list of abbreviations.

Also, what is the difference between Q (o-dopaquinone) and putatively its protonated form "QH". The species that are released from the enzyme's active centre are Q and water, leading me to the conclusion that QH = Q.

The difference between Q and QH is in the protonation of the amino group. In QH the amino group is as NH_3^+ and in Q, this group is as NH_2. Therefore, they are different molecules.

In addition, there is no release of a single proton in the reaction of 2 x o-dopaquinone -> L-Dopa + L-Dopachrome. The amount of hydrogen atoms is the same on both sides of the reaction and so is the charge. The tyrosinase reaction does not acidify its reaction medium and neither does the chemical evolution of o-quinones.

To avoid these potential contradictions from pestering the reader please add the structures of the involved species for both Scheme 1 and Scheme 2.

Following the indications of the Reviewer, two new figures have been added corresponding to Scheme 1 and 2 in their structural form. In Figure 1B, we can see the release of a proton from the NH_3^+ group caused by the cyclization of *o*-quinone to leucodopachrome. In addition, another *o*-quinone molecule oxidizes leucodopachrome, leading to stoichiometry: $2QH \rightarrow D+DC+H^+$.

7) Scheme 2:

tyra, L-Mme y sin [8,10-13]: Please use english conjunctions.

We have change them to normal conjuctions.

8) 2.3 Computational docking:

(Page 7) PDB entry 2Y9W depicts isoform 3 of the A. bisporus tyrosinase.

It has been included “isoform 3” in the description of the PDB entry 2Y9W (page 8).

9) 3. Action of tyrosinase on different types of monophenols: (Page 8) The first premise is formulated too strict: As long as the starting population of Eox is not zero your model will work. In practice you will almost never encounter a preparation with exactly 30% Eox. The release of D from the active site (premise 3) is not strictly required as long as the evolution of the oxidised substrate does produce D.

We agree with the Reviewer with the first premise that may be there is a certain percent Eox in the native enzyme, not necessarily 30%.

On the other hand, regarding premise 3, what the Reviewer says has been highlighted: “evolution of the oxidised substrate does produce D”.

10) (Page 9) "The V283 residue is close to the aromatic ring …": Please mention the corresponding enzyme. You choose tyrosinase 3 from A. bisporus, but as your model should apply to any tyrosinase this may not be obvious to the reader.

Also, consider adding something like "of the substrate" to specify which 'aromatic ring' you refer to.

(Page 9) "… almost parallel to the imidazole ring of the H263 residue…": You should mention that this is one of the three histidines forming the CuB site.

The suggestions proposed by the Reviewer have been included in page 10.

11) 3.1. The physiological substrate L-tyrosine and related compounds. Substrates Type A:

(Page 10) "The docking from M to the Eox form is shown in Fig. 3, …": There is some evidence that prior to o-hydroxylation the incoming monophenol needs to be deprotonated. e.g. References [1-3].

The suggestions proposed by the Reviewer have been added.

12) 3.2.1. Enzymatic action of tyrosinase on hydroquinone. Substrates Type B: (Page 11) "… an attempt has been made to replace D-Arb [42] and more recently thiamydol (isobutyl amido thiazolyl resorcinol), which is a stronger inhibitor …": There was no incentive to replace D-Arb, but rather to replace HQ with D-Arb. The somewhat new inhibitor for the human tyrosinase is called "Thiamidol".

We agree with the Reviewer that deoxyarbutin replaces HQ. Thiamidol has also been ammended. This compound has been described as a potent inhibitor of human tyrosinase.

13) (Page 11) "… showing that the thiamydol preparation achieves better results [44].": This study reports no significant difference between the two tested treatment regimes.

We agree with the Reviewer that the two treatments offer similar results and have changed them in the manuscript.

14) (Page 12) "HQ has been used since ancient times as a depigmenting agent, interpreting its mechanism of action as reducing o-Q.": That may be a bit of an overstatement since HQ was discovered in 1844. It is, however, present in propolis which has been used, among many other medical applications, as a "skin care product" for quite some time, e.g. in ancient Egypt for the preparation of mummies.

This paragraph has been rewritten following the directions of the Reviewer.

15) (Page 12) "The simplest mechanism that can be proposed to explain the action of tyrosinase on HQ is:": How did you ascertain that this is the simplest possible mechanism? Related to that, what is your criteria for simplicity of a reaction mechanism?

This paragraph has been corrected following the directions of the Reviewer.

16) (Page 12) "…HPB, until all enzyme is found as Em which is inactive on HQ.": Is it possible to determine the share of Eox in E0 of a tyrosinase preparation using the reaction on HQ in the presence of a catalase?

In our opinion, the action of tyrosinase on HQ in the absence of hydrogen peroxide could not be used to assess the amount of Eox that exists in the enzyme preparation, since the product formation is very small and the molar absorptivity coefficient of HPB is very low too.

17) 3.2.2. Action of tyrosinase on 4-tert-butylphenol. Substrates Type C: (Page 14) "…K1TBF ≅ 20 μM and kcatapp = 4.56 ± 0.2 s−1 [38].": Please use english conjunctions.

The correction has been made.

18) Scheme 6:

peroxyde -> peroxide

Peroxyde has been changed to peroxide.

19) 3.2.3. Action of tyrosinase on deoxyarbutin. Substrates Type D: (Page 18) "The calculated dissociation equilibrium constants from the docking conformations (Kd) agree quite well with our experimental values for the KM values (Tables 1 and 2).": Please add a column with the values of the binding energies used to calculate the dissociation constants to Table 1. For easier comparison it would be more convenient if both tables (1 and 2) were using the same order of substrates.

With the exception of L-dopa there is more than an order of magnitude between the corresponding theoretical dissociation constants and measured Km values.

As you use Km in the rest of the manuscript: KM -> Km.

A column with the values of the free energies of binding has been included in Table 1. In Table 2 the order of the substrates has been changed to match Table 1.

The Reviewer is correct in stating that the values of theoretical dissociation constants are greater than the values of Km determined experimentally. We have

modified this aspect indicating that the values, for the oxy form, follow the same order, that is: D > HQ ≈ M > D-Arb ≈ 4-TBF.
KM has been modified by Km both at work and in the Supporting Information.

20) Table 1:

You should probably mention that the reported distances are in Ångström.

It is now been included in the header of Table 1.

21) Table 2:

At which temperature were these parameters determined?

The experiments were made at 25ºC.

22) Equation S14A:

What is K1 (equilibrium constant)? I believe it should be k1 (rate constant). Please correct this also in all the following equations.

According to Scheme 2, K1 is the dissociation constant of the EmM complex, which is an inactive form, and, in equation S14A, it is indicated that the catalytic constant is apparent.

23) SUPPORTING INFORMATION: B

It would be more convenient if these definitions were integrated in SI A, immediately before the respective equations that the defined symbols are used in.

Following the Reviewer's suggestions, the analytical expressions of the parameters of the different rate equations have been taken to Supporting Information A, immediately before the respective equations.

Reviewer #2:

We want to acknowledge Reviewer 2's comments about our work. All suggested changes and corrections contributed to the improvement of the Review. We will now answer to each of his/her comments:

General. This is an interesting and well-written review about the kinetic mechanism of tyrosinase. The reactivity with different monophenols is well explained and supported by accurate developed kinetic schemes. Docking is used to indicate critical distances between ligands and copper ions in the active site. The paper is a useful work of reference for the biocatalysis community.

Suggestions for textual improvements:

P3 (Abbreviations):
hidroquinone → hydroquinone
hydroxyhidroquinone → hydroxyhydroquinone
constante .... → dissociation constant EmTBF complex
michaelis constant → Michaelis constant
sinephrine → synephrine
etoxyphenol → ethoxyphenol
3-hydroxyalcohol benzylic → 3-hydroxybenzyl alcohol
L-α-methyl tyrosine → L-α-methyltyrosine
All abbreviations have been changed to Reviewer's suggestions.

1) P5: compounds (substrates) need to be written in full when mentioned for the first time.

We added full name when mentioned for the first time.

2) P6: L-Mme and sin

The correction indicated by the Reviewer has been made.

3) P11: remove second (isobutyl amido thiazolyl resorcinol)

The correction indicated by the Reviewer has been made.

4) P14: replace similar to HQ such as to: similar to HQ are:

The correction indicated by the Reviewer has been made.

5) P16: the use of D-Arb up to 3%...makes this concentration dangerous

The correction indicated by the Reviewer has been made.

6) P17: to be hydroxylated at the ortho carbon atom

The change indicated by the Reviewer has been made.

7) P17: are oriented with both hydroxyl groups toward the oxo group

The changes indicated by the Reviewer have been made.

8) P17/18: This conformation of D facilitates oxidation of the o-diphenol to the o-quinone (diphenolase activity). However, in the met form.....bringing the hydroxyl groups close to....(Fig. S2AC).

All the indications of the Reviewer have been carried out.

9) P18: This influence can only be considered for the monophenols....

The change indicated by the Reviewer has been made.

10) P18: The concentration of o-diphenol....is very low.

The changes indicated by the Reviewer have been made.

11) P19: As described for the different mechanisms...

The change indicated by the Reviewer has been made.

12) P19: from the fungus Neurospora crassa

It has been modified as indicated by the Reviewer.

13) P19: the Michaelis constants for molecular oxygen in the presence of monophenols should be very low.

The change indicated by the Reviewer has been made.

14) P20: the Michaelis constants for molecular oxygen are slightly higher

The changes indicated by the Reviewer have been made.

15) P21: trigger the catalytic reaction on these diphenols.

It has been changed by Em to Ed and the latter with oxygen to Eox, which can act on monophenols.

16) P22: J.M.M. received funding from....

We have added the funding received by J.M.M. in the acknowledgement section.

17) P33: legend Table 1: from selected ligand groups....The calculated dissociation constants of the enzyme-ligand complexes in the oxy- and met forms are also indicated.

Changes have been made in Table 1.

18) P34: legend table 2: Kinetic parameters characterizing.....Hydroquinone.....a) Considering the kinetic parameters for the reaction of Ed with O2.

Corrections have been made in Table 2.

19) P35: methoxyphenol, ethoxyphenol

The change indicated by the Reviewer has been made.

20) Fig. 1: Chemical structures of the aromatic compounds....

This change has been added to the figure caption, which is now figure 3.

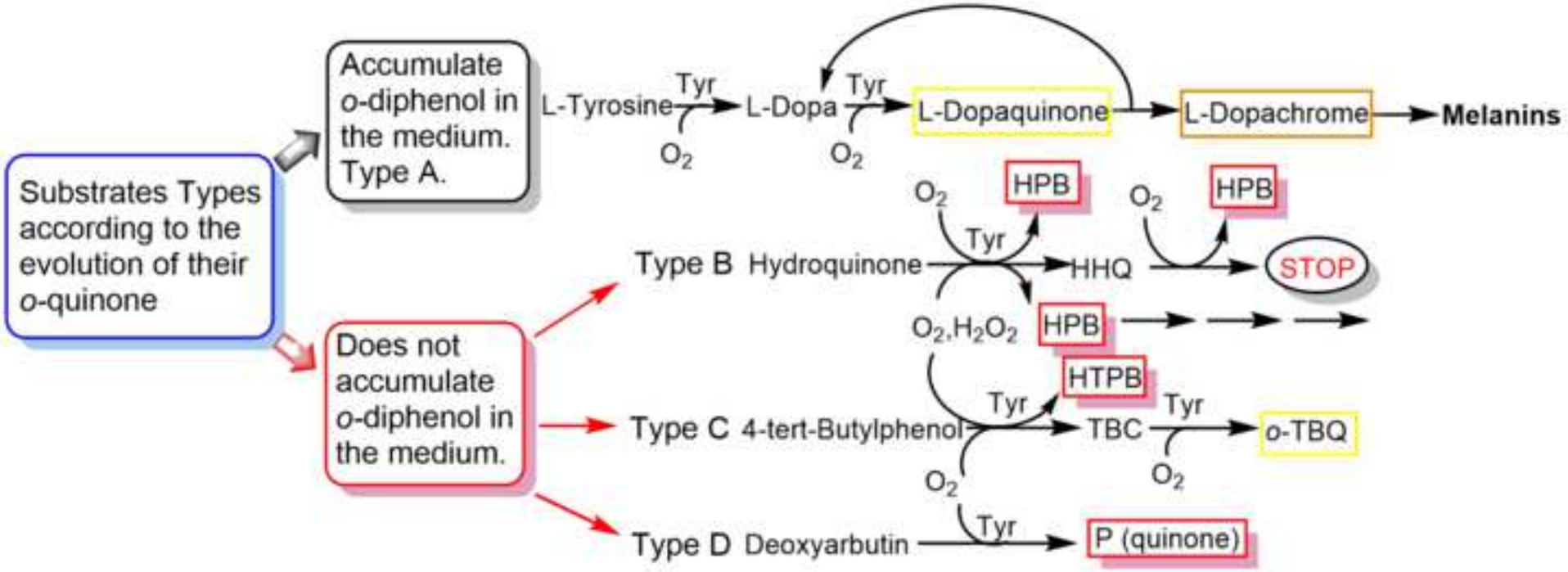
21) Table 2 and Figs. 1,2,4,6,8 and 9: indicate reaction temperatura

Table 2 indicates the temperature of 25ºC.

22) For each simulation refer to the equation of the corresponding Scheme.

Following the Reviewer's suggestions, the Scheme and the corresponding equation have been indicated in each simulation.

Graphical Abstract



Highlights

- Tyrosinase can hydroxylate a broad range of monophenols.
- In Type A substrates, the *o*-quinone evolution generates *o*-diphenol in the medium.
- In Types B, C and D substrates, the evolution doesn't accumulate *o*-diphenol.
- Hydrogen peroxide transforms Em to Eox and the enzyme has hydroxylating activity.
- In presence of monophenols, Michaelis constant for oxygen is very low.

SUPPORTING INFORMATION
CONSIDERATIONS ABOUT THE KINETIC MECHANISM OF TYROSINASE IN ITS ACTION ON MONOPHENOLS: A REVIEW

Pablo García-Molina, Francisco García-Molina, Jose Antonio Teruel-Puche, José Neptuno Rodríguez-López, Francisco García-Cánovas, José Luis Muñoz-Muñoz

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1. Tyrosinase action on different substrates.

1.1. Action of tyrosinase on L-tyrosine. Substrates type A.

Kinetic analysis.

The steady-state rate equation for diphenolase activity of tyrosinase.

The kinetic mechanism for this activity is shown in Scheme 1. The steady-state speed must comply with (the disappearance speed of *o*-diphenol (D) ($-V_{ss}^{D}$), must be equal to the rate of accumulation of dopachrome (DC) ($V_{ss}^{D,DC}$), and equal to the rate of oxygen disappearance ($-V_{ss}^{D,O_2}$). Analytical expressions for α_1 and β_0 - β_3 are a function of the rate constants implicated in the mechanism and they are obtained applying the programs described in [1], being:

$\alpha_1 = 2k_2k_3k_8k_6k_7$

$\beta_0 = k_2k_3k_{-8}(k_7 + k_{-6})$

$\beta_1 = k_2k_3k_6k_7$

$\beta_2 = k_8k_6k_7(k_{-2} + k_3) + k_2k_3k_8(k_7 + k_{-6})$

$\beta_3 = k_2k_8k_6(k_7 + k_3)$

$$-V_{ss}^{D,D} = V_{ss}^{D,DC} = -V_{ss}^{D,O_2} = \frac{\alpha_1[O_2]_0[D]_0[E]_0}{2(\beta_0+\beta_1[D]_0+\beta_2[O_2]_0+\beta_3[O_2]_0[D]_0)} \quad \text{(S1)}$$

Considering that the concentration of oxygen is saturating [2,3] $[O_2]_0 \rightarrow \infty$, the equation (S1) can be simplified as follows:

$$V_{ss}^{D,DC} = \frac{\alpha_1[D]_0[E]_0}{2(\beta_2+\beta_3[D]_0)} \quad \text{(S2)}$$

Substituting the values of α_1, β_2 and β_3 in equation (S2), and considering that,

$$k_3 \gg k_7, k_7 \gg k_{-6} \text{ y } k_3 \gg k_{-2}$$

we get:

$$V_{ss}^{D,DC} = \frac{V_{max}^{D,DC}[D]_0}{K_M^D+[D]_0} \quad \text{(S3)}$$

with:

$$V_{max}^{D,DC} = \frac{k_3k_7}{k_3+k_7}[E]_0 \cong k_7[E]_0 \quad \text{(S4)}$$

with:

$$k_{cat} \cong k_7 \quad \text{(S5)}$$

The binding of D to Em is easier than to Eox, so, $k_2 \gg k_6$ and K_m^D results in [4]:

$$K_m^D = \frac{k_7}{k_6} \quad \text{(S6)}$$

If in equation (S1), $[D]_0 \rightarrow \infty$, the expression of the Michaelis constant for oxygen is obtained in presence of D:

$$K_m^{D,O_2} = \frac{k_7}{k_8} \quad \text{(S7)}$$

The steady-state rate equation for monophenolase activity of tyrosinase.

The kinetic mechanism of this activity is indicated in Scheme 2. The steady-state rate is: (disappearance rate of M = formation rate of DC)

$$-V_{ss}^{M,M} = +V_{ss}^{M,DC} \quad \text{(S8)}$$

With respect to oxygen, it is:

$$-V_{ss}^{M,O_2} = -1{,}5\ V_{ss}^{M,M} = +1{,}5\ V_{ss}^{M.DC} \quad \text{(S9)}$$

Using the program described in [1], we can obtain equation (S10), and the parameters $\alpha_1 - \alpha_2$ and $\beta_1 - \beta_8$, are:

$\alpha_1 = K_1 k_2 k_3 k_8 k_4 k_5 (k_{-6} + k_7)$

$\alpha_2 = 2K_1 k_2 k_3 k_8 k_6 k_7 (k_{-4} + k_5)$

$\beta_1 = K_1 k_2 k_3 k_{-8} (k_{-4} + k_5)(k_{-6} + k_7)$

$\beta_2 = K_1 k_2 k_3 k_4 k_5 (k_{-6} + k_7)$

$\beta_3 = K_1 k_2 k_3 k_6 k_7 (k_{-4} + k_5)$

$\beta_4 = K_1 k_{-2} k_8 k_4 k_5 (k_{-6} + k_7)$

$\beta_5 = K_1 k_3 k_8 (k_{-4} + k_5)[k_6 (k_5 + k_7) + k_2 (k_7 + k_{-6})]$

$\beta_6 = k_{-2} k_8 k_4 k_5 (k_{-6} + k_7)$

$\beta_7 = k_8 [k_6 k_7 (k_{-4} + k_5)(k_{-2} + k_3) + K_1 k_2 k_4 (k_{-6} + k_7)(k_5 + k_3)]$

$\beta_8 = K_1 k_2 k_8 k_6 (k_{-4} + k_5)(k_7 + k_3)$

$$V_{ss}^{M,DC} = \frac{[\alpha_1 [O_2]_0 [D]_0 [M]_0 + \alpha_2 [O_2]_0 [D]_0^2][E]_0}{2\left[\begin{matrix}\beta_1 [D]_0 + \beta_2 [D]_0 [M]_0 + \beta_3 [D]_0^2 + \beta_4 [O_2]_0 [M]_0 \\ + \beta_5 [O_2]_0 [D]_0 + \beta_6 [O_2]_0 [M]_0^2 + \beta_7 [O_2]_0 [D]_0 [M]_0 + \beta_8 [O_2]_0 [D]_0^2\end{matrix}\right]} \quad \text{(S10)}$$

When $[O_2]_0 \rightarrow \infty$ [2,3]

$$V_{ss}^{M,DC} = \frac{[\alpha_1[D]_0[M]_0+\alpha_2[D]_0^2][E]_0}{2[\beta_4[M]_0+\beta_5[D]_0+\beta_6[M]_0^3+\beta_7[D]_0[M]_0+\beta_8[D]_0^2]} \quad (S11)$$

and:

$$\frac{[D]_{ss}}{[M]_{ss}} = \frac{k_4k_5(k_{-6}+k_7)}{2k_7k_6(k_{-4}+k_5)} = \frac{V_{max}^{M,DC}K_m^{D,DC}}{2V_{max}^{D,DC}K_m^{M,DC}} = R \quad (S12)$$

Substituting equation (S12) in equation (S10) and considering the values of the expressions for $\alpha_1 - \alpha_2$ and $\beta_4 - \beta_8$, we can obtain:

$$V_{ss}^{M,DC} = \frac{V_{max}^{M}[M]_0}{K_m^{M,DC}+[M]_0} = \frac{k_{cat_{app}}^{M,DC}[M]_0[E]_0}{K_m^{M,DC}+[M]_0} \quad (S13)$$

Where:

$$k_{cat_{app}}^{M,DC} = \frac{2K_1k_2k_3k_4k_5k_7}{4k_{-2}k_5k_6k_7+2k_3k_7(k_5k_6+K_1k_2k_4)+K_1k_2k_3k_4k_5} \quad (S14)$$

and as $k_2 \gg k_6$, results:

$$K_m^M = \frac{k_5}{k_4} \quad (S15)$$

From equation (S10), considering that $[M]_0 \rightarrow \infty$, results:

$$V_{ss}^{M,DC} = \frac{V_{max}^{M,DC}[O_2]_0}{K_m^{M,O_2}+[O_2]_0} = \frac{k_{cat_{app}}^{M,DC}[O_2]_0[E]_0}{K_m^{M,O_2}+[O_2]_0} \quad (S16)$$

The analytical expression for K_m^{M,O_2} is:

$$K_m^{M,O_2} = \frac{3k_{cat_{app}}^{M,DC}}{2k_8} \quad (S17)$$

If in equation (S13) we replace $[M]_0$ by the relation described in equation (S12), we obtain equation (S18) in which the Michaelis constant of tyrosinase is defined for *o*-diphenol in the presence of monophenol, resulting:

$$V_{SS}^{M,DC} = \frac{V_{max}^{M,DC}[D]_{ss}}{K_m^{M,DC}R+[D]_{ss}} = \frac{V_{max}^{M,DC}[D]_{ss}}{K_m^{D(M)}+[D]_{ss}} \quad (S18)$$

where $K_m^{D(M)}$, is the Michaelis constant for D in the presence of M [5]. It can be expressed based on the kinetic parameters K_m^D, $V_{max}^{M,Cr}$ and $V_{max}^{D,Cr}$, according to:

$$K_m^{D(M)} = \frac{V_{max}^{M,Cr}K_m^D}{2V_{max}^{D,Cr}} \quad (S19)$$

The relationship between the $K_m^{O_2}$ for monophenols and *o*-diphenols and the maximum rates obtained, either by measuring the oxygen consumption or the dopachrome formation, are shown in the equations (S20) and (S21) [2,3].

$$\frac{V_{max}^{M,DC} K_m^{D,O_2}}{V_{max}^{D,DC} K_m^{M,O_2}} = \frac{2}{3} \quad \text{(S20)}$$

$$\frac{V_{max}^{M,O_2} K_m^{D,O_2}}{V_{max}^{D,O_2} K_m^{M,O_2}} = 1 \quad \text{(S21)}$$

Differential equations corresponding to the diphenolase and monophenolase activities of tyrosinase.

