Ferrocenyl, Alkyl, and Aryl-Pyrido[2,3-*d*]Pyrimidines as Vasorelaxant of Smooth Muscle of Rat Aorta via cAMP Conservation Through Phosphodiesterase Inhibition

Ivonne Arellano,[a]* Fernando Rodríguez-Ramos,[b] Martín González-Andrade,[c] Andrés Navarrete,[c] Manju Sharma,[d] Noé Rosas,[a] and Pankaj Sharma[a]*

[a]Instituto de Química, Universidad Nacional Autónoma de México, México, Distrito Federal, 04510, México
[b]Departamento de Ciencias Naturales, DCNI, Universidad Autónoma Metropolitana, Unidad Cuajimalpa, México, Distrito Federal, 05300, México
[c]Facultad de Medicina, Departamento de Bioquímica, Universidad Nacional Autónoma de México, México, Distrito Federal, 04510, México
[d]Ingeniería Bioquímica, Instituto Tecnológico Superior de Atlixco, Atlixco, Puebla, México
*E-mail: ivonnearj@gmail.com; pankajsh@unam.mx

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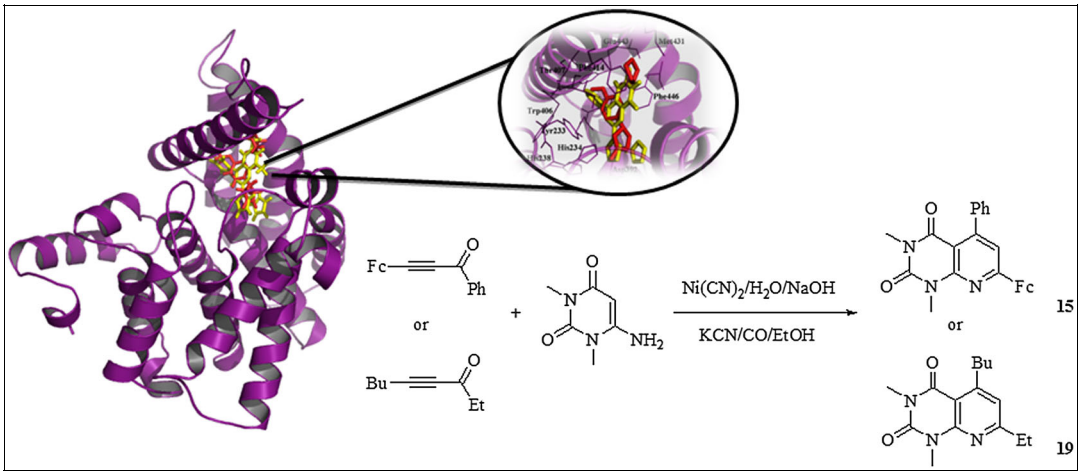


New pyrido[2,3-*d*]pyrimidines **11**, **12**, **13,** and **21** have been synthesized. The vasorelaxant effect on smooth muscle isolated from rat aorta, via PDEs inhibition, of these compounds along with other pyrido [2,3-*d*]pyrimidines **14-20** reported earlier by our group, has also been determined. These pyrido[2,3-*d*]pyrimidines **11–21** were synthesized by the reaction of ferrocenyl-ethynyl ketones (**1–4**) or α-alkynyl ketones (**5–10**) with 6-amino-1,3-dimethyluracil using $[Ni(CN)_4]^{-4}$ as an active catalytic species, formed *in situ* in a $Ni(CN)_2$/NaOH/H_2O/CO/KCN aqueous system. Evaluation of the vasorelaxant effect of compounds **11–21** demonstrated that all compounds relax the tissue in a concentration-dependent manner. The structural changes do not alter the effectiveness; however, there are differences related to potency expressed as EC_{50}. Compounds **12** (7-ferrocenyl-1,3-dimethyl-5-(*m*-tolyl)-pyrido[2,3-*d*]pyrimidine) and **13** (7-ferrocenyl-1,3-dipropyl-5-(4-metoxyphenyl)-pyrido[2,3-*d*]pyrimidine) were the most potent compounds, even more than rolipram, reference drug; the EC_{50} was 0.41 ± 0.02 μM and 0.81 ± 0.11 μM for **12** and **13,** correspondingly. The EC_{50} of compounds **15** (7-ferrocenyl-1,3-dimethyl-5-phenyl-pyrido[2,3-*d*]pyrimidine), **14** (7-ferrocenyl-5-(3,5-dimethoxyphenyl)-1,3-dimethylpyrido[2,3-*d*]pyrimidine), and **19** (5-*n*-butyl-7-ethyl-1,3-dimethylpyrido[2,3-*d*]pyrimidine) was similar to EC_{50} of rolipram. Compounds **11–21** significantly induce concentration-dependent vasorelaxation in endothelium-intact aortic rings. In addition, the relaxation responses to each compound in either endothelium-intact or endothelium denuded aortic rings were comparable, suggesting that removal of the functional endothelium has no significant influence on its intrinsic vasorelaxant activity. *In vitro* capability of conserving cyclic-AMP or cyclic-GMP (adenosine and guanosine 3′, 5′-cyclic monophosphate) via PDE inhibition for compounds **12–15** and **19** was evaluated. Compounds **15** and **19** show the highest percent inhibition effect (94.83% and 83.98%, respectively) for the decomposition of c-AMP. Docking studies showed that the compound **15** was selective for the inhibition of PDE-4.

