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Synthesis, docking study and relaxant effect of 2-alkyl and 2-naphthylchromones on rat aorta and guinea-pig trachea through phosphodiesterase inhibition

Fernando Rodríguez-Ramos [a,*], Andrés Navarrete [b], Martín González-Andrade [c], Carlos Alarcón [a], Alejandro Aguilera-Cruz [a], Adelfo Reyes-Ramírez [a,d,*]

[a] Unidad Multidisciplinaria de Investigación Experimental, Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, México DF 09230, Mexico
[b] Facultad de Química, Departamento de Farmacia. Universidad Nacional Autónoma de México, México DF 04510, Mexico
[c] Instituto Nacional de Medicina Genómica, Secretaria de Salud, México DF 14610, Mexico
[d] Instituto de Química, Universidad Nacional Autónoma de México, México DF 04510, Mexico

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ABSTRACT

Chromone (**4**), which form the base structure of various flavonoids isolated as natural products, is capable of relaxing smooth muscle. This is relevant to the treatment of high blood pressure, asthma and chronic obstructive pulmonary disease. The former disorder involves the contraction of vascular smooth muscle (VSM), and the latter two bronchoconstriction of airway smooth muscle (ASM). One of the principal mechanisms by which flavonoids relax muscle tissue is the inhibition of phosphodiesterases (PDEs), present in both VSM and ASM. Therefore, a study was designed to analyze the structure–activity relationship of chromone derivatives in vaso- and bronchorelaxation through the inhibition of PDE. Docking studies showed that these chromones bind at the catalytic site of PDEs. Consequently, we synthesized analogs of chromones substituted at position C-2 with alkyl and naphthyl groups. These compounds were synthesized from 2-hydroxyacetophenone and acyl chlorides in the presence of DBU and pyridine, modifying the methodology reported for the synthesis of 3-acylchromones by changing the reaction temperature from 80 to 30 °C and using methylene chloride as solvent, yielding the corresponding phenolic esters **10a**–**10h**. These compounds were cyclized with an equivalent of DBU, pyridine as solvent, and heated at reflux temperature, yielding the chromones **11a**–**11h**. Evaluation of the vasorelaxant effect of **4**, **11a**–**11h** on rat aorta demonstrated that potency decreases with branched alkyl groups. Whereas the EC_{50} of compound **11d** (substituted by an *n*-hexyl group) was 8.64 ± 0.39 μM, that of **11f** (substituted by an isobutyl group) was 14.58 ± 0.64 μM. Contrarily, the effectiveness of the compound is directly proportional to the length of the alkyl chain, as evidenced by the increase in maximal effect of compound **11c** versus **11d** (66% versus 100%) and **11e** versus **11f** (60% versus 96%). With an aromatic group like naphthyl as the C-2 substituent, the effectiveness was only 43%. All compounds tested on guinea pig trachea showed less than 55% effectiveness. Compounds **4**, **11a**–**11h** were evaluated as PDE inhibitors *in vitro*, with **11d** showing the greatest effect (73%), corroborating the importance of a long alkyl chain, which inhibits the decomposition of cGMP. Docking studies showed that the compound **11d** was selective for the inhibition of PDE-5.

1. Introduction

Flavonoids have shown important pharmacological activity as relaxant agents of vascular smooth muscle (VSM) [1] and airway smooth muscle (ASM) [2]. One of the pharmacological action mechanisms reported for these compounds is via inhibition of phosphodiesterase (PDE) [2,3]. These effects are important in the treatment of diseases involving VSM contraction, such as arterial hypertension [3,4], as well as disorders involving ASM contraction, such as asthma and chronic obstructive pulmonary disease (COPD) [2,4]. Whereas the use of PDE-4 inhibitors is well-documented as a therapeutic alternative in the treatment of asthma and COPD [5], it is still under study for VSM contraction. There is a remarkable interest in the potential use of PDE-5 inhibitors in combination with others drugs for the treatment of systemic hypertension [6].

* Corresponding authors. Address: Facultad de Estudios Superiores Zaragoza. Unidad Multidisciplinaria de Investigación Experimental. Universidad Nacional Autónoma de México. Batalla 5 de Mayo esquina Fuerte de Loreto, Ejército de Oriente, Iztapalapa 09230, México D.F. Mexico. Fax: +52 57736330.
E-mail addresses: dr.frodriguezr@comunidad.unam.mx (F. Rodríguez-Ramos), adelfo@puma2.zaragoza.unam.mx (A. Reyes-Ramírez).

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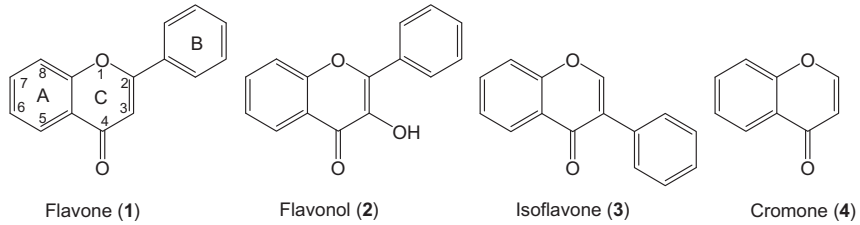


Fig. 1. Structure of some flavonoids related with relaxant effect on ASM and VSM smooth muscle and PDE inhibition.

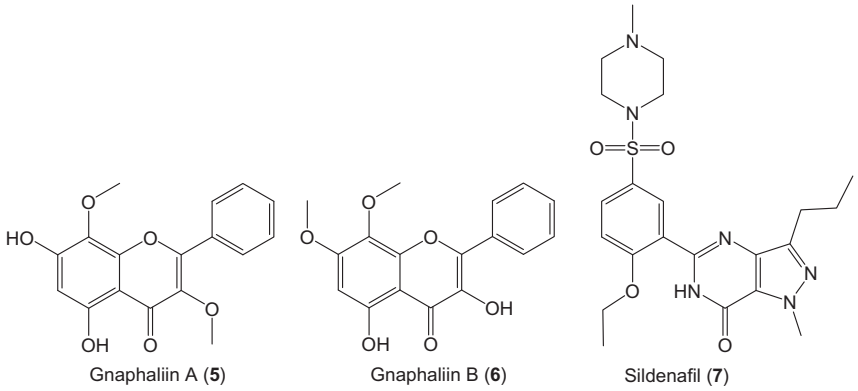


Fig. 2. Some representative examples of selective PDE-5 inhibitors.

with special interest in the treatment of pulmonary arterial hypertension [7].

It has been reported that the flavonoids, including flavones (**1**), flavonoles (**2**) and isoflavones (**3**), have a relaxant effect on smooth muscle. The chemical structure of these compounds is related to chromone (**4**), formed by the A and C rings of flavones (**1**) (Fig 1). Although the synthesis of chromones has been reported in multiple works by easy and efficient methods [8–10], the ability of these compounds to relax VSM or ASM has been little studied. In a previous study we isolated the flavones gnaphaliin A (**5**) and gnaphaliin B (**6**) from *Gnaphalium liebmannii* as vasorelaxant molecules in rat aorta (Fig 2). According to the docking study, these flavones bind at the catalytic site of phosphodiesterase type 5 through an unsubstituted aromatic ring at position C-2 of the chromone [4]. Computational studies also show that sildenafil (**7**; PDE-5 inhibitor) [5] interacts in the same catalytic site with a propyl group in position C-3 of the pyrazole [4,3-*d*]pyrimidine ring (Fig 2) [4]. Therefore, we synthesized analogs of chromones substituted only at position C-2 with alkyl and naphthyl groups to evaluate their relaxing effect on VSM and ASM. Through the inhibition of PDE and docking studies we explored the inhibitory activity of PDE-3, PDE-4 and PDE-5 by test compounds.