Diphenolase activity.

The mechanism of the diphenolase activity of tyrosinase (Scheme 1), considering that the *o*-quinone evolves to DC, involves the following differential equations, which were used to carry out the numerical integration [6]:

$$[\dot{Em}] = k_{-2}[EmD] + k_7[EoxD] - k_2[D][Em]$$
$$[\dot{Ed}] = k_3[EmD] + k_{-8}[Eox] - k_8[O_2][Ed]$$
$$[\dot{Eox}] = k_8[O_2][Ed] + k_{-6}[EoxD] - k_6[D][Eox] - k_{-8}[Eox]$$
$$[\dot{EmD}] = k_2[D][Em] - k_{-2}[EmD] - k_3[EmD]$$
$$[\dot{EoxD}] = k_6[D][Eox] - k_7[EoxD] - k_{-6}[EoxD] \quad \text{(S22)}$$
$$[\dot{Q}] = k_3[EmD] + k_7[EoxD] - 2k_{10}[Q]$$
$$[\dot{D}] = k_{-2}[EmD] + k_{-6}[EoxD] - k_2[D][Em] - k_6[D][Eox] + k_{10}[Q]$$
$$[\dot{DC}] = k_{10}[Q]$$
$$[\dot{O_2}] = k_{-8}[Eox] - k_8[O_2][Ed]$$

Monophenolase activity.

The mechanism of the monophenolase activity of tyrosinase (Scheme 2), considering that the *o*-quinone evolves to DC. The differential equations involved in the evolution of the different enzymatic species in these mechanisms are:

$$[\dot{Em}] = k_{-2}[EmD] + k_7[EoxD] + k_{-1}[EmM] - k_2[D][Em] - k_1[M][Em]$$
$$[\dot{Ed}] = k_3[EmD] + k_{-8}[Eox] - k_8[O_2][Ed]$$
$$[\dot{Eox}] = k_8[O_2][Ed] + k_{-6}[EoxD] + k_{-4}[EoxM] - k_{-8}[EoxD] - k_6[D][Eox] - k_4[M][Eox]$$
$$[\dot{EmD}] = k_2[D][Em] + k_5[EoxM] - k_{-2}[EmD] - k_3[EmD]$$
$$[\dot{EoxD}] = k_6[D][Eox] - k_7[EoxD] - k_{-6}[EoxD]$$

$$\left[\dot{\mathrm{EoxM}}\right] = k_4[M][Eox] - k_{-4}[EoxM] - k_5[EoxM]$$

$$\left[\dot{\mathrm{EmM}}\right] = k_1[M][Em] - k_{-1}[EmM] \quad \text{(S23)}$$

$$\left[\dot{Q}\right] = k_3[EmD] + k_7[EoxD] - 2k_{10}[Q]$$

$$\left[\dot{D}\right] = k_{-2}[EmD] + k_{-6}[EoxD] - k_2[D][Em] - k_6[D][Eox] + k_{10}[Q]$$

$$\left[\dot{DC}\right] = k_{10}[Q]$$

$$\left[\dot{M}\right] = k_{-1}[EmM] + k_{-4}[EoxM] - k_1[M][Em] - k_{-4}[M][Eox]$$

$$\left[\dot{O_2}\right] = k_{-8}[Eox] - k_8[O_2][Ed]$$

1.2. Action of tyrosinase on hydroquinone. Substrates type B.

Kinetic analysis.

Steady-state rate equation in the presence of hydrogen peroxide.

The analytical expression for the product appearance rate hydroxyparabenzoquinone (HPB) in the steady-state and the analytical expressions for the parameters α_1 and $\beta_1 - \beta_7$ can be obtained with the program described in [1], being:

$$\alpha_1 = K_1 k_9 k_8 k_4 k_5 (k_2 + k_3)$$

$$\beta_1 = K_1 k_9 k_{-8} (k_{-4} + k_5)(k_{-2} + k_3)$$

$$\beta_2 = K_1 k_8 k_{-9} (k_{-4} + k_5)(k_{-2} + k_3)$$

$$\beta_3 = K_1 k_9 k_3 k_4 k_5$$

$$\beta_4 = k_8 k_{-9} (k_{-4} + k_5)(k_{-2} + k_3) + K_1 k_{-2} k_8 k_4 k_5$$

$$\beta_5 = K_1 k_9 k_8 (k_{-4} + k_5)(k_{-2} + k_3)$$

$$\beta_6 = k_{-2} k_4 k_8 k_5$$

$$\beta_7 = K_1 k_9 k_8 k_4 (k_{-2} + k_3 + k_5)$$

$$V_{ss}^{HPB} = \frac{\alpha_1[O_2]_0[H_2O_2]_0[HQ]_0[E]_0}{\beta_1[H_2O_2]_0 + \beta_2[O_2]_0 + \beta_3[H_2O_2]_0[HQ]_0 + \beta_4[O_2]_0[HQ]_0 + \beta_5[O_2]_0[H_2O_2]_0 + \beta_6[O_2]_0[HQ]_0^2 + \beta_7[O_2]_0[H_2O_2]_0[HQ]_0} \quad \text{(S24)}$$

Considering that the concentration of oxygen is saturating [2,3] $[O_2]_0 \to \infty$, the equation (S24) can be simplified as follows:

$$V_{ss}^{HPB} = \frac{\alpha_1[H_2O_2]_0[HQ]_0[E]_0}{\beta_2 + \beta_4[HQ]_0 + \beta_5[H_2O_2]_0 + \beta_6[HQ]_0^2 + \beta_7[H_2O_2]_0[HQ]_0} \quad \text{(S25)}$$

Substituting the expressions for α_1 and $\beta_2 - \beta_7$, we can obtain:

$$V_{ss}^{HPB} = \frac{V_{max}^{app}[H_2O_2]_0}{K_m^{app}+[H_2O_2]_0} \quad \text{(S26)}$$

$$V_{max}^{app} = \frac{\frac{\alpha_1}{\beta_7}[HQ]_0}{\frac{\beta_5}{\beta_7}+[HQ]_0} = \frac{k_{cat}^{HQ}[HQ]_0}{K_m^{HQ}+[HQ]_0} \quad \text{(S27)}$$

with,

$$k_{cat}^{HQ} = k_5 \quad \text{(S28)}$$

$$K_m^{HQ} = \frac{k_5}{k_4} \quad \text{(S29)}$$

If in equation (S24), we consider that $[HQ]_0$ and $[H_2O_2] \rightarrow \infty$, K_m^{HQ,O_2} expression can be obtained:

$$K_m^{HQ,O_2} \cong \frac{k_5}{k_8} \quad \text{(S30)}$$

Steady-state rate equation in absence of hydrogen peroxide.

In these conditions, the enzyme has no activity, because Eox cannot be regenerated, and the reaction stops.

Differential equations corresponding to the action of tyrosinase on hydroquinone in absence of hydrogen peroxide.

$$\begin{aligned}
[\dot{EmHQ}] &= k_1[Em][HQ] - k_{-1}[EmHQ] \\
[\dot{Em}] &= k_{-2}[EmHHQ] + k_{-9}[Eox] + k_{-1}[EmHQ] - k_1[Em][HQ] \\
[\dot{EmHHQ}] &= k_5[EoxHQ] - k_{-2}[EmHHQ] - k_3[EmHHQ] \\
[\dot{EoxHQ}] &= k_4[Eox][HQ] - k_{-4}[EoxHQ] - k_5[EoxHQ] \\
[\dot{Ed}] &= k_3[EmHHQ] + k_{-8}[Eox] - k_8[O_2][Ed] \\
[\dot{Eox}] &= k_8[Ed][O_2] + k_{-4}[EoxHQ] - k_{-9}[Eox] - k_{-8}[Eox] - k_4[Eox][HQ] \\
[\dot{HPB}] &= k_{-2}[EmHHQ] + k_3[EmHHQ]
\end{aligned} \quad \text{(S31)}$$

Differential equations corresponding to the action of tyrosinase on hydroquinone in the presence of hydrogen peroxide.

$$\begin{aligned}
[\dot{EmHQ}] &= k_1[Em][HQ] - k_{-1}[EmHQ] \\
[\dot{Em}] &= k_{-2}[EmHHQ] + k_{-9}[Eox] + k_{-1}[EmHQ] - k_1[Em][HQ] - k_9[Em][H_2O_2]
\end{aligned}$$

$$\left[\mathrm{Em\dot{H}HQ}\right] = k_5[\mathrm{EoxHQ}] - k_{-2}[\mathrm{EmHHQ}] - k_3[\mathrm{EmHHQ}]$$

$$\left[\mathrm{Eox\dot{H}Q}\right] = k_4[\mathrm{Eox}][\mathrm{HQ}] - k_{-4}[\mathrm{EoxHQ}] - k_5[\mathrm{EoxHQ}] \qquad \text{(S32)}$$

$$\left[\mathrm{\dot{E}d}\right] = k_3[\mathrm{EmHHQ}] + k_{-8}[\mathrm{Eox}] - k_8[\mathrm{O_2}][\mathrm{Ed}]$$

$$\left[\mathrm{E\dot{o}x}\right] = k_9[\mathrm{Em}][\mathrm{H_2O_2}] + k_8[\mathrm{Ed}][\mathrm{O_2}] + k_{-4}[\mathrm{EoxHQ}] - k_{-9}[\mathrm{Eox}] - k_{-8}[\mathrm{Eox}] - k_4[\mathrm{Eox}][\mathrm{HQ}]$$

$$\left[\mathrm{H\dot{P}B}\right] = k_{-2}[\mathrm{EmHHQ}] + k_3[\mathrm{EmHHQ}]$$

1.3. Action of tyrosinase on 4-tert-butylphenol. Substrates type C.

Kinetic analysis.

Pseudo-steady-state rate equation in absence of hydrogen peroxide.

In absence of hydrogen peroxide, the enzymatic form Eox hydroxylates 4-TBF to TBC and, at the same time, Em and Eox convert TBC *o*-TBQ, according to the mechanism described in Scheme 5.

The system reaches a pseudo-steady-state and, using the program described in [1], we can obtain the rate equation and the analytical expressions for the parameters $\alpha_1 - \alpha_2$ and $\beta_1 - \beta_8$, being:

$$\alpha_1 = K_1 k_2 k_3 k_8 k_4 k_5 (k_{-6} + k_7)$$

$$\alpha_2 = 2K_1 k_2 k_3 k_8 k_6 k_7 (k_{-4} + k_5)$$

$$\beta_1 = K_1 k_2 k_3 k_{-8} (k_{-6} + k_7)(k_{-4} + k_5)$$

$$\beta_2 = K_1 k_2 k_3 k_4 k_5 (k_{-6} + k_7)$$

$$\beta_3 = K_1 k_2 k_3 k_6 k_7 (k_5 + k_{-4})$$

$$\beta_4 = K_1 k_{-2} k_8 k_4 k_5 (k_{-6} + k_7)$$

$$\beta_5 = K_1 k_8 (k_{-4} + k_5)[k_6 k_7 (k_{-2} + k_3) + k_2 k_3 (k_{-6} + k_7)]$$

$$\beta_6 = k_{-2} k_8 k_4 k_5 (k_7 + k_{-6})$$

$$\beta_7 = k_8 k_6 k_7 (k_{-4} + k_5)(k_{-2} + k_3) + K_1 k_2 k_8 k_4 (k_{-6} + k_7)(k_5 + k_3)$$

$$\beta_8 = K_1 k_2 k_6 k_8 (k_{-4} + k_5)(k_3 + k_7)$$

$$V_{ss}^{o-TBQ} = \frac{[\alpha_1[O_2]_0[TBC]_0[TBF]_0 + \alpha_2[O_2]_0[TBC]_0^2][E]_0}{\beta_1[TBC]_0 + \beta_2[TBC]_0[TBF]_0 + \beta_3[TBC]_0^2 + \beta_4[O_2]_0[TBF]_0 + \beta_5[O_2]_0[TBC]_0 + \beta_6[O_2]_0[TBF]_0^2 + \beta_7[O_2]_0[TBC]_0[TBF]_0 + \beta_8[O_2]_0[TBC]_0^2} \qquad \text{(S33)}$$

Considering that the oxygen concentration is saturated [2,3] and that the TBC concentration is at most stoichiometric with Eox concentration and in turn this is a fraction of Eo, $[\mathrm{Eox}] = f[\mathrm{E}]_0$, results $[\mathrm{TBC}] = f[\mathrm{E}]_0$, therefore the rate is:

$$V_{ss}^{o-TBQ} = \frac{\alpha_1 f[E]_0^2}{\beta_4 + \beta_6[TBF]_0} \quad \text{(S34)}$$

Substituting α_1, β_4 and β_6 into (S34), results:

$$V_{ss}^{o-TBQ} = \frac{k_3 \frac{K_1}{K_2} f[E]_0^2}{K_1 + [TBF]_0} \quad \text{(S35)}$$

Pseudo-steady-state rate equation in the presence of hydrogen peroxide.

In presence of hydrogen peroxide, the rate can be obtained measuring in the isosbestic point between *o*-TBQ and the hydroxylated derived compound. The analytical expression for the rate equation and the parameters $\alpha_1 - \alpha_5$ and $\beta_1 - \beta_{14}$ [1], are:

$$\alpha_1 = K_1 k_2 k_3 k_8 k_{-9}(k_{-4} + k_5)(k_{-6} + k_7)$$
$$\alpha_2 = K_1 k_2 k_3 k_8 k_4 k_5 (k_{-6} + k_7)$$
$$\alpha_3 = 2K_1 k_2 k_3 k_8 k_6 k_7 (k_{-4} + k_5)$$
$$\alpha_4 = K_1 k_9 k_3 k_8 k_4 k_5 (k_{-6} + k_7)$$
$$\alpha_5 = K_1 k_9 k_8 k_6 k_7 (k_{-4} + k_5)(k_{-2} + k_3)$$
$$\beta_1 = K_1 k_2 k_3 (k_{-4} + k_5)(k_{-6} + k_7)(k_{-8} + k_{-9})$$
$$\beta_2 = K_1 k_9 k_{-8} (k_{-4} + k_5)(k_{-6} + k_7)(k_{-2} + k_3)$$
$$\beta_3 = K_1 k_8 k_{-9} (k_{-2} + k_3)(k_{-4} + k_5)(k_{-6} + k_7)$$
$$\beta_4 = K_1 k_2 k_3 k_4 k_5 (k_7 + k_{-6})$$
$$\beta_5 = K_1 k_2 k_3 k_6 k_7 (k_{-4} + k_5)$$
$$\beta_6 = K_1 k_3 k_9 k_4 k_5 (k_7 + k_{-6})$$
$$\beta_7 = [k_8 k_{-9}(k_{-4} + k_5)(k_{-2} + k_3) + K_1 k_{-2} k_8 k_4 k_5](k_{-6} + k_7)$$
$$\beta_8 = K_1 k_8 (k_{-4} + k_5)[k_7(k_{-2} k_4 + k_3 k_6) + k_2(k_{-9} + k_3)] + k_{-6} k_2 (k_{-9} + k_3)$$
$$\beta_9 = K_1 k_9 k_8 (k_{-4} + k_5)(k_{-6} + k_7)(k_{-2} + k_3)$$
$$\beta_{10} = k_{-2} k_8 k_4 k_5 (k_{-6} + k_7)$$
$$\beta_{11} = k_8 k_6 k_7 (k_{-4} + k_5)(k_{-2} + k_3) + K_1 k_2 k_8 k_4 (k_5 + k_3)(k_{-6} + k_7)$$
$$\beta_{12} = K_1 k_2 k_8 (k_{-4} + k_5)(k_6 k_7 + k_2 k_3)$$
$$\beta_{13} = K_1 k_9 k_8 k_4 (k_{-6} + k_7)(k_5 + k_{-2} + k_3)$$
$$\beta_{14} = K_1 k_9 k_8 k_6 (k_{-4} + k_5)(k_{-2} + k_3)$$

$$V_{ss}^{o-TBQ} = \frac{\begin{bmatrix}\alpha_1[O_2]_0[TBC]_0+\alpha_2[O_2]_0[TBC]_0[TBF]_0 \\ +\alpha_3[O_2]_0[TBC]_0^2+\alpha_4[O_2]_0[H_2O_2]_0[TBF]_0+\alpha_5[O_2]_0[H_2O_2]_0[TBC]_0\end{bmatrix}[E]_0}{\begin{matrix}\beta_1[TBC]_0+\beta_2[H_2O_2]_0+\beta_3[O_2]_0+\beta_4[TBC]_0[TBF]_0 \\ +\beta_5[TBC]_0^2+\beta_6[H_2O_2]_0[TBF]_0+\beta_7[O_2]_0[TBF]_0+\beta_8[O_2]_0[TBC]_0 \\ +\beta_9[O_2]_0[H_2O_2]_0+\beta_{10}[O_2]_0[TBF]_0^2+\beta_{11}[O_2]_0[TBC]_0[TBF]_0 \\ +\beta_{12}[O_2]_0[TBC]_0^2+\beta_{13}[O_2]_0[H_2O_2]_0[TBF]_0+\beta_{14}[O_2]_0[H_2O_2]_0[TBC]_0\end{matrix}} \quad \text{(S36)}$$

Considering that the oxygen concentration is saturated [2,3] and that the TBC accumulation in the reaction medium is related with $[TBF]_0$, results:

$$V_{ss}^{o-TBQ} = \frac{\begin{bmatrix}\alpha_1[TBC]_0+\alpha_2[TBC]_0[TBF]_0+\alpha_3[TBC]_0^2 \\ +\alpha_4[H_2O_2]_0[TBF]_0+\alpha_5[H_2O_2]_0[TBC]_0\end{bmatrix}[E]_0}{\begin{matrix}\beta_3+\beta_4[TBC]_0[TBF]_0+\beta_5[TBC]_0^2+\beta_6[H_2O_2]_0[TBF]_0 \\ +\beta_7[O_2]_0[TBF]_0+\beta_8[O_2]_0[TBC]_0+\beta_9[O_2]_0[H_2O_2]_0+\beta_{10}[O_2]_0[TBF]_0^2 \\ +\beta_{11}[O_2]_0[TBC]_0[TBF]_0+\beta_{12}[O_2]_0[TBC]_0^2 \\ +\beta_{13}[O_2]_0[H_2O_2]_0[TBF]_0+\beta_{14}[O_2]_0[H_2O_2]_0[TBC]_0\end{matrix}} \quad \text{(S37)}$$

If we consider that $[O_2]_0$ and $[H_2O_2] \rightarrow \infty$ in equation (S36), we can define:

$$k_{cat}^{TBF} \cong k_5 \quad \text{(S38)}$$

$$K_m^{TBF} \cong \frac{k_5}{k_4} \quad \text{(S39)}$$

If we consider that $[TBF]_0$ and $[H_2O_2] \rightarrow \infty$ in equation (S36), we can define:

$$K_m^{TBF,O_2} = \frac{k_5}{k_8} \quad \text{(S40)}$$

Differential equations corresponding to the action of tyrosinase on 4-tert-butylphenol in absence of hydrogen peroxide.

$$\begin{aligned}
[\dot{EmTBF}] &= k_1[TBF][Em] - k_{-1}[EmTBF] \\
[\dot{Em}] &= k_{-1}[EmTBF] + k_{-2}[EmTBC] + k_7[EoxTBC] - k_2[TBC][Em] - k_1[Em][TBF] \\
[\dot{EmTBC}] &= k_2[TBC][Em] - k_{-2}[EmTBC] - k_3[EmTBC] + k_5[EoxTBF] \\
[\dot{Ed}] &= k_3[EmTBC] + k_{-8}[Eox] - k_8[O_2][Ed] \\
[\dot{Eox}] &= k_8[Ed][O_2] + k_{-6}[EoxTBC] + k_{-4}[EoxTBF] - k_{-8}[Eox] - k_6[Eox][TBC] \\
&\quad - k_4[TBF][Eox] \\
[\dot{EoxTBC}] &= k_6[TBC][Eox] - k_{-6}[EoxTBC] - k_7[EoxTBC] \\
[\dot{EoxTBF}] &= k_4[TBF][Eox] - k_{-4}[EoxTBF] - k_5[EoxTBF]
\end{aligned} \quad \text{(S41)}$$

$$[\dot{\mathrm{TBC}}] = \mathrm{k}_{-2}[\mathrm{EmTBC}] + \mathrm{k}_{-6}[\mathrm{EoxTBC}] - \mathrm{k}_2[\mathrm{TBC}][\mathrm{Em}] - \mathrm{k}_6[\mathrm{TBC}][\mathrm{Eox}]$$

$$[o - \dot{\mathrm{T}}\mathrm{BQ}] = \mathrm{k}_3[\mathrm{EmTBC}] + \mathrm{k}_7[\mathrm{EoxTBC}]$$

Differential equations corresponding to the action of tyrosinase on 4-tert-butylphenol in the presence of hydrogen peroxide.

The differential equations are, in general terms, like the described in absence of hydrogen peroxide, excluding the equations described for the enzymatic forms Em and Eox. The variation of *o*-TBQ and TBC is also influenced by the concentrations of hydrogen peroxide according to:

$$[\mathrm{Em}\dot{\mathrm{T}}\mathrm{BF}] = \mathrm{k}_1[\mathrm{TBF}][\mathrm{Em}] - \mathrm{k}_{-1}[\mathrm{EmTBF}]$$

$$[\dot{\mathrm{E}}\mathrm{m}] = \mathrm{k}_{-1}[\mathrm{EmTBF}] + \mathrm{k}_{-2}[\mathrm{EmTBC}] + \mathrm{k}_7[\mathrm{EoxTBC}] + \mathrm{k}_{-9}[\mathrm{Eox}] - \mathrm{k}_9[\mathrm{H_2O_2}][\mathrm{Em}] - \mathrm{k}_2[\mathrm{TBC}][\mathrm{Em}] - \mathrm{k}_1[\mathrm{TBF}][\mathrm{Em}]$$

$$[\mathrm{Em}\dot{\mathrm{T}}\mathrm{BC}] = \mathrm{k}_2[\mathrm{TBC}][\mathrm{Em}] - \mathrm{k}_{-2}[\mathrm{EmTBC}] - \mathrm{k}_3[\mathrm{EmTBC}] + \mathrm{k}_5[\mathrm{EoxTBF}]$$

$$[\dot{\mathrm{E}}\mathrm{d}] = \mathrm{k}_3[\mathrm{EmTBC}] + \mathrm{k}_{-8}[\mathrm{Eox}] - \mathrm{k}_8[\mathrm{O_2}][\mathrm{Ed}]$$

$$[\dot{\mathrm{E}}\mathrm{ox}] = \mathrm{k}_8[\mathrm{O_2}][\mathrm{Ed}] + \mathrm{k}_{-6}[\mathrm{EoxTBC}] + \mathrm{k}_{-4}[\mathrm{EoxTBF}] + \mathrm{k}_9[\mathrm{H_2O_2}][\mathrm{Em}] - \mathrm{k}_{-8}[\mathrm{Eox}] - \mathrm{k}_6[\mathrm{TBC}][\mathrm{Eox}] - \mathrm{k}_4[\mathrm{TBF}][\mathrm{Eox}] - \mathrm{k}_{-9}[\mathrm{Eox}]$$

$$[\mathrm{Eox}\dot{\mathrm{T}}\mathrm{BC}] = \mathrm{k}_6[\mathrm{TBC}][\mathrm{Eox}] - \mathrm{k}_{-6}[\mathrm{EoxTBC}] - \mathrm{k}_7[\mathrm{EoxTBC}] \qquad \text{(S42)}$$

$$[\mathrm{Eox}\dot{\mathrm{T}}\mathrm{BF}] = \mathrm{k}_4[\mathrm{TBF}][\mathrm{Eox}] - \mathrm{k}_{-4}[\mathrm{EoxTBF}] - \mathrm{k}_5[\mathrm{EoxTBF}]$$

$$[\dot{\mathrm{T}}\mathrm{BC}] = \mathrm{k}_{-2}[\mathrm{EmTBC}] + \mathrm{k}_{-6}[\mathrm{EoxTBC}] + \mathrm{k}[\mathrm{H_2O_2}][o - \mathrm{TBQ}] - \mathrm{k}_2[\mathrm{TBC}][\mathrm{Em}] - \mathrm{k}_6[\mathrm{TBC}][\mathrm{Eox}]$$

$$[o - \dot{\mathrm{T}}\mathrm{BQ}] = \mathrm{k}_3[\mathrm{EmTBC}] + \mathrm{k}_7[\mathrm{EoxTBC}] - 2\mathrm{k}[\mathrm{H_2O_2}][o - \mathrm{TBQ}]$$

$$[p - \dot{\mathrm{Q}}] = \mathrm{k}[\mathrm{H_2O_2}][o - \mathrm{TBQ}] \quad \mathrm{D}$$

1.4. Action of tyrosinase on deoxyarbutin. Substrates type D.

Kinetic analysis.

