Journal of Enzyme Inhibition and Medicinal Chemistry, 2002 VOL. 17 (4), pp. 207–218

Review Article

Tyrosinase Inhibition: General and Applied Aspects

ANTONIO RESCIGNO*, FRANCESCA SOLLAI, BRUNELLA PISU, AUGUSTO RINALDI and ENRICO SANJUST

Cattedra di Chimica Biologica, Università di Cagliari, Cittadella Universitaria, 09042 Monserrato (CA), Italy

(Received 15 May 2002)

The active site of tyrosinase is described with a view to depicting its interactions with substrates and inhibitors. Occurrence and mechanism(s) of tyrosinase-mediated browning of agrofood products are reviewed, with regard to both enzymic and chemical reactions, and their control, modulation, and inhibition. Technical and applicational implications are discussed.

Keywords: Tyrosinase; PPO; Inhibitors; Melanin; Melanogenesis

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase catalysing the *o*-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity), and the oxidation of catechols to the corresponding *o*-quinones (diphenolase or catecholase activity). Tyrosinases are widespread in the living world, where they are often referred to as phenolases, phenol oxidases, polyphenol oxidases, catechol oxidases, depending on the particular source or also on the Authors who have described any particular enzyme. The term tyrosinase is usually adopted for the animal and human enzymes, and refers to the 'typical' substrate, tyrosine. Polyphenoloxidase is perhaps the most suitable general definition, and will be used as the acronym PPO through this review.

Also the enzyme extracted from the champignon mushroom *Agaricus bisporus* is usually referred to as tyrosinase, and its high homology with the mammalian ones renders it well suited as a model for studies on melanogenesis.[1,2] This enzyme has been thoroughly characterised.[3] Another interesting fungal PPO is that purified from *Neurospora crassa*, which is suitable for both mechanistic and applicative studies.[3]

PPOs contain a di-copper centre, strictly resembling that of hemocyanins (HC).[4,5] HCs are found in the haemolymph of several *Mollusca* and *Arthropoda*, where they act as oxygen carriers. HCs show a high tendency to form supramolecular aggregates, whereas many (but not all)[6] PPOs do not show any quaternary structure.

The di-copper centre of PPOs has been the target of many structural studies.[4] Moreover, PPO has been suggested to be the ancestor protein of HC as it is present in very primitive organisms. However, di-copper centres in PPOs and HCs are similar but not identical.[7] In particular, X-ray spectroscopy has shown that conformational changes among the three forms (*met*, *oxy* and *deoxy*) of PPO strictly parallel those of their HC counterparts. On the other hand, this centre shows a lower symmetry in the enzyme, and a rapid interchange among a number of conformational sub-states has been suggested. Moreover, the metal cluster in HC is shielded by a PHE residue,[8] which prevents any interaction with phenols and catechols. Limited proteolysis of HCs evokes PPO activity[9] by removing a peptide which prevents protein/substrate interactions. Although in both HC and PPO the copper ions are co-ordinated by three nitrogen atoms, pertinent to three imidazole rings of three HIS residues, some evidence exists in favour of a certain inequivalence between these ions in the enzyme.

The di-copper centre of PPO can physiologically exist in three forms[10] (Figure 1): i) *met* form,

*Corresponding author. E-mail: rescigno@unica.it

ISSN 1475-6366 print/ISSN 1475-6374 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/1475636021000010923

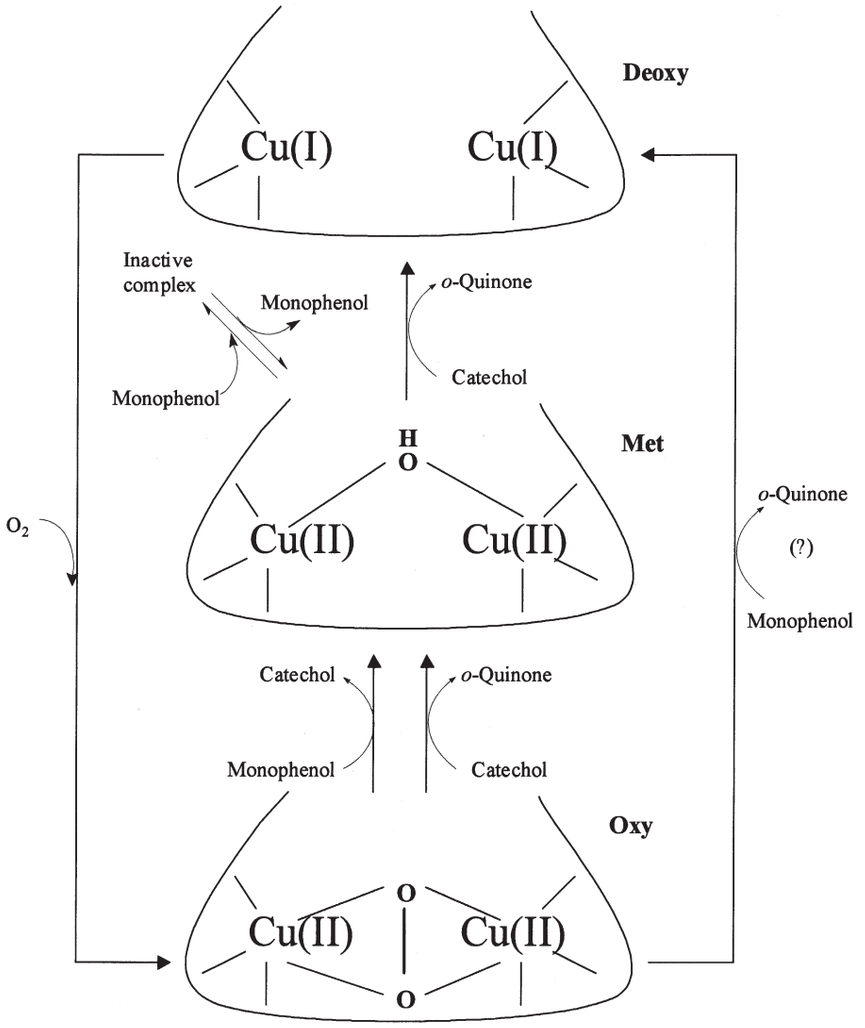

FIGURE 1 The three physiological states of the active site of PPO: from the top to bottom, the cuprous, *deoxy* form, and the cupric forms, respectively *met* and *oxy*.

containing two copper(II) ions antiferromagnetically coupled and therefore EPR-non-detectable. These ions are tri-co-ordinated, and each roughly occupies the apex of a tetrahedron, whose other three apices are formed by the above mentioned nitrogen atoms. A hydroxide ion most probably forms a bridge between the metal ions; ii) *oxy* form, as the *met* form, but containing a peroxo bridge between the two copper (II) ions, with a $\mu - \eta^2 : \eta^2$ geometry. Therefore, the copper ions are penta-co-ordinated, with a distorted square-pyramidal geometry. A relatively high electron density is present in the σ^* peroxide orbital, so that O–O bond is comparatively weak and prone to cleavage.[5] A greater affinity of *oxy* PPO towards catechol substrates compared to the *met* form has been suggested;[11] iii) *deoxy* form, containing two copper(I) ions (with a co-ordination arrangement similar to that of the *met* form, but without the hydroxide bridge) which are obviously EPR-silent.

