Chalcone dimethylallyltransferase from *Morus nigra* cell cultures. Substrate specificity studies

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Abstract **A new prenyltransferase (PT) enzyme derived from the microsomal fractions of cell cultures of *Morus nigra* was shown to be able to prenylate exclusively chalcones with a 2′,4′-dihydroxy substitution and the isoflavone genistein. Computational studies were performed to shed some light on the relationship between the structure of the substrate and the enzymatic activity. PT requires divalent cations, particularly Mg^{2+}, to be effective. The apparent K_m values for γ,γ-dimethylallyldiphosphate and 2′,4′-dihydroxychalcone were 63 and 142 μM, respectively. The maximum activity of the enzyme was expressed during the first 10 days of cell growth.**
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Key words: Prenyltransferase; γ,γ-Dimethylallyldiphosphate; Product specificity; Molecular modeling

1. Introduction

The term prenyltransferase (PT) defines all those enzymes that catalyze the transfer of prenyl groups to a wide variety of acceptors (isoprenoid groups, aromatic compounds, proteins etc.). PTs are widely distributed in all the kingdoms of life and participate in most of the metabolic routes leading to side chains of respiratory coenzymes [1], carotenoids, terpenes and polymers such as rubbers [2,3]. The PT enzymes are also involved in the post-translational modification of proteins, assuming a key role in some carcinogenic mechanisms [4–6].

Recently, these enzymes are receiving growing interest for their potential use as biocatalysts in the formation of C–C bonds, a reaction as important as difficult in organic chemistry; only some aldolases and lyases have been previously used [7].

However, PTs are peculiar enzymes because they not only create a new C–C bond, but also introduce a double bond in the framework of the final product, a feature which is often associated with the activation or the enhancement of the biological properties [8–10]: for instance, prenylated chalcones have been shown to be interesting compounds as modulators of some biological activities [8,11–13]. Moreover, the chemical synthesis of prenylated aromatic compounds is quite difficult to achieve in good yield and with the regio- or stereospecificity essential for biological activity and usually requires the use of protective groups. Aromatic PTs are in this view very interesting enzymes and some of them have been purified, characterized [14] and cloned [15] and others studied in flavonoid biogenic pathways [16–18]. The possibility of manipulating such an enzymatic catalyst represents an interesting tool for the organic synthesis of biologically active compounds. This paper deals with a study on the specificity and some biochemical characteristics of the PT from *Morus nigra* cell cultures [19].

2. Materials and methods

2.1. Cell culture

Calluses of *M. nigra* were grown on MS 62 basal medium solidified with agar (0.8%) and supplemented with 2,4-dihydrochlorophenoxyacetic acid (2,4-D; 0.25 ppm), naphthalene acetic acid (0.1 ppm), kinetin (0.75 ppm) and sucrose 3% [15]. Calluses (4 g fresh weight) were transferred in a liquid medium (65 ml) with the same composition as above, enriched with casein hydrolysate (0.1%). The flasks were harvested under continuous stirring (125 rpm) in the dark at 25°C and subcultured in fresh medium every 25 days.

2.2. Extraction and localization of the PT activity

Cells of *M. nigra* were collected at 7–10 days old and filtered on a glass filter under vacuum to be homogenized at 4°C in buffer A (Tris–HCl 50 mM, pH 7.5, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 10% of sucrose) in a (w/v) ratio of 1:1 (1 ml of buffer/1 g of cell), first in a ceramic potter in the presence of polyvinylpyrrolidone, and then with an Ultra Turrax homogenizer. The resulting material was centrifuged at 13 000 rpm for 22 min at 4°C. This supernatant (3 ml) was used as crude extract for further purification by centrifugation at 100 000 × *g* for 50 min at 4°C, obtaining a sticky greenish pellet. This was washed with distilled and deionized H_2O, resuspended with buffer B (Tris–HCl 50 mM, pH 7.5, 10 mM DTT, 0.5 ml final volume) and transferred in a 1.5 ml vial to have a more homogeneous solution with the aid of a micro homogenizer. Each preparation, containing 0.8–1.0 mg/ml of protein, was used for a single biotransformation test.

