Enhanced Substituted Resorcinol Hydrophobicity Augments Tyrosinase Inhibition Potency

Soliman Khatib,[†,‡] Ohad Nerya,[†] Ramadan Musa,[†] Snait Tamir,[†,‡] Tal Peter,[‡] and Jacob Vaya[†,‡,*]

The Laboratory of Natural Medicinal Compounds, MIGAL - Galilee Technology Center, P.O. Box 831, Kiryat Shmona 11016, Israel, and Department of Biotechnology, Tel-Hai Academic College, Israel

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The objective of the present study was to investigate to what extent the addition of hydrophobic residues to a 2,4-resorcinol derivative would contribute to their tyrosinase inhibitory potency. Hence, 3-(2,4-dihydroxyphenyl)propionic acid, isolated from *Ficus carica*, was transformed into esters, and the relationship between the structure of these esters to their mushroom tyrosinase inhibition activity was explored. The enzyme crystallographic structure, published recently (Matoba, Y. et al. *J. Biol. Chem.* **2006**, *281*, 8981−8990) was docked with the new esters, and their calculated free energy (FE) and docking energy (DE) were compared with the experimental IC_{50} values, providing good correlations. The observed IC_{50} of the isopropyl ester was 0.07 μM, and its interaction with the enzyme binding site appears to be composed of four hydrogen bonds and two hydrophobic interactions. It may be concluded that the addition of a hydrophobic moiety to 2,4-resorcinol derivatives augments tyrosinase inhibitory potency as was predicted from the modeling study.

Introduction

Tyrosinase is a copper-containing enzyme, widely distributed in nature. It has a diverse role in agriculture, the cosmetic industry, and medicine, is involved in the browning of fruits and vegetables[2] and in the formation of brown pigments (melanins), and is responsible for mammalian skin and hair color.[3] Tyrosinase is also linked to Parkinson's and other neurodegenerative diseases,[4,5] oxidizing excess dopamine to produce DOPA quinones, highly reactive compounds that induce neuronal damage and cell death.

The crystallographic structure of tyrosinase has been established recently,[1] enabling a close look at its three-dimensional structure and a better understanding of its mechanism of action. The enzyme catalyzes two reactions in the melanin biosynthesis pathway involving molecular oxygen: the hydroxylation of monophenols to *o*-phenols, and the oxidation of the *o*-phenols to *o*-quinones, which further polymerize spontaneously into melanin.

Tyrosinase inhibitors have become increasingly important, and a number of naturally occurring ones have been described. Most consist of a polyphenol structure with poor (IC_{50} > 100 μM)[6] to moderate (IC_{50} = 10−100 μM) activity[7] and a few with high inhibition potency (IC_{50} < 10 μM).[8] Reports on the relation between the structure of such polyphenol derivatives and their enzyme inhibition have emphasized the contribution of the 2,4-substituted resorcinol structure and the significance of the location of their hydroxyl groups on both aromatic rings, with a preference for a 4-substituted B ring rather than a substituted A ring in regard to the potency.[8−10] Resorcinol residues, such as in flavonoids, in 4-alkyl-substituted resorcinols,[7,11−13] in stilbenes[3,6,14] and in chalcones[8] have been investigated elsewhere as tyrosinase inhibitors. Among the various 4-alkyl-substituted resorcinols, the 4-hexyl derivative has been found to be a superior inhibitor to the 4-dodecyl- or 4-ethylresorcinol, and slightly less effective than kojic acid.[11] In reports on the inhibitory effect of dodecyl gallate, it has been debated that its enzyme inhibition is derived, in part, from the interaction of the dodecyl hydrophobic moiety with the hydrophobic domain of the enzyme.[15] This finding emphasized the possibility that a hydrophobic moiety may also contribute to the inhibition.

As part of our study in search of new biologically active agents from plants and fungi, we have isolated a tyrosinase inhibitor from fig leaves and fruits. The structure of the active compound has been elucidated as 3-(2,4-dihydroxyphenyl)-propionic acid (DPPA[a]). Some of the biochemical properties of this compound have been characterized previously,[16,17] including its tyrosinase inhibition. Apparently, DPPA is a competitive inhibitor of tyrosinase, with reported IC_{50} of 3.2 μM. In a recent study, aliquots of DPPA (1 μg/mL) reduced the browning of slide mushrooms by 50%.[2] The new tyrosinase three-dimensional structure revealed the presence of a hydrophobic protein pocket adjoining the binuclear copper active site.

