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Effect of natural and synthetic benzyl benzoates on calmodulin

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Abstract

The present investigation describes the effect of the spasmolytic benzylbenzoates **1**–**9** from *Brickellia veronicifolia* on CaM using a functional *in vitro* enzymatic assay. Bovine brain PDE1 was used as a monitoring enzyme. The most active natural inhibitors of the system CaM–PDE1 were benzyl benzoates **3**–**5**, which inhibited the activity of PDE1 in a concentration-dependent manner. In addition, three series of analogs of compound **4**, compounds **10a**–**32a**, were prepared and assayed. The benzyl benzoates from the first series, namely **10a**–**24a**, possess no substituents on ring B but different number and position of hydroxyl or methoxy groups in ring A. The second group (**25**–**32a**), on the other hand, possesses an A ring identical to that on compound **4**, but different substituents in Ring B. The most active compounds were **14a**, **15a** and **30a**. These compounds were two to six times more potent than chlorpromazine, a well known CaM inhibitor. Benzyl benzoates **14a** and **15a** have methoxyl groups at C-2/C-4 and C-3/C-4 in ring A, respectively; while **30a**, in addition to the methoxyl groups at C-2/C-6 of ring A, hold a benzoyloxy moiety at C-3′ of ring B. Kinetic studies revealed that compounds **3**, **4**, **14a**, **15a** and **30a** behave as competitive CaM antagonists.

Keywords: Asteraceae; *Brickellia veronicifolia*; Benzyl benzoates; Calmodulin; *c*AMP phosphodiesterase; PDE1

1. Introduction

In a previous investigation on the medicinal species *Brickellia veronicifolia* (Kunth) Gray, formerly *B. veronicaefolia* (Kunth) Gray (Asteraceae), a series of spasmolytic benzyl benzoates were discovered (Rivero-Cruz et al., 2005). These secondary metabolites provoked a significant relaxation of the spontaneous contractions of the guinea-pig ileum with a higher efficacy than papaverine, a well known spasmolytic agent of therapeutic use (Rivero-Cruz et al., 2005).

During the course of the investigation of the mode of action of the benzyl benzoates isolated from *B. veronicifolia*, as potential smooth muscle relaxants, the role of the regulatory protein calmodulin (CaM) as a molecular target was explored. This was because several events that take place during smooth muscle contraction–relaxation events are regulated by CaM-dependent mechanisms (Webb, 2003). The Ca^{2+}–CaM complex activates myosin light chain kinase (MLCK) by association with the catalytic subunit of the enzyme; the active MLCK catalyzes phosphorylation of the regulatory light chain (LC) subunits of myosin (MLC20); phosphorylated MLC20 activates myosin ATPase, thus triggering cycling of the myosin heads (cross-bridges) along the actin filaments, resulting in contraction of the smooth muscle. Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by activation of the mechanism leading to contraction inhibition. For example, a decrease in intracellular level of Ca^{2+} or an interference with CaM induces a dissociation of the Ca^{2+}–CaM–MLCK complex, resulting in increased activity of myosin light chain phosphatase (MLCP) activity, dephosphorylation of the MLC20 and thus relaxation of the smooth muscle (Webb, 2003). Indeed some CaM antagonists such as W-7,

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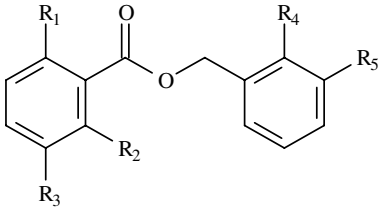


	R_1	R_2	R_3	R_4	R_5
1	OCH_3	OCH_3	H	H	β-D-glucopiranosyloxy
2	OCH_3	OCH_3	H	H	OH
3	OH	H	H	OCH_3	H
4	OCH_3	OCH_3	H	H	H
5	OH	OCH_3	H	H	OCH_3
6	OH	OCH_3	H	H	H
7	OCH_3	OCH_3	OCH_3	H	H
8	OH	OCH_3	OCH_3	H	H
9	OCH_3	OCH_3	H	H	OCH_3

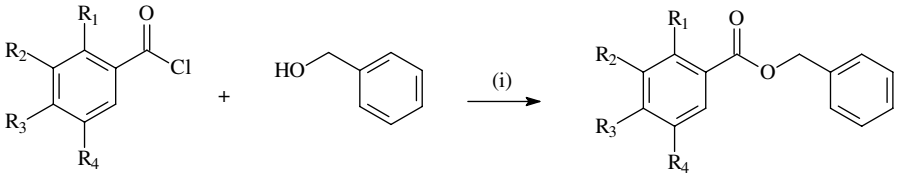
Fig. 1. Benzyl benzoates from *Brickellia veronicaefolia.*

N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide, trifluoperazine and chlorpromazine provoke smooth muscle relaxation. On the other hand, CaM modulates activity of other important proteins participating in the signal transduction events during the smooth muscle contraction–relaxation events, such as CaM kinase II, CaM sensitive-phosphodiesterase (PDE1), the nitric oxide synthases, adenylate cyclases 1 and 8, and several ion channels, notably voltage-gated Ca^{2+} channels, among others (Chin and Means, 2000; Means, 2000). Thus the present investigation was undertaken to evaluate the effect of the spasmolytic benzylbenzoates **1**–**9** (Fig. 1) from *B. veronicifolia* on CaM using a functional in vitro enzymatic assay. In addition, to start the analysis of structure–activity relationship a series of analogs of compound **4** were prepared and assayed. Bovine brain PDE1 was used as a monitoring enzyme.

2. Results and discussion

2.1. Synthesis

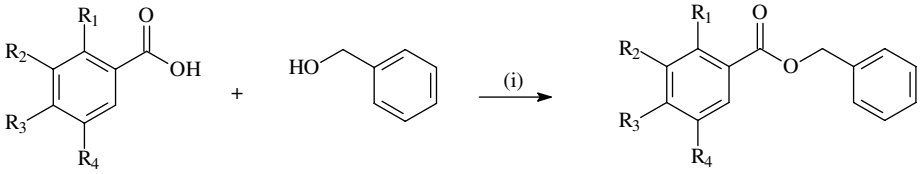
The preparation of compounds **10a**–**32a** was carried out as depicted in Schemes 1–3. The first group of compounds possessing an unsubstituted ring B (**10a**–**17a**) were prepared by condensation of benzyl alcohol with the appropriate acid chlorides **10**–**17** in the presence of triethylamine (Dhimitruka and SantaLucia, 2006). Benzyl benzoates **18a**–**24a** were obtained by the reaction of a suitable benzoic acid derivative (**18**–**24**) with benzyl alcohol in the presence of 1,1′-carbonyldiimidazole (DCC) (Cutler, 1997). Benzyl benzoates **25a**–**28a** were prepared following the same strategy but using 2,6-dimethoxy benzoyl chloride and a suitable *m*-substituted benzyl alcohol derivative (**25**–**28**). Compound **29a** was obtained by



