Selective Inhibition of Rat Heart cAMP Phosphodiesterases by Lipophilic *C*-Methyl-2-phenyl-4*H*-1-benzopyran-4-ones (*C*-Methylflavones)

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A series of eight methoxylated *C*-methyl-2-phenyl-4*H*-1-benzopyran-4-ones 3, 6, 10—15 was evaluated as inhibitors of rat heart cytosolic cyclic nucleotide phosphodiesterase (PDE). The 2-(3,4-dimethoxyphenyl)-5,7-dimethoxy-3,8-dimethyl-4*H*-1-benzopyran-4-one (3) and the 2-(4-methoxyphenyl)-5,7-dimethoxy-3,8-dimethyl-4*H*-1-benzopyran-4-one (10) have never been previously described. Inhibition was performed on the whole cytosolic preparation and on the four PDE isoforms after HPLC purification. The flavones 3, 6, 10, 13 and 14 were selective and potent inhibitors of the isoforms, namely ROI (rolipram-sensitive) and CGI (cGMP-sensitive) PDEs specifically hydrolyzing cAMP. The di-*C*-methylflavones 3 and 13 have been shown to be potent inhibitors of these two isoforms, with IC_{50} values in the micromolar range.

Key words *C*-methylflavone; phosphodiesterase inhibitor; structure–activity relationship; 3-methylflavone; flavonoid

Cyclic nucleotide phosphodiesterases (PDEs), the enzymes hydrolyzing cAMP and cGMP, regulate various physiological responses such as platelet aggregation,[1] vascular relaxation[2] and cardiac muscle contraction.[3] It has now become evident that at least five isoenzymes may be present, depending on tissues and species.[4] Considering heart tissue, flavonoids (a family of polyphenolic antioxidants), reported to selectively inhibit PDE from different tissues,[5-11] are known to display cardiac effects.[12,13] As an example, it has recently been reported that their presence in regularly consumed foods may reduce the risk of death from coronary heart disease in elderly men.[14] In particular, the potential cardiovascular effects of luteolin may be attributed to the inhibition of cAMP PDE.[15] Most of the flavonoids assayed for their ability to inhibit PDE were polyhydroxylated.[16] However, of the few studies concerning the inhibition of various PDE isoforms, our previous reports included flavonoids with various polyether groups which have been found to be more potent and more selective inhibitors than the corresponding polyhydroxylated equivalents.[7,8,10] Additionaly, the introduction of lipophilic substituents should facilitate the penetration of such compounds into the cell and/or increase their affinity for the plasma membrane.

Our work dealt with the inhibition of rat heart PDE isoforms by *C*-methylflavones, a group of flavonoids, which although found commonly in plants, have to date never been assayed.[17] Among the eight flavones studied, seven are methylethers of mono- or di-*C*-methylluteolin **3**, **6**,[18] **13**,[19] **14** and apigenin **10**, **12**, **15**,[20] whereas the last one, **11**[21-24] used as a reference compound, is not *C*-methylated. The synthesis of the flavones **12**—**15** is reported elsewhere.[25] Although having been previously described, we report here the synthesis of the flavone **6**, as the published melting point is not consistent with our value. The flavones **3** and **10** have never been described, nor have their corresponding diaroylmethanes, **2** and **9**. The inhibitions were evaluated on the various PDE isoforms after HPLC purification. Rat heart tissue was chosen as a starting material because it contains four of the five families of PDE isoforms, and in particular the two different subtypes of specific cAMP PDE.

Chemical Synthesis The 3-methylflavones **3**, **6** and **10** possess a phloroglucin-like A-ring with additional substitutions introduced by *O*- and *C*-methylation. They were synthesized according to the pathways outlined in the Chart 1. Methylation of **1**[26] afforded **2**, whereas methylation of **4**[27] yielded a mixture of the diaroylmethylmethanes **2** and **5** which were further dehydrated into the flavones **3** and **6**,[18] respectively. Identical treatments, applied to the mixture of **7a**, **7b**, **8a** and **8b**[25] led to the flavone **10** *via* the diaroylmethylmethane **9**. The structure of our products was established from ^{1}H-NMR, ^{13}C-NMR and MS (EI or FAB$^+$) spectral data, in agreement with literature.[28,29]

