STUDIES IN CELL SUSPENSION CULTURES OF *CASSIA DIDYMOBOTRYA*. PART VI.[1] THE BIOTRANSFORMATION OF CHALCONES TO AURONES AND AURONOLS

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Abstract - Cell-free extracts derived from tissue cultures of *Cassia didymobotrya,* which previously had been reported to convert 4-hydroxychalcones to flavones and biflavanones, catalyze the biotransformation of 2-hydroxychalcones to aurones and auronols. The aurone was shown to be the direct precursor of auronol.

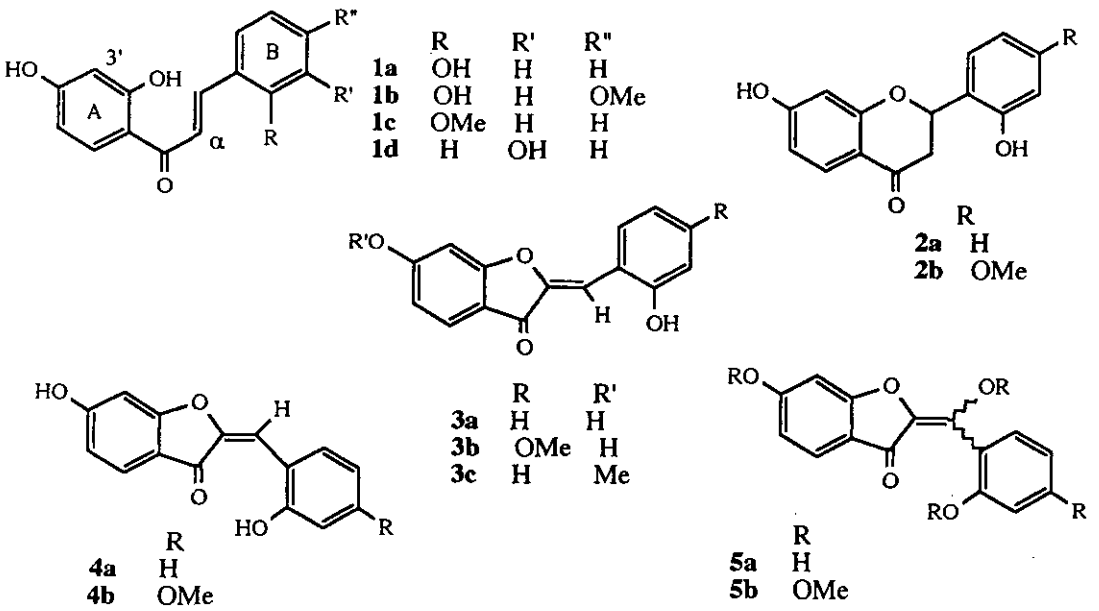
Older (29 days) cell cultures of *Cassia didymobotrya* were shown to provide a cell-free extract containing a polyphenol oxidase (PPO), which can effectively catalyze the conversion of 4-hydroxychalcones to the corresponding flavones and 3,3'- biflavanones.[2]

In this paper we describe our further studies on the substrate specificity of the PPO derived from the above cell cultures. These studies were focused on chalcones possessing a free hydroxyl group at C-2 in ring B. The substrate, 2, 2',4'-trihydroxychalcone (**1a**) gave, after 8 h incubation with the cell-free extract, containing a catalytic amount of H_2O_2 , a mixture of compounds (**2a-5a**, Scheme 1) in a 69% overall yield. The ^1H nmr spectrum of compound (**2a**) (8%), slightly less polar than **1a**, showed the AXY system typical of H-2 and H_2-3 protons of flavanones and was thus identified with 7,2'-dihydroxyflavanone in the racemic (α_D 0) form. The bioconversion to **2a** cannot be attributed to the presence of a chalcone-flavanone isomerase in the cell-free extract, since it was shown previously that this enzyme, obtained from 22-day-old cells, not only affords optically active flavanones but also was not able to biotransform C-2 hydroxylated chalcone (**1a**).[3]

The compounds (**3a**) (26%) and (**4a**) (10%) showed very similar spectral data. In the ^1H nmr spectrum the signals of the aromatic protons showed the same multiplicity as in the starting chalcone (**1a**), but no signal of chelated OH was present. Moreover, only one singlet at δ 7.29 was attributable to an olefinic proton. These data suggest the presence of both a ring and a double bond, still coniugated to the B ring, as confirmed by the chemical shift of the signal attributed in the chalcone to H-6.