These three forms of PPO account for both the mono- and di-phenolase activities, although some enzymes have been described which are the unable to act on monophenols[8,12] and therefore referred to as catecholases. On the other hand, these catecholases have been defined as 'general' phenolases, whose monophenolase activity was lost as a result of too drastic purification procedures or was not found as a consequence of inappropriate assays.[5]

PPO in its 'resting' state is mainly the *met* form, which is unable to act on monophenols.[5] On the contrary, this form oxidises catechols to the corresponding *o*-quinones, with concomitant reduction to the *deoxy* form. This latter is converted by molecular oxygen to the *oxy* form, still capable of oxidising the diphenol while turning again to the *met* state. It is also worth noting that other reducing agents are well capable of drawing the *met* enzyme into the *deoxy* one: therefore, the classification of PPO as a monooxygenase is fully justified, and catechols

could be considered as a particular class of 'external' reductants for the enzyme. In most cases, a diphenol is necessary as the reducing agent to obtain the *deoxy* form, the only one capable of reacting with molecular oxygen and continuing in the catalytic action.[5] For this reason the monophenolase activity presents a characteristic lag time which exists until a sufficient amount of catechol (needed to reduce the *met* form to the *deoxy* one) is produced by the small amount of the *oxy* form generally present in the resting enzyme preparations. Obviously, this lag time cannot be observed (or is proportionally reduced) in the presence of small amounts of catechols, reducing agents, or traces of transition metal ions such as Fe^{2+}. Both experimental data and theoretical considerations are in favour of an allosteric mechanism to explain the two activities of PPO[5] have been overwhelmingly ruled out by more coherent observations that satisfactorily explain the whole PPO mechanism by means of an integrated kinetic model. In this latter model, one catalytic centre could alternatively interact with both mono- or di-phenols, behaving therefore as mutual competitive inhibitors, with diphenols always being much better substrates than their monophenol counterparts for a given PPO.[5] The formation of a relatively stable adduct between the monophenol substrate and the *met* form of the enzyme should be pointed out;[5] this complex maintains a considerable enzyme fraction out of the catalytic cycle until a sufficient amount of catechol is produced. The set of kinetic equations derived from this model has no analytical solutions unless the steady state treatment is assumed for the catechol concentration; in other words, the lag time only ends when catechol formation rate (from monophenol and *oxy* PPO) equals its destruction rate (from catechol itself and both *oxy* and *met* forms to yield the *o*-quinone). A somewhat different mechanism, in which a direct formation of the *o*-quinone (i.e. without the catechol intermediacy) from the monophenol by the *oxy* PPO arises could be excluded when the enzymic reaction is studied in the presence of a substrate, 4-*tert*-butylphenol, leading to a stable quinone: also in this case a well-defined lag time is observed.[5] Very recent work[13,14] has confirmed this view. However, the dilemma between the two hypotheses is still unresolved: when 4-hydroxy-2,5,6-trimethylanisole is used as a substrate, the lag time is not observed. This has been interpreted as proof of the direct generation of the corresponding *o*-quinone, without any catechol intermediacy, taking into account that the *o*-quinone cannot undergo any reductive nucleophilic addition, further leading to catechol generation by means of a redox reaction.[15] Similar conclusions have been drawn from experiments, in which a substrate, whose *o*-quinone counterpart cannot revert to the corresponding catechol due to its change to a relatively stable quinone methide, did not show any lag time when subjected to mushroom PPO.[10] A low but significant laccase contamination is systematic in most commercial mushroom PPOs,[16] so experimental data have to been carefully evaluated to avoid misleading conclusions. In fact PPO and laccase are very often present at the same time in several sources and moreover they share most phenol and polyphenol substrates.

Recent studies[17,18] have been focused on *Streptomyces antibioticus* PPO. Advanced spectrometric techniques have pointed out the importance of the unsymmetrical co-ordination field of the di-copper centre, though the proposed catalytic mechanism does not account for the following features of the reaction: first, the electron-excessive phenoxide ion is quite inert towards nucleophilic attack; second, such an attack is in contrast with the well-known general behaviour of all PPOs, that are best active towards electron-rich substrates and slightly active or inactive towards electron-deficient ones. Therefore, the most probable mechanism implies the heterolytic cleavage of the peroxide bridge, concomitant with an attack of the electrophilic oxygen atom at the *ortho* position of the phenol substrate, while the nucleophilic one turns to the hydroxide bridge of the resulting *met* PPO, and the substrate is released as the corresponding catechol. The alternative hypothesis, excluding any catechol release and implying the direct formation of the *o*-quinone while the enzyme is reduced to its deoxy form, is also in full accordance with the proposed electrophilic oxygenation mechanism.[3]

However, the possibility of a substrate binding to one copper ion at the catalytic site, whereas the other one remains free, could suggest the possibility of an allosteric activation mechanism, involving the second copper ion and the activating catechol. The conclusion, not too encouraging, is that in spite of the huge mass of experimental and speculative work on the topic, a definitive choice between allosteric and kinetic explanations for the catalytic mechanism of PPO still cannot be made.

PPO SUBSTRATES

In principle, any simple monophenol and the corresponding catechol could behave as a PPO substrate, taking into due account that catechols are quinonised much faster than monophenols, owing to K_S as well as k_{cat} differences. As a general rule, bulky and/or crowded substituents too close to the phenolic hydroxyl prevent or at least render more difficult any enzyme/substrate interaction.[19] Apart from this remarkable limitation, among homologous mono- and di-phenol series, a clear correlation between the electron-donating power of substituents

and ease of enzymic action can be seen, and therefore phenols (and of course catechols), substituted with electron-withdrawing functional groups such as $-NO_2$, $-COOH$, $-CHO$ etc tend to behave as competitive inhibitors rather than substrates for PPO.[19] This is a convincing indication of an electrophilic character for the oxygenation mechanism of monophenols. Accordingly, 4-hydroxyanisole (hydroquinone monomethylether, 4-methoxyphenol) is an excellent monophenol substrate for PPO.[19,20] Hydroquinone (quinol, 1,4-dihydroxybenzene) had been described as a PPO inhibitor, but was shown later to be a substrate.[19] However, an increasing oxidation rate for catechols, with the increasing electron-withdrawing power of substituents in the *para* position, has been reported for some plant enzymes.[21]

Generally speaking, human and animal PPO tend to be relatively specific for L-tyrosine and L-DOPA, whereas a wider substrate range (regardless of stereochemistry) is known for plant and fungal enzymes.[21]

Despite being exceptionally hindered monophenols, tyrosine residues in proteins[19] behave sometimes as PPO substrates; the same has been found for certain peptides.[22] The DOPA residues arising are in turn quinonised to dopaquinone residues, capable of undergoing nucleophilic attack and therefore leading to deep cross-linking. This can lead to very strong fibrous structures such as mussel byssus.[19] When properly positioned, cysteine residues can react with the dopaquinone ring, leading to protein-bound 5-cystein-*S*-yl-DOPA (Figure 2), which appears to no longer be a substrate for the enzyme. It is worth noting that PPO action towards proteins other than those forming byssus is not indiscriminate,[23,24] so various degrees of chemical modification and activity loss (in the case of enzymes) have been noted. When chemical modification occurs, cysteinyldopa is found after complete proteolysis. Interestingly, nucleophilic attack of dopaquinone by primary amines takes place at the 6- position rather than at the 5- position.

The case of *o*-aminophenols is interesting as these compounds are often PPO substrates;[19] 2-amino-3-hydroxybenzoic (3-hydroxyanthranilic) acid has been recently studied and it was concluded that, besides being a (poor) PPO substrate, it behaves as a catechol both in stimulating the hydroxylase activity of the enzyme towards monophenolic substrates and in yielding the corresponding *o*-quinoneimine carboxylic acid as the product.[25] Also aromatic amines and some more complex aminoaromatic compounds behave as monophenols when subjected to PPO; however, though showing affinities comparable to those of monophenols, they are very slowly hydroxylated.[19] On the other hand, a putative (on the basis of its structure) *o*-aminophenol-type substrate, 3-amino-tyrosine, has been shown to be a PPO competitive reversible inhibitor.[26] However, at low tyrosine concentrations, the inhibition by 3-aminotyrosine turns to a mixed type (competitive/noncompetitive) one. This substance appears to belong to the most potent and selective PPO inhibitors.

