

Chalcones as potent tyrosinase inhibitors: the importance of a 2,4-substituted resorcinol moiety

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Abstract—Compounds, which inhibit tyrosinase, could be effective as depigmenting agents. We have introduced a group of mono-, di-, tri- and tetra-substituted hydroxychalcones as effective tyrosinase inhibitors, showing that the most important factor determining tyrosinase inhibition efficiency is the position of the hydroxyl group(s) rather their number. The aim of the present study was to investigate the contribution of the different functional groups of the tetrahydroxychalcones to their inhibitory potency, with a view to optimizing the design of whitening agents. Four tetrahydroxychalcones were evaluated, the commercially available Butein and other three were synthesized, and their inhibitory effect on tyrosinase was tested. Results showed that a 2,4-substituted resorcinol subunit on ring B contributed the most to inhibitory potency. Changing the resorcinol substitute to position 3,5- or placing it on ring A significantly diminished the inhibitory effect of the compounds. A catechol subunit on ring A acted as a metal chelator (in the presence of copper ions) and as a competitive inhibitor (in the presence of tyrosinase), while a catechol on ring B oxidized to *o*-quinone (in the presence of both copper ions and tyrosinase). Three of the compounds also demonstrated antioxidant activity, which may contribute to the prevention of pigmentation. An examination of correlations between inhibitory activity and physical properties of the chalcones tested (such as dissociation energy and molecular planarity) showed positive correlation with the moment dipole value in the *Y*-axis, which may be used as an indicator of the inhibitory potential of new molecules. The present study revealed two very active tyrosinase inhibitors, 2,4,3',4'-hydroxychalcone and 2,4,2',4'-hydroxychalcone (with IC₅₀ of 0.2 and 0.02 μM, respectively). Structure-related activity studies added some understanding of the role and contribution of different functional groups associated with tyrosinase inhibitors.

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1. Introduction

Tyrosinase is a copper-containing enzyme widely distributed in nature, catalyzes two key reactions in the melanin biosynthesis pathway. Its inhibition is one of the major strategy in developing new whitening agents. Many tyrosinase inhibitors (Table 1) are polyphenol derivatives of flavonoids or of *trans*-stilbene (*t*-stilbene), such as resveratrol and its derivatives, which have been investigated intensively.^{1,2} They are usually constructed from one of two distinct substructures, which dictate their mechanism of tyrosinase inhibition: containing either a 4-substituted resorcinol moiety, or catechol. The 4-substituted resorcinol group has been

reported as potent in tyrosinase inhibition,³ and their structure–activity relationships and inhibition mechanisms have been examined.⁴ It was suggested that 4-substituted resorcinol-type inhibitors bind to the enzyme binuclear active site. The catechol structure, with two OH groups at *o*-positions, may behave as a chelator to the copper ions in the tyrosinase.¹ These polyphenols generally compete in inhibition with tyrosinase.

trans-Stilbene derivatives contain two aromatic rings, separated by two carbon atoms, connected by a double bond in *trans* configuration. Another group of compounds, with a similar structure to *t*-stilbene, is the chalcones, which are widely distributed in higher plants.^{5,6} Chalcones also consist of two aromatic rings in *trans* configuration, separated by three carbons, of which two are connected by double bond and the third is a carbonyl group. Recently, we have demonstrated

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Table 1. Tyrosinase inhibitors—data from the literature

Inhibitor	Structure	Substrate	Tyrosinase inhibition (IC ₅₀ , μ M)
Artocarbene ¹⁹		L-Tyrosine	2.45
Chlorophorin ¹⁹		L-Tyrosine	2.6
Norartocarpanone ¹⁹		L-Tyrosine	1.76
4-Propylresorcinol ⁴		L-Tyrosine	0.91
Glabridin ⁷		L-Tyrosine	0.09
3,4-Dihydroxybenzonitrile ²⁰		L-DOPA	45
Kojic Acid ²⁰		L-DOPA L-Tyrosine	22 8.66

that chalcones, like *t*-stilbene, are potential inhibitors of tyrosinase⁷ and the position of the hydroxyl(s) attached to the chalcone rings are of major importance in that activity.⁸ In the present study we designed, synthesized and tested new chalcones, constructed from substituted resorcinol at one aromatic ring and a catechol moiety at the second aromatic ring (A or B) of the molecule. Our hypothesis was that a molecular structure containing, within the same compound, a resorcinol moiety (with the ability to penetrate to the enzyme active site and inhibit it), together with a catechol moiety (which, if properly designed, may chelate the copper ions present in the active site), would exert potent tyrosinase inhibition. In addition, these compounds contain phenolic hydroxyls, which can prevent pigmentation resulting from auto-oxidative processes. The relation between the structure and different physical properties of these chalcones to their tyrosinase inhibitory effects, as well as their inhibition of melanin formation in human melanocyte cells, were investigated, and the contribution of each functional group to that activity was characterized.

2. Results

2.1. 3,4,2',4'-Tetrahydroxy-*t*-stilbene as tyrosinase inhibitor

In order to test our hypothesis on tyrosinase inhibitors, which combine the function of being a competitive inhibitor (which many of the resorcinol derivatives are) with that of a chelating agents, we chose a *t*-stilbene that contains resorcinol on one aromatic ring and a catechol moiety on the other: 3,4,2',4'-tetrahydroxy *t*-stilbene (compound I). As shown in Table 2, compound I exhibited higher tyrosinase inhibitory activity than other known inhibitors⁹ with IC₅₀ of 1.5 μ M.

