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Action of Tyrosinase on Ortho-Substituted Phenols: Possible Influence on Browning and Melanogenesis

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Supporting Information

ABSTRACT: The action of tyrosinase on ortho-substituted monophenols (thymol, carvacrol, guaiacol, butylated hydroxyanisole, eugenol, and isoeugenol) was studied. These monophenols inhibit melanogenesis because they act as alternative substrates to L-tyrosine and L-Dopa in the monophenolase and diphenolase activities, respectively, despite the steric hindrance on the part of the substituent in ortho position with respect to the hydroxyl group. We kinetically characterize the action of tyrosinase on these substrates and assess its possible effect on browning and melanognesis. In general, these compounds are poor substrates of the enzyme, with high Michaelis constant values, K_m, and low catalytic constant values, k_{cat}, so that the catalytic efficiency k_{cat}/K_m is low: thymol, 161 ± 4 M^{-1} s^{-1}; carvacrol, 95 ± 7 M^{-1} s^{-1}; guaiacol, 1160 ± 101 M^{-1} s^{-1}.

KEYWORDS: *tyrosinase, melanogenesis, monophenols, thymol, carvacrol*

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a binuclear copper-containing enzyme that catalyzes the conversion of a monophenol (L-tyrosine) and/or *o*-diphenol (L-Dopa) into the corresponding *o*-quinone (*o*-dopaquinone), which is further converted into melanin. Melanin is a pigment that is ubiquitously present in living organisms from all phyla. Since tyrosinase is the key enzyme in the first step of melanin biosynthesis, the enzyme is frequently associated with pigmentation.[1]

The conversion of *o*-dopaquinone into melanin involves a series of enzymatic and chemical reactions. The *o*-dopaquinone must first lose a proton from its amino group, whose nitrogen makes a nucleophilic attack on the quinone ring. Following a Michael intramolecular 1,4 addition, this process leads to the formation of leucodopachrome, which is then oxidized by another molecule of *o*-dopaquinone, regenerating L-Dopa in the medium and forming dopachrome. This last compound can be attacked by enzymes related to tyrosinase (TRP1) and (TRP2), or the same reactions can occur chemically.[2−4]

Apart from this, undesirable browning is a considerable problem in the food industry and for cosmetics users. For this reason, antibrowning reagents are frequently used and there is keen competition to find natural alternatives. In this respect, natural compounds such as thymol and carvacrol, which are constituents of thyme, an herb of the genus *Thymus*, have been described as having antimicrobial[5−7] and antioxidant[8−10] activities, both acting as inhibitors of melanin biosynthesis.[11] The inhibition of melanogenesis by thymol has been attributed to the inhibition of the redox reaction between dopaquinone and leucodopachrome without any interaction with tyrosinase.[11]

In previous studies, we studied the kinetics of the reactions that occur between the *o*-quinones generated enzymatically by tyrosinase from L-Dopa,[2] dopamine,[12] α-methyl-L-Dopa,[13] noradrenaline,[14] adrenaline,[15] and isoproterenol[16] and their respective aminochromes. These studies showed that in the evolution of *o*-quinones to aminochromes, leucoaminochrome-type compounds are formed. These are rapidly oxidized by the *o*-quinones so that it would seem difficult for this reaction to be inhibited by ortho-substituted monophenols like thymol and carvacrol.

Compounds extracted from *Thymus* are very interesting for their antioxidant, antimicrobial, and recently discovered antimelanogenic properties.[11] The aim of this study was to characterize the antimelanogenic mechanism of these compounds and to provide information on the behavior of the ortho-substituted monophenols during melanogenesis and browning.

MATERIALS AND METHODS

Enzyme Source. Mushroom tyrosinase or polyphenol oxidaze (*o*-diphenol, oxygen-oxidoreductase, EC 1.14.18.1, 4276 U/mg) was supplied by Sigma (Madrid, Spain). The enzyme was purified as previously described.[17] Protein concentration was determined by Bradford's method using bovine serum albumin as standard.[18]

Reagents. 4-Hydroxyphenylalanine (L-tyrosine), 3,4-dihydroxyphenylalanine (L-Dopa), 2-isopropyl-5-methylphenol (thymol), 5-isopropyl-2-methylphenol (carvacrol), 2-methoxyphenol (guaiacol), 4-allyl-2-methoxyphenol (eugenol), 2-methoxy-4-propenylphenol (iso-

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eugenol), butylated hydroxyanisole (BHA), 3-methoxycatechol (3MC), 3-isopropyl-6-methylcatechol (3I6MC), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH), *N*,*N*-dimethylformamide (DMF), aluminum oxide, ammonium acetate, and 4-*tert*-butylcatechol (TBC) were all from Sigma (Madrid, Spain). The corresponding structures of the monophenols studied are shown in (Figure 1). The chemicals were of analytical grade.