	R_1	R_2	R_3	R_4
10	H	H	H	H
11	OCH_3	H	H	H
12	H	OCH_3	H	H
13	H	H	OCH_3	H
14	OCH_3	H	OCH_3	H
15	H	OCH_3	OCH_3	H
16	H	OCH_3	H	OCH_3
17	H	OCH_3	OCH_3	OCH_3

	R_1	R_2	R_3	R_4
10a	H	H	H	H
11a	OCH_3	H	H	H
12a	H	OCH_3	H	H
13a	H	H	OCH_3	H
14a	OCH_3	H	OCH_3	H
15a	H	OCH_3	OCH_3	H
16a	H	OCH_3	H	OCH_3
17a	H	OCH_3	OCH_3	OCH_3

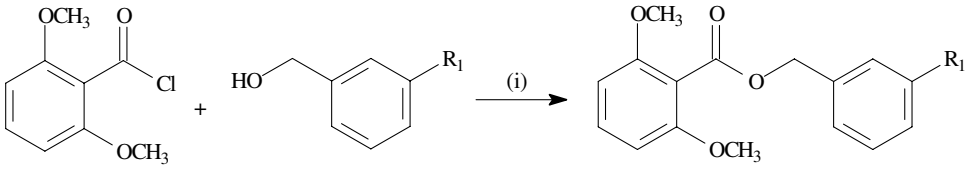
Scheme 1. Preparation of compounds **10a**–**17a**: (i) ACN, $N(CH_2CH_3)_3$, stirred during 3 h, r.t.

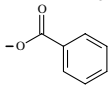
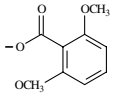
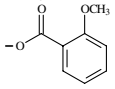


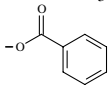
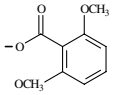
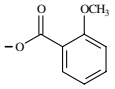
	R_1	R_2	R_3	R_4
18	OH	H	H	H
19	H	OH	H	H
20	H	H	OH	H
21	OH	OH	H	H
22	H	OH	OH	H
23	H	OH	H	OH
24	H	OH	OH	OH

	R_1	R_2	R_3	R_4
18a	OH	H	H	H
19a	H	OH	H	H
20a	H	H	OH	H
21a	OH	OH	H	H
22a	H	OH	OH	H
23a	H	OH	H	OH
24a	H	OH	OH	OH

Scheme 2. Preparation of compounds **18a**–**24a**: (i) ACN, $N(CH_2CH_3)_3$, 1,1′-carbonyldiimidazole, stirred during 3 h, 65 °C.



	R_1
25	Cl
26	NH_2
27	NO_2
28	OCH_3
29	$OCOCH_3$
30	
31	
32	

	R_1
25a	Cl
26a	NH_2
27a	NO_2
28a	OCH_3
29a	$OCOCH_3$
30a	
31a	
32a	

Scheme 3. Preparation of compounds **25a**–**32a**: (i) ACN, $N(CH_2CH_3)_3$, 1,1′-carbonyldiimidazole, stirred during 3 h, r.t.

acetylation with pyridine and acetic anhydride (Ac_2O) of natural product **2**. Finally, products **30a**–**32a** possessing an ester group at C-3′ in the benzyl alcohol moiety were prepared by esterification of the 3′-hydroxybenzyl-2,6-dimethoxybenzoate, and the appropriate benzoyl, 2,6-dimethoxy benzoyl or *o*-anisoyl chlorides (**30**–**32**), respectively, in the presence of triethylamine (Dhimitruka and SantaLucia, 2006). The spectroscopic properties of compounds **10a** **11a**, **18a**, **13a** and **28a** were identical to those previously described (Bohlman et al., 1977; Kodpinid et al., 1984). The remaining compounds are newly synthesized and their structures were determined by analysis of their HRMS, IR, ^{1}H NMR, ^{13}C NMR and NOESY spectra.

2.2. Evaluation of the activity of benzylbenzoates on the enzyme bovine brain CaM-dependent phosphodiesterase

In vitro PDE1 activity was established through a functional coupled enzymatic reaction by measuring, spectrophotocolorimetrically at 655 nm, the amount of inorganic phosphorous released by the hydrolysis of 5′-AMP. The latter compound is generated by the hydrolysis of *c*AMP by the action of PDE1. The resulting 5′-AMP is then converted to adenosine and inorganic phosphorous in the presence of a 5′-nucleotidase (Sharma and Wang, 1979). The assay was performed by triplicate and repeated at least six times. The 50% inhibition concentration (IC_{50}) values of compounds **1**–**10** and **11a**–**32a** on

PDE1 are summarized in Table 1, which includes also their relative potency in relation to chlorpromazine, a well known CaM inhibitor (Harmat et al., 2000; Vasta and Beavo, 2004). According to the data in Table 1, the most active natural inhibitors of the system CaM–PDE1 were benzyl benzoates **3**–**5**. Compound **3** was almost two times more potent (relative potency = 1.8) than the positive control, but **4** and **5** were as active as chlorpromazine with a relative potency of 1. The three compounds inhibited the activity of PDE1 in a concentration-dependent manner.

Since compound **4** showed a similar efficacy *in vitro* to chlorpromazine, a series of analogs were designed in order to attempt to enhance its activity. These compounds were designed considering the commercial availability of suitable acids, acid chlorides or benzyl alcohols. The modifications were designed to generate valuable information regarding the weight on the enzymatic activity of the position and number of oxygenated substituents of ring A, as well as the presence of different type of substituents in C-3′ of ring B. Thus, the first series (**10a**–**24a**) possesses no substituents on ring B but have a different number and positions of hydroxyl or methoxy groups in ring A. The second group (**25a**–**32a**), on the other hand, possesses an A ring identical to that of compound **4**, but different substituents on ring B. The most active compounds of the first series were **14a**, **15a**, **19a**, **21a** and **24a**. As the lead molecule **4**, all these compounds had two oxygenated substituents in ring A, except for **24a** which has three oxygenated substituents; however, when the substituents were methoxy groups and were located in C-2/C-4 as in **14a**, or C-3/C-4 as in **15a**, a higher inhibitory activity was found. These compounds were three and two times more active than chlorpromazine, respectively.