On the basis of data from literature[7] and our lab, including a modeling study,[8] we hypothesize that compounds of 2,4-resorcinol structure can fit the binuclear copper active site well, and that in the presence of a hydrophobic subunit connected to the resorcinol aromatic ring, may interact with the hydrophobic enzyme pocket, leading to increased inhibitor−enzyme binding affinity, and thus to an improved compound activity. In the present study, on the basis of the new tyrosinase three-dimensional structure,[1] we have aimed to form DPPA esters by reactions of DPPA with hydrophobic alcohols of various structures and to examine the above hypothesis.

Material and Methods

Chemicals and Reagents. Tyrosinase (EC 1.14.18.1, with an activity of 6680 units/mg, T7755) and the DPPA standard were purchased from Sigma. 3-(2,4-Dihydroxyphenyl)propionic acid

* Corresponding author. Tel: 972-4-6953512. Fax: 972-4-6944980. E-mail: vaya@migal.org.il.
† MIGAL - Galilee Technology Center.
‡ Tel-Hai Academic College.

[a] Abbreviations: FE, free energy; DE, docking energy; DPPA, 3-(2,4-dihydroxyphenyl)propionic acid; EtOH, ethanol; THF, tetrahydrofuran; ADT, AutoDock Tools program; RP, reverse phase; ES, electrospray ionization; R_{ij}, equilibrium separation between two nucleus; eps_{ij}, pairwise potential energy; GA, genetic algorithm; K_i, inhibition constant; MOPAC, semiempirical molecular orbital package; PM3, semiempirical Hamiltonian.

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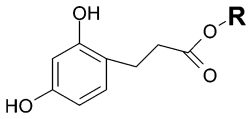
Table 1. Docking and Free Energies Calculated by Using AutoDock Program. A Comparison between Experimental and Calculated IC_{50} Values



	R	docking energy (kcal/mol)	free energy (kcal/mol)	IC_{50} (μM) experimental	IC_{50} (μM) calculated[a]
1	H	−6.21	−5.43	1.85	0.40
2	ethyl	−7.41	−5.88	0.22	0.27
3	isopropyl	−8.01	−6.45	0.07	0.12
4	octyl	−7.98	−4.29	0.70	0.70
5	*tert*-butyl	−7.59	−6.9	14.87	0.0025
6	2,2-dimethylpropyl	−8.19	−6.27	0.18	0.17
7	3,3,5,5-tetramethyl-4-heptyl	−6.17	−6.73	0.15	0.05

[a] Calculated IC_{50} values were generated from free energy values by the following equation, FE = 3.738 × (IC_{50}) − 6.909 (generated from a regression curve relating free energy to experimental IC_{50}).

(DPPA, **1**) was isolated from fig leaves, as described below. Solvents used were of HPLC grade.

Isolation and Structure Determination of DPPA from Fig Leaves and Fruits. Dry leaves (380 g) from fig trees (*Ficus carica* L.) were extracted twice, each time with 760 mL of 70% ethanol in water. The crude extract (28.12 g, 7.4% yield) exhibited 80% inhibition of the mushroom tyrosinase activity, at a concentration of 0.032 mg/mL.

The dry extract was dissolved in 100 mL of 20% methanol in water and centrifuged (12000 rpm, 4 °C) to remove insoluble materials, and the supernatant was collected. The filtrate was chromatographed on a silica gel column (silica gel S, 0.032−0063) and eluted with a solvent mixture gradient (0% to 100% methanol/dichloromethane). A sample from each fraction was examined for tyrosinase inhibition, and the active fractions were collected and rechromatographed on a similar form of silica gel. Fractions with active compounds were then separated on an HPLC preparative RP-18 column, using a gradient of eluents (5% to 30% acetonitrile/water), followed by an additional separation on sephadex LH-25, with methanol as an eluent, to afford a pure active compound (28 mg). The structure of the pure isolated active compound was elucidated as 3-(2,4-dihydroxyphenyl)- propionic acid (DPPA) by a conventional analytical method. UV−vis spectra (EtOH three bands at λ_{max} = 210, 225, 280 nm); IR(KBr) ν = 3387, 2933, 1701, 1605, 1515; NMR 6.94 (1H, d, J = 8.18 Hz), 6.38 (1H, d, J = 2.60 Hz), 6.27 (1H, dd, J = 8.18, 2.60 Hz), 2.79 (2H, t, J = 7.44 Hz), 2.56 (2H, t, J = 7.80 Hz).