INTRODUCTION

Pyrido[2,3-*d*]pyrimidines are versatile compounds with pharmacological effects as anti-hypertensive [1], analgesic, anti-inflammatory [2], vasorelaxant [3], cytotoxic [4], antiviral [5], antibacterial [6], antihistaminic [7], as protein kinases [8], tyrosine kinases [9], anti-proliferative CDK2 [10], and phosphodiesterases (PDEs) enzymes inhibitors, specifically, types 4 (PDE-4) [11,12] and 7 (PDE-7) [13]. PDEs are enzymes in charge for the hydrolysis of

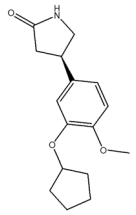


Figure 1. Rolipram.

intracellular cAMP or cGMP to AMP and GMP, respectively. Inhibition of these enzymes causes the increase of mentioned second messengers, inducing a vasorelaxant effect in a variety of blood vessels [14] and plays an important role for controlling the blood pressure [15] and is used for the development of new anti-hypertensive drugs [16,17].

Previously, we have reported the synthesis of some pyrido[2,3-*d*]pyrimidines analogs by an efficient and simple way through heterocyclization of α-ketoalkynes or ferrocenyl ethynyl ketones with 6-amino-1,3-dimethyluracil using $[Ni(CN)_4]^{-4}$ as an active catalytic species, formed *in situ* by a $Ni(CN)_2/NaOH/H_2O/CO/KCN$ aqueous system [18,19].

On the other side, many ferrocenyl compounds have demonstrated biological activities as cytotoxic, antitumor, antimalarial, antifungal, antioxidant, and antineoplastic [20–23]. It is also known that ferrocenyl group has been used widely in the design or redesign of drugs as phenyl bioisostere so it is worthwhile to functionalize pyrido[2,3-*d*]pyrimidines with a ferrocenyl group. In this work, several analogs of these compounds (some of them contain ferrocenyl group in the molecule) were synthesized to explore their activity as vasorelaxant through the inhibition of PDEs and to analyze the structure–activity relationship. Rolipram (Figure 1), a selective PDE-4 inhibitor that has shown to conserve cAMP levels in vascular smooth muscle [24], was used as a reference drug.

Table 1

Ethynyl ketones obtained to synthesize pyrido[2,3-*d*] pyrimidines.

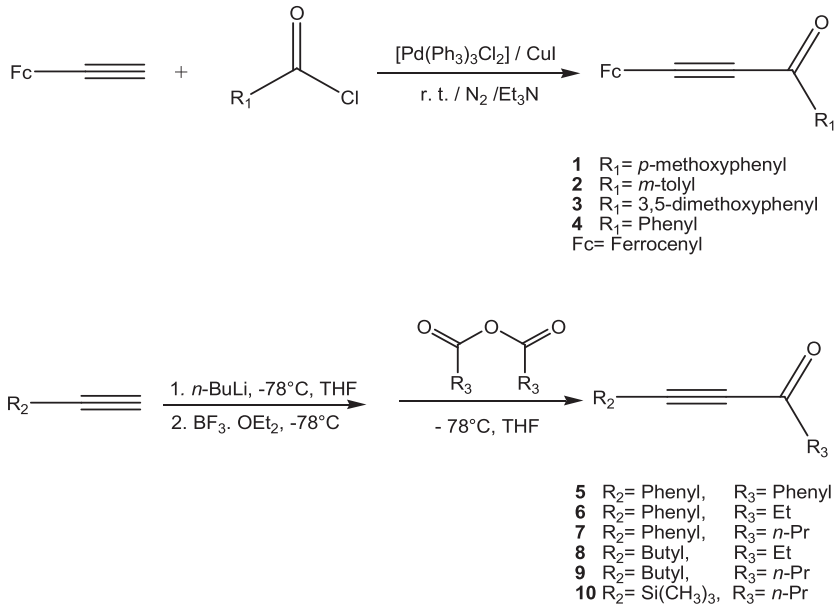
Compound	*R*	R_1	Ref.*
1	Ferrocenyl	*p*-methoxyphenyl	26
2	Ferrocenyl	*m*-tolyl	26
3	Ferrocenyl	3,5-dimethoxyphenyl	19
4	Ferrocenyl	Phenyl	19
5	Phenyl	Phenyl	27
6	Phenyl	Ethyl	18
7	Phenyl	Propyl	18
8	Butyl	Ethyl	18
9	Butyl	Propyl	18
10	$(CH_3)_3Si$	Propyl	28

*Ref., reference.
To obtain pyrido[2,3-*d*]pyrimidines **11** and **13**, compound **1** was used.

