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Chalcones as potent tyrosinase inhibitors: the effect of hydroxyl positions and numbers

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Abstract

The inhibition of tyrosinase is one of the major strategies to treat hyperpigmentation. Various limitations are associated with many of these inhibitors, such as high cytotoxicity, poor skin penetration and low stability in formulations. In continuation of our previous study [J. Agric. Food Chem. 51 (2003) 1201], showing that isoliquiritigenin chalcone (ILC) is a potent tyrosinase inhibitor, the present study aims to characterize the chalcone family as new tyrosinase inhibitors, and demonstrate their potential whitening potency. Nine mono-, di-, tri- and tetrahydroxychalcones were tested as inhibitors of tyrosinase mono- and diphenolase activities, showing that the most important factor in their efficacy is the location of the hydroxyl groups on both aromatic rings, with a significant preference to a 4-substituted B ring, rather than a substituted A ring. Neither the number of hydroxyls nor the presence of a catechol moiety on ring B correlated with increasing tyrosinase inhibition potency. 4-Hydroxychalcone (4-HC), ILC and Butein inhibited tyrosinase and shortened the lag period of enzyme monophenolase activity from about 490 min (control) to 30 min (ILC). As pigmentation also results from auto-oxidation, the antioxidant activity of 4-HC, ILC and Butein, were tested. Results showed that chalcones are also potent antioxidants, with Butein the most potent. We may conclude that chalcones are potentially potent new depigmentation agents, with their double effect of reduction and antioxidant activity. A deeper understanding of the relation between their structures to their potency will contribute to designing the optimal agents.

Keywords: Tyrosinase; Inhibitor; Whitening agent; Chalcones; SAR

1. Introduction

Tyrosinase (monophenol monooxygenase, E:C:1.14. 18.1), also known as polyphenol oxidase (Whitaker, 1995), is a copper-containing enzyme widely distributed in nature. It catalyzes two reactions involving molecular oxygen in the melanin biosynthesis pathway: the hydroxylation of monophenols to *o*-phenols (monophenolase activity), and the oxidation of the *o*-phenols to *o*-quinones (diphenolase activity). These quinones are highly reactive and tend to polymerize spontaneously to form brown pigments of high molecular weight (melanins), which determine the color of mammalian skin and hair (Seo et al., 2003). Quinones can also react with amino acids and proteins and thus enhance the development of brown color.

Various dermatological disorders, such as melasama, age spots and sites of actinic damage, arise from the accumulation of an excessive level of epidermal pigmentation. Tyrosinase inhibitors have become increasingly important in medication (Seo et al., 2003) and in cosmetics (Maeda and Fukuda, 1991) to prevent hyperpigmentation, by inhibiting enzymatic oxidation. A number of naturally occurring tyrosinase inhibitors have been described, the majority consisting of a phenol structure or of metal chelating agents (Mayer, 1987; Mayer and Harel, 1979; Passi and Nazzaro-Porro, 1981; Pifferi et al., 1974). Some of these inhibitors suffer from number of limitations, such as low activity, high toxicity, and insufficient penetrative ability. Other potentially active agents, such as kojic acid and arbutin, have not

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yet been demonstrated as clinically efficient (Briganti et al., 2003). Hydroquinone, a widely used skin lightening agent, is a compound considered to be cytotoxic to melanocytes, and hence potentially mitogenic (Briganti et al., 2003; Dooley, 1997; Frenk, 1995; Hermanns et al., 2000). A possible way to overcome some of these limitations could be the use of a combination of compounds in a mixture, which may act as whitening agents through different mechanisms and in a complementary way, such as combining tyrosinase inhibitors with efficient antioxidants, together with antiperoxidases.