Synthesis of DPPA Esters. DPPA ethyl (**2**), isopropyl (**3**), octyl (**4**), *tert*-butyl (**5**), 2,2-dimethylpropyl (**6**), and 3,3,5,5-tetramethyl-4-heptyl (**7**) esters (Table 1) were synthesized by conventional procedures. Briefly, 20 mg (0.11mmol) of DPPA were dissolved in 5 mL of the respective alcohol (for esters **2**−**6**), 100 μL H_2SO_4 (98%) was added, and the resulting solution was heated to either reflux or 100 °C for 2 h. A slight modification was employed for the synthesis of ester **7**. Briefly, 20 mg (0.11mmole) of DPPA was reacted with 20 mg (0.22 mmol) of 3,3,5,5-tetramethyl-4-heptanol in 1 mL of dry THF, followed by the addition of 50 μL of H_2SO_4. After the solution had been stirred under argon for 24 h at 37 °C, 5 mL of water was added and each ester was extracted three times with 10 mL of chloroform. The chloroform was evaporated under reduced pressure, and the product was purified by flash chromatography (silica gel, hexane:ethyl acetate, 70:30 as solvents), affording purified esters, with a 75−85% yield. The products were injected into the LC-MS under the electrospray ionization mode (ES^-), resulting in the expected molecular ion at m/z = 181, 209, 223, 293, 237, 251, and 335 (M − 1) for compounds **1**−**7**, respectively.

^{1}H NMR Data. DPPA ethyl ester [3-(2,4-dihydroxyphenyl)-propionic acid ethyl ester, (compound **2**] (acetone-d_6): δ 6.9 (1H, d, J = 8.20 Hz); 6.38 (1H, d, J = 2.60 Hz); 6.27 (1H, dd, J = 8.18, 2.20 Hz); 3.58 (2H, q, J = 6.70 Hz); 2.79 (2H, t, J = 7.80 Hz), 2.56 (2H, t, J = 7.40 Hz); 1.12 (3H, t, J = 6.70 Hz).

DPPA isopropyl ester [3-(2,4-dihydroxyphenyl)propionic acid isopropyl ester, compound **3**] (acetone-d_6): δ 6.9 (1H, dd, J = 8.18, 6.30 Hz), 6.37 (1H, d, J = 2.23 Hz), 6.28 (1H, dt, J = 8.56, 2.20 Hz), 4.9 (1H, sept., J = 6.33 Hz), 2.79 (2H, t, J = 7.80 Hz), 2.55 (2H, t, J = 7.44 Hz), 1.19 (6H, d, J = 6.33 Hz).

DPPA octyl ester [3-(2,4-dihydroxyphenyl)propionic acid octyl ester, compound **4**] (acetone-d_6): δ 7.02 (1H, d, J = 7.80 Hz); 6.5 (1H, d, J = 2.23 Hz); 6.4 (1H, dd, J = 8.18, 2.23 Hz); 3.72 (2H, t, J = 7.07 Hz); 2.92 (2H, t, J = 7.80 Hz); 2.69 (2H, t, J = 7.80 Hz); 1−1.7 (15H, m).

DPPA *tert*-butyl ester [3-(2,4-dihydroxyphenyl)propionic acid *tert*-butyl ester, compound **5**] (acetone-d_6): δ 6.89 (1H, d, J = 8.18 Hz); 6.38 (1H, d, J = 2.23 Hz); 6.28 (1H, dd, J = 8.10, 2.23 Hz); 2.77 (2H, t, J = 8.20 Hz); 2.54 (2H, t, J = 7.815 Hz); 1.5 (9H, s).

DPPA 2,2-dimethylpropyl ester [3-(2,4-dihydroxyphenyl)-propionic acid 2,2-dimethylpropyl ester, compound **6**] (acetone-d_6): δ 6.89 (1H, d, J = 8.18 Hz); 6.38 (1H, d, J = 2.23 Hz); 6.28 (1H, dd, J = 8.10, 2.23 Hz); 3.74 (2H, s); 2.82 (2H, t, J = 8.20 Hz); 2.59 (2H, t, J = 7.815 Hz); 0.9 (9H, s).

3,3,5,5-Tetramethyl-4-heptyl ester [3-(2,4-dihydroxyphenyl)-propionic acid 3,3,5,5-tetramethyl-4-heptyl ester, compound **7**] ($CDCl_3$): δ 6.87 (1H, d, J = 8.10 Hz); 6.34 (1H, d, J = 2.40 Hz); 6.32 (1H, dd, J = 8.10, 2.70 Hz); 3.74 (1H, s); 2.9 (2H, t, J = 8.00 Hz); 2.66 (2H, t, J = 8.00 Hz); 1.2 (4H, q, J = 7.20 Hz); 0.77 (12H, s); 0.735 (6H, t, J = 7.50 Hz).

