PEROXIDASE FROM CELL CULTURES OF *CASSIA DIDYMOBOTRYA*: A REVIEW AND COMPARISON WITH HORSERADISH PEROXIDASE[1]

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Abstract – The catalytic activity on flavonoids and dibenzylbutanolides and the specificity of the peroxidase isolated from 29d-old cell cultures of *Cassia didymobotrya* are reported. Studies on several substrates in comparison with horseradish peroxidase revealed peculiar activities and suggested the enzyme to be involved in the lignification process.

Peroxidases are ubiquitous in nature and are associated with diverse and crucial biological roles.[2] Hemoglobin, cytochromes P-450 and peroxidases all contain protoporphyrin IX-heme as the prosthetic group; however, their function and reactivities are quite different. Hemoglobin is the oxygen transporting protein in mammals, whereas the cytochromes P-450 are enzymes involved in a series of reactions (hydroxylation, epoxidation, phenol oxidative coupling etc.).[3]

Among peroxidases some, like ascorbate peroxidase, may act as H_2O_2 scavengers, whereas others, such as horseradish peroxidase (HRP), catalyse free-radical oligomerizations and polimerizations of electron rich aromatics.[4] Peroxidases are also involved in lignin biosynthesis, in plant-defense mechanism and plant-hormone regulation.[5]

Plant tissue-cultures provide a powerful tool to investigate the functions, the activity and the specificity of such enzymes.

Cell cultures of *Cassia didymobotrya* were shown, in fact, to provide a cell-free extract containing peroxidase enzymes.[6] A set of experiments, with guaiacol as a marker, allowed to establish that the highest peroxidase activity was confined in 29 day old cells.[6]

In this paper, first, we report some results obtained when peroxidase enzymes derived from 29 day old cell of *Cassia didymobotrya* were used to catalyse the biotransformation of different substituted chalcones and dibenzylbutanolides as precursors. Secondly, we compare the above results with those obtained when the same precursors were treated with HRP.

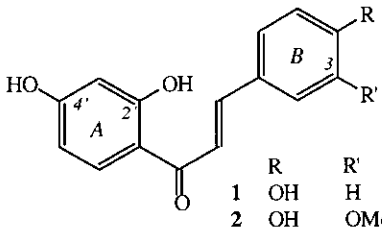
On consideration of the presence of flavonoids both in the mother plant and suspended cells, as well as of the prominent role of chalcones in flavonoid biogenesis, our early studies were focused on the biotransformation of a number of synthetic chalcones.[6,7]

When 4,2',4'-trihydroxychalcone (**1**) was incubated (1 mg of substrate *vs* 1 mg/mL of enzyme, Tris-HCl buffer (50 mM, pH 7.7-8.0 T = 37°C) with the peroxidase mixture derived from the above cell cultures, the products (**1a**) (18%), (**1b**) (12%) and (**1c**) (15%) were obtained after 1 h of incubation.

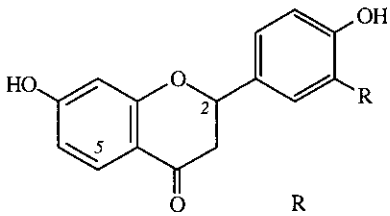
The two biflavanones (**1a**) and (**1b**) were assigned the racemate and the *meso* form, respectively, as determined by ^{1}H NMR studies with a chiral shift reagent of the corresponding methyl ethers.[8]

In order to verify whether the chalcone (**1**) was the direct precursors of the biflavanones (**1a/1b**), the flavone (**1c**) and the synthetic flavanone (**1d**) were incubated separately with the cell free extract. Since both the substrates were recovered without any conversion, it was clear that the chalcone (**1**) proceeds *via* two independent pathways to **1a/1b** and **1c**. The mechanism reported in Scheme 1 was thus proposed for the conversion to biflavanones: the enzyme initially converts **1** to a *p*-quinone methide radical intermediate (**1e**), which then dimerizes to **1f**, and the latter, finally, undergoes cyclization to **1a/1b**. Conversely, the enzymatic biotransformation of **1** to **1c** could well correspond to the Pelter mechanism for flavone formation.[9] In order to provide further data about the possible generality of the above process, we investigated the enzyme-catalysed conversion of substrate (**2**), possessing an additional methoxy group in ring B. Chalcone (**2**) gave the corresponding products (**2a-2d**) in comparable yields.[6]