2. Results and discussion

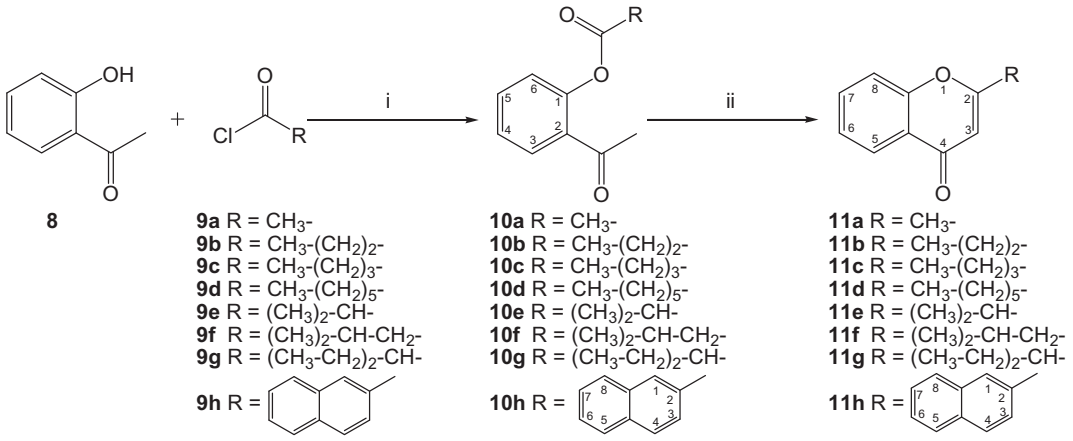
2.1. Chemistry

With the goal of comparing the biological effect of the substituted and the unsubstituted structures, we synthetized chromone (**4**) by the same route previously reported [11]. The chromones substituted at the C-2 position were synthesized from 2-hydroxyacetophenone and acyl chlorides in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and pyridine, as was reported by Ganguly et al. [9] They obtained 3-acyl-chromones in one step without obtaining the rearrangement of Baker–Venkataraman, yielding compound 1,3-diketone, followed by subsequent cyclization under heating with concentrated sulfuric acid. In this study we modified the reaction conditions by reducing the temperature from 80 to 30 °C and using methylene chloride as solvent, which yielded the corresponding phenolic esters **10a**–**10h**. These compounds were cyclized through the use of an equivalent of DBU, pyridine as solvent and reflux, obtaining chromones **11a**–**11h**. Like the previous methodology, this process showed no rearrangement of Baker–Venkataraman (see Scheme 1).

2.2. Vasorelaxant activity of C-2 substituted chromones in rat aorta

Chromones **4** and **11a**–**11h** were tested for their vasorelaxant activity in rat aortic rings pre-contracted with norepinephrine (0.1 μM) [4]. Fig. 3, shows the concentration–response curves in which only **11d** and **11f** efficiently relaxed the tissue, with EC_{50} values of 8.64 ± 0.39 μM (r^2 = 0.902) and 14.58 ± 0.64 μM (r^2 = 0.907), respectively. Drugs used as reference were sildenafil [a PDE-5 inhibitor; EC_{50} = 0.46 ± 0.01 μM (r^2 = 0.946)] [5,12], rolipram [a PDE-4 inhibitor; EC_{50} = 3.54 ± 0.06 μM (r^2 = 0.926)] [5], enoximone [a PDE-3 inhibitor; EC_{50} = 0.92 ± 0.03 μM (r^2 = 0.956)] [2], and aminophylline [an unspecific PDE inhibitor EC_{50} = 4.46 ± 0.41 μM (r^2 = 0.911)] [13]. Table 1 shows the percent of maximal relaxation at a concentration of 100 μM for all compounds tested.

Structure–activity analysis of the vasorelaxant effect was carried out for compounds **4** and **11a**–**11h**. Compound **4**, the base structure of this study, showed a vasorelaxant effect of only 29%. Thus this effect was assigned to the chromone structure. When we added a methyl group at position C-2 (**11a**), the vasorelaxant effect was of 42%. The higher relaxing effect for compound **11a** likely owes itself to the substitution of the C-2 position of chromone structure with an alkyl group. Whereas the substitution of the C-2 position with an *n*-propyl group (**11b**) did not lead to any significant difference in vasorelaxation, substitution with an *n*-butyl group (**11c**) did indeed increase the effect to 66%. Compound **11d** (with 6 atoms of carbon in the alkyl chain), increased



Scheme 1. Reagents and conditions: (i) DBU, Pyridine, CH_2Cl_2, 30 °C, 6 h; and (ii) DBU, Pyridine, reflux, 12 h.

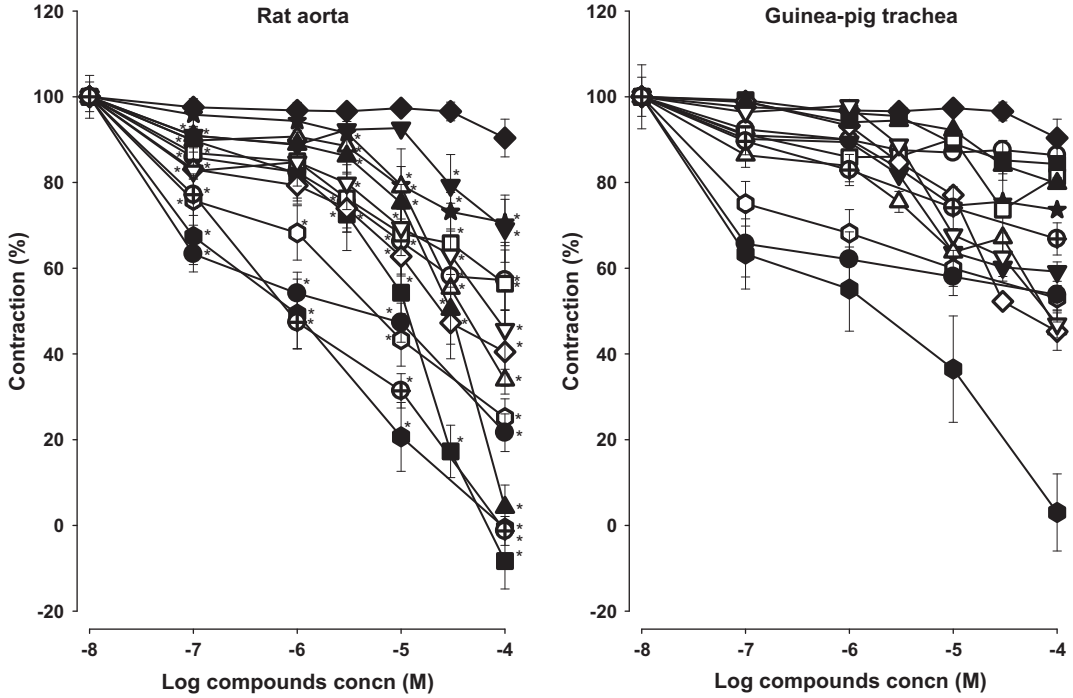


Fig. 3. Relaxant effect on smooth muscle of compounds **4** (★), **11a** (○), **11b** (▼), **11c** (△), **11d** (■), **11e** (◇), **11f** (▲), **11g** (▽),**11h** (□), sildenafil (⬢), rolipram (●), enoximone (⊕), aminophylline (⬡) and control (⧫). Each point represents the mean ± SEM, $n = 6$. $^{*}p < 0.05$, significantly different compared with the respective control at the same time (Dunnett's *t*-test after analysis of variance).

the effect to 100%, meaning that a greater length of this chain at C-2 promotes the vasorelaxant effect of the chromone. With the non-linear alkyl substituent at C-2 in **11e** and **11g**, the vasorelaxant effect was 59% and 54%, respectively. Although the effects of **11e** and **11g** were not significantly different from each other or with respect to the effect of **11c**, they were more effective than **4**, **11a** and **11b**. Therefore we decided to increase the chain length of the isopropyl carbon of **11e** to obtain **11f**, which increased the effect to 95%, representing the same level of relaxation as that induced by **11d**. However **11d** was 1.6 times more potent than **11f**, and sildenafil was 18 times more potent than the compound **11d**.

Considering the structure–activity studies of the vasorelaxant effect of flavonoids [2,3,14], we decided to synthetize compound **11h**, substituted with a naphthyl group at the C-2 position. This group is aromatic and more voluminous than the benzene ring in flavonoids. Compound **11h** at 100 μM showed a relaxant effect of 43%, compared to 29% by compound **4** at the same concentration. Ajay reported [14] that **1** induces about 70% of relaxation at a concentration of 100 μM, and that 100% was achieved at 300 μM. Thus there is a remarkable difference in activity between compound **4** with an unsubstituted chromone structure, compound **11h** with the chromone structure substituted by naphthyl group at the C-2 position, and compound **1** with the chromone structure substituted by a phenyl group at the C-2 position. Among these three structures, the best was that of compound **1**, which was less effective than **11d** and **11f**. The latter compounds are substituted by an alkyl group. These results demonstrate that the chromones **4** and **11a**–**11h** are molecules whose vasorelaxant effectiveness and potency varies according to the modification of the alkyl substituent at position C-2 on the aromatic ring.