Only a few compounds belonging to the 3-methylflavone group have been previously described, having been synthesized either from propiophenone (leading directly to 3-methyl derivatives,[30]) or by methylation of diaroylmethanes.[31,32] But the latter reaction had never been performed on 2,6-dihydroxylated diaroylmethanes which, at neutral pH, exist in cyclic hemiketal forms *e.g.* 2,5-dihydroxyflavanones.[33] Consequently, we compared the methylation products of the hemiketal compound **4** and the non-hemiketal-forming diaroylmethanes **1** and the **7a**, **7b**, **8a**, **8b** mixture.[25] In the usual manner, associated with the obvious *O*-methylation, *C*-methylation occurs mostly on the methylene group located between the two carbonyl groups. As opposed to diaroylmethanes, this methylene group is not present in the 2,5-dihydroxyflavanones, except when the heterocycle becomes opened in an alkaline medium. Thus, the corresponding diaroylmethylmethanes were obtained: **2** from **1**, **5** from **4**, and **9** from the **7a**, **7b**, **8a**, **8b** mixture. Moreover, a concomitant nuclear methylation occurred but only from **4**, affording diaroylmethylmethane **2** as a minor product. It was the first time to observe a secondary A-ring nuclear methylation of diaroylmethane. This result may be closely

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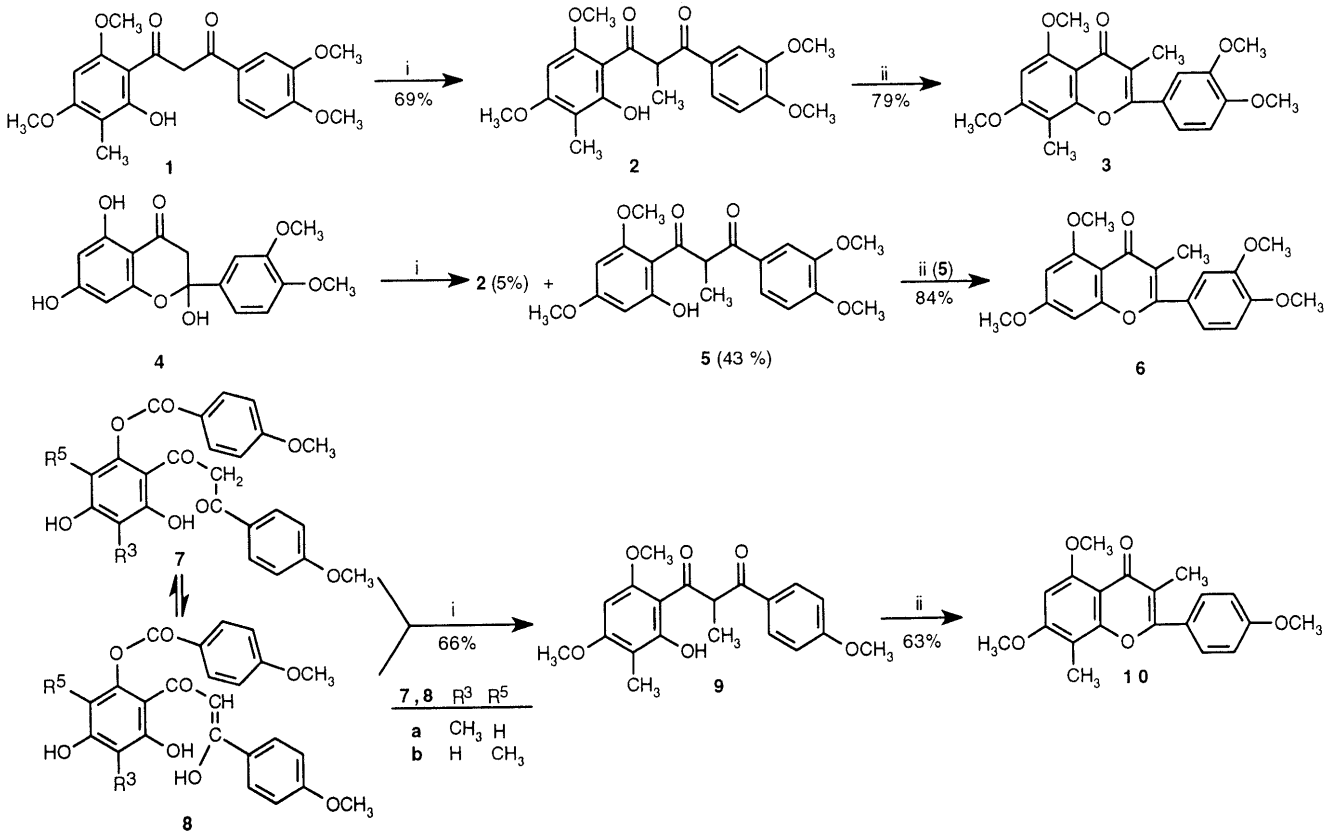
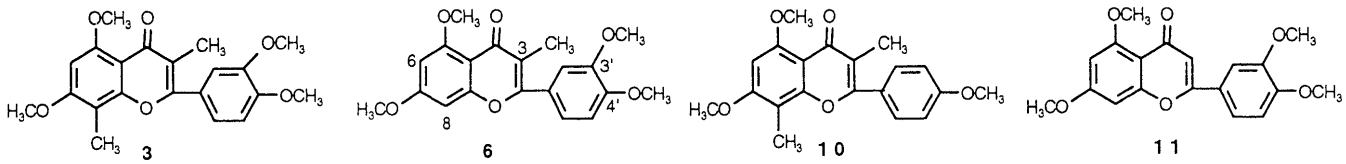


Chart 1.

i: CH_3I, anhydrous K_2CO_3, acetone, reflux, 2 h; ii: AcOH, 96% H_2SO_4 two drops, reflux, 10 min.