2.2. Chalcones as tyrosinase inhibitors

Having demonstrated that *t*-stilbene with the two subunits is indeed active, we then synthesized chalcones containing both resorcinol and catechol. Unlike *t*-stilbene, the two aromatic rings in chalcones are not sym-

Table 2. The inhibitory effect of *t*-stilbene and chalcones on mushroom tyrosinase activities

Compound	Structure	L-Tyrosine (IC ₅₀ , μ M)	L-Dopa (IC ₅₀ , μ M)
3,4,2,4- <i>trans</i> -Stilbene I		1.5	—
3,4,2',4'-HC (Butein) II		29.3	>100
2,4,3',4'-HC III		0.2	7.5
3,5,3',4'-HC IV		31.68	>1000
2,4,2',4'-HC V		0.02	90

A solution of tyrosinase (50 units/mL), tested compound (0.005–50 μ M) and L-tyrosine (1 mM) or L-DOPA (6 mM) in 200 μ L PBS (50 mM, pH 6.5) was prepared and its optical density at 492 nm was measured after 20 min.

metric, thus chalcones containing the catechol functional group either on ring A or ring B were tested (compounds II–IV) (Table 2).

The inhibition of the tyrosinase was tested on both enzyme activities stages: L-tyrosine as substrate to L-DOPA and the oxidation of the later to *o*-quinone. Compounds II–V demonstrated higher tyrosinase inhibitory activity with the first stage than that of the second stage. Butein (compound II), with a catechol subunit on ring B and resorcinol on ring A (positions 2' and 4'), inhibited the first stage with an IC₅₀ of 29.3 μ M and presented poor inhibition of the second stage. The inhibitory activity of compound III (2,4,3',4'-HC), the opposite structure to Butein, increased inhibition approximately 150-fold, with IC₅₀ of 0.2 μ M at the first stage, and with IC₅₀ of 7.5 μ M for the second stage. In compound IV (3,5,3',4'-HC), the OH groups in the resorcinol moiety on ring B are located on positions 3 and 5 (compared with positions 2 and 4 in compound III), keeping the catechol subunit at the same location as in compound III. This change decreased the tyrosinase inhibitory effect to about the same degree as that obtained with Butein (IC₅₀ of 31.7 μ M), with practically no inhibition of the second stage.

When the chalcone molecule was constructed with resorcinol moieties on both rings A and B, at positions 2, 4 and 2', 4' (compound V), tyrosinase inhibitory activity increased to IC₅₀ of 0.02 μ M for the first stage, and to 90 μ M for the second stage.

First stage activity of tyrosinase is characterized by a lag time, followed by an increase in reaction rate.² Figure 1

depicts the change in OD at 475 nm as a function of time due to the formation of *o*-quinone in the absence (control) or the presence of compounds II–V. As expected, in the presence of tyrosinase inhibitors, the lag phase was prolonged from 440 s in the control to 787 s with the addition of compound IV. Furthermore, compounds

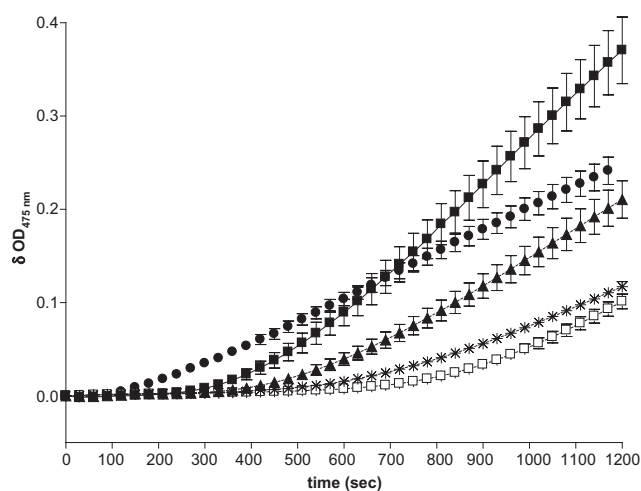


Figure 1. Lag period of L-dopaquinone formation. L-Tyrosine (1 mM) was incubated with tyrosinase in the absence (■—control) or with the addition of chalcones II–V, each at twice its IC₅₀ value; Butein (●, compound II) 50 μ M, 2,4,3',4'-HC (*, compound III) 0.4 μ M, 3,5,3',4'-HC (□, compound IV) 50 μ M and 2,4,2',4'-HC (▲, compound V) 0.04 μ M, and the change in the OD at 475 nm were recorded as a function of time. Ethanol was used as a control. Values are means $\pm$ SE of three separate experiments.

III–V also reduced the reaction rate (the slope of the curve) of the *o*-quinone formation by 80–60% versus control. Contrary to the above effect, Butein decreased the lag time to 220 s a phenomena, which will be discussed further in Discussion section.