Table 1
Relaxant effects of compounds synthetized and phosphodiesterase inhibitors on VSM and ASM.

Compound (100 μM)	Maximal relaxation in smooth muscle (%)	
	Rat aorta	Guinea-pig trachea
4	29.33 ± 5.40	31.39 ± 2.86
11a	42.78 ± 7.05	15.11 ± 1.14
11b	30.77 ± 7.83	40.78 ± 2.26
11c	66.09 ± 1.37	54.17 ± 0.71
11d	108.31 ± 6.52	15.68 ± 1.19
11e	59.52 ± 9.83	54.78 ± 4.36
11f	95.79 ± 5.22	20.21 ± 1.32
11g	54.28 ± 9.27	52.99 ± 3.28
11h	43.62 ± 9.66	18.65 ± 3.34
Sildenafil	100.63 ± 1.40	96.97 ± 8.99
Rolipram	78.37 ± 4.39	46.19 ± 6.34
Enoximone	101.28 ± 3.37	33.15 ± 3.75
Aminophylline	74.84 ± 4.36	46. 97 ± 2.95

Percent values are presented as the mean ± SEM of six experiments.

2.3. Bronchorelaxant activity of C-2 substituted chromones in guinea-pig trachea

A relaxing effect higher than 50% on ASM isolated from guinea pig trachea (Fig. 3) was not achieved by any of the following compounds at 100 μM: **4**, **11a**, **11b**, **11d**, **11f** or **11h**. Compounds **11c**, **11e** and **11g** were the most effective in this tissue (Table 1). The reference drugs used in this evaluation were sildenafil, rolipram and enoximone (inhibitors of the PDE-5, PDE-4 and PDE-3, respectively) [2,5,12], as well as aminophylline (an unspecific-PDE inhibitor used in asthma treatment) [13]. Aminophylline and rolipram at 100 μM showed a maximum relaxant effect in the same range (30–50%) as compounds **4** and **11b** (Fig. 1). Sildenafil was the most potent (EC_{50} = 0.60 ± 0.34 μM, r^2 = 0.917) and effective (100% maximum relaxant effect) in this tissue. Enoximone demonstrated 33% effectiveness, similar to **4**. The lack of any trend in the structural-activity relationship shown by the compounds in this tissue suggests that alkyl and naphthyl substituents at C-2 do not increase the relaxant effect in ASM.

Rolipram (inhibitor of PDE-4) was not capable of reaching the maximum relaxant effect, due to the sensitivity of the tissue and the concentration used of the contractile drug [4]. It has been reported that aminophylline reaches the maximum relaxant effect in this tissue at a concentration of 10 mM [15], which suggests that inhibitors of PDEs require a greater concentration on guinea-pig trachea to reach the maximum effect [4].

2.4. PDE inhibition

Cyclic nucleotide phosphodiesterases (PDEs) are amply distributed in mammalian tissues. Their principal function is to influence in cellular signaling, principally in inflammatory processes by means of the degradation of secondary messengers adenosine and guanosine 3′,5′-cyclic monophosphate (cAMP, cGMP) by a stereospecific hydrolysis of the P-O3′ bond to produce 5′-AMP and 5′-GMP, respectively [16,17]. An evaluation was made of compounds **4**, **11a**–**11h** as inhibitors of PDEs. The reference compounds employed were enoximone and rolipram (selective inhibitors of PDE-3 and PDE-4, respectively, which reduce the decomposition of cAMP and cGMP, having a greater effect on the former) [17,18] as well as 3-isobutyl-1-methylxanthine (IBMX, an unspecific PDE inhibitor) [18] and sildenafil (an inhibitor of PDE-5 that increases cGMP levels) [12]. The latter reference drug was the most potent compound tested herein (Fig. 4). It is important to point out that the increase in the concentration of cAMP and/or cGMP causes relaxation of smooth vascular muscle tissue [18].

Compounds **11d** and **11f** were the most effective as inhibitors of the decomposition of cAMP and cGMP (Fig. 4). Compound **11d** showed 59% inhibition of cAMP and 73% for cGMP, thus showing a significant difference between the two. This result suggests that **11d** provokes vasorelaxation by inhibition of PDEs, with greater selectivity for PDEs with a cGMP substrate, such as is the case with PDE-5. It is accepted that in vascular tissue it is principally PDE-3 and PDE-5 that are involved in this mechanism [17,19]. However, PDE-3 efficiently decomposes cAMP [17], while **11d** inhibits the decomposition of cGMP. Thus the latter compound shows a mechanism similar to a PDE-5 inhibitor. Compound **11f** was active, showing an inhibition of the decomposition of cAMP and cGMP by 61% and 58%, respectively. However, the selectivity of this compound was less than **11d**. This suggests that **11f** acts as an unspecific inhibitor of PDEs. The rest of the compound evaluated, **4**, **11a**–**11c**, **11e** and **11g**–**11h**, showed inhibitory activity in the range of 30–50% on PDEs with cAMP as a substrate and 24–45% with cGMP as a substrate (Fig. 4). These results demonstrate that the synthesized chromones have inhibitory activity on PDEs with effectiveness similar to that shown on vasorelaxation (Fig. 3; Table 1).

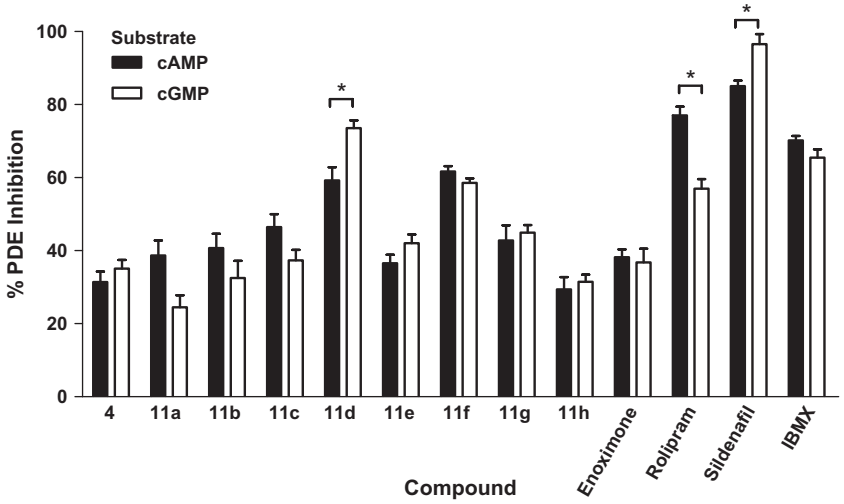


Fig. 4. Percentage PDE inhibition of compounds **4**, **11a**–**11h** using a colorimetric assay. The basis for the assay is the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase. Each bar represents the mean ± SEM, $n = 3$. $^{*}p < 0.05$, significant difference compared the inhibition of phosphodiesterases with different substrate at the same time (Dunnett's *t*-test after analysis of variance). The final concentration of the compounds and reference drugs used were 40 μM, except sildenafil which was tested to 0.1 μM.

Table 2
Theoretical parameters of phosphodiesterase inhibitors. Predicted activity values of the studied compounds.

	PDE-3		PDE-4		PDE-5	
	K_i	EFEB	K_i	EFEB	K_i	EFEB
Compound						
4	89.84	−5.52	32.26	−6.13	41.4	−5.98
11a	10.9	−6.77	6.23	−7.1	13.68	−6.64
11b	5.75	−7.15	3.15	−7.31	6.25	−7.1
11c	8025	−6.94	1.99	−7.78	2.69	−7.6
11d	2.67	−7.6	0.828	−8.3	0.478	−8.62
11e	8.08	−6.95	2.73	−7.59	4.93	−7.24
11f	6.14	−7.11	1.71	−7.87	2.15	−7.73
11g	2.86	−7.56	1.24	−8.06	1.94	−7.79
11h	0.159	−9.27	0.031	−10.24	0.025	−10.37
Sildenafil	0.00169	−11.97	0.00725	−11.1	987.62 pM	−12.29
Rolipram	0.784	−8.33	0.39228	−8.74	0.72996	−8.37
Enoximone	4.99	−7.23	4.92	−7.24	3.84	−7.39

K_i: Estimate inhibition constant (μM). EFEB: estimating free energy of binding (kcal/mol).

The reference compounds in this study showed the expected behavior, according to the information in the literature. That is, there was the expected selectivity for their reported substrates (Fig. 4) [12,17,18].