In the second series, the most active benzyl benzoates were those possessing a benzoyloxy moiety (**30a**–**32a**) at C-3′. In turn, in this subgroup the most active was compound **30a** which was six times more active than chlorpromazine.

In order to obtain further evidence of the involvement of CaM in the inhibition of CaM–PDE1, a kinetic analysis of the inhibition of the activity of PDE1 was assessed using different amounts of CaM in the presence of different concentrations of the most active compounds (**3**, **4**, **14a**, **15a** and **30a**). The inhibition constants (K_I's) of **3**, **4**, **14a**, **15a** and **30a** were calculated using non-linear fits of the data with the equation indicated in Section 4. In this case, the Dixon plots approach was not used because after subtracting the activity found at saturating inhibitor concentration for each Ca^{2+}–CaM concentration employed, the curvature in the Dixon plot was not completely eliminated; furthermore, this procedure is hard to apply when the tested compounds have limited solubility, because estimation of the baseline value requires extrapolation to infinite inhibitor concentration and introduces an additional source of error in the calculation. The K_I values for **3**, **4**, **14a**, **15a** and **30a** were 13.43 ± 0.1, 18.42 ± 0.21, 6.46 ± 1.52, 15.32 ± 0.80 and 1.59 ± 0.10 μM, respectively. The data

Table 1
Inhibition of CaM–PDE1 by compounds **1**–**9** and **10a**–**32a**

Compound	IC_{50} (μM)	Chlorpromazine	Potency
1	>100	11.68 ± 4.76	–
2	67.51 ± 12.36	6.09 ± 2.35	0.1
3	10.61 ± 2.84	19.35 ± 2.97	1.8
4	12.34 ± 4.67	12 31 ± 4.76	1.0
5	14.59 ± 5.27	14.61 ± 3.55	1.0
6	>100	19.35 ± 2.97	–
7	23.73 ± 9.02	11.15 ± 3.65	0.5
8	21.01 ± 3.19	11.15 ± 3.65	0.5
9	46.94 ± 11.18	11.68 ± 4.76	0.2
10a	6.49 ± 2.55	1.47 ± 0.37	0.23
11a	10.20 ± 1.90	1.47 ± 0.37	0.14
12a	24.74 ± 5.79	17.4 ± 7.46	0.71
13a	33.12 ± 5.80	3.97 ± 1.66	0.12
14a	2.29 ± 2.44	17.4 ± 7.46	3.33
15a	2.21 ± 0.51	3.97 ± 1.66	1.79
16a	4.23 ± 1.22	2.12 ± 0.74	0.50
17a	25.10 ± 1.54	17.4 ± 7.46	0.70
18a	>65	3.97 ± 1.66	–
19a	3.35 ± 0.66	3.97 ± 1.66	1.18
20a	17.81 ± 4.47	3.97 ± 1.66	0.22
21a	3.5 ± 1.34	3.97 ± 1.66	1.13
22a	4.13 ± 1.05	3.97 ± 1.66	0.96
23a	14.9 ± 3.11	3.97 ± 1.66	0.26
24a	2.78 ± 0.54	3.97 ± 1.66	1.42
25a	5.14 ± 2.33	2.12 ± 0.74	0.41
26a	28.3 ± 8.50	2.12 ± 0.74	0.07
27a	15.8 ± 10.00	2.12 ± 0.74	0.13
28a	>65	2.12 ± 0.74	–
29a	36.11 ± 17.99	13.37 ± 3.55	0.37
30a	2.05 ± 1.08	13.37 ± 3.55	6.51
31a	15.32 ± 4.57	13.37 ± 3.55	0.87
32a	>100	11.68 ± 4.76	–

Values as means ± SEM ($n = 6$).
Potency was obtained by the formula: IC_{50} (chlorpromazine)/IC_{50} (compound), assuming a value of 1.00 for chlorpromazine.

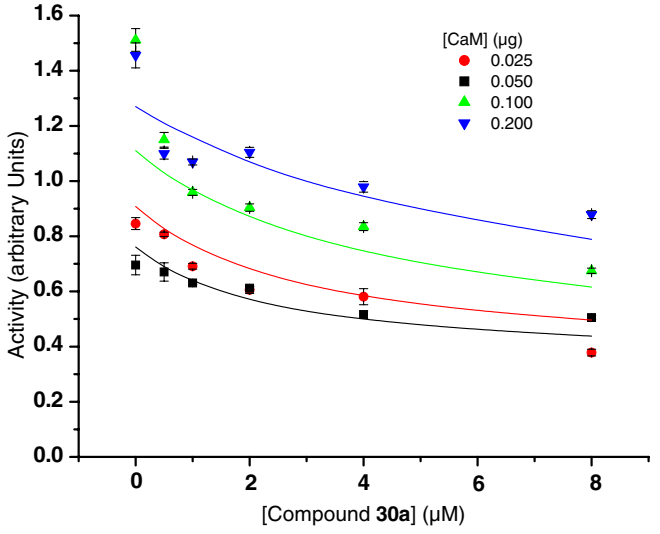


Fig. 2. Kinetic analysis of inhibition by **30a** of PDE1 activity. Kinetic analysis was done by means of a non-linear fit (see Section 4).

are consistent with benzyl benzoates being competitive CaM antagonists. As an example the induced inhibition of CaM-activated PDE1 for **30a** is illustrated in Fig. 2.

3. Conclusion

The present study indicates that benzyl benzoates are novel competitive calmodulin antagonists and the involvement of CaM as a molecular target for their smooth muscle relaxatory effect is highly probable. If the compounds compete with CaM for the PDE1 recognition site, or if they just interact with the protein changing its active conformation, remains an open question.

4. Experimental

4.1. General

Melting point determinations were carried out on a Fisher–Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks or neat on a Perkin–Elmer 599B spectrophotometer. NMR spectra including COSY, NOESY, HMBC and HMQC experiments were recorded in $CDCl_3$ on a Varian VXR-300S, spectrometer either at 300 (^{1}H) or 75.4 (^{13}C) MHz, or on a Bruker DMX500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C), using tetramethylsilane (TMS) as an internal standard. MS were obtained on a JEOL JMS-AX505HA mass spectrometer. Open Column chromatography employed silica gel 60 (70–230 mesh, Merck). The progress of all reactions was monitored by thin layer chromatography (TLC) which was performed on Merck silica gel $60F_{254}$ aluminum sheets.