2.3. The chelating ability of compounds II–V

The interactions of each chalcone with Cu^{+2} , at pH 6.5, was assessed by UV/vis spectroscopy, measuring bathochromic shifts as a result of complex formation between

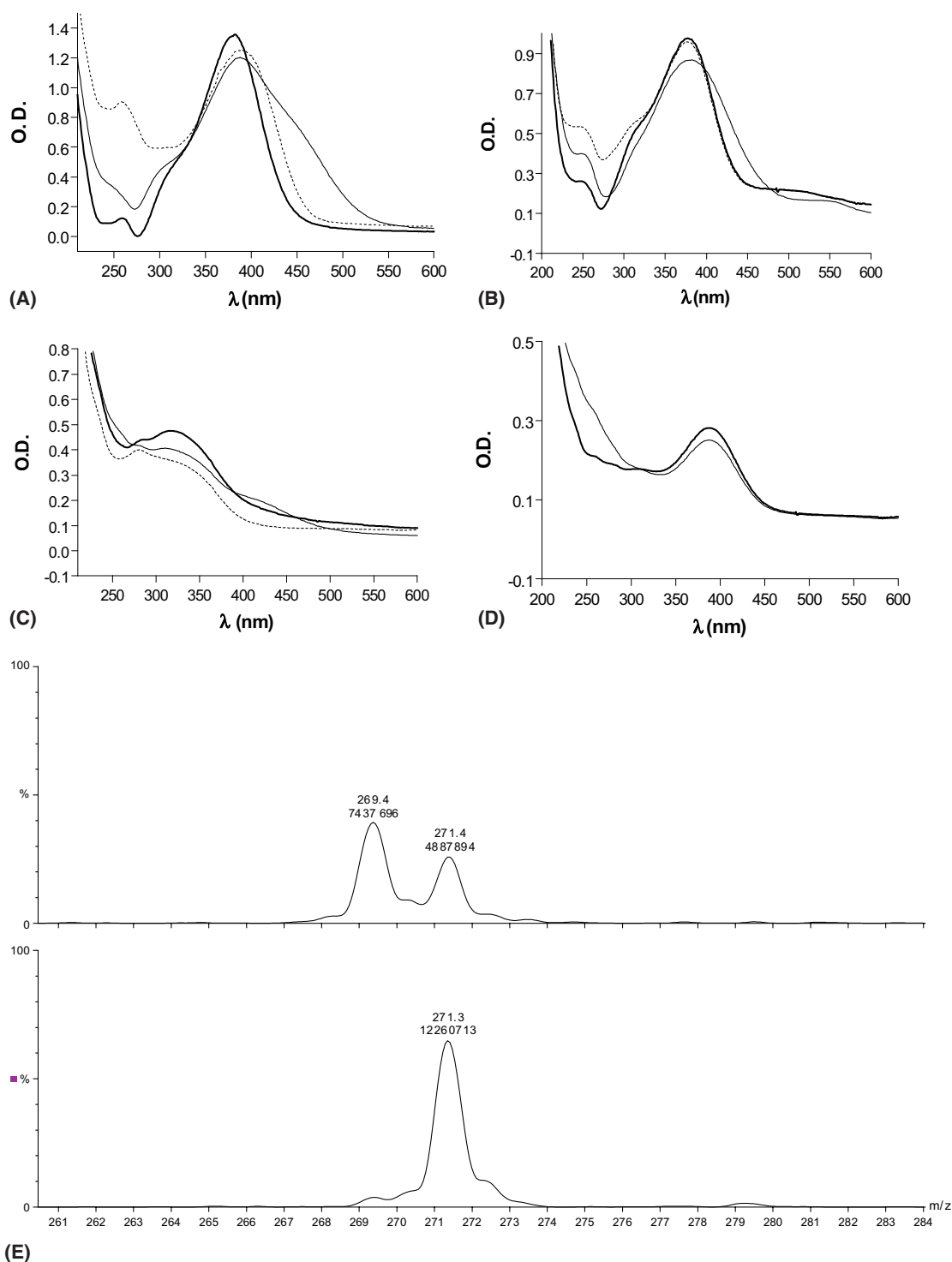


Figure 2. Copper ions chelating ability of chalcones II–V. The UV–vis spectra of Butein II (A), 2,4,3',4'-HC (III) (B), 3,5,3',4'-HC (IV) (C) and 2,4,2',4'-HC (V) (D) at a $50 \mu\text{M}$ solution of phosphate-buffered saline (PBS) each, at pH = 6.5 (thick solid line). Spectrum recorded again with the addition of $100 \mu\text{M}$ CuSO_4 (thin solid line), and again with the addition of EDTA $250 \mu\text{M}$ (dotted line). The mass spectrum of Butein (II) before (E, below) and after the addition of copper ions (E, above) using negative ion electrospray ionization method.

the copper ion and the tested compounds, and by LC–MS analysis, following changes in m/z values. A solution of each compound (50 μM) in PBS buffer was scanned with or without CuSO_4 (100 μM), and the spectra obtained were compared. The characteristic maximum at 382 nm of Butein shifted to 388 nm with the addition of copper ions (Fig. 2A); on addition 250 μM EDTA, the shifted λ_{max} remained at 388 nm. LC–MS of Butein showed molecular ion at m/z 271 ($M-1$, negative electrospray ionization), whereas upon injection of the reaction mixture after the addition of copper ions, the molecular ion shifted by two mass units giving m/z of 269 ($M-1$) (Fig. 2E). Similar results were obtained with chalcone III, showing λ_{max} of 378 nm; with the addition of copper ions the λ_{max} shifted to 382 nm (Fig. 2B), but it returned to its original value (378 nm) with the addition of EDTA (250 μM). LC–MS analysis of the mixture of the chalcone III with copper ions did not show any change in the m/z value as the reaction proceeded. Upon the addition of copper ions to chalcone IV, λ_{max} at 317 nm shifted to give a shoulder at 400 nm, which was re-absorbed to λ_{max} of 317 with the addition of EDTA (Fig. 2C). The MS analysis of compound IV with copper ions did not show any change in the mass of the peak of the parent compound. The UV/vis spectrum of chalcone V was not affected by the addition of copper ions (Fig. 2D).