Ortho-quinones are the products, arising from PPO action whenever a monophenol or a catechol are oxidised. However, *o*-quinones are usually highly reactive towards nucleophiles, so only seldom can these species be isolated as stable products and used, owing to their typical absorption of visible light, for activity measurements. The most obvious nucleophile is water, or more correctly hydroxide ion;[27] a hydroxyquinol then arises, quite prone to autoxidation and potentially again a substrate for the enzyme (Figure 3). The resulting hydroxyquinone is usually unstable, and leads to a complex mixture of unidentified products, most probably oligomers and/or polymers. A noticeable exception is 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone, which is remarkably stable and can be readily isolated.[27] α-Substituted 4-hydroxy- and 3,4-dihydroxy-toluenes regularly form the expected *o*-quinones that rapidly isomerise to the corresponding hydroxyquinone methides (hydroxyquinomethanes) when any electron-withdrawing group is bound to the benzylic carbon.[28] Some quinone methides are relatively stable and photometrically detectable for quantitative analysis; others can undergo a complex series of condensation/oxidation reactions, leading to structurally complicated products. The cases of caffeic and 2,3-dihydrocaffeic acids[29] have been the target of detailed studies. Insect PPOs act on 3,4-dihydroxymandelic acid leading to decarboxylation and production of 3,4-dihydroxybenzaldehyde. In this

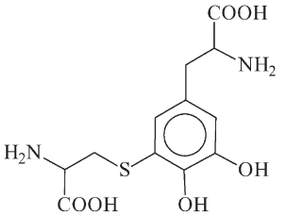

FIGURE 2 The structure of *S*-cystein-5-yl-DOPA.

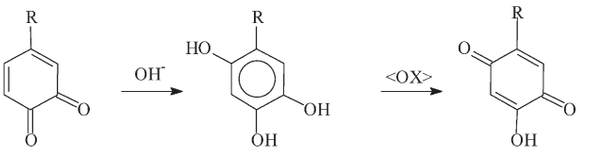

FIGURE 3 From the left to right: an *o*-quinone undergoes a reductive hydroxylation to a hydroxyquinol which is in turn oxidised to a hydroxyquinone.

case, the intermediacy of a quinone methide is evident.[32]

The formation of quinone methides has been in general overestimated[30] since commercial mushroom PPO, the enzyme most widely used for substrate specificity studies, is normally contaminated by noticeable amounts of extraneous activities, namely laccase.[16]

When a PPO substrate, either mono- or diphenolic, contains a sidechain (usually in the 4- position relative to the phenolic hydroxyl) bearing a nucleophilic moiety such as $-NH_2$, $-SH$, $-COO^-$, etc the formed *o*-quinone tends to undergo an intramolecular attack leading to a bicyclic catechol, which is again a PPO substrate, and in addition can give rise to a redox exchange with the still non-cyclised quinone. The extent of such cyclisation reactions is pH-dependent, so for example in the case of tyrosine and DOPA is severely reduced at low pH values, where the aminogroup exists as the conjugate acid and is therefore unable to carry out the nucleophilic attack. The ability attitude to intramolecular cyclisation following quinonisation is one of the most outstanding features of several PPO substrates and is of the highest importance with respect to the metabolism of phenolics. Within this consideration, the case of *ar*-fluorotyrosines is particularly intriguing.[31] Thus, 3-fluorotyrosine, which is a strong poison since it is metabolised to fluoroacetate in the liver, is converted to the relatively stable 5-fluoro-dopaquinone through 5-fluorodopa. By contrast, PPO action on 2-fluorotyrosine leads to fluoride ion release and 'regular' melanogenesis (*see below*). In that case, 6-fluorodopaquinone is the final product of enzyme action (through 6-fluorodopa), and it undergoes an intramolecular nucleophilic attack with fluoride substitution (*see above*).

A quite similar mechanism operates when 6-fluoro-3,4-dihydroxyphenylacetic acid is subjected to PPO action. This is regularly quinonised by PPO, then loses fluoride and cyclises going towards the expected fate of its non-fluorinated counterpart.[32]

INHIBITORS

Taking into due account the observation that in the case of competitive inhibition a sharp distinction between substrates and inhibitors is impossible (this is particularly true for PPO), a huge number of inhibitors have been described. However, the definition of 'PPO inhibitor' is sometimes misleading: many authors use that terminology with reference to melanogenesis inhibitors, whose action mainly resides in some interference in melanin formation, regardless of any direct inhibitor/enzyme interaction. This could perhaps be the case of some isonitrile antibiotics, reported as tyrosinase inhibitors, but whose kinetic characterisation is only preliminary.[33] Moreover, many putative inhibitors are examined in the presence of tyrosine or DOPA as the enzyme substrate, and activity is assessed in terms of dopachrome formation. Apart from the (common) cases of dopachrome removal by the 'inhibitor', many phenolics are PPO substrates whose quinoid reaction products absorb in a spectral range different from that of dopachrome. When these phenolics show a good affinity for the enzyme, dopachrome formation is prevented and they could be mistakenly classified as inhibitors.

Among 'true' inhibitors, a distinction could be made between copper chelators (competitive with respect to oxygen) and substrate analogues (competitive towards phenol and/or diphenol substrates). However, such a classification is purely indicative, as many inhibitors cannot be ascribed to a particular group and many of them behave as mixed-type inhibitors (competitive/noncompetitive).

Copper chelators (Figure 4) occupy an important position, although for more compounds the exact mechanism by which they exert their action has not been explained. Their action depends on the redox status of the PPO active site, as it is common knowledge that the coordination chemistries of cuprous and cupric ions are sharply different. Tropolone (**a**) is among the best PPO inhibitors,[19,34] but its mode of action (copper chelation and/or competition with substrates) has not been conclusively elucidated. Its usefulness to provide evidence for PPO among other phenol-oxidising enzymes has been assessed.[34]

Benzhydroxamic (**b**) and salicylhydroxamic (**c**) acids (and presumably other hydroxamic acids) clearly act as copper chelators, and are therefore PPO inhibitors.[19,35] The hydroxylamine derivative *N*-hydroxyglycine (**d**) effectively inhibits laccase but not PPO.[35] Hydroxylamine itself has an unpredictable effect as it inhibits apple PPO but not banana and mushroom enzymes.[36]

Some hydrazine derivatives have been examined as potential PPO inhibitors for banana, apple and mushroom enzymes.[36] Agaritine (*β*-*N*-*γ*-L-glutamyl-4-hydroxymethyl-phenylhydrazine, **e**), a common constituent of *Agaricus bisporus* as well as other species of *Agaricus* sporocarps, was found to be an effective PPO inhibitor with a somewhat complex kinetic behaviour with respect to monophenolase and diphenolase activities.[37] The compound is also very effective as an *o*-quinone scavenger.