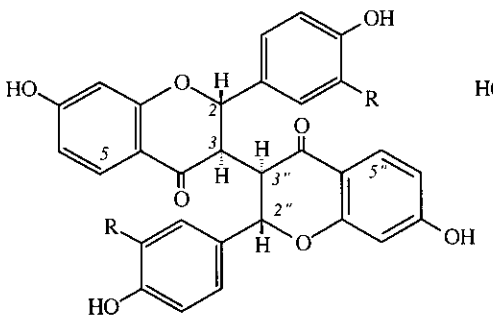
Afterwards, the studies were focused on the substrate specificity using chalcones possessing a free hydroxy group a C-2.[10] The substrate (2,2',4'-trihydroxychalcone) (**3**) gave, after 8 h incubation with the peroxidase derived from 29-d old cells of *Cassia didymobotrya*, in the presence of H_2O_2 as cofactor, the aurones (**3a**) (*Z*-form, 26%) and (**3b**) (*E*-form, likely an artefact, 10%), the auronol (**3c**) (25%) and the racemic flavanone (**3d**) (8%).[7] The auronol (**3c**) was shown to arise from further biotransformation of **3a**. In fact, when the aurone (**3a**) was re-incubated with the cell-free extract, conversion to **3c** occurred. While the mechanism of enzymatic conversion of aurone (**3a**) to auronol (**3c**) is not clear, the formation of the aurone (**3a**) can be explained by a free radical mechanism (Scheme 2) when the "enzymatic activation" promoted by cell-free extract occurs *via* the *o*-quinone methides (**3e**) and (**3f**). The latter is expected to



	R	R'
1	OH	H
2	OH	OMe
5	H	OH

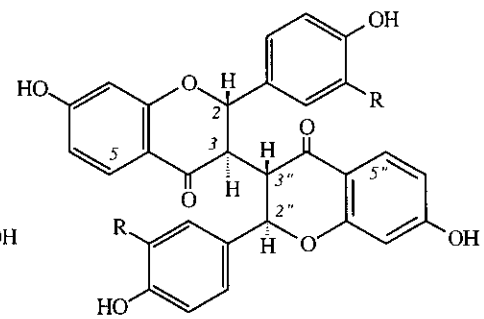


	R
1d	H
2d	OMe

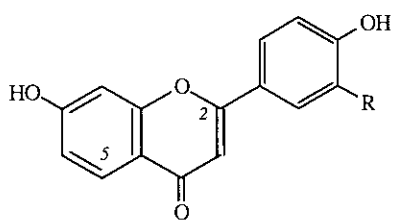


	R
1a	H
2a	OMe

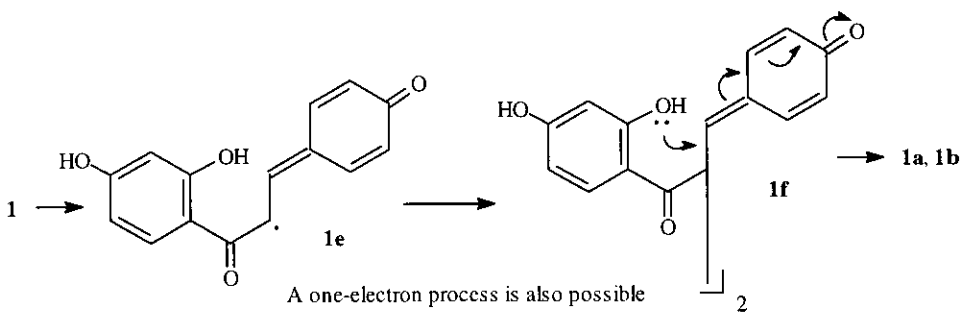
only one enantiomer represented



	R
1b	H
2b	OMe



	R
1c	H
2c	OMe



Scheme 1. Biotransformation products of 4-hydroxychalcones and proposed mechanism

undergo, as a possible reaction mode, the cyclization by a 5-*exo* ring closure, to yield **3a**. Notably, the same intermediate (**3e**) may give the flavanone (**3d**), *via* 6-*endo* closure.