2.4. The effect of chalcones III–V on melanin biosynthesis in human melanocytes

Table 3 presents the effect of glabridin, a known tyrosinase inhibitor,¹⁰ in comparison to chalcones III–V, on melanin biosynthesis in melanocyte cultures. Glabridin (10 μM) decreased melanin synthesis to about 41% and had no cytotoxic effect. Compound III (5 μM) inhibited melanin synthesis to about 63%, with 88% cell survival. Compound V (5 μM) inhibited melanin synthesis to about 55% and had no cytotoxic effect. Butein (5 μM) was cytotoxic (20% survival, data not shown).

2.5. Radical scavenging capacity of compounds II–V

Phenols may exert antioxidant properties, due to their ability to donate an electron or chelate metals. In the present study, chalcones II–V represent polyphenols and thus may perform antioxidant activity which prevents or delays pigmentation resulting from auto-oxidation. The antioxidant activity of chalcones II–V was tested by measuring their ability to donate an electron to the free radical compound DPPH, monitoring

Table 3. The inhibitory effect of glabridin and chalcones III and V on melanin biosynthesis in G361 human melanocytes

Compounds (concn)	Melanin inhibition (%)	Survival (%)
Glabridin 10 μM	41 $\pm$ 7	94.6 $\pm$ 9
2,4,3',4'-HC (III) 5 μM	62.5 $\pm$ 8	87.8 $\pm$ 11
2,4,2',4'-HC (V) 5 μM	55 $\pm$ 14	93.3 $\pm$ 12

Melanocyte cells grown in McCoy's medium (Bet-Hae'emek) were incubated with compounds added 24 h after cell seeding. The cells were harvested after 4–5 days, and treated with 1 N NaOH. Melanin content was measured in a spectrophotometer at 405 nm and protein content was measured, using Bradford reagent at 595 nm.

Table 4. The radical scavenging capacity of chalcones

Compounds	DPPH reduction ratio (DPPH/antioxidant (mole/mole))
Ethanol	0
Trolox	0.32
Butein, 3,4,2',4'-HC (II)	7.87
2,4,3',4'-HC (III)	5.46
3,5,3',4'-HC (IV)	3.4
2,4,2',4'-HC (V)	Not active

Trolox (positive control), 2,4,3',4'-HC (III), 3,5,3',4'-HC (IV) and 2,4,2',4'-HC (V), each at 10 μM , and Butein (II) at 2 μM in methanol were added to 63 μM DPPH. The absorption at 515 nm was recorded after 420 min incubation at 37 °C. Ethanol was used as control.

changes in absorption at 515 nm after 7 h incubation, due to elimination of the DPPH radical. The reactions were carried out under excess DPPH, in order to determine the number of electrons (the ratio of mole DPPH/mole antioxidant) each compound may donate to DPPH (Table 4). Results demonstrate that Butein donated the highest number of electron to the DPPH (8). Compounds III, IV and trolox donated 5.5, 3.4 and 0.32 electrons, respectively. Compound V failed to donate any electron to DPPH under these experimental conditions.

3. Discussion

In the present article we aimed to explore the depigmentation potential of hydroxylated chalcones with four substituted OH groups. These were studied as new inhibitors of melanin formation, in relation to their structure. The new compounds included chalcones constructed from a resorcinol moiety on one aromatic ring and a catechol substructure on the other, to evaluate the possibility of combining two types of inhibitory mechanisms in a single molecule, that is, binding to the enzyme active site and thus being competitive to tyrosine and chelating the copper ion(s) within the active site. As a model system, the validity of such a combination was first examined with the *t*-stilbene class of compounds.

Two well-known tyrosinase inhibitors of the *t*-stilbene class are resveratrol (4',3,5-trihydroxy-*t*-stilbene), found in grapes, with IC_{50} of 54.6 μM , and oxyresveratrol (2',4',3,5-tetrahydroxy-*t*-stilbene), extracted from *Morus alba*, exerting stronger tyrosinase inhibition with IC_{50} of 1.2 μM .⁹ Neither resveratrol nor oxyresveratrol contain an aromatic ring with a catechol subunit. In accordance to our hypothesis, a model molecule of the *t*-stilbene class, containing both subunits was selected, exerting tyrosinase inhibition with IC_{50} of 1.5 μM , superior to resveratrol and similar to that of oxyresveratrol. These results are in agreement with previous studies, suggesting that the number of the hydroxyl groups attached to *t*-stilbenes have an important role in determining their activity.^{3,4,9} Compound I is the first compound of this *t*-stilbene class containing both resorcinol and catechol moieties to be tested as tyrosinase inhibitor.