2.5. Molecular docking

PDE-3 and PDE-4 have been isolated in ASM from guinea-pig trachea [2], as have PDE-3 and PDE-5 in VSM from rat aorta [18,19]. Therefore, we investigated the binding mode of compounds **4** and **11a**–**11h** to PDE-3, PDE-4, and PDE-5 by docking studies with the AUTODOCK 4.0.2 program [20,21].

The structures of compounds **4** and **11a**–**11h** were built using the program HyperChem 8 release. Initially, the ligands were docked to the entire protein, and then the best conformations were docked in a smaller area (grid) in order to refine the results. The compounds analyzed bound to the pocket corresponding to one or more of the sites of PDE-3, PDE-4 and PDE-5 (K_i values are indicated in Table 2). All of compounds exhibited affinity to the proteins, mainly to PDE-4 (compounds **4**, **11a**–**11c** and **11e**–**11g**) and PDE-5 (compounds **11d** and **11h**). In relation to the reference drugs, sildenafil showed the proven affinity for PDE-5, rolipram for PDE-4, and enoximone for PDE-3 PDE-4 and PDE-5 (the greater affinity of the latter was for PDE-5).

As aforementioned, the inhibition of PDEs produces an increase in the levels of cAMP and/or cGMP, depending on the selectivity of a compound for a certain type of PDE [16–19]. The increase in concentration of one or both of these secondary messengers provokes relaxation of smooth vascular muscle tissue [4]. This could well be

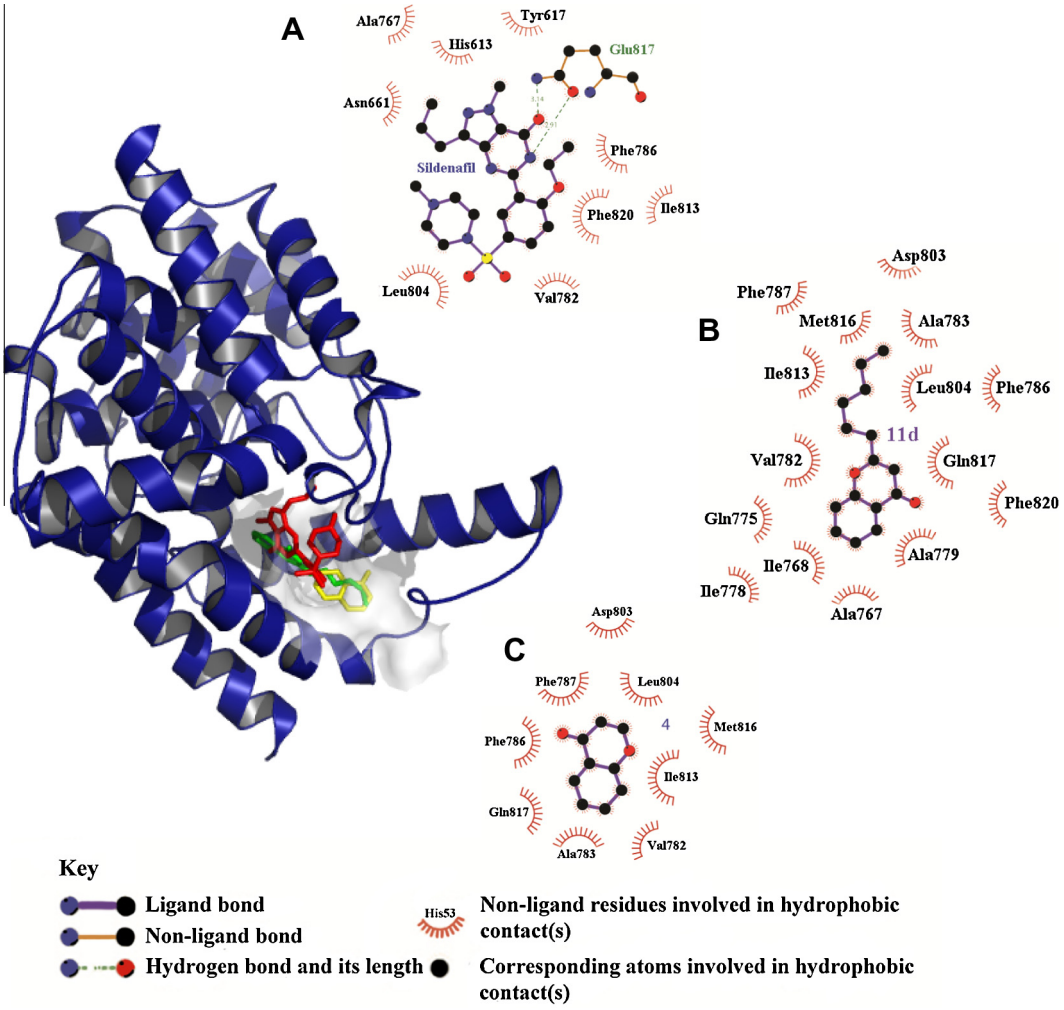


Fig. 5. Binding model of sildenafil, **11d** and **4** with PDE-5. On the left shows the complex in blue cartoon the PDE-5; red, green, and yellow stick the sildenafil, **11d** and **4**, respectively. The pocket of binding is showed in white surface. Right shows the 2D representation of PDE-5 interactions resulting from the docking, with residues indicated for Silderafil (A), **11d** (B), and **4**(C). The images ware made whit PyMOL and LIGPLOT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

one of the mechanisms of action of the tested compounds on rat aorta in the present study, taking into account their vasorelaxant effect (Fig. 3; Table 1), *in vitro* PDEs inhibition (Fig. 4) and the predictions from the docking study (Table 2). The *in silico* studies showed that these compounds have affinity for the catalytic site of PDE-3, PDE-4 and PDE-5 (to a greater or lesser extent depending on the *Ki* value).

Compound **11d** showed the greatest theoretical inhibitory potential (K_i) at the catalytic site of PDE-5 (Table 2) of the alkyl compounds. However, it also has inhibitory potential at the catalytic site of PDE-4 and PDE-3, even though the K_i value at these sites are 1.7-fold and 5.6-fold less, respectively, than that at PDE-5. This result has good correlation with the *in vitro* assays, in which **11d** demonstrated greater selectivity for the inhibition of cGMP decomposition. The results of the docking study of the present study thus suggest that **11d** exercises its vasorelaxant effect on PDE-5.

On the other hand, the docking studies on compound **11f** showed a slightly less potent vasorelaxant effect than **11d** (Fig. 3; Table 1), since the K_i values of **11f** for PDE-3 and PDE-5 are 3.6 and 1.2-fold less, respectively, than that for PDE-4 (Table 2). These differences in K_i values are less for **11f** than such differences found for **11d**, indicating a more limited range of inhibitory effect and a lower selectivity for the former compound than for the latter. Likewise, regarding the inhibitory effect of **11f** on PDEs (Fig. 4), there is no significant difference between the percentage of inhibition of cAMP or cGMP decomposition, indicating a lack of selectivity.

Indeed, the effect of **11f** is similar to that of non-selective inhibitors, such as aminophylline (Fig. 3) and IBMX (Fig. 4). The latter two drugs are capable of increasing the levels of cAMP and cGMP and of inducing relaxation of vascular smooth muscle tissue [4].

To exemplify the structure–activity relationship of chromones substituted at position C-2 and inhibition of PDEs, a theoretical analysis was carried out for chromone **4** (without substituents) and **11d** (with C-2 hexyl substituent) in relation to PDE-5. Fig. 5 shows the theoretical binding model of sildenafil, **11d** and **4** superimposed on the X-ray structure of PDE-5. It can be seen that compounds **11d** and **4** bind to the enzyme at the same pocket as sildenafil. The pyrazolopyrimidinone group of sildenafil interacts with His613, Tyr617, Asn661, Ala767, and Glu817. The O1 and N4 atoms of pyrazolopyrimidinone form two hydrogen bonds with NE2 and OE1 of Gln817, respectively. The ethoxyphenyl group interacts through van der Waals forces with Val782, Phe786, Ile813, and Phe820. Finally, Val782 and Leu804 make contact with the methylpiperazine group of the drug.