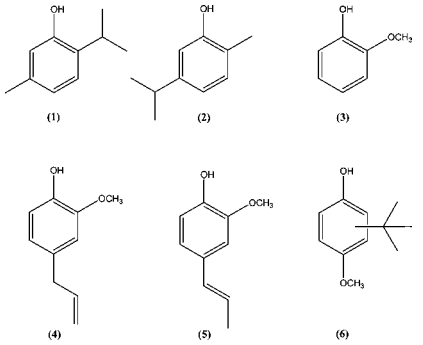

Figure 1. Structures of the compounds used in the present study: **1**, thymol (2-isopropyl-5-methylphenol); **2**, carvacrol (5-isopropyl-2-methylphenol); **3**, guaiacol (2-methoxyphenol); **4**, eugenol (4-allyl-2-methoxyphenol); **5**, isoeugenol (2-methoxy-4-propenylphenol); **6**, BHA (butylated hydroxyanisole).

Spectrophotometric Assays. These enzymatic assays were carried out with a Perkin-Elmer Lambda-35 spectrophotometer (Waltham, MA, U.S.), online-interfaced with a PC computer, where the kinetic data were recorded, stored, and later analyzed. The monophenolase activity of tyrosinase on L-tyrosine was followed by measuring the accumulation of dopachrome at pH 7.0, wavelength 475 nm (ε = 3500 M^{-1} cm^{-1}).[19] The conditions of the assay are specified in the corresponding figure captions.

The enzymatic activity of the tyrosinase on the monophenols thymol (**1**) and carvacrol (**2**) was followed by measuring the formation of the *o*-quinone corresponding to 3I6MC. The molar absorptivity coefficient was determined by oxidizing the 3I6MC at pH 7.0 with excess sodium periodate and measuring at 437 nm (ε = 2166 M^{-1} cm^{-1}). The assays involving monophenolase activity on **1** and **2** were carried out by adding catalytic quantities of 3I6MC at a constant ratio [3I6MC]/[monophenol] = 0.055.[20]

In the case of guaiacol (**3**), the enzymatic activity was followed by measuring the formation of the *o*-quinone at the isosbestic point that occurs during its evolution when it is generated through the oxidation of 3-methoxycatechol (3MC),[21] at a wavelength of 337 nm with a molar absorptivity coefficient ε = 1555 M^{-1} cm^{-1}. The assays of enzymatic activity on guaiacol were made by adding catalytic quantities of 3MC at a constant ratio, [3MC]/[guaiacol] = 0.06.[20,21]

As for the monophenols eugenol (**4**), isoeugenol (**5**), and butylated hydroxyanisole (**6**), the corresponding *o*-diphenol is not commercially available, and so it is difficult to obtain the molar absorptivity coefficient of the *o*-quinone. It is known that MBTH generates colored adducts with a high molar absorptivity coefficient by means of a nucleophilic attack on the *o*-quinones,[22,23] and given the low enzyme activity on these compounds, this property is used to follow the enzyme's activity.

The diphenolase activity of tyrosinase was followed by measuring the accumulation of 4-*tert*-butyl-*o*-benzoquinone at pH 7 in the case of TBC, at 400 nm (ε = 1150 M^{-1} cm^{-1}).[19] In the case of L-Dopa, the accumulation of dopachrome at pH 7.0 was measured at a wavelength of 475 nm (ε = 3600 M^{-1} cm^{-1}).[19]

The experimental conditions were 30 mM phosphate buffer, pH 7.0, at 25 °C. The monophenols studied were purified of possible *o*-diphenol contamination by passing through a 1 cm diameter column containing 2 g of aluminum oxide suspended in 0.5 M ammonium acetate, pH 6.1.[24]

Oxymetric Assays. Oxygen evolution was measured with a Clark-type electrode coupled to a Hansatech Oxygraph (King's Lynn, Norfolk, U.K.). The equipment was calibrated using the tyrosinase/4-*tert*-butylcatechol method.[25] Nitrogen was bubbled through the reaction medium to remove the oxygen when necessary. The reaction medium contained 30 mM sodium phosphate buffer, pH 7.0. The medium was stirred constantly, and the temperature was kept at 25 ± 0.1 °C using a Haake D1G circulating water bath.

Kinetic Analysis Data. For tyrosinase, the initial rate values (V_0) were calculated from triplicate measurements at each reducing substrate concentration, and V_0 vs $[S]_o$ data were adjusted to the Michaelis–Menten equation through the Sigma Plot 9.0 program for Windows,[26] obtaining the maximum rate (V_{max}) and Michaelis constant (K_m) when possible. In those cases where it is not possible to experimentally achieve saturation, the apparent second-order constant k_{cat}/K_m is obtained.