Table 1. Inhibition of Rat Heart PDEs by the Flavones **3**, **6**, **10** and **11**

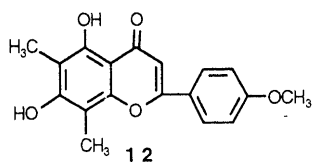


Compound	IC_{50} (μM) or percentage of inhibition at 36 μM								
	Unseparated cytosolic form			CAM PDE cGMP		CGS PDE cAMP		ROI PDE cAMP	CGI PDE cAMP
	cAMP	cGMP	A/G	+EGTA	Ca^{2+}/CAM		+cGMP		
3	1.0 (0.8—1.4)	47%	0.03	41%	37.8 (12.7—112)	29%	3.9 (1.5—9.8)	0.14 (0.03—0.64)	0.59 (0.27—1.3)
6	3.5 (2.4—5.0)	9.5 (8.2—10.9)	0.37	13.2 (10.2—16.9)	13.1 (11.5—15.0)	29%	4.5 (2.9—7.1)	1.8 (1.5—2.1)	3.1 (2.3—4.2)
10	2.9 (1.0—8.2)	39.3 (22.7—67.8)	0.07	43%	34%	18%	28.0 (15.1—52.1)	1.2 (0.6—2.6)	3.5 (0.9—14.0)
11	20.5 (14.2—29.6)	65.6 (44.3—97.0)	0.32						
Rolipram								3.3 (2.3—4.6)	237 (128—442)
Milrinone								9.6 (7.6—12.1)	1.7 (0.6—5.3)
cGMP								26%	2.1 (0.2—17.9)

linked to the phloroglucin-like substitution, as had previously been proposed for flavonol[34] and flavone[35] nuclear methylation. It is noteworthy that among the three flavones we synthesized, two are novel compounds, **3** and **10**, bearing two *C*-methyl groups at both the 3- and 8-positions, a type of substitution which had never been found either in natural or in synthetic flavones.

Inhibition Study The inhibition of PDE by the eight flavones **3**, **6**, **10**—**15** was studied on the crude rate heart cytosolic fraction and on the purified isoforms.

Table 2. Inhibition of Rat Heart PDEs by the Flavones **12**—**15**



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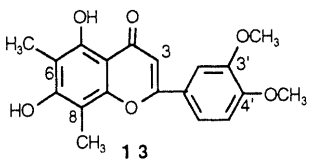

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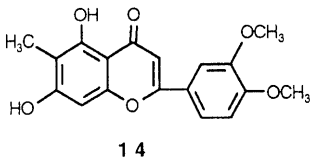

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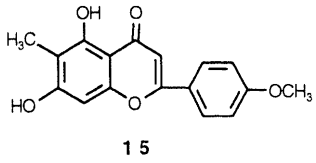

15

Compound	IC_{50} (μM) or percentage of inhibition at 36 μM								
	Unseparated cytosolic form			CAM PDE cGMP		CGS PDE cAMP		ROI PDE cAMP	CGI PDE cAMP
	cAMP	cGMP	A/G	+EGTA	Ca^{2+}/CAM	+cGMP			
12	12%	36%							
13	7.1 (3.2—15.7)	5.0 (3.5—7.0)	1.42	1.3 (0.4—4.1)	4.6 (2.3—9.3)	0%	14.3 (6.6—31.1)	1.3 (1.2—1.4)	0.6 (0.4—1.2)
14	17.5 (12.9—23.7)	28.0 (19.2—40.9)	0.63	7.2 (4.6—11.2)	15.5 (7.5—32.2)	0%	39.7 (21.5—73.3)	5.7 (4.2—7.7)	6.1 (3.6—10.4)
15	0%	10%							
Rolipram								3.3 (2.3—4.6)	237 (128—442)
Milrinone								9.6 (7.6—12.1)	1.7 (0.6—5.3)
cGMP								26%	2.1 (0.2—17.9)