The chromones **4** and **11d** interact with the same residues as sildenafil (Fig. 5), but unlike the reference compound, these two test compounds only interact by van der Waals forces. Compound **4** interacts with Val782, Ala783, Phe786, Phe787, Asp803, Leu804, Ile812, Met816, and Gln817. Compound **11d** interacts with Ile768, Ala767, Gln775, Ile778, Ala779, Val782, Ala783, Phe786, Phe787, Asp803, Leu804, Ile813, Met816, Gln817, and Phe820. The predicted affinity difference is due to the type and number of interactions shown by the compounds. In the case of sildenafil (K_i = 987.62 pM), the interactions have both van der Waals and hydrogen bonds, the latter providing greater stability and therefore better affinity. For compound **11d** (K_i = 0.478 μM), the longer linear chain at C-2 provides a greater number of interactions compared to **4** (K_i = 41.4 μM), which is directly reflected in the predicted inhibition constant. In the case of compound **11d**, the experimental data and the docking study both indicate that it is a PDE-5 inhibitor.

3. Conclusion

All of compounds synthetized, **4**, **11a**–**11h**, proved to have a vasorelaxant effect. The study showed that for effectiveness greater than 90%, it is necessary for a compound to have an alkyl lineal chain of six carbons or an isobutyl group at the C-2 position. Accordingly, compounds **11d** and **11f** were the most effective and potent. On the other hand, the compounds that had substituents with a shorter alkyl chain (**11a**–**11c**), a bulky alkyl chain (**11e**–**11g**), or an aromatic group like naphthyl (**11h**), were the least effective and potent. Although **11d** and **11f** were not more potent than the four reference drugs (sildenafil, rolipram, enoximone or aminophylline), they were similar to sildenafil and enoximone in effectiveness and better in this sense than rolipram and aminophylline. The *in vitro* assays demonstrated that compounds **11d** and **11f** have inhibitory activity on PDEs. It turns out that **11d** is the most effective inhibitor of the decomposition of cGMP, and that **11f** has an unspecific inhibitory activity on the decomposition of cAMP and cGMP. Based on the docking study, it was predicted that **11d** is selective for PDE-5. Further studies are needed with the isolated organ model in order to determine whether the herein synthesized compounds also act as vasorelaxants by other pharmacological mechanisms besides the inhibition of PDEs. Finally, since hypertension is one of the most common diseases related to VSM contraction, and the therapeutic agents used for treatment modulate such contraction, the current results suggest that chromones **11d** and **11f** hold promise as antihypertensive agents that selectively relax VSM.

4. Experimental

4.1. Chemistry

^{1}H and ^{13}C spectra were recorded on a JEOL GX300 instrument, at 300 MHz for ^{1}H and 75 MHz for ^{13}C, using $CDCl_3$ as solvent. MS spectra were obtained using a JEOL JMSAX505HA spectrometer. Melting points were determined using an Electro-thermal Mel-Temp capillary apparatus. All starting materials were purchased from Aldrich (>98% purity) and used without further purification.

4.1.1. Chromone 4

Compound **4** was synthetized as described in a previously report [11], with some changes. To 2-hydroxyacetophenone (**8**, 12.8 mmol) was added ethyl formate (93 mmol) in a flask fitted with a reflux condenser. Then small portions (maintained in *n*-hexane) of powdered sodium (0.75 g altogether) were gradually added. Finally, more ethyl formate (1.5 g) and powdered sodium (0.25 g) were added to the reaction mixture, which was refluxed overnight. Ice and water were carefully added to the reaction and then acidified with hydrochloric acid at 10%. The resulting solution was extracted with ethyl acetate. The isolated organic layer was concentrated in vacuum and yielded a crystalline precipitate, which was crystallized from *n*-hexane and ethyl acetate (7:3) (Mp 106 °C, yield 60%). An alcoholic solution of the substance was red due to the presence of ferric chloride, or yellow with sulfuric acid. The corresponding properties were previously reported [11]. 0.86 g of solid obtained from the above reaction was heated on an oil-bath under reflux with 30 mL of aqueous sulfuric acid (16 mL of 98% sulfuric acid mixed with 70 mL of water) for thirty minutes. After cooling, the solution was neutralized with a saturated sodium bicarbonate solution and extracted with ethyl acetate; the product extracted was purified by flash-column chromatography.

Colorless crystals (*n*-hexane/ethyl acetate 8:2). Mp 51 °C; yield 80%. The properties correspond to a previous report [11]. ^{1}H and ^{13}C NMR and EIMS data are included. ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 8.18 (1H, dd, J = 7.9, 1.5 Hz, H-5), 7.83 (1H, d, J = 6.3 Hz, H-2), 7.64 (1H, m, H-7), 7.42 (1H, m, H-8), 7.36 (1H, m, H-6), 6.31 (1H, d, J = 6 Hz, H-3). ^{13}C NMR (75 MHz, $CDCl_3$, ppm):

δ 177.7 (C-4), 156.6 (C-8a), 155.4 (C-2), 133.8 (C-7), 125.9 (C-5), 125.3 (C-4a), 124.9 (C-6), 118.2 (C-8), 113.0 (C-3). EIMS *m*/*z* (% relative intensity): 146 [M$^{+\cdot}$] (76), 121 (100), 92 (63).

4.1.2. Phenolic esters 10a–10h

Phenolic esters were synthetized as described in a previous report [9]. Acyl chloride (8.88 mmol) was added dropwise under N_2-atmosphere to a solution of 2-hydroxyacetophenone (2.69 mmol) in anhydrous pyridine. Finally, DBU (10.7 mmol) was added dropwise to the reaction mixture, which turned into a solid. To this solid 20 mL of anhydrous dichloromethane was added, and the mixture was heated at a temperature of 30 °C for 6–7 h (monitored by TLC). The reaction mixture was washed with ice water, acidified to pH 3–4 using hydrochloric acid at 10%, extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by silica-gel flash column chromatography using *n*-hexane–EtOAc (8:1) as eluent. ^{1}H NMR data for compounds **10b**, **10e** and **10f** were previously reported [22]. ^{13}C NMR and EIMS data are included. Compounds **10c**, **10d** and **10g** are novel.

4.1.2.1. 2-Acetylphenyl acetate 10a. White crystals (*n*-hexane–ethyl acetate; 9:1). Mp 71 °C; yield 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.82 (1H, dd, *J* = 7.8, 1.8 Hz, H-3), 7.54 (1H, m, H-5), 7.33 (1H, ddd, *J* = 7.8, 7.7, 1.2 Hz, H-4), 7.12 (1H, dd, *J* = 8.0, 0.9 Hz, H-6), 2.56 (3H, s, C$\underline{H}_3$CO), 2.35 (3H, s, C$\underline{H}_3$CO$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.4 (CO), 169.4 (CO$_2$), 148.9 (C-1), 133.3 (C-5), 130.2 (C-3), 125.9 (C-2), 123.7 (C-4), 117.6 (C-6), 29.2 (C$\underline{H}_3$CO), 22.0 (C$\underline{H}_3$-CO$_2$). EIMS *m*/*z* (% relative intensity): 178 [M$^{+\cdot}$] (1.2), 136 (40), 121 (100), 43 (12).

4.1.2.2. 2-Acetylphenyl butyrate 10b. Yellow oil; yield: 93%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (1H, dd, *J* = 7.7, 1.8 Hz, H-3), 7.51 (1H, ddd, *J* = 8.1, 7.8, 1.8 Hz, H-5), 7.30 (1H, ddd, *J* = 7.8, 7.7, 1.2 Hz, H-4), 7.09 (1H, dd, *J* = 8.1, 1.2 Hz, H-6), 2.59 (2H, t, *J* = 7.5 Hz, C$\underline{H}_2$CO$_2$), 2.53 (3H, s, C$\underline{H}_3$CO), 1.85–1.73 (2H, m, CH$_3$C$\underline{H}_2$), 1.04 (3H, t, *J* = 7.5 Hz, C$\underline{H}_3$CH$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.7 (CO), 172.1 (CO$_2$), 149.1 (C-1), 133.4 (C-5), 131.0 (C-3), 130.2 (C-2), 126.0 (C-4), 123.9 (C-6), 36.2 (C$\underline{H}_2$CO$_2$), 29.5 (C$\underline{H}_3$CO), 18.1 (CH$_3$-C$\underline{H}_2$), 13.7 (C$\underline{H}_3$CH$_2$). EIMS *m*/*z* (% relative intensity): 206 [M$^{+\cdot}$] (2.8), 136 (98), 121 (65), 71 (100).