Table 1. Percentage Inhibition of Monophenolase and Diphenolase Activities of Tyrosinase in the Presence of Different Ortho-Substituted Monophenols and δ_1 for C-1 of the Benzene Ring of Ortho-Substituted Monophenols

ortho-substituted monophenol	δ_1 (ppm)	monophenolase activity (%)	diphenolase activity (%)
none		0	0
1	154.60	52.46	29.62
2	154.36	62.30	27.67
3	145.54	55.74	7.08
4	143.73	79.24	68.44
5	144.73	91.81	69.42
6	150.06	4.92	16.50

Determination of ^{13}C NMR Chemical Shift. The carbon chemical shifts given in Table 1 were obtained from the corresponding ^{13}C NMR spectra, which were recorded at 298 K on a Bruker Avance 400 Hz instrument employing buffered solutions (at pH 7.0) of pure samples in D_2O.[27,28]

RESULTS AND DISCUSSION

Effect of Ortho-Substituted Monophenols on Tyrosinase Activity. To study the action of the monophenols described in (Figure 1) on the monophenolase and diphenolase activities of tyrosinase, the effect of different concentrations of **1** on the formation of dopachrome from L-tyrosine is depicted in (Figure 2). This experiment is similar to that described by Satooka and Kubo.[11] Note that by use of low concentrations of L-tyrosine (0.1 mM) and very long measuring times, the system does not reach the steady-state and dopachrome is only accumulated as a result of a balance between its formation and destruction rates, since (as indicated in Supporting Information) the dopachrome evolves toward indoles.[29]

To study enzymatic inhibition, it is convenient to work with systems in their steady-state. However, note in Figure 2A how the presence of **1** (curves b–d) diminishes the accumulation of dopachrome with respect to curve a as a result of the decreased formation rate, possibly due to inhibition. In a shorter time represented in Figure 2B, the accumulation of dopachrome when tyrosinase acts on L-tyrosine (curve a) is described while the addition of **1** (curve b) decreases the velocity of the same, and the joint addition of **1** and **6** (curve c) apparently reduces part of the inhibition caused by **1**. In other words, **6** apparently activates dopachrome accumulation in the medium (an aspect that will be discussed below).

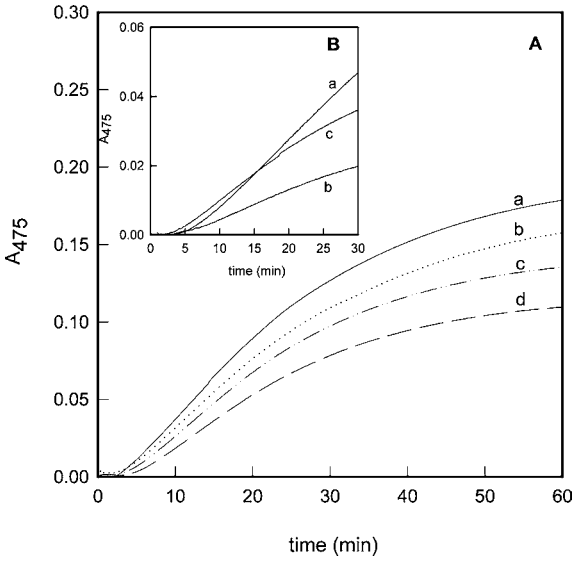

Figure 2. (A) Action of tyrosinase on L-tyrosine: recording of the accumulation of dopachrome at 475 nm in the action of tyrosinase, 4 nM, on L-tyrosine (0.1 mM) in the absence (a) and presence of **1** (b–d). Curves b–d were obtained in the same conditions as curve a but in the presence of **1** (mM): 0.1 (b), 0.2 (c), and 0.3 (d). (B) Accumulation of dopachrome at 475 nm in the action of tyrosinase, 1.2 nM, on L-tyrosine (0.1 mM) in the absence of **1** (a). Curve b was obtained in the same conditions as curve a in the presence of 0.1 mM **1**. Curve c was obtained in the same conditions as curve b but adding 0.1 mM **6**.