An oxazolidinethione found in various plants, barbarine (**f**) which is a simple phenyl derivative, was shown to be a potent noncompetitive PPO inhibitor.[38]

Some hydroxylated heterocyclic compounds can bind at the di-copper centre but are not processed by

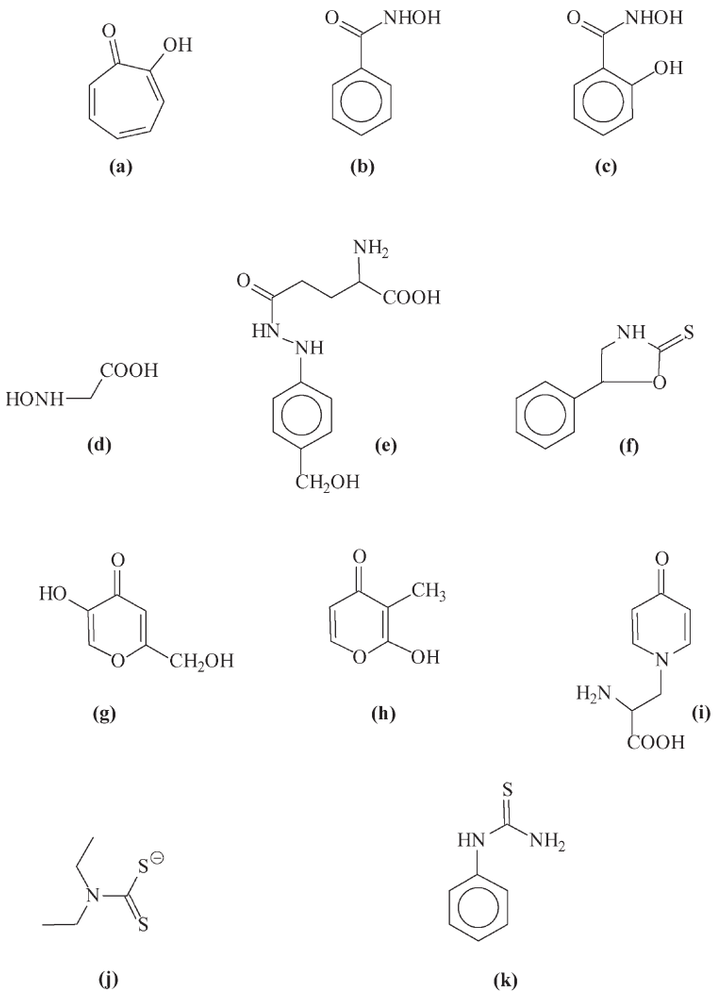

FIGURE 4 The structures of some PPO inhibitors, that presumably act as copper chelators: (**a**) tropolone; (**b**) benzhydroxamic acid; (**c**) salicylhydroxamic acid; (**d**) *N*-hydroxyglycine; (**e**) agaritine; (**f**) barbarine; (**g**) kojic acid; (**h**) maltol; (**i**) mimosine; (**j**) diethyldithiocarbamate; (**k**) phenylthiourea.

the enzyme. Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone, **g**) is an antibiotic substance[11] produced by various *Aspergillus* and *Penicillium* moulds and is widely used as a cosmetic whitening agent. The substance has proven to be a 'slow binding', competitive inhibitor of PPO.[39–42] It is worth noting its structural analogy with tropolone. Despite the same analogy, the 'inhibitory' activity of a kojic acid analogue, maltol (3-hydroxy-2-methyl-4-pyrone, **h**) has been shown to be an artefact:[42] the compound does not affect oxygen consumption whereas it counteracts *o*-quinone formation from catechols. It was concluded that most probably maltol is a mere quinone scavenger.

Mimosine (*N*-2′-amino-2′carboxy-ethyl-3-hydroxy-pyrid-4-one, **i**) is a plant non-proteic aminoacid also showing a clear similarity with both maltol and kojic acid. It is also a 'slow binding' competitive PPO inhibitor.[11]

Simple chemical species capable of binding to copper, such as cyanide, azide, and halide ions, as expected behave as purely competitive inhibitors towards dioxygen binding, even if sharp differences have been seen among PPOs from different sources.[36]

Somewhat surprisingly, thioamides behave unpredictably with respect to the chemical structure of the single substance considered, so the well known heavy metal chelator, diethyldithiocarbamate (**j**) acts as a competitive inhibitor with respect to phenolic substrates, whereas phenylthiourea (**k**) gives rise to noncompetitive inhibition.[19]

Among substrate analogues (Figure 5), resorcinol (1,3-dihydroxybenzene, **l**) inhibits PPO but not laccase;[36] some 4-substituted resorcinols are even more effective. Among these, 4-hexyl-resorcinol is a potent PPO inhibitor, whereas it is much less effective towards laccase.[35,43,44] Also 4-chloro-resorcinol is

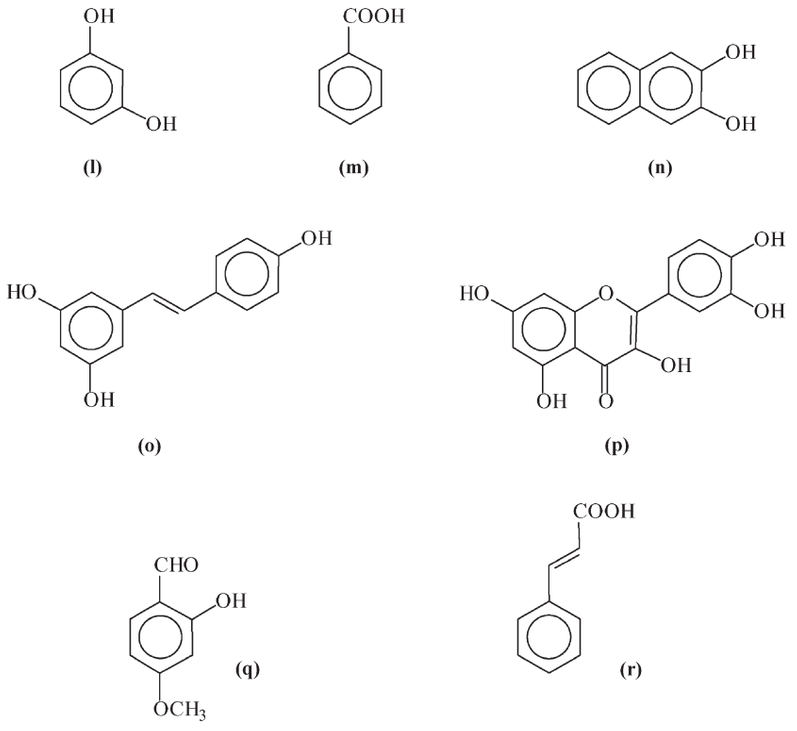

FIGURE 5 Some other PPO inhibitors: (**l**) resorcinol; (**m**) benzoic acid; (**n**) 2,3-dihydroxynaphthalene; (**o**) resveratrol; (**p**) quercetin; (**q**) 2-hydroxy-4-methoxybenzaldehyde; (**r**) cinnamic acid.

a strong PPO inhibitor, whereas orcinol (5-methylresorcinol) has only a little effect. Starting from the consideration that both resorcinol and benzoic acid (**m**, *see below*) behave as inhibitors, inhibitory properties of some isomeric resorcylic acids were tested. Among these, only *β*-resorcylic (2,4-dihydroxybenzoic) acid was found as expected to be a potent inhibitor, whereas the other isomers were void of any significant effect.

Simple catechols generally behave as both substrates and enzyme activators. However, when their quinonisation is chemically difficult or even impossible, they could behave as competitive inhibitors. This is the case of the already cited 4-nitro and 4-cyano catechols, even if in some cases they are (poor) substrates. The most interesting compound of this type is 2,3-dihydroxynaphthalene (**n**). This particular bicyclic catechol is highly resistant towards quinonisation (quinone formation must imply the sacrifice of most resonance energy) and behaves as a very effective PPO inhibitor.[21] Interestingly, it is a good laccase substrate (E. Sanjust, unpublished observation). More recently, (**n**) has been described as void of any inhibitory action towards mushroom PPO.[44]

Some other phenolics, lacking any structural analogy and therefore forming a rather heterogeneous group, have proved to be PPO inhibitors. Among these, resveratrol (**o**) family has drawn some interest and some naturally occurring compounds, of varying type and extent of chemical substitution, have been compared as regards their relative inhibitory potencies.[45,46]

Flavonoids are phenolics of plant origin, whose structure is in principle compatible with roles of both substrates and (presumably competitive) inhibitors. Detailed studies[47,48] have shown that some bioflavonoids (but not all!), and in particular quercetin (**p**) are in fact rather potent inhibitors, even if their exact mode of action has not been fully elucidated. Interestingly, the hydroxy substituent at the 4′- position is essential for inhibitory activity.