Steady-state rate equation.

Applying the steady-state approach to the mechanism described in Scheme 7 and using the program described in [1], we can obtain the rate equation and the parameters α_1 and $\beta_0 - \beta_3$, being:

$$\alpha_1 = k_3 k_4 k_5 k_8$$

$$\beta_0 = (k_{-5} + k_3)(k_{-4} + k_5)$$

$$\beta_1 = k_3 k_4 k_5$$

$$\beta_2 = k_3 k_8 (k_{-4} + k_5)$$

$$\beta_3 = k_4 k_8 (k_3 + k_5)$$

$$V_{ss}^{P} = \frac{\alpha_1 [D-Arb]_0 [O_2]_0 [Eox]_0}{\beta_0 + \beta_1 [D-Arb]_0 + \beta_2 [O_2]_0 + \beta_3 [O_2]_0 [D-Arb]_0} \quad \text{(S43)}$$

Substituting the expressions for α_1, $\beta_0 - \beta_3$ in equation (S43) and considering that the oxygen concentration is saturated [2,3], we can obtain:

$$V_{ss}^{P} = \frac{k_5 [D-Arb]_0 [Eox]_0}{\frac{k_5}{k_4} + [D-Arb]_0} \quad \text{(S44)}$$

with:

$$k_{cat}^{D-Arb} = k_5 \quad \text{(S45)}$$

$$K_M^{D-Arb} = \frac{k_5}{k_4} \quad \text{(S46)}$$

If we consider that $[D - Arb]_0 \rightarrow \infty$ in equation (S43), we can define the Michaelis constants for oxygen in presence D-Arb:

$$K_m^{D-Arb,O_2} = \frac{k_5}{k_4} \quad \text{(S47)}$$

Differential equations corresponding to the action of tyrosinase on deoxyarbutin.

$$[\dot{Ed}] = k_{-8}[Eox] + k_3[EmD - ArbOH] - k_8[O_2][Ed]$$

$$[\dot{Eox}] = k_8[Ed][O_2] + k_{-4}[EoxD - Arb] - k_{-8}[Eox] - k_4[D - Arb][Eox]$$

$$[\dot{EoxD - Arb}] = k_4[D - Arb][Eox] - k_{-4}[EoxD - Arb] - k_5[EoxD - Arb] \quad \text{(S48A)}$$

$$[\dot{EmD - ArbOH}] = k_5[EoxD - Arb] - k_3[EmD - ArbOH]$$

$$[\dot{P}] = k_3[EmD - ArbOH]$$

FIGURES

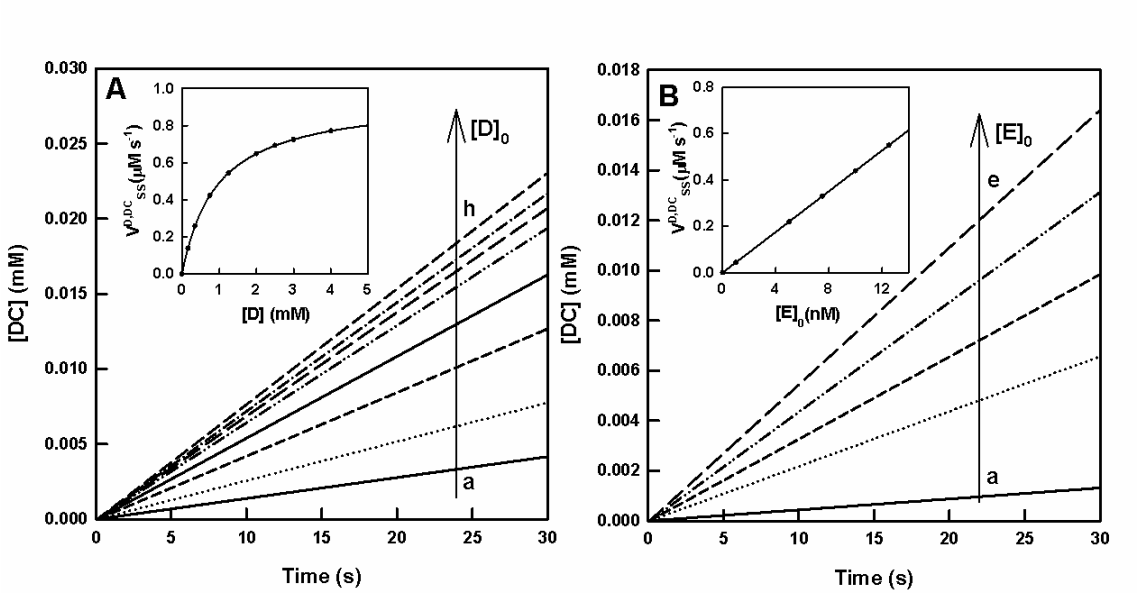

Fig. S1. Simulated curves of tyrosinase action on L-dopa (Scheme 1, equation (S22)). A. Effect of the substrate concentration. Representation of the concentration of product $[\mathrm{DC}]$ (mM) *vs.* time (s). The conditions were: $[\mathrm{E}]_0 = 10\ \mathrm{nM}$, $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$. $[\mathrm{D}]_0$ was varied according to (mM): a) 0.16, b) 0.35, c) 0.75, d) 2, e) 2.50, f) 3 and g) 4. Inset, representation of $\mathrm{V_{ss}^{DC}}\ (\mu\mathrm{M\ s^{-1}})$ *vs.* $[\mathrm{D}]_0\ (\mathrm{mM})$. B. Effect of the enzyme concentration. Representation of the concentration of product $[\mathrm{DC}]$ (mM) *vs.* time (s). The conditions were: $[\mathrm{D}]_0 = 0.8\ \mathrm{mM}$ and the enzyme was varied according to (nM): a) 1, b) 5, c) 7.5, d) 10 and e) 12.5. Inset, representation of $\mathrm{V_{ss}^{DC}}\ (\mu\mathrm{M\ s^{-1}})$ *vs.* $[\mathrm{E}]_0\ (\mathrm{nM})$. The conditions and the rate constants were: $[\mathrm{O_2}]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$, $\mathrm{k_2} = 8\mathrm{x}10^{6}\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-2}} = 10\ \mathrm{s^{-1}}$, $\mathrm{k_3} = 1000\ \mathrm{s^{-1}}$, $\mathrm{k_6} = 1.33\mathrm{x}10^{5}\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-6}} = 10\ \mathrm{s^{-1}}$, $\mathrm{k_7} = 107\ \mathrm{s^{-1}}$, $\mathrm{k_8} = 2.3\mathrm{x}10^{7}\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-8}} = 1.07\mathrm{x}10^{3}\ \mathrm{s^{-1}}$ and $\mathrm{k_{10}} = 10\ \mathrm{s^{-1}}$.

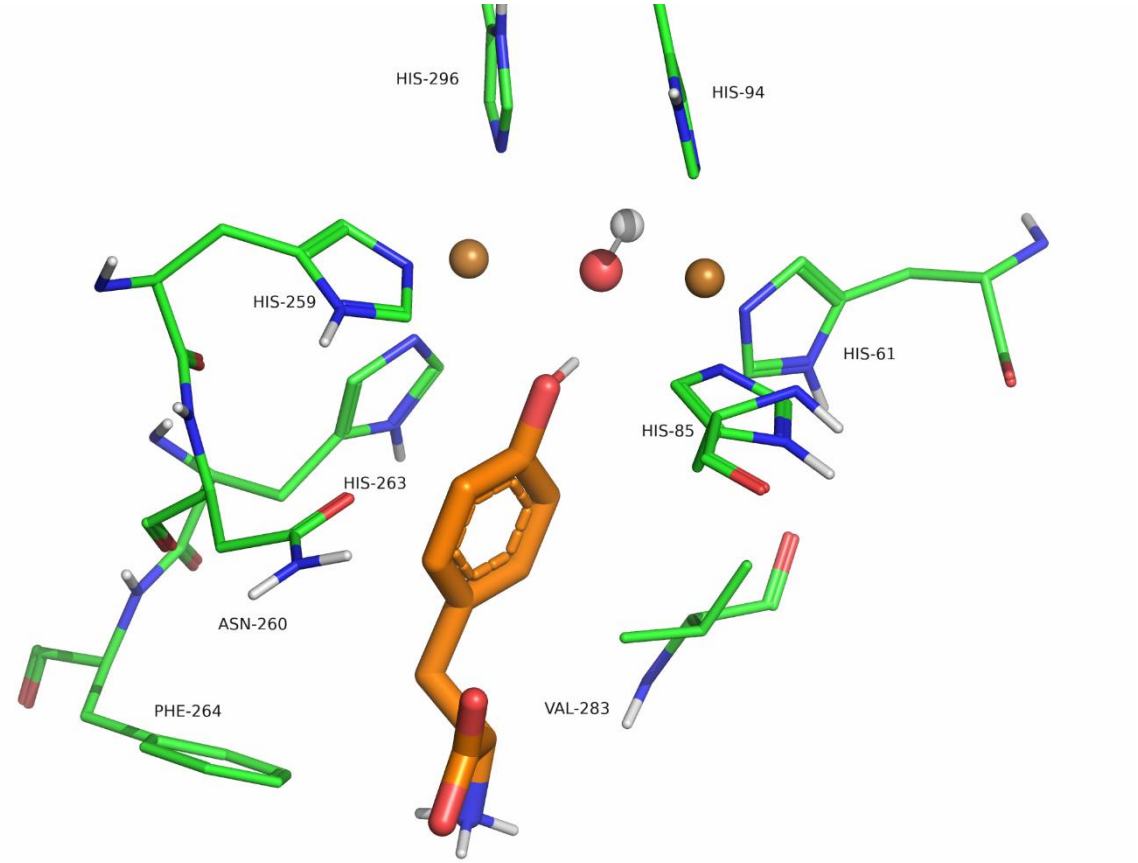

Fig. S2A. Computational docking of L-tyrosine in the zwitterionic form at the active site of met form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

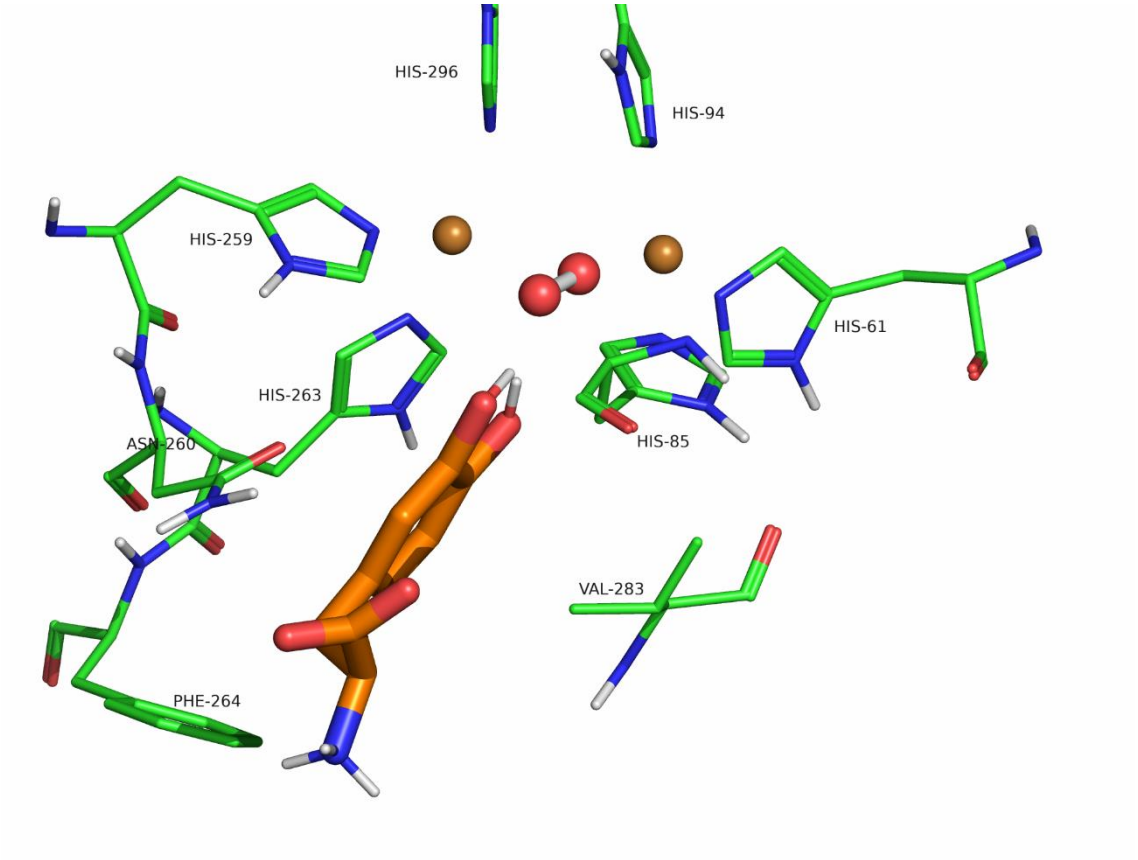

Fig. S2B. Computational docking of L-dopa at the active site of oxy form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

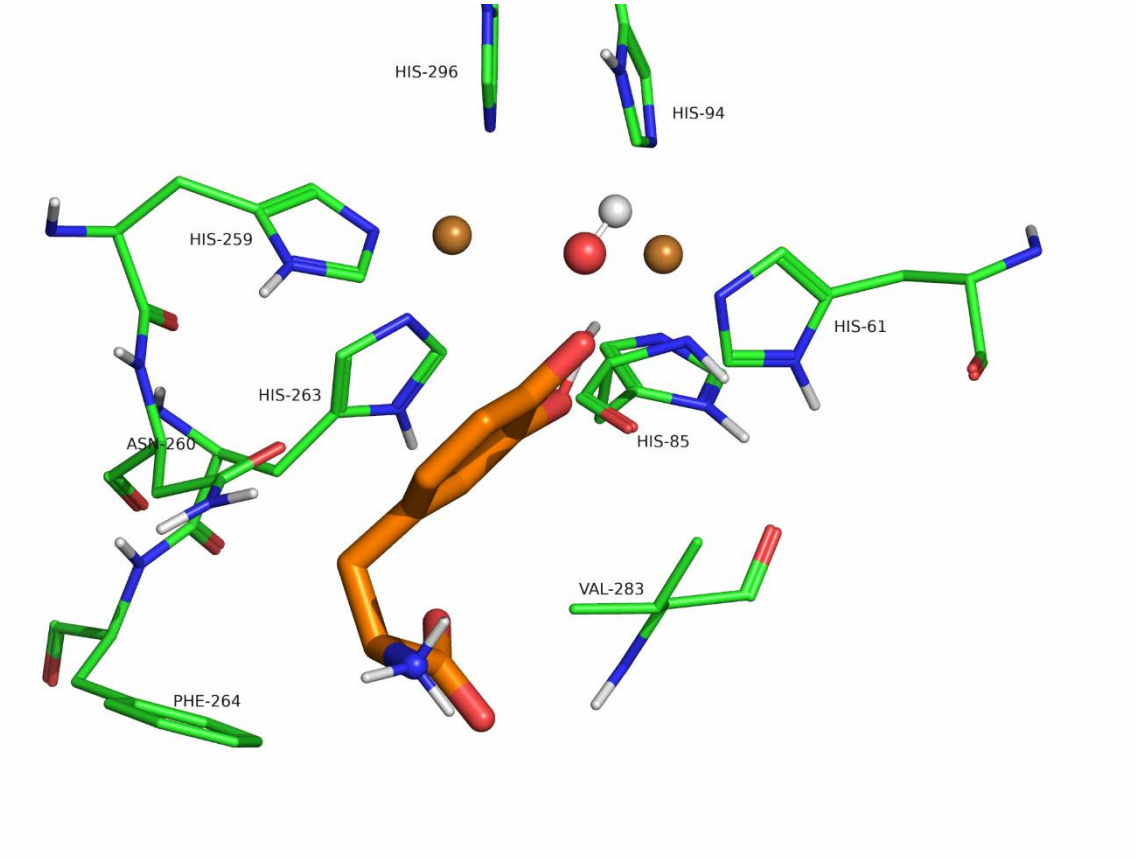

Fig. S2C. Computational docking of L-dopa at the active site of met form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

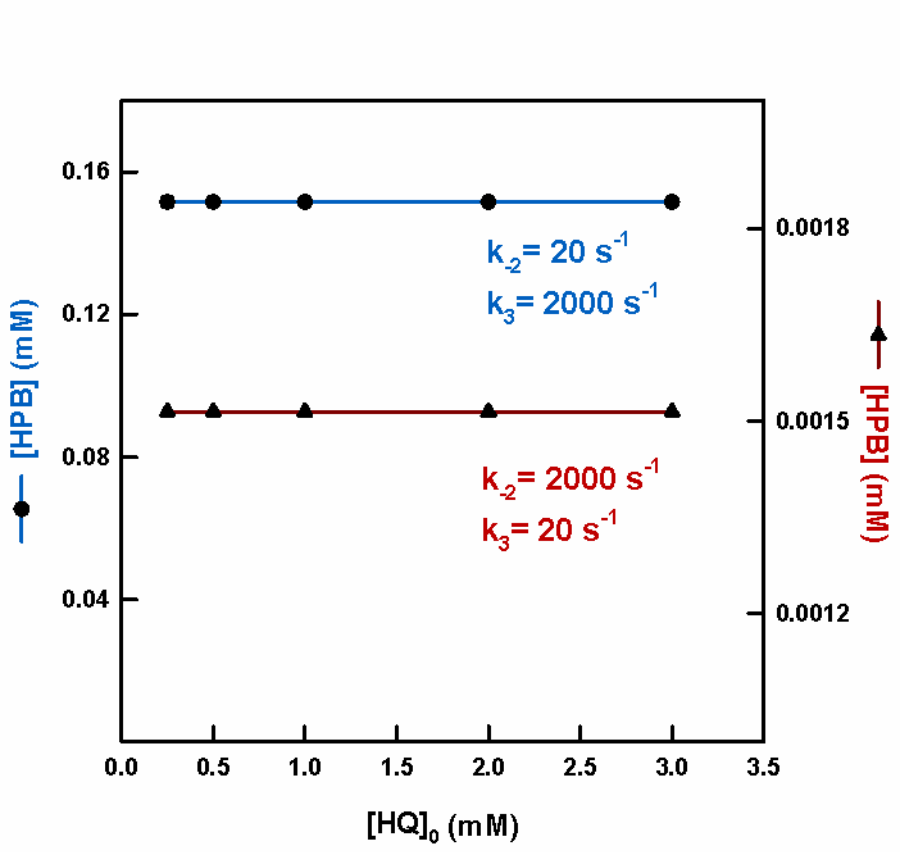

Fig. S3. Simulated curves of tyrosinase action on hydroquinone in absence of hydrogen peroxide (Scheme 3, equation (S31)). Effect of substrate concentration. Representation of the concentration of product $[\mathrm{HPB}]$ (mM) *vs.* $[\mathrm{HQ}]_0$ (mM). The experimental conditions were: $[\mathrm{E}]_0 = 5\ \mu\mathrm{M}$, $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$. $[\mathrm{HQ}]_0$ was varied according to (mM): a) 0.25, b) 0.5, c) 1, d) 2 and e) 3. The conditions and the rate constants were: $[\mathrm{O_2}]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$, $\mathrm{k_1} = 10^4\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-1}} = 45.6\ \mathrm{s^{-1}}$, $\mathrm{k_4} = 9.2\mathrm{x}10^4\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-4}} = 10\ \mathrm{s^{-1}}$, $\mathrm{k_5} = 23\ \mathrm{s^{-1}}$, $\mathrm{k_8} = 2.3\mathrm{x}10^7\ \mathrm{M^{-1}s^{-1}}$ and $\mathrm{k_{-8}} = 1.07\mathrm{x}10^3\ \mathrm{s^{-1}}$. (•) $\mathrm{k_{-2}} = 20\ \mathrm{s^{-1}}$ and $\mathrm{k_3} = 2000\ \mathrm{s^{-1}}$; (▲) $\mathrm{k_{-2}} = 2000\ \mathrm{s^{-1}}$ and $\mathrm{k_3} = 20\ \mathrm{s^{-1}}$.