Chalcones are a group of compounds widely present in higher plants (Star and Mabry, 1971; Stevens et al., 2000), which may combine these various activities. They contain two aromatic rings with an unsaturated chain. Many biological activities have been attributed to this group, such as anticancer (Satyanarayana and Rao, 1993; Shibata, 1994), anti-inflammatory, antipyretic and analgesic (Satyanarayana and Rao, 1993), cytotoxic in vitro (Dhar, 1981), bactericidal, insecticidal, anti-fungal (Satyanarayana and Rao, 1993), antioxidant (Ruby et al., 1995; Vaya et al., 1997) and phytoestrogenic activities (Maggiolini et al., 2002; Tamir et al., 2001). In our previous work (Vaya et al., 1997), two chalcones were isolated from licorice root, isoliquiritigenin (2′,4′,4-OH chalcone, ILC) and isoprenylchalcone (IPC), the former presenting antioxidant and phytoestrogenic activities (Tamir et al., 2001) and the latter not active in the tests carried out. The aim of the present study was to explore the potential of chalcones as whitening agents: this group of compounds has not been studied before in this context, despite their structural similarity to *t*-stilbene, a well-known tyrosinase inhibitor. The inhibitory activity of a series of chalcones was set against their structure and their antioxidant potency (which can contribute to prevent pigmentation resulting from non-enzymatic oxidation). Such a comparison may lead to the design of new whitening agents which overcome some of the limitations associated with existing depigmentation compounds.

2. Results

2.1. Inhibition of tyrosinase activity by chalcones

The inhibitory effect of tyrosinase by nine chalcones was examined at both enzyme activity stages: the hydroxylation of tyrosine to L-dopa, and the oxidation of the L-dopa to L-dopa quinone. A summary of the results obtained, shown in Table 1, demonstrates that when either no hydroxyl group is attached to the chalcone skeleton, or when one or two hydroxyls are present only on ring A of the chalcone moiety, the compounds are practically inactive. Thus, chalcone and 2′-HC did not inhibit tyrosinase at 50 μM, while 4′-HC and 2′,4′-HC showed only low activity (11% and 14% inhibition, respectively). The results shown in Table 1 also demonstrate that substitution of a hydroxyl group on chalcone ring B significantly increased inhibition potency. Thus 4-HC and, 2′,4′,4-HC presented an IC50 of 21.8 and 8.1 μM, respectively. The presence of hydroxyl at position 2′ eliminated the inhibitory effect of hydroxyl 4: hence, 2′,4-HC was practically inactive (10% inhibition at 50 μM). The presence of a catechol structure on ring B (2′,4′,3,4-HC, Butein) maintained the inhibitory effect, leading to an IC50 of 29.3 μM, despite the presence of a hydroxyl at position 2′. The addition of two isoprenyl groups to positions 2′ and 3 (IPC) totally negated this effect.

The inhibitory effect of chalcones 4-HC, 2′,4′,4-HC and 2′,4′,3,4-HC on tyrosinase activity with L-dopa as substrate was much lower than when L-tyrosine was used as substrate. Increasing the concentration of inhibitors to 100 μM did not affect L-dopa oxidation (data not shown).

2.2. Kinetic parameters of the effect of 4-HC, ILC and Butein on the monophenolase activity of tyrosinase

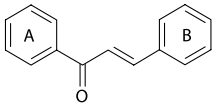
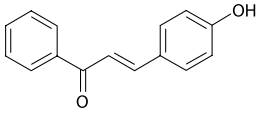
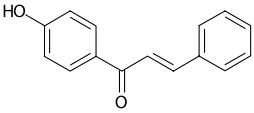
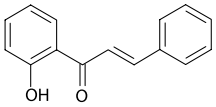
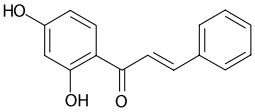
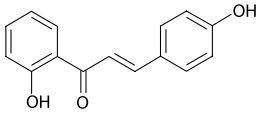
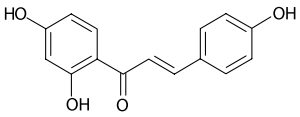
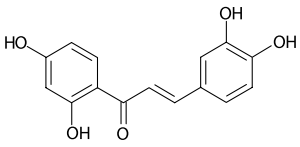
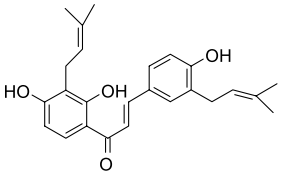
The monophenolase activity of tyrosinase is characterized by a lag period, followed by an increasing reaction rate, with dopachrome formation (Cabanes et al., 1987). In the present study, the effect of the three active compounds on enzyme lag period was examined (Table 2). Contrary to benzoic acid, cumic acid, ascorbic acid and anisaldehyde (Kubo and Kinst-Hori, 1998a,b; Xiaodan et al., 2003), the chalcones did not prolong the lag period as expected, but rather reduced it, relative to the control (Table 2). Thus, the lag period decreased from 440 s in the control to 222, 220 and 30 s for Butein, 4-HC and ILC, respectively. In parallel to lag-time changes, all chalcones significantly decreased the reaction rate of dopa-quinones formation, by 90–57% (Table 2).