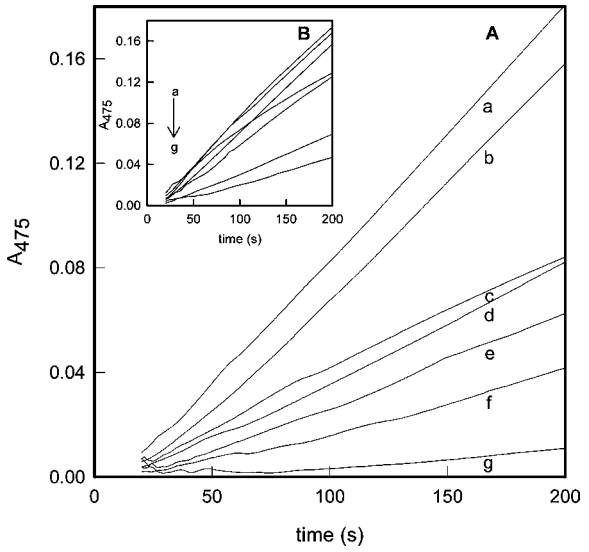

Figure 3. (A) Effect of the monophenols studied on the activities of tyrosinase with their physiological substrates, L-tyrosine and L-Dopa. In the monophenolase activity, the accumulation of dopachrome with time was measured from the action of tyrosinase (15 nM) on L-tyrosine (0.26 mM) (a). The rest of the experiments were in the same conditions as (a) but with the ortho-substituted monophenols at the same concentration (2.75 mM): **6** (b), **1** (c), **3** (d), **2** (e) **4** (f), **5** (g). (B) Diphenolase activity. The accumulation of dopachrome with time was measured from the action of tyrosinase (3 nM) on L-Dopa (0.5 mM) (a). The rest of the experiments were carried out in the same conditions as (a) and with the ortho-substituted monophenols at the same concentration (2.75 mM): **6** (b), **1** (c), **3** (d), **2** (e), **4** (f), and **5** (g).

Chracterization of Ortho-Substituted Monophenols as Possible Inhibitors of Tyrosinase. The inhibition of monophenolase and diphenolase activities of tyrosinase in the steady state is shown in (Figure 3A and Figure 3B), and the effect of the addition of the different *o*-monophenols studied at the same concentration is also shown. Note the inhibiting effect of both activities on the formation of dopachrome (Table 1).

In the case of monophenolase activity toward L-tyrosine (Figure 3A), the increase in absorbance was measured at 475 nm, adding the *o*-diphenol (L-Dopa) necessary for the system to reach the steady state at $t = 0$, in accordance with the ratio $D/M = R = 0.034$.[20] Note that in the particular case of **6** addition, there is an apparent activation (Figure 3, curve b).

The inhibition the monophenolase and diphenolase activities of tyrosinase by ortho-substituted monophenols (Figure 3A and Figure 3B) may be related to the value of the chemical shift, δ, of the carbon atom supporting the hydroxyl group, and furthermore, possible steric hindrance should be taken into account. For example, **4** has a lower δ_1 (see Table 1) and should therefore bind more closely to the enzyme, but the allyl group may demonstrate steric hindrance. However, **5** has a slightly higher δ_1 but suffers less steric hindrance by its propenyl group and reaches the active site of the enzyme more readily, inhibiting its activity. Meanwhile, the inhibition exercised by **3** on both activities is less than that by **4** and **5** since δ_1 is higher. According to the NMR values (Table 1), the sequence of inhibition should be **1** < **2** < **3** < **5** < **4** and, taking into account the steric hindrance, the sequence of inhibition should be **1** < **2** < **3** < **4** < **5**, since **4** and **5** have very close NMR values and the steric hindrance of the group in C-4 of **4** is greater than that of **5**, which has a double conjugated bond with the aromatic ring (Table 1).

It is emphasized that the inhibition % (Table 1) is always greater in the case of monophenolase than diphenolase activity, which may be explained by the action mechanism of the enzyme (Supporting Information) with $D' = 0$ and $M = 0$. Thus, in the diphenolase activity in the catalytic cycle, practically all the enzymes are in the oxy forms (Supporting Information) with $D' = 0$ and $M = 0$, while in the case of monophenolase, activity of the enzyme is equally shared between oxy and met forms, both of which are accessible to the monophenol, although there is greater affinity toward the met form because the base B_1 would accept the proton from the hydroxyl group, favoring the binding.[17,21]

Oxygen consumption during the action of tyrosinase on L-tyrosine is shown in Figure 4, curve a, where it can be seen that the steady state is reached after a lag period. The addition of an inhibitor (curve b) such as benzoic acid lengthens the lag period and slows the action rate of the enzyme. Note that compounds **1** and **4** (curves c and d) inhibit the enzyme and lengthen the lag, since they act as tyrosinase inhibitors.