Butein (compound II), a commercial compound with a catechol subunit on ring B (positions 3 and 4) and resorcinol on ring A (positions 2' and 4') was found to inhibit the first stage of tyrosine oxidation with IC_{50} of 29.3 μ M. Compound III has the opposite structure to Butein. This compound markedly enhanced the inhibitory effect at the first stage (IC_{50} of 0.2 μ M). These results emphasize the major role of 2,4-substituted resorcinol substructure on ring B. Replacing the resorcinol substituted positions from 2 and 4 (in compound III) to positions 3 and 5 (compound IV—a novel chalcone), and keeping the catechol subunit on ring A at the same location in both compounds III and IV, resulted in a decreased inhibitory potency to about the same degree as Butein (IC_{50} of 31.68 μ M), with practically no inhibition at the second stage. These results demonstrate that resorcinol construction on ring B is not sufficient: it requires this resorcinol moiety to be at position 2 and 4, rather than at position 3 and 5.

Substitution of the chalcone molecule with resorcinol moieties on both rings A and B, at positions 2 and 4 and 2' and 4' (V) resulted with the formation of the most effective tyrosinase inhibitor known to us so far, with IC_{50} of 0.02 μ M. Comparing the tyrosinase inhibitory activity of chalcone (III) with *t*-stilbene (I), both sharing same functional groups, 3',4'-substituted catechol and 2,4-substituted resorcinol, revealed that the chalcone is 7.5 times more active than the corresponding *t*-stilbene, emphasizing the enhanced potential of the chalcone subclass as new whitening agents.

Monophenolase activity of tyrosinase is characterized by a lag time, influenced by various factors such as substrate and enzyme concentration, enzyme source, pH of the medium, presence of a hydrogen donor such as L-DOPA or other reducing agents or transition metal ions.² With the addition of exogenous reductants (ascorbate, hydroxylamine or hydroquinone), this lag period can be shortened and re-extended with the addition of

inhibitors.¹¹ In the present study, compounds III–V prolonged the lag phase, while Butein decreased it by half. This finding may suggest that chalcones III, IV and V, with four OH groups, do not mimic diphenol action in reducing copper (II) to copper (I) and regenerating it, and thus shorten the lag time. On the other hand, Butein may acts as reducing agent, donates electrons and converts to quinone. This possibility was further studied by injecting the reaction mixture to LC–MS and analyzing the products formed, and by calculating differences in the heat of formation. The results of both tests are discussed below (Table 5).

The depigmenting action of kojic acid, used in cosmetics, is attributed to its ability to chelate copper ion in the tyrosinase active site.¹² Similarly, chalcones II–IV demonstrated chelating ability, which may contribute to their inhibitory potency. In contrast, compound V did not chelate copper ions, suggesting the necessity of a catechol structure for chelation properties. The addition of EDTA to Butein did not reverse the reaction as it did in compound III and IV, suggesting that copper ions oxidized Butein to *o*-quinone, an assumption supported by the LC–MS analysis.

Tyrosinase was added to Butein, instead of copper ions (in both cases an immediate colour change to brown-red anticipated). An LC–MS analysis of the reaction mixture showed that with the enzyme addition to Butein, a new compound was formed with reduction of two mass units. This result may confirm that Butein was either oxidized to *o*-quinone by tyrosinase or copper ions, or it was converted to aurone derivatives, a reaction which may occur spontaneously.^{13,14} These possibilities were further investigated. Thus, under stirring Butein in ethanol during a week at room temperature in open glass no change occurred (based on LC/MS/MS analysis). FTIR analysis of the reaction product showed a single sharp peak at 1626 cm^{-1} for the carbonyl group, while that of Butein has three peaks

Table 5. The relation between tyrosinase inhibitory potency of chalcones II–V and their physical parameters

Compound (IC_{50} , μ M)	Planarity	Dipole moment (Debye) <i>X</i> -, <i>Y</i> - and <i>Z</i> -axes				$\Delta\Delta H_f$, kcal/mol (bond dissociation energy)
		<i>X</i>	<i>Y</i>	<i>Z</i>	Total	
II (29.3)	+	2.02	2.46	0	3.18	4-OH = 32.5 3-OH = 30.2 2'-OH = 42.2 4'-OH = 38.9
III (0.2)	–	1.27	3.83	— 2.08	4.54	4-OH = 35.1 2-OH = 34.2 3'-OH = 31.2 4'-OH = 30.5
IV (31.68)	–	2.64	2.74	— 2.87	4.77	3-OH = 36.2 5-OH = 36.0 3'-OH = 31.3 4'-OH = 30.7
V (0.02)	+	3.02	4.78	0	5.65	4-OH = 35.5 2-OH = 34.6 2'-OH = 44.7 4'-OH = 38.6

Potency was correlated to the planarity of the molecule [(+) planar, (–) not planar], to their total moment dipole (μ) or their *X*-, *Y*- or *Z*-axes values and to the O–H bond dissociation energy ($\Delta\Delta H_f$). These parameters were obtained by generating semi-empirical calculations, using MOPAC program and PM3 Hamiltonian.