2.3. Biotransformations

In biotransformation studies with whole cells (feeding experiments) the precursors were dissolved in the minimal amount of an ethanol/sterile water (1:1) solution. The cells were inoculated when their fresh

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Abbreviations: PT, prenyltransferase; 2,4-D, 2,4-dihydrochlorophenoxyacetic acid; DTT, dithiothreitol; DMAPP, dimethylallylpyrophosphate

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weight was 10 g under sterile conditions. After 3 days of incubation the cells were collected (fresh weight ca. 10 g), filtered under vacuum on a glass filter, lyophilized and then homogenized with an Ultra Turrax device using as solvent ethyl acetate in a 1:1 (w/v) ratio. The organic layer was dried with anhydrous Na_2SO_4 and evaporated. For every extraction three flasks of the same experiment were used.

For the reactions in which microsomal preparations were employed, the following conditions were used: the suspended pellet in buffer B (0.5 ml), with a final protein concentration of 0.8–1.0 mg/ml, was kept at 30°C. The cofactors and substrates were added: Mg^{2+} (1 mM), γ,γ-dimethylallylpyrophosphate (DMAPP; 60 μM) and the substrate (30 μM) in that order. After 1 h of incubation the reaction was stopped and the mixture extracted with ethyl acetate. The residue was analyzed by thin layer (TLC) and high performance liquid chromatography (HPLC), as described below.

2.4. Evaluation of the maximum enzymatic activity and kinetic studies

Cells of *M. nigra* of different ages were collected every 2 days and filtered under vacuum. The assays were carried out in the same manner as described in Section 2.3 with microsomal fractions using 2′,4′-dihydroxychalcone (**1**) as enzyme marker. The percentage of isocordoin (**1a**) formed, calculated by HPLC peak areas, was used to quantify the enzymatic activity. For kinetic studies microsomal fractions were employed as enzyme source. Varying concentrations (0.01–1 mM) of compound **1** versus fixed concentrations (1 mM) of DMAPP and, conversely, varying concentrations of DMAPP (0.015–2.5 mM) versus fixed concentrations (1 mM) of **1**, were used to calculate K_m values by a Lineweaver–Burk plot.

2.5. Computational studies

Calculations and graphics manipulations were performed on Iris 4D/35 and Indigo R4000 Silicon Graphics workstations, using the software packages InsightII and Discover of MSI (Molecular Simulations, San Diego, CA, USA).

2.6. Analysis of the reaction products

The prenylation of **1**, both for determination of PT activity and for kinetic studies, was followed by HPLC with a RP-18 column (100×4.6 mm I.D., 5 μm), eluting with MeOH/H_2O 80:20 in isocratic conditions and with a flow rate of 1 ml/min. The wavelength of the UV detector was set at 300 nm. The amounts of **1** and of **1a** were calculated by extrapolation from calibration curves.

Silica gel TLC plates from Merck, developed with chloroform/ethyl acetate mixtures, in a ratio depending on the polarity of the compounds, were used to monitor the reactions and to separate the biotransformation products.

2.7. Synthesis of substrates and identification of products

The syntheses and the spectral and physical data of chalcones **1**–**3** [20] and **4** and **5** [21] have been previously reported, while chalcones **6**–**9** were prepared following the general procedure: the appropriate acetophenone (6.6 mM) and benzaldehyde (2 ml) in MeOH (10 ml) and KOH/H_2O (10 g/10 ml) were held at reflux for 45 min. The reaction mixture was poured into ice/HCl 6 N and the precipitate was washed with abundant water and purified on a silica gel column.

Compounds **10**, **11**, **12** and **16** were purchased from Sigma-Aldrich (Buchs, Switzerland), compounds **13**, **14** and **15** were a kind gift of Prof. F. Delle Monache (Istituto di Chimica del Riconoscimento Molecolare, Rome, Italy).

DMAPP was synthesized according to the literature [22].

Melting points were determined with a Köfler apparatus and are uncorrected. ^{1}H NMR spectra were run at 300 MHz at room temperature with TMS as internal standard. EI-MS were performed by direct inlet at 70 eV, while for ESI-MS spectra MeOH (I) or MeOH/0.1% HCOOH (II) solutions were used.