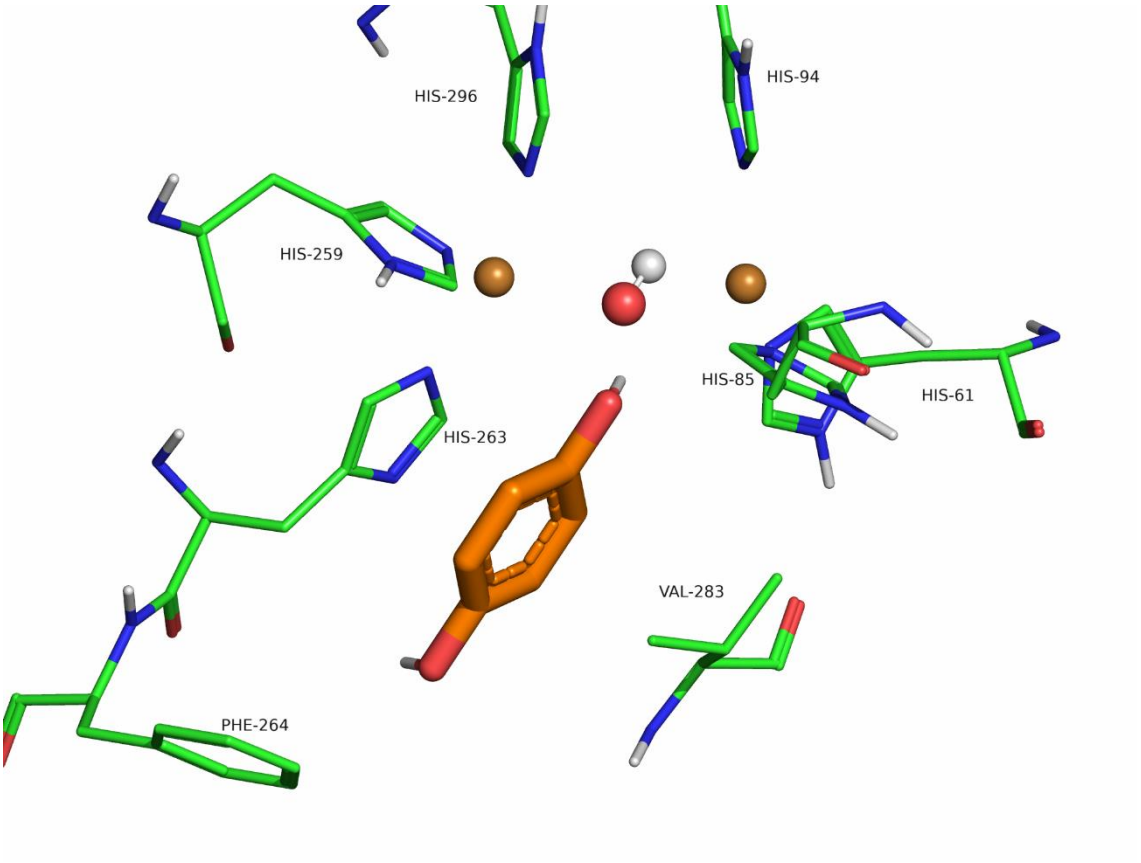

Fig. S4. Computational docking of hydroquinone at the active site of met form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen and carbon = green in tyrosinase and orange in the ligand.

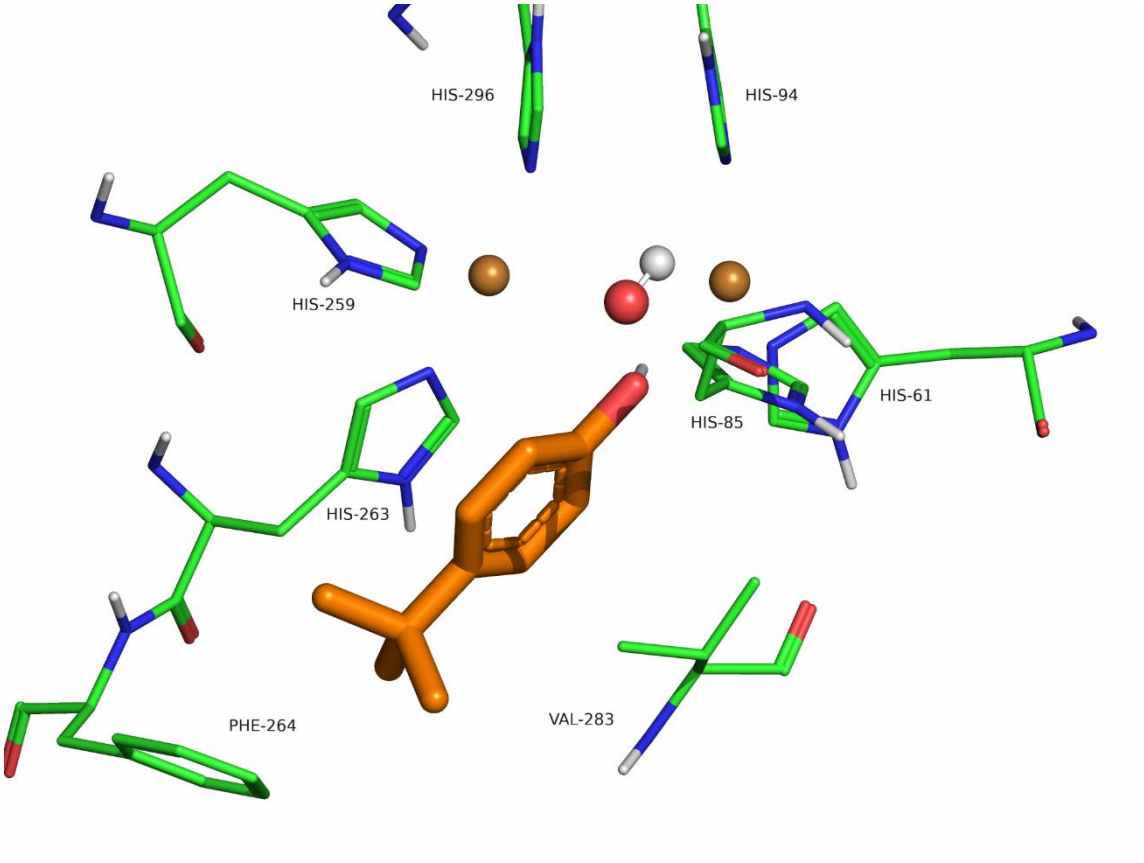

Fig. S5. Computational docking of 4-tert-butylphenol at the active site of met form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

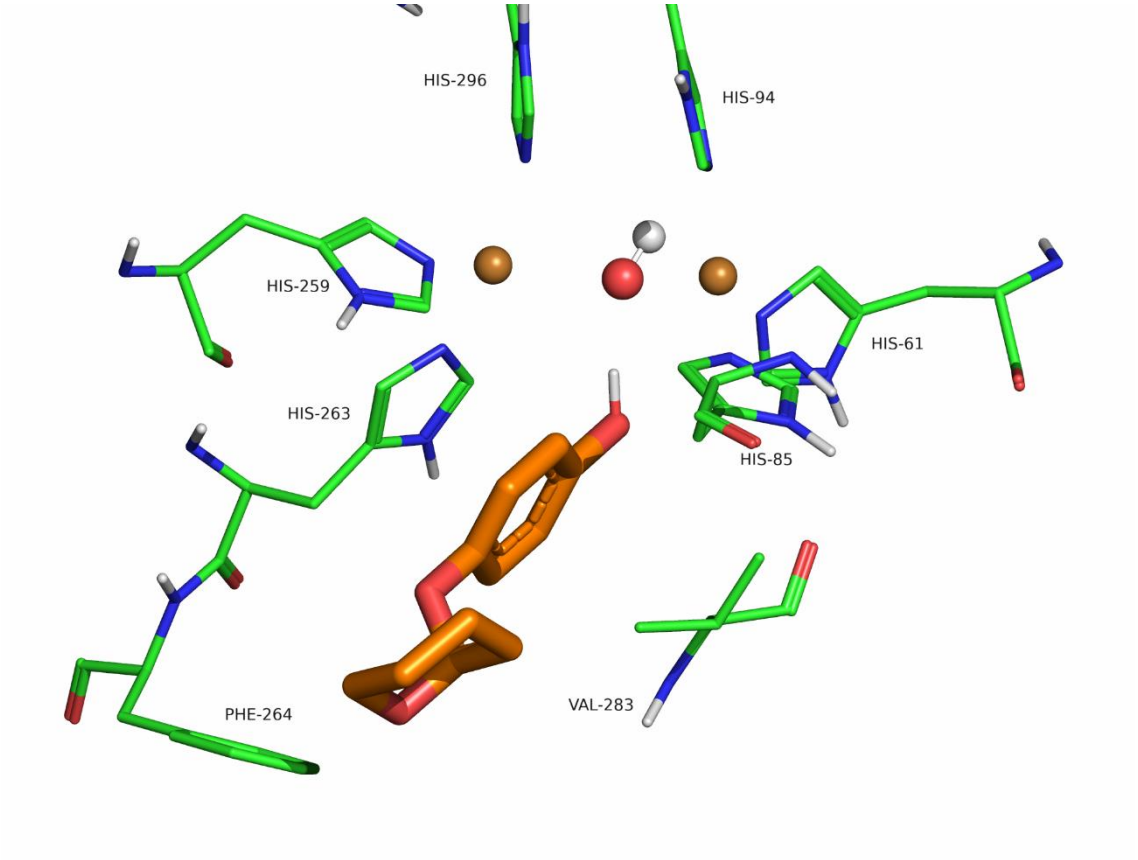

Fig. S6. Computational docking of deoxyarbutin at the active site of met form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CONSIDERATIONS ABOUT THE KINETIC MECHANISM OF TYROSINASE IN ITS ACTION ON MONOPHENOLS: A REVIEW

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Highlights

- Tyrosinase can hydroxylate a broad range of monophenols.
- In Type A substrates, the *o*-quinone evolution generates *o*-diphenol in the medium.
- In Types B, C and D substrates, the evolution does not accumulate *o*-diphenol.
- Hydrogen peroxide transforms Em to Eox and the enzyme has hydroxylating activity.
- In presence of monophenols, Michaelis constant for oxygen is very low.

ABSTRACT

The mechanism of action of tyrosinase on monophenols is complex, several processes overlap in time, such as the hydroxylation of monophenols to *o*-diphenols, the oxidation of these to *o*-quinones and the evolution of the latter towards melanin. The enzyme's mechanism of action is unique but depending on the chemical nature of the substrate it may show different exceptions. In this review we want to dissect the kinetic mechanism for the action of the enzyme on: a) L-tyrosine, the physiological substrate for mammalian tyrosinase, and related compounds, whose *o*-quinones in their chemical evolution accumulate *o*-diphenol in the medium (Type A). b) Substrates that cannot accumulate *o*-diphenol in the medium because it is easily oxidized and they need the presence of hydrogen peroxide for the enzyme to show activity, such as hydroquinone and related compounds (Type B). c) Substrates that release *o*-diphenol into the medium and the enzyme oxidizes it generating a stable *o*-quinone and therefore does not generate more *o*-diphenol in the medium, as is the case of 4-tert-butylphenol and related compounds (Type C). d) Substrates that do not release or generate *o*-diphenol in the medium, as is the case with deoxyarbutin, which produces a stable *o*-quinone (Type D). The different mechanisms that explain the enzymatic activity are proposed, a kinetic analysis is established for each mechanism and by means of numerical integration results are obtained that are discussed and compared with experimental data. To help and support the results and discussion, molecular docking for substrates (L-tyrosine, hydroquinone, 4-tert-butylphenol, and deoxyarbutin) to both the oxy and met forms of tyrosinase was carried out.

Abbreviations: Enzyme species: Tyr, tyrosinase; Em, met-tyrosinase; EmD, met-tyrosinase/dopa complex; Ed, deoxy-tyrosinase; Eox, oxy-tyrosinase; EoxD, oxy-tyrosinase/dopa complex; EmM, met-tyrosinase/tyrosine complex; EoxM, oxy-tyrosinase/tyrosine complex; EmHQ, met-tyrosinase/hydroquinone complex; EmHHQ, met-tyrosinase/hydroxyhydroquinone complex; EoxHQ, oxy-tyrosinase/hydroquinone complex; EmTBF, met-tyrosinase/4-tertbutylphenol complex; EmTBC, met-tyrosinase/4-tertbutylcatechol complex; EoxTBF, oxy-tyrosinase/4-tertbutylphenol complex; EoxTBC, oxy-tyrosinase/4-tertbutylcatechol complex; EoxD-Arb, oxy-tyrosinase/deoxyarbutin complex; EmD-ArbOH, met-tyrosinase/hydroxydeoxyarbutin complex. **Kinetic parameters:** k_a, deprotonation constant of QH; k_{-a}, protonation constant of Q; K_a, ionization constant for amino group of QH; k_c, specific rate constant for the ring closure of Q to leukodopachrome (intramolecular 1,4 Michael addition) ;K_m^D, Michaelis constant for (D); K_m^{D,O_2}, Michaelis constant for (O_2) in the presence of D; $V_{max}^{D,DC}$, maximum rate of tyrosinase acting on D, calculating by measuring DC; K_m^M, Michaelis constant for (M); K_m^{M,O_2}, Michaelis constant for (O_2) in the presence of M; $K_m^{D(M)}$, Michaelis constant for (D), in the presence of M; $V_{max}^{M,DC}$, maximum rate of tyrosinase acting on M, calculating by measuring DC; K_m^{HQ}, Michaelis constant for (HQ); K_m^{HQ,O_2}, Michaelis constant for (O_2), in the presence of HQ; $V_{max}^{HQ,HPB}$, maximum rate of tyrosinase acting on HQ, calculating by measuring HPB; K_1^{TBF}, dissociation constant of the complex EmTBF; K_m^{TBF}, michaelis constant for (TBF); K_m^{TBF,O_2}, Michaelis constant for (O_2), in the presence of TBF; $V_{max}^{TBF,HTPB}$, maximum rate of tyrosinase acting on TBF, calculating by measuring HTPB; K_m^{D-Arb}, michaelis constant for (D-Arb); K_m^{D-Arb,O_2}, Michaelis constant for (O_2), in the presence of D-Arb; $V_{max}^{D-Arb,P}$, ,maximum rate of tyrosinase acting on D-Arb, calculating by measuring P; τ, lag period. **Physicochemical parameter:** δ, ^{13}C NMR chemical shifts. **Substrates: Monophenols: Type A:** M, L-tyrosine; tyra, tyramine; L-Mme, L-tyrosine methyl ester; sin, synephrine; 4-HA, 4-hydroxyanisole; 4-EP, 4-etoxyphenol; 4-HBA, 4-hydroxybenzyl alcohol; 3-HA, 3-hydroxyanisole; 3-HBA, 3-hydroxybenzyl alcohol; PHPPA, *p*-hydroxyphenylpropionic acid; PHPAA, *p*-hydroxyphenylacetic acid; L-α-mM, L-α-methyltyrosine. **Type B:** HQ, hydroquinone; R, resorcinol; 4-ER, 4-

ethylresorcinol; 2-MR, 2-methylresorcinol; 4-MR, 4-methylresorcinol; BR, 4-*n*-butylresorcinol; HR, 4-hexylresorcinol; OR, oxyresveratrol. **Type C:** 4-TBF, 4-tert-butylphenol; 4-HA, 4-hydroxyanisole; 4-EP, 4-ethoxyphenol. **Type D:** D-Arb, Deoxyarbutin. ***o*-Diphenols:** D, L-dopa; HHQ, hydroxyhydroquinone; TBC, 4-tert-butylcatechol. **Products:** DC, dopachrome; Q, quinone product; QH, *o*-dopaquinone-H^+ (in the amine group); *o*-Q, *o*-dopaquinone; HPB, hydroxy-*p*-benzoquinone; *o*-TBQ, *o*-tert-butylquinone; HTPB, 2-hydroxy-5-tert-butyl-*p*-benzoquinone; P, *o*-quinone derived from the oxidation of D-ArbOH. **Reagents:** MBTH, 3-methyl-2-benzothiazolinone hydrazone.

KEYWORDS: tyrosinase, polyphenol oxidase, monophenols, kinetic mechanism, L-tyrosine.

1. Introduction

Tyrosinase (EC 1.14.18.1), is a cuproprotein that catalyzes the hydroxylation of monophenols to *o*-diphenols through an electrophilic aromatic substitution [1–3] and the oxidation of these to *o*-quinones with the participation of molecular oxygen [4]. The enzyme is involved in the browning of fruits and vegetables [5]. It also participates in the pigmentation of the skin, eye, ear [6].

The active site contains copper that is found in the catalytic cycle in three forms actives in the stationary-phase, depending on its oxidation state: met-tyrosinase in the $Cu^{+2}Cu^{+2}$ form, deoxy-tyrosinase in the $Cu^{+1}Cu^{+1}$ form and oxy-tyrosinase with peroxide in the Cu active site, $Cu^{+2}Cu^{+2}\,O_2^{-2}$ and the inactivated form deact-tyrosinase with $Cu^{0}Cu^{+2}$ [7,8]. All three active forms occur in both diphenolase and monophenolase activity and are represented in Schemes 1 and 2 for diphenolase and monophenolase activity respectively and in its structural form in the Figs. 1 and 2 [9,10].

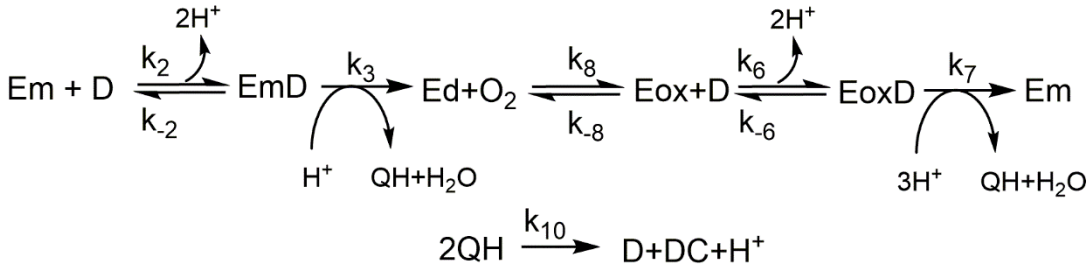
In this review, we consider the enzymatic action on four groups of monophenols studied: a) In the first case, there would be the substrates that originate *o*-quinones whose chemical evolution regenerates *o*-diphenol in the medium, as is the case of the physiological substrate for mammalian tyrosinase, L-tyrosine (M) and related compounds (tyramine (tyra), L-tyrosine methyl ester (L-Mme) and sinephrine (sin)) [9,11–14]. Also in this case would be those substrates whose *o*-quinones, in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) or L-serine, come to generate *o*-diphenol in the medium as is the case of 4-hydroxyanisole (4-HA), 4-etoxyphenol (4-EP), 3-hydroxyanisole (3-HA), 4-hydroxybenzyl alcohol (4-HBA), 3-hydroxybenzyl alcohol (3-HBA), *p*-hydroxyphenylacetic acid (PHPPA), L-α-methyl tyrosine (L-α-mM), and phenol in the case of MBTH and *p*-cresol in the case of L-serine [15–21].

The other three cases correspond to substrates whose quinones in their evolution do not generate *o*-diphenol in the medium or if it is released by the enzyme, it is easily oxidized: hydroquinone (HQ), 4-tert-butylphenol (4-TBF) and deoxyarbutin (D-Arb).

The enzyme tyrosinase shows two activities: diphenolase and monophenolase. In this case, we also consider the diphenolase activity on L-dopa (D) because it helps to understand the monophenolase activity on M,

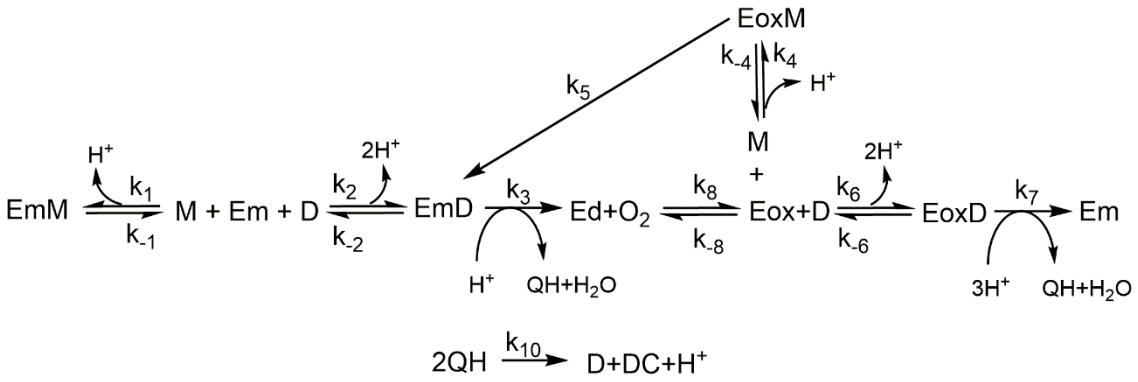
through the instability of *o*-quinone that leads to the accumulation of D in the medium.

The kinetic scheme for the diphenolase activity is:



Scheme 1. Diphenolase activity of tyrosinase.

For the monophenolase activity, the kinetic scheme would be:



Scheme 2. Monophenolase and diphenolase activities of tyrosinase.

The mechanisms described above correspond to the substrates M / D and related compounds such as: tyra, L-Mme and sin [9,11–14], note that in this case, since the evolution of *o*-dopaquinone-H^+ (QH) regenerates *o*-diphenol in the medium and the system is self-activated in the case of M through a delay period, τ [9]. We also include here the substrates whose evolution in the presence of MBTH or L-serine generate *o*-diphenol in the medium. These mechanisms were analyzed in detail in different studies [15–21].

In this review, other monophenols have been selected that have some special characteristics regarding the chemistry of their hydroxylated and quinone derivatives: b) HQ [22], the main characteristic is that the action of the enzyme does not accumulate net *o*-diphenol in the medium, due to the easy oxidation of the *o*-diphenol released hydroxyhydroquinone (HHQ), also in this section we can consider resorcinol (R), 4-ethylresorcinol (4-ER), 2-methylresorcinol (2-MR), 4-methylresorcinol (4-MR), 4-*n*-butylresorcinol (BR), 4-hexylresorcinol (HR) and

oxyresveratrol (OR), c) 4-TBF [23], tyrosinase releases 4-tert-butylcatechol (TBC) to the medium and oxidizes it, generating in the catalysis *o*-tert-butylquinone (*o*-TBQ) which is stable, in addition in this group we can consider: 4-HA and 4-EP and d) D-Arb [24], neither the action of the enzyme nor the evolution of *o*-quinone generate *o*-diphenol in the reaction medium, because *o*-quinone is quite stable.

Through this approach, an attempt is made to confirm the validity of the proposed kinetic mechanism to explain the action of tyrosinase on monophenols [25]. The proposed mechanism has a series of premises. When a substrate does not comply with them, deviations in kinetic behavior arise, as occurs with: HQ, 4-TBF, D-Arb and related compounds. The deviations observed for some monophenols and their explanation at the level of the kinetic mechanism confirm the validity of the kinetic mechanisms proposed for tyrosinase in its action on *o*-diphenols and monophenols.

2. Numerical integration, data analysis and computational docking

2.1. Simulation assays

The simulated progress curves were obtained by numerical integration of the non-linear set of differential equations corresponding to each kinetic scheme (see Supporting Information), using a PC-compatible Computer programme (WES) [26,27].

2.2. Analysis data

The initial rates were represented and fitted to the Michaelis-Menten equation using the Sigma Plot 9.0 program for Windows [28].

2.3. Computational docking

Molecular docking was carried out around the active site of mushroom tyrosinase with all ligands studied. Their chemical structures information were obtained from the PubChem Substance and Compound database [29]. The molecular structure of tyrosinase was taken from the Protein Databank [30] (PDB ID:2Y9W, Chain A), corresponding to the deoxy-form of isoform 3 of tyrosinase from *Agaricus bisporus*. The met and oxy forms of tyrosinase were built as previously described [31]. Gasteiger's partial charges and rotatable bonds were

assigned by AutoDockTools4 software [32,33]. AutoDock 4.2.6 software package was employed for docking calculation [33]. Grid parameter files were built using AutoGrid 4.2.6 [34]. The grid box was centered at the copper ions with a grid size of 35x35x35 grid points (x, y and z), with an spacing of 0.375 Å. Other AutoDock parameters were used with default values. PyMOL 2.3.0 (Schrödinger) was employed to edit and inspect the molecule structures and docked conformations [35].