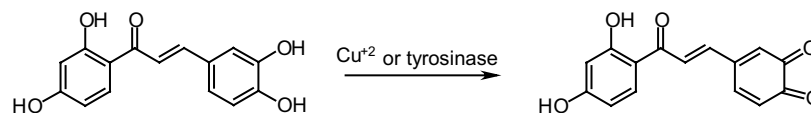


Figure 3. Oxidation of Butein to *o*-diquinone in the present of copper ions or tyrosinase.

at 1635, 1597 and 1558 cm^{-1} (data not shown), all together correspond to formation of quinone rather than aurone derivatives. We may thus conclude that a catechol subunit on ring A chelates copper ions (as observed in compounds III and IV), while a catechol subunit on ring B is oxidized by copper ions to its *o*-quinone derivatives (compound II, Fig. 3). Desirable skin-lightening agents should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce tyrosinase activity. They should exhibit low cytotoxicity, and be nonmutagenic.¹⁵ In the present study the two most active inhibitory chalcones (compounds III and V) were further tested for their ability to inhibit melanin synthesis in human melanocytes. Both were active, with low toxicity: compound III, at 5 μM , decreased melanin synthesis to about 63%, and compound V, at 5 μM , decreased it to about 55%. These results further encourage the possibility of using chalcones as new depigmentation agents.

Browning of food, beverage and skin could be the result of an enzymatic reaction of polyphenols oxidases and/or a result of the auto-oxidation of polyunsaturated fatty acids and proteins. Chalcones containing phenolic and catecholic derivatives may exhibit antioxidant activity as a result of their ability to donate an electron (or hydrogen atom) and/or chelate transition metals, such as copper or ferrous ions, and thereby eliminating reactive oxygen and nitrogen species (ROS and RNS) and decay-free radical propagation reactions: both reactions, when strong, are known to induce melanin synthesis.¹⁶ In the present study, chalcones II–V demonstrated antioxidant activity, which may contribute to the inhibition of melanin biosynthesis in cells.

We then tried to search for some explanations for the data gathered, especially trying to correlate tyrosinase inhibition by chalcones with physical parameters associated with chalcone structure. Such parameters were their molecular planarity, O–H bond dissociation energy, and their moment dipole (μ), using MOPAC program and PM3 semi-empirical Hamiltonian. A summary of the results is shown in Table 5. As expected, chalcones with a OH group at position 2' (ring A) are planar molecules, due to a hydrogen bond formation between the 2'-OH group and the adjacent carbonyl group. No correlation was anticipated between the planarity of the molecules and their inhibitory potencies. The heat of formation differences ($\Delta\Delta H_f$)^{17,18} between each possible phenoxyl radical and its parent chalcones (bond dissociation energy) correlated with antioxidant activity, but not with tyrosinase inhibition potency (Table 5). Compounds II, III and IV all contain a catechol moiety, and thus have at least one O–H with low dissociation energy, which can relatively, easily donate a hydrogen atom ($\Delta\Delta H_f \sim 30\text{kcal/mole}$) and thus act as a potent anti-

oxidant; while in compound V the lowest $\Delta\Delta H_f$ was 34.5kcal/mole, which resulted in an absence of antioxidant activity (testing a donation of hydrogen atom to DPPH).

The moment dipole (μ) of a molecule can be a relevant parameter for evaluating the interaction efficiency of ligand/inhibitor with an enzyme active site. A correlation of the total (μ) of each of the four chalcones under study with their tyrosinase inhibition was also shown to be not indicative. Thus, Butein differed significantly in its total (μ) from compound IV, despite their similar tyrosinase inhibition. We then separated the total (μ) values into their three X, Y and Z-axes. Such a process is pertinent, since ligands are oriented inside an enzyme active site in a relatively rigid position, and free rotation of the ligand molecule is usually not possible. This separation revealed a positive correlation between tyrosinase inhibition potency and the (μ) of the compounds with their Y-axis (Table 5). Thus, Butein and compound IV have similar inhibitory activity and similar (μ) values, (2.46 and 2.74 Debye), while compounds III and V have higher (μ) values in the Y-axis (3.83 and 4.78 Debye, respectively), and correspondingly higher inhibitory potency (IC_{50} of 0.2 and 0.02 μM). For drawing conclusions, this last finding has to be supported by many additional examples of compounds.

The collected data from our studies analyzing the relationship between the chemical structures of chalcones and their tyrosinase inhibition potency, led us to design and synthesis the 2',4',2,4-tetrahydroxychalcone, the most powerful inhibitor known to date. The presence of two functional resorcinol units, both in 2- and 4-positions, found to contribute the most to chalcone for tyrosinase inhibition potency while the presence of catechol moiety either on ring A or on ring B did not contribute much to the inhibitory potency.

4. Materials and methods

4.1. Chemicals and reagents

Tyrosinase (EC1.14.18.1, Sigma Product T7755 with an activity of 6680 units/mg). Chalcones were synthesized according to procedure described below. Butein (compound II) and other reagents and solvents were purchased from Aldrich–Sigma and used as received unless otherwise noted.

4.2. Inhibition of tyrosinase activity

Potassium phosphate buffer (0.07 mL, 50 mM) at pH 6.5, 0.03 mL tyrosinase (333 units/mL) and 2 μL of the tested compounds (0.5–500 μM), dissolved in absolute ethanol

were inserted into 96-well plates. After 5 min incubation at room temperature, 0.1 mL L-tyrosine (2 mM) or 12 mM L-DOPA were added and incubated for additional 20 min. The optical density of the samples at 492 nm were measured (Elisa SLT Lab instruments Co. A-5082) relative to control containing ethanol (2 μ L) and without inhibitor, demonstrating a linear colour change with time during the 20 min of the experiment.