Scheme 1

Chalcones studied and their biotransformation products



Accordingly the mass spectra of products (**3a**) and (**4a**) (both showing $[M]^+$ at *m/z* 254) revealed that an oxidation had occurred. Flavones, isoflavones and aurones correspond to the above requirements, but the olefinic proton in flavones occurs at different values (*ca* 6.3 ppm)[4] while the uv spectra of isoflavones do not show absorption bands at *ca* . 380 nm. Conversely, the uv and ^{13}C nmr spectra, the latter compared in Table1 with the spectrum of 2'-hydroxy-6-methoxyaurone (**3c**),[5] were consistent with an aurone skeleton. In particular, compound (**3a**) was assigned the structure (Z)-6, 2'-dihydroxyaurone, because of the highfield value of the H-6' proton (δ 8.23 *vs* δ 8.59 in **4a**) [6] as well as the higher field resonance of the C-β carbon (δ 105.4 *vs* 115.9).[5] Consequently, compound (**4a**), which is formed from **3a** during work-up and purification of the reaction mixture, as shown by tlc, was assigned the structure (E)-6,2'-dihydroxyaurone.

In the ^1H nmr spectrum of the most polar compound (**5a**) (25%) no signal for olefinic protons is present, while the $[M]^+$ in the mass spectrum was shifted to *m/z* 270 (16 mu). Therefore, compound (**5a**) was assigned the structure 6,2',β-trihydroxyaurone.

The structure was confirmed by the formation of a trimethoxy derivative (**5b**) with CH_2N_2. The auronol (**5a**) arises from further biotransformation of **3a.** In fact, when the aurone (**3a**) was incubated with the cell-free extract of 29-day-old cultures, conversion to **5a** occurred.

The formation of the aurone (**3a**) can be explained by a free-radical mechanism (Scheme 2) where the "enzymatic activation" promoted by the cell-free extract occurs *via* radicals (**6a**) and (**7a**). The latter is expected to undergo, as one possible reaction mode, the cyclization by a 5-*exo* ring closure, to yield **3a**. Notably, the same intermediate (**7a**) may give the flavanone (**2a**), *via* 6-*endo* closure.

Table 1. ^{13}C nmr spectral data of aurones*

Position	**3a**	**3c**	**4a**	**5a**
2	148.36	146.81	149.12	147.78
β	105 .37	105.90	115.94	145.53
3	182.57	181.77	179.64	178.84
3a	114.89	114.41	117.86	115.82
4	126.55	24.99	126.70	127.22
5	113.53	111.96	113.26	113.90
6	169.11	167.71	168.42	164.12
7	99.55	96.56	98.75	102.61
7a	166.72	166.87	168.42	159.38
1'	120.45	119.02	120.26	122.36
2'	157.71	157.15	157.11	158.50
3'	116.44	115.60	116.60	119.06
4'	132.25	131.13	132.58	131.26
5'	120.92	119.30	120.20	120.18
6'	131.94	130.93	132.58	128.56

*75 MHz; TMS as int. stand.; solvents: **3a, 4a**: Me_2CO-d_6; **5a**: C_5D_5N; **3c**: $CDCl_3$/DMSO-d_6, 3:1

The mechanism of enzymatic conversion of aurone (**3a**) to auronol (**5a**) is not known. Plausible pathways may involve either direct hydroxylation or initial epoxidation followed by epoxide-ring opening and dehydration.

In order to provide further data about the possible generality of the above process, we investigated the enzyme-catalyzed conversion of substrate (**1b**), possessing an additional methoxy group in ring B.