It seems then that ortho-substituted monophenols inhibit both tyrosinase activities (Figure 3). Moreover, Figure 5 shows that this inhibition affects the enzyme and not the set of chemical reactions that occur from *o*-dopaquinone to dopachrome (see Supporting Information) in which the oxidation/reduction reaction between *o*-dopaquinone and leucodopachrome occurs. To confirm the above, L-dopa was oxidized in the presence and absence of **1** by periodate in deficiency and in excess. When carrying out the oxidation by sodium periodate in deficiency, the quantity of dopachrome accumulated was half that of the periodate, since the stoichiometry between *o*-dopaquinone and dopachrome is

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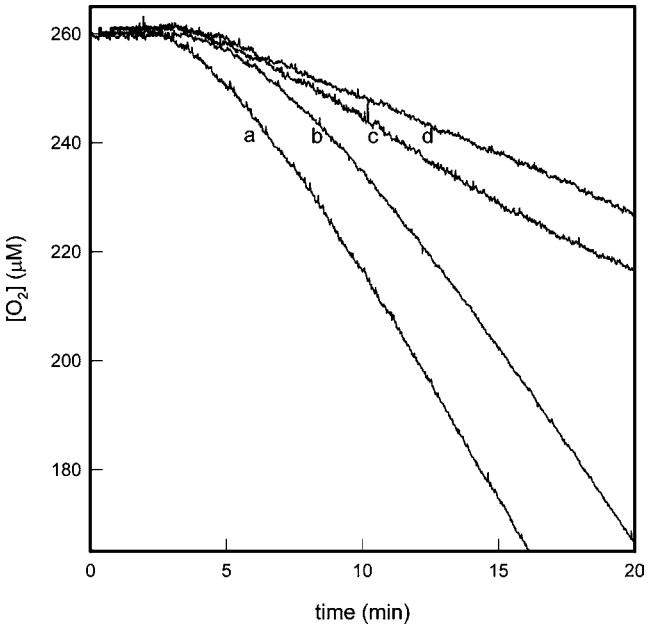

Figure 4. Oxymetric recordings of oxygen consumption during the action of tyrosinase on L-tyrosine. Action of tyrosinase (4 nM) on L-tyrosine (0.26 mM) (a). Action of different compounds (2.5 mM) was measured in the experimental conditions of curve a but using benzoic acid (b), compound **1** (c), and compound **4** (d).

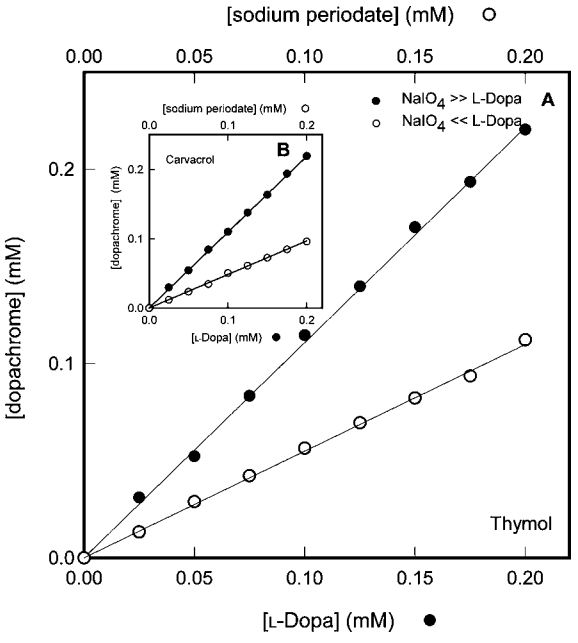

Figure 5. (A) Representation of the dopachrome accumulated during the oxidation of L-Dopa (the concentrations of L-Dopa are shown in the figure) by sodium periodate in the presence of **1**: (●) oxidation of L-Dopa by sodium periodate in excess (2 mM), where the concentrations of L-Dopa are shown in the figure; (○) oxidation of L-Dopa (2 mM) by sodium periodate in deficiency, where the concentrations of periodate are shown in the figure and, in both cases, the concentration of **1** was 1 mM. (B) Oxidation of L-Dopa (the concentrations of L-Dopa are shown in the figure) in the presence of **2**: (●) excess of sodium periodate (2 mM); (○) oxidation of L-Dopa (2 mM) by sodium periodate in deficiency (the concentrations of sodium periodate are shown in the figure). The concentration of **2** in both cases was 1 mM.