Tyrosinase Inhibitory Effect of DPPA Derivatives. Potassium phosphate buffer (70 μL, 50 mM) at pH 6.5, 30 μL of tyrosinase (final concentration of 50 units/mL), and 3 μL of the tested compounds dissolved in absolute ethanol (final concentration of 5−100 μM) were added to wells of 96-well plates. After 10 min incubation at 25 °C, L-tyrosine (final concentration of 1 mM) was added. The optical density (450 nm) of the samples was measured (ELISA SLT Lab Instruments Co. A-5082) every 5 min, relative to the control, which contained ethanol (2 μL and without inhibitor), demonstrating a linear color change with time. The IC_{50} was calculated at the linear zone of the curve (20 min with L-tyrosine, as the substrate).

Preincubation of Compounds 6, 7, and DPPA with Tyrosinase. Either compouns **5** or **7** or DPPA was tested, with and without preincubation, with the enzyme. Each of these compounds was added separately to the enzymatic reaction, as described above, at concentrations that inhibit 80% of the enzyme activity (0.5, 0.3, and 10 μM for compounds **5**, **7**, and DPPA, respectively). After 10 min of incubation, the substrate (L-tyrosine, 1.0−3.75 mM) was added. The formation of DOPA-quinone was followed by an absorption measurement at 450 nm, 20 min after the addition of L-tyrosine.

Sample Dialysis. DPPA (10 μM) was incubated with tyrosinase for 20 min, followed by dialysis (dialysis cassette, 10000 MWCO, Pierce) overnight. The activity of the enzyme was measured after dialysis and compared to that of the enzyme under the same conditions before dialysis. After dialysis, the presence of free DPPA, was also tested by LC-MS at the ES^- mode (LC- Waters 2790 HPLC, with the Waters Photodiode Array Detector 996, attached to the Micromass Quattro Ultima MS). The protein quantity was calculated by Bradford analysis, before and after the dialysis.

Molecular Docking: Preparation of the Enzyme. The crystal structure of the tyrosinase oxy form, complexed with a Caddie Protein and prepared by the addition of hydrogen peroxide, was retrieved from the Protein Data Bank.[1] The enzyme was prepared for docking by the ADT program (AutoDock Tools program), an accessory program that allows the user to interact with AutoDock[18−20] from a graphic user interface. Water, heteroatoms, and the Caddie Protein were removed from the protein PDB file. Polar hydrogen

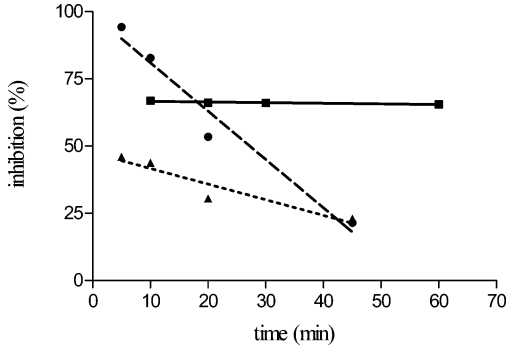


Figure 1. Inhibition of mushroom tyrosinase by three natural compounds, each at concentration at or above 50% inhibition: (■) 2 μM 3-(2,4-dihydroxyphenyl)propionic acid (DPPA), (●) 7 μM glabrene, and (▲) 0.07 μM glabridin.

atoms were added and Kollman united atomic charges assigned. The solvation parameters were added by the Addsol program (part of the ADT program). The two copper ions and the oxygen atoms of the enzyme active sites were returned to their original sites. The "C" letter of the copper ions was changed to "M", and the solvation parameters of the copper ions and peroxide oxygen were fixed to zero.

Preparation of Ligands. The ligands were constructed by "CS Chemdraw Pro" and "CS ChemBats3D Pro" and optimized to their minimum energy with the MOPAC program and the PM3 semiempirical Hamiltonian.[21] Rotatable bonds and charges in the ligands were assigned by the ADT program.

Grid Parameters. The grid points were set to the catalytic site of the enzyme. The number of grid points in xyz was set to 40, 40, 40, the spacing value equivalent to 0.375, and the grid center to −14.041, −13.964, 21.713.

The copper van der Waals parameters, which were obtained from the "Quanta 3.0 Parameters Handbook" are atom type M equilibrium separation between the nucleus of the two copper ions, $R_{ij} = 1.27$ A^0, and pairwise potential energy, $eps_{ij} = 0.06$.