2-Hydroxy-4-methoxybenzaldehyde (**q**) has been isolated[49] from the African medicinal plants, *Mondia whitei*, *Rhus vulgaris*, and *Sclerocarya caffra*, and shown to be a partially competitive, potent PPO inhibitor. Other isomeric hydroxy-methoxybenzaldehydes, where the hydroxy substituent is not in the *ortho* position with respect to the aldehyde function, were found to be slightly active or completely inactive as PPO inhibitors. To explain this behaviour, the formation of an aromatic SCHIFF base, stabilised with an additional hydrogen bridge, with an essential LYS residue of the enzyme, has been suggested.[49] However, further studies involving other aldehydes, well capable of forming SCHIFF bases but unable to bind to the di-copper centre, indicate in favour of a partial competition with the substrate.

Cinnamic (**r**), 4-hydroxycinnamic (*p*-coumaric), and 4-methoxycinnamic acids give rise to a mixed type (competitive/noncompetitive) inhibition.[50] The inhibitory power of cinnamic and *p*-methoxycinnamic acids (both lacking any phenol-type hydroxy substituent) is also a convincing proof of their mode of action (a relatively specific interaction of the carboxy group with the di-copper centre). This point of view is corroborated by comparative observations on a number of substituted cinnamic acids[36] for which the relationship between substitution and inhibitory activity has been assessed.[36] The same study has also shown sharp differences among PPOs of different sources so that 3,4-dihydroxycinnamic (caffeic) acid is a competitive inhibitor for certain enzymes and a substrate for others. Earlier work[21] had suggested a specific 'inhibitor site', topologically distinct from the catalytic one, for cinnamic acid(s) binding on the basis of kinetic considerations.

Not only cinnamic, but also benzoic acids have been shown to be more or less effective PPO inhibitors.[19] In particular, benzoic acid itself is a competitive inhibitor towards cresolase activity of *A. bisporus* PPO, whereas it shows a mixed type (competitive/noncompetitive) towards catecholase activity.[51] Benzoic acid behaviour towards various PPOs is somewhat complicated and has been studied in some detail.[52] The type of inhibition is usually competitive with respect to catechol substrate, and mixed (competitive / noncompetitive) with respect to oxygen. Interestingly, inhibition is strictly pH-dependent, but regardless of the particular pK value of the considered acid. It rather depends on the protonated vs. deprotonated status of a HIS residue, close to the di-copper centre and possibly involved in metal binding. This hypothesis is in reasonable agreement with the results obtained by working with oxalic and sorbic acids.[36] Aliphatic dicarboxylic acids ranging between C8 and C13 have been studied in some detail as they could arise from unsaturated fatty acid metabolism. In particular, azelaic (1,7-heptanedicarboxylic acid) acid is so effective an inhibitor that has been successfully proposed as a therapeutical agent against some pathological pigmentation disorders. However, it has been shown later that metabolic effects of azelaic acid go far beyond a mere PPO inhibition.[19]

A number of different heterocyclic compounds, mainly of natural origin, have been shown to behave as more or less selective PPO inhibitors, although more have been described as 'melanogenesis inhibitors' without further mechanistic specifications. This is the case of the very effective 4-*trans*-cinnamoyl-3,6-dihydroxy-1-methoxy-6-methyl-9-(1-methylethyl)-5a,6,7,8,9,9a-hexahydrodibenzofuran (Figure 6), isolated from the bark of *Lindera umbellata*.[52]

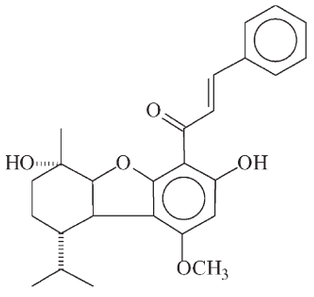

FIGURE 6 The structure of 4-*trans*-cinnamoyl-3,6-dihydroxy-1-methoxy-6-methyl-9-(1-methylethyl)-5a,6,7,8,9,9a-hexahydrodibenzofuran from *Lindera umbellata*.

Another class of inhibitors comprises substances whose relation with the active site of PPO is not apparent. However, these inhibitors are often extremely effective and selective in their action, so are among the most promising for therapeutical and/or technological applications. For example, variations in PPO activity in housefly *Musca domestica* depend strongly also on time-dependent variations in a specific inhibitor, a DOPA-containing polypeptide. This inhibitor is well present in the pupal stage but quickly disappears in adult insects.[53] The presence of DOPA is not a compulsory requisite for inhibitory activity: thus, the cyclooctapeptide pseudostellarin G from the plant *Pseudostellaria heterophylla* is void of DOPA but exerts a noticeable PPO inhibition.[54]

MELANOGENESIS

Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, i.e. melanins (after the classic Greek '*Melas*, *Melanos*' meaning 'black'). Formation of a wide variety of dark or nearly black pigments relies upon oxidative polymerisation of small phenolic precursors, passing through *o*-quinoidal intermediates, and is conceptually well distinct from lignin biosynthesis which starts from three well-defined phenolic monomers, i.e. the *p*-hydroxycinnamyl alcohols coumarylic, coniferylic, and sinapylic. In a more proper sense the definition of melanins should be reserved to those polymeric pigments arising from oxidative polymerisation of tyrosine, under tyrosinase catalysis. When tyrosine is the sole monomer involved in the melanogenetic process the resulting melanin is named eumelanin, whereas related brownish pigments, containing some cysteine-derived moieties into their molecular backbone, are termed pheomelanins (after the classic Greek *Phaios*, meaning 'brown'). Other 'melanins', relying on phenolic monomers different from tyrosine, should be termed allomelanins.[19]

Generally speaking, melanins have been considered as a particular case of a much more general oxidative/polymerisative pathway of the metabolism of phenolics.[19]

The physiopathological importance of eumelanin is obvious so the substance has drawn and nowadays still draws much attention and its biosynthesis is reasonably well understood.[5] A detailed discussion on melanin biogenesis, properties and physiopathological functions is far beyond the scope of this article; however, most findings ascertained for eumelanin biosynthesis could reasonably be extended to allomelanins.

Browning and/or blackening phenomena, both spontaneous or arising from mechanical, physical, chemical and/or biological injuries, are also widespread among plants and fungi, and are usually related to oxidative polymerisation, conceptually similar to melanogenesis *sensu stricto*. The main difference resides in the fact that allomelanins substantially do not contain dopaquinone-derived motifs as the main monomers in their structure and, on the contrary, are based on other quinoid building blocks.

