STUDIES IN CELL SUSPENSION CULTURES OF CASSIA DIDYMOBOTRYA. PART III.[1] THE BIOTRANSFORMATION OF CHALCONES TO FLAVONES AND BIFLAVANONES

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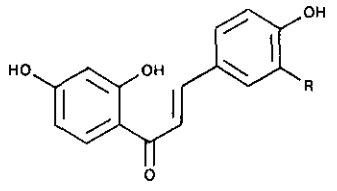
Abstract - Older (29 day) cell cultures of Cassia didymobotrya are shown to possess enzymes which can effectively catalyze the conversion of chalcones to flavone and novel biflavanone products. The substrates 2',4,4'-trihydroxychalcone and 3-methoxy-2,4,4'-trihydroxychalcone were evaluated in terms of bioconversion yields versus time of incubation, and the effect, if any, of H_2O_2 and horseradish peroxidase on such bioconversions. Comments concerning the nature of the intermediates involved are presented.

In the accompanying publication[1] we described our studies with cell free extract (C.F.E.) derived from Cassia didymobotrya cell cultures and the ability of such systems to isomerize chalcones to (-) 2S-flavanones.

In studying the above process we had observed that other products besides flavanones were obtained from the biotransformation of chalcones 1 and 2 (chalcones 10 and 16, accompanying publication), both possessing a free hydroxyl group at C-4 in ring B.



1 R=H

2 R=OMe

Since it is well known that "polyphenol oxidases" are widely distributed in the plant kingdom,[2] it was thought that they could be also present in our cell suspension cultures. These enzymes are known to catalyze[3] phenol oxidation and are usually divided into three main classes: i) tyrosinases or o-diphenol oxidases, ii) laccase or p-diphenol oxidases, and iii) peroxidases. The last family is dependant on H_2O_2, while the first two appear not to require this cofactor.

This publication deals with our studies on the nature of these enzymes and on their substrate specificity. In our initial studies, a series of experiments were performed in order to establish the variation of the polyphenol oxidases produced at different ages of the cultures. Guaiacol (2-methoxyphenol) was used as substrate.

Table 1 shows that such enzymatic activity was higher in older cells, specifically cells grown for 29 days.

Table 1

Age of cells (days)	0		15		22		29	
Expt	%*	Time** (min)	%	Time (min)	%	Time (min)	%	Time (min)
A	4	60	5	10	15	60	59	30
B	5	60	22	60	42	60	68	60
C	7	60	17	10	33	10	41	10
D	7	60	10	60	35	60	40	60

pH = 6.5 ~ 7.0; temperature = 37 °C

A: Cell free extract (C.F.E.) B: C.F.E. + H_2O_2

C: C.F.E. + horseradish peroxidase (HRP) D: C.F.E. + H_2O_2 + HRP

* % of transformed guaiacol

** optimum time of conversion

In the accompanying publication,[1] we had shown that maximum accumulation of the chalcone - flavanone isomerase (CHFI) enzymes occurred in 22 day old cultures, it was now appropriate to compare the competition of these two types of enzyme systems for the substrate chalcone at the various time periods (0, 15, 22 and 29 days). In order to distinguish the two possible types of enzymatic conversions, cyanide was added since it is known[4] that this ion inhibits polyphenol oxydase (PPO) activity.

Therefore, the relative extent of CHFI conversion versus that for PPO activity could be readily demonstrated. For this study, 2',4,4'-trihydroxychalcone (1) was utilized as substrate. Table 2 summarizes the data in the bioconversion of 1 with an enzyme system derived from 29 day old cells. It is noted in experiment A, in which only the cell produced enzyme system is employed, that 1 is utilized to the extent of 60 % in 3 hour incubation and, as Scheme 1 indicates, products arising from PPO type of activity are produced. We could detect no evidence of CHFI activity, that is, no flavanone products. In experiment B, where H_2O_2 is added as cofactor, there is an increase in the yield of compounds 3 and 4 as well as other unaccounted products but no change in yields of 5 and 7. In similar fashion, addition of commercial horseradish peroxidase (HRP, Experiment C) increases the yield of 3 and 4 and, in particular, a substancial increase in yields of other unaccounted products. Experiment D confirms the data concerning the overall effects