When the chalcone (**4**) was used as a substrate, compounds (**4a-4d**), analogous to those obtained from **3**, were formed, very likely by the same mechanism, unaffected by the presence of the 4-OMe substituent.

The importance of the nature and the position of the C-2-hydroxyl group for the specificity of the enzyme was also stressed by the finding that no biotransformation occurred when chalcones (**5**) and (**6**), possessing in the B ring C-2-methoxy and C-3-hydroxyl groups, respectively, were treated with the enzymatic mixture. Obviously, radical formation (for example **3e**, Scheme 2) is not possible and thus the corresponding cyclization does not occur. The free-radical mechanism was also supported by the loss of activity of the enzyme in the presence of ascorbic acid.

The next step was to compare the activity of the peroxidase from cell cultures of *Cassia didymobotrya* (CDP-29 since now, for simplicity) with that of horse radish peroxidase (HRP, type VI, Sigma).

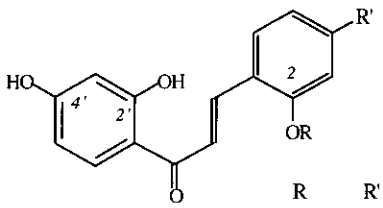
A series of experiments (details in the EXPERIMENTAL) with the chalcones (**1-6**) revealed that HRP possessed a similar catalytic activity *vs* compounds (**1**) and (**2**), giving the same products as CDP-29 with a comparable yield, except the flavanones (**1d**) and (**2d**) (*ca.* 5%). As expected, chalcones (**5**) and (**6**) gave no reaction with HRP. By contrast, when chalcones (**3**) and (**4**) were treated with HRP, neither the starting precursors could be recovered, nor any conversion product could be isolated after 3 h.

It must be remembered that 4,2',4'-trihydroxychalcone was reported to give different product by treatment with HRP,[8] but in different conditions (temperature, concentrations of enzyme and substrate).

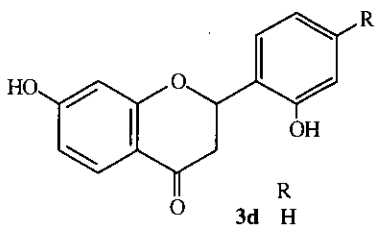
Following our studies on the applicability of CDP-29 to exogenous substrates, the enzyme-system was applied to dibenzylbutanolides, structural analogues of the late stage intermediate proposed by Dewick and coworkers[12] in the biosynthetic pathway leading to podophyllotoxins. The cyclization of such precursors to aryltetralin lignans is a process, which is expected to involve radical intermediates, usually generated by peroxidase enzymes.[13,14] The precursors (**7-9**), synthesized according to the published procedures,[15,16] had all the prerequisites to give the *p*-quinone methide intermediates (like **7g**).

After 30 min of incubation at 37°C, **7** was completely biotransformed. As usual, a control experiment was also performed in order to determine if H_2O_2, in the absence of our peroxidase system, was able to transform **7** under the conditions used in the biotransformation: extraction of the reaction mixture gave a 95% recovery of material consisting exclusively of **7**.

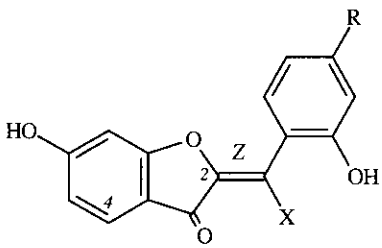
From the biotransformation mixture with CDP-29, three products were isolated and assigned the structures (**7a-7c**).[17] With the aim to shed some light on the biosynthetic relationship, if any, between the three products isolated from the biotransformation of **7**, the compounds (**7a**, **7b** and **7c**) were incubated in separate experiments, with our peroxidase system under the same conditions as those used for the conversion of **7**. After 24 h of incubation, **7a** was not biotransformed at all, whereas **7c** was degraded into