2′,4′-Dihydroxy-3,4-methylenedioxychalcone (**6**; yield 68%): mp 191–2°C; ^{1}H NMR (Me_2CO-d_6): δ 8.18 (d, ^3J(H,H) = 9 Hz, 1H, H-6′), 7.81 (d, ^3J(H,H) = 16 Hz, 1H, H-α), 7.78 (d, ^3J(H,H) = 16 Hz, 1H, H-β), 7.28 (dd, ^3J(H,H) = 8.5 Hz and 2 Hz,1H, H-6), 7.42 (d, ^3J(H,H) = 2 Hz, 1H, H-2),7.03 (d, ^3J(H,H) = 8.5 Hz,1H, H-5), 6.49 (dd, ^3J(H,H) = 9 Hz and 2 Hz, 1H, H-5′), 6.38 (d, ^3J(H,H) = 2 Hz, 1H, H-3′), 6.12 (s, 2H, O-CH_2-O); ESI-MS (II): 285 $[MH]^+$.

4′-Hydroxychalcone (**7**: yield 46%): mp 156–7°C; ^{1}H NMR ($CDCl_3$): δ 8.01 (d, ^3J(H,H) = 8.5 Hz, 1H, H-2′), 7.81 (d, ^3J(H,H) = 16 Hz, 1H, H-α), 7.64 (m, 2H, H-2 and H-6), 7.55 (d, ^3J(H,H) = 16 Hz, 1H, H-β), 7.42 (m, 3H, H-3, H-4 and H-5), 6.96 (d, ^3J(H,H) = 8.5 Hz, 2H, H-5′, H-3′), 6.65 (br s, 1H, OH); EI-MS *m/z* (rel. int.): 224 (65) $[M]^+$, 223 (58), 196 (25) $[M-CO]^+$, 195 (26), 131 (32) [M−A ring]$^+$, 121 (100) [M−B ring]$^+$, 103 (75) [B ring]$^+$, 93 (52) [A ring]$^+$.

2′-Hydroxychalcone (**8**, yield 78%): 78–9°C; ^{1}H NMR ($CDCl_3$): δ 7.92 (dd, ^3J(H,H) = 8 Hz and 2 Hz, 1H; H-6′), 7.91 (d, ^3J(H,H) = 16 Hz, 1H-α), 7.66 (d, ^3J(H,H) = 16 Hz, H-β), 7.65 (m, 2H, H-2, H-6), 7.49 (td, ^3J(H,H) = 7.5 Hz and 2 Hz, 1H, H-4′), 7.43 (m, 3H, H-3, H-5 and H-4), 7.03 (br d, ^3J(H,H) = 8 Hz, 1H, H-3′), 6.94 (br t, ^3J(H,H) = 7.5 Hz, H-5′); EI-MS *m/z* (rel. int.): 224 (49) $[M]^+$, 223 (38), 196 (5) $[M-CO]^+$, 131 (12) [M−A ring]$^+$, 121 (100) [M−B ring]$^+$, 103 (65) [B ring]$^+$, 93 (38) [A ring]$^+$.

2′,4′-Dimethoxychalcone (**9**, yield 88%): mp 67–8°C; ^{1}H NMR (Me_2CO-d_6): δ 7.77 (d, ^3J (H,H) = 8.5 Hz, 1H, H-6′), 7.67 (d, ^3J(H,H) = 16 Hz, 1H, H-α), 7.60 (m, 2H, H-2, H-6), 7.56 (m, 3H, H-3, H-4, H-5), 7.56 (d, J = 16 Hz, 1H, H-β), 6.57 (dd, ^3J(H,H) = 8.5 Hz and 2.5 Hz, 1H, H-5′), 6.51 (d, ^3J(H,H) = 2.5 Hz, 1H, H-3′), 3.91 (s, 3H, OMe), 3.88 (s, 3H, OMe); ESI-MS (I) *m/z* (rel. int.): 291 (100) $[MNa]^+$, 269 (16) $[MH]^+$, 193 (4) [MH−B ring]$^+$, 165 (8) $[A-CO]^+$.

The structures of the biotransformation products were assigned on the basis of ^{1}H NMR and mass spectral data. The presence of the prenyl chain was suggested by the appropriate signals in the ^{1}H NMR spectrum for two methyls (3H broad singlets in the range of δ 1.6–1.8), a methylene (2H doublet at ca. δ 3.4) and a methine group (1H broad triplet at ca. δ 5.3). The prenylation was confirmed by the (68 mu) shift of the molecular peak in the mass spectrum and by a series of fragments showing the typical losses (15, 43 and 55 mu) of the prenyl chain either from the molecular ion or from the ion (*a*) corresponding to the A ring. The location of the chain is immediately indicated in 2′,4′-chalcones by the simplification of the H-5′ signal, which becomes an *ortho*-coupled doublet. In the isoflavanone **16a** the substitution site was determined both by the ^{1}H NMR resonance value (δ 3.37 for a C-6 prenyl) of the methylene group in Me_2CO-d_6 [23] and by a delayed (20 min) bathochromic shift in the UV spectrum after addition of $AlCl_3$ [24].