2:1, and as shown previously,[3] the slope of the straight line obtained by regression of [dopachrome] vs [periodate] is 0.5 (Figure 5A). When the oxidation was carried out with excess periodate, dopachrome accumulation was stoichiometric with the periodate and, in this case, the stoichiometry between the *o*-quinone and dopachrome was 1:1,[3] and so the slope of the straight line was unity in the absence and presence of **1** (Figure 5A). This result indicates that **1** does not inhibit the chemical reactions that occur after the *o*-dopaquinone. Similar experiments carried out in the presence of **2** provided the same results (Figure 5**B**), and the rest of the monophenols behaved similarly (results not shown). The inhibitory action of **4** on the oxidation of an *o*-diphenol like hydroxychavicol by tyrosinase has been described previously.[30]

Oxidation of *N*-Acetyl-L-tyrosine and *N*-Acetyl-L-tyrosine Ethyl Ester by Tyrosinase. The amino group of the L-tyrosine derivate, *N*-acetyl-L-tyrosine, is acetylated, which hinders *o*-quinone cyclation,[31] and so little *o*-quinone is transformed into the aminochrome form, although when different concentrations of **1** (Figure 6) and **2** (result not shown) are used, the lag is shortened and the enzymatic activity is inhibited. The results suggest that these monophenols do not take part in the series of chemical reactions that occur from *o*-dopaquinone to dopachrome, but act directly in the enzymatic step, as will be discussed below. Note that the lag phase is shorter in the presence of **1** (Figure 6A, curves b–d), since this compound makes a nucleophilic attack on position 6 of the *N*-acetyl-L-*o*-dopaquinone (see Figure 7) through the oxygen of the hydroxyl (see Table 1). Upon oxidation by another molecule of the *o*-quinone, this adduct generates an *o*-diphenol in the medium in addition to that formed as a result of the oxidation by another *o*-quinone molecule of the cycling compound of *N*-acetyl-L-*o*-dopaquinone. However, although the lag is shortened, the enzyme is inhibited, confirming once again the inhibitory action of these compounds (Figure 6A, curves b–d). When **6** is added (Figure 6A, curve e), the lag diminishes but total absorbance increases.

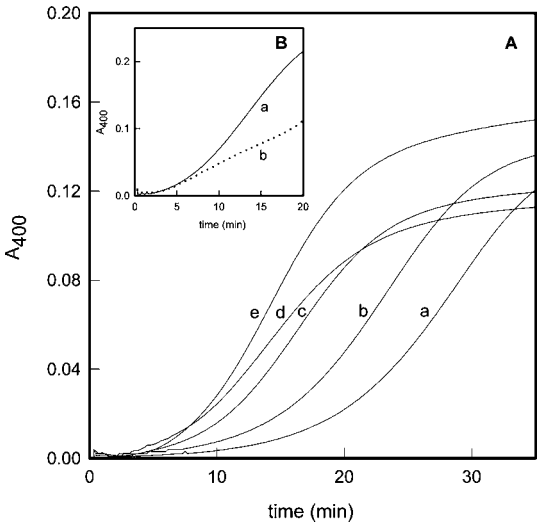

Figure 6. (A) Oxidation of *N*-acetyl-L-tyrosine (0.1 mM) by tyrosinase (6 nM). The concentrations of **1** (mM) were 0 (a), 0.1 (b), 0.2 (c), and 0.4 (d). In recording (e), 0.1 mM **6** was added. (B) Oxidation of *N*-acetyl-L-tyrosine ethyl ester (0.5 mM) by tyrosinase (12 nM). The concentrations of **1** (mM) were 0 (a) and 3.24 (b).

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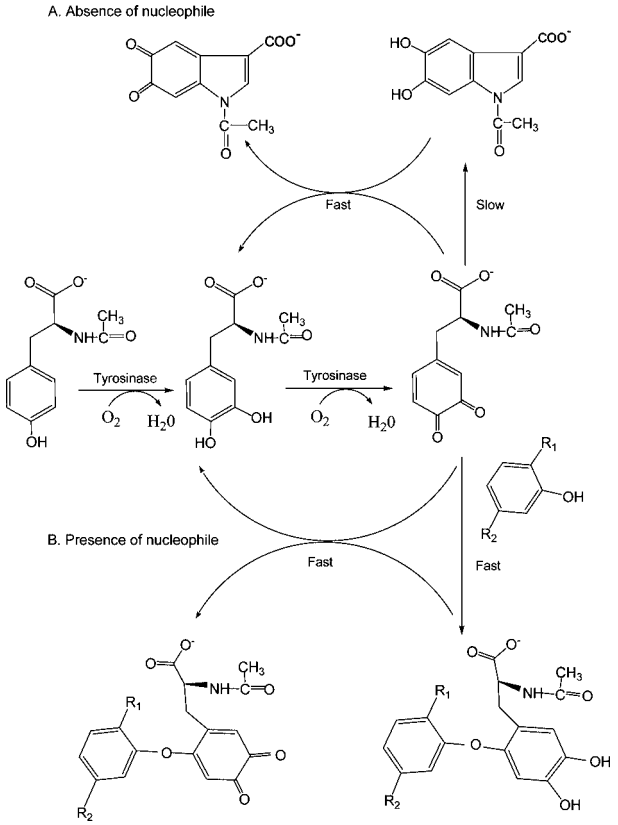

Figure 7. Oxidation of *N*-acetyl-L-tyrosine by tyrosinase.