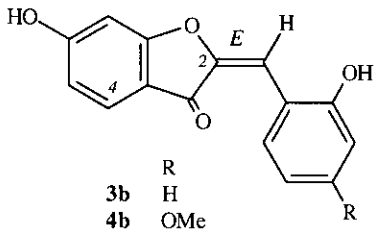
	R	R'
3	H	H
4	H	OMe
6	Me	H



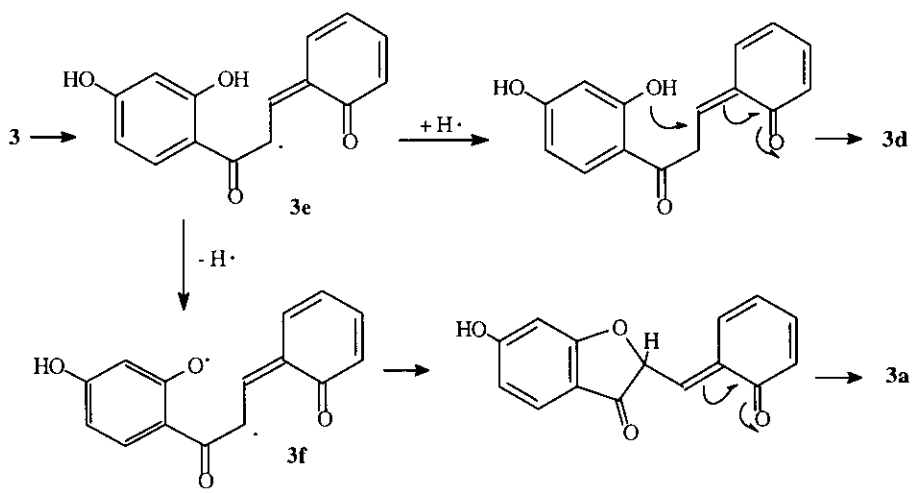
	R
3d	H
4d	OMe



	R	X
3a	H	H
3c	H	OH
4a	OMe	H
4c	OMe	OH



	R
3b	H
4b	OMe



Scheme 2. Biotransformation products of 2-hydroxychalcones and proposed mechanism

non-isolable products when a larger reaction time was employed. Compound (**7b**) was converted, after 30 min of incubation, to **7c**, very likely, *via* an intermolecular nucleophilic addition (Michael type) of the C-7' hydroxyl group to the activated double bond at C-2 - C-7". The above results suggested that the key intermediate in the biotransformation process of precursor (**7**) is **7d** and the latter, *via* plausible pathways[17] allows the formation of **7a** and **7b** to **7c**. On the other hand, treatment of precursors (**8**) and (**9**) with CDP-29 gave only the cyclized products (**8a**) (62 %) and (**9a**) (75 %), respectively.

In order to provide further proof for the free radical mechanism, the importance of the two phenolic groups (in C-3' and C-4") within the dibenzylbutanolide system was investigated. After 24 h incubation of the methyl derivatives (**10**) and (**11**) with CDP-29 unreacted starting materials were completely recovered. Conversely, the butanolide (**12**) bearing a methylenedioxy function in ring A, but a free 4'-hydroxyl group in ring , was incubated to our enzyme-system, and did not give any cyclization product but was converted by the enzyme mixture into **12b** and **12c**.[17] Notably, the ketone (**13**), an intermediate in the synthesis of **11**, gave with CDP-29 exclusively the uncyclized compound (**13b**) (50%).

The substrates (**7**,**8** and **9**) treated with HRP (Experimental) gave only the cyclized corresponding compounds (**7a**) (15%), (**8a**) (15%) and (**9a**) (20%), not only in yields lower than those with CDP-29, but also after 24 h incubation. These results are in agreement with those obtained by Kutney and coworkers with similar substrates.[15] Moreover, both **12** and **13** gave no conversion product with HRP, confirming that the dehydrogenation reaction to **12b** and **13b** is peculiar of CDP-29.

Various natural compounds were also incubated with both CDP and HRP (Table 1). The highest activity of CDP-29 was expressed towards coniferyl alcohol and ferulic acid, whereas no activity was expressed towards hydroquinones, in which CDP-29 markedly differs from HRP.[8]

The peroxidase from cell cultures of *Cassia didymobotrya* is characterized by a high specificity towards natural compounds belonging to the lignin formation pathway and, notably, bearing a ferulic type substitution on the aromatic ring, like chalcone (**2**).