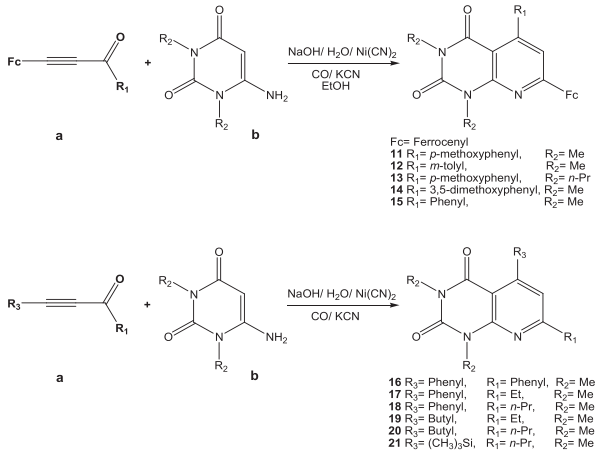
RESULTS AND DISCUSSION

Chemistry. Pyrido[2,3-*d*]pyrimidines were obtained through a heterocyclization reaction between α-

Scheme 1. Synthesis of ethynyl ketones. This figure is available in colour online at wileyonlinelibrary.com/journal/jhet



Scheme 2. Synthesis of pyrido[2,3-*d*]pyrimidines.



ketoalkynes or ferrocenyl ethynyl ketones and 6-amino-1,3-dimethyluracil. Ferrocenyl ethynyl ketones were obtained from ethynylferrocene and acyl chlorides via a palladium-catalyzed coupling reaction as reported earlier by our group (**1–4**) [19]. α-ketoalkynes were obtained from an alkynide, formed by an alkyne, *n*-BuLi, and BF_3OEt_2, and an anhydride in an inert atmosphere at −78°C (**5–10**) (Scheme 1) [25]. The compounds obtained in this work are shown in Table 1.

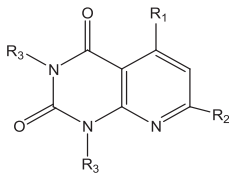
Pyrido[2,3-*d*]pyrimidines substituted at the C-7 position by a ferrocenyl group (**11–15**) were obtained from ferrocenyl ethynyl ketones and 6-amino-1,3-dimethyluracil (**11**, **12**, **14,** and **15**) or 6-amino-1,3-dipropyluracil (**13**) using a nickel aqueous catalytic system, as reported earlier [19]. Compounds that are substituted at the C-5 and C-7 positions by aryl or alkyl groups (**16–21**) were obtained by using α-ketoalkynes and 6-amino-1,3-dimethyluracil [18]. Compound **21** was obtained from the heterocyclization of silyl-α-ketoalkyne and 6-amino-1,3-dimethyluracil. Compounds **11**, **12**, **13,** and **21** are new and have not been reported earlier (Scheme 2) and were characterized by various physico-chemical methods. This method allows the easy preparation of several pyrido[2,3-*d*]pyrimidines-5,7 disubstituted in water under mild conditions.

Vasorelaxant effect of ferrocenyl, alkyl, and aryl-pyrido [2,3-d]pyrimidines on rat aorta. Pyrido[2,3-*d*] pyrimidines **11–21** (Table 2) were tested for their vasorelaxant activity in rat aortic rings pre-contracted with norepinephrine (NE) (0.1 μM). Figure 2 shows the concentration–response curves of these compounds. Rolipram, a PDE-4 inhibitor, was used as a reference drug. It is noted that all compounds relax the tissues in a concentration-dependent manner. All compounds present vasodilator effect, which indicates that the pyrido[2,3-*d*] pyrimidine ring retains its effect and does not lose it even if it is substituted at N-1 and N-3 positions with a methyl group (compounds **11**, **12,** and **14–21**) or a propyl group as in compound **13**.

EC_{50} values for compounds **11–21** along with the EC_{50} value for rolipram are shown in Table 2. These values are in the following descendent potency order

Table 2

Pyrido[2,3-*d*]pyrimidines obtained and evaluated by their vasodilator effect in rat aortic rings (results expressed as EC_{50}).

Compound			Yield or reference	EC_{50} [μM]	
	R_1	R_2		With endothelium	Without endothelium
11	*p*-methoxyphenyl	Ferrocenyl	70%	9.83 ± 2.28	10.84 ± 2.50
12	*m*-tolyl	Ferrocenyl	70%	0.41 ± 0.02	0.36 ± 0.06
13*	*p*-methoxyphenyl	Ferrocenyl	70%	0.81 ± 0.11	0.80 ± 0.01
14	3,5-dimethoxyphenyl	Ferrocenyl	Ref. 18	1.45 ± 0.39	1.78 ± 0.80
15	Phenyl	Ferrocenyl	Ref. 18	2.00 ± 0.71	1.81 ± 0.45
16	Phenyl	Phenyl	65%	3.13 ± 0.16	3.52 ± 0.25
17	Phenyl	Ethyl	Ref. 17	3.61 ± 0.74	3.63 ± 0.83
18	Phenyl	Propyl	Ref. 17	2.31 ± 0.77	2.57 ± 0.79
19	Butyl	Ethyl	Ref. 17	1.60 ± 0.36	1.80 ± 0.26
20	Butyl	Propyl	Ref. 17	5.00 ± 0.44	4.14 ± 0.33
21	H	Propyl	70%	5.63 ± 1.16	4.31 ± 0.59
Rolipram	NA	NA	NA	1.821 ± 0.72	NE