4.2. Natural products and synthetic precursors

The natural products 3′-(β-D-glucopyranosyloxy)benzyl-2,6-dimethoxybenzoate (**1**), 3′-hydroxybenzyl-2,6-dimethoxybenzoate (**2**), 2′-methoxybenzyl-2-hydroxybenzoate (**3**), benzyl 2,6-dimethoxybenzoate (**4**), 3′-methoxybenzyl 2-hydroxy-6-methoxybenzoate (**5**), benzyl 2-hydroxy-6-methoxybenzoate (**6**), benzyl 2,5,6-trimethoxybenzoate (**7**), benzyl 2-hydroxy-5,6-dimethoxybenzoate (**8**) and 3′-methoxybenzyl 2,6-dimethoxybenzoate (**9**) were isolated from *B. veronicifolia* as previously described (Rivero-Cruz et al., 2005). Benzyl alcohol, acid chlorides **10**–**17**, acids **18**–**24**, 3-chlorobenzyl alcohol (**25**), 3-aminobenzyl alcohol (**26**), 3-nitrobenzyl alcohol (**27**), 3-methoxybenzyl alcohol (**28**), benzoyl, 2,6-dimethoxy benzoyl and *o*-anisoyl chlorides (**30**–**32**) were purchased from Sigma (St. Louis, MO).

4.3. Preparation of analogs *10a–17a*

To solutions of benzyl alcohol (9.24 mmol, 1000 mg) and the corresponding acid chlorides **10**–**17** (18.5 mmol of each) in CH_3CN (10 mL), Et_3N (9.24 mmol, 935 mg) was added drop wise. The reaction mixture was stirred at room temperature for 3 h. Next the resulting product was poured in to cold H_2O and extracted with CH_2Cl_2 (3×15 mL). The organic phase was then dried over Na_2SO_4 and concentrated *in vacuo*. In each case, the crude reaction mixtures were purified by open column chromatography on silica gel (~100 g) eluting with hexane–CH_2Cl_2 (1:1). The resulting pure products were **10a** (930 mg, 65 %) as a transparent oil, **11a** (673 mg, 56%) as an oil, **12a** (762 mg, 60%) as an oil, **13a** (900 mg, 64%) as an oil, **14a** (140 mg, 15%, m.p. 50–52 °C) as a crystalline solid, **15a** (160 mg, 15%) as an oil, **16a** (600 mg, 64 %) as an oil and **17a** as a white solid (1050 mg, 75%, m.p. 61–62 °C). The spectroscopic properties of compounds **10a**, **11a** and **13a** were identical to those previously described in the literature (Bohlman et al., 1977; Kodpinid et al., 1984).

4.3.1. Benzyl 3-methoxybenzoate (*12a*)

Colorless oil; IR (KBr), υ_{max} 1720, 1276, 1226, 1103, 1045, 754 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.67 (1H, dd, $J = 7.8$, 1.5 Hz, H-6), 7.60 (1H, dd, $J = 1.5$, 1.2 Hz, H-2), 7.44 (2H, m, H-2′ and H-6′), 7.37 (2H, m, H-3′ and H-5′), 7.35 (1H, m, H-4′), 7.01 (1H, ddd, $J = 8.4$, 2.7, 1.2 Hz, H-4), 5.36 (2H, s, H-7′), 3.83 (3H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 166.3 (C-7), 159.5 (C-3), 136.0 (C-1′), 131.4 (C-1), 129.4 (C-5), 128.6 (C-3′ and C-5′), 128.2 (C-4′), 128.1 (C-2′ and C-6′), 122.1 (C-6), 119.5 (C-4), 114. 2 (C-2), 66.7 (C-7′), 55.4 (–OMe). EIMS *m/z* 242 [M$^+$ (100)], 224 (18), 135 (60), 91 (15). HRMS (EI) for $C_{15}H_{14}O_3$ (M$^+$): Calc.: 242.2751. Found: 242.2749.

4.3.2. Benzyl 2,4-dimethoxybenzoate (*14a*)

White crystalline solid, m.p. 50–52 °C; IR (KBr) υ_{max} 1693, 1269, 1146, 767 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.9 (1H, d, $J = 7.8$ Hz, H-6), 7.45 (2H, m, H-2′ and H-6′), 7.38 (2H, m, H-3′ and H-5′), 7.31 (1H, m, H-4′), 5.32 (2H, s, H-7′), 3.81 (6H, s, –OMe). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.2 (C-7), 164.4 (C-4), 161.9 (C-2), 136.5 (C-1′), 133.9 (C-6), 128.4 (C-3′ and C-5′), 128 (C-2′ and C-6′), 127.9 (C-4′), 104.5 (C-1 and C-5), 99 (C-3), 66.6 (C-7′), 55.9 (–OMe), 55.2 (–OMe). EIMS *m/z* 272 [M$^+$ (38)], 165 (100), 138 (19), 91 (45), 77 (6). HRMS (EI) for $C_{16}H_{16}O_4$ (M$^+$): Calc.: 272.3014. Found: 272.3011.

4.3.3. Benzyl 3,4-dimethoxybenzoate (*15a*)

Colorless oil; IR (KBr) υ_{max} 1710, 1270, 1221, 1024, 762 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.73 (1H, dd, $J = 8.4$, 2.1 Hz, H-6), 7.58 (1H, d, $J = 1.8$ Hz, H-2), 7.45 (2H, m, H-2′ and H-6′), 7.39 (2H, m, H-3′ and H-5′), 7.34 (1H, m, H-4′), 6.88 (1H, d, $J = 8.4$ Hz, H-5), 5.38 (2H, s, H-7′), 3.94 (6H, s, –OMe). ^{13}C NMR ($CDCl_3$,75 MHz) δ 166.2 (C-7), 153.0 (C-4), 148.6 (C-3), 136.2 (C-1′), 128.5 (C-3′ and C-5′), 128.1 (C-4′), 128.0

(C-2′ and C-6′), 123.7 (C-6), 122.56 (C-1), 112.0 (C-2), 110.2 (C-5), 66.5 (C-7′), 56.0 (–OMe). EIMS *m/z* 272 [M^+ (70)], 165 (100), 91 (78), 77 (11). HRMS (EI) for $C_{16}H_{16}O_4$ (M^+): Calc.: 272.3014. Found: 272.2960.