Finally, in the ^{1}H NMR spectra of dihydrochalcones (2 mu higher M^+) the signals for two methylene groups (triplets at ca. δ 3.3 and 3.0) replace those of the olefinic protons.

The structures of compounds **1a** [19] and **2a** [25] were assigned by comparison (spectral and physical data) with authentic samples.

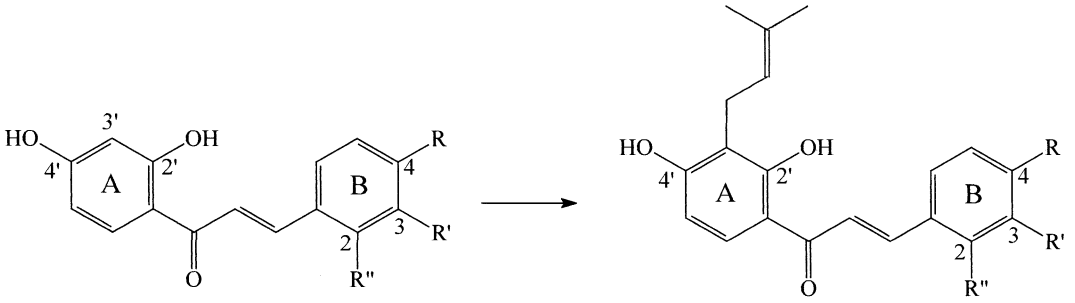
2′,4,4′-Trihydroxy-3-methoxy-3′-[2-methylbut-3-enyl]-chalcone (**3a**): vitreous solid; ^{1}H NMR (Me_2CO-d_6): δ 7.83 (d, ^3J(H,H) = 16 Hz, 1H, H-α), 7.74 (d, ^3J(H,H) = 8.5 Hz, 1H, H-6′), 7.44 (d, ^3J(H,H) = 16 Hz, 1H, H-β), 7.24 (dd, ^3J(H,H) = 8 Hz, 1H, H-6), 7.13 (d, ^3J(H,H) = 2.5 Hz, 1H, H-2), 6.97 (d, ^3J(H,H) = 8.5 Hz, 1H, H-5), 6.43 (d, ^3J(H,H) = 8.5 Hz, 1H, H-5′), 5.3 (br t, ^3J(H,H) = 7 Hz, CH), 3.98 (s, 3H, OMe), 3.49 (d, ^3J(H,H) = 7 Hz, 2H, CH_2), 1.83 br s, 3H, Me), 1.78 (br s, 3H, Me). EI-MS *m/z*: 354 (100) $[M]^+$, 339 (6) $[M-15]^+$, 321 (19) $[M-15-18]^+$, 311 (72) $[M-43]^+$, 299 (20) $[M-55]^+$, 205 (19) $[a+H]^+$, 204 (17) $[a]^+$, 189 (17) $[a-Me]^+$, 161 (30) $[a-43]^+$, 150 (43) $[b]^+$, 149 (66) $[a-55]^+$.

4′-Hydroxydihydrochalcone (**7a**): vitreous solid; ^{1}H NMR ($CDCl_3$): δ 7.91 (d, ^3J(H,H) = 8.5 Hz, 2H, H-2′, H-6′), 7.34–7.2 (m, 5H, H-2, H-3, H-4, H-5, H-6), 6.87 (d, ^3J(H,H) = 8.5 Hz, 2H, H-3′, H-5′), 3.25 (br t, ^3J(H,H) = 7.5 Hz, 2H, H_2-α), 3.05 (br t, ^3J(H,H) = 7.5 Hz, 2H, H_2-β); ESI-MS (I) *m/z*: 227 $[MH]^+$.