4.3. Inhibition of melanin synthesis in human melanocytes

Melanocytes (G361) cells were seeded in 24-well plates, and grown in McCoys medium (Bet-Hae'emek). 24 h after seeding, the chalcones were added to a final concentration of 5 μ M (total volume 0.5 mL). After four or five days the cells were harvested, using trypsin, and washed once with PBS. The cells in each well were dissolved in 1 mL 1 N NaOH. Melanin content was measured in a spectrophotometer at 405 nm. Protein content was measured, using Bradford reagent at 595 nm.

4.4. Radical donation capacity of chalcones

The radical donation ability of the chalcones was measured, with trolox as a positive control, using a mixture with 2,2-diphenyl-1-picrylhydrazyl (DPPH): 9 μ L of the compounds (compounds III–V and trolox, 2 mM each and compound II—Butein—0.4 mM), dissolved in ethanol, was added to a 63 μ M DPPH solution dissolved in methanol (63 μ M). The final volume of the reaction was 1.8 mL. The absorbance at 515 nm was measured at different time interval until the reaction reached a plateau. The donation capacity of the product was then calculated as the molar ratio of a mole of DPPH/mole of the compound.

4.5. The ability of chalcones to chelate copper ions

Stock solution of each chalcone (2 mM) was prepared in ethanol. Then 50 μ M solution was prepared in a cuvette containing PBS (50 mM, pH = 6.5) and the sample absorbance was recorded in the 200–800 nm range. A repeated scan was recorded after addition of 100 μ M CuSO₄ and a third spectrum after the addition of EDTA (250 μ M).

4.6. HPLC, GC–MS and LC–MS analysis

HPLC analysis was performed on an instrument connected to a diode array detector (HP-1100) and equipped with a reverse phase column (C-8) (125 mm length; 4 mm diameter) of 5 μ M particles. The mobile phase was a mixture of acetonitrile and water with a flow of 1 mL/min and a gradient starting with 20% acetonitrile in water and increasing in concentration to 80% acetonitrile for 10 min, and then to 97% for an additional 10 min. GC–MS analysis was performed by means of an HP gas chromatograph, Model 5890 Series II (Waldbronn, Germany), fitted with an HP-5 trace analysis capillary column (column 0.32 mm I.D. 0.25 mm film thickness, 5% phenyl methyl silicone), with a mass selective detector, Model 5972 (Waldbronn, Germany),

linked to an HP ChemStation data system. Helium was used as carrier gas, at a flow rate of 0.5 mL/min, pressure 10.4 psi and at a linear velocity of 26.8 cm/s. The MS transfer line was maintained at 280 °C. The injector was set at 300 °C, the detector at 280 °C and the column heated at a gradient starting at 130 °C, increasing to 250 °C at 10 °/min and then at 5 °/min to 300 °C and maintained for an additional 15 min at 300 °C. LC–MS measurements was performed by using LC–MS (Waters 2790 HPLC, with Waters photodiode array detector 996 and micromass Quattro ultima MS).

4.7. Synthesis of 2,4,3',4'-tetrahydrochalcone (compound III)

3',4'-Dimethoxyacetophenone: To a stirred mixture of 3',4'-dihydroxyacetophenone (100 mg, 0.66 mmole) and anhydrous K₂CO₃ (5 g, 36 mmole) in dry acetone (10 mL) 1 mL, 16 mmole MeI was added. The mixture was heated at reflux for 45 min, then cooled to room temperature, filtered and evaporated under reduced pressure. The residue was dissolved in CH₂Cl₂, washed by two portions of water, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The product was purified by flash chromatography (silica, hexane/ethylacetate 4:1, v/v). Removal of the solvent gave a 93% yield of the product, 3',4'-dimethoxyacetophenone (110 mg, 0.61 mmole). The product was detected by HPLC as one peak, with a retention time of 5.2 min, and identified by GC–MS (m/z = 180 M⁺; 165 M⁺–CH₃; 137 M⁺–COCH₃).

2,4-Dimethoxybenzaldehyde: using the same procedure as above afforded 100 mg of 2,4-dimethoxybenzaldehyde (0.60 mmole, 85% yield). The product purity was determined by HPLC as one peak with a retention time of 6.3 min, and identified by GC–MS with m/z = 166 M⁺; 149 M⁺–M⁺–CH₃–2H; 120 M⁺–CH₃–COCH₃–2H.

2,4,3',4'-Tetramethoxychalcone: To a stirred solution of 3',4'-dimethoxyacetophenone (24 mg 0.133 mmole) and 2,4-dimethoxybenzaldehyde (25 mg, 0.15 mmole) in 2 mL ethanol at 0 °C was added a solution of NaOH (40%) in water (150 μ L). The mixture was stirred overnight at 4 °C, and then extracted with two portions of ethylacetate and the organic layer was separated and evaporated under reduced pressure. The residue after evaporation was purified by flash chromatography (silica, hexane/ethylacetate 7:3 v/v). Removal of the solvent afforded 3',4',2,4-tetramethoxychalcone, 31 mg, 0.095 mmole (70% yield). The HPLC of the product has a retention time of 8.85 min.