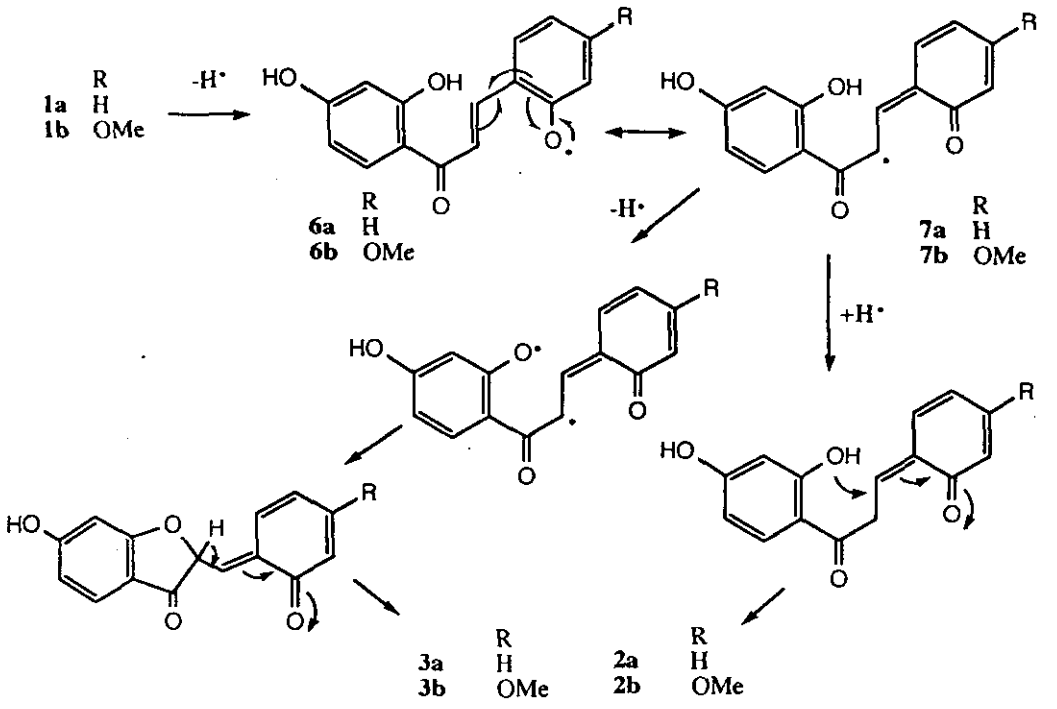
Compounds (**2b-5b**), analogous to those obtained by **1a**, were formed, very likely, by the same mechanism of Scheme 2.

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 (**1c**) and (**1d**), possessing in the B ring C-2-methoxyl and C-3 hydroxyl groups, respectively, were treated with the enzymatic mixture. Obviously, radical formation (for example, **6a** in 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.

In contrast to the C-4 hydroxyl substituted chalcones studied earlier,[2] where cyclization to the products was achieved with a cell-free extract without H_2O_2 as cofactor, the present products can be obtained only when H_2O_2 is added to the crude cell-free extract, thus revealing the active enzyme being very likely a peroxidase.

Scheme 2

Proposed mechanism of formation of **2a** and **3a**



EXPERIMENTAL

Cell suspension cultures

Growth, optimization of cell cultures and cell-free extraction (CFE) procedure are reported in reference 3.

Synthesis of chalcones

Resacetophenone (1 g, 6.5 mmol) and the appropriate benzaldehyde (10 mmol) in MeOH (20 ml), KOH (10 g) and H_2O (10 ml) were held at reflux for 45 min. Standard work-up and purification by silica gel column chromatography gave the following results (elution system and yield): **1a** ($CHCl_3$-MeOH, 98:2; 0.92 g, 55%), mp 185-186 °C; **1b** ($CHCl_3$-MeOH, 98:2; 0.85 g, 45%), vitreous solid; **1c** (CH_2Cl_2-EtOAc, 4:1; 1.6 g, 90%), mp 192-193 °C; **1d** (CH_2Cl_2-EtOAc, 9:1; 1.6 g, 95%), mp 215 °C.

2,2',4'-Trihydroxychalcone (**1a**):

Uv λmax (MeOH) nm (log ε): 208 (4.13), 252 (3.90), 308 (3.92), 368 (4.11); ^{1}H nmr: δ 13.73 (1H, s, 2'-OH), 9.52 (2H, br s, 2-OH, 4'-OH), 8.29 (1H, d, J = 16 Hz, H-α), 8.09 (1H, d, J = 9 Hz, H-6'), 7.98 (1H, d, J = 16 Hz, H-β), 7.84 (1H, dd, J = 8 and 1.5 Hz, H-6), 7.30 (1H, br dt, J = 7.5 and 1.5 Hz, H-4), 7.02 (1H, dd, J = 8 and 1.5 Hz, H-3), 6.93 (1H, br dt, J = 7.5 and 1.5 Hz, H-5), 6.50 (1H, dd, J = 9 and 2.5 Hz, H-5'), 6.40 (1H, d, J = 2.5 Hz, H-3');^{13} C nmr: δ 193.13 (s, C=O), 167.54 (s, C-4'), 165.53 (s, C-2'), 157.96 (s, C-2), 140.38 (d, C-α), 133.20 (d, C-6') 132.74 (d, C-6), 129.92 (d, C-4), 122.77 (d, C-1), 120.90 (d, C-3), 120.79 (d, C-5), 117.06 (d, C-β), 114.46 (s, C-1'), 108.74 (d, C-5'), 103.73 (d, C-3'); EIms *m/z* (rel. int.): 256 $[M]^+$ (7), 238 $[M - H_2O]^+$ (84), 163 [M - ring B]$^+$ (10), 137 [a + H]$^+$ (100), 120 [b]$^+$ (31), 108 (13). Anal. Calcd for $C_{15}H_{12}O_4$: C, 70.29; H, 4.72. Found: C, 70.05; H, 4.95.