*For compound **13**, R_3 = *n*-Propyl. For compounds **11**, **12**, **14–21**, R_3 = Me.
EC_{50} [μM], half maximal effective concentration. Each value represents the mean ± SEM of at least six experiments.
NA, do not apply; NE, was not evaluated; Ref., reference.

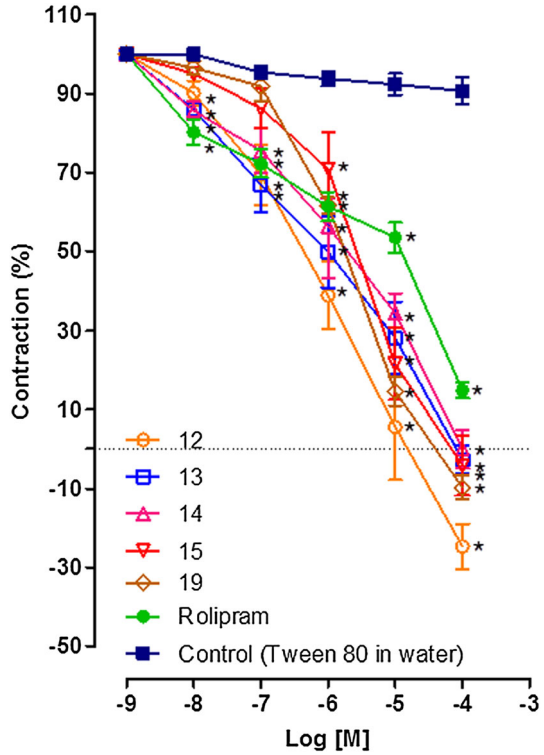


Figure 2. Concentration–response curves of pyrido[2,3-*d*]pyrimidines **12–15**, **19**, and rolipram. Each point represents the mean ± SEM ($n = 6$). *$p < 0.05$, significant difference compared with the respective control (Dunnett's *t*-test after analysis of variance).

12 > **13** > **14** > **19** > rolipram > **15** > **18** > **16** > **17** > **20** - **21** > **11**. It is observed that **15** and **19** have a similar potency as of rolipram. From the statistical analysis of maximum effect for compounds **11–21**, it is concluded that structural changes do not alter the effectiveness; however, there are difference related to potency expressed as EC_{50}. It was observed that compounds **11–21** significantly induce concentration-dependent vasorelaxation in endothelium-intact aortic rings (Table 2). Figure 2 shows concentration–response curves of compounds **12–15**, **19,** and rolipram for a better understanding. In addition, the relaxation responses to each compound in either endothelium-intact or endothelium denuded aortic rings were comparable (Table 2), suggesting that removal of the functional endothelium does not influence significantly on its intrinsic vasorelaxing activity.

The structural analysis showed that compound **11**, substituted with a ferrocenyl group at C-7 and *p*-methoxyphenyl at C-5, was less potent as vasorelaxant; however, its effectiveness is not very different from the other compounds, except from compound **12**. On comparing compound **13** analogous to **11**, it was inferred that a longer chain at positions N-1 and N-3 promotes the vasodilatory effect of pyrido[2,3-*d*]pyrimidines. When the position C-7 is substituted with a propyl group as in compounds **18**, **20,** and **21**, the relaxing power is slightly higher for compound **20,** which is substituted by an *n*-butyl group at C-5 than compound **21,** which is unsubstituted at C-5. This indicates that the substitution at C-5 position of the ring has a plus effect on the activity and it is better if substituent is a phenyl group (compound **18**). A similar tendency is observed in the relaxing effects for compounds **16** and **17**, which is greater than the relaxing effects for compounds **20** and **21**, confirming that the presence of aromatic rings at C-5 increases the relaxing potency.

In the case of compounds **15** and **18,** which have aromatic ring at C-5, whereas C-7 position is substituted with a ferrocenyl group and propyl group, respectively, an increased relaxing effect was observed. When the aromatic ring at C-5 of compound **15** was replaced with a 3,5-dimethoxyphenyl group as in compound **14**, an improvement of vasorelaxant effect was observed, which indicates that substitution on phenyl group at position C-5 is preferred. Compounds **12** and **13** are the most potent, which are ferrocenyl-substituted at C-7 position. Compounds **15** and **19** show a similar potency as shown by the reference drug rolipram.