Table 2

Expt	Time of reaction (hours)	% Chalcone (1) converted*	% of products formed (Scheme 1)			
			3	4	5	7
A	3	60	5	5	15	30
B	3	79	8	9	15	30
C	1	100	8	9	15	30
D	1	100	8	9	15	30

A,B,C,D: see Table 1

* Conversion of 1 was determined by hplc employing 1 as internal standard for purposes of calibration

of H_2O_2 and HRP in the bioconversion of 1. In summary, the known flavone 5[5] and novel compound 7 are truly products resulting from cell-produced enzymes reacting with substrate 1.

In order to establish the structure of 7, an extensive study involving spectroscopic methods was undertaken. Mass spectrometry (see Experimental) established the molecular formula $C_{30}H_{22}O_8$ and Chart 1 summarizes the most important fragmentations (fragments at m/z 375 and 137) strongly suggesting the α,α'-type of linkage between the flavanone moieties as shown in structure 7.

The doublets at δ 5.95 (2 x 1H, J = 12 Hz) and δ 2.72 (2 x 1H, J = 12 Hz) in the ^{1}H nmr spectrum were attributed to the trans-diaxial C-2 and C-3 protons respectively,

Scheme 1

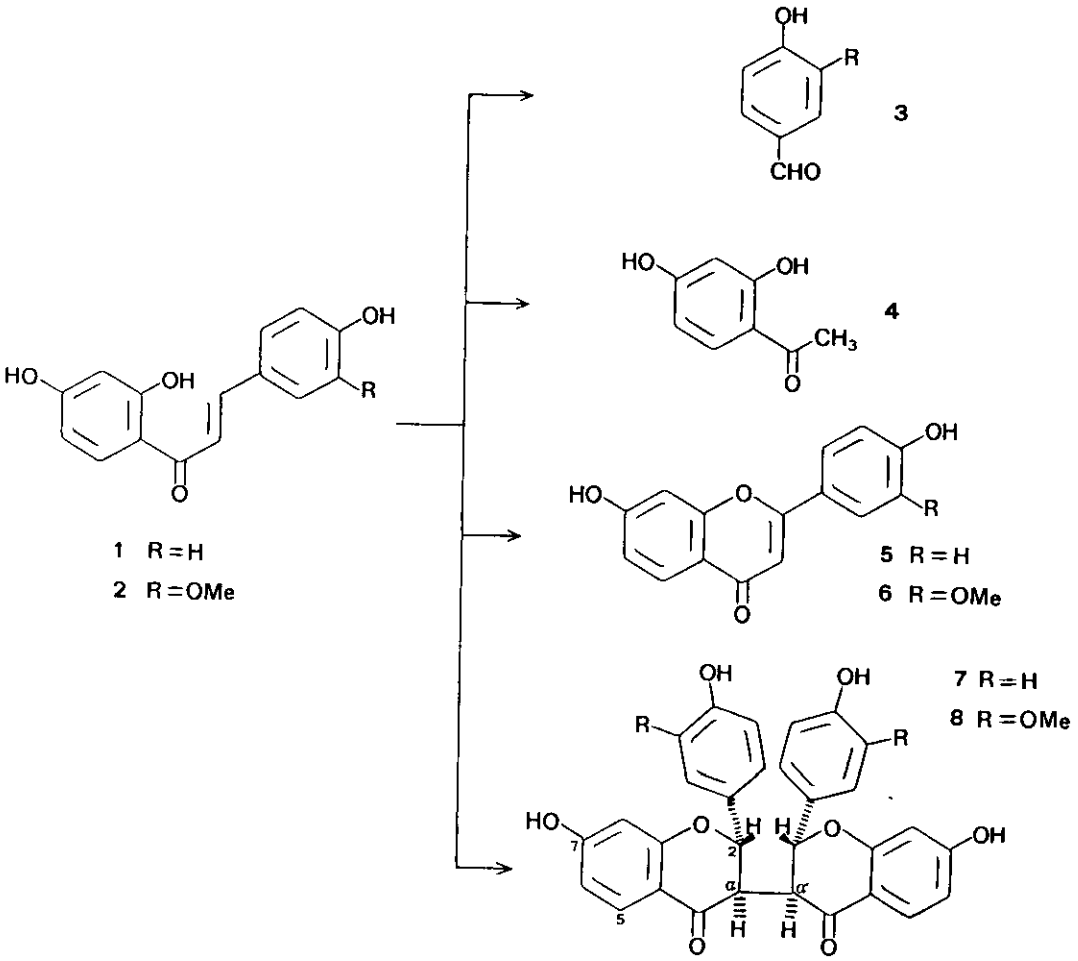
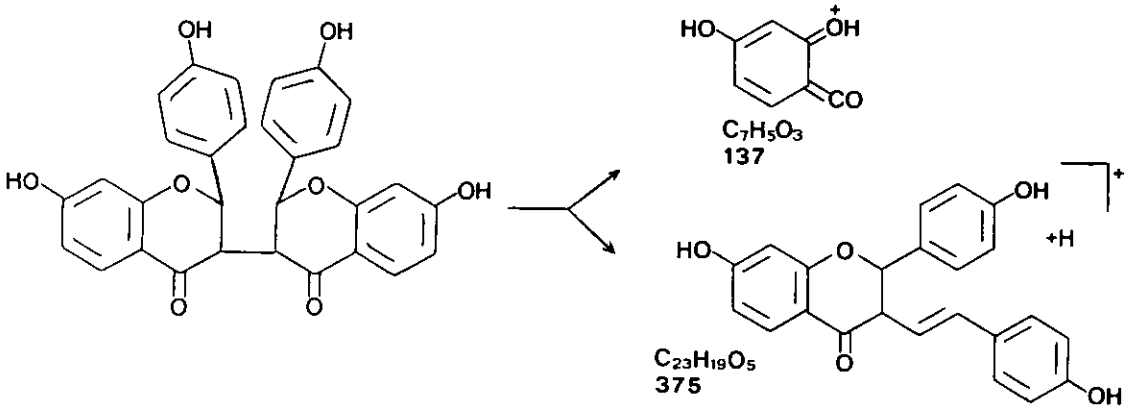
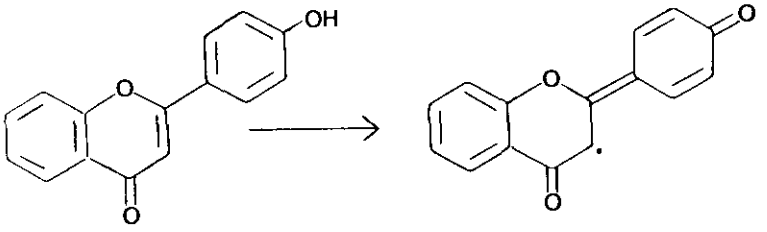


Chart 1



while all the other signals in the aromatic region were coincident with those of liquiritigenin (4',7-dihydroxyflavanone), representing the monomeric unit of 7. These assignments were supported by the ^{13}C nmr data (see Experimental). Since α_D = 0 the biflavanone must be either a racemic or a mesomeric form; only the latter is represented in the structure 7. The mechanistic rational for the enzymatic transformation of 1 to 5 and 7 is of interest. In an earlier study involving phenolic oxydation of flavonoids with potassium ferricyanide, Pelter and coworkers[6] proposed that the chalcone undergoes cyclization to flavone via a radical process and the flavone (9) thus formed (Scheme 2) undergoes conversion to intermediate 10,