3. Action of tyrosinase on different types of monophenols

The kinetic mechanism of action of tyrosinase on *o*-diphenols (diphenolase activity) is well established [7,9]. It is represented in Scheme 1 for the general case, in which the QH evolves towards dopachrome (DC) with a defined stoichiometry [36].

In the mechanism of action of tyrosinase on monophenols for its correct kinetic interpretation requires that some starting premises are met [25]:

1. The enzyme, in its native form, needs to have a certain percentage in its Eox form.
2. There is a dead path in the union of M to the form Em, originating the inactive complex EmM (Scheme 2).
3. When the Eox form hydroxylates M to D, passing the EoxM complex to EmD, there are two possibilities which happen simultaneously: to release D or to oxidize it (Scheme 2), as long as the evolution of the oxidation product of D, *o*-quinone, generates D in the medium.
4. For the system to reach a steady-state, a certain concentration of D must accumulate in the medium.
5. Because of the above, there is a lag in the accumulation of the DC product.

The different types of substrates considered are shown in Fig. 3. When the kinetics of the substrate considered does not meet any of the five premises described, the kinetic behavior is separated from that proposed by our group [25].

All ligands studied have some structural properties in common, such as, for example, they have at least one aromatic ring with a phenolic group, and one carbon atom of the aromatic ring in ortho position to the phenol group. These

similarities make the interactions of the ligands in the active center similar, sharing interactions with the same residues.

Thus, we can describe hydrogen bonding interactions from the phenolic group to the oxo group of the oxy form and to the hydroxyl group of the met form. Especially in the met form o-diphenols can interact also with copper atoms through electrostatic interactions, orienting the molecule to facilitate the subsequent oxidation of the o-diphenol group to o-quinone. In the oxy form, the oxo group is close enough to the carbon atom in ortho to the phenol group to allow hydroxylation in ortho position by the monophenolase activity.

The V283 residue (in isoform 3 of tyrosinase from *Agaricus bisporus*) is close to the aromatic ring anchoring the aromatic ring position through hydrophobic interactions. Furthermore, the aromatic ring of the substrate is oriented almost parallel to the imidazole ring of the H263 residue (one of the His residues forming the CuB site) through π-π interactions between its electron clouds of p orbitals. Both interactions help to position the ligand molecule toward the binuclear copper active site.

All these interactions and distances have been summarized in Table 1.

3.1. Substrate L-tyrosine and related compounds. Substrates Type A

The first substrates kinetically studied in detail were M and D [9]. The action of tyrosinase on D is shown in Scheme 1 [36]. The evolution of QH allows the regeneration of D in the medium and this fact is not very important in this activity (diphenolase), however it is key in the monophenolase activity (Scheme 2), because the generation of D from *o*-quinones is essential for the system to reach the steady state after a delay period, τ [25]. In the Supporting Information, the steady-state rate equations are shown for both diphenolase activity, equation (S1), and for monophenolase, equation (S2), applying these equations the kinetic parameters of the enzyme for M and D are obtained (see Table 2) [37]. The set of differential equations that describe these mechanisms are also detailed, the numerical integration of these equations for reasonable values of the rate constants allows obtaining the results of Figs. S1A and S1B for diphenolase activity and those of Figs. 4A and 4B for monophenolase activity. Figs. S1A and 4A show the variation of product concentration (DC) with time. Fig. 4A shows a delay period that varies with the substrate concentration, according to Scheme 2.

In Fig. 4A inset the accumulated D in the medium is represented, with respect to the substrate concentration M. Note the proportionality between the concentration of accumulated D and the concentration of M, this leads to the delay period being longer as M increases, since the system must accumulate more D. In addition, the variation of V_{ss}^{DC} respecting $[M]_0$ is also shown.

The study of the variation of the enzyme concentration is shown in Fig. S1B for diphenolase activity and in Fig. 4B for monophenolase activity. In Fig. 4B the lag period decreases with increasing enzyme concentration. Fig. 4B inset shows the values of the concentration of D accumulated in the medium, with respect to the enzyme concentration. Note the constancy of the D value that does not depend on the enzyme concentration and therefore the lag period decreases with increasing enzyme concentration. Moreover, the variation of V_{ss}^{DC} vs. $[E]_0$ is also shown.

Thus, the data obtained through numerical integration of the Scheme 1 and Scheme 2 mechanisms are in agreement with the experimental results [9] and confirm the validity of the proposed mechanism [25].

The docking from M to the Eox form is shown in Fig. 5, the distance to the ortho position of hydroxylation is 2.8 Ångström (Table 1), there is some evidence that prior to *o*-hydroxylation the incoming monophenol needs to be deprotonated [1–3] and on the other hand, the docking from M to the Em form and from D to the Eox and Em forms are shown in Figs. S2A-S2C of the Supporting Information. It is shown in Fig. 5 that the Eox form can hydroxylate in the ortho position to M and to oxidize to D (Fig. S2B). However, the Em form cannot hydroxylate M (Fig. S2A), however it does oxidize D (Fig. S2C) (Table 1).

Therefore, the case of the mechanism of action on the substrate M meets the basic requirements of the mechanism for monophenolase and diphenolase activities and in the case of monophenolase activity, the conditions described above are met [25].

3.2. Deviations of the kinetic mechanism for monophenolase activity of tyrosinase

Deviations of the kinetic mechanism for the monophenolase activity of tyrosinase can occur if the substrate under study does not meet some of the

premises of the general case described above. Lack of accumulation of *o*-diphenol in the medium, it can occur for several reasons:

i) *o*-Diphenol is very unstable and is oxidized by the oxygen in the medium, this is the case of HQ and related substrates (Fig. 3). The HHQ derivative that is generated in the passage from EoxHQ to EmHHQ, when released, is oxidized by the oxygen in the medium [38].

ii) Another case that may occur is that *o*-diphenol is released and is enzymatically oxidized, originating a very stable *o*-quinone, as is the case with *o*-TBQ, this would correspond to the substrate 4-TBF and related substrates (Fig. 3) [23,39].

Kinetic deviations of the mechanism of action of tyrosinase due to the lack of accumulation of *o*-diphenol in the medium, or else the *o*-diphenol released generates a stable *o*-quinone. We will consider three cases: HQ, 4-TBF, and D-Arb.

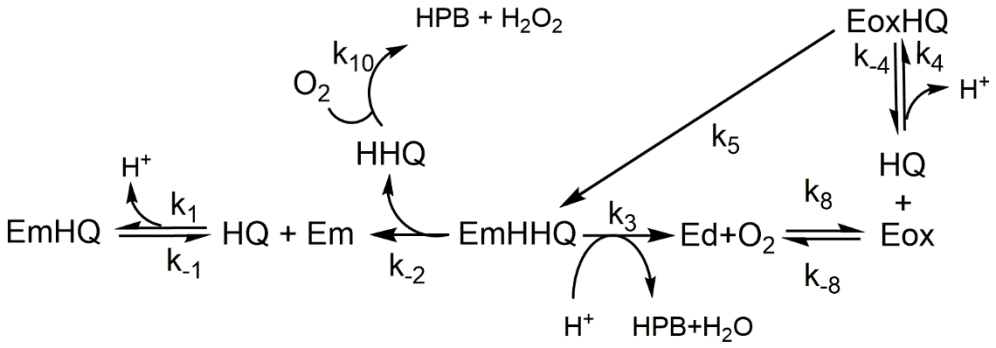
3.2.1. Enzymatic action of tyrosinase on hydroquinone. Substrates Type B

HQ has a chemical structure (Fig. 3) that indicates that it could be an alternative substrate to M for tyrosinase, as it was proposed some time ago [22], however, years later it was proposed that it was not a substrate for the enzyme [40]. Later it was clearly demonstrated that HQ is a tyrosinase substrate in the presence of hydrogen peroxide, ascorbic acid and with catalytic amounts of TBC [38,41]. This property of HQ may be the origin of its adverse effects, although it has been widely used as a depigmenting agent [42]. Due to the adverse effects of HQ, an attempt has been made to replace D-Arb [43]. More recently thiamidol (isobutyl amido thiazolyl resorcinol), has been proposed as a potent inhibitor of human tyrosinase, stronger than HQ, and with a reversible mechanism [44]. A comparative study for the treatment of facial melasma has recently been published between a 0.2% thiamidol preparation versus a 4% HQ preparation, showing similar results obtained. [45].

HQ is a compound known from long time ago with multiple uses, such as depigmenting agent, showing its action mechanism through reduction of QH and blocking the melanin biosynthesis. Today it is well known that HQ can act as an alternative substrate for the enzyme. Just as HQ can be oxidized by tyrosinase, it has been suggested that the adverse effects caused by this substrate may be

due to the fact that HQ is a tyrosinase substrate [22]. HQ can cause ochronosis [46] and is possibly carcinogenic [47], hence it follows that many of the adverse effects are due to this property [22,38,41,48].

The mechanism to explain the action of tyrosinase on HQ is:



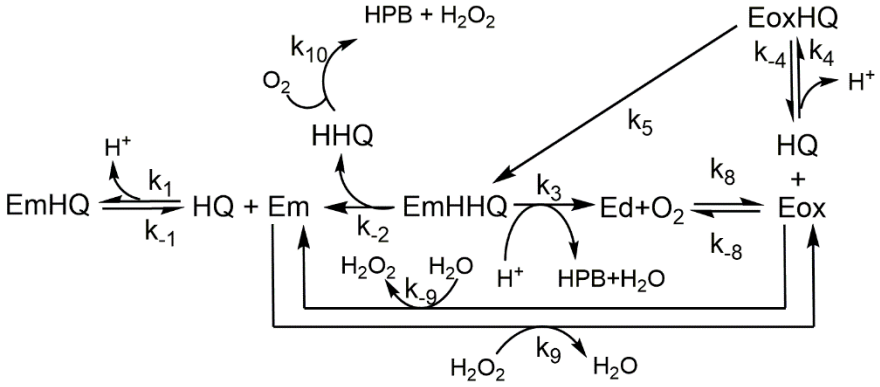
Scheme 3. Action of tyrosinase on hydroquinone.

The hydroxy compound of HQ, HHQ, is unstable and easily oxidizable, it does not accumulate in the medium and therefore the system stops, the catalytic cycle cannot be closed and therefore HQ has not been described as a substrate until recently (see Table 2) [38,41]. The numerical integration of the Scheme 3 mechanism is shown in Fig. S3. The enzyme shows activity on HQ, through the steps governed by k_3 and k_{-2}, it is released as a product, in the first case HPB and in the second case HHQ that is rapidly oxidized by the O_2 of the medium to HPB, until all enzyme is found as Em which is inactive on HQ. In Fig. S3, the effect of substrate concentration, HQ, is shown. The product concentration does not vary for fixed values of k_{-2} and k_3, however, theoretically it does change when these values vary. This simulation shows that the stage governed by k_{-2} must be significant with respect to the one governed by k_3, that is, it indirectly confirms the release of o-diphenol from the EmHHQ complex, otherwise more HPB product would be formed and would be measurable spectrophotometrically.

The formation of hydrogen peroxide in the auto-oxidation of *o*-diphenols has been described if we add D to the medium [49,50]. The formation of hydrogen peroxide by tyrosinase has also been proposed [51,52]. Our group showed that this compound is formed and accumulates in the medium through non-enzymatic reactions [53]. The accumulation of large amounts of hydrogen peroxide by human tumor cells has also been shown [54]. Thus, a contribution of hydrogen peroxide can originate, which can initiate the action of tyrosinase through the

passage of Em to Eoxy, on HQ and other monophenols [55]. Furthermore, hydrogen peroxide helps to reveal alternative substrates for tyrosinase [56].

The mechanism described in Scheme 3 does not allow HHQ to accumulate in the medium, thus tyrosinase does not show activity, apparently, on HQ. However, the addition of hydrogen peroxide achieves the transformation of Em into Eox and thus closes the catalytic cycle (Scheme 4) [38,41].



Scheme 4. Action of tyrosinase on hydroquinone with hydrogen peroxyde in the medium.

The steady-state rate equation in the presence of hydrogen peroxide is shown in the equation (S24). The numerical integration of the set of differential equations that define the mechanism of HQ in the presence of hydrogen peroxide, described in the equation (S32), provide the recordings shown in Figs. 6A-6C. These recordings meet the fundamental dependencies on the concentration of HQ (Fig. 6A), enzyme (Fig. 6B) and H_2O_2 (Fig. 6C).

The most significant characteristic of the action of tyrosinase on HQ is that none of the experimental records described [38,41], as well as the numerical integrations presented in Figs. 6A-6C show lag, since in this case the *o*-diphenol that should accumulating HHQ does not because it is oxidized to HPB. The introduction of hydrogen peroxide that achieves the transformation from Em to Eox, causes the kinetic behaviour to have typical dependencies of an enzymatic reaction, that is, the steady-state speed increases with increasing enzyme and substrate concentration. The dependence of the HPB product concentration on the hydrogen peroxide concentration shows that no intermediate product accumulates in the medium since they do not show a delay period, this case is different from the action of the enzyme on 4-TBF, as will be discussed later.

It is also possible to close the catalytic cycle in the action of tyrosinase on HQ in the presence of ascorbic acid [38] and of TBC in catalytic amounts [41].

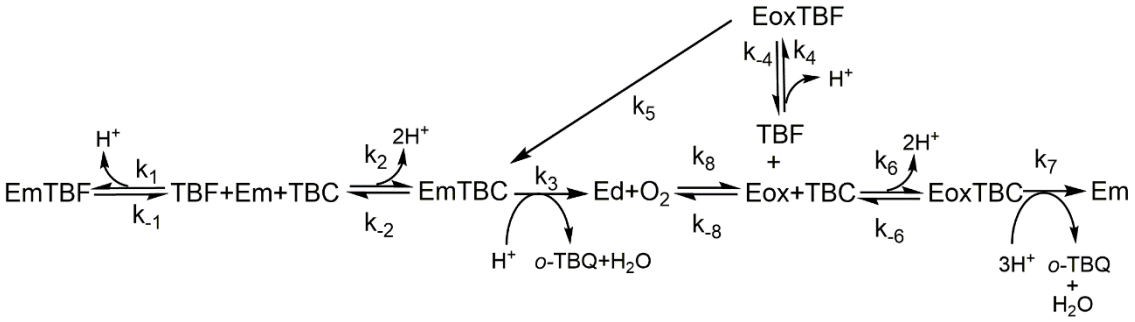
In Fig. 7, the docking of HQ with tyrosinase is shown, the possibility of hydroxylation is real in the Eox form, however the Em form cannot carry out this reaction (Fig. S4) (Table 1).

The study of the action of tyrosinase on HQ does not fulfill one of the premises described in the model proposed by our group, since it does not allow the accumulation of HHQ in the medium. In the study of the action of tyrosinase on HQ, a deviation of the kinetic behavior of the general mechanism is revealed. Other compounds described as inhibitors, but which in the presence of H_2O_2 are substrates of the enzyme with a mechanism similar to HQ are: BR [57], R, 4-ER, 2-MR and 4-MR [58], HR [59] and OR [60].

3.2.2. Action of tyrosinase on 4-tert-butylphenol. Substrates Type C

The kinetics of the action of tyrosinase on 4-TBF was described long ago, resulting in atypical dependencies of the speed of the pseudo steady-state, this was inversely proportional to the substrate concentration and depended on the square of the enzyme concentration [23] (Scheme 5 and equation (S35)). A more detailed study was published years later, obtaining the following kinetic information (Table 2): $K_1^{TBF} \cong 20\ \mu M$ and $k_{cat}^{app} = 4.56 \pm 0.2\ s^{-1}$ [39]. Taking advantage of the stability of *o*-TBQ, 4-TBF was used to demonstrate the enzymatic release of TBC to the medium [61]. Furthermore, 4-TBF was shown not to induce suicidal inactivation of tyrosinase [62]. Due to the high stability of *o*-TBQ originating from 4-TBF and TBC, they are cytotoxic and can cause leukoderma [63–65].

In this case, from the action of tyrosinase on 4-TBF, TBC accumulates in the medium, which is oxidized by the enzyme, originating *o*-TBQ, which is practically stable on the measurement time scale and does not evolve accumulating TBC in the medium and thus the system only reaches a pseudo steady-state [23,39].



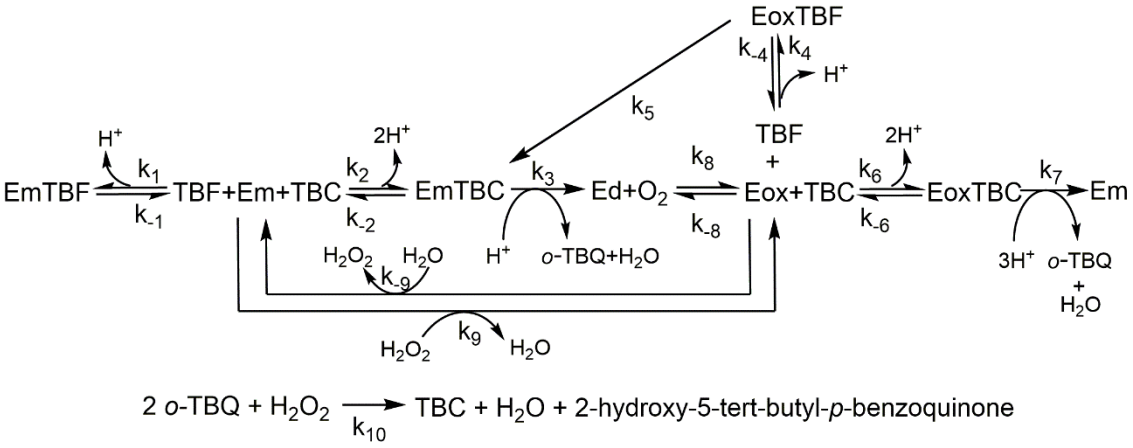
Scheme 5. Action of tyrosinase on 4-tert-butylphenol.

The numerical integration of the set of differential equations that define the Scheme 5 mechanism are described in the Supporting Information. The dependencies of the rate of the pseudo steady-state with respect to the concentration of 4-TBF is expressed in Fig. 8A and with respect to the enzyme concentration in Fig. 8B. These dependencies deviate from the kinetic behavior of the mechanism proposed by our group.

Therefore, the mechanism of the action of tyrosinase on 4-TBF does not fulfill the conditions described for the general mechanism, and therefore it gives an anomalous behavior. Other monophenols that give this type of behavior are: 4-HA and 4-EP [39].

Regarding docking, Fig. 9 shows how the Eox form is capable of hydroxylating 4-TBF (Table 1), however the Em form is not (Fig. S5) (Table 1).

In the presence of hydrogen peroxide, the rate increases and the previous dependencies on the concentration of substrate and enzyme disappear (a value of $K_M^{TBF} = 16\ \mu M$ has been described) (see Table 2) [66]. On the other hand, the speed and the delay period that arises become dependent on hydrogen peroxide according to Scheme 6 [66]. H_2O_2 achieves the passage from met to oxy form (Scheme 6) (Figs. 10A, 10B and 10C).



Scheme 6. Action of tyrosinase on 4-tert-butylphenol with hydrogen peroxide in the medium.

It is important with respect to Scheme 5, to highlight that in this case TBC accumulates in the reaction medium when simulating the behaviour of the system. Note that in this case, the TBC is not easily oxidized by the oxygen in the solution, as in the case of Scheme 3 with the released HHQ. This accumulation of TBC in the medium increases with the concentration of hydrogen peroxide as shown in Fig. 10C inset. This accumulation of TBC gives rise to the presence of a lag in the accumulation of product like that which occurs in Figs. 4A and 4B in the case of M when the concentrations of substrate and enzyme vary.

3.2.3. Action of tyrosinase on deoxyarbutin. Substrates Type D

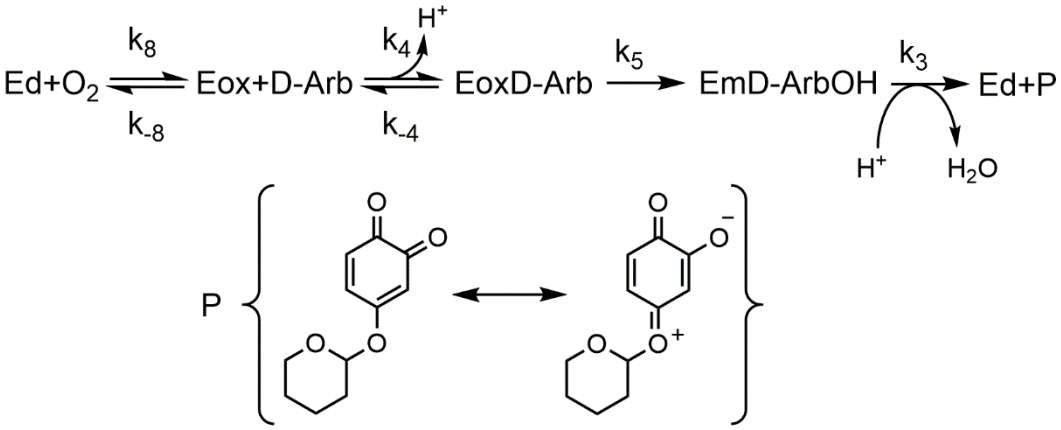
D-Arb is a monophenol derived from HQ, this monophenol is very hydrophobic and its exit from the active site in the form of *o*-diphenol is delayed and therefore, the enzyme that is like Em can oxidize it to *o*-quinone [67].

The action of tyrosinase on D-Arb implies that *o*-diphenol is not released into the medium from the hydroxylated met-tyrosinase-deoxyarbutin complex (EmD-ArbOH, Scheme 7).

In 2005, D-Arb was described as a potent and reversible inhibitor of tyrosinase, even more so than HQ and Arb [24,68]. Later, D-Arb was described as a safe and effective depigmenting agent in its inhibition of tyrosinase [69]. D-Arb could be an alternative to HQ [43] although it has been shown that D-Arb can release HQ [70]. Regarding the safety of the D-Arb compared with arbutins, the Scientific Committee on Consumer Safety (SCCS) has stated that, although the use D-Arb up to 3% has been considered safe, the possible formation of HQ makes this concentration dangerous [71]. D-Arb has been compared with HQ

regarding toxicity, resulting in the one with less toxicity [72]. New anhydrous emulsion formulations have been developed to increase the stability of D-Arb [73]. D-Arb has recently been described as a tyrosinase substrate (see Table 2) [67].

In the action of tyrosinase on D-Arb, *o*-diphenol is not released into the medium. This fact will make this monophenol radically separate from the behavior predicted by our kinetic mechanism of the action of tyrosinase on monophenols. Thus, the product appearance will not show a lag period. The behavior is reduced to that of a michaelian-type enzyme but acting only with the concentration of the Eox form present, according to the mechanism proposed in (Scheme 7). The dependence of the substrate concentration (Fig. 11A) and with respect to the enzyme (Fig. 11B), correspond to a michaelian-type enzyme. The D-Arb docking is shown in Fig. 12, in its union with the Eox form, where the hydroxylation capacity is shown, with respect to the Em form shown in Fig. S6 (Table 1).



Scheme 7. Action mechanism of tyrosinase on deoxyarbutin.