Experiments with the *N*-acetyl-L-tyrosine ethyl ester (Figure 6B) point to the shortening of the lag period before *o*-quinone accumulation (curve a), since the nucleophilic attack of the nitrogen of the *o*-quinone on the ring by a Michael intramolecular 1,4 addition occurs more quickly than in the case of *N*-acetyl-L-tyrosine when the withdrawing effect exercised by the acid group of the *N*-acetyl-L-tyrosine diminishes and before *o*-diphenol accumulates in the medium. The inhibitory effect of **1** can be seen in Figure 6B, curve b. In this case, too, the lag phase is shortened. The effect of monophenols such as **1** and **2** in the action of tyrosinase on the *N*-acetyl-L-tyrosine at concentrations that permit the steady state to be reached is shown in (Figure 8). In recording (a), the system reaches the steady state after a very long lag period, since the *o*-quinone cyclation constant is very small, and so the *o*-diphenol accumulates very slowly in the medium. In the presence of **1** (curve b), the lag is shorter and the *o*-diphenol accumulates more rapidly but the *o*-quinone is formed from the *N*-acetyl-L-tyrosine more slowly because the enzyme is inhibited. When the **1** concentration is increased (curve c), the lag is shorter than in curve a and the rate of *o*-quinone accumulation is lower. Similar results were obtained with **2** (Figure 7B).

From all the above, it can be seen that the group of ortho-substituted monophenols studied in this work act as inhibitors of the monophenolase and diphenolase activities of tyrosinase (Figure 3A and Figure 3B) and not as inhibitors of the sequence of chemical reactions that occur from *o*-dopaquinone to dopachrome. Since these compounds were seen to bind to the enzyme, inhibiting the formation of dopachrome, we investigated whether they could act as tyrosinase substrates.

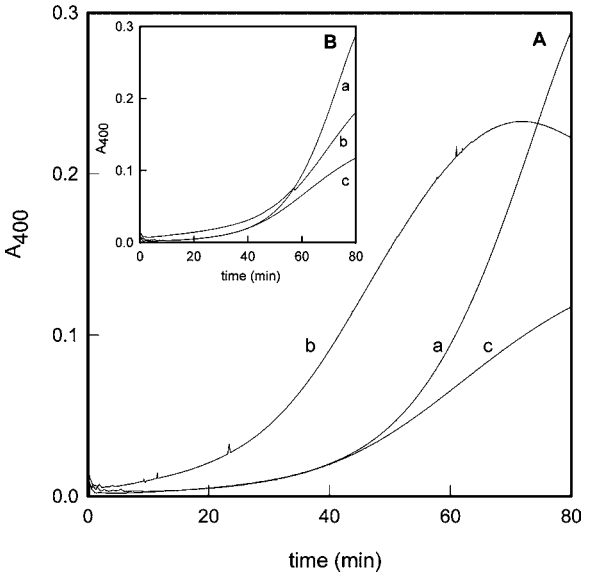

Figure 8. Inhibition of tyrosinase (3.5 nM) by **1** and **2** in its action on *N*-acetyl-L-tyrosine (0.5 mM). (A) Action of **1**. The **1** concentrations (mM) were 0 (a), 1.6 (b), and 3.2 (c). (B) Action of **2**. The **2** concentrations (mM) were 0 (a), 1.6 (b), and 3.2 (c).

Characterization of Ortho-Substituted Monophenols as Substrates of Tyrosinase. The ortho-substituted monophenols act as substrates of tyrosinase (Supporting Information) and so would act as alternative substrates to L-tyrosine and L-Dopa in the melanin biosynthesis pathway, giving rise to *o*-quinones other than *o*-dopaquinone and so inhibiting the physiological pathway of melanin formation through dopachrome. The analytical expressions for the dopachrome formation rates are shown in the Supporting Information, where the inhibitory action of an alternative substrate can be seen.[32] The kinetic characterization of these monophenols as tyrosinase substrates was made spectrophotometrically. In the cases of **1** and **2**, a small quantity of the corresponding *o*-diphenol (3I6MC) was added and the appearance of the *o*-quinone was measured at λ = 437 nm, the same for both substrates (see Materials and Methods and Supporting Information). From this figure, it can be seen that the enzyme is not saturated by these substrates, and nonlinear regression to the Michaelis equation gives k_{cat}/K_m (Table 2). Note that **2** is a worse substrate than **1**; it could be due to steric hindrance.