2,2',4'-Trihydroxy-4-methoxychalcone (**1b**):

^{1}H Nmr: δ 13.77 (1H, s, 2'-OH), 9.53, 9.47 (1H each, br s, 2-OH, 4'-OH), 8.24 (1H, d, J = 15.5 Hz, H-α), 8.05 (1H, d, J = 9 Hz, H-6'), 7.85 (1H, d, J = 15.5 Hz, H-β), 7.78 (1H, d, J = 8 Hz, H-6), 6.57 (1H, d, J = 2 Hz, H-5), 6.55 (1H, dd, J = 8 and 2 Hz, H-3), 6.48 (1H, dd, J = 9 and 2.5 Hz, H-5'), 6.38 (1H, d, J = 2.5 Hz, H-3'7, 3.81 (3H, s, OMe); ^{13}C nmr: δ 193.13 (s, CO), 167.48 (s, C-4'), 165.37 (s, C-α'), 164.09 (s, C-4), 159.67 (s, C-2), 140.59 (d, C-α), 132.97 (d, C-6'), 131.47 (d, C-6), 118.07 (d, C-β), 116.06 (s, C-1), 114.52 (s, C-1'), 108.58 (d, C-5'), 107.46 (d, C-5), 103.72 (d, C-3'), 102.12 (d, C-3), 55.67 (q, OMe); EIms *m/z* (rel. int.): 286 $[M]^+$ (5), 268 $[M - H_2O]^+$ (96), 163 [M - ring B]$^+$ (12), 150 $[b]^+$ (50), 137 $[a + H]^+$ (100). Anal. Calcd for $C_{16}H_{14}O_5$: C, 67.11; H,4.93. Found: C, 66.95; H, 5.04.

2',4'-Dihydroxy-4-methoxychalcone (**1c**):

^{1}H Nmr: δ 13.57 (1H, s, 2'-OH), 9.61 (1H, br s, 4'-OH), 8.24 (1H, d, J = 16 Hz, H-α), 8.11 (1H, d, J = 9Hz, H-6'), 7.94 (1H, d, J = 16 Hz, H-β), 7.90 (1H, br d, J = 7.5 Hz, H-6), 7.45 (1H, br t, J = 8 Hz, H-4), 7.12 (1H, br d, J = 8 Hz, H-3), 7.03 (1H, br t, J = 7.5 Hz, H-5), 6.49 (1H, dd, J = 9 and 2.5 Hz, H-5'), 6.39 (1H, d, J = 2.5 Hz, H-3'), 3.97 (3H, s, OMe); ^{13}C nmr: δ 193.04 (s, CO), 167.62 (s, C-4'), 165.71 (s, C-2'), 159.71 (s, C-2), 139.79 (d, C-α), 133.39 (d, C-6'), 133.02 (d, C-4), 129.67 (d, C-6), 124.38 (s, C-1), 121.55 (d, C-β), 121.50 (d, C-5), 114.49 (s, C-1'), 112.39 (d, C-3), 108.79 (d, C-5'), 103.74 (d, C-3'), 56.08 (q,, OMe). EIms *m/z* (rel. int.): 270 $[M]^+$ (24), 137 $[a + H]^+$ (100), 134 $[b]^+$ (46). Anal. Calcd for $C_{16}H_{14}O_4$: C, 71.09; H, 5.22. Found: C, 71.01; H, 5.33.