These findings suggest that the enzyme is involved in lignification processes of the cell wall.

By purification the peroxidase was shown to be a glycoprotein with a pI 3.5 and a molecular mass approx. 43 kDa by SDS/PAGE and 50 kDa by gel filtration. The *N*-terminal sequence was very similar to those of other plant peroxidases.[8]

Preliminary results showed that the purified isoenzyme was still able to catalyze the biotranformation of chalcones (**1**) and (**2**) exclusively to the corresponding biflavanones (**1a**) and (**2a**).

ACKNOWLEDGEMENTS

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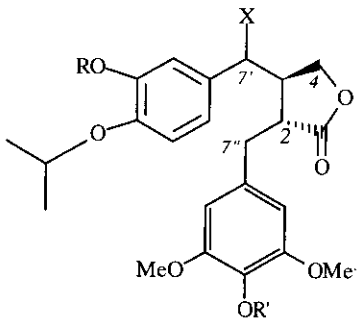
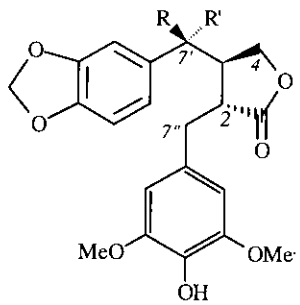
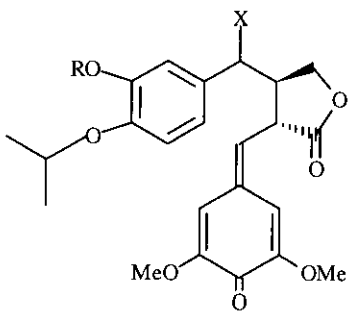


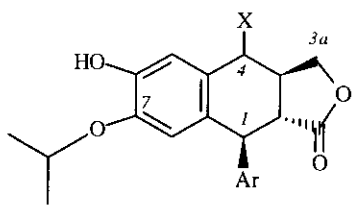
	R	R'	X
7	H	H	◢OH
8	H	H	⋯OH
9	H	H	H
10	H	Me	◢OH
11	Me	Me	◢OH



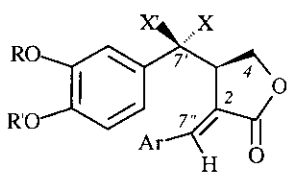
	R	R'
12	H	OH
13	=O	



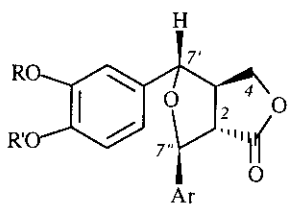
7d



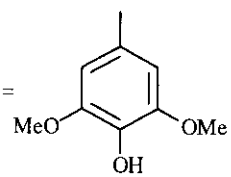
	X
7a	◢OH
8a	⋯OH
9a	H



	X	X'	R	R'
7b	H	OH	H	
12b	OH	H	-CH 2-	
13b	=O		-CH 2-	



	R	R'
7c	H	
12c	-CH 2-	

Ar =  throughout

Scheme 3. Biotransformation products of dibenzylbutanolides and possible intermediate

Table 1. Summary of experiments and conversion products obtained by catalysis of HRP and CDP-29

Substrate	Products*	
	HRP	CDP
1	**1a, 1b, 1c, 1d**	**1a, 1b, 1c**
2	**2a, 2b, 2c, 2d**	**2a, 2b, 2c**
3	n. i.	**3a, 3b, 3c, 3d**
4	n. i.	**4a, 4b, 4c, 4d**
5, 6	-	-
7	**7a**	**7a, 7b, 7c**
8	**8a**	**8a**
9	**9a**	**9a**
10, 11	-	-
12	-	**12b**
13	-	**13b**
Guaiacol	n. i.	n. i.
Coniferyl alcohol	n. i.	n. i.
Ferulic acid	n. i.	n. i.
Caffeic acid	n. i.	n. i.
Coumaric acid	-	n. i.
Vanillin	n. i.	n. i.
Ascorbic acid	n. i.	n. i.
NADH	n. i.	n. i.
Tyrosine	-	n. i.