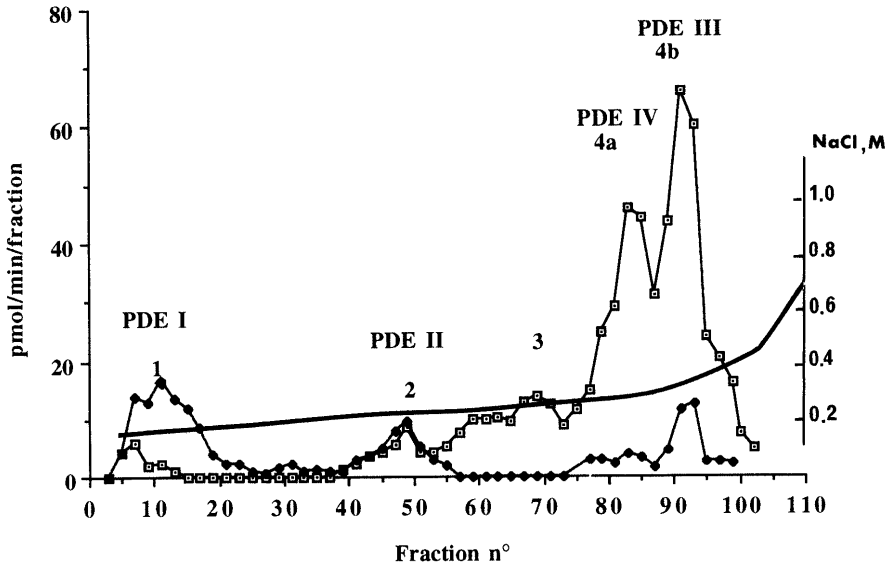

Fig. 1. HPLC Profile of Phosphodiesterase Activities in the Soluble Fraction of Rat Ventricle Eluted from Mono-Q Column by NaCl Gradient

The phosphodiesterase activity was assayed with 0.25 mM cAMP (▣) or cGMP (■) as a substrate.

Inhibition of the Cytosolic Fraction: With this crude preparation, some structurally-dependent differences could be observed (Tables 1 and 2). The flavones **11**, **12** and **15** were poor inhibitors of cAMP or cGMP PDE activities. Of the five other ones, the partially methoxylated flavone **14** was the least effective, whereas the fully methoxylated **3** and **10** appeared to be strong and selective inhibitors, as demonstrated by the IC_{50} cAMP/IC_{50} cGMP ratios far lower than 1.

Inhibition of the Isolated Isoforms: Figure 1 shows the profile of the HPLC purification of the crude cytosolic fraction from which four distinct peaks could be derived.[4,36] The cGMP hydrolyzing activity of peak 1, being stimulated by exogenous Ca^{2+}-calmodulin, was thus used as a source of PDE I (CAM PDE). Peak 2 hydrolyzed both cAMP and cGMP; 5 μM cAMP hydrolysis was stimulated by the addition of 5 μM cGMP. This peak was used as a source of PDE II (CGS PDE). The activity eluted in peaks 3 and 4 preferentially hydrolyzed cAMP. Peaks 3 and 4a were sensitive to rolipram and far less sensitive to cGMP inhibition, whereas peak 4b was markedly inhibited by cGMP (IC_{50} = 2.1 μM) but was insensitive to rolipram (IC_{50} = 237 μM). Alternatively, peak 4b was more sensitive to milrinone (IC_{50} = 1.7 μM) than peak 4a (IC_{50} = 9.6 μM). Thus, peaks 4a and 4b were used as a source of PDE IV [rolipram-sensitive (ROI PDE)] and PDE III [cGMP-sensitive (CGI PDE)], respectively.

Inhibition by the five flavones **3**, **6**, **10**, **13**, **14**, active on crude cytosolic PDE activity, was examined on each

isolated fraction. The results confirmed the preliminary ones obtained with the crude extract. Whereas the flavones **3** and **10** did not inhibit CAM PDE activity (peak 1), in the experimental concentration range, the other three (**6**, **13** and **14**) inhibited this activity (stimulated or not) in a similar manner, suggesting that these compounds have no anti-calcium properties. Such a result had previously been obtained from rat heart PDE isoforms with another series of *O*-substituted flavonoids,[8] but in this case, anti-calmodulin activity was demonstrated on the activities of the rat brain isoforms.[10] In our present study, the flavone **13** was the most effective compound for PDE I inhibition. Considering the CGS PDE of peak 2, none of the five flavones had any effect, except in presence of cGMP, suggesting an interaction with the allosteric site rather than with the catalytic one. The flavones **3** and **6** were the most potent inhibitors, with IC_{50} values of 3.9 μM and 4.5 μM, respectively. The activities of both cAMP specific isoforms, ROI and CGI PDEs (peaks 4a and 4b), were greatly affected by the five above-mentioned flavones with IC_{50} values ranging from 0.14 to 6.1 μM. Amongst these, the flavone **3** proved to be even more potent than rolipram, with an IC_{50} value of 0.14 μM for the inhibition of the ROI PDE isoform. In conclusion, with the exception of flavone **13**, which is a potent inhibitor of all PDE isoforms, but without any marked selectivity, the flavones studied inhibited Ca^{2+}-dependent enzyme activity (PDE I) less efficiently than the Ca^{2+}-independent ones (PDE II, III and IV).