Docking. Ligand docking was carried out with the AutoDock 3.0.5 Lamarckian Genetic Algorithm (GA). The approximate binding free energies, calculated by this program, are based on an empirical function, derived by linear regression analysis of protein−ligand complexes with known binding constants. This function includes terms for changes in energy due to van der Waals, hydrogen bonds, and electrostatic forces, as well as ligand torsion and desolvation. The docked energy also includes the ligand internal energy or the intramolecular interaction energy of the ligand. All of the parameters were assigned the default values, implemented by the program.

Analysis of Results. All of the docking results were visualized and analyzed with ADT and Chimera software. The lowest docked energy was taken as the best-docked conformation of the compound for the macromolecule.

Results

Isolation of DPPA. A 70% ethanolic extract from dry fig leaves exhibited potent inhibition of the mushroom tyrosinase. The active compound from the extract was identified as DPPA, which was identical to the authentic sample of this compound. The characteristic DPPA inhibitory effect on mushroom tyrosinase, in comparison to other two known inhibitors isolated from licorice root,[13] is shown in Figure 1. The results demonstrate that while the DPPA inhibitory level remained stable during the first hour of incubation those of the other two inhibitors declined rapidly.

Tyrosinase Inhibitory Level of DPPA Esters. In order to assess the effect of the increasing hydrophobicity of DPPA derivatives on enzyme inhibition, DPPA ethyl (**2**), isopropyl (**3**) and octyl (**4**) esters were synthesized (Table 1) and their activities determined, 0.22 μM, 0.07 μM, and 0.7 μM, respectively, as compared to the IC_{50} of DPPA, 1.9 μM. These results may indicate that increased inhibitory potency does not necessarily correlate with the increase in the carbon chain length, but rather may be related to greater ester bulkiness.

At this point, we questioned if such a performance could be predicted theoretically, prior to inhibitor synthesis, by calculating parameters related to interactions between the inhibitor and the enzyme's amino acids, within the enzyme core. Thus, parameters such as docking and free energy were calculated using the AutoDock program, as detailed in Materials and Methods. The crystallographic structure of the enzyme was acquired from newly available data.[1] The structure of each potential inhibitor, requiring minimum energy, as determined by the MOPAC program,[21] and according to the energy calculations of the enzyme−inhibitor complexes, was used. A summary of the results, which is shown in Table 1, implies that both parameters, the docking and free energy, were useful predictors of the expected inhibitory potency. The values of the energy level of both parameters decreased with the elevation of the observed inhibitor activity (experimental IC_{50}) and correlated with the expected IC_{50}. On the basis of the above data of the free energy, three additional potential inhibitors, with a variety of bulky esters, were docked. The predicted FE values of the compounds, **5** (*tert*-butyl ester of DPPA), **6** (2,2-dimethylpropyl ester), and **7** (1-(1,1-dimethylpropyl)-2,2-dimethylbutyl ester), were −6.9, −6.27, and −6.73 kcal/mol, respectively (Table 1) and from ploting a regression curve of free energy and docking energy versus the experimental IC_{50}, the expected IC_{50} for compounds **5**, **6**, and **7** were calculated to be 0.0025, 0.17, and 0.05 μM, respectively. These three compounds, **5**, **6**, and **7**, were synthesized and their observed IC_{50} values found to be 14.90, 0.18, and 0.15, respectively. A new regression curve was calculated relating the above energies values to the experimental IC_{50} of all esters synthesized including those of compounds **5**, **6**, and **7**, excluding the *tert*-butyl ester of the DPPA, afforded an R^2 of 0.94 and 0.76, respectively, with P values of 0.0063 and 0.0525, respectively.

The effect of Preincubation of Each of the Compounds, 6, 7, and DPPA with Tyrosinase. The tyrosinase inhibitory effect of the two synthetic DPPA esters, compounds **6** and **7** (share a resorcinol aromatic ring), were tested with and without preincubation in the presence of the enzyme. Thus, DPPA **1** (at 10 μM) or compound **6** (at 0.5 μM) or **7** (at 0.3 μM) were each incubated for 10 min with the enzyme alone, before the addition of tyrosine. The formation of DOPA-quinone was recorded after 20 min. In order to assess the effect of competition between the inhibitors and tyrosine, these experiments were repeated with increasing tyrosine concentrations from 1 to 3.75 mM, and the inhibition level of the enzyme−inhibitor complex tested. Results summarized in Figure 2 I−III show that without preincubation with the enzyme, the inhibitory levels of the compounds, **6** (Figure 2II) and **7** (Figure 2III), decreased with the increase in tyrosine concentration. Similarly, the inhibitory effect of DPPA (**Figure 2**I) dropped in the presence of 1, 2, and 2.5 mM tyrosine, but increased again at 3 and 3.75 mM tyrosine. When these inhibitors were preincubated with the enzyme for 10 min prior to the addition of tyrosine, the inhibitory potency of ester **6** (0.5 μM) remained the same even in the presence of 3.75 mM tyrosine, and that of ester **7** (0.3 μM) diminished slightly with the rise in tyrosine concentration, from 97% inhibition with 1 mM tyrosine to 86% inhibition with 3.75 mM tyrosine. Such a preincubation of DPPA with tyrosinase only slightly affected its inhibitory behavior.