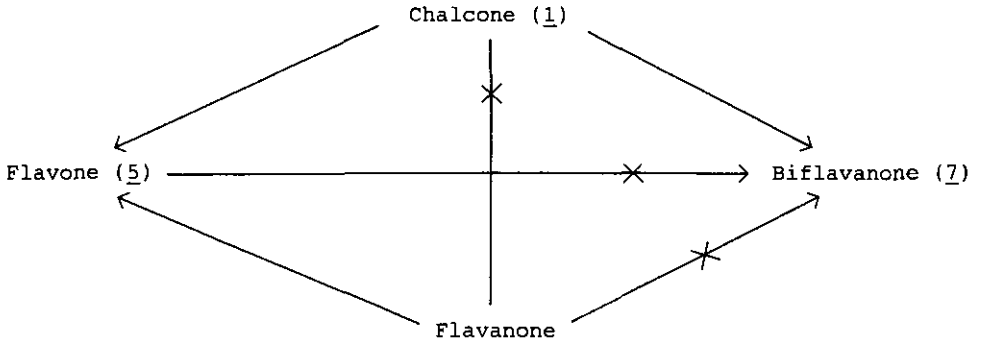
Scheme 2



9 10

which can, in turn, afford biflavanone products. This rational is of direct relevance to the enzyme-catalyzed conversion of 1 to 7. Various possible routes to 7 can be considered (Scheme 3) and these were evaluated with our cell free extract.

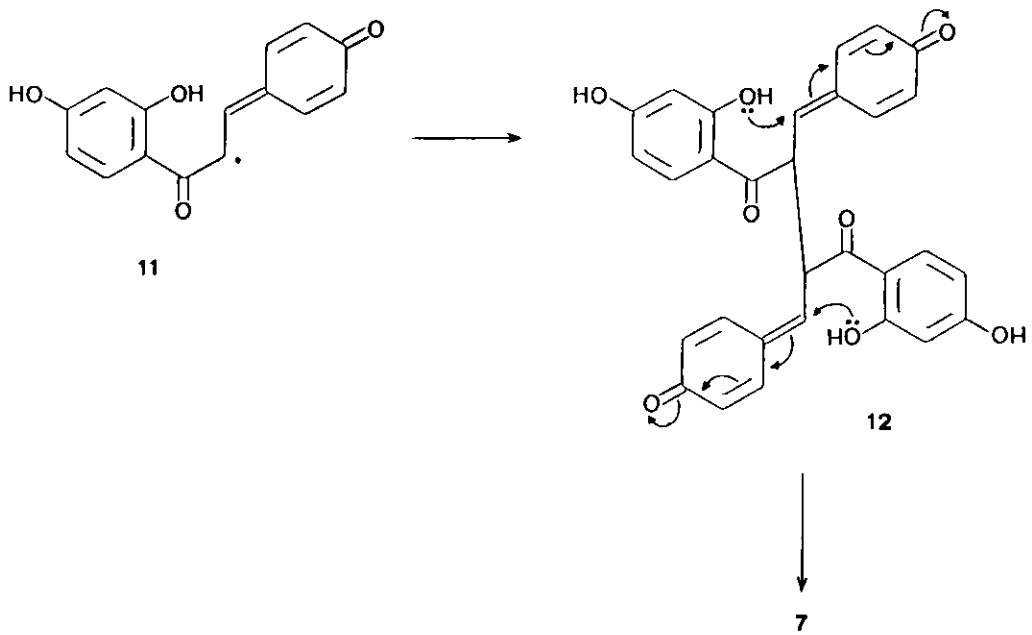
Scheme 3



The above results have revealed that the chalcone substrate 1 does not provide flavanone products with the cell free extract exhibiting PPO type of activity. Furthermore when liquiritigenin (4',7-dihydroxyflavanone) was incubated with our cell free extract derived from the 29 day old cultures, the biflavanone product 7 was not formed. This result thereby excludes a flavanone intermediate as a precursor to 7. When the flavone 5 was similarly incubated with the cell free extract, 7 was not formed. It is therefore clear that the chalcone 1 proceeds via two independent pathways to 5 and 7. Therefore the enzyme-catalyzed process to 7 is not in accord with the mechanistic pathway proposed by Pelter in his chemical studies.[6] On the other hand, the enzymatic conversion of 1 to 5 could well correspond to the Pelter mechanism for flavone formation.[6]