4.1.2.3. 2-Acetylphenyl pentanoate 10c. Yellow oil; yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (1H, dd, *J* = 7.8, 1.8 Hz, H-3), 7.54–7.48 (1H, m, H-5), 7.30 (1H, ddd, *J* = 7.6, 7.5, 1.2 Hz, H-4), 7.08 (1H, dd, *J* = 8.1, 1.2 Hz, H-6), 2.61 (2H, t, *J* = 7.7 Hz, C$\underline{H}_2$CO$_2$), 2.53 (3H, s, C$\underline{H}_3$CO), 1.79–1.69 (2H, m, C$\underline{H}_2$CH$_2$CO$_2$), 1.50–1.38 (2H, m, CH$_3$C$\underline{H}_2$), 0.96 (3H, t, *J* = 7.4 Hz, C$\underline{H}_3$CH$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.7 (CO), 172.3 (CO$_2$), 149.2 (C-1), 133.4 (C-5), 131.1 (C-3), 130.2 (C-2), 126.0 (C-4), 124.0 (C-6), 34.1 (C$\underline{H}_2$CO$_2$), 29.5 (C$\underline{H}_3$CO), 26.7 (C$\underline{H}_2$CH$_2$CO$_2$), 22.3 (CH$_3$C$\underline{H}_2$), 13.8 (C$\underline{H}_3$CH$_2$). EIMS *m*/*z* (% relative intensity): 220 [M$^{+\cdot}$] (5), 136 (15), 121 (60), 85 (100).

4.1.2.4. 2-Acetylphenyl heptanoate 10d. Yellow oil; yield 98%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (1H, dd, *J* = 7.8, 1.5 Hz, H-3), 7.55–7.49 (1H, m, H-5), 7.31 (1H, ddd, *J* = 7.8, 7.7, 1.2 Hz, H-4), 7.10 (1H, dd, *J* = 8.0, 1.2 Hz, H-6), 2.61 (2H, t, *J* = 7.5 Hz, C$\underline{H}_2$CO$_2$), 2.54 (3H, s, C$\underline{H}_3$CO), 1.81–1.68 (2H, m, C$\underline{H}_2$CH$_2$CO$_2$), 1.39–1.30 (6H, m, CH$_3$(C$\underline{H}_2$)$_3$), 0.90 (3H, t, *J* = 7.1 Hz, C$\underline{H}_3$CH$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.7 (CO), 172.3 (CO$_2$), 149.2 (C-1), 133.4 (C-5), 131.1 (C-3), 130.2 (C-2), 126.0 (C-4), 123.9 (C-6), 34.4 (C$\underline{H}_2$CO$_2$), 31.5 (CH$_3$CH$_2$C$\underline{H}_2$), 29.5 (CH$_3$CO), 28.8 (C$\underline{H}_2$(CH$_2$)$_2$CO$_2$), 24.6 (C$\underline{H}_2$CH$_2$-CO$_2$), 22.5 (CH$_3$C$\underline{H}_2$), 14.1 (C$\underline{H}_3$CH$_2$). EIMS *m*/*z* (% relative intensity): 248 [M$^{+\cdot}$] (1.5), 136 (35), 113 (100), 85 (15).

4.1.2.5. 2-Acetylphenyl isobutyrate 10e. Yellow oil; yield 92%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (1H, dd, *J* = 7.8, 1.5 Hz, H-3), 7.53–7.48 (1H, m, H-5), 7.30 (1H, ddd, *J* = 7.7, 7.5, 1.2 Hz, H-4), 7.07 (1H, dd, *J* = 8.1, 0.9 Hz, H-6), 2.91–2.77 (1H, m, C$\underline{H}$CO$_2$), 2.53 (3H, s, C$\underline{H}_3$CO), 1.34 (6H, d, *J* = 7.2 Hz, (C$\underline{H}_3$)$_2$CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.8 (CO), 175.4 (CO$_2$), 149.2 (C-1), 133.3 (C-5), 131.3 (C-3), 130.1 (C-2), 125.9 (C-4), 123.7 (C-6), 34.3 (C$\underline{H}$CO$_2$), 29.5 (C$\underline{H}_3$-CO), 18.8 ((C$\underline{H}_3$)$_2$CH). EIMS *m*/*z* (% relative intensity): 206 [M$^{+\cdot}$] (1.3), 121 (100), 71 (78), 43 (34).

4.1.2.6. 2-Acetylphenyl 3-methylbutanoate 10f. Yellow oil; yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (1H, dd, *J* = 7.8, 1.8 Hz, H-3), 7.55–7.49 (1H, m, H-5), 7.31 (1H, ddd, *J* = 7.7, 7.5, 2.1 Hz, H-4), 7.10 (1H, dd, *J* = 8.1, 1.9 Hz, H-6), 2.55 (3H, s, C$\underline{H}_3$CO), 2.50 (2H, d, *J* = 6.9 Hz, C$\underline{H}_2$CO$_2$), 2.33–2.19 (1H, m, (CH$_3$)$_2$C$\underline{H}$), 1.07 (6H, d, *J* = 6.6 Hz, (C$\underline{H}_3$)$_2$CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.6 (CO), 171.4 (CO$_2$), 149.0 (C-1), 133.2 (C-5), 131.1 (C-3), 130.0 (C-2), 125.9 (C-4), 123.7 (C-6), 43.2 (C$\underline{H}_2$CO$_2$), 29.4 (C$\underline{H}_3$CO), 25.3 ((CH$_3$)$_2$-C$\underline{H}$), 22.4 ((C$\underline{H}_3$)$_2$CH). EIMS *m*/*z* (% relative intensity): 220 [M$^{+\cdot}$] (1.5), 136 (32), 121 (45), 85 (100).

4.1.2.7. 2-Acetylphenyl 2-ethylbutanoate 10g. Yellow oil; yield 93%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (1H, dd, *J* = 7.8, 1.8 Hz, H-3), 7.53–7.47 (1H, m, H-5), 7.29 (1H, ddd, *J* = 7.6, 7.4, 0.9 Hz, H-4), 7.08 (1H, dd, *J* = 8.1, 1.2 Hz, H-6), 2.55 (3H, s, C$\underline{H}_3$CO), 2.51 (1H, m, (C$\underline{H}$CO$_2$), 1.92–1.61 (4H, m, (CH$_3$C$\underline{H}_2$)$_2$CH), 1.02 (6H, t, *J* = 7.5 Hz, (C$\underline{H}_3$CH$_2$)$_2$CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 198.1 (CO), 174.5 (CO$_2$), 148.9 (C-1), 133.0 (C-5), 131.9 (C-3), 129.8 (C-2), 125.9 (C-4), 123.7 (C-6), 48.6 (C$\underline{H}$CO$_2$), 29.7 (C$\underline{H}_3$CO), 24.5 ((CH$_3$-C$\underline{H}_2$)$_2$CH), 11.8 ((C$\underline{H}_3$CH$_2$)$_2$CH). EIMS *m*/*z* (% relative intensity): 234 [M$^{+\cdot}$] (2), 136(15), 121 (100), 99 (94), 71(79).

4.1.2.8. 2-Acetylphenyl 2-naphthoate 10h. Colorless crystals (*n*-hexane–ethyl acetate; 8:2). Mp 110 °C; yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.81 (1H, s, NaphH-1), 8.21 (1H, dd, *J* = 8.5, 1.5 Hz, NaphH-3), 8.02–7.87 (4H, m, PhH-3, NaphH-4, NaphH-5, NaphH-8), 7.66–7.55 (3H, m, PhH-5, NaphH-6, NaphH-7), 7.39 (1H, ddd, *J* = 7.6, 7.5, 1.2 Hz, PhH-4), 7.29 (1H, dd, *J* = 8.1, 1.2 Hz, PhH-6), 2.56 (3H, s, C$\underline{H}_3$CO). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.7 (CO), 165.4 (CO$_2$), 149.5 (C-1), 136.0 (NaphC-4), 133.5 (C-5), 132.6 (NaphC-8a), 132.3 (NaphC-1), 131.4 (NaphC-8), 130.3 (NaphC-3), 129.6 (NaphC-2), 128.8 (NaphC-6), 128.6 (NaphC-4), 127.9 (NaphC-5), 126.9 (C-2), 126.5 (NaphC-7), 126.3 (NaphC-3), 125.5 (C-4), 124.0 (NaphC-6), 29.9 (C$\underline{H}_3$CO). EIMS *m*/*z* (% relative intensity): 290 [M$^{+\cdot}$] (5), 155 (100), 127 (55).