The ability of most phenolics from plants and fungi to quinonise and then polymerise to brown polymers is general: therefore, to distinguish among many concomitant browning mechanisms *in vivo* is very often a discouraging task. Even very simple phenolics can participate well in browning, so for example 4-methylcatechol gives the corresponding *o*-quinone which is non-enzymically quickly converted to 4-methyl-1,2,5-trihydroxybenzene, which in turn is oxidised to a pink hydroxyquinone.[55] This slowly changes to an unidentified, insoluble, black product. This example could well be generalised, as a huge variety of phenolics are present in fungi as well as in plants. Among these, several are autooxidisable, in other words are capable of reacting with molecular oxygen without any enzymic assistance but eventually, under transition metal catalysis, leading to quinoid products. The reaction pathways for autoxidations are complex and vary as well when different phenolics are considered,[56] invariably going through semiquinoid intermediates and reactive oxygen species release. The whole process tends to be highly pH-sensitive and is favoured by alkaline conditions, with a sharp rate increase above pH 10 (this corresponds to the pK value of most phenolic hydroxyl groups). Autoxidation of naturally occurring phenolics is a topic outside the scope of this article, so attention will be focused on PPO-assisted oxidation. Plant polyphenols have been the target of several studies, so their classification, occurrence, structural aspects, reactivity, biochemistry and biogenesis have been repeatedly reviewed[57] and are currently the target of intense studies.[58]

For reasons of clarity, plant phenolics can be divided into some main subclasses: (i) phloroglucinol derivatives; (ii) 4-hydroxycinnamic acids (mainly coumaric, caffeic, ferulic and sinapic acids); (iii) flavonoids; (iv) leucoanthocyanins (yielding cyanidins upon dehydration and oxidation under acid catalysis); (v) tannins, derived from gallic acid.

Among this huge variety of substances, some have proven to be PPO inhibitors (*see above*) whereas others are presumably substrates;[21] however, as regards their enzymological relationship (if any) with PPO of the large majority, this is still unknown.

Some important properties of plant PPOs must at this point be adequately emphasised to understand the extreme complexity of the relationship between PPOs and their substrates: (i) subcellular location of the enzyme—it may be particulate and/or soluble; (ii) latency of PPO and its activation mechanism (s). The activation of latent forms of PPO, often by means of limited proteolysis,[59] is a common feature of both fungal and plant enzymes—this is strictly related to huge variations in PPO levels at different stages of growth; (iii) presence of endogenous inhibitors and their variation in concentration with time and/or various physiopathological conditions; (iv) different compartmentation of PPO and PPO substrates, which prevents enzymic reaction unless the plant tissues are damaged.

Availability of potential PPO substrates could well be efficiently controlled in plant tissues by means of reversible glycosylation and/or methylation reactions.[60]

Adverse effects of excess dietary phenolics towards nutrient digestion are well established,[61] however their beneficial properties as antioxidant and anticancer agents have been more recently stressed so the conclusion that they are essential or at least very useful to health can reasonably be reached and is a generally accepted statement. On the other hand, phenolic-derived quinoid oxidation products, arising from both non-enzymic and enzymic oxidation, need to be considered. It is common knowledge that they act as radical scavengers but this beneficial feature is largely overwhelmed by their inclination to undergo nucleophilic attack leading to irreversible covalent adducts. Copolymerisation and precipitation of proteins by quinones leads to a substantial decrease in the nutritional value of food; also digestive enzymes are adversely affected by quinones, as well as other proteins, peptides, aminoacids etc. The dilemma, as to whether a certain action is just depending on a given phenolic, or rather arises from its quinoid counterpart, has to be considered as still *sub judice* in most cases.

However, a major concern in avoiding browning is of economical nature, as consumers' reluctance to buy and eat discoloured vegetables and

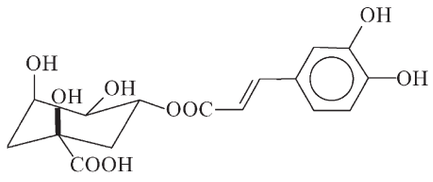

FIGURE 7 The structure of chlorogenic acid.

mushrooms is a well-established and presumably unchangeable fact. Obviously, studies have been focused on widely consumed products, such as potatoes[62] and mushrooms.[63] Chlorogenic acid (5-*O*-caffeoylquinic acid, Figure 7) is a common phenolic in plants[62] and represents up to 90% of total phenolics in potatoes.[62] It is a good catecholic substrate/activator for PPO. Generally speaking, increases in both PPO and phenolics (mainly chlorogenic acid) concentrations are seen as a response to infections. The potato physiological defence mechanism based on PPO action towards phenolics is rather selective and seems to be substantially ineffective under many circumstances, where its main effect is limited to browning of the affected tissues.[62]

Different varieties of the champignon mushrooms *Agaricus bisporus* owing to their high commercial interest have been carefully studied[37,64–67] to gain insight into their browning mechanism(s). Tyrosine and DOPA concentrations are relatively high in sporocarps (the edible part of the fungal organism) as well as is that of γ-*N*-glutamyl-4-hydroxyaniline. All these substances are good substrates for mushroom PPO, so both action and effectiveness of the physiological inhibitor agaritine[37,65] deserve further study.

Generally speaking, enzymic browning could be prevented by following two alternative strategies: a) selection of cultivars, featuring low PPO and/or phenolics concentrations, and b) use of proper processing procedures, capable of inhibiting melanogenesis, without significant alteration in maintaining product safety. The former choice is in the field of molecular biology and biotechnology, whereas the latter can well take advantage from a judicious use of PPO inhibitors. PPO inhibition can obviously be attained by means of thermal inactivation, which is usually irreversible but unavoidably leads to significant organoleptic changes. Another possibility, well known also to housewives, is the use of acidic juices, and in particular lemon juice: the obtained low pH effectively prevents PPO actions and renders autoxidation slower. The high content of ascorbic acid is important as this compound is well known for its effectiveness in rapidly reducing most quinones that are responsible for the browning process. Ascorbic acid has been long considered as a PPO inhibitor, whereas it has been conclusively classified as a melanogenesis inhibitor continuously reducing *o*-quinones, arising from PPO action. Interestingly, for this reason at low concentrations the compound has got a *stimulating* action towards PPO, by favouring catechol formation by *o*-quinone and therefore abolishing the lag time. Ascorbic acid analogues have been proposed[63] in association with a bleaching solution of hydrogen peroxide to extend the shelf-life of fresh *Agaricus* mushrooms.

The enzymic browning of apples is widely known and has recently drawn attention[68–70] with respect to the phenolic substrates responsible for the process.[71] A deep involvement of 4-methylcatechol and of epicatechin has been shown for the first stage of the browning, whereas caffeic and chlorogenic acids become more and more important in the late stages. Browning is effectively inhibited by both coumaric and 2,4-dihydroxybenzoic acids.

Enzymic browning has been also studied in pears,[72] radishes[73] and apricots.[74] In the last case, an interesting favourable action of browning prevention was found for β-carotene. This inhibits PPO and moreover hinders chlorogenic acid quinonisation. β-Carotene is partly isomerised to the *cis* isomer during its action.

Among the huge number of compounds, potentially useful as PPO and/or melanogenesis inhibitors, maltol and kojic acid attracted much attention,[42] being naturally occurring compounds devoid of significant toxicity. As noted above, maltol is not a true PPO inhibitor, but rather prevents product accumulation in the reaction medium by reacting with *o*-quinones. On the contrary, the strictly related compound kojic acid, in addition to bleaching *o*-quinones, is a very effective PPO inhibitor. The bleaching mechanism of quinones by kojic acid seems to be related to a redox reaction leading to an oxidised yellow derivative of the inhibitor.[75] This derivative could be identified by means of its UV-VIS absorption spectrum, and the proposed redox mechanism is supported by the observation that the same yellow product could be formed when kojic acid is oxidised by hydrogen peroxide in the presence of horseradish peroxidase. The inhibitory activity of kojic acid towards a number of PPO, both of animal and plant origin, has been largely assessed.[11,39–41] The health aspects of kojic acid use as a food additive have been recently re-examined and reviewed[76] and the compound was found to be quite safe.