Some important structural features have to be underlined. Firstly, the influence of the 3′-methoxy group appears to be very complex. Comparison of the inhibitory activity of flavone **12** with respect to **13** shows that the loss of this methoxy substituent drastically reduces the potency to inhibit each form of PDE. Similarly, the flavone **15** does not inhibit PDE activities, whereas **14** is quite efficient. In contrast, the loss of this 3′-methoxy group in the case of the flavone **3** leading to **10**, only induces a moderate increase in IC_{50} values for the inhibition of ROI and CGI PDEs, with flavone **10** actually appearing to be the more specific of these isoforms. Secondly, when examining the influence of the 8-*C*-methyl group, its loss, concomitant with the loss of another substituent (flavones **6** and **14** compared with **11** and **15**), dramatically decreases (for **11**) or fully abolishes (for **15**) the inhibition. This was not the case for the flavones **3** and **13**, as the flavones **6** and **14** still possess IC_{50} values in the micromolar range. All these above-mentioned observations suggest that the less substituted flavones (**11**, **12** and **15**) are rather inactive, whereas the hexasubstituted flavones (**3** and **13**) are potent inhibitors of the PDE isoforms. It may also be noted that the flavone **3**, with a *C*-methyl group at the 3-position and four methoxy groups, is a more potent and selective inhibitor of the ROI and CGI PDEs than the flavone **13** with only two methoxy groups and a *C*-methyl group at the 6-position. In contrast, analogues of **3** (Table 1) with changes in substitution were quite unaffected by these modifications, for example, comparing **10** with **12** and **6** with **14**. Methylation of all hydroxy groups together with the presence of a *C*-methyl group at the 3-position seems to be the more favorable structural condition for the inhibition of each form of PDE.

Detailed comparison of our results with previous reports is difficult, because only few studies have been devoted to the selective inhibition of purified isoforms. We have previously reported the inhibitory potency of *O*-substituted flavonoids towards the various PDE isoforms present in rat heart[8] and rat brain[10] isolated by isoelectric fractionation. In the case of quercetin derivatives we arrived at similar conclusions. Indeed, the highest selectivity for cAMP hydrolysis inhibition was observed with fully substituted quercetin when substituents were methyl or ethyl groups. It is noteworthy that the introduction of *C*-methyl substituents helped maintain the inhibition specificity since our present results are in agreement with those previously reported, showing that the flavonoids preferentially inhibit the cAMP-specific PDE isoforms.[5-9,15] Thus, among the five *C*-methylflavones we assayed, **3** and **13** were highly potent inhibitors of the ROI and CGI PDE, with **3** showing a weak selectivity for the ROI PDE.

The discovery of potent inhibitors of these two cAMP specific PDE isoforms should allow a better understanding of their role in cardiac diseases. Indeed, in cardiac tissue, as CGI PDE inhibitors exert a positive inotropic effect, CGI PDE may be considered a molecular target for some cardiotonic drugs. In contrast, ROI PDE inhibitors were reported to increase cAMP level without producing any inotrope effects. However Muller *et al.*[37] have shown that in the presence of cAMP-dependent positive inotropic agents, PDE IV inhibitors also induce a positive inotropic effect. This suggests that ROI PDE may play an important role in the regulation of cardiac contraction under physiological conditions. The potent and specific inhibition of both cAMP specific isoenzymes could partly explain the cardiac effects of some flavonoids, as previously reported.[12,13]

Experimental

Melting points were measured with a Leitz melting point apparatus and are uncorrected. TLC were carried out on Merck F_{254} silica gel plates. ^{1}H-NMR spectra were recorded at 300 MHz, in DMSO-d_6 or $CDCl_3$ solution with $SiMe_4$ as an internal standard, on an AM300 Bruker spectrometer. Chemical shifts were expressed in δ-scale (ppm) and J values in Hz (J_o, J_m, J_{vic}=*ortho*, *meta*, *vicinal* couplings). ^{13}C-NMR spectra were recorded in DMSO-d_6 (internal standard) at 50 MHz on an AC200 Bruker spectrometer. Fast-atom bombardment (FAB^+) and electron impact (EI, 70 eV) mass spectra were recorded on VG ZAB2-SEQ and VG305 spectrometers, respectively.