Inhibition of degradation of cAMP or cGMP via phosphodiesterases inhibition. Compounds **12–15** and **19** were evaluated as inhibitors of PDEs, as these compounds presented the most potent vasorelaxant effect. A colorimetric enzymatic assay was carried out, in which these compounds were capable to avoid the degradation of secondary messengers (cAMP and cGMP) via PDEs inhibition. Rolipram was used as a reference compound, which is a selective inhibitor of PDE-4. Table 3 presents the percentage inhibition of degradation of cAMP or cGMP by compounds **12–15** and **19**. All the tested compounds inhibit the action of PDE, and the observed changes depend on the substrate used, and in fact, the compounds inhibit the degradation of cAMP. When cAMP was used as a substrate, all compounds presented inhibitory effect from 30% to 95%, unlike when cGMP

Table 3

Percentage inhibition of degradation of cAMP or cGMP via PDE inhibition.

	Substrate	
Compound	cAMP	cGMP
12	31.24	20.13
13	30.00	17.53
14	50.83	16.73
15	94.83	5.68
19	83.98	6.38
Rolipram	70.43	16.56

Each percentage inhibition shows the maximum inhibition effect of compounds to 100 μM.

was used as a substrate presenting inhibitory effect of approximately 6% and 20%. Compounds **12** and **13** present the best vasorelaxant effect, inhibiting the degradation of cAMP (31.24% and 30.00%, respectively), which indicates that their relaxant effect occurs through another mechanism.

It has been well documented that the presence of PDE-1, PDE-3, PDE-4, and PDE-5 in vascular smooth muscle tissue [14,29] and an increase in the concentration of cAMP causes relaxation of vascular smooth muscle [29] tissue, where the pyrido[2,3-d]pyrimidines **15** and **19** showed a similar activity than rolipram. This result suggests that **15** and **19** have a vasorelaxant effect on smooth muscle isolated from rat aorta via inhibition of cAMP-dependent PDEs.

Molecular docking. According to the results of the enzyme inhibition, it was observed that compounds **12–15** and **19** are more efficient to inhibit degradation of cAMP, and its binding to the cAMP-dependent PDEs was predicted using a docking study. Docking studies were carried out with the AUTODOCK 4.0.2 program, and compounds **12–15** and **19** were evaluated by their capability to bind PDE-3 and PDE-4 cleavage to obtain the theory K_i. These enzymes were used as they are cAMP-dependent and present in a considerable extent in rat aorta.

The structures of compounds **12–15** and **19** were built using the program HyperChem 8 release and optimized using the Gaussian software. 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 and PDE-4 (K_i values are indicated in Table 4). All compounds exhibited affinity to the proteins. In relation to the reference drugs, enoximone (PDE-3 Inhibitor) and rolipram (PDE-4 inhibitor) showed the proven affinity for their respective PDE.

In silico studies showed that compound **15** binds to the catalytic site of PDE-4, in a similar way as shown by rolipram. Besides, both compounds have a minor *Ki* value for PDE-3 (0.77 and 0.99 fold less, respectively, than for PDE-4). Compound **19** has affinity for the catalytic site of PDE-3. These facts demonstrate a good correlation with the *in vitro* assays. Thus, compound **15**, which is substituted by a ferrocenyl group at C-7 position and by a six-membered aromatic ring at C-5 position, is the most effective inhibitor of degradation of cAMP (Table 3) and shows a similar vasorelaxant effect as rolipram (Table 2) and the best *Ki* value for PDE-4. In the same way, compound **19**, which is substituted by alkyl chains at C-5 and C-7 positions, shows a best *Ki* value for PDE-3 than for PDE-4. This explains the fact that in *in vitro* PDE assay, **19** is more effective than rolipram and **15** is more effective than **19**. Figure 3 shows the results of docking study. Compound **15** and rolipram bound to the same catalytic site formed by the amino acids Tyr233, His234, His238, Asp392, Leu393, Asn395, Pro396, Tyr403, Trp406, Thr407, Ile410, Phe414, Met431, Ser442, Gln443, Phe445, and Phe446.

Table 4

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

	PDE-3		PDE-4	
Compound	K_i (nM)	EFEB (kcal/mol)	K_i (nM)	EFEB (kcal/mol)
12	90.04	−9.61	151.32	−9.30
13	131.84	−9.39	87.72	−9.63
14	1110	−8.12	444.26	−8.67
15	127.36	−9.41	71.56	−9.75
19	894.61	−8.25	1240	−8.06
Rolipram	784.38	−8.33	392.28	−8.74
Enoximone	3840	−7.39	4920	−7.24

Ki, estimate inhibition constant (μM). EFEB, estimating free energy of binding (Kcal/Mol).

CONCLUSION

New pyrido[2,3-*d*]pyrimidines **11**, **12**, **13,** and **21** have been synthesized and characterized. Vasorelaxant effect via PDEs inhibition of these compounds along with known pyrido[2,3-*d*]pyrimidines **14–20** has also been determined. Apparently, there is no clear trends in potency from the different substitution patterns, compounds **15** and **19** are the most potent PDE inhibitors and are more effective than rolipram. Docking study demonstrates that compound **15** has an affinity for the catalytic site of PDE-4, and compound **19** has an affinity for the catalytic site of PDE-3, and these results have a correlation with their vasorelaxant effects. Even though compounds **12** and **13** were presented as the most potent vasorelaxant substances, nevertheless, they are not so effective to inhibit degradation of cAMP or cGMP via PDE-inhibition suggesting that **12** and **13** present their relaxant effect through another pharmacological action mechanism.