3,2',4'-Trihydroxychalcone (**1d**):

^{1}H Nmr: δ 13.48 (1H,s, 2'-0H), 9.60 (2H, br s, 3-OH, 4'-OH), 8.16 (1H, d, J = 8 Hz, H-6'), 7.88 (1H, d, J = 15.5 Hz, H-α), 7.80 (1H, d, J = 15.5 Hz, H-β), 7.33 (1H, dd, J = 8 and 2 Hz, H-6), 7.29 (1H, t, J = 7.5 Hz, H-5), 7.28 (1H, d, J = 2 Hz, H-2), 6.95 (1H, dt, J = 7.5 and 2 Hz, H-4), 6.49 (1H, d, J = 2 Hz, H-3'); ^{13}C nmr: δ 192.77 (s, CO), 167.62 (s, C-4'), 165.85 (s, C-2'), 158.70 (s, C-3), 144..90 (d, C-α), 137.26 (s, C-1), 133.53 (d, C-6'), 130.79 (d, C-5), 121.61, 121.07 (d each, C-2, C-6), 118.59 (d, C-β), 116.03 (d, C-4), 114.42 (s, C-1'), 108.84 (d, C-5'), 103.72 (d, C-3'); EIms *m/z* (rel. int.): 256 $[M]^+$ (12), 163 [M - ring B]$^+$ (14), 137 $[a + H]^+$ (100), 120 $[b]^+$ (19). Anal. Calcd for $C_{15}H_{12}O_4$: C, 70.29; H, 4.72. Found: C, 70.03; H, 5.01.

CFE assays. Standard preparation

Enzyme preparation (1.8 mg of protein/ml) was diluted to 2 ml with 0.1M Tris-HCl (pH 7.7-8.0) at 37 °C and chalcone (1 mg) in 2-methoxyethanol (0.2 ml) and H_2O_2 (2.05 x 10-3 mmol) were added. The conversion of substrate was monitored by reverse-phase (C-18) hplc, with the uv detector fixed at the λmax of each chalcone. The eluting system was MeOH-H_2O (gradient from 60:40 to 90:10 in 22 min with a flow rate of 0.9 ml/min). Large scale experiments were carried out with 50 mg of substrate.

The reaction mixture of (**1a**) by preparative tlc on silica gel with CH_2Cl_2 - EtOAc - MeOH, 90:7:3 as eluant, gave **2a** (4 mg; 8 %; mp 191-192 °C), **3a** (13 mg; 26 %; mp > 320 °C), **4a** (5 mg; 10 %; mp > 320 °C), **5a** (13 mg; 25 %; mp > 320 °C).

7,2'-Dihydroxyflavanone (**2a**):

Uv λmax (MeOH) nm (log ε): 217 (4.13), 276 (3.95), 310 (3.64); ^{1}H nmr: δ 7.77 (1H, d, J =8.5 Hz, H-5), 7.57 (1H, dd, J = 8 and 2 Hz, H-6'), 7.22 (1H, br dt, J = 8 and 2 Hz, H-4'), 6.96 (1H, dd, J = 8 and 1.5 Hz, H-3'), 6.95 (1H, br dt, J = 8 and 1.5 Hz, H-5'), 6.60 (1H, dd, J = 8.5 and 2.5 Hz, H-6), 6.48 (1H, d, J = 2.5 Hz, H-8), 5.92 (1H, dd, J = 13 and 3 Hz, H-2), 2.97 (1H, dd, J = 16.5 and 13 Hz, H-3ax), 2.80 (1H, dd, J = 16.5 and 3 Hz, H-3eq); ^{13}C nmr: δ 190.56 (s, C=O), 165.16 (s, C-7), 164.71 (s, C-8a), 156.06 (s, C-2'), 129.99, 129.51 (d each, C-5, C-6'), 128.43 (s, C-1'), 127.61 (d, C-4'), 116.20 (d, C-3'), 116.17 (s, C-4a), 111.21 (d, C-6), 103.63 (d, C-8), 75.92 (d, C-2), 43.67 (t, C-3); EIms *m/z* (rel. int.): 256 M^+ (20), 238 $[M - H_2O]^+$ (69), 149 (16), 137 $[a + H]^+$ (100), 120 $[b]^+$ (35), 108 (23). Anal. Calcd for $C_{15}H_{12}O_4$: C, 70.29; H, 4.72. Found: C, 70.27; H, 4.81.