4.3.4. Benzyl 3,5-dimethoxybenzoate (*16a*)

Colorless oil; IR (KBr) υ_{max} 1719, 1597, 1228, 1206, 1157, 766 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.44 (2H, m, H-2′ and H-6′), 7.37 (2H, m, H-3′ and H-5′), 7.34 (1H, m, H-4′), 7.22 (2H, d, $J = 2.4$ Hz, H-2 and H-6), 6.65 (1H, t, $J = 2.4$ Hz, H-4), 5.36 (2H, s, H-7′), 3.82 (6H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.2 (C-7), 160.7 (C-3 and C-5), 136.0 (C-1′), 132.1 (C-1), 128.6 (C-3′ and C-5′), 128.2 (C-4′), 128.1 (C-2′ and C-6′), 107.4 (C-2 and C-6), 105.7 (C-4), 66.8 (C-7′), 55.6 (–OMe). EIMS *m/z* 272 [M^+ (100)], 165 (47), 138 (44), 91 (47). HRMS (EI) for $C_{16}H_{16}O_4$ (M^+): Calc.: 272.3014. Found: 272.2988.

4.3.5. Benzyl 3,4,5-trimethoxybenzoate (*17a*)

Colorless glassy solid, m.p. 61–62 °C; IR (KBr) υ_{max} 1709, 1330, 1128, 994, 762 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.44 (2H, m, H-2′ and H-6′), 7.39 (2H, m, H-3′ and H-5′), 7.34 (1H, m, H-4′), 7.35 (2H, m, H-2 and H-6), 5.36 (2H, s, H-7′), 3.9 (9H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.1 (C-7), 152.9 (C-3 and C-5), 142.3 (C-4), 136.1 (C-1′), 128.6 (C-3′ and C-5′), 128.5 (C-4′), 128.2 (C-2′ and C-6′), 124.1 (C-1), 106.9 (C-2 and C-6), 66.8 (C-7′), 60.9 (–OMe), 56.2 (-OMe). EIMS *m/z* 302 [M^+ (100)], 195 (62), 168 (15), 153 (18), 91 (47). HRMS (EI) for $C_{17}H_{18}O_5$ (M^+): Calc.: 302.3277. Found: 302.3275.

4.4. Preparation of analogs *18a–24a*

To the acids **18–24** (2.5 mmol each) in CH_3CN (15 mL), were added 2.5 mmol (405.5 mg) of 1,1′-carbonyldiimidazole (DCC). The reaction mixtures were stirred for 1 h at 50 °C until complete dissolution of the solids was achieved. Next benzyl alcohol (2.5 mmol, 270.4 mg) were added to the mixtures and stirred for 3 h at 65 °C. In each case, the resulting product was poured in to cold water and extracted with CH_2Cl_2 (3×20 mL). The organic phases were successively extracted with 5% HCl 1 N (3×20 mL), 5% $NaHCO_3$ (3×20 mL) and H_2O (3×20 mL). The final organic phases were then dried (Na_2SO_4) and concentrated *in vacuo* to yield crude products **18a–24a**. The crude products were finally purified by open on silica gel CC (100 g), eluting with hexane-CH_2Cl_2 1:1 to afford **18a** (970 mg, 64%) as an oil, **19a** (40 mg, 10%) as an oil, **20a** (800 mg, 50%, m.p. 119–121 °C) as a white crystalline solid, **21a** (700 mg, 46%, m.p. 54–55 °C) as a white crystalline solid, **22a** (880 mg, 67%, m.p. 112–114 °C) as a white crystalline solid, **23a** (960 mg, 71%, m.p. 132–133 °C) as a white crystalline solid and **24a** (830 mg, 61%, m.p. 145–149 °C) as a white crystalline solid. The spectroscopic parameters of **18a** were identical to those previously reported (Kodpinid et al., 1984).

4.4.1. Benzyl 3-hydroxybenzoate (*19a*)

Colorless oil; IR (KBr) υ_{max} 3391, 1694, 1290, 754 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.7 (1H, s, –OH), 7.52 (1H, ddd, $J = 7.6$, 1.5 Hz, H-6), 7.51 (1H, d, $J = 2.7$ Hz, H-2), 7.50 (2H, m, H-2′ and H-6′), 7.40 (2H, m, H-3′ and H-5′), 7.36 (1H, m, H-4′), 7.33 (1H, t, $J = 7.8$ Hz, H-5), 7.09 (1H, ddd, $J = 8.1$, 2.7 and 1.2 Hz, H-4), 5.35 (1H, s, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.6 (C-7), 158.5 (C-3), 137.5 (C-1′), 132.5 (C-1), 130.7 (C-5), 129.5 (C-3′ and C-5), 129.1 (C-2′, C-4′ and C-6′), 121.5 (C-6), 121.1 (C-4), 116.8 (C-2), 67.1 (C-7′). EIMS *m/z* 228 [M^+ (61)], 210 (22), 121 (100), 91 (69), 65 (12). HRMS (EI) for $C_{14}H_{12}O_3$(M^+): Calc.: 228.2481. Found: 228.2479.

4.4.2. Benzyl 4-hydroxybenzoate (*20a*)

White crystalline solid, m.p. 119–121 °C; IR (KBr) υ_{max} 3423, 1710, 1269, 1162, 761, 693 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.17 (1H, d, $J = 8.7$ Hz, H-6), 8.14 (1H, d, $J = 8.7$ Hz, H-2), 7.46 (2H, m, H-2′ and H-6′), 7.39 (2H, m, H-3′ and H-5′), 7.33 (1H, m, H-4′), 6.95 (2H, d, $J = 8.7$ Hz, H-3 and H-5), 5.39 (2H, s, $J = 7.8$ Hz, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.9 (C-7), 164.3 (C-4), 137.4 (C-1′), 132 (C-2 and C-6), 129.0 (C-3′ and C-5′), 128.6 (C-2′ and C-6′), 128.5 (C-4′), 123.1 (C-1), 114.3 (C-3), 114.2 (C-5), 66.4 (C-7′). EIMS *m/z* 228 [M^+ (25)], 121 (100), 91 (49), 65 (14). HRMS (EI) for $C_{14}H_{12}O_3$(M^+): Calc.: 228.2481. Found: 228.2476.