Finally, because hypertension is one of the most common diseases related with vascular smooth muscle contraction, and the therapeutic strategies to combat the consequent damage to the vascular endothelium are generally aimed at modulating the molecular and biochemical mechanisms underlying this dysfunction, such as vascular smooth muscle contraction, the pyrido[2,3-*d*]pyrimidines are suggested as possible effective antihypertensive agents as vasodilators via PDE inhibition.

EXPERIMENTAL

Chemistry. 6-amino-1,3-dimethyluracil and 6-amino-1,3-diprophyluracil were purchased from Aldrich. ^{1}H and ^{13}C spectra were recorded on a JEOL GX-300 instrument (Tokyo, Japan), 300 MHz for ^{1}H and 75 MHz for ^{13}C using $CDCl_3$ as solvent. IR

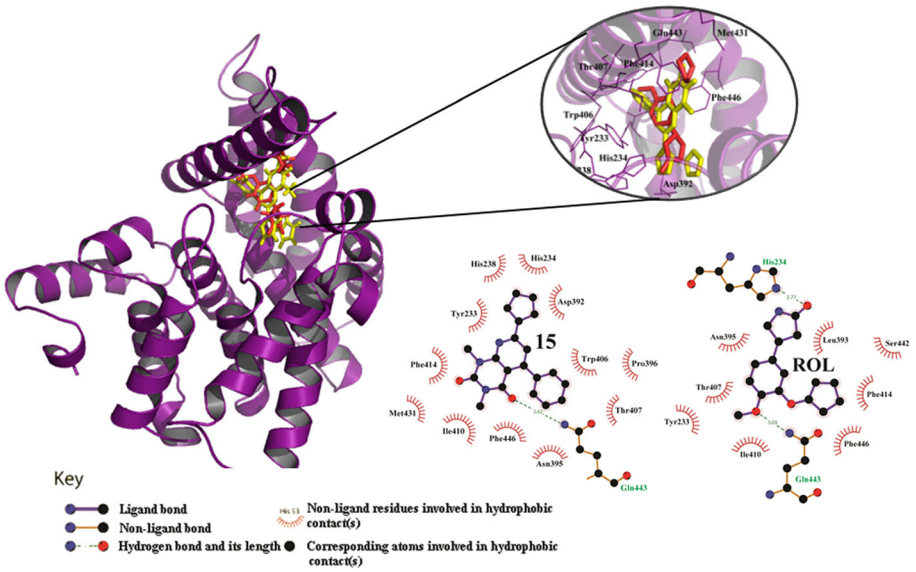


Figure 3. Structural models of PDE4-15 and PDE4 = rolipram complex. On the right shows the complex in magenta cartoon, yellow, and red stick the PDE4, **15**, and rolipram, respectively. The 2D representation of PDE4-15 and PDE4-rolipram complexes resulting from the docking is shown. The images were made with PyMOL and LIGPLOT.

spectra were recorded in film on a Nicolet FT 5SX spectrophotometer (Madison, Wisconsin, USA). Mass spectra were obtained using a JEOL JMSAX505HA spectrometer (Tokyo, Japan). Ferrocenyl-ethynyl ketones were prepared according to the previous report [19]. α-alkynyl ketones were obtained as follows.

In N_2 atmosphere, 40 mL of dried THF was added to a flask which was kept in a bath of acetone-dried ice to −78°C. About 30 mmol of the corresponding alkyl compound and 30 mmol of Bu-Li were then added. After 30 min, 30 mmol of BF_3OEt_2 and 45 mmol of the corresponding anhydride were added. After stirring for 15 min, reaction was quenching using 2 N NaOH solution (50 mL). The crude product was purified by flash chromatography using ethyl acetate/hexane (90:10) as eluent.

General procedure. ***Pyrido[2,3-d]pyrimidines.*** A 5N NaOH solution (50 mL) was degassed and saturated with CO under atmospheric pressure for 30 min, 2 mmol of $Ni(CN)_2.4H_2O$ was added to the solution, the mixture was kept at room temperature overnight, with stirring and slow bubbling of CO, until a pale yellow solution was obtained. Addition of 15 mmol of KCN resulted in a color change to orange. The catalytic species $[Ni(CN)_4]^{-4}$ is obtained when an excess of KCN is added to an alkaline solution of $Ni(CN)_2$ in CO atmosphere. After stirring for 0.5 h, the corresponding ketoalkyne (10 mmol) and the corresponding 6-amino-uracil (10 mmol) were added. The evolution of the reaction was followed by TLC. At the end of the reaction, ethyl acetate was used to extract the product. After evaporation of the solvent followed by drying over $MgSO_4$, the crude product was purified by flash chromatography using ethyl acetate/hexane (80:20) as eluent.