Thiol-containing compounds (mercaptans) are well known melanogenesis inhibitors. They are of particular interest for several reasons: (i) several compounds belonging to this class are widespread in Nature, and devoid of significant toxicity; some are, on the contrary, important constituents of living organisms (cysteine, glutathione); (ii) mere

reductants (such as ascorbic acid) reduce *o*-quinone to catechols, that are re-oxidised by PPO. The melanogenetic process is therefore slowed until all the reductant is consumed, and then it goes at its original rate. On the contrary, mercaptans tend to covalently attack the quinone ring, leading to catechols, bearing an organic sulphide function bonded to the 3-position on the resulting aromatic ring. These hindered products are usually non-substrates for PPO so melanogenesis is irreversibly stopped, even if these catechols are sometimes sensitive to autoxidation.[24] However, when methyl-mercaptan is the thiol, the 3-methylthiocatechols arising from non-enzymic nucleophilic attack on *o*-quinones are not hindered sufficiently to prevent their further quinonisation by PPO. The products undergo a nucleophilic attack by another methyl-mercaptan molecule, leading to relatively stable 3,6-*bis*-methylthio-catechols.[77] Deliberate, PPO-assisted quinonisation of catechols such as chlorogenic acid has been proposed to effectively remove foul odours (arising from volatile thiols) from polluted air and from manure.[77]

Usually, the aim of applying such reactions is the prevention of browning, and cysteine should be the best candidate, owing to its cheapness, water solubility, and safety: it is officially recognised and generally accepted as a safe product.[78] However this aminoacid is readily autoxidisable, leading to cystine which is useless as a browning preventer. To overcome this drawback *N*-acetylcysteine, which is much more stable, has been proposed.[78] It is well known as a virtually non-toxic drug (mucus fluidiser and paracetamol poisoning antidote) and is as effective as cysteine in browning prevention.[78] It also shows antimutagenic activity and is a good cysteine source for both animals and humans.[78] Both cysteine and *N*-acetylcysteine have got their own tastes and odours, therefore concentrations must be chosen to avoid excessive influence on organoleptic properties. Moreover their ability in penetrating through tissues is limited: protection against browning in deep tissues is therefore difficult or impossible.

Enzymic browning of vegetables, fruits and mushrooms is a phenomenon which although undesirable (from both commercial and nutritional considerations) is largely unavoidable: selection of cultivars, low in PPO and/or phenolics, leads to products less sensitive to enzymic browning but devoid of the beneficial features linked to a high phenolic content, and probably more sensitive to a number of pests. Treatment of food with inhibitors more or less alters the organoleptic properties, and moreover many inhibitors are still *sub judice* as regards the safety of their use. Much experimental and applied work is required to give reasonable answers to the questions regarding enzymic browning prevention and its effects on commercial value, storage life and safety of treated food.

References

[1] Schoot-Uiterkamp, A.J.M. and Mason, H.S. (1973) *Proc. Natl Acad. Sci. USA* **70**, 993–996.
[2] Nishioka, K. (1978) *Eur. J. Biochem.* **85**, 137–146.
[3] Burton, S.G. (1994) *Catal. Today* **22**, 459–487.
[4] Della Longa, S., Ascone, I., Bianconi, A., Bonfigli, A., Congiu Castellano, A., Zarivi, O. and Miranda, M. (1996) *J. Biol. Chem.* **271**, 21025–21030.
[5] Sánchez-Ferrer, Á., Rodríguez-López, J.N., García-Cánovas, F. and García-Carmona, F. (1995) *Biochim. Biophys. Acta* **1247**, 1–11.
[6] Orlow, S.J., Bao-Kang, Z., Chakraborty, A.K., Dricker, M., Pifko-Hirst, S. and Pawelek, J.M. (1994) *J. Investig. Dermatol.* **103**, 196–201.
[7] Eicken, C., Krebs, B. and Sacchettini, J.C. (1999) *Curr. Opin. Struct. Biol.* **9**, 677–683.
[8] Eicken, C., Zippel, F., Büldt-Karentzopoulos, K. and Krebs, B. (1998) *FEBS Lett.* **436**, 293–299.
[9] Decker, H. and Tuczek, F. (2000) *TIBS* **25**, 392–397.
[10] Cooksey, C.J., Garratt, P.J., Land, E.J., Ramsden, C.A. and Riley, P.A. (1998) *Biochem. J.* **333**, 685–691.
[11] Galindo, J.D., Pedreño, E., García-Carmona, F., García-Cánovas, F., Solano, F. and Lozano, J.A. (1983) *Int. J. Biochem.* **15**, 1455–1461.
[12] Ben-Shalom, N., Kahn, V., Harel, E. and Mayer, A.M. (1977) *Phytochemistry* **16**, 1153–1158.
[13] Rodríguez-López, J.N., Fenoll, L.G., Peñalver, M.J., García-Ruiz, P.A., Varón, R., Martínez-Ortíz, F., García-Cánovas, F. and Tudela, J. (2001) *Biochim. Biophys. Acta* **1548**, 238–256.
[14] Fenoll, L.G., Rodríguez-López, J.N., García-Sevilla, F., García-Ruiz, P.A., Varón, R., García-Cánovas, F. and Tudela, J. (2001) *Biochim. Biophys. Acta* **1548**, 1–22.
[15] Naish-Byfield, S. and Riley, P.A. (1998) *Pigment Cell Res.* **11**, 94–97.
[16] Sugumaran, M. and Bolton, J.L. (1998) *Arch. Biochem. Biophys.* **353**, 207–212.
[17] Bubacco, L., Salgado, J., Tepper, A.W.J.W., Vijgenboom, E. and Canters, G.W. (1999) *FEBS Lett.* **442**, 215–220.
[18] Van Gastel, M., Bubacco, L., Groenen, E.J.J., Vijgenboom, E. and Canters, G. (2000) *FEBS Lett.* **474**, 228–232.
[19] Prota, G. (1992) *Melanins and Melanogenesis* (Academic Press, New York).
[20] Espín, J.C., Tudela, J. and García-Cánovas, F. (1998) *Anal. Biochem.* **259**, 118–126.
[21] Mayer, A.M. and Harel, E. (1979) *Phytochemistry* **18**, 193–215.
[22] Rescigno, A., Porcu, M.C., Olianas, A., Rinaldi, A.C., Sanjust, E., Cocco, D. and Rinaldi, A. (1995) *Biochem. Mol. Biol. Int.* **37**, 319–327.
[23] Cory, J.G. and Frieden, E. (1967) *Biochemistry* **6**, 121–126.
[24] Ito, S., Kato, T., Shinpo, K. and Fujita, K. (1984) *Biochem. J.* **222**, 407–411.
[25] Rescigno, A., Sanjust, E., Soddu, G., Rinaldi, A.C., Sollai, F., Curreli, N. and Rinaldi, A. (1998) *Biochim. Biophys. Acta* **1384**, 268–276.
[26] Maddaluno, J.F. and Faull, K.F. (1988) *Experientia* **44**, 885–887.
[27] Rinaldi, A.C., Porcu, M.C., Oliva, S., Curreli, N., Rescigno, A., Sollai, F., Rinaldi, A., Finazzi-Agrò, A., Sanjust, E. (1998) *Eur. J. Biochem.* **251**, 91–97.
[28] Sugumaran, M., Duggaraju, P., Jayachandran, E. and Kirk, K.L. (1999) *Arch. Biochem. Biophys.* **371**, 98–106.
[29] Pompel, A., Fischer, H., Meiwes, D., Büldt-Karentzopoulos, C., Magrini, A., Eicken, C., Gerdemann, C. and Krebs, B. (1999) *FEBS Lett.* **445**, 103–110.
[30] Krol, E.S. and Bolton, J.L. (1997) *Chem.-Biol. Interact.* **104**, 11–27.
[31] Phillips, R.S., Fletcher, J.G., Von Tersch, R.L. and Kirk, K.L. (1990) *Arch. Biochem. Biophys.* **276**, 65–69.
[32] Sugumaran, M. and Lipke, H. (1983) *FEBS Lett.* **155**, 65–68.
[33] Lee, C.H., Koshino, H., Chung, M.C., Lee, H.J. and Kho, Y.K. (1995) *J. Antibiot.* **48**, 1168–1170.