Discussion

Among the various tyrosinase inhibitors reported in literature, those containing a resorcinol subunit are of special interest, since they are usually highly active (IC_{50} < 10 μM). The 2,4-resorcinol derivatives are distributed among the stilbene, flavonoid, and chalcone families and are significantly more potent as inhibitors than the 3,5-substituted resorcinol derivatives. For example, among the stilbene family, oxyresveratrol (2′,4′,3,5-tetrahydroxy-*trans*-stilbene) has an IC_{50} of 1.2 μM, whereas resveratrol (4′,3,5-trihydroxy-*trans*-stilbene) has an IC_{50} of 54.6 μM.[22] These differences in the IC_{50} values are not necessarily a result of the additional hydroxyl group found in oxyresveratrol, as compared to resveratrol. It has been reported by others that in the case of polyphenols, such as *N*-benzylbenzamides[9] and chalcones,[10] the major determining factor of their inhibitory efficacy is the location and not the number of the hydroxyl groups.

Natural and synthetic substituted 2,4-resorcinol compounds, such as artocarbene and chlorophorin (*trans*-stilbene), norartocarpanone (flavanone),[7,23] and chalcones[8] are all constructed from a 4-substituted resorcinol skeleton and have IC_{50} values of <10 μM. In the present study, DPPA, with a 2, 4-dihydroxy resorcinol skeleton demonstrated a high tyrosinase inhibitory potency (1.9 μM), which decayed very slowly with time **(Figure 1)**. We questioned if the addition of a hydrophobic moiety to the basic structure of the DPPA would increase enzyme-binding affinity. A new 2,4-dihydroxystilbene, isolated from *Chlorophora excelsa* by Shimizu et al.,[24] and chlorophorin contain an additional substituted octyl side chain at position 3 of the aromatic ring, unlike the new stilbene, which contains, in addition, a hydroxyl group on carbon 7″ of the octyl residue. Both compounds were inhibitors of tyrosinase with an IC_{50} of 96 μM in the case of the latter, and 1.9 μM for the more hydrophobic compound (chlorophorin). In another study, the inhibitory effect of the 4-substituted resorcinol, e.g., 4-hexylresorcinol, was found to be more active in preventing L-DOPA oxidation to DOPA-quinone than was 4-ethylresorcinol,[11] with the less hydrophobic side chain. Similarly, when studying the inhibitory effect of *p*-alkylbenzoic acids, it was found that with the increase in the length of the alkyl side chain, the tyrosinase inhibitory effect improved.[25] In the present study, the docked 2,4-substituted resorcinol derivative (compound **3)** appeared to fit well in the enzyme active site **(**Figure 3), close to the copper ion couple. Figure 3 demonstrates that the two resorcinol hydroxyl groups seem to form three hydrogen bonds, two with the nitrogen atoms of the amino acids, Arg 55 and Trp 184, on the side chains, and one with the carboxylic group of the Glu (182). An additional hydrogen bond is located between the carbonyl oxygen of DPPA and the imidazole hydrogen of His 190. Furthermore, hydrophobic interactions of compound **3** are also possible between its resorcinol aromatic ring and Ile 42, as well as between the isopropyl ester and Ala 202. Other hydrophobic interactions may occur if one of the two methyl groups of the isopropyl ester is replaced by a long chain, which may interact with Ile 42 and Met 43 in the enzyme hydrophobic pocket **(**Figure 3B**)**; such a compound will be considered for future synthesis.