1-(2-Hydroxy-4,6-dimethoxy-3-methylphenyl)-3-(3,4-dimethoxyphenyl)-2-methylpropane-1,3-dione (2) To a solution of **1**[26] (1.25 g, 3.3 mmol) in anhydrous acetone (20 ml) was added CH_3I (2 ml, 33 mmol) and anhydrous K_2CO_3 (2.6 g, 19 mmol). The mixture was refluxed for 2 h, cooled and filtered off. The salts were washed with acetone and all the acetonic fractions were concentrated to dryness. The solid residue obtained, washed with H_2O, recrystallized from benzene and then AcOEt, yielded the title compound (0.9 g, 69%). On the other hand, the benzene mother liquors of **5** were concentrated to dryness. The residue, recrystallized from AcOEt, gave the title compound (0.02 g, 5%) after recrystallizations from benzene and then AcOEt, mp 185—187 °C. *Rf* 0.44 (benzene–acetone–hexane, 7:2:1). UV λ_{max}^{EtOH} nm (log ε): 220 (sh), 227 (3.34), λ_{min}: 248 (2.67). FAB^+-MS *m/z*: 389 (MH^+). ^{1}H-NMR ($CDCl_3$) δ: 1.50 (d, 3H, J_{vic} =7.0 Hz, CH–C<u>H</u>$_3$), 2.01 (s, 3H, 3-CH_3), 3.42, 3.85, 3.92, 3.97 (4s, 12H, 3′,4,4′,6-OCH_3), 5.34 (q, 1H, J_{vic}=7.0 Hz, C<u>H</u>–CH_3), 5.85 (s, 1H, 5-H), 6.94 (d, 1H, J_o=8.4 Hz, 5′-H), 7.58 (d, 1H, J_m=2.0 Hz, 2′-H), 7.63 (dd, 1H, J_o=8.4 Hz, J_m=2.0 Hz, 6′-H), 13.80 (s, 1H, 2-OH, H/D exchange with D_2O). *Anal.* Calcd for $C_{21}H_{24}O_7$: C,

64.93; H, 6.23. Found: C, 64.90; H, 6.26.

2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-3,8-dimethyl-4*H*-1-benzopyran-4-one (3) A solution of **2** (0.2 g, 0.5 mmol) in AcOH (2 ml) and two drops of 96% H_2SO_4 was refluxed for 10 min. After dilution, the solution was cooled at 0 °C for 1 h. The white floculent precipitate obtained, filtered off and washed with H_2O, yielded the title compound (0.15 g, 79%) after recrystallizations from AcOH and then benzene, mp 217—218 °C. *Rf* 0.41 ($CHCl_3$–MeOH, 8:2). UV λ_{max}^{EtOH} nm (log ε): 248 (sh), 261 (4.20), λ_{min}: 236 (4.33), 285 (4.11). EI-MS *m/z* (rel. int. %): 371 (23), 370 (M^+, 100), 369 (47), 351 (24), 340 (25), 324 (17), 171 (20). ^{1}H-NMR ($CDCl_3$) δ: 1.98 (s, 3H, 8-CH_3), 2.13 (s, 3H, 3-CH_3), 3.81, 3.83, 3.87, 3.94 (4s, 12H, 3′,4′,5,7-OCH_3), 6.61 (s, 1H, 6-H), 7.11 (d, 1H, J_o=8.4 Hz, 5′-H), 7.27 (d, 1H, J_m=2.0 Hz, 2′-H), 7.28 (dd, 1H, J_o=8.4 Hz, J_m=2.0 Hz, 6′-H). ^{13}C-NMR (DMSO-d_6) δ: 7.6 (8-CH_3), 11.5 (3-CH_3), 55.6, 55.7 (5,7-OCH_3), 56.0 (3′,4′-OCH_3), 92.3 (C6), 104.0 (C8), 106.6 (C10), 11.4 (C2′), 112.1 (C5′), 115.7 (C3), 122.0 (C6′), 125.3 (C1′), 148.4 (C3′), 150.3 (C4′), 155.6 (C5), 157.4 (C9), 158.4 (C7), 160.7 (C2), 176.3 (C4). *Anal.* Calcd for $C_{21}H_{22}O_6$: C, 68.09; H, 5.99. Found: C, 68.06; H, 6.01.