As a result of our studies, we propose that the PPO-type of enzymes initially convert 1 to a p-quinonemethide radical intermediate 11 (Scheme 4) which then dimerizes to 12 and the latter undergoes cyclization to 7. The cyclization of 12 to 7 could involve either a two-electron conjugate addition, as shown, or a one-electron process since our results do not bear direct evidence on either of these alternatives.

Scheme 4



In order to provide further data about the possible generality of the above

process, we investigated the enzyme-catalyzed conversion of substrate 2, possessing an additional methoxyl group in ring B. Table 3 summarizes the results.

Table 3

Expt	Time of reaction (hours)	% Chalcone (2) converted*	% of products formed (Scheme 1)			
			3 (R=OCH$_3$)	4	6	8
A	1	100	10	10	20	45
B	1	100	15	15	20	45
C	1	100	15	15	20	45
D	1	100	15	15	20	45

A, B, C, D: see Table 1

* Conversion of 2 was determined by hplc employing 2 as internal standard for purposes of calibration.

In this instance, substrate 2 is more rapidly consumed (1 H versus 3 h for 1) and the yields of the known flavone 6[7] and the biflavanone 8 are higher. Again H_2O_2 and HRP play no role in affording conversions of 2 to 6 and 8 but simply alter the yields of 3 (R = OCH_3) and 4 presumably via a retro-aldol like process. It is also clear that the account for products [3 (R = OCH_3), 4, 6, and 8] comprises 85 % - 95 % of the final product mixture so that "other" products (for example, 45 % in Experiment A, Table 2) are minimized in the bioconversion of 2 when compared to 1. In summary, the cell free extract isolated from 29 day old cultures does not appear to be of the "peroxidase" type since H_2O_2 is not involved in the bioconversions. Further studies on the nature of these enzymes are underway.

EXPERIMENTAL

Cell suspension cultures

Growth, optimization of the suspended cell cultures and cell free extraction (C.F.E.) procedure are reported in the accompanying publication.

Assays and Polyphenol-oxidases:

1.2 μM of guaiacol were added to the following solutions:

A) C.F.E. (1 mg/ml): B) C.F.E. (1 mg/ml) and H_2O_2 (0.2 μM); C) C.F.E. (1 mg/ml) and horseradish peroxidase (HRP, 0.01 μM); D) C.F.E. (1 mg/ml), H_2O_2 (0.2 μM) and HRP (0.01 μM); E) H_2O_2 (0.2 μM); F) HRP (0.01 μM); G) H_2O_2 (0.2 μM) and HRP (0.01 μM)

To all the solutions 1 ml of tris-HCl buffer was added.

The pH was kept in a range of 7.7-8.0 and the temperature at 37 °C.

The enzymatic reaction was followed by hplc, checking with uv detector at 275 nm every 10 min for 120 min. The conversion of guaiacol was determined by hplc, employing guaiacol as internal standard for purposes of calibration.

The experiments E, F and G were considered as blanks. All the above experiments were performed to the C.F.E. of 0, 15, 22 and 29 day old cultures.

C.F.E. assays. Standard method

Assays were done in tris-HCl (pH 7.7-8.0, 50 mM) at 37 °C.