Docking calculations revealed that the inhibitory effect of DPPA esters somewhat correlated with increasing bulkiness of the hydrophobic side chain. The DPPA isopropyl ester was discovered to be a more potent inhibitor than the ethyl or octyl esters. This finding is in agreement with that of Kubo,[15] who studied the inhibitory effect of the gallic acid ester, and showed that geranyl gallate and decahydro-2-naphthyl gallate with their bulky esters are 6-fold better inhibitors of tyrosinase than dodecyl gallate with the linear chain. This is assuming that this preferential inhibitory effect of the bulky portion is apparently due to a better match of the hydrophobic protein pocket surrounding the binuclear copper active site. The present study further elucidated the effect of the increased bulkiness of the hydrophobic group. We questioned if such an effect could be predicted from the calculated parameters related to the energy of the interaction between the potential inhibitor and the enzyme. Several DPPA esters were selected, their structures were drawn, and the conformation with the minimum energy was chosen, using the MOPAC program. Each of these esters was allowed to interact with the three-dimensional structure of the enzyme[1] by means of the docking program. The interactions with the minimum energy were selected. According to these calculations,

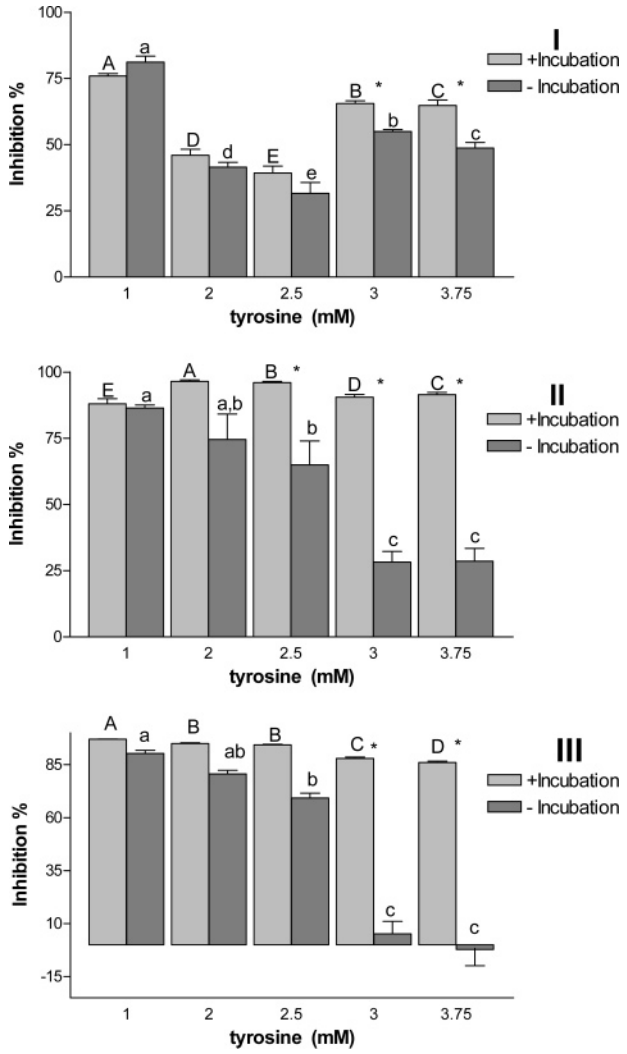


Figure 2. The effect of preincubation of enzyme inhibitors with tyrosinase. Tyrosinase was incubated either with 10 μM DPPA (I), with 0.5 μM compound **6** (II), or with 0.3 μM compound **7** (III) (each at concentration exhibiting 80% inhibition) for 10 min, before the addition of tyrosine (1−3.75 mM). Twenty minutes after the addition of tyrosine, the level of DOPA-quinone formation was monitored spectrophotometrically at 450 nm. Letters represent significant differences (p < 0.05, according to Duncan's multiple range test) among the results of % enzyme inhibition obtained at the various concentrations of tyrosine (uppercase, with preincubation with enzyme; lower case, without preincubation with enzyme). *Significance of p < 0.05 according to *t*-test between the results obtained with preincubation and without preincubation with the enzyme at the same concentration.