(Z)-6,2'-Dihydroxyaurone (**3a**):

Uv λmax (MeOH) nm (log ε): 248 (3.76), 265 (3.71), 376 (4.14); ^{1}H nmr: δ 8.23 (1H, dd, J = 8 and 2 Hz, H-6'), 7.63 (1H, d, J = 8.5 Hz, H-4), 7.34 (1H, s, H-β), 7.27 (1H, br dt, J = 7.5 and 2 Hz, H-4'), 7.01 (1H, dd, J = 8 and 1.5 Hz, H-3'), 6.99 (1H, br dt, J = 7.5 and 1.5 Hz, H-5'), 6.86 (1H, d, J= 1.5 Hz, H-7), 6.81 (1H, dd, J = 8.5 and 1.5 Hz, H-5); ^{13}C nmr in Table 1; EIms *m/z* (rel. int.); 254 $[M]^+$ (48), 237 $[M - OH]^+$ (55), 137 $[a + H]^+$ (100), 120 $[b]^+$ (38), 118 (83). Anal. Calcd for $C_{15}H_{10}O_4$: C, 70.85; H, 3.97. Found: C, 70.27; H, 4.81.

(E)-6,2'-Dihydroxyaurone (**4a**):

Uv λmax (MeOH) nm (log ε): 249 (3.74), 266 (3.76), 373 (4.02); ^{1}H nmr: δ 8.59 (1H, dd, J = 8 and 2 Hz, H-6'), 7.62 (1H, d, J = 8.5 Hz, H-4), 7.34 (1H, s, H-β), 7.29 (1H, br dt, J = 7.5 and 2 Hz, H-4'), 6.98 (1H, dd, J = 8 and 1.5 Hz, H-3), 6.92 (1H, br dt, J = 7.5 and 1.5 Hz, H-5'), 6.76 (1H, dd, J = 8.5 and 2 Hz, H-5), 6.69 (1H, d, J = 2 Hz, H-7); ^{13}C nmr in Table 1; EIms *m/z* (rel. int): 254 $[M]^+$ (29), 237 $[M - OH]^+$ (23), 137 $[a + H]^+$ (100), 120 $[b]^+$ (65), 118 (52). Anal. Calcd for $C_{15}H_{10}O_4$: C, 70.85; H, 3.97. Found: C, 70.72; H, 4.03.

(Z)-6,2',β-Trihydroxyaurone (**5a**):

Uv λmax (MeOH) nm (log ε): 249 (4.12), 290sh (3.90), 310sh (3.90), 322 (3.91), 388 (4.05); ^{1}H nmr: δ 8.00 (1H, d, J = 9 Hz, H-4), 7.78 (1H, dd, J = 8 and 2 Hz, H-6'), 7.23 (1H, br dt, J = 7.5 and 2 Hz, H-5), 7.01 (1H, d, J= 2.2 Hz, H-7), 6.95 (1H, dd, J = 9 and 2.2 Hz, H-5), 6.87 (1H, br dt, J = 7.5 and 1 Hz, H-5'), 6.86 (1H, dd, J= 8 and 1 Hz, H-3'); ^{13}C nmr in Table 1; EIms *m/z* (rel. int.): 270 $[M]^+$ (76), 253 $[M - OH]^+$ (100), 208 (73), 149 (40), 137 $[a + H]^+$ (53), 120 $[b]^+$ (49). Anal. Calcd for $C_{15}H_{10}O_5$: C, 66.65; H, 3.72. Found: C, 66.44; H, 4.03.

6,2',β-Trimethoxyaurone (**5c**, with CH_2N_2; oil):

Uv λmax (MeOH) nm (log ε): 206 (4.47), 252sh (3.81), 306 (354); ^{1}H nmr: δ 8.19 (1H, d, J = 9 Hz, H-4), 7.50 (1H, dd, J = 8 and 1.5 Hz, H- 6'), 7.46 (1H, dt, J = 8 and 1.5 Hz, H-4'), 7.08 (1H, dt, J = 8 and 1 Hz, H-5'), 7.05 (1H, dd, J = 8 and 1 Hz, H-3'), 6.98 (1H, dd, J = 9 and 2.5 Hz, H-5), 6.80 (1H, d, J = 2.5 Hz, H-7), 3.89, 3.86, 3.82 (3H each, s, 3 x OMe); EIms *m/z* (rel. int.): 312 $[M]^+$ (79), 311 $[M - H]^+$ (74), $[M - Me]^+$ (23), 281 $[M - OMe]^+$ (100), 269 $[297 - CO]^+$ (10), 263 (25), 253 $[281 - CO]^+$ (12), 251 $[281 - OCH_2]^+$ (12), 211 (16), 151 (53), 135 (19). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.21; H, 5.17. Found: C, 69.02; H, 5.01.

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