Table 2. Kinetic Parameters and Constants Characterizing the Oxidation of Thymol, Carvacrol, and Guaiacol by Tyrosinase

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
1			161 ± 4
2			95 ± 7
3	5.40 ± 0.14	4.65 ± 0.39	1160 ± 101

In the case of **3** (Supporting Information) catalytic quantities of its *o*-diphenol, 3-methoxycatechol, were added and the reaction was followed at λ = 337 nm, the isosbestic point in the evolution of *o*-methoxyquinone[21] (see Materials and Methods). The spectrophotometric recordings of *o*-quinone formation are shown in Supporting Information and, based on these, the Supporting Information shows the steady-state rate vs **3** concentration. As indicated in Materials and Methods, the

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data analysis provides the values of k_{cat} and K_m (see Table 2). From the values corresponding to **1**, **2**, and **3**, it is deduced that the values of k_{cat} are, as expected, very low and that the presence of the group that is ortho with respect to the hydroxyl group means that the affinity of the enzyme for these compounds is slight so that the value of K_m is high (Table 2).

As for the monophenols **4**, **5**, and **6**, the corresponding *o*-diphenol is not commercially available and a small quantity of TBC was added, almost eliminating the lag period. However, it was not possible to obtain reliable results concerning oxygen consumption because of the poor signal, even at high enzyme concentrations. For this reason, MBTH was added and the absorbance was measured spectrophotometrically, since MBTH reacts with the *o*-quinones generated by the action of the enzyme on these substrates, producing adducts with a high coefficient of molar absorptivity.[22] The results are shown in Supporting Information, which also demonstrate that these ortho-substituted monophenols act as substrates of the enzyme. It has been proposed that phenols with an *o*-methoxy group are not transformed to quinones by tyrosinase and that their structural similarity to catechols may result in nonproductive binding and inhibition of the tyrosinase.[30] However, the results depicted in Supporting Information reveal that these compounds act as substrates of the enzyme regardless of whether the *o*-quinone is transformed into a methide quinone.[29] Moreover, it has recently been demonstrated that **3** is transformed into 3-methoxyquinone, which is reduced by ascorbic acid to 3-methoxycatechol.[33]

This evidence that substituted *o*-phenols act as tyrosinase substrates may help explain the results concerning the formation of dopachrome described in Figure 3 and in Table 1 for the monophenolase and diphenolase activities, respectively. The *o*-diphenol (L-Dopa) acts as cofactor in the activity on ortho-substituted monophenols (Figure 3B).

The spectra for dopachrome and the *o*-quinone of 3-methoxycatechol are shown in Supporting Information). Note the overlap at λ = 475 nm, which in the case of **3** translates into an increase in absorbance when the inhibition of diphenolase is studied (Figure 3B). The *o*-diphenols act as cofactors in the monophenolase activity, apparently decreasing the inhibition.[34] A similar situation is presented in Supporting Information, which shows the overlap of the *o*-quinone of the 3-isopropyl-6-methylcatechol (3I6MC) and dopachrome, which leads to an apparent diminution in the inhibition of diphenolase activity when thymol and carvacrol are studied.[34] Such effects could explain the reduced inhibition of diphenolase activity compared with monophenolase activity (Table 1).

As for **6** in Supporting Information, the structure of the *o*-quinone and the possibility the resonance due to the presence of the methoxyl group, which would impart the reddish color, would explain the possible activation (Figure 2B, curve c). Also, the structure of the *o*-quinone originated after the attack of **6** on the *o*-quinone of the *N*-acetyl-L-tyrosine, according to Figure 7, is shown (Supporting Information). The resonance means that the adduct in this case is reddish in color, explaining the result of Figure 6A, curve e. Furthermore, Supporting Information shows how the dopachrome spectrum is deformed because of the presence of the resonant forms of the *o*-quinone of **6** (Supporting Information). These effects may explain the apparent activation of the **6**.

The substrates **4** and **5** according to Table 1 have a high inhibitory capacity on both monophenolase and diphenolase activities, which indicates that there is no overlap between the spectra of their *o*-quinones and dopachrome. The data represented in Table 1 show that the inhibition of the monophenolase activity on L-tyrosine by ortho-substituted monophenols is much more pronounced, since little activating *o*-diphenol exists in the steady state.

In conclusion, the action of tyrosinase on a group of ortho-substituted monophenols reveals that these compounds are substrates of the enzyme despite the steric hindrance that exists in their binding to the active site. In the specific cases of **1** and **2**, to their known antimicrobial and antioxidant properties should be added an inhibitory mechanism of melanin synthesis, since they act as alternative substrates of L-tyrosine and L-Dopa and inhibit the accumulation of dopachrome because of the formation of other *o*-quinones, which may be responsible for the oxidative stress-related toxicity mechanism of **1** and may explain why it acts as a pro-oxidant rather than as an antioxidant at high concentrations.[35]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Kinetic analysis of the proposed mechanisms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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