4.1.3. Chromones 11a–11h

Compounds **11a**–**11h** were prepared in a similar manner as compounds **10a**–**10h**, starting from the corresponding phenolic ester with 1.0 eq of DBU and pyridine as solvent to reflux at 80 °C for 6 h. The reaction was stopped by adding ice, adjusted to pH 3–4, extracted with ethyl acetate and purified with a flash chromatographic column eluted with *n*-hexane–ethyl acetate (8:2). Compounds **11a** [23], **11c**–**11d** [24], **11e** [25] and **11h** [26] have been previously reported. Compound **11g** is new.

4.1.3.1. 2-Methyl-4H-chromen-4-one 11a. Yellow crystals (*n*-hexane/ethyl acetate; 8:2). Mp 65 °C; yield 23%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.61 (1H, dd, *J* = 8.1, 1.5 Hz, H-5), 7.56–7.50 (1H, m, H-7), 7.35–7.26 (2H, m, H-6, H-8), 6.30 (1H, s, H-3), 2.44 (3H, s, CH$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.7 (C-4), 153.5 (C-2), 152.2 (C-8a), 131.7 (C-7), 124.5 (C-5), 124.1 (C-4a), 119.9 (C-6), 117.1 (C-8), 115.1 (C-3), 18.6 (CH$_3$). EIMS *m*/*z* (% relative intensity): 160 [M$^{+\cdot}$] (89), 145 (2), 131 (100).

4.1.3.2. 2-Propyl-4H-chromen-4-one 11b. Yellow oil; yield 25%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.18 (1H, dd, J = 8.0, 1.8 Hz, H-5), 7.66–7.58 (1H, m, H-7), 7.46–7.34 (2H, m, H-6, H-8), 6.18 (1H, s, H-3), 2.60 (2H, t, J = 7.5 Hz, C$\underline{H}_2$CH$_2$CH$_3$), 1.84–1.72 (2H, m, CH$_2$C$\underline{H}_2$CH$_3$), 1.03 (3H, t, J = 7.5 Hz, CH$_2$CH$_2$C$\underline{H}_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.4 (C-4), 169.5 (C-2), 156.5 (C-8a), 133.4 (C-7), 125.6 (C-5), 124.8 (C-4a), 123.7 (C-6), 117.8 (C-8), 109.9 (C-3), 36.2 (C$\underline{H}_2$CH$_2$CH$_3$), 20.1 (CH$_2$C$\underline{H}_2$CH$_3$), 13.5 (CH$_2$CH$_2$C$\underline{H}_3$). EIMS m/z (% relative intensity): 188 [M$^{+\cdot}$] (100), 160 (95), 145 (37), 43 (10).

4.1.3.3. 2-Butyl-4H-chromen-4-one 11c. Yellow oil; yield 75%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.14 (1H, dd, J = 8.1, 1.8 Hz, H-5), 7.62–7.56 (1H, m, H-7), 7.39–7.30 (2H, m, H-6, H-8), 6.13 (1H, s, H-3), 2.58 (2H, t, J = 7.5 Hz, C$\underline{H}_2$(CH$_2$)$_2$CH$_3$), 1.73–1.63 (2H, m, CH$_2$C$\underline{H}_2$CH$_2$), 1.45–1.33 (2H, m, C$\underline{H}_2$CH$_3$), 0.92 (3H, t, J = 7.2 Hz, CH$_2$C$\underline{H}_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.5 (C-4), 170.0 (C-2), 156.7 (C-8a), 133.5 (C-7), 125.8 (C-5), 125.0, (C-4a), 123.9 (C-6), 118.0 (C-8), 110.0 (C-3), 34.2 (C$\underline{H}_2$(CH$_2$)$_2$CH$_3$), 29.0 (CH$_2$C$\underline{H}_2$CH$_2$), 22.3 (C$\underline{H}_2$CH$_3$), 13.9 (CH$_2$C$\underline{H}_3$). EIMS m/z (% relative intensity): 202 [M$^{+\cdot}$] (19), 160 (100), 173 (9), 145 (3), 57 (2.5).

4.1.3.4. 2-Hexyl-4H-chromen-4-one 11d. Yellow oil; yield 45%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.14 (1H, dd, J = 7.8, 1.8 Hz, H-5), 7.62–7.56 (1H, m, H-7), 7.40–7.32 (2H, m, H-6, -8), 6.13 (1H, s, H-3), 2.57 (2H, t, J = 7.5 Hz, C$\underline{H}_2$(CH$_2$)$_4$CH$_3$), 1.74–1.64 (2H, m, C$\underline{H}_2$CH$_2$CH$_3$), 1.33–1.25 (6H, m, CH$_2$C$\underline{H}_2$C$\underline{H}_2$CH$_2$C$\underline{H}_2$CH$_3$), 0.85 (3H, t, J = 6 Hz, CH$_2$C$\underline{H}_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.6 (C-4), 170.0 (C-2), 156.7 (C-8a), 133.6 (C-7), 125.9 (C-5), 125.0 (C-4a), 123.9 (C-6), 118.0 (C-8), 110.0 (C-3), 34.5 (C$\underline{H}_2$(CH$_2$)$_4$CH$_3$), 31.6 (C$\underline{H}_2$CH$_2$CH$_3$), 28.8 (C$\underline{H}_2$(CH$_2$)$_2$CH$_3$), 26.9 (CH$_2$C$\underline{H}_2$(CH$_2$)$_3$CH$_3$), 22.6 (C$\underline{H}_2$CH$_3$), 14.2 (CH$_2$C$\underline{H}_3$). EIMS m/z (% relative intensity): 230 [M$^{+\cdot}$] (100), 201 (7), 173 (9), 160 (87), 145 (5), 121 (80), 85 (3).

4.1.3.5. 2-Isopropyl-4H-chromen-4-one 11e. Yellow crystal (*n*-hexane/ethyl acetate; 8:2). Mp 43 °C; yield 66%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.14 (1H, dd, J = 8.0, 1.8 Hz, H-5), 7.63–7.57 (1H, m, H-7), 7.41–7.30 (2H, m, H-6, H-8), 6.15 (1H, s, H-3), 2.87–2.78 (1H, m, C$\underline{H}$(CH$_3$)$_2$), 1.29 (6H, d, J = 6.6 Hz, CH(C$\underline{H}_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.9 (C-4), 174.4 (C-2), 156.7 (C-8a), 133.6 (C-7), 125.8 (C-5), 125.0 (C-4a), 123.9 (C-6), 118.0 (C-8), 107.8 (C-3), 33.5 (C$\underline{H}$(CH$_3$)$_2$), 20.3 (CH(C$\underline{H}_3$)$_2$). EIMS m/z (% relative intensity): 188 [M$^{+\cdot}$] (100), 173 (30), 145 (60), 121 (27), 43 (4).

4.1.3.6. 2-Isobutyl-4H-chromen-4-one 11f. Yellow oil; yield 21%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.17 (1H, dd, J = 8.1, 1.8 Hz, H-5), 7.66–7.58 (1H, m, H-7), 7.52–7.45 (2H, m, H-6, H-8), 6.16 (1H, s, H-3), 2.47 (2H, d, J = 7.2 Hz, C$\underline{H}_2$CH), 2.19–2.06 (1H, m, CH$_2$C$\underline{H}$), 0.98 (6H, d, J = 6.6 Hz, CH(C$\underline{H}_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.9 (C-4), 169.6 (C-2), 163.3 (C-8a), 131.1 (C-7), 129.0 (C-5), 125.9 (C-4a), 121.2 (C-6), 115.6 (C-8), 110.9 (C-3), 50.6 (C$\underline{H}_2$CH), 24.5 (CH$_2$C$\underline{H}$), 23.6 (CH(C$\underline{H}_3$)$_2$). EIMS m/z (% relative intensity): 202 [M$^{+\cdot}$] (45), 160 (95), 149 (100), 145 (7), 121 (44), 57 (28).