The relation between tyrosinase inhibition by 4-HC, ILC and Butein was further tested with increased tyrosine concentration. The Lineweaver–Burk plot obtained (Fig. 1) shows that Butein exhibited competitive inhibition with K_I value of 1.41 mM and V_{max} of 5×10^{-3} δOD/min, while 4-HC and ILC exhibited semi-competitive inhibition with K_Is values of 15.97 and 2.5 mM, respectively. The K_M value was 0.62 mM and the V_{max} of the control was 5.4×10^{-3} δOD/min.

2.3. The antioxidant activity 4-HC, Butein and ILC

Browning of food and beverage may be the result of an enzymatic reaction of polyphenol oxidases and/or auto-oxidation of polyunsaturated fatty acids and proteins (Seo et al., 2003). Compounds with phenolic hydroxyl(s) may exhibit antioxidant activity, due to their ability to donate an electron (or hydrogen atom) and/or

Table 1
The effect of various chalcones on mushroom tyrosinase monophenolase activity

Compound name	Structure	Inhibition at 50 μM (%)	IC50 (μM)
Chalcone		3	
4-Hydroxychalcone (4-HC)		71	21.8
4′-Hydroxychalcone (4′-HC)		11	
2′-Hydroxychalcone (2′-HC)		4	
2′,4′-Dihydroxychalcone (2′,4′-HC)		14	
2′,4-Dihydroxychalcone (2′,4-HC)		10	
2′,4′,4-Trihydroxychalcone (2′,4′,4-HC) (ILC)		67	8.1
2′,4′,3,4-Tetrahydroxychalcone 2′,4′,3,4-ch (Butein)		77	29.3
2′,4′,4-Trihydroxychalcone-3′,3-di-isoprenyl chalcone (IPC)		not active	

Inhibition of L-tyrosine oxidation to dopachrome by various chalcones was measured at 492 nm. Ethanol was used as a control. Data presented as percent inhibition.

chelate transition metals, such as copper or ferrous ions, and thus decay free radical reactions and eliminate reactive oxygen or nitrogen species (ROS, RNS) (Vaya and Aviram, 2001). Chalcones may at the same time exert antioxidant activity, due their phenolic hydroxyls contents. We therefore tested the ability of these chalcones to prevent auto-oxidation of unsaturated fatty acids, by measuring their ability to prevent linoleic acid oxidation, using the conjugated dienes formation (CD) method (Esterbauer et al., 1989). Trolox (water-soluble vitamin E derivatives) was used as a positive control. There was no increase in the time-lag with 4-HC and

Table 2
The effect of chalcones on tyrosinase activity

Compound	Lag phase (s)	Velocity (δOD 475 nm s^{-1})
EtOH (control)	440	3.33×10^{-4}
Butein	222	1.42×10^{-4}
4-HC	220	0.33×10^{-4}
ILC	30	0.75×10^{-4}

Enzyme activity was tested in the presence of L-tyrosine, as substrate, and 4-HC or Butein at 50 μM each, or ILC at 25 μM, using a spectrophotometer to measure reaction progress at 475 nm. Ethanol was used as a control. Values are means of three separate experiments.