All docking figures (Figs. 5, 7, 9, 12, S2A, S4-S6) show the docking conformations of the substrates studied corresponding to the lower energy conformations in the active site of tyrosinase which could lead to substrate catalysis. Only some residues of the catalytic center have been shown for the sake of clarity. It is interesting to note that all monophenols are well oriented in the oxy form to be hydroxylated at the ortho carbon atom (Table 1, and Figs. 5, 7, 9, 12, S2A, S4-S6). However, diphenols such as D, in the oxy form are oriented with both hydroxyl groups toward the oxo group, being the aromatic ring not parallel to the imidazole ring of H263 residue (Table 1 and Figs. S2B, S2C). This conformation of D facilitates oxidation of the *o*-diphenol to QH (diphenolase

activity). However, in the met form the phenyl ring of D is almost parallel to H263 allowing π-π interaction bringing the hydroxyl groups close to the copper atoms to drive diphenolase activity (Fig. S2C).

The calculated dissociation equilibrium constants from the docking conformations (K_d) for Eox follow the same order than the values obtained experimentally for the K_m (D > HQ ≈ M > D-Arb ≈ 4-TBF) (Tables 1 and 2).

Thus, the behavior of different types of monophenols helps to confirm the mechanism of action of the enzyme. The mechanism is universal, and is fulfilled by monophenols that, through the evolution of their *o*-quinones, allow *o*-diphenol to accumulate in the medium. All the monophenols studied in this work fulfill the fact that the formation of the EmM dead-path complex exists. The other requirement, the accumulation of *o*-diphenol in the medium from the evolution of *o*-quinones, is only fulfilled by the substrate M and related compounds (tyra, L-Mme and sin), as well as the monophenols that are studied in the presence of MBTH (4-HA, 3-HA, 4-HBA, 3-HBA, 4-EP, 3-HBA, PHPPA and PHPAA, L-α-mM and phenol) or L-serine (*p*-cresol). The monophenols described in this work: HQ, 4-TBF and D-Arb give atypical behaviors with respect to the general mechanism (Scheme 2).

3.3. Influence of the presence of o-diphenol on the kinetics of action of tyrosinase on monophenols

This influence can only be considered for the monophenols described in Type A. In these cases, *o*-diphenol accumulates in the medium, either by the chemical reactions that take place from the *o*-quinone or by the oxidation of an adduct between *o*-quinone and a nucleophilic reagent, such as MBTH, by another molecule of *o*-quinone. The concentration of *o*-diphenol that the system has to accumulate to reach steady-state is very low, on the micromolar order, so the enzyme apparently shows two different affinities for *o*-diphenol; when the enzyme acts only in the presence of *o*-diphenol, it does so by showing a K_m^D, equation (S6) and when it acts on monophenol, it accumulates *o*-diphenol in the medium, showing an affinity $K_m^{D(M)}$, equation (S19) (Michaelis constant on *o*-diphenol in the presence of monophenol) [74], where $K_m^{D(M)} \ll K_m^D$. This does not mean that the enzyme has two binding sites for D: in our mechanism [25], there is only one site

for the binding of substrate in the enzyme for M and D, although other authors have proposed two different sites [52]. It should be remembered that these kinetic constants are not binding constants but Michaelis constants with a kinetic meaning. The value of the parameter $K_m^{D(M)}$ can be determined experimentally or through the analytical expression described by equation (S19). Table 2 shows these data for mushroom tyrosinase.

3.4. Influence of oxygen on the catalytic action of tyrosinase on monophenols

As described for the different mechanisms proposed in this review, tyrosinase needs oxygen to hydroxylate monophenols and oxidize *o*-diphenols. In Type A, C and D substrates, this aspect is clear [9,23,67]. However, in Type B substrates, another substrate is necessary, either hydrogen peroxide that passes the met-tyrosinase form to oxy-tyrosinase [38,41,55,56], or reducers such as ascorbic acid or catalytic diphenol in the presence of ascorbic acid, to achieve the transformation from met-tyrosinase to deoxy-tyrosinase [41].

The kinetic characterization of oxygen as a tyrosinase substrate has been difficult especially in the action of the enzyme on monophenols.

More affordable has been the study of oxygen in the case of *o*-diphenols. The first studies with French-prunes tyrosinase [75] and later with mushroom tyrosinase [76], showed a variation in the oxygen Michaelis constant according to the structure of *o*-diphenol studied respectively. It was also described in the study with tyrosinase from the fungus *Neurospora crassa* [77].

The mechanism of action of tyrosinase has been previously mentioned in (24). If we consider the M / D pair, they overlap in the reaction time of hydroxylation of M to D, the oxidation of D to QH and the chemical evolution of this QH towards DC carrying with it the generation of D in the medium [9]. Thus, the first studies on the Michaelis constant for oxygen were carried out with tyrosinase from the bacterium *Streptomyces glaucescens* [78], finding that in the case of *o*-diphenols the activity showed a strong dependence on oxygen concentration, but in the case of monophenols were practically independent, indicating that the Michaelis constants for oxygen in the presence of monophenols should be very low. On the other hand, studies with the graphe

enzyme in its action on monophenols, propose that the mechanism should be different from that of *o*-diphenols [79].

We addressed the kinetic characterization of mushroom tyrosinase in its catalytic action on *o*-diphenols and monophenols in reference [10]. In the case of *o*-diphenols [10,37], the Michaelis constants for molecular oxygen are slightly higher and their determination can be made by the initial rate method or by using the integrated Michaelis equation [10]. However, in the case of monophenols, due to the low values of K_m^{M,O_2}, these methods cannot be used as demonstrated in [10]. Therefore, the analytical expressions for K_m^{M,O_2} must be applied as a function of the rest of the kinetic parameters of the enzyme, equations (S20 or S21) [10,37].

From the physiological point of view, the most important thing is that the enzyme is saturated with oxygen when it acts on monophenols even though its concentration is low; therefore, it does not kinetically limit the melanin biosynthesis pathway.

3.5. Influence of monophenols on the kinetics of suicide inactivation of tyrosinase.

The process of suicide inactivation or mechanism-based inhibitors of tyrosinase has been known for a long time [80–82]. In addition, tyrosinase from different sources has been described in its action on *o*-diphenols [80,83,84]. Our group has carried out kinetic studies of suicide inactivation processes with mushroom and frog skin tyrosinase [85–87] and with peroxidases from different sources [88].

Several models have been proposed to explain the suicide inactivation of tyrosinase, the two oldest proposals are: *i*) the so-called “cresolase presentation” in which *o*-diphenol is presented in the active center of the enzyme as a monophenol and the enzyme hydroxylates it in ortho position [89–93], *ii*) the one proposed by our group that fundamentally consists of the transfer of a proton to hydroperoxide instead of to a base.

These two models have several aspects in common: a) the enzyme is not inactivated in monophenolase activity, b) suicidal inactivation occurs through the Eox form, c) in both models a (Cu^{+2}) atom from the active center passes to (Cu^{0})

and it is released to the medium. However, the first model predicts that suicidal inactivation would only occur when the enzyme acts on *o*-diphenols. The experimental demonstration that tyrosinase is inactivated when acting on triphenols such as: pyrogallol, gallic acid and methyl gallate ruins that hypothesis [94]. Furthermore, the experimental demonstration of tyrosinase inactivation when it acts on 3,6-difluorocatechol, 3-isopropyl-6-methylcatechol and 3-tert-butyl-6-methylcatechol invalidates the first model too [95].

A model has recently been proposed that, according to the authors, measures the singlet oxygen generated by the suicide inactivation pathway [96]. The fact that tyrosinase suffers suicide inactivation when it acts on ascorbic acid [97] would leave out the two alternative mechanisms to our model, because this singlet oxygen would react with L-ascorbic acid. A scheme of our mechanism of suicide inactivation is shown in Fig. 13A (for *o*-diphenols and triphenols), showing that the C-2 ($\delta_{C\text{-}2} > \delta_{C\text{-}1}$) hydroxyl group transferred the proton to the protonated peroxide, which acts as a general base. Now, there is coplanarity between the oxygen of C-1, the copper atom and the substrate ring and this coplanarity of orbitals allows the oxidation of the substrate to *o*-quinone and the reduction of the copper atom (Cu^{+2}), giving rise to (Cu^{0}), which would be released, thus inactivating the enzyme [98]. In Fig. 13B, it is shown the same process for L-ascorbic acid. In this case, C-3 ($\delta_{C\text{-}3} > \delta_{C\text{-}2}$) hydroxyl group transferred the proton to the protonated peroxide [97].

Note that tyrosinase does not undergo suicide inactivation when it acts on monophenols, in this case there is a unique proton transfer as has been shown in [1–3]. However, when the action of tyrosinase on M is studied by measuring the accumulation of dopachrome or the consumption of oxygen a deviation from linearity is observed, this is due to the inactivation of the enzyme on the accumulated *o*-diphenol [99]. The same occurs when tyrosinase acts on 4-TBF and catalytic TBC is added, again suicide inactivation is observed by the action of the enzyme on TBC [62]. When the monophenols studied are M or related compounds, the enzyme undergoes suicide inactivation. In the case of working on monophenols such as HQ [38], *o*-diphenol does not accumulate in the medium and therefore the enzyme does not commit suicide, the same happens with D-Arb [67].

In summary, monophenols (M) protect tyrosinase from suicide inactivation, forming a complex with the form Em (EmM) and with the form Eox (EoxM) (Fig 13), thus not all the enzyme is exposed to this inactivation process. Therefore, tyrosinase undergoes suicidal inactivation by acting on some substrates, in such a way that the formation of cytotoxic products by the enzyme is controlled. The monophenols protect the enzyme from being totally inactivated.

4. Conclusion

The kinetic mechanism of tyrosinase is complex, involving enzymatically catalyzed chemical stages and purely chemical stages, overlapping in time. Diphenolase activity is simple, although it corresponds to a tri-substrate mechanism, two *o*-diphenols and one oxygen, and two *o*-quinones and two water molecules are released. However, the monophenolase activity is more complex. In order for the speed of action of the enzyme to reach a steady-state, a series of aspects are required to be fulfilled, as described in this review. This complexity has led to the description of many monophenols as tyrosinase inhibitors. This aspect must be taken care of, since these compounds, when the chemical conditions of the environment change, can behave as alternative substrates to M. The change in conditions can be caused by the presence of hydrogen peroxide as described with HQ and 4-TBF. Furthermore, the presence of *o*-diphenols such as D can achieve the transformation from Em to Ed and this, with the help of oxygen, to Eox, which can act on monophenols. Thus, the use of compounds with free hydroxyl as inhibitors must be controlled and verified, first, that they are not alternative substrates to M. With respect to the other substrate, O_2, the enzyme is saturated at low concentrations due to the low Michaelis constants with respect to this substrate.

ACKNOWLEDGMENTS

This work was partially supported by the grant from Fundacion Séneca, the Region de Murcia (FS-RM) (20809/PI/18). J. M.-M. only receives funding from internal grants at Northumbria University for consumables and reagents.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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Table 1 Distances from selected ligand groups to the oxy and met forms of tyrosinase. Minimum distances in Ångström are shown from selected ligand groups to tyrosinase in the active site. The calculated dissociation constants (mM) and the free energy of binding (kcal/mol) of the enzyme-ligand complexes in the oxy- and met forms are also indicated.

		Tyrosinase forms											
		oxy						**met**					
Ligands		**Peroxide**	**Cu**	**V283**	**H263**	**K_d**	**ΔG**	**OH**	**Cu**	**V283**	**H263**	**K_d**	**ΔG**
M	OH (phenol)	1.9	4.0	-	-			1.7	2.7	-	-		
	C-ortho	2.8	3.8	-	-	3.3	-3.38	-	-	-	-	2.6	-3.52
	Phenyl ring	-	-	2.7	3.2			-	-	2.6	3.4		
HQ	OH (phenol)	1.7	4.0	-	-			1.7	3.2	-	-		
	C-ortho	3.4	4.2	-	-	3	-3,43	-	-	-	-	2	−3.68
	Phenyl ring	-	-	2.2	3.5			-	-	2.5	3.4		
4-TBF	OH (phenol)	1.8	4.1	-	-			1.6	2.4	-	-		
	C-ortho	2.7	3.7	-	-	1	-4.08	-	-	-	-	0.2	−5.03
	Phenyl ring	-	-	2.9	3.1			-	-	2.6	3.4		
D-Arb	OH (phenol)	2	3.9	-	-			1.7	3.7	-	-		
	C-ortho	2.9	4	-	-	1.08	-4.04	-	-	-	-	1.33	−3.92
	Phenyl ring	-	-	2.4	3.4			-	-	2.6	3.3		
D	OH (phenol)	1.5	3.5	-	-			1.6	2.6	-	-		
	C-ortho	4.7	5.5	-	-	4.2	−3.24	-	-	-	-	0.8	−4.22
	Phenyl ring	-	-	3.2	3.3			-	-	3.2	2.7		

Table 2. Kinetic parameters characterizing the reactions of tyrosinase form mushroom on monophenols, *o*-diphenols and oxygen at 25ºC.

Substrate	K_m^M (μM)	k_{cat}^M (s^{-1})	K_m^{M,O_2} (μM)	K_m^D (μM)	k_{cat}^D (s^{-1})	K_m^{D,O_2} (μM)	$K_m^{D,(M)}$ (μM)	R	References
L-tyrosine	210 ± 10	7.9 ± 0.3	0.52 ± 0.11				29.4 ± 5.6	0.14 ± 0.02	[10,37,74]
Hydroquinone	250 ± 40	23.0 ± 1.1	1 ± 0.2 (a)						[38,41]
4-tert-butylphenol	16	---	---						[23,39,66]
Deoxyarbutin	33 ± 4	1.95 ± 0.06	0.08 ± 0.02 (a)						[67]
L-dopa				800 ± 30	107.4 ± 3.1	4.6 ± 0.9			[10,37]

(a) Considering the kinetic parameters for the reaction of Ed with O_2. $k_{+8} = (2.3 \pm 0.4)\ x\ 10^7\ M^{-1}s^{-1}$ [100] and $K_m^{HQ,O_2} = \frac{k_{cat}^{HQ}}{k_8}$ and $K_m^{D-Arb,O_2} = \frac{k_{cat}^{D-Arb}}{k_8}$ equations (S30) and (S47) respectively.

Figures

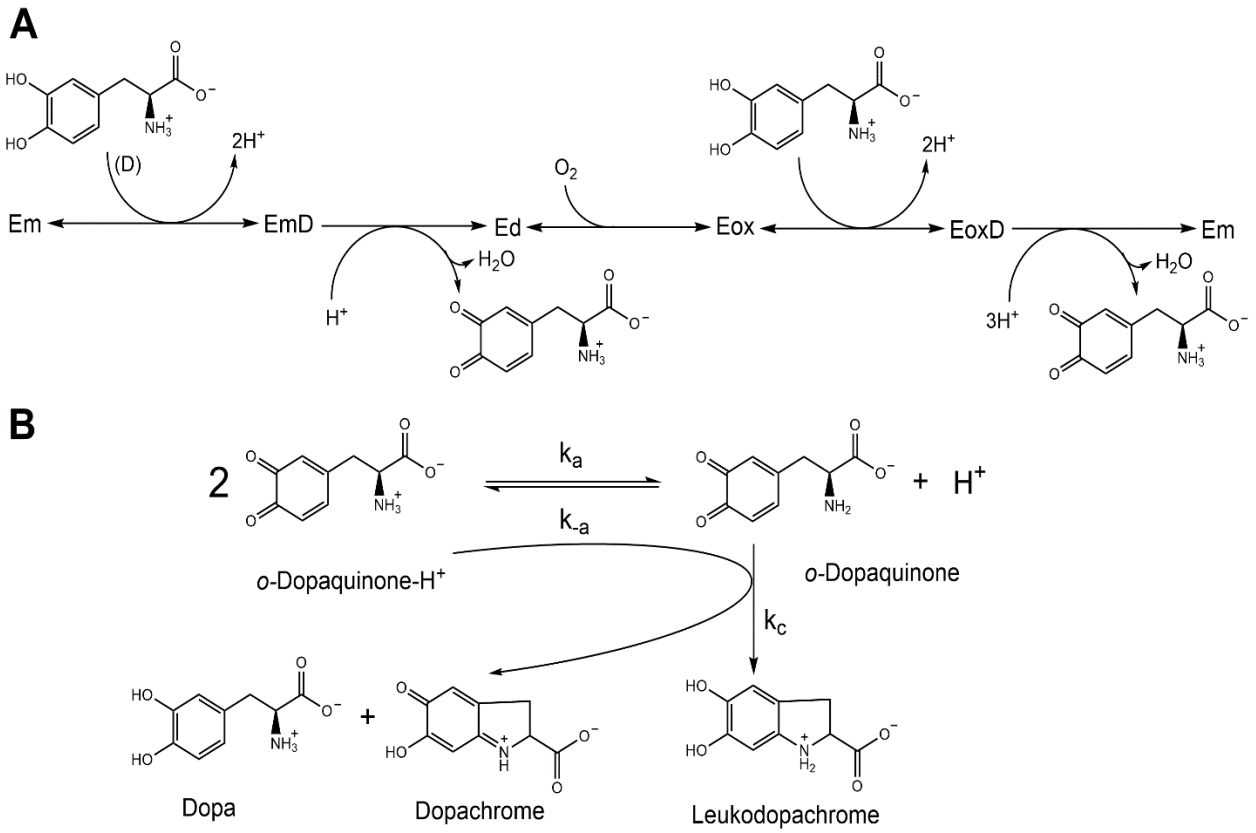

Fig. 1. Diphenolase activity of tyrosinase. A. Enzymatic steps. B. Purely chemical steps of evolution of *o*-dopaquinone-H^+.

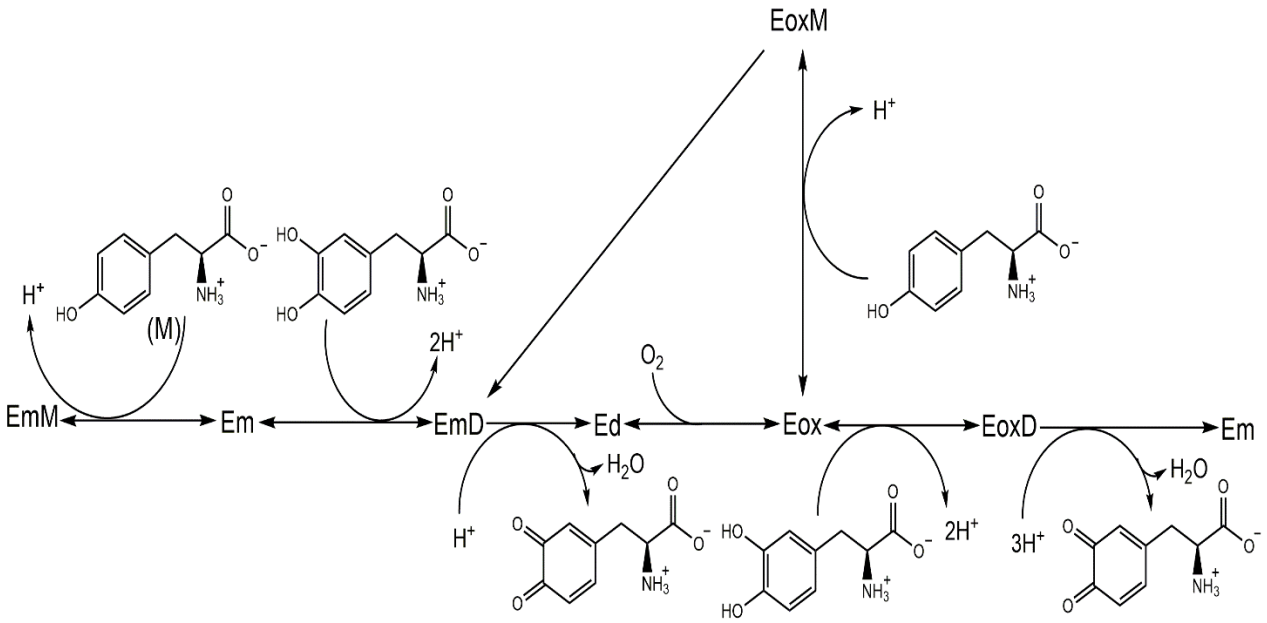

Fig. 2. Monophenolase and diphenolase activities of tyrosinase. Enzymatic steps. Purely chemical steps of the evolution of *o*-dopaquinone-H^+ are the same than the described in Fig. 1B.

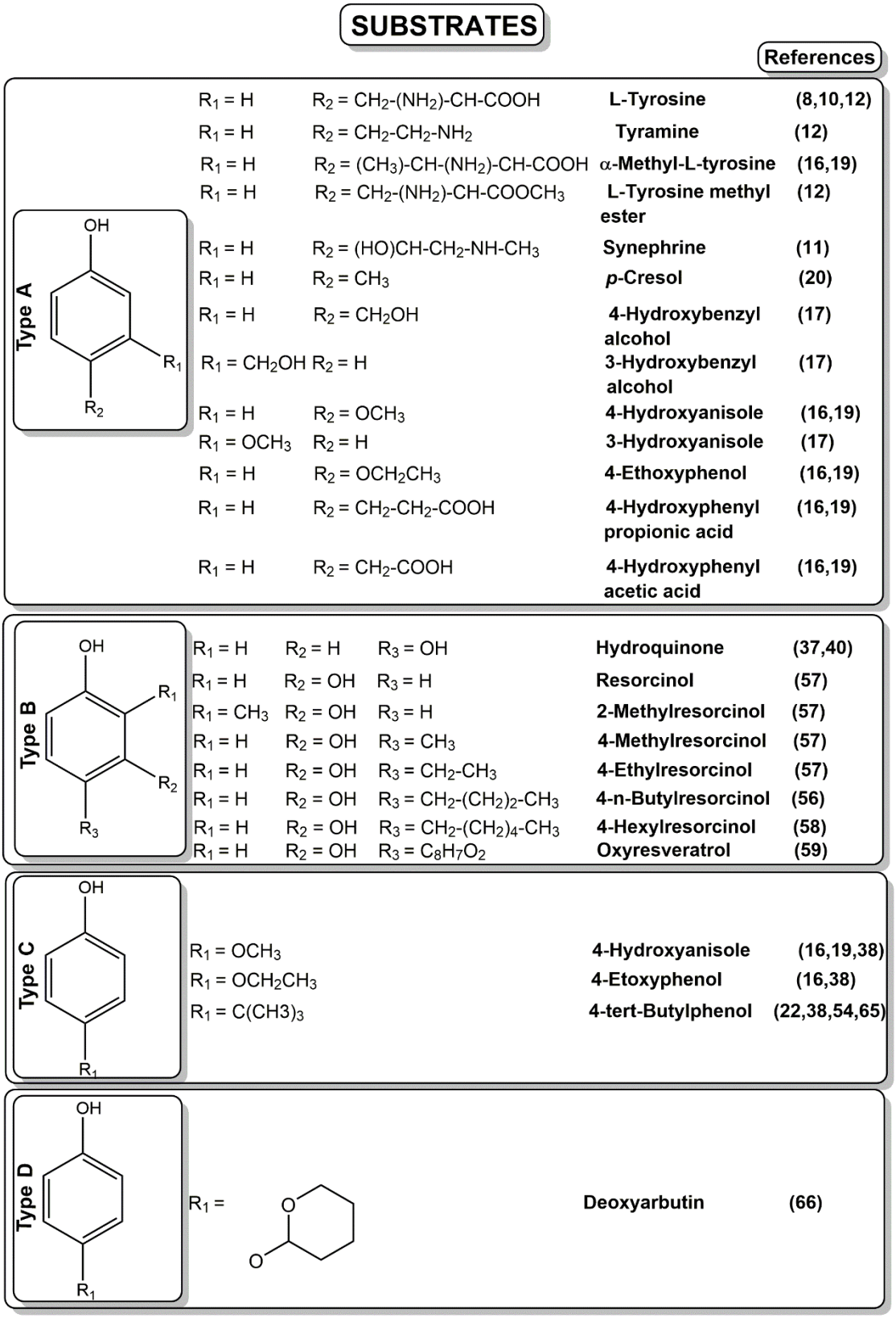

Fig. 3. Chemical structures of the aromatic compounds described in this review.