2′-Hydroxydihydrochalcone (**7a**): ^{1}H NMR ($CDCl_3$): δ 7.85 (d, ^3J(H,H) = 9 Hz, 1H, H-6′), 7.47 (td, ^3J(H,H) = 8 and 2 Hz, H-4′), 7.33 (br d, ^3J(H,H) = 5 and 7 Hz, 2H, H-2, H-6), 7.29 (br t, ^3J(H,H) = 7 Hz, 2H, H-3, H-5), 7.25 (br t, ^3J(H,H) = 8 Hz, 1H, H-4), 6.99 (br d, ^3J(H,H) = 8 Hz, H-3′), 6.89 (br t, ^3J(H,H) = 8 Hz, 1H, H-5′), 3.34 (t, ^3J(H,H) = 7.5 Hz, 2H, H_2-α), 3.08 (t, ^3J(H,H) = 7.5 Hz, 2H, H_2-β); ESI-MS (I) *m/z* (rel. int.): 249 (100) $[MNa]^+$, 227 (26) $[MH]^+$.

2′,4′-Dimethoxydihydrochalcone (**9a**): ^{1}H NMR (Me_2CO-d_6): δ 7.74 (d, ^3J(H,H) = 8.5 Hz, 1H, H-6′), 7.25–7.08 (m, 5H, H-2, H-3, H-4, H-5), 6.46 (dd, ^3J(H,H) = 8.5 Hz and 2.5 Hz, 1H, H-5′), 6.38 (d, ^3J(H,H) = 2.5 Hz, 1H, H-3′), 3.80 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.28(t, ^3J(H,H) = 8 Hz, 2H, H_2-α), 2.93 (t, ^3J(H,H) = 8 Hz, 2H, H_2-β). EI-MS *m/z* (rel. int.): 270 (24) $[M]^+$, 165 (100) $[A-CO]^+$, 138 (11) $[A-H]^+$, 122 (7) $[M-A-H]^+$; ESI-MS (I) *m/z*

Table 1
Specificity experiments with 2′,4′-dihydroxychalcones diversely substituted in ring B



Precursor	Product	R	R'	R"	Yields of prenylation (%)	
					Microsomes	Whole Cells
1	**1a**	H	H	H	85	80
2	**2a**	OH	H	H	80	70
3	**3a**	OH	OCH_3	H	75	60
4	-	H	OH	H	0	0
5	-	H	H	OH	0	0
6	-	O-CH_2-O		H	0	0

The yields refer to the overall amount of prenylated compounds obtained after a suitable time of incubation.

(rel. int.): 293 (100) $[MNa]^+$, 271 (12) $[MH]^+$, 165 (7) $[A\text{-}CO]^+$, 105 (5) $[B\text{-}CH_2\text{-}CH_2]^+$.

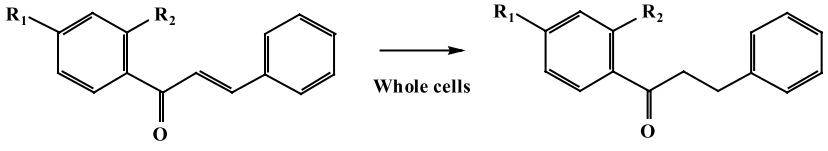
The structure of compound **16a** was confirmed by comparison (spectral and physical data) with an authentic specimen [24].

3. Results and discussion

3.1. Enzyme specificity

We have found that cell cultures of *M. nigra* were able to convert in very good yields 2′,4′-dihydroxychalcone (**1**) in the anticancer derivative isocordoin (**1a**) [19]. Further studies on the catalytic properties of the PT enzyme system were performed both with whole cells in feeding experiments and with a preparation obtained by ultracentrifugation of the crude extract and corresponding to the microsomal fraction.

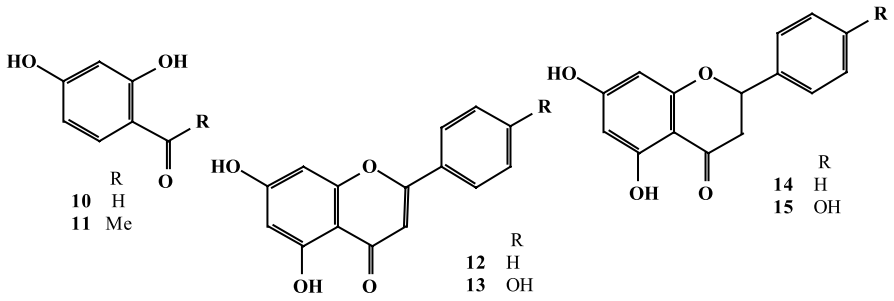
In a first set of experiments, nine diversely substituted chalcones were used as substrates. The results, summarized in Table 1, revealed that prenylation had occurred with chalcones **1**, **2**, **3**, bearing two hydroxyl groups (C-2′, C-4′) on ring A.