1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(3,4-dimethoxyphenyl)-2-methylpropane-1,3-dione (5) A solution of **4**[27] (0.4 g, 1.2 mmol) in anhydrous acetone (10 ml), CH_3I (0.75 ml, 12 mmol) and anhydrous K_2CO_3 (1 g, 7.2 mmol), treated as described for the preparation of **2**, yielded the title compound (0.195 g, 43%) after recrystallizations from benzene and then AcOEt, mp 221—222 °C. *Rf* 0.52 (benzene–acetone–hexane, 7:2:1). UV λ_{max}^{EtOH} nm (log ε): 227 (5.95), 290 (4.39), λ_{min}: 254 (3.62). FAB$^+$-MS *m/z*: 375 (MH^+). ^{1}H-NMR ($CDCl_3$) δ: 1.50 (d, 3H, J_{vic}=7.0 Hz, CH-C<u>H</u>$_3$), 3.36, 3.80, 3.92, 3.97 (4s, 12H, 3′,4,4′,6-OCH_3), 5.34 (q, 1H, J_{vic}=7.0 Hz, C<u>H</u>-CH_3), 5.82, 6.09 (2d, 2H, J_m=2.4 Hz, 3,5-H), 6.94 (d, 1H, J_o=8.4 Hz, 5′-H), 7.57 (d, 1H, J_m=2.4 Hz, 2′-H), 7.62 (dd, 1H, J_o=8.4 Hz, J_m=2.4 Hz, 6′-H), 13.90 (s, 1H, 2-OH, H/D exchange with D_2O). *Anal.* Calcd for $C_{20}H_{22}O_7$: C, 64.16; H, 5.92. Found: C, 64.11; H, 5.95.

2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-3-methyl-4*H*-1-benzopyran-4-one (6) A solution of **5** (0.2 g, 0.53 mmol) in AcOH (2 ml) and 2 drops of 96% H_2SO_4, treated as described for the preparation of **3**, yielded the title compound (0.16 g, 84%) after recrystallizations from AcOEt and then benzene, mp 185—187 °C (lit.[18] mp 167 °C). *Rf* 0.60 ($CHCl_3$–MeOH, 8:2). UV λ_{max}^{EtOH} nm (log ε): 240 (4.39), 256 (sh), 315 (4.22), λ_{min}: 274 (4.02). EI-MS *m/z* (rel. int. %): 357 (2), 356 (M^+, 100), 341 (11), 339 (10), 337 (24), 327 (19), 325 (28), 310 (18), 164 (18). ^{1}H-NMR ($CDCl_3$) δ: 2.11 (s, 3H, 3-CH_3), 3.88, 3.95, 3.96 (4s, 12H, 3′,4′,5,7-OCH_3), 6.35 (d, 1H, J_m=2.0 Hz, 6-H), 6.45 (d, 1H, J_m=2.0 Hz, 8-H), 6.97 (d, 1H, J_o=8.3 Hz, 5′-H), 7.13 (d, 1H, J_m=2.0 Hz, 2′-H), 7.21 (dd, 1H, J_o=8.3 Hz, J_m=2.0 Hz, 6′-H). *Anal.* Calcd for $C_{20}H_{20}O_6$: C, 67.40; H, 5.66. Found: C, 67.35; H, 5.61.

1-(2-Hydroxy-4,6-dimethoxy-3-methylphenyl)-3-(4-methoxyphenyl)-2-methylpropane-1,3-dione (9) A mixture of **7a**, **7b**, **8a**, **8b**[25] (0.05 g, 0.11 mmol) dissolved in anhydrous acetone (3 ml), CH_3I (0.07 ml, 1.1 mmol) and anhydrous K_2CO_3 (0.09 g, 0.65 mmol), treated as described for the preparation of **2**, furnished the title compound (0.026 g, 66%) after two recrystallizations from benzene, mp 172—173 °C. *Rf* 0.64 (benzene–acetone–hexane, 9:1:1). UV λ_{max}^{EtOH} nm (log ε): 238 (sh), 284 (4.55), λ_{min}: 244 (4.00). FAB$^+$-MS *m/z*: 359 (MH^+). ^{1}H-NMR (DMSO-d_6) δ: 1.30 (d, 3H, J_{vic}=7.0 Hz, CH-C<u>H</u>$_3$), 1.91 (s, 3H, 3-CH_3), 3.35, 3.86, 3.87 (3s, 9H, 4,4′,6-OCH_3), 5.53 (q, 1H, J_{vic}=7.0 Hz, C<u>H</u>-CH_3), 6.11 (s, 1H, 5-H), 7.11 (2d, 2H, J_o=8.8 Hz, 3′,5′-H), 8.02 (2d, 2H, J_o=8.8 Hz, 2′,6′-H), 13.88 (s, 1H, 2-OH, H/D exchange with D_2O). *Anal.* Calcd for $C_{20}H_{22}O_6$: C, 67.02; H, 6.19. Found: C, 66.99; H, 6.15.