*HRP was less active with substrates (1-4, 8 and 9), whereas CDP-29 was less active with caffeic acid and vanillin. In the other cases the two enzymes showed a comparable activity. n.i. the products were not identified

EXPERIMENTAL

Biotransformation of chalcones with HRP

A set of experiments as indicated below were performed

Exp.	Chalcone (mg)	HRP (μL)	3% H_2O_2 (μL)	Time (hr)
1	1	400	400	1
2	1	400	400	4
3	1	400	400	8
4	1	400	400	24
5	1	400	-	24
6	1	-	400	24
7	1	200	200	8
8	1	200	200	24
9	1	100	100	4
10	1	100	100	24
11	2	400	400	24

The chalcone was dissolved in MeOH/H_2O, 1:1 (2 mg /mL). HRP was diluted 1 to 100 with Tris buffer (pH 7.4). The mixture was diluted to 20 mL with Tris buffer (pH 7.4) and kept at 37 °C for the time indicated. The reaction was sospended by addition of ethyl acetate and extraction (x3) with the same solvent. The residues were analyzed on TLC silica gel plates, adequately pooled and purified by prep TLC (with CH_2Cl_2-EtOAc-MeOH mixtures, mainly 90:7:3).

Biotransformation of substrates (8, 9 and 13) by CDP-29

Butanolides (2 mg) were incubated at 37 °C with CDP-29 (10 mL, 5.6 U/mL), Tris HCl buffer (5 mL, pH 7.5) and H_2O_2 12.3 mM (120 μL). After 30 min the reaction mixture was extracted with EtOAc. The pooled extracts of several small-scale biotransformations gave by column chromatography on silica gel (with hexane/EtOAc/MeOH, 50:47:3) the products: **8a** (62%), **9a** (74%) and **13b** (50%).

1-(4-Hydroxy-3,5-dimethoxyphenyl)-4β,6-dihydroxy-3-hydroxymethyl-7-isopropyloxy-1,2,3,4-tetrahydro-2-naphthoic acid γ -lactone (**8a**): mp 245-246 °C; ^{1}H NMR (Me_2CO-d_6): δ 7.43, 7.06 (1H each, s, exchanged by D_2O, 6-OH, 4'-OH), 7.14 (1H, s, H-5), 6.55 (2H, H-2", H-6"), 6.39 (1H, s, H-8), 4.94 (1H, dd, J = 10 and 7.5 Hz, H-4), 4.74 (1H, d, J = 7.5 Hz), 4.57 (1H, dd, J = 8.5 and 7 Hz, H-3aa), 4.27 (1H, m, J = 6 Hz, OCH), 4.16 (1H, dd, J = 10 and 8.5 Hz, H-3ab), 4.06 (1H, d, J = 11 Hz, H-1), 3.75 (6H, s, 2 x OMe), 2.95 (1H, dd, J = 14 and 11 Hz, H-2), 2.60 (1H, dtd, J = 14, 10 and 7 Hz, H-3), 1.19, 1.09 (3H each, d, J = 6 Hz, 2 x Me); ^{13}C NMR (Me_2CO-d_6): δ 175.59 (s, CO), 148.50 (s, C-3', C-5'), 147.30 (s, C-7), 145.08 (s, C-6), 135.59, 135.22 (s each, C-1', C-4'), 134.91, 131.69 (s each, C-9, C-10), 116.65 (d, C-8), 113.36 (d, C-5), 108.03 (d, C-2', C-6'), 71.83, 71.66 (d each, C-4, OCH), 70.46 (t, CH_2O), 56.65 (q, OMe), 48.31, 47.36 (d each, C-1, C-2), 46.46 (d, C-3), 22.09, 21.78 (q each, 2 x Me); MS *m/z* (rel. int.): 430 (85) $[M]^+$, 388 (100) $[M - C_3H_6]^+$, 273 (19), 167 (5), 154 (76) [ring B]$^+$, 139 (6).