7-Ferrocenyl-1,3-dimethyl-5-(4-methoxyphenyl)-pyrido[2,3-d]pyrimidine-2,4-dione (11). The product was obtained as red crystals in a 70% yield (mp 243–244°C); mass spectrum IE: *m/z* (%): 481 (6) $M^{+\cdot}$, 416 (3), 91 (100); IR (film, cm^{-1}): 1702, 1656 (C=O), 1545 (C=N); ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 3.38 (*s*, 3H,$CH_3N(2)$), 3.81 (*s*, 3H, $CH_3N(1)$), 3.88 (*s*, 3H, OCH_3), 4.07 (*s*, 5H, cp′-ring), 4.52 (*t*, 2H, *J* = 1.9 Hz, cp-ring), 5.00 (*t*, 2H, *J* = 1.9 Hz, cp-ring), 7.00 (*d*, 2H, *J* = 10.8 Hz, 3,5-C_6H_4), 7.30 (*d*, 2H, *J* = 8.6 Hz, 2,6-C_6H_4), 8.13 (*s*, 1H, C=CH); ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 29.1 (N(2)CH_3), 31.7 (N(1)CH_3), 55.6 (OCH_3), 69.6 (C–C, cp-ring), 70.4 (C–H, cp′-ring), 70.9 (C–H, cp-ring), 79.4 (C–H, cp-ring), 113.8 (C6), 119.4 (C 3,5-C_6H_4), 130.6 (C 2,6-C_6H_4) 132.5 (*C*1-C_6H_4) 150.9 (*C*2), 151.3 (*C*7), 154.7 (*C*5), 157.3 (*C*9), 160.1 (*C*4).

7-Ferrocenyl-1,3-dimethyl-5-(m-tolyl)-pyrido[2,3-d]pyrimidine-2,4-dione (12). The product was obtained as orange powder in 70% yield (mp 249.5–250°C); mass spectrum IE: *m/z*(%): 465 (97) $M^{+\cdot}$, 400 (100); IR (film, cm^{-1}): 1700, 1657 (C=O), 1548 (C=N); ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 2.50 (*s*, 3H, Ph-CH_3), 3.40 (*s*, 3H,$CH_3N(2)$), 3.84 (*s*, 3H, $CH_3N(1)$), 4.16 (*s*, 5H, cp′-ring), 4.47 (*t*, 2H, *J* = 1.8 Hz, cp-ring), 4.72 (*t*, 2H, *J* = 1.8 Hz, cp-ring), 7.34 (*d*, 1H, *J* = 7.3 Hz, 4-C_6H_4), 7.45 (*t*, 1H, *J* = 7.5 Hz, 3-C_6H_4), 7.95 (*d*, 2H, *J* = 8.8 Hz, 2,6-C_6H_4), 8.15 (*s*, 1H, C=CH);^{13}C NMR (75 MHz, $CDCl_3$, ppm): 21.9 (Ph*C*H_3), 28.6 (N(2)*C*H_3), 30.5 (N(1)*C*H_3),68.5 (*C*–C, cp-ring), 70.5 (*C*–H, cp′-ring), 72.7 (*C*–H, cp-ring), 84.2 (*C*–H, cp-ring), 107.6 (C=*C*H), 120.6 (*C*2-C_6H_4), 124.6 (*C*3-C_6H_4), 128.2 (*C*4-C_6H_4), 129.3 (*C*6-C_6H_4), 131.6 (*C*5-C_6H_4), 138.9 (*C*1-C_6H_4), 151.6 (*C*2), 151.9 (*C*7), 154.4 (*C*5), 157.9 (*C*9), 160.7 (*C*4).

7-Ferrocenyl-1,3-dipropyl-5-(4-methoxyphenyl)-pyrido[2,3-d]pyrimidine-2,4-dione (13). The product was obtained as orange powder in 70% yield (mp 166–167°C); mass spectrum IE: *m/z* (%): 537 (32) $M^{+\cdot}$, 472 (23), 132 (100); IR (KBr, cm^{-1}): 1703, 1660 (C=O), 1542 (C=N); ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 0.92 (*t*, 3H, *J* = 7.4 Hz, N(2)($CH_2CH_2CH_3$), 1.05 (*t*, 3H, *J* = 7.4 Hz, N(1)($CH_2CH_2CH_3$), 1.65 (*sex*, 2H, *J* = 7.5 Hz, N(2)($CH_2CH_2CH_3$), 1.85 (*sex*, 2H, *J* = 7.4 Hz, N(1)($CH_2CH_2CH_3$), 3.91 (*s*, 3H, OCH_3), 3.97 (*m*, 2H, N(2)($CH_2CH_2CH_3$), 4.16 (*s*, 5H cp′-ring), 4.43 (*m*, 2H, N(1)($CH_2CH_2CH_3$), 4.45 (*t*, 2H, *J* = 1.9 Hz, cpring), 4.68 (*t*, 2H, *J* = 1.8 Hz, cp-ring), 7.07 (*d*, 2H, *J* = 9.1, 3,5-C_6H_4), 8.08 (*s*, 1H, C=C*H*), 8.12 (*d*, 2H, *J* = 8.9, 2,6-C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$, δ in ppm): 11.4 (N(2)$CH_2CH_2CH_3$), 11.6 (N(1)$CH_2CH_2CH_3$), 21.2 (N(2)$CH_2CH_2CH_3$),