2,4,3',4'-Tetrahydrochalcone (compound III): Boron tribromide BBr₃ (100 μ L) was added to a stirred solution of 3',4',2,4-tetramethoxychalcone (23 mg, 0.07 mmole) in dried dichloromethane (10 mL) at 0 °C. The mixture was stirred overnight at 4 °C, extracted with two portions of ethylacetate and evaporated under reduced pressure. The product was purified by reverse phase chromatography (C18, acetonitrile/water 1:4, v/v) and the solvent was removed by lyophilizer. Two products were isolated.

(1) 2,4,3',4'-Trihydroxymonomethoxychalcone (2mg, 0.007mmole—10% yield), which has a retention time of 5.7 min by HPLC, and identified by LC–MS in direct injection method (ES^- ; 285.3 $\text{M}-\text{H}^+$) and by UV/vis spectrum (λ_{max} of 374 nm, ($\epsilon_{(\text{mM}^{-1} \text{cm}^{-1})} = 33.0$)).

(2) 2,4,3',4'-Tetrahydroxychalcone (4mg, 0.01 mmole—21% yield), with a retention time of 4.18 min in HPLC, and identified by LC–MS in direct injection method (ES^- ; m/z of 271.1 $\text{M}-1$) and by UV/vis spectrum with λ_{max} 378 nm ($\epsilon_{(\text{mM}^{-1} \text{cm}^{-1})} = 22.12$).

4.8. Synthesis of 3,5,3',4'-tetrahydroxychalcone (compound IV)

3,5-Dimethoxybenzaldehyde: the same procedure was used as with 3',4'-dimethoxyacetophenone to afford 124 mg product (0.74 mmole—90% yield). The product has a retention time of 6.8 min in HPLC, and was identified by GC–MS ($m/z = 166 \text{ M}^+$; $135 \text{ M}^+ - \text{OCH}_3$; $122 \text{ M}^+ - \text{CH}_3 - \text{COCH}_3$).

3,5,3',4'-Tetramethoxychalcone: The same procedure as with 3',4',2,4-tetramethoxychalcone was used to give 44 mg product (0.134 mmole—70% yield). This product has a retention time of 9.15 min in HPLC, and was identified by proton NMR (^1H NMR) (CDCl_3 , 200 MHz): δ_{ppm} ; 7.66 (m, 2H); 7.51 (d, 1H); 7.48 (d, 1H); 6.89 (d, 1H); 6.76 (2s, 2H); 6.50 (s, 1H); 3.95 (s, 6H); 3.82 (s, 6H).

3,5,3',4'-Tetrahydroxychalcone (compound IV): The same procedure as with 3',4',2,4-tetrahydroxychalcone was used to give 3',4',3,5-tetrahydroxychalcone (3 mg, 0.01 mmole—25% yield) which has a retention time of 3.99 min in HPLC, and was identified by LC–MS in direct injection mode (ES^- ; m/z of 271.1 $\text{M}-1$) and by UV/vis spectrum with λ_{max} of 330 nm ($\epsilon_{(\text{mM}^{-1} \text{cm}^{-1})} = 9.16$).

4.9. Synthesis of 2,4,2',4'-tetrahydroxychalcone (compound V)

2',4'-Dimethoxyacetophenone: the same procedure was used as for 3',4'-dimethoxy acetophenone to afford 100 mg product (0.56 mmole—85% yield). The product has a retention time of 6.7 min in HPLC, and was identified by GC–MS ($m/z = 180 \text{ M}^+$, $165 \text{ M}^+ - \text{CH}_3$, $122 \text{ M}^+ - \text{CH}_3 - \text{COCH}_3$).

2,4,2',4'-Tetramethoxychalcone: The same procedure was used as for 3',4',2,4-tetramethoxychalcone, except that the NaOH solution (40% in water) was added at room temperature and the reaction mixture was stirred overnight at room temperature to give 10 mg 2',4',2,4-tetramethoxychalcone (0.03 mmole—50% yield). The product has a retention time of 9.34 min in HPLC, and was identified by proton NMR (CDCl_3 , 200 MHz): δ_{ppm} ; 7.87 (d, 1H); 7.70 (d, 1H); 7.48 (dd, 2H); 6.49 (m, 4H); 3.83 (3s, 12H).

2,4,2',4'-Tetrahydroxychalcone (compound V): The same procedure as for 3',4',2,4-tetrahydroxychalcone was used, except that the reaction mixture was stirred overnight at room temperature. The product was purified

by flash chromatography (silica, hexane/ethylacetate 50:50, v/v) and by additional chromatography, using a reverse phase column (semi-preparative column), resulting in two pure compounds.

(1) One with a retention time of 7.5 min which was identified by LC–MS (ES^- ; m/z of 285.3 $\text{M}-1$), and by UV/vis spectrum (λ_{max} of 390 nm ($\epsilon_{(\text{mM}^{-1} \text{cm}^{-1})} = 24.32$)) as 2',4',2,4-trihydroxy monomethoxychalcone (10 mg, 0.035 mmole) (39% yield) and

(2) a second product with a retention time of 5.74 min in HPLC, which was identified by LC–MS (ES^-) with m/z of 271.7 ($\text{M}-1$) and UV/vis spectrum with λ_{max} of 390 nm ($\epsilon_{(\text{mM}^{-1} \text{cm}^{-1})} = 17.32$) as 2',4',2,4-tetrahydroxychalcone (10 mg, 0.037 mmole—41% yield).

No attempt was made to improve the yields in the above synthesis.

References and notes

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