	R_1	R_2
7	OH	H
8	H	OH
9	OMe	OMe

	R_1	R_2
7a	OH	H
8a	H	OH
9a	OMe	OMe

Scheme 1. Biotransformation reactions of chalcones **7**, **8** and **9** upon feeding experiments with whole cells of *M. nigra*. The corresponding yields were 20% (**7a**), 25% (**8a**) and 40% (**9a**). When microsomes were employed as enzyme source, no kind of reaction product was detected



Scheme 2. Unreactive substrates in trials with both cell suspension cultures (feeding experiments) and microsomal fractions.

Among them, the position of the substituents in ring B appeared to be critical for the prenylation. A hydroxyl group in C-4 (**2**, **3**) did not modify the fate of the reaction as for unsubstituted chalcone **1**. By contrast, the presence of a hydroxyl group in C-2 (**5**) or C-3 (**4**), as well as of a methylenedioxy bridge between C-3 and C-4 (**6**), inhibited the reaction.

Notably, when 2′-hydroxychalcone (**8**) and 4′-hydroxychalcone (**7**) were inoculated in the cells, they did not undergo prenylation, but gave the corresponding dihydro derivatives **8a** and **7a**, with 25 and 20% yields, respectively (Scheme 1); similarly 2′,4′-dimethoxychalcone **9** gave the dihydrochalcone **9a** (Scheme 1) in fair yield (40%).

These results suggest that another enzymatic activity is present in *M. nigra* cell cultures which is responsible for the formation of these compounds. It should be noted that when the same substrates were employed with the microsomal fraction, neither prenylation nor dehydrogenation took place indicating that the two enzyme systems are independent and that PT does not recognize these chalcones at all.

To explore whether the whole chalcone structure was determinant for the enzyme recognition, simpler compounds than **1** with an identical substitution pattern in ring A, i.e. 2′,4′-dihydroxy-benzaldehyde (**10**) and 2′,4′-dihydroxy-acetophenone (**11**), were used as substrates both in vivo (feeding) and in vitro (microsomes) trials. Since no reaction occurred with **10** or **11** either, the importance of the B ring of chalcones for the interaction with the active site of the PT and a possible stabilization of the enzyme–substrate complex was confirmed.

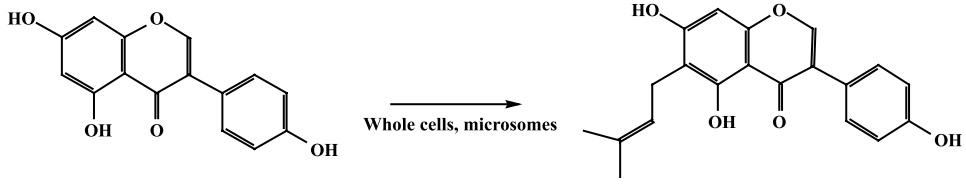
In order to verify whether the PT from *M. nigra* cell cultures was able to biotransform flavonoids other than chalcones, the cells were fed with flavones **12**, **13** and flavanones **14**, **15** (Scheme 2), but no prenylated compound was isolated in any case, the starting flavonoids being completely recovered from the reaction mixtures. The same results were obtained when **12**–**15** were used for reactions with microsomal preparations. By contrast, genistein (5,7-dihydroxy-isoflavone, **16**), as a substrate, gave the corresponding 6-prenyl derivative **16a** (Scheme 3) both in feeding experiments (30% yield) and with microsomes (40% yield). These results must be emphasized, because the PT enzyme system was able to recognize a compound exogenous for *M. nigra* either in vivo or in vitro.

To explain these findings, the structural similarities between 2′,4′-dihydroxychalcone (**1**), chosen as representative of the whole set of active chalcones, genistein (**16**) and 5,7-dihydroxyflavone (**12**) were investigated by a 3D-comparative study.

The three corresponding gas-phase lowest energy conformers obtained by a conformational analyses performed in a 3.0 kcal/mol energetic window (Search/Compare module) [26] were superimposed, as depicted in Fig. 1.

The aromatic ring A of **1** was fitted atom by atom with ring A of genistein (**16**), so that the positions 2′ and 4′ of the former were coincident with positions 5 and 7 of the latter, while the remaining moiety was free to be oriented in the 3D space [27].