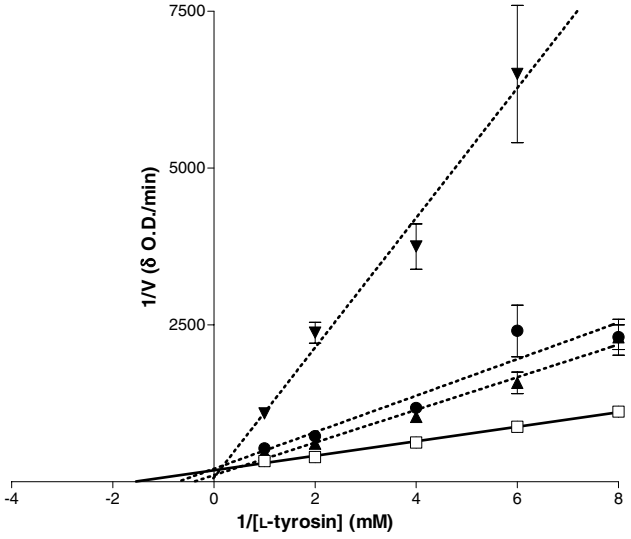


Fig. 1. Lineweaver–Burk plots of mushroom tyrosinase. With L-tyrosine as a substrate was measured at 492 nm in the presence of: 4-HC 25 μM (▼); ILC 8 μM (▲); Butein 25 μM (●); ethanol (□) as control. Values are means ± SD of four separate experiments.

ILC, relative to the control (measuring absorbance at 234 nm), whereas Trolox at 10 μM prolonged the time-lag to 80 min (Fig. 2). Butein, which differs from ILC by having an additional hydroxyl group at position 3 of ring B chalcones (forming a catechol moiety) gave significantly higher antioxidant activity, with a lag time >4 h at 10 μM concentration, and 49 min at 0.1 μM.

3. Discussion

In the present study, the relation between the structure of nine different chalcones was compared to their tyrosinase inhibition activity, showing that the position of the hydroxyl groups attached to the A and B aromatic rings is of major importance, while hydroxylation on ring B contributes markedly more to inhibition than when it is on ring A. Butein, an effective tyrosinase inhibitor, was also able to delay linoleic acid auto-oxidation, as shown by conjugated diene (CD) formation.

The tyrosinase inhibition activity of the unsubstituted chalcones, 2′-HC, 4′-HC and 2′,4′-HC, was tested, each at 50 μM concentration, demonstrating practically no activity (Table 1). These compounds are all substituted with one or two OH groups on ring A. The compound 4-HC, in which ring B is substituted at position 4, presented 71% inhibition at 50 μM. In similar experiments, performed by Ohguchi et al. (2003), testing the tyrosinase inhibition effects of *t*-stilbene derivatives substituted with OH groups (1–4 OH groups), the inhibitory potency was shown to increase with the number of OH groups. In the present study, increasing the OH number on chalcone aromatic rings did not necessarily lead to enhanced inhibitory potency: the OH in position 4 (ring B) was the major factor affecting potency. Chalcones