2-(4-Methoxyphenyl)-5,7-dimethoxy-3,8-dimethyl-4*H*-1-benzopyran-4-one (10) A solution of **9** (0.05 g, 0.14 mmol) in AcOH (4 ml), treated as described for the preparation of **3**, yielded the title compound (0.03 g, 63%) after two recrystallizations from AcOH, mp 172—173 °C. *Rf* 0.57 ($CHCl_3$–MeOH, 8:2). UV λ_{max}^{EtOH} nm (log ε): 261 (4.49), λ_{min}: 241 (4.18). FAB$^+$-MS *m/z*: 341 (MH^+). ^{1}H-NMR (DMSO-d_6) δ: 1.96 (s, 3H, 8-CH_3), 2.11 (s, 3H, 3-CH_3), 3.83, 3.87, 3.93 (3s, 9H, 5,7,4′-OCH_3), 6.60 (s, 1H, 6-H), 7.10 (2d, 2H, J_o=8.8, 3′,5′-H), 7.67 (2d, 2H, J_o=8.8, 2′,6′-H). ^{13}C-NMR (DMSO-d_6) δ: 7.7 (8-CH_3), 11.5 (3-CH_3), 55.4, 56.1 (4′,5,7-OCH_3), 92.5 (C6), 104.6 (C8), 106.6 (C10), 114.0 (C3′,5′), 115.6 (C3), 125.2 (C1′), 130.4 (C2′,6′), 155.6 (C5), 157.3 (C9), 158.5 (C7), 160.5 (C4′), 160.7 (C2), 176.2 (C4). *Anal.* Calcd for $C_{20}H_{20}O_5$: C, 70.57; H, 5.92. Found: C, 70.51; H, 5.94.

Tissue Preparation Hearts from male Sprague Dawley rats weighing 250—300 g were perfused with 0.15 M NaCl through the aorta to remove blood. The ventricles were minced and homogenized in a glass–glass Potter–Elvejhem homogenizer with 5 volumes (v/w) of 10 mM Tris–HCl buffer (pH 7.5) containing 0.32 M sucrose, 1 mM EDTA, 5 mM dithiothreitol and 0.1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was then centrifuged at 105000 × *g* for 1 h and stored at −75 °C until injection on the HPLC column.

HPLC Resolution of Cyclic Nucleotide PDE 105000 × *g* supernatant fractions from the ventricle (12 mg prot. in 4 ml) were applied to a Mono Q HPLC column which had been previously equilibrated with buffer A (50 mM Tris, 2 mM EDTA, 14 mM mercaptoethanol and 0.1 mM PMSF). PDE activity was then eluted at 1 ml/min using step-by-step and linear gradients of NaCl in buffer A (15 ml 0.16 M NaCl, 30 ml from 0.16 to 0.23 M NaCl, 5 ml 0.23 M NaCl, 30 ml from 0.23 to 0.29 M NaCl, 5 ml 0.29 M NaCl, 15 ml from 0.29 to 0.50 M NaCl and 15 ml from 0.5 to 1 M NaCl). Fractions (1 ml) were collected and kept frozen at −75 °C with 20% glycerol. The separation was performed at 4 °C.

PDE Assay PDE activity was assayed by a two-step radioisotopic method modified by Prigent *et al.*[38] with the following substrate conditions: 0.25 μM cAMP which reflects mainly the activity of cAMP PDE isoforms, 0.25 μM cGMP in the presence of either 1 mM EGTA or 100 U calmodulin plus 1 mM $CaCl_2$, which reflects the activity of the calmodulin-stimulated isoform. The cGMP-stimulated PDE activity was measured with 5 μM cAMP as a substrate in the presence or absence of 5 μM cGMP as an allosteric effector. PDE activities were expressed as pmol of hydrolyzed cyclic nucleotides per min per fraction. Flavones were dissolved in DMSO at a final concentration of 1% which had no significant effect on any of the PDE activities. IC_{50} values were calculated by plotting the percentage of the residual enzymatic activity *versus* the logarithmic concentration of each flavone. (95% Confidence limits were determined by linear regression analysis according to the method of Hubert.[39]) Each measurement was performed three times, with a standard error lower than 10%.

Materials [8-^{3}H]cAMP (28 Ci/mmol), [8-^{3}H]cGMP (15 Ci/mmol) and [U^{14}C]adenosine (571 mCi/mmol) were obtained from Amersham Les Ullis, France. [U^{14}C]Guanosine (528 mCi/mmol) was from Du Pont de Nemours, Paris, France. Unlabelled cyclic nucleotides, 5′-nucleotidase (Ophiophagus hannah venom) and milrinone were from Sigma, L'Isle d'Abeau, France. Rolipram was a gift from Schering, Berlin, Germany.

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