A very good structural similarity was observed between compounds **1** and **16** (Fig. 1). Genistein appeared to possess a rigid structure in which ring B was rotated around 49° with respect to the condensed bicyclic system because of a steric repulsion between the carbonyl oxygen atom and the 3D related hydrogen (H-2′ or H-6′). Analogously, the extended system of π electrons of 2′,4′-dihydroxychalcone forced the molecule to adopt a quite planar conformation in which ring B was rotated around 46° with respect to ring A, as a result of three rotations around the single bonds connecting ring B to ring A. The main rotation of the three was the one involving the bond connecting ring B to the carbonyl group (36°). Cumulatively, the metabolite pattern produced from both plant and cell cultures of *M. nigra* [25], the experimental results of the biotransformations, and the molecular modeling studies suggest that only one kind of PT is present in *M. nigra* cells and not a series of PT enzymes, as reported elsewhere [28–30]. For this peculiar enzyme we propose the name of chalcone-PT.



16 16a

Scheme 3.

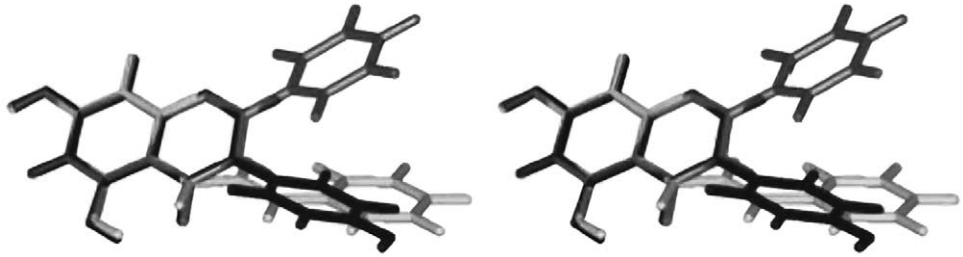


Fig. 1. Stereo view of the superimposition of genistein (**16**) (black), 2′,4′-dihydroxychalcone (**1**) (light gray) and 5,7-dihydroxyflavone (**12**) (dark gray).

3.2. Enzyme localization and partial characterization

A suspension culture was grown from calluses of *M. nigra* [19] using a MS62 medium enriched with sucrose (3%) and a triad of hormones: 2,4-D (0.2 ppm), naphthalene acetic acid (0.1 ppm), and kinetin (0.75 ppm). The medium was selected after trials with different media, because it was associated with the maximum enzymatic activity, the latter being expressed in the first 10 days of growth (Fig. 2). Since many γ,γ-dimethylallyl transferases have been reported [14,16] to be tightly associated with the microsomal fraction, a series of experiments were performed with the pellets obtained after ultracentrifugation of crude extract. Attempts to solubilize the PT with detergents such as Triton X-100 or CHAPS, and also with strong ionic solutions (NaCl 1 M), resulted in a very low recovery of the enzymatic activity (Table 2); these findings could be explained either by a strong interaction with microsomal membranes or by a loss of activity after the detergent treatment and subsequent suspension. However, even in the best case of recovery of enzymatic activity in the supernatant obtained with octyl-β-D-glucopyranoside (Table 2), the enzymatic activity was lost 1–2 h after the treatment. This aspect needs further investigation in view of the enzyme purification.

As for other PTs [28–31], the reactions performed with the microsomes required the presence of a divalent cation. In our case Mg^{2+} at a concentration of 1 mM gave the best results in term of yields of **1a**. In a set of experiments, performed with other cations such as Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} at the fixed concentration of 1 mM, smaller activity values, as compared with Mg^{2+} (100%), were obtained for Mn^{2+} (80%), Co^{2+} (28%), Ca^{2+} (11%), Ni^{2+} (11%), and Zn^{2+}(0%) indicating that Mg^{2+} and Mn^{2+} are the most suitable cations for the enzyme as observed in other PTs [14,17,18,32].

In all the successful reactions isocordoin (**1a**) was the only product to be obtained, suggesting that the different cations modify the reaction rate, but not the enzyme specificity [33].

The optimum pH was found to be ca. 7.5 in a Tris–HCl buffer, even though at pH 9 activity was still retained; the enzyme activity was well maintained even though the microsomal fractions were kept at −20°C for several weeks.