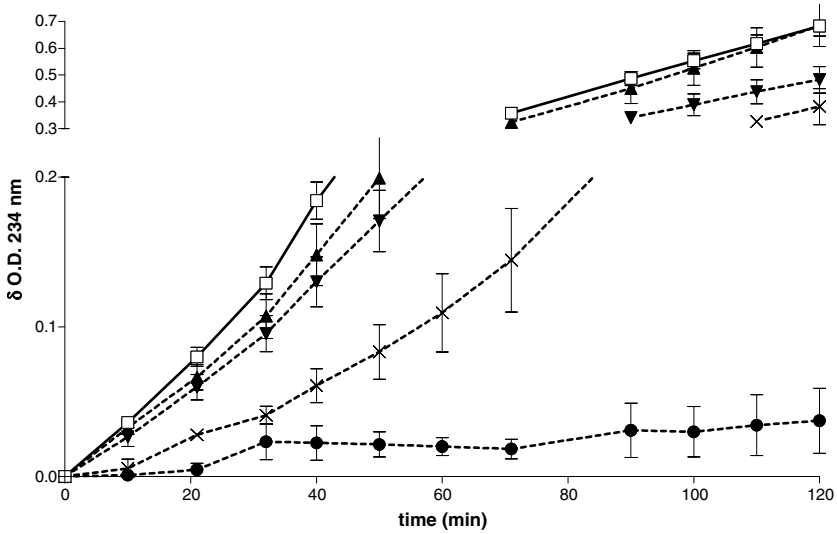


Fig. 2. The antioxidant activity of trolox (x), 4-HC (▼), ILC (▲) and Butein (●) at 10 μM each (measuring conjugated dienes formation of linoleic acid at 200 mg/l) was measured at 234 nm. Ethanol (□) was use as control. Values are means ± SD of three separate experiments.

and *t*-stilbene differ in respect of the symmetry of their aromatic rings. While in *t*-stilbene both aromatic rings are symmetric toward the two olefinic carbons, ring A in chalcones is attached to a carbonyl group, whereas ring B is connected to vinylic carbon, exhibiting two distinct aromatic rings whose substituted OH location is of significant importance to tyrosinase inhibition. The different effects associated with OH location on rings A or B may also be explained by the structural similarity between 4-HC and the amino acid tyrosine, the natural ligand tyrosinase (Fig. 3), forming a similar skeleton and thus possibly acting as a competitive substrate to tyrosine.

Surprisingly, as shown in Table 1, the addition of a second OH to 4-HC at position 2′ (ring A) negated tyrosinase inhibition activity, as observed in 2′,4-HC, which was practically inactive. The effect of such chemical modification on the molecule structure was examined, using semi-empiric PM3 calculations (data not shown). A major change in molecular conformation was observed, resulting from the formation of a hydrogen bond between OH 2′ and its adjacent carbonyl group. The addition of 2′ OH, resulted in a change in the non-planar structure of 4-HC (ring A is out of plane, with a torsion angle of 47°) to the inactive planar structure of 2′,4-HC. With the addition of a third OH group (2′,4′,4-HC, ILC) and a fourth OH group (Butein), tyrosinase inhibition was restored (Nerya et al., 2003), although these molecules are planar (Table 1). Butein, with a resorcinol sub-structure on ring A and a catechol moiety on ring B, did not enhance inhibition, compared with 4-HC (IC50 of 29.3 and 21.8 μM, respectively), despite the fourth OH, which again emphasizes that the major factor determining tyrosinase inhibition activity is not the number of OH groups attached to the chalcone skeleton, but rather their location on B ring. The addition of two isoprenyl groups to ILC, one on each aromatic ring, forming IPC (Table 1), abolished tyrosinase inhibition, possibly as a result of the introduction of steric hindrance, which does not permit competition with tyrosine.

Tyrosinase catalyzes two reactions, the hydroxylation of monophenols to *o*-phenols (L-dopa), using oxygen, and further oxidizing them to *o*-quinone, again using oxygen. The monophenolase activity of the enzyme requires the catechol substructure (L-dopa) as cofactor, presumably to donate electrons to the oxidized Cu(II)

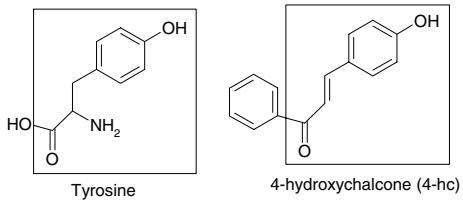


Fig. 3. The structural similarity of tyrosine and 4-HC.