4.4.3. Benzyl 2,3-dihydroxybenzoate (*21a*)

White crystalline solid, m.p. 54–55 °C; IR (KBr) υ_{max} 3219, 1694, 1251, 742 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 10.88 (1H, s, –OH), 7.44 (2H, m, H-2′ and H-6′), 7.42 (1H, dd, $J = 8.4$, 1.5 Hz, H-6), 7.38 (2H, m, H-3′ and H-5′), 7.36 (1H, m, H-4′), 7.11 (1H, dd, $J = 8.1$, 1.8 Hz, H-4), 6.79 (1H, dd, $J = 8.1$ Hz, H-5), 5.63 (1H, s, OH), 5.39 (2H, s, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.1 (C-7), 148.9 (C-2), 145.0 (C-3), 135.1 (C-1′), 128.7 (C-3′ and C-5′), 128.6 (C-4′), 128.2 (C-2′ and C-6′), 120.7 (C-6), 119.9 (C-4), 119.2 (C-5), 112.4 (C-1), 67.1 (C-7′). EIMS *m/z* 244 [M^+ (20)], 91 (100), 65 (10). HRMS (EI) for $C_{14}H_{12}O_4$(M^+): Calc.: 244.2474. Found: 244.2469.

4.4.4. Benzyl 3,4-dihydroxybenzoate (*22a*)

White crystalline solid, m.p. 112–114 °C; IR (KBr) υ_{max} 3219, 1690, 1599, 1339, 1245, 752 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.45 (2H, s, –OH), 7.43 (2H, m, H-2′ and H-6′), 7.38 (2H, m, H-3′ and H-5′), 7.32 (1H, m, H-4′), 7.01 (2H, d, $J = 2.4$ Hz, H-2 and H-6), 6.79 (1H, d, $J = 8.1$ Hz, H-5), 5.28 (2H, s, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.15 (C-7), 149.8 (C-3 and C-4), 138.6 (C-1′), 132.1 (C-1), 129.2 (C-3′ and C-5′), 128.7 (C-2′, C-4′ and C-6′), 109.2 (C-2 and C-6), 108.1 (C-5), 67.5 (C-7′). EIMS *m/z* 244 [M^+ (20)], 91 (100), 65 (10). HRMS (EI) for $C_{14}H_{12}O_4$(M^+). Calc.: 244.2474. Found: 244.2478.

*4.4.5. Benzyl 3,5-dihydroxybenzoate (**23a**)*

White crystalline solid, m.p. 132–133 °C; IR (KBr) υ_{max} 3372, 1693, 1602, 1341 1237, 766 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.60 (2H, s, –OH), 7.49 (2H, m, H-2′ and H-6′), 7.41 (2H, m, H-3′ and H-5′), 7.35 (1H, m, H-4′), 7.04 (2H, d, $J = 2.4$ Hz, H-2 and H-6), 6.59 (1H, dd, $J = 2.4$, H-4), 5.32 (2H, s, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.5 (C-7), 159.6 (C-3 and C-5), 137.6 (C-1′), 133.1 (C-1), 129.4 (C-3′ and C-5′), 129.0 (C-2′, C-4 ′ and C-6′), 108.7 (C-2 and C-6), 108.1 (C-4), 67.1 (C-7′). EIMS *m/z* 244 [M^+ (100)], 226 (14), 137 (96), 91 (94), 65 (8). HRMS (EI) for $C_{14}H_{12}O_4(M^+)$: Calc.: 244.2474. Found: 244.2469.

*4.4.6. Benzyl 3,4,5-trihydroxybenzoate (**24a**)*

White crystalline solid, m.p. 145–149 °C; IR (KBr) υ_{max} 3372, 1693, 1602, 1341 1237, 766 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.19 (3H, s, –OH), 7.46 (2H, m, H-2′ and H-6′), 7.40 (2H, m, H-3′ and H-5′), 7.35 (1H, m, H-4′), 7.14 (2H, s, H-2 and H-6), 5.27 (1H, s, H-7′). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.4 (C-7), 146.1 (C-3 and C-5), 138.9 (C-4), 137.8 (C-1′), 129.3 (C-3′ and C-5′), 128.8 (C-2′ and C-6′), 128.7 (C-4′), 121.6 (C-1), 109.8 (C-2 and C-6), 66.6 (C-7′). EIMS *m/z* 260 [M^+ (100)], 226 (24), 137 (75), 91 (91), 65 (54). HRMS (EI) for $C_{14}H_{12}O_5$ (M^+): Calc.: 260.2467. Found: 260.2471.

*4.5. Preparation of analogs **25a–28a***

To solutions of 2,6-dimethoxybenzoyl chloride (5.0 mmol, 1003.1 mg) in CH_3CN (10 mL) were added (2.5 mmol) 3-chlorobenzyl alcohol (**25**), 3-aminobenzyl alcohol (**26**), 3-nitrobenzyl alcohol (**27**) or 3-methoxybenzyl alcohol (**28**), respectively. Then, Et_3N (1 equiv.) was added dropwise. Each reaction mixture was stirred at room temperature for 3 h. Next the resulting product was poured into cold H_2O and extracted with CH_2Cl_2 (3×15 mL). The crude products were purified by silica gel CC (100 g) to afford the pure compounds: **25a** (220 mg, 25%) as an oil; eluant: hexane–CH_2Cl_2 (1:1); **26a** (510 mg, 50%) as an oil; eluant: hexane–CH_2Cl_2 (1:1), **27a** (985 mg, 90%, mp 130–135 °C) as a yellow crystalline solid; eluant: hexane–CH_2Cl_2(6:4) and **28a** (30 mg, 15%) as an oil; eluant: hexane–CH_2Cl_2 (7:3). The spectroscopic properties of **28a** were identical to those previously described (Bohlman et al., 1977).

*4.5.1. 3′-Chlorobenzyl 2,6-dimethoxybenzoate (**25a**)*

Colorless oil; IR (KBr) υ_{max} 1729, 1255, 1114, 782 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28 (1H, dd, $J = 8.7$ Hz, H-2′ and H-4′), 7.15 (1H, dd, $J = 8.7$ Hz, H-4), 7.16 (1H, d, H-5′), 6.93 (1H, d, $J = 7.8$ Hz, H-6′), 6.58 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.37 (2H, s, H-7′), 3.83 (6H, s, –OMe). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2 (C-7), 157.5 (C-2 and C-6), 138.3 (C-1′), 134.3 (C-3′), 131.3 (C-4), 129.6 (C-5′), 128.0 (C-4′), 127.8 (C-2′), 125.6 (C-6′), 112.7 (C-1), 103.9 (C-3 and C-5), 65.6 (C-7′), 55.9 (–OMe). HRMS (EI) for $C_{16}H_{15}ClO_4(M^+)$: Calc.: 306.7461. Found: 306.7459.