For incubations, enzyme preparations (1 mg of proteins / 1 ml) was diluted to 2 ml with buffer and added 1 mg of chalcones (1 and 2) dissolved in 2-methoxyethanol (0.2 ml). The enzyme activity was not dependent on cofactors. The conversion of chalcones was monitored by C-18 reverse phase hplc, checking with uv detector at the maximum of absorbance for each chalcone. The percentage of the biotransformed chalcones was monitored by hplc and calculated by using the internal standard (chalcones 1 and 2) depending on the experiments. The eluting system used was MeOH/H_2O (65/35) enriched with 1% of TFA and the flow rate was 1 ml / min. Large scale experiment was carried out with 120 mg of substrate. Solvent, C.F.E. and buffer were added proportionally to standard method. The reaction mixtures were purified by chromatography on silica gel columns with various solvent systems.

Di-(7,4'-dihydroxyflavanon-3-yl), 7; $C_{30}H_{22}O_8$, oil, α_D=0°; uv: λ_{max}^{MeOH} nm: 232, 278, 314; λ_{max}^{NaOAc} nm: 250 sh., 282, 325 sh, 334; λ_{max}^{NaOMe} nm: 250, 300 sh, 325 sh, 334; ^1H nmr (Me_2CO-d_6): δ 7.73 (2 x 1H, d, J=9 Hz, H-5), 6.97 (2 x 2H, d, J=9 Hz, H-2', H-6'), 6.80 (2 x 2H, d, J=9 Hz, H-3', H-5'), 6.51 (2 x 1H, dd, J=9 and 2.2 Hz,H-6), 6.31 (2 x 1H, d, J=2.2 Hz, H-8), 5.95 (2 x 1H, d, J=12 Hz, H-2), 2.72 (2 x 1H, d, J=12 Hz, H-3). ^{13}C nmr (Me_2CO-d_6): δ 191.62 (C-4), 165.38 (C-7), 164.19 (C-9), 159.05 (C-4'), 129.81 (C-5), 129.08 (C-1'), 130.10 (C-2', C-6'), 116.19 (C-3', C-5'), 111.45 (C-10), 111.24 (C-6), 103.35 (C-8), 85.00 (C-2), 51.80 (C-3); FAB-MS, m/z (%): 511 $[M+H]^+$ (40), 483 $[M-CO+H]^+$ (5), 375 $[C_{23}H_{18}O_5]^+$ (19), 256 $[C_{15}H_{12}O_4]^+$ (60), 255 $[M/2]^+$ (72), 137 $[C_7H_5O_3]^+$ (100)

Di-(7,4'-dihydroxy-3'-methoxyflavanon-3-yl), 8; $C_{32}H_{26}O_{10}$, oil, α_D=0°; uv: λ_{max}^{MeOH} nm: 278.9, 373.3; λ_{max}^{NaOAc} nm: 279.9, 331.9, 345 sh; ^1H nmr (Me_2CO-d_6): δ 7.80 (2 x 1H, d, J = 9 Hz, H-5), 6.88 (2 x 1H, d, J = 9 Hz, H-5'), 6.79 (2 x 1H, dd, J = 2.1 and 9 Hz, H-6'), 6.62 (2 x 1H, dd, J = 2.1 and 9 Hz, H-6), 6.34 (2 x 1H, d, J = 2.1 Hz, H-8), 6.30 (2 x 1H, d, J = 2.1 Hz, H-2'), 5.94 (2 x 1H, d, J = 12 Hz, H-2),

3.71 Hz (2 x 3H, s, OCH_3), 2.74 (2 x 1H, d, J = 12 Hz, H-3); ^{13}C nmr (Me_2CO-d_6): δ 191.66 (C-4), 165.32 (C-7), 164.08 (C-9), 148.47, 148.25 (C-3', C-4'), 129.91 C-1'), 129.78 (C-5), 121.66 (C-6'), 115.21 (C-5'), 111.44 (C-10), 111.19 (C-6), 110.80 (C-2'), 103.39 (C-8), 85.24 (C-2), 55.88 (OMe), 52.01 (C-3); FAB-MS m/z (%): 571 $[M+H]^+$, 543 $[M-CO+H]^+$, 434 $[C_{25}H_{21}O_7+H]^+$, 285 $[M/2]^+$, 137 $[C_7H_5O_3]^+$

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