[34] Kahn, V. (1985) *Phytochemistry* **24**, 915–920.
[35] Flurkey, W.H., Ratcliff, B., Lopez, L., Kuglin, J. and Dawley, R.M. (1995) Chapter 6, In: Lee, C.Y. and Whitaker, J.R., eds, *Enzymatic Browning and its Prevention* (American Chemical Society, Washington DC) pp 81–89.
[36] Ferrar, P.H. and Walker, J.R.L. (1996) *J. Food Biochem.* **20**, 15–30.
[37] Espín, J.C., Jolivet, S. and Wichers, H.J. (1998) *J. Agric. Food Chem.* **46**, 2976–2980.
[38] Seo, B., Yun, J., Lee, S., Kim, M., Hwang, K., Min, K.R., Kim, Y. and Moon, D. (1999) *Planta Med.* **65**, 683–686.
[39] Chen, J.S., Wei, C. and Marshall, M.R. (1991) *J. Agric. Food Chem.* **39**, 1897–1901.
[40] Chen, J.S., Wei, C., Rolle, R.S., Otwell, W.S., Balaban, M.O. and Marshall, M.R. (1991) *J. Agric. Food Chem.* **39**, 1396–1401.
[41] Kahn, V., Ben-Shalom, N. and Zakin, V. (1997) *J. Agric. Food Chem.* **45**, 4460–4465.
[42] Kahn, V. (1995) Chapter 22, In: Lee, C.Y. and Whitaker, J.R., eds, *Enzymatic Browning and its Prevention* (American Chemical Society, Washington DC), pp 277–294.
[43] Dawley, R.M. and Flurkey, W.H. (1993) *Phytochemistry* **33**, 281–284.
[44] Dawley, R.M. and Flurkey, W.H. (1993) *J. Food Sci.* **58**, 609–610.
[45] Shin, N.-H., Ryu, S.Y., Choi, E.J., Kang, S.-H., Chang, I.-M., Min, K.R. and Kim, Y. (1998) *Biochem. Biophys. Res. Commun.* **243**, 801–803.
[46] Iida, K., Hase, K., Shimomura, K., Sudo, S., Kadota, S. and Namba, T. (1995) *Planta Med.* **61**, 425–428.
[47] Kubo, I., Kinst-Hori, I., Ishiguro, K., Chaudhuri, S.K., Sanchez, Y. and Ogura, T. (1994) *Bioorg. Med. Chem. Lett.* **4**, 1443–1446.
[48] Kubo, I., Yokokawa, Y. and Kinst-Hori, I. (1995) *J. Nat. Prod.* **58**, 739–743.
[49] Kubo, I. and Kinst-Hori, I. (1999) *Planta Med.* **65**, 19–22.
[50] Lim, J.-Y., Ishiguro, K. and Kubo, I. (1999) *Phytother. Res.* **13**, 371–375.
[51] Menon, S., Fleck, R.W., Yong, G. and Strothkamp, K.G. (1990) *Arch. Biochem. Biophys.* **280**, 27–32.
[52] Mimaki, Y., Kamei Ama, A., Sashida, Y., Miyata, Y. and Fujii, A. (1995) *Chem. Pharm. Bull.* **43**, 893–895.
[53] Daquinag, A.C., Nakamura, S., Takao, T., Shimonishi, Y. and Tsukamoto, T. (1995) *Proc. Natl Acad. Sci. USA* **92**, 2964–2968.
[54] Morita, H., Kobata, H., Takeya, K. and Itokawa, H. (1994) *Tetrahedron Lett.* **35**, 3563–3564.
[55] Rinaldi, A.C., Porcu, M.C., Curreli, N., Rescigno, A., Finazzi-Agrò, A., Pedersen, J.Z., Rinaldi, A. and Sanjust, E. (1995) *Biochem. Biophys. Res. Commun.* **214**, 559–567.
[56] Rescigno, A., Rinaldi, A.C. and Sanjust, E. (1998) *Biochem. Pharmacol.* **56**, 1089–1096.
[57] Haslam, E. (1989) *Plant Polyphenols. Vegetable Tannins Revisited* (Cambridge University Press, Cambridge).
[58] Nakayama, T., Sato, T., Fukui, Y., Yonekura-Sakakibara, K., Hayashi, H., Tanaka, Y., Kusumi, T. and Nishino, T. (2001) *FEBS Lett.* **499**, 107–111.
[59] Núñez-Delicado, E., Bru, R., Sánchez-Ferrer, A. and García-Carmona, F. (1996) *J. Chromatogr. B* **680**, 105–112.
[60] Poulton, J.E. (1981) Chapter 22, In: Stumpf, P.K. and Conn, E.E., eds, *The Biochemistry of Plants* (Academic Press, New York) **Vol. 7**, pp 668–723.
[61] Jung, H.G. and Fahey, Jr, G.C. (1983) *J. Anim. Sci.* **57**, 206–219.
[62] Friedman, M. (1997) *J. Agric. Food Chem.* **45**, 1523–1540.
[63] Sapers, G.M., Miller, R.L., Pilizota, V. and Kamp, F. (2001) *J. Food Sci.* **66**, 362–366.
[64] Jolivet, S., Arpin, N., Wichers, H.J. and Pellon, G. (1998) *Mycol. Res.* **102**, 1459–1483.
[65] Espín, J.C., Jolivet, S., Overeem, A. and Wichers, H.J. (1999) *Phytochemistry* **50**, 555–563.
[66] Zhang, X. and Flurkey, W.H. (1997) *J. Food Sci.* **62**, 97–100.
[67] Ratcliffe, B., Flurkey, W.H., Kuglin, J. and Dawley, R. (1994) *J. Food Sci.* **59**, 824–827.
[68] Rocha, A.M.C.N. and Morais, A.M.M.B. (2001) *Int. J. Food Sci. Technol.* **36**, 425–432.
[69] Subaric, D., Pilizota, V., Lovric, T., Vukovic, R. and Erceg, A. (2001) *Acta Aliment.* **30**, 81–87.
[70] Ibarz, A., Pagan, J. and Garza, S. (2000) *J. Sci. Food Agric.* **80**, 1162–1168.
[71] Bajaj, K.L., Debethencourt, D., Junquera, B. and Gonzalez-San José, M.L. (1997) *J. Food Sci. Technol.* **34**, 296–302.
[72] Larrigaudière, C., Pinto, E., Lentheric, I. and Vendrell, M. (2001) *J. Hortic. Sci. Biotechnol.* **76**, 157–162.
[73] Fukuoka, N. and Enomoto, T. (2001) *Plant Sci.* **161**, 117–124.
[74] De Rigal, D., Gauillard, F. and Richard-Forget, F. (2000) *J. Sci. Food Agric.* **80**, 763–768.
[75] Kahn, V. (1995) *Pigment Cell Res.* **8**, 234–240.
[76] Burdock, F.A., Soni, M.G. and Carabin, I.G. (2001) *Reg. Toxicol. Pharmacol.* **33**, 80–101.
[77] Negishi, O. and Ozawa, T. (1997) *Biosci. Biotech. Biochem.* **61**, 2080–2084.
[78] Friedman, M. (1996) *J. Agric. Food Chem.* **44**, 631–653.