*4.5.2. 3′-Aminobenzyl 2,6-dimethoxybenzoate (**26a**)*

Colorless glassy solid; IR (KBr) υ_{max} 1727, 1258, 1111, 734 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.32 (1H, dd, $J = 8.7$ and 8.4 Hz, H-4), 6.99 (1H, d, H-5′), 6.42 (2H, d, $J = 8.1$ Hz, H-2′ and H-4′), 6.73 (1H, d, $J = 7.8$ Hz, H-6′), 6.59 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.48 (2H, s, H-7′), 3.85 (6H, s, –OMe). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.1 (C-7), 157.5 (C-2 and C-6), 148.5 (C-3′), 138.6 (C-1′), 133.3 (C-6′), 131.5 (C-4), 129.3 (C-5′), 122.8 (C-4′), 122.3 (C-2′), 103.9 (C-1, C-3 and C-5), 65.0 (C-7′), 55.9 (–OMe). HRMS (EI) for $C_{16}H_{15}NO_6(M^+)$: Calc.: 317.2945. Found: 317.2950.

*4.5.3. 3′-Nitrobenzyl 2,6-dimethoxybenzoate (**27a**)*

White crystalline solid, m.p. 130–135 °C; IR (KBr) υ_{max} 1727, 1258, 1111, 734 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.39 (1H, m, H-2′), 8.17 (1H, brd, $J = 8.1$ Hz, H-4′), 7.73 (1H, dddt, $J = 7.8$, 1.7, 1.7, 0.6 Hz, H-6′), 7.54 (1H, dd, $J = 8.1$, 7.8 Hz, H-5′), 7.32 (1H, dd, $J = 8.7$ and 8.4 Hz, H-4), 6.59 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.48 (2H, s, H-7′), 3.85 (6H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.1 (C-7), 157.5 (C-2 and C-6), 148.5 (C-3′), 138.6 (C-1′), 133.3 (C-6′), 131.5 (C-4), 129.3 (C-5′), 122.8 (C-4′), 122.3 (C-2′), 103.9 (C-1, C-3 and C-5), 65.0 (C-7′), 55.9 (–OMe). HRMS (EI) for $C_{16}H_{15}NO_6(M^+)$: Calc.: 317.2987. Found: 317.2982.

*4.5.4. 3′-Methoxybenzyl 2,6-dimethoxybenzoate (**28a**)*

Colorless oil; IR (KBr) υ_{max} 1735, 1267, 1118, 730 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28 (1H, t, $J = 8.4$ Hz, H-4), 7.27 (1H, d, $J = 8.4$ Hz, H-5′), 7.01 (1H, d, $J = 7.9$ Hz, H-6′), 6.86 (1H, d, $J = 2.7$ Hz, H-4′), 6.83 (1H, m, H-2′), 6.55 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.36 (2H, s, H-7′), 3.81 (6H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.4 (C-7), 159.7 (C-3′), 157.5 (C-2 and C-6), 137.7 (C-1′), 131.1 (C-4), 129.3 (C-5′), 120.2 (C-6′), 113.5 (C-2′, C-1 and C-4′), 103.9 (C-3 and C-5), 66.6 (C-7′), 55.9 (-OMe). HRMS (EI) for $C_{17}H_{18}O_5(M^+)$: Calc.: 302.1154. Found: 302.1149.

*4.6. Preparation of analogs **29a–32a***

A solution of **2** (200 mg) in pyridine (2 mL) and Ac_2O (2 mL) was kept at room temperature for 48 h, diluted with CH_2Cl_2 (10 mL), washed with 1 N HCl (3×15 mL), saturated $NaHCO_3$ solution (3×15 mL), dried (Na_2SO_4) and evaporated to dryness, affording the acetate **29a** (159 mg) as a colorless oil, IR (KBr) υ_{max} 1749, 1256, 1113, 732 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.29 (1H, d, $J = 7.8$ Hz, H-4), 7.17 (1H, brd, $J = 8.1$ Hz, H-5′), 7.01 (2H, d, $J = 8.1$ Hz, H-4′ and H-6′), 6.98 (1H, d, H-2′), 6.54 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.55 (2H, s, H-7′), 2.12 (3H, s, -OCOMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.3 (–OCOMe), 163.1 (C-7), 159.5 (C-2 and C-6), 151.5

(C-3′), 140.6 (C-1′), 135.5 (C-4), 123.3 (C-6′), 129.3 (C-5′), 120.8 (C-4′), 119.3 (C-2′), 104.9 (C-1, C-3 and C-5), 65.0 (C-7′), 23.6 (–OCOMe). HRMS (EI) for $C_{18}H_{18}O_6(M^+)$: Calc.: 330.3342. Found: 330.3344.

To individual solutions of 3′-hydroxybenzyl-2,6-dimethoxybenzoate (720 mg, 2.5 mmol) in CH_3CN (10 mL) and the corresponding acid chlorides **30**–**32** (5 mmol). Then Et_3N (2.5 mmol, 253.0 mg) was added dropwise. The reaction mixture was stirred at room temperature for 3 h. Next the resulting product was poured into cold H_2O and extracted with CH_2Cl_2 (3×15 mL). The organic phase was then dried over Na_2SO_4 and concentrated *in vacuo*. The crude products were purified by on silica gel CC (100 g), eluting with CH_2Cl_2 in all cases, to yield the synthetic derivatives **30a**–**32a**. **30a** (335 mg; 47%); **31a** (359 mg; 50%) and **32a** (331.2 mg; 46%).

4.6.1. 3′-(benzoyl)-benzyl 2,6-dimethoxybenzoate (*30a*)

Colorless oil; IR (KBr) υ_{max} 1729, 1270, 1126, 738 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.51 (2H, d, $J = 8.1$ Hz, H-3″ and H-5″), 7.41 (1H, d, $J = 7.8$ Hz, H-4″), 7.08 (3H, m, H-2′, H-4′ and H-6′), 7.26 (1H, d, $J = 7.9$ Hz, H-5′), 6.44 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.51 (2H, s, H-7′), 3.75 (12H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.0 (C-7), 165.0 (C-7″), 162.3 (C-2 and C-6), 151.5 (C-3′), 141.6 (C-1′), 135.5 (C-4), 134.3 (C-4″), 131.3 (C-1″, C-2″ and C-6″), 129.3 (C-5′), 128.3 (C-3″ and C-5″), 124.3 (C-6′), 120.3 (C-4′), 118.8 (C-2′), 114.9 (C-3″), 106.6 (C-3 and C-5), 101.9 (C-1), 68.1 (C-7′), 55.9 (-OMe). HRMS (EI) for $C_{23}H_{22}O_7(M^+)$: Calc.: 410.4198. Found: 410.4192.