1-(4-Hydroxy-3,5-dimethoxyphenyl)-6-hydroxy-3-hydroxymethyl-7-isopropyloxy-1,2,3,4-tetrahydro-2-naphthoic acid γ -lactone (**9a**): mp 210-211 °C; ^{1}H NMR (Me_2CO-d_6): δ 7.40, 7.06 (1H each, s, exchanged by D_2O, 6-OH, 4'-OH), 6.63 (1H, s, H-5), 6.56 (2H, s, H-2", H-6"), 6.41 (1H, s, H-8), 4.49 (1H, dd, J = 8.5 and 6.5 Hz, H-3aa), 4.24 (1H, m, J = 6 Hz, OCH), 4.02 (1H, dd, J = 10.5 and 8.5 Hz, H-3ab), 4.01 (1H, d, J = 11 Hz, H-1), 3.76 (6H, s, 2 x OMe), 2.93 (2H, br d, J = 8.5 Hz, H_2-4), 2.77 (1H, dd, J = 14 and 11 Hz, H-2), 2.60 (1H, ddtd, J = 14, 10.5, 8.5 and 6.5 Hz, H-3), 1.18, 1.08 (3H each, d, J = 6Hz, 2 x Me); ^{13}C NMR (Me_2CO-d_6): δ 176.12 (s, CO), 148.48 (s, C-3', C-5'), 147.14 (s, C-7), 144.51 (s, C-6), 135.56, 135.41 (s each, C-1", C-4"), 131.66, 129.16 (s each, C-9, C-10), 117.53 (d, C-5), 115.96 (d, C-8), 108.07 (d, C-2', C-6'), 71.93 (d, OCH), 71.47 (t, CH_2O), 56.64 (q, OMe), 48.56, 47.05 (d each, C-1, C-2), 40.90 (d, C-3), 32.70 (t, C-4), 22.10, 21.77 (q each, 2 x Me); MS *m/z* (rel. int.): 414 (96) $[M]^+$, 372 (100) $[M - C_3H_6]^+$, 257 (4), 167 (11), 154 (59) [ring B]$^+$, 139 (5), 123 (4).

(E)-2,2'-(4-Hydroxy-3,,5-dimethoxybenzal)-3-(3,4-methylenedioxybenzoyl)butanolide (**13b**): amorphous solid; ^{1}H NMR ($CDCl_3$): δ 7.73 (1H, d, J = 2 Hz, H-7"), 7.54 (1H, dd, J = 8 and 2 Hz, H-6'), 7.39 (1H, d, J = 2 Hz, H-2'), 6.92 (1H, d, J = 8 Hz, H-5'), 6.56 (2H, s, H-2", H-6"), 6.11, 6.10 (1H each, d, J = 1.2 Hz, OCH_2O), 5.13 (1H, ddd, J = 9.5, 4.5 and 2 Hz, H-3), 4.74 (1H, t, J = 9.5 Hz, H-3aa), 4.39 (1H, dd, J = 9.5 and 4.5 Hz, H-3ab), 3.64 (6H, s, 2 x OMe); ^{13}C NMR ($CDCl_3$): δ 195.31 (s, CO), 172.19 (s, OCO), 148.93, 147.02 (s each, C-3', C-4'), 144.50 (s, C-3", C-5"), 140.53 (d, C-7"), 129.52, 128.14 (s each, C-1", C-4"), 125.10 (s, C-1'), 124.73 (d, C-6'), 120.44 (s, C-2), 108.28, 108.21 (s each, C-2', C-5'), 107.05

(d, C-2", C-6"), 102.34 (t, OCH_2O), 67.48 (t, C-4), 55.95 (q, OMe), 47.03 (d, C-3); MS *m/z* (rel. int.): 398 (40) $[M]^+$, 349 (8), 231 (9), 167 (36) [ring B]$^+$, 149 (100) [ring A]$^+$, 121 (18) [149 –CO]$^+$.

Biotransformation of dibenzylbutanolides with HRP

A set of experiments as those listed above for chalcones were performed for dibenzylbutanolides, except that 2 mg of substrate (dissolved in 0.5 mL of methoxyethanol) were used in each experiment. Work-up was the same, but an eluent hexane/EtOAc/MeOH, 50:47:3 was used for TLC and prep TLC.

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