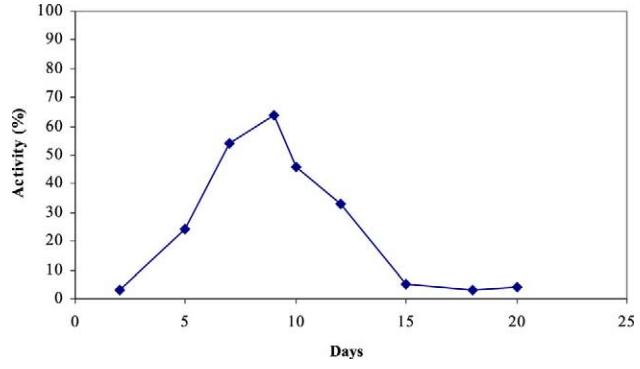


Fig. 2. PT activity during aging of cell suspension cultures. The PT activity has been calculated as percentage of prenylation of 2′,4′-dihydroxychalcone (**1**) employing microsomal fractions as enzyme source. Cells were collected and extracted at 2, 5, 7, 9, 10, 12, 15, 18, and 20 days.

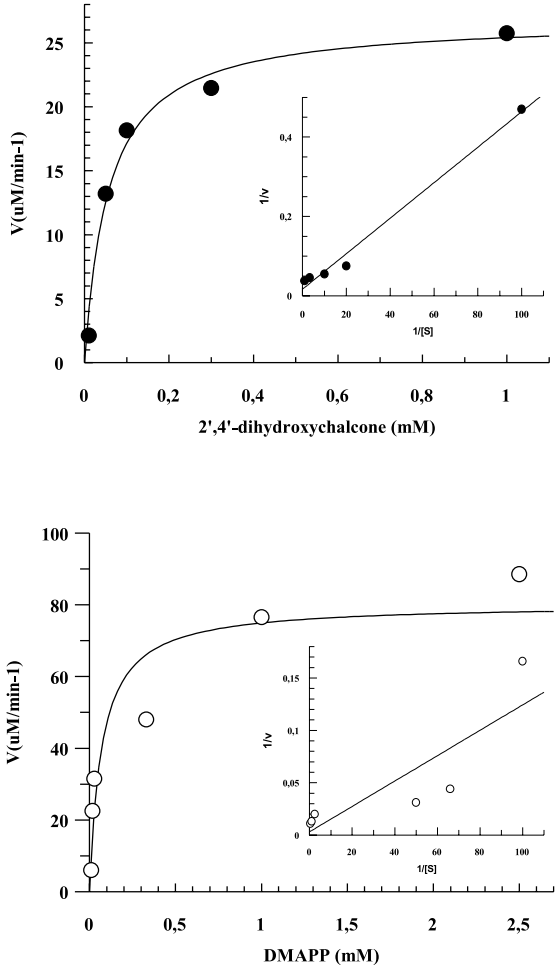


Fig. 3. Dependence of PT activity on the concentration of chalcone (**1**) (upper graph) and DMAPP (lower graph). The apparent K_m values were calculated from the Lineweaver–Burk plots (insets) with different concentrations (0.015–2.5 mM) of DMAPP and (0.01–1 mM) chalcone (**1**). Microsomal fractions were employed for both experiments.

Table 2
Effects of different detergents at various concentrations in solubilizing PT activity

Solution	Concentration	Activity (%)	
		Pellet	Supernatant
Triton X-100	0.2%	94	6
CHAPS	0.2%	95	5
Digitonin	0.2%	85	15
Octyl-β-D-glucopyranoside	0.1%	57	43
NaCl	0.5 and 1 M	98	nd

Triton X-100 was used at 0.2%, 0.5%, 0.75% and 2%. The same amounts were used for CHAPS and digitonin, while octyl-β-D-glucopyranoside was employed at 0.05%, 0.1%, 0.2% and 0.5%. The table indicates the detergent concentration for which activity was highest. Enzyme activity is expressed as the percentage of bioconversion of **1** and **1a** after 45 min of reaction.

The apparent kinetic parameters of the enzyme were obtained via HPLC measurements, with microsomal fractions by calculation of a Lineweaver–Burk plot. As a result, apparent K_m values of 63 and 142 μM were obtained for γ,γ-dimethylallyldiphosphate and 2′,4′-dihydroxychalcone, respectively (Fig. 3).

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