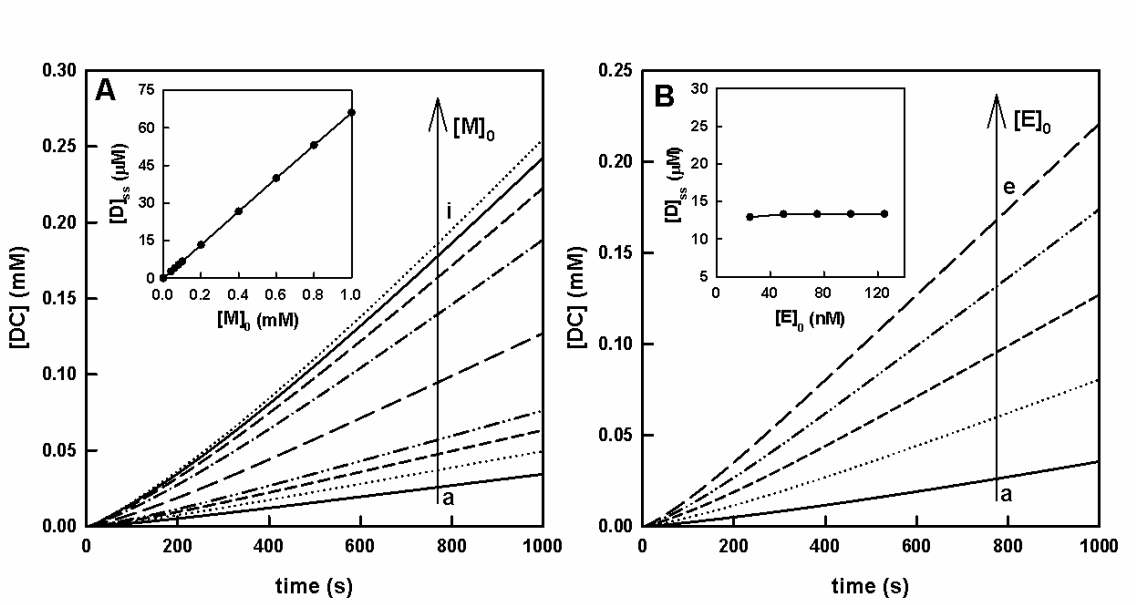

Figure 4

Fig. 4. Simulated curves of tyrosinase action on L-tyrosine (Scheme 2, equation (S23). (A). Effect of substrate concentration variation. Representation of the product concentration $[\mathrm{DC}]$ (mM) vs. time (s). Experimental conditions were: $[\mathrm{M}]_0$ was (mM): a) 0.04, b) 0.06, c) 0.08, d) 0.1, e) 0.2, f) 0.4, g) 0.6, h) 0.8 and i) 1. Enzyme concentration was $[\mathrm{E}]_0 = 50\ \mathrm{nM}$, with $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$ and $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$. Inset. Representation of $[\mathrm{D}]_{\mathrm{ss}}$ vs. $[\mathrm{M}]_0$. (B). Effect of enzyme concentration variation. Representation of product concentration $[\mathrm{DC}]$ (mM) vs. time (s). The conditions were: the enzyme $[\mathrm{E}]_0$ was (nM): a) 10, b) 50, c) 75, d) 100 and e) 125 with $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$ and $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$ $[\mathrm{M}]_0$ concentration was 0.2 mM. Inset. Representation of $[\mathrm{D}]_{\mathrm{ss}}$ vs. $[\mathrm{E}]_0$. The oxygen concentration was $[\mathrm{O_2}]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$ and the rate constants values were: $\mathrm{k}_1 = 3.2\mathrm{x}10^5\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-1} = 10.8\ \mathrm{s}^{-1}$, $\mathrm{k}_2 = 8\mathrm{x}10^6\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-2} = 10\ \mathrm{s}^{-1}$, $\mathrm{k}_3 = 1000\ \mathrm{s}^{-1}$, $\mathrm{k}_4 = 3.7\mathrm{x}10^4\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-4} = 10\ \mathrm{s}^{-1}$, $\mathrm{k}_5 = 7.8\ \mathrm{s}^{-1}$, $\mathrm{k}_6 = 1.33\ 10^5\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-6} = 10\ \mathrm{s}^{-1}$,$\mathrm{k}_7 = 107\ \mathrm{s}^{-1}$, $\mathrm{k}_8 = 2.3\mathrm{x}10^7\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-8} = 1.07\mathrm{x}10^3\ \mathrm{s}^{-1}$, $\mathrm{k}_{10} = 10\ \mathrm{s}^{-1}$.

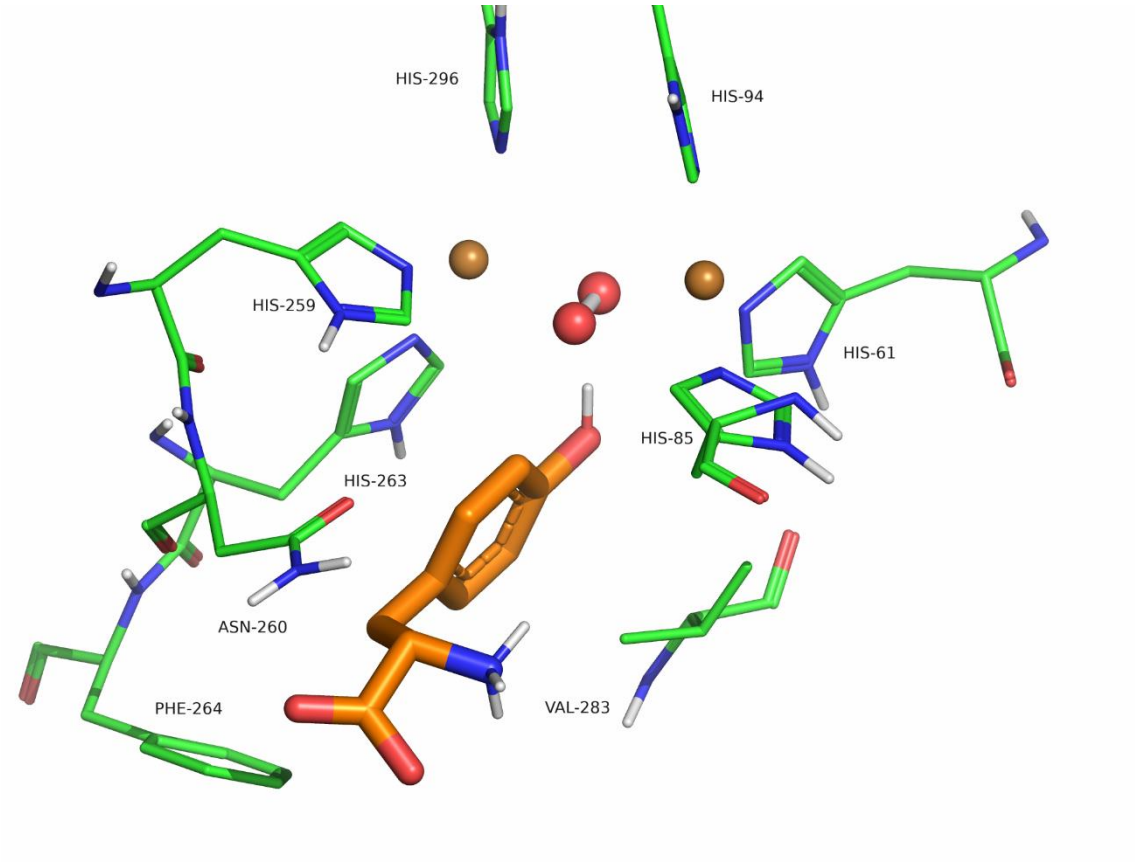

Fig. 5. Computational docking of L-tyrosine in the zwitterionic form at the active site of Eox form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

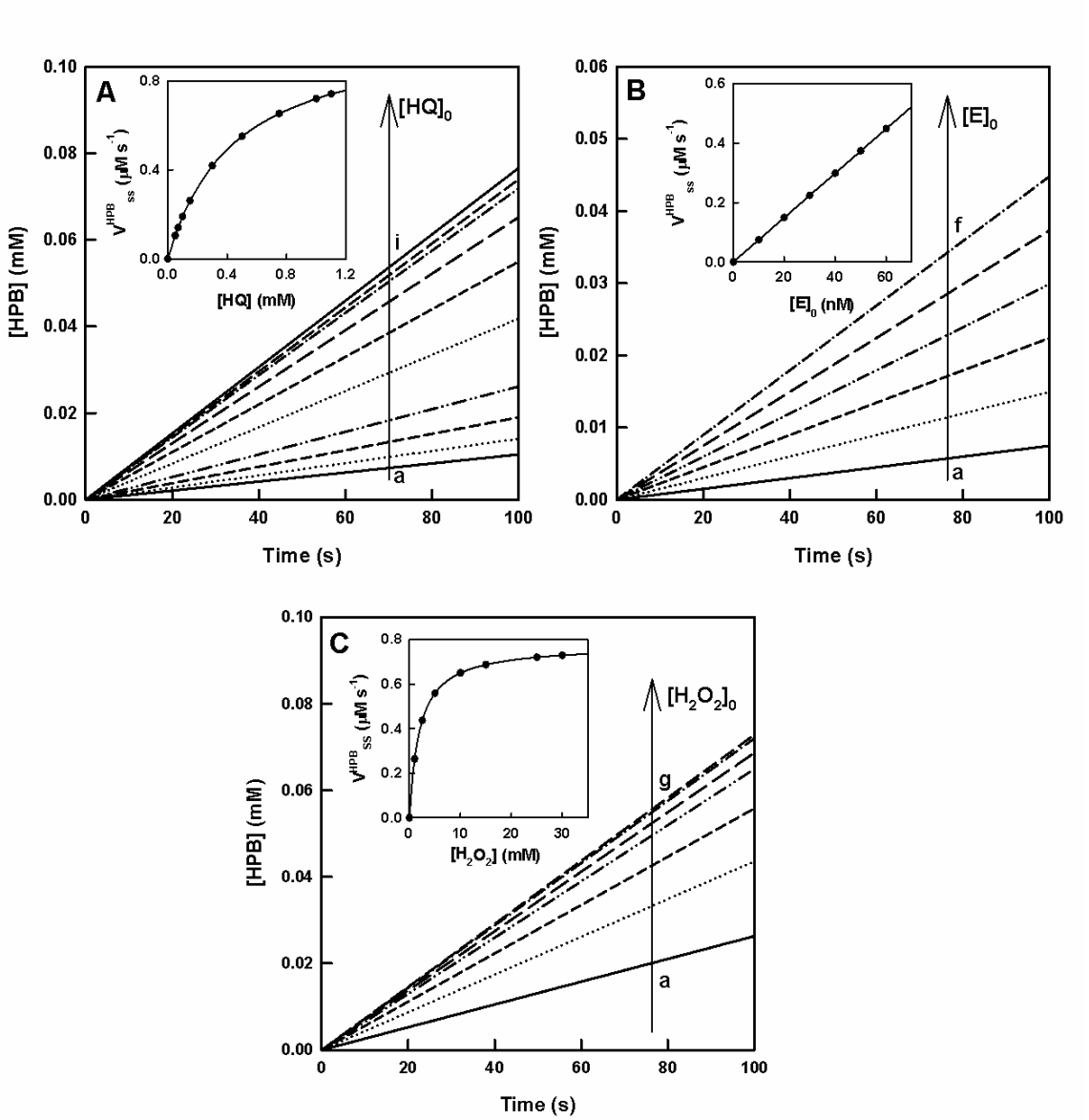

Figure 6

Fig. 6. Simulated curves of tyrosinase action on hydroquinone in presence of hydrogen peroxide (Scheme 4, equation S32). (A). Effect of HQ concentration. Representation of the product concentration $[\mathrm{HPB}]$ (mM) vs. time (s). The experimental conditions were: $[\mathrm{E}]_0 = 50\ \mathrm{nM}$, $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{H_2O_2}]_0 = 25\ \mathrm{mM}$, HQ was varied according to (mM): a) 0.05, b) 0.07, c) 0.1, d) 0.3, e) 0.5, f) 0.75, g) 1, h) 1.1, and i) 1.25. (B). Effect of $[\mathrm{E}]_0$ concentration variation. Representation of product concentration $[\mathrm{HPB}]$ (mM) vs. time (s). The experimental conditions were: $[\mathrm{HQ}]_0 = 0.25\ \mathrm{mM}$, $[\mathrm{H_2O_2}]_0 = 25\ \mathrm{mM}$ and $[\mathrm{E}]_0$ was varied according to (nM): a) 10, b) 20, c) 30, d) 40, e) 50 and f) 60. (C). Effect of $[\mathrm{H_2O_2}]_0$. Concentration variation. Representation of the product concentration $[\mathrm{HPB}]$ (mM) vs. time (s). The experimental conditions were: $[\mathrm{E}]_0 =$

$50\ \mathrm{nM}$, $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{HQ}]_0 = 1\ \mathrm{mM}$ and $[\mathrm{H_2O_2}]_0$ was varied according to (mM): a) 1, b) 2.5, c) 5, d) 10, e) 15, f) 25 and g) 30. The oxygen concentration was $[\mathrm{O_2}]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$ and the rate constants values were: $\mathrm{k_1} = 10^4\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-1}} = 45.6\ \mathrm{s^{-1}}$, $\mathrm{k_{-2}} = 200\ \mathrm{s^{-1}}$, $\mathrm{k_3} = 200\ \mathrm{s^{-1}}$, $\mathrm{k_4} = 9.2\mathrm{x}10^4\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-4}} = 10\ \mathrm{s^{-1}}$, $\mathrm{k_5} = 23\ \mathrm{s^{-1}}$,$\mathrm{k_8} = 2.3\mathrm{x}10^7\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-8}} = 1.07\mathrm{x}10^3\ \mathrm{s^{-1}}$, $\mathrm{k_9} = 2\mathrm{x}10^4\ \mathrm{M^{-1}s^{-1}}$ y $\mathrm{k_{-9}} = 100\ \mathrm{s^{-1}}$.

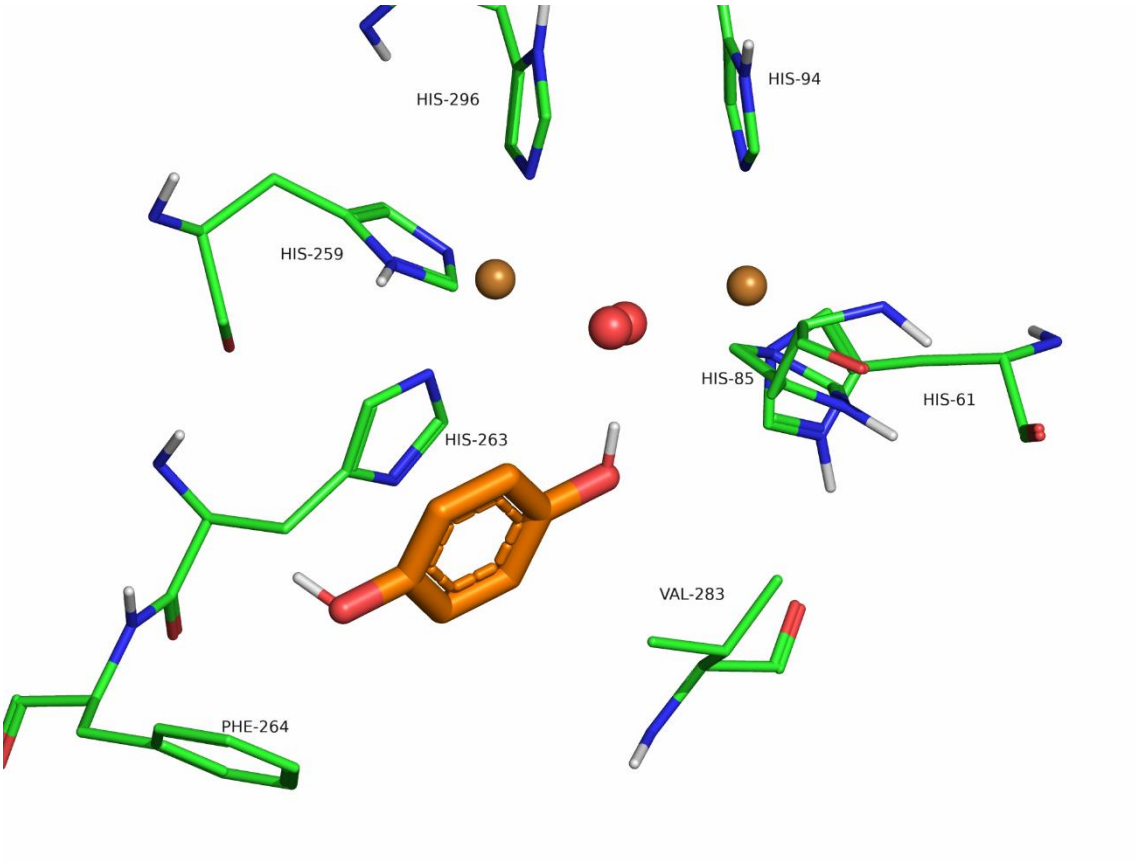

Fig. 7. Computational docking of hydroquinone at the active site of Eox form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

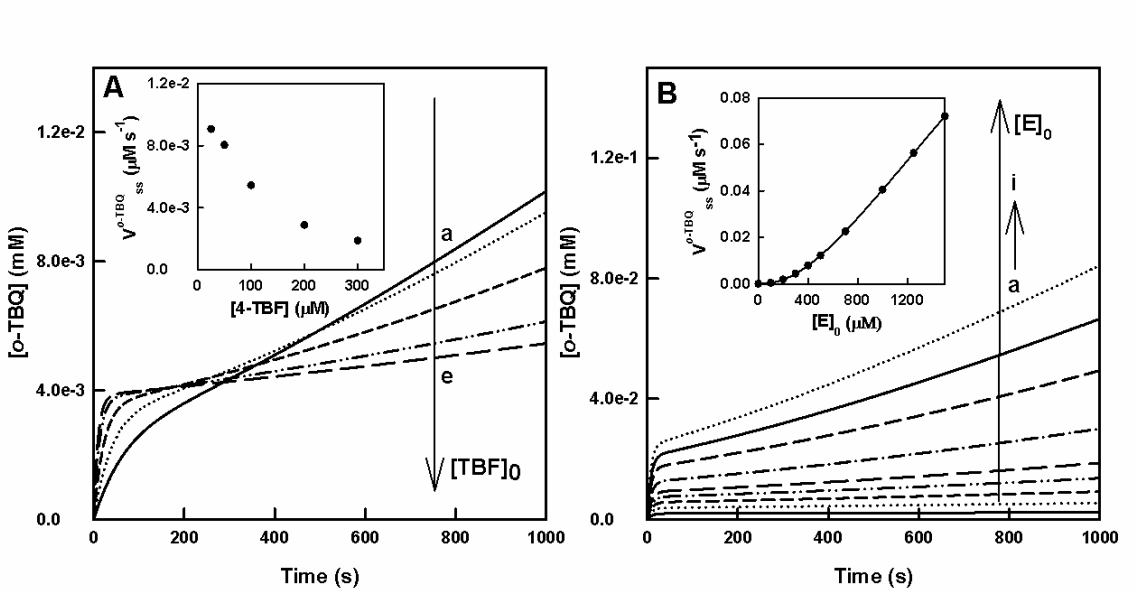

Figure 8

Fig. 8. Simulated curves of tyrosinase action on 4-tert-butylphenol (absence of hydrogen peroxide) (Scheme 5, equation (S41)). (A). Effect of substrate concentration variation. Representation of the product concentration $[o-\mathrm{TBQ}]$ (mM) vs. time (s). The experimental conditions were: $[\mathrm{E}]_0 = 200\ \mathrm{nM}$, $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$. $[\mathrm{TBF}]_0$ concentration was varied according to (μM): a) 25, b) 50, c) 100, d) 200 and e) 300. Inset. Representation of $\mathrm{V}_{\mathrm{ss}}^{o-\mathrm{TBQ}}$ vs. $[\mathrm{TBF}]_0$. (B) Effect of enzyme concentration variation. Representation of the product concentration $[o-\mathrm{TBQ}]$ (mM) vs. time (s). The experimental conditions were: $[\mathrm{TBF}]_0 = 300\ \mu\mathrm{M}$ and the enzyme was varied according to (nM): a) 100, b) 200, c) 300, d) 400, e) 500, f) 700, g) 1000, h) 1250 and i) 1500, keeping always the ratio $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$. Inset. Representation of $\mathrm{V}_{\mathrm{ss}}^{o-\mathrm{TBQ}}$ vs. $[\mathrm{E}]_0$. The oxygen concentration was $[\mathrm{O}_2]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$ and the rate constants values were: $\mathrm{k}_1 = 10^6\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-1} = 20\ \mathrm{s}^{-1}$, $\mathrm{k}_2 = 3\mathrm{x}10^5\ \mathrm{s}^{-1}$, $\mathrm{k}_{-2} = 200\ \mathrm{s}^{-1}$, $\mathrm{k}_3 = 200\ \mathrm{s}^{-1}$, $\mathrm{k}_4 = 9.2\mathrm{x}10^4\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-4} = 10\ \mathrm{s}^{-1}$, $\mathrm{k}_5 = 23\ \mathrm{s}^{-1}$, $\mathrm{k}_6 = 2\mathrm{x}10^5\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, $\mathrm{k}_{-6} = 10\ \mathrm{s}^{-1}$, $\mathrm{k}_7 = 680\ \mathrm{s}^{-1}$, $\mathrm{k}_8 = 2.3\mathrm{x}10^7\ \mathrm{M}^{-1}\mathrm{s}^{-1}$ y $\mathrm{k}_{-8} = 1.07\mathrm{x}10^3\ \mathrm{s}^{-1}$.