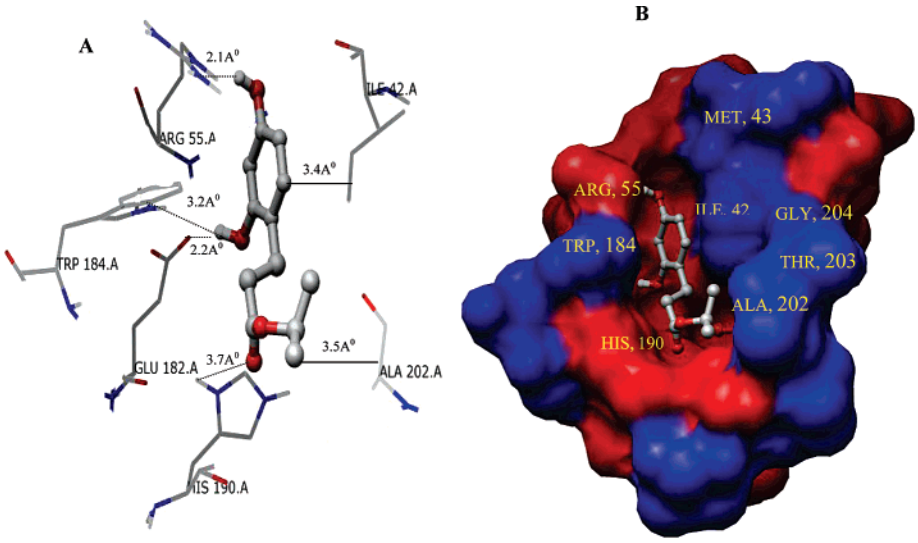


Figure 3. Isopropyl 3-(2,4-dihydroxyphenyl)propionate (compound **3**) interaction with the tridimensional tyrosinase binding site (Matoba, Y. et al. *J. Biol. Chem.* **2006**, *281*, 8981). (A) Lowest energy AutoDock of compound **3** in the tyrosinase active site. The hydrophilic and hydrophobic interactions of the inhibitor with the enzyme's amino acids at the enzyme binding site are shown together with their distance; red = oxygen, blue = nitrogen, and gray = carbon. (B) Surface representation of tyrosinase with the enzyme; hydrophilic (red) and hydrophobic (blue) zones docked with the DPPA isopropyl ester are shown. The cleft to which compound **3** is bound is the enzyme-binding site.

three potentially promising esters of DPPA were chosen: compound **5** (containing a *tert*-butyl ester), compound **6** (with a 1-[1,1-dimethylpropyl]-2,2-dimethylbutyl ester), and compound **7** (with a 3,3,5,5-tetramethyl-4-heptyl ester). These esters were synthesized and their tyrosinase inhibitory effects (observed IC_{50}) tested and compared with the predicted IC_{50}. All three compounds were expected to demonstrate an $IC_{50} < 1.0$ μM, and compounds **6** and **7** indeed inhibited the enzyme at the IC_{50} values of 0.18 and 0.15 μM, respectively. However, compound **5** had an IC_{50} of 14.9 μM, much higher than the predicted value; possibly such predictions are allowed only with primary or secondary alcohols as in the case of compounds **2**−**4**, **6**, and **7**, but not with tertiary alcohols, as in the case of the DPPA *tert*-butyl ester (**5**). The correlation between the observed IC_{50} values and the predicted values afforded a regression curve with the R^2 of 0.94 and P value <0.01, based on the free energy values.

The incubation of DPPA (10 μM) or DPPA esters (each at concentrations inhibiting 80% of the enzyme activity [0.5 and 0.3 μM for **6** and **7**, respectively]), with the enzyme and with increasing tyrosine concentrations (1−3.75 mM), resulted, as expected, in an inverse relationship, i.e., the inhibition decreased with an elevation in tyrosine concentration. When the enzyme was first preincubated with either compound, **6** or **7**, for 10 min, different results were anticipated **(**Figure 2). The preincubation of the enzyme with the ester almost entirely prevented the entrance of the tyrosine, supplemented in increasing doses, into the enzyme active site. This could be explained by the additional contribution of the hydrophobic bulky group to the inhibition constant (K_i) by increasing van der Waals forces with the enzyme hydrophobic core and thus preventing effective competition with tyrosine. Compounds **6** and **7** were discovered to be more potent than DPPA, with and without preincubation with the enzyme, at all tyrosine concentration ranges tested (Figure 2).

The present investigation emphasizes the importance of the 2,4-resorcinol skeleton in potent tyrosinase inhibitors and the extent to which a lipophilic moiety, combined with the resorcinol skeleton, can contribute to this activity. It was demonstrated that additional lipophilic groups affect both the inhibition potency, as well as the ability of the tyrosine to compete with the inhibitors. Such a lipophilic unit, that contains a minor bulky group, was a preferred inhibitor over a long chain or highly bulky functional moiety, as it may interact with the enzyme hydrophobic pocket and augment binding affinity. The lowest energy AutoDock of compound **3** at the tyrosinase active site revealed that the 2,4-resorcinol unit fits the enzyme-binding site well, forming four hydrogen bonds and one lipophilic interaction. By means of such docking methodology, one may design new inhibitors with additional van der Waals forces (with Met 43 and Thr 203) and hydrogen bonds (toward His 190) and thus enhanced binding affinity. Further study is required to synthesize such inhibitors and elucidate the type of inhibition that occurs.

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