(*met*-state) and reduce it to Cu(I), which is the enzymatic form, capable of binding molecular oxygen in a peroxy state (Solomon and Lowery, 1993). This enzyme activity is characterized by a time-lag (Lerner et al., 1949) in which the initial rate of oxidation is slow and gradually accelerates, until the accumulation of the *o*-phenols reaches its equilibrium. The presence of compounds with redox potential can shorten the time-lag, or even eliminate it (Robb, 1984). In the present study, the three active inhibitors shown in Table 1 were tested for their effect on the tyrosinase lag period. It was found that all three chalcones reduced the lag, relative to control (Table 2), possibly due to their reducing character. Butein and 4-HC were the least effective, and ILC the most pronounced (reducing the time-lag to 30 s, compared with 440 s in the control). In parallel to a decreased time-lag, all three chalcones reduced the tyrosinase oxidation rate, with 4-HC the most active (Table 2). With the addition of Butein to the enzyme, an immediate change of orange to red color was observed, a change which was not recorded with the other chalcones tested. It was thought that Butein is immediately oxidized to the appropriate *o*-quinone under these conditions. To confirm this hypothesis, the reaction mixture was injected to LC/MS (negative electrospray ionization), giving two peaks: un-reacted Butein (m/z 271, M-1), and a second, with m/z of 269 corresponding to either oxidized Butein product, e.g., to the *o*-quinone or to the formation of aurone derivatives. Further characterization of the reaction between chalcones and tyrosinase will be studied.

Antioxidants or compounds with redox properties can prevent or delay pigmentation by different mechanisms: by scavenging ROS and RNS, known to induce melanin synthesis (Seo et al., 2003), or by reducing *o*-quinones or other intermediates in the melanin biosynthesis, and thus delaying oxidative polymerization (Karg et al., 1993). In the present study, the antioxidative properties of 4-HC, Butein and ILC were tested by evaluating their ability to delay linoleic acid oxidation, measuring conjugated diene formation (Esterbauer et al., 1989). Butein was found to be the strongest antioxidant, delaying linoleic acid oxidation by over 4 h at 10 μM concentration, probably due to the presence of a catechol substructure, which is known to significantly enhance polyphenol antioxidant activity (Vaya et al., 2003) (Fig. 2). Butein is also a powerful electron or hydrogen atom donor to stable free radicals, such as DPPH (Tsuchiya et al., 1985), whereas 4-HC and ILC are not able to donate hydrogen atoms (data not shown), possibly due to their high oxido/redox potential. The potency of tyrosinase inhibition and the antioxidant behavior of the active chalcones both share chemical features, such as the presence of phenolic hydroxyls and the ability to donate electrons. These two biological activities are complementary properties of potential

whitening agents. We believe that chemically modified chalcones could be used in the design of improved depigmentation agents.

4. Materials and methods

4.1. Chemicals and reagents

Tyrosinase (EC1.14.18.1, Sigma Product T7755, with an activity of 6680 units/mg), Butein and trolox (a synthetic, hydrophilic derivate of vitamin E), were purchased from Sigma. The chalcones, 4-hydroxychalcone (4-HC), 4′-hydroxychalcone (4′-HC), 2′-hydroxychalcone (2′-HC), 2′,4′-dihydroxychalcone (2′,4′-HC), 4,2′-dihydroxychalcone (4,2′-HC), 4,2′,4′-trihydroxychalcone (ILC) and 2′,4′,4-trihydroxychalcone-3,3′-diisoprenyl chalcone (IPC) were purchased from Indofine Chemical Co.

4.2. Tyrosinase assay

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 (2–500 mM), were dissolved in absolute ethanol, and 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. The optical density at 492 nm was measured (Elisa SLT Labinstruments Co. A-5082).

4.3. Antioxidant activity of 4-HC, ILC using linoleic acid

The antioxidant activity of 4-HC, ILC, trolox, and Butein (10 μM, each) was determined. Due to the high activity of Butein, this compound was re-tested at a concentration of 0.1 μM. Ten microliters of $CuSO_4$, at 2 mM concentration in DDW, and 5 μl of the tested compounds were added to freshly prepared linoleic acid (0.2 mg) in PBS buffer (50 mM at pH 7.0) with tween 20. This buffer was prepared by dissolving 3.6 ml tween 20 in 100 ml chloroform. Tween 20 solution (2.9 ml) was added to 17.5 ml PBS buffer, after evaporation of the chloroform. The final volume of the reaction was 2 ml. The increase in absorption at 234 nm, as a result of the formation of conjugated diene hydroperoxides, was monitored.

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