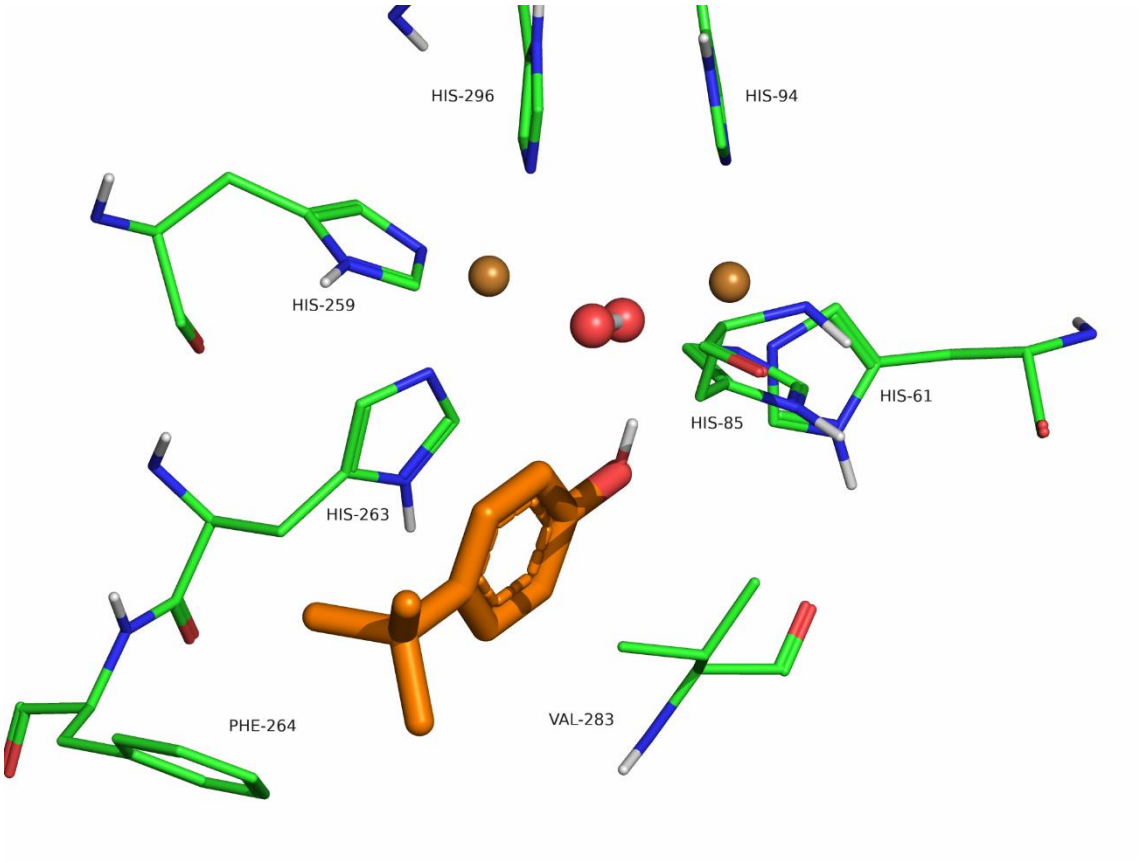

Fig. 9. Computational docking of 4-tert-butylphenol at the active site of Eox form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

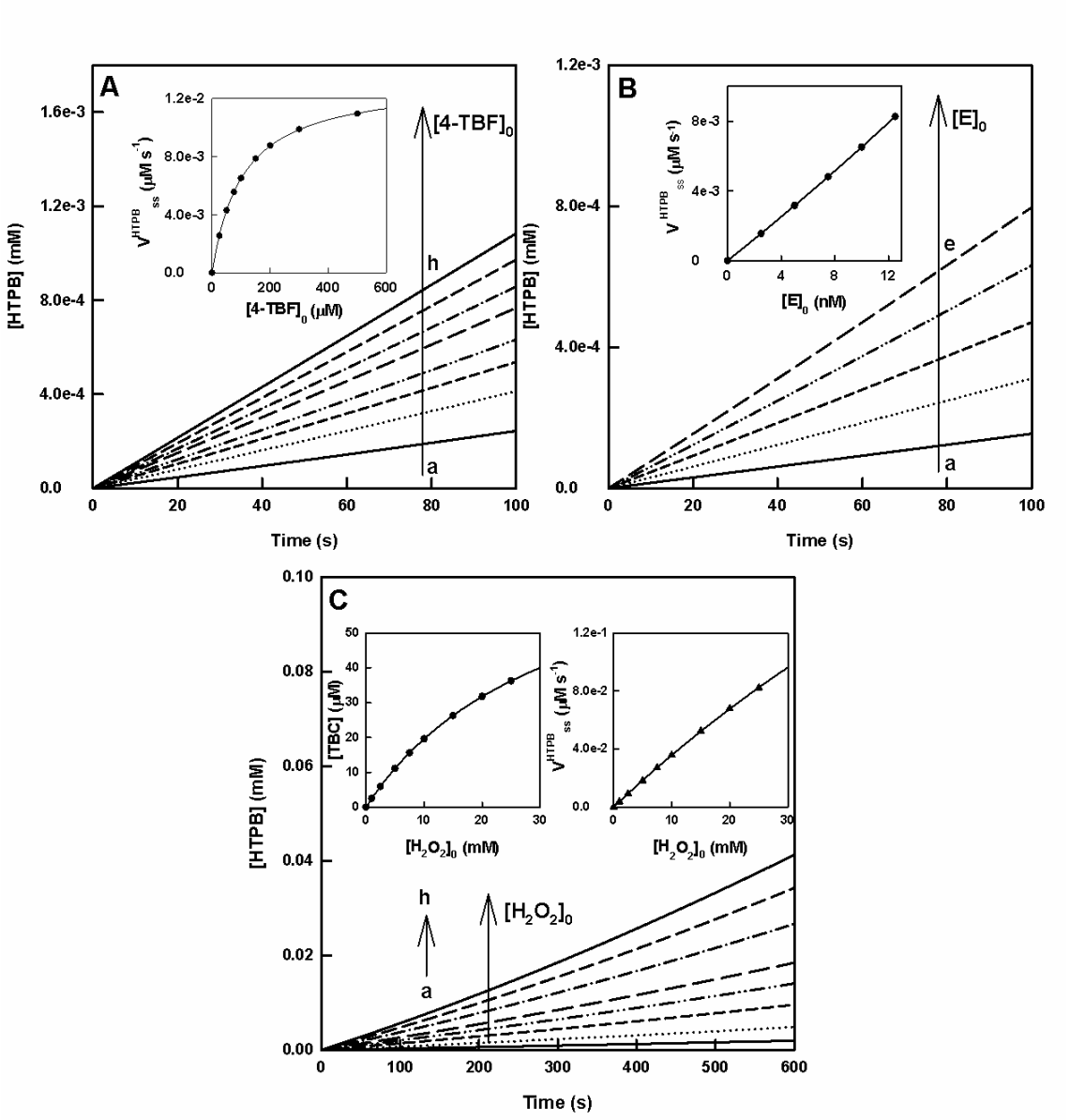

Figure 10

Fig. 10. Simulated curves of tyrosinase action on 4-tert-butylphenol in presence of hydrogen peroxide (Scheme 6, equation (S42)). (A). Effect of substrate concentration variation. Representation of the product concentration $[2-\text{hydroxy}-5-\text{tert}-\text{butyl}-\text{p}-\text{benzoquinone}]$ [HTPB] (mM) *vs.* time (s). The conditions were: $[\text{E}]_0 = 100$ nM, $[\text{Eox}]_0 = 0.3$ x $[\text{E}]_0$, $[\text{Em}]_0 = 0.7$ x $[\text{E}]_0$, $[\text{H}_2\text{O}_2]_0 = 25$ mM and the TBF was varied according to (μM): a) 25, b) 50, c) 75, d) 100, e) 150, f) 200, g) 300 and h) 500. Inset. Representation of $\text{V}_{\text{ss}}^{o-\text{TBQ}}$ *vs.* $[\text{TBF}]_0$. (B) Effect of enzyme concentration variation. Representation of the product concentration [HTPB] (mM) vs. time (s). The conditions were: $[\text{TBF}]_0$ = 100 μM and $[\text{H}_2\text{O}_2]_0 = 25$ mM. The $[\text{E}]_0$ concentration was varied according to (nM): a)

2.5, b) 5, c) 7.5, d) 10 y e) 12.5. (C). Effect of $[H_2O_2]_0$ variation. Representation of the product concentration [HTPB] (mM) vs. time (s). The conditions were: $[E]_0 = 50\ nM$, $[Eox]_0 = 0.3\ x\ [E]_0$, $[Em]_0 = 0.7\ x\ [E]_0$, $[TBF]_0 = 500\ \mu M$ and $[H_2O_2]_0$ was varied according to (mM): a) 1, b) 2.5, c) 5, d) 7.5, e) 10, f) 15, g) 20 and h) 25. The oxygen concentration was $[O_2]_0 = 0.26x10^{-3}M$ and the rate constants values were: $k_1 = 10^6\ M^{-1}s^{-1}$, $k_{-1} = 20\ s^{-1}$, $k_2 = 3x10^5\ s^{-1}$, $k_{-2} = 200\ s^{-1}$, $k_3 = 200\ s^{-1}$, $k_4 = 9.2x10^4\ M^{-1}s^{-1}$, $k_{-4} = 10\ s^{-1}$, $k_5 = 23\ s^{-1}$, $k_6 = 2x10^5\ M^{-1}s^{-1}$, $k_{-6} = 10\ s^{-1}$, $k_7 = 680\ s^{-1}$, $k_8 = 2.3x10^7\ M^{-1}s^{-1}$, $k_{-8} = 1.07x10^3\ s^{-1}$, $k_9 = 2x10^4\ M^{-1}s^{-1}$ y $k_{-9} = 100\ s^{-1}$.

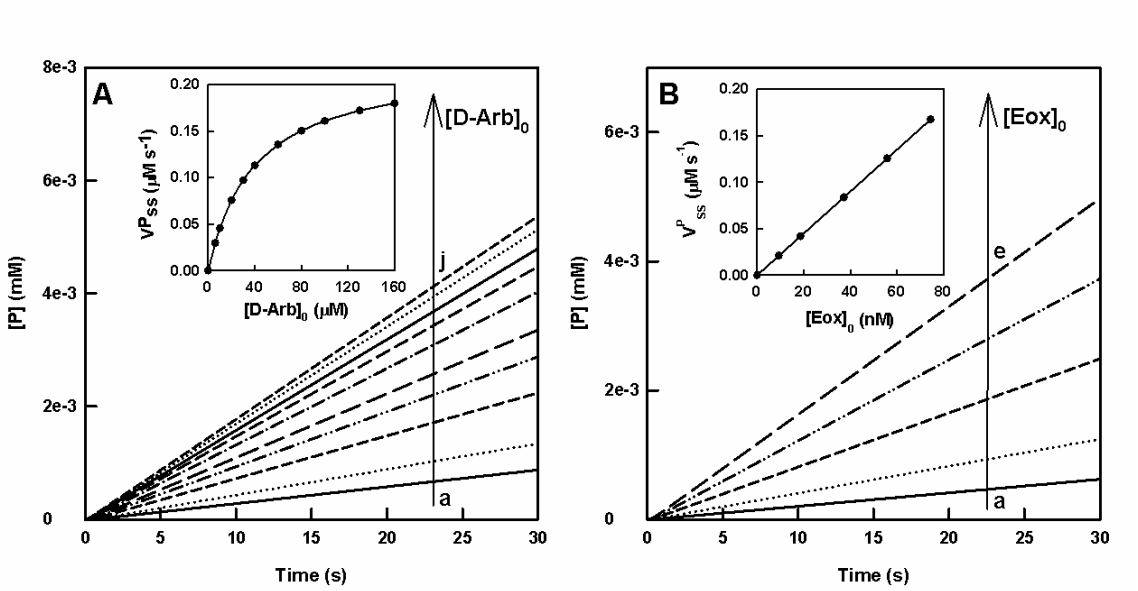

Figure 11

Fig. 11. Simulated curves of tyrosinase action on deoxyarbutin (Scheme 7, equation S48). (A). Effect of substrate concentration variation. Representation of the product concentration $[\mathrm{P}]$ (mM) *vs.* time (s). The experimental conditions were: $[\mathrm{E}]_0$ was 500 nM, with $[\mathrm{Eox}]_0 = 0.3\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{Em}]_0 = 0.7\ \mathrm{x}\ [\mathrm{E}]_0$, $[\mathrm{D-Arb}]_0$ was varied according to (μM): a) 6, b) 10, c) 20, d) 30, e) 40, f) 60, g) 80, h) 100, i) 130 and j) 160. Inset. Representation of $\mathrm{V_{ss}^P}$ (μM $\mathrm{s^{-1}}$) *vs.* $[\mathrm{D-Arb}]_0$ (μM). (B). Efffect of enzyme concentration variation. Representation of the product concentration $[\mathrm{P}]$ (mM) *vs.* time (s). The experimental conditions were: $[\mathrm{D-Arb}]_0 = 50$ μM, and the enzyme concentration was varied according to (nM): a) 25, b) 50, c) 100, d) 150 and e) 200. Inset. Representation of $\mathrm{V_{ss}^P}$ (μM $\mathrm{s^{-1}}$) *vs.* $[\mathrm{E}]_0$ (nM). The oxygen concentration was $[\mathrm{O_2}]_0 = 0.26\mathrm{x}10^{-3}\mathrm{M}$ and the rate constants values were: $\mathrm{k_8} = 2.3\mathrm{x}10^7\ \mathrm{M^{-1}s^{-1}}$, $\mathrm{k_{-8}} = 1.07\mathrm{x}10^3\ \mathrm{s^{-1}}$, $\mathrm{k_4} = 4.5\mathrm{x}10^4\ \mathrm{M^{-1}s^{-1}}, \mathrm{k_{-4}} = 1\mathrm{s^{-1}}, \mathrm{k_5} = 1.5\mathrm{s^{-1}}$ y $\mathrm{k_3} = 300\mathrm{s^{-1}}$.

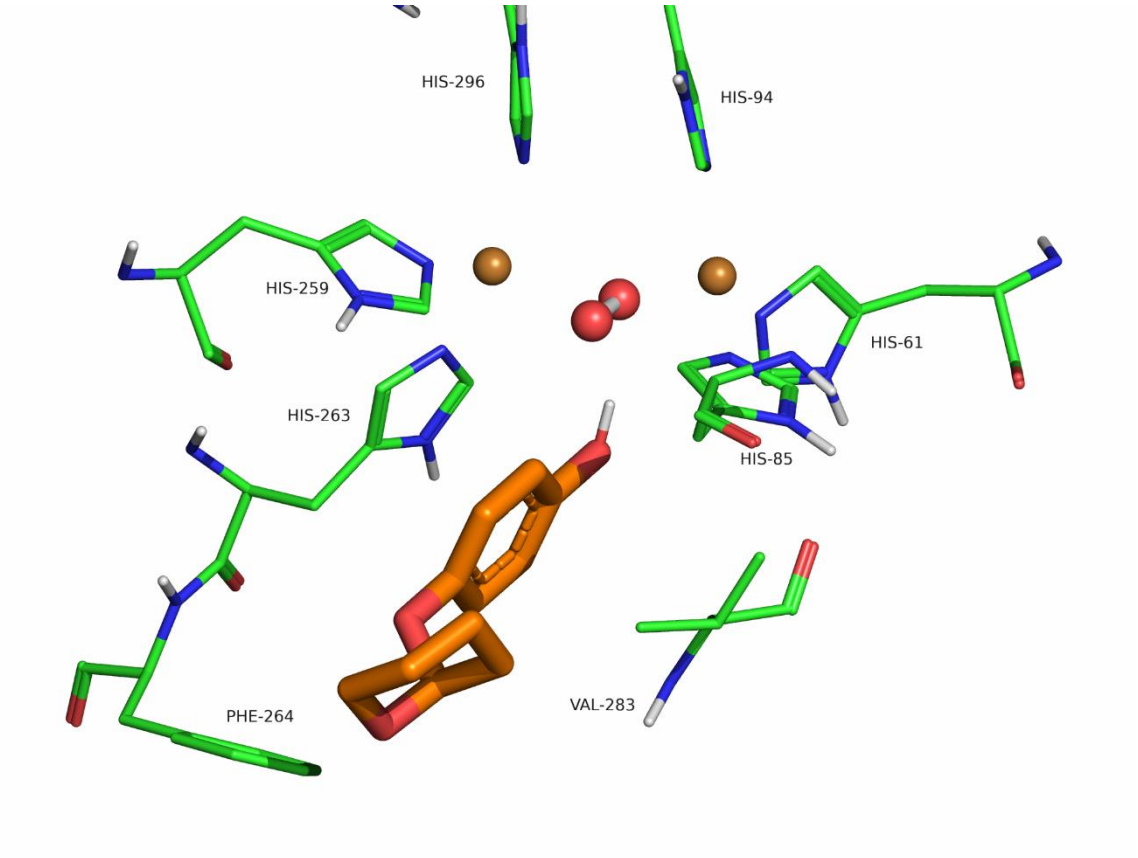

Fig. 12. Computational docking of deoxyarbutin at the active site of Eox form of tyrosinase. The atom colours are as follows: oxygen = red, nitrogen = blue, copper = brown, white = polar hydrogen, and carbon = green in tyrosinase and orange in the ligand.

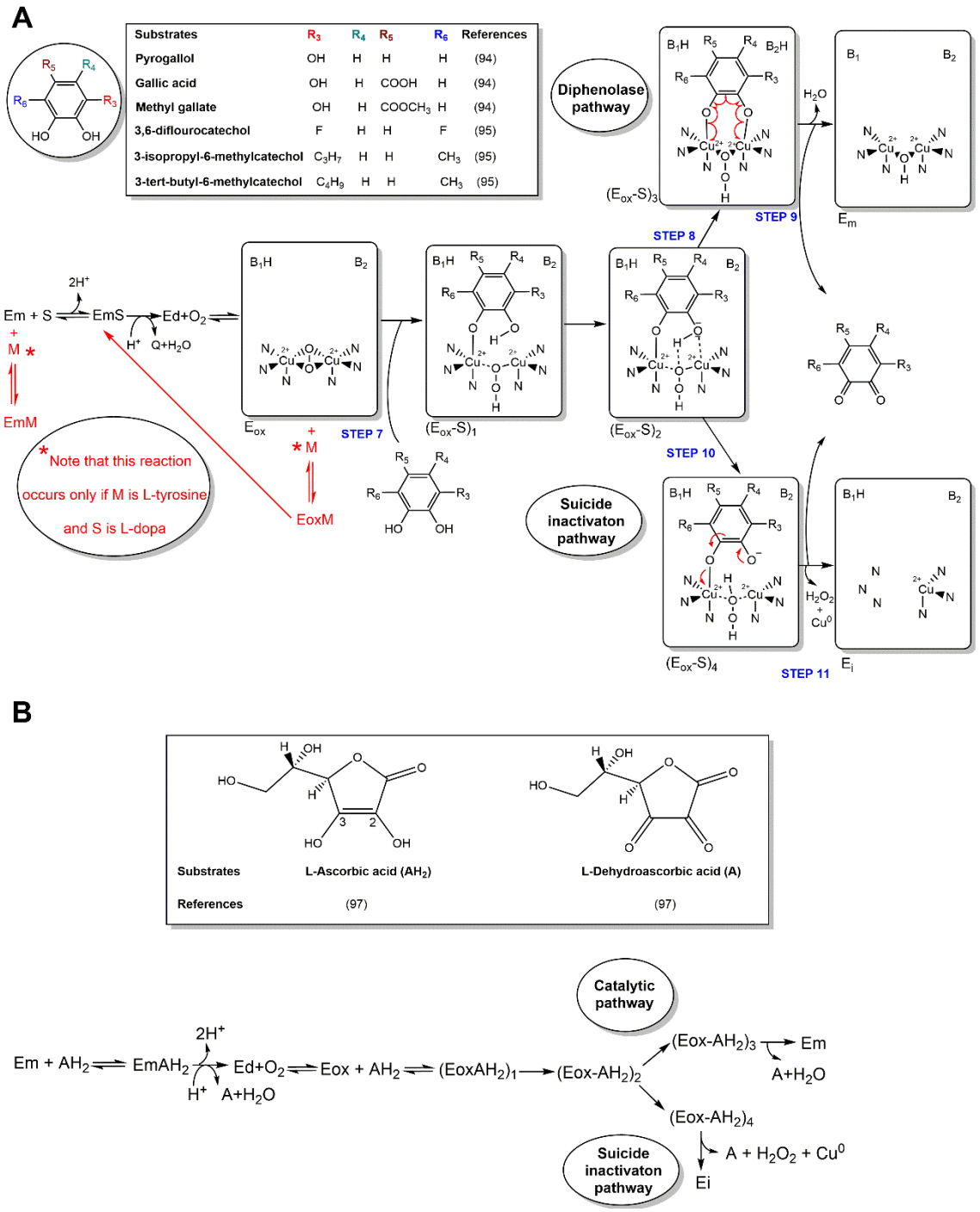

Fig. 13. Kinetic mechanism proposed to explain the suicidal inactivation of tyrosinase in the action on its substrates. A. Suicidal inactivation in action on diphenols and triphenols. The Em form reacts with the substrate to form the EmS complex, quinone is released and the Ed form is produced. The enzyme then binds oxygen giving rise to Eox. In the next step, another molecule of *o*-diphenol or triphenol enters and oxy-tyrosinase / substrate complex is formed $(Eox\text{-}S)_1$. This substrate is axially bound to a (CuB) atom, through the nucleophilic attack of the C-1 hydroxyl group, with the proton of C-1 hydroxyl group transferred to

the peroxide group, being this step the limiting in the catalysis. The proton from the C-2 hydroxyl group forms a hydrogen bridge bond with the oxygen of the hydroperoxide forming the oxy-tyrosinase / substrate complex $(Eox\text{-}S)_2$. Subsequently, the oxygen atom of the second hydroxyl group, bound to the carbon atom with the highest δ, carries out a nucleophilic attack on the other copper atom (CuA) and the proton is transferred to the base B_2, forming $(Eox\text{-}S)_3$, oxy-tyrosinase / substrate complex axially bound to the two (Cu^{+2}) atoms. The coplanarity of orbitals between different atoms enables concerted oxidation/reduction, that is, catalysis. Another possibility is the formation of $(Eox\text{-}S)_4$, oxy-tyrosinase / substrate complex axially bound to (CuB) atom, the deprotonated hydroxyl group of C-2 and the protonated hydroperoxide. The coplanarity respect to (CuB) enables the oxidation/reduction and (Cu^{+2}) is transformed into (Cu^{0}) releasing it from the active site, with H_2O_2 and *o*-quinone. This process results in the inactivation of the enzyme (Ei).

B. Suicidal inactivation in the action of tyrosinase on L-ascorbic acid. The Em form binds to the substrate AH_2, oxidizes it to A and the Ed form is originated, this binds oxygen forming Eox. Subsequently, Eox binds a molecule of AH_2 forming the complex $(Eox\text{-}AH_2)_1$, oxy-tyrosinase / substrate complex axially bound to a (CuB) atom with the proton of C-2 hydroxyl group transferred to the peroxide group. Starting from intermediate $(Eox\text{-}AH_2)_2$ and following a similar sequence described in Fig. 13A, two routes can be given: catalysis $(Eox\text{-}AH_2)_3$ or suicide inactivation $(Eox\text{-}AH_2)_4$, originating (Ei).