4.6.2. 3′-(2″,6″-dimethoxybenzoyl)-benzyl 2,6-dimethoxybenzoate (*31a*)

Colorless oil; IR (KBr) υ_{max} 1729, 1268, 1121, 735 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.51 (2H, d, $J = 8.1$ Hz, H-3″ and H-5″), 7.41 (1H, d, $J = 7.8$ Hz, H-4″), 7.08 (3H, m, H-2′, H-4′ and H-6′), 7.26 (1H, d, $J = 7.9$ Hz, H-5′), 6.44 (2H, d, $J = 8.4$ Hz, H-3 and H-5), 5.51 (2H, s, H-7′), 3.75 (12H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.0 (C-7), 160.3 (C-2, C-6, C-2″ and C-6″), 151.5 (C-3′), 146.6 (C-1′), 136.3 (C-4″), 135.5 (C-4), 131.3 (C-6″), 129.3 (C-5′), 124.3 (C-6′), 120.3 (C-4′), 118.8 (C-2′), 114.9 (C-3″), 108.6 (C-3″ and C-5″), 105.6 (C-3 and C-5), 101.9 (C-1 and C-1″), 68.1 (C-7′), 55.9 (–OMe). HRMS (EI) for $C_{25}H_{24}O_8(M^+)$: Calc.: 452.4566. Found: 452.4558.

4.6.3. 3′-(2″-methoxybenzoyl)-benzyl 2,6-dimethoxybenzoate (*32a*)

White crystalline solid, m.p. 91–92 °C; IR (KBr) υ_{max} 1729, 1260, 759 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.03 (1H, d, $J = 9.0$ Hz, H-6″), 7.41 (1H, d, $J = 8.1$ Hz, H-4″), 7.08 (3H, m, H-2′, H-4′ and H-6′), 6.98 (2H, d, $J = 9.0$ Hz, H-3″ and H-5″), 7.29 (1H, d, $J = 8.1$ Hz, H-5′), 7.21 (1H, d, $J = 9.0$ Hz, H-4), 7.28 (1H, dd, $J = 8.7$ Hz, H-4), 6.46 (2H, d, $J = 8.7$ Hz, H-3 and H-5), 5.41 (2H, s, H-7′), 3.89 (3H, s, -OMe), 3.75 (6H, s, –OMe). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.2 (C-7 and C-7″), 160.3 (C-2, C-6 and C-2″), 151.5 (C-3′), 139.6 (C-1′), 135.5 (C-4), 135.3 (C-4″), 132.3 (C-1″), 131.3 (C-6″), 129.3 (C-5′), 122.6 (C-6′ and C-5″), 120.8 (C-4′), 114.9 (C-3″), 103.9 (C-1, C-3 and C-5), 67.0 (C-7′), 56.5 (–OMe). HRMS (EI) for $C_{24}H_{22}O_7(M^+)$: Calc.: 422.4308. Found: 422.4311.

4.7. Bioassay

Phosphodiesterase activity was measured according to the method described by Sharma and Wang (1979) with some modifications. Bovine brain CaM (0.083 μg) was incubated with 0.015 units of CaM-deficient–CaM-dependent *c*AMP from bovine brain during 30 min in 40 μL of assay solution containing 0.063 units of 5′-nucleotidase, 45 mM Tris–HCl, 5.6 mM magnesium acetate, 45 mM imidazole, 2.5 mM calcium chloride, and 10 μM bovine serum albumin (BSA), pH 7.0. Compounds were then added to the assay medium at 1, 2, 4, 7, 13, 20, 32, 50 and 65 μM in ACN, and the samples were incubated during 30 min. Then, 10 μL of 10.8 mM *c*AMP was added to start the assay. After 15 min, the assay was stopped by the addition of 190 μL of malachite green solution. All the above steps were carried out at 30 °C. The phosphodiesterase reaction was coupled to the 5′-nucleotidase (*Crotalus atrox* venom from Sigma) reaction; the amount of inorganic phosphate released, measured spectrophotometrically at 655 nm, correlated with the activity of the PDE1. The experiments to determine the inhibition constant (K_I) were performed as described above but in the presence of four different concentrations of CaM (10, 20, 40 y 80 ng/mL). For the estimation of the K_I values a global non-linear fits of the data was performed using the following equation:

$$V_0 \frac{\left[\frac{C_T}{K_A^{APP}} + C_B\left(1 + \frac{[I]}{K_I}\right)\right]}{\left(1 + \frac{[I]}{K_I}\right) + \frac{C_T}{K_A^{APP}} + C_B\left(1 + \frac{[I]}{K_I}\right)}$$

$$C_B = \left(\frac{[\mathrm{BSA}]}{K_{\mathrm{BSA}}}\right)\left(\frac{[c\mathrm{AMP}]}{K_{c\mathrm{AMP}}}\right), \quad \text{and}$$

$$\frac{C_T}{K_A^{APP}} = K_A\left(\frac{[c\mathrm{AMP}]}{K_{c\mathrm{AMP}}}\right)$$

where C_B and K_A^{APP} are adjustable parameters. C_T is the effective total concentration of Ca^{2+}–CaM, $[I]$ is the inhibitor concentration, K_I is the inhibition constant, K_A is the dissociation constant of the Ca^{2+}–CaM–PDE1 complex, being K_{BSA} the dissociation constant for the BSA. PDE complex and K_{cAMP} the dissociation constant of *c*AMP from the Ca^{2+}–CaM–PDE1–*c*AMP complex. This equation assumes high *c*AMP concentrations relative to the K_{cAMP}. This equation considers the slight but perceptible stimulation of PDE1 by high concentrations of BSA. Such basal level of activity, in turn, dependent on the amount of free PDE1, which varies with the amount of active Ca^{2+}–CaM. Though this interference disappears in the absence of BSA, and the equation reduces to competitive inhibi-

tion, BSA is required to minimize the negative effects of organic solvents (ACN in this case) on PDE1 activity.

To measure the basal activity of PDE1, a second set of experiments were performed as described above, except that CaM was not added to the reaction mixture.

All the results are expressed as the mean of at least six experiments ± SEM. The results are expressed as IC_{50} (concentration inhibiting by 50% the activity of the enzyme) values, which were determined from the analysis of the concentration-effect curves. The concentration-response graphics were analyzed using a curve-fitting program (Microcal Origin 3.0, Origin Lab Corporation, Northampton, MA). The global fits were performed using the Sigma Stat 2.0 statistic package (Jendel Scientific, San Rafael, CA).

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