4.1.3.7. 2-(Pentan-3-yl)-4H-chromen-4-one 11g. Yellow oil; yield 43%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.16 (1H, dd, J = 8.1, 1.8 Hz, H-5), 7.63–7.57 (1H, m, H-7), 7.41–7.34 (2H, m, H-6, -8), 6.15 (1H, s, H-3), 2.38–2.28 (1H, m, C$\underline{H}$(CH$_2$CH$_3$)$_2$), 1.73–1.60 (4H, m, CH(C$\underline{H}_2$CH$_3$)$_2$, 0.87 (6H, t, J = 7.5 Hz, CH(CH$_2$C$\underline{H}_3$)$_2$. ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.5 (C-4), 172.2 (C-2), 156.7 (C-8a), 133.5 (C-7), 125.9 (C-5), 125.1 (C-4a), 124.1 (C-6), 118.1 (C-8), 110.7 (C-3), 48.4 (C$\underline{H}$(CH$_2$CH$_3$)$_2$), 22.8 (CH(C$\underline{H}_2$CH$_3$)$_2$), 12.3 (CH(CH$_2$C$\underline{H}_3$)$_2$). EIMS m/z (% relative intensity): 216 [M$^{+\cdot}$] (100), 188 (63), 173 (47), 121 (42), 71 (10).

4.1.3.8. 2-(naphthalen-3-yl)-4H-chromen-4-one 11h. Yellow crystals (*n*-hexane/ethyl acetate; 8:2). Mp 148 °C; yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.43 (1H, s, NaphH-1), 8.24 (1H, dd, J = 7.9, 1.5 Hz, NaphH-5), 7.96–7.84 (4H, m, NaphH-8, H-5, NaphH-4, H-7), 7.72–7.67 (1H, m, NaphH-3), 7.61–7.54 (3H, m, NaphH-6, NaphH-7, H-6), 7.43–7.38 (1H, m, H-8), 6.92(1H, s, H-3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.3 (C-4), 163.2 (C-2), 156.2 (C-8a), 134.6 (C-7), 133.7 (NaphC-2), 132.8 (NaphC-8a), 130.8 (NaphC-4a), 128.9 (C-5), 128.8 (NaphC-4), 127.9 (NaphC-8), 127.7 (NaphC-5), 127.0 (NaphC-7), 126.8 (NaphC-6), 125.6 (NaphC-1), 125.1 (C-4a), 123.9 (C- NaphC-3), 122.4 (C-6), 118.0 (C-8), 107.8 (C-3). EIMS m/z (% relative intensity): 272 [M$^{+\cdot}$] (70), 160 (100), 127 (4).

4.2. Vasorelaxant and bronchorelaxant procedure

Acetylcholine chloride, carbamylcholine chloride (carbachol), (-)-norepinephrine, rolipram, enoximone and aminophylline were purchased from Sigma. Sildenafil citrate was used as the USP reference standard. The drugs were suspended in 0.05% Tween 80 in distilled water. The final concentration of Tween 80 was trace (less than 0.0005%) and did not affect the tracheal or vascular response. The drug solutions or suspensions were freshly prepared a few minutes before each use. Male guinea-pigs (Hartley) weighing 400–450 g were obtained from Biosupply S.A de C.V. (México). Male Wistar rats weighing 200–300 g were obtained from the Animal House of the Graduate Studies and Research Department of the U.N.A.M. (FES-Zaragoza, Mexico). Both species were maintained at a constant room temperature (22 ± 2 °C) and submitted to a12 h light/dark cycle with free access to food and water. Procedures involving animal care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with international rules on the care and use of laboratory animals. Furthermore, clearance for conducting the studies was obtained from the Ethics Committee for the Use of Animals in Pharmacological and Toxicological Testing of the Chemistry Faculty, UNAM.

Male Wistar rats were euthanized in a CO_2 chamber and the thoracic aorta was removed and immediately immersed in a Krebs solution at 37 °C. After removal of excess connective tissue and fat, the aorta was divided into eight small rings of about 2 mm in length. Each aorta ring was hung between two nichrome hooks inserted into the lumen, and placed in a 10 mL organ bath containing Krebs solution (composition in mM: NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}2H_2O$ 2.5, $NaHCO_3$ 25 and glucose 11.1) at 37 °C and bubbled constantly with 5% CO_2-95% O_2. Isometric tension was recorded through an eight-channel Biopack System polygraph MP100 via a Grass FT 03E force transducer. The data was digitalized and analyzed by means of software for data acquisition (Acknowledge 3.9.0).

Tissues were placed under a resting tension of 4.0 g and allowed to stabilize for 60 min. They were washed with fresh Krebs solution at 15 min intervals before starting the experiments. After a stabilization period the rings were contracted with (−)-norepinephrine (0.1 μM) two times at 30 min intervals. The integrity of the endothelium was verified by the relaxant response to acetylcholine chloride (10 μM), with intact endothelium showing a relaxation of over 84%, and denuded aorta exhibiting no relaxation. Therefore, all experiments were performed with the endothelium intact. Thirty minutes after the tissues were contracted with (−)-norepinephrine (0.1 μM), cumulative concentrations of synthetized compounds or reference drugs were added to the bath until yielding the required aorta relaxant effects, then allowed to reach a steady state at each concentration. The concentration required for relaxing 50% of the aorta pre-contracted with (−)-norepinephrine was expressed as EC_{50}. Guinea pigs were euthanized by intraperitoneal injection with an overdose of sodium pentobarbital (95 mg kg^{-1}). The tissue stabilization period and recording of data were carried out as described for rat aorta preparation. After the stabilization

period rings were contracted twice with acetylcholine chloride (30 μm) at 30-min intervals and then washed with fresh Krebs solution. After 30 min, the tissues were contracted with carbachol (3 μm), and then cumulative concentrations of compounds or reference drugs were added to measure the relaxant effect [4].

4.3. Data analysis

The EC_{50} values for the relaxant effect were calculated by linear regression [27]. All values are shown as the mean ± SEM of at least six experiments. The differences among obtained values were statistically calculated by one-way analysis of variance, and then determined by Dunnett's *t*-test, with $p < 0.05$ considered statistically significant [28].

4.4. Cyclic nucleotide phosphodiesterase assay

Chromones **4** and **11a**–**11h** were tested for their capacity to inhibit cyclic nucleotide phosphodiesterase (PDE) and therefore the decomposition of cAMP and cGMP. The catalytic pocket where the hydrolysis takes place is a highly conserved region and has several residues that are absolutely conserved across PDE families [16]. The enzyme inhibition assay was performed using a BIOMOL cyclic nucleotide PDE assay kit provided by Enzo Life Sciences, consisting of a colorimetric non-radioactive assay designed in a microplate format. The basis for this assay is the cleavage of cAMP or cGMP by a cyclic nucleotide PDE. The 5′-nucleotide released is further cleaved into the nucleoside and phosphate by the 5′-nucleotidase enzyme. The phosphate released as a result of the enzymatic cleavage was quantified using BIOMOL Green reagent in a modified malachite green assay [29], which can be used to screen inhibitors and modulators of cyclic nucleotide PDE activity. The compounds and drugs were tested at 40 μM, except sildenafil, which was tested at 0.1 μM. The colorimetric detection method used involved a reader of ELX-800 plates, and the resulting data was processed with Gen 5 microplate software. The percentage of inhibition was calculated by taking the absorbance of the respective control group as 100% of PDE enzymatic activity.

4.5. Computational method

To generate more accurate and physically realistic models of the proteins (PDB code 1S02, 1RO6, and 2H42 to PDE-3, PDE-4, and PDE-5, respectively), after several iterations of rebuilding and refinement, a final all-atom refinement of the PDEs was performed with the idealization application of the Rosetta3.1 release [30]. This application rebuilds molecules using ideal bond lengths, bond angles and torsion angles. The proteins and ligands were further prepared using AutoDockTools 1.5.4 (http://mgltools.scripps.edu/). To the proteins were added polar hydrogen atoms and Kollman united-atom partial charges. Gasteiger-Marsilli formalism charges and rotatable groups were automatically assigned to the ligands, as were the active torsions. Blind docking was carried out using AutoDock4 version 4.2 software (http://autodock.scripps.edu/) [20,21] with the default parameters for the Lamarkian genetic algorithm with local search, the number of individuals in a population (150), the maximum number of energy evaluations (2.5 million), the maximum number of generations (27,000), the rate of gene mutation (0.02) and the rate of crossover (0.8). There were 1000 runs for docking. The initial grid box size was 60 Å × 60 Å × 60 Å in the *x*, *y* and *z* dimensions. The refined docking analysis was performed in a smaller grid box of 30 Å × 30 Å × 30 Å, centered on the ligand. All calculations were made using a parallel distributed memory supercomputer (Kanbalam, Center for Computing and Information and Communication Technology, UNAM) which contains 1368 processors AMD Opteron, around 3 terabyte of memory and 160 terabyte of storage (http://www.super.unam.mx/).

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