21.4 (N(1)$CH_2CH_2CH_3$), 43.4 (N(2)$CH_2CH_2CH_3$), 44.8 (N(1)$CH_2CH_2CH_3$), 55.6 (OCH_3), 68.8 (*C*–C, cp-ring), 70.2 (*C*–H, cp′-ring), 72.2 (*C*–H, cp-ring), 84.9 (*C*–H, cp-ring), 114.6 (C6), 119.3 (C 3,5-C_6H_4), 128.7 (C 2,6-C_6H_4), 130.1 (C1-C_6H_4), 151.6 (C2), 154.0 (C7), 154.9 (C5), 157.2 (C9), 160.5 (C4), 161.7 (C4-C_6H_4).

7-propyl-1,3-dimethylpyrido[2,3-d]pyrimidine-2,4-dione (21). The product was obtained as white needles in 70% yield (mp 86–86.5°C); mass spectrum IE: *m*/*z* (%): 233 (14) $M^{+\cdot}$, 205 (100); ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 0.91 (*t*, 3H, CH_3), 1.72 (*m*, 2H, $CH_3CH_2CH_2$), 2.74 (*t*, 2H, $CH_3CH_2CH_2$), 3.40 (*s*, 3H, N(1)CH_3), 3.65 (*s*, 3H, N(2)CH_3), 6.96 (*d*, 1H, CH=C*H*) 8.26 (*d*, 1H, C*H*=CH); ^{13}C NMR (75 MHz, $CDCl_3$, δ in ppm): 13.8 ($CH_3CH_2CH_2$), 22.3 ($CH_3CH_2CH_2$), 28.3 (N(2)CH_3), 29.3 (N(1)CH_3), 40.6 ($CH_3CH_2CH_2$), 108.2 (C7), 118.2 (C6), 137.5 (C5), 150.5 (C10), 151.0 (C9), 161.6 [N(*C*=O)N], 168.4 [N(*C*=O)C].

Other pyrido[2,3-*d*]pyrimidines **14**, **15**, **16**, **17**, **18**, **19**, and **20** were synthesized according to our earlier reports. *7-Ferrocenyl-5-(3,5-dimethoxyphenyl)-1,3-dimethylpyrido[2,3-d]pyrimidine-2,4-dione* (**14**) and *7-Ferrocenyl-1,3-dimethyl-5-phenyl-pyrido[2,3-d]pyrimidine-2,4-dione* (**15**) [19]. *1,3-dimethyl-5,7-diphenylpyrido[2,3-d]pyrimidine-2,4-dione* (**16**); [30] *7-Ethyl-1,3-dimethyl-5-phenylpyrido[2,3-d]pyrimidine-2,4-dione* (**17**); *1,3-Dimethyl-5-phenyl-7-propylpyrido[2,3-d]pyrimidine-2,4-dione* (**18**); *5-n-Butyl-7-ethyl-1,3-dimethylpyrido[2,3-d]pyrimidine-2,4-dione* (**19**) and *5-n-Butyl-1,3-dimethyl-7-n-propylpyrido[2,3-d]pyrimidine-2,4-dione* (**20**) [18].

Vasorelaxant procedure. Acetylcholine chloride (ACh), (−) NE, rolipram, and enoximone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sildenafil citrate was used as USP reference standard. The drugs were suspended in 0.05% Tween 80 in distilled water. The final concentration of Tween 80 was in trace (less than 0.0005%) and did not affect the tracheal or vascular response. The drug solution or suspensions was freshly prepared each time few minutes before the experimentation. Male rat (Wistar) weighting 200–300 g was obtained from Harlan México (México). Animals were maintained at constant room temperature (22 ± 2°C) and submitted to 12 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; Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio) 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, Facultad de Química, UNAM. Male Wistar rats were euthanized in a CO_2 chamber, and thoracic aorta was removed and immediately immersed in a Krebs solution at 37°C. After removal the excess of 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, 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 were digitalized and analyzed by mean 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, and they were washed with fresh Krebs solution at 15-min interval before starting the experiments. After stabilization period, the rings were contracted with NE (0.1 μM) two times at 30-min interval. The integrity of the endothelium was verified by the relaxant response to ACh (10 μM); intact endothelium showed relaxation over 84%, the endothelium absence (denuded aorta) did not relax with Ach. About 30 min after the tissues were contracted with NE (0.1 μM), cumulative concentrations of synthesized compounds or reference drugs were added to the bath to yield the required aorta relaxant effects and allowed to reach a steady state at each concentration. The concentration required to relax 50% of the aorta pre-contracted with NE that was expressed as EC_{50}.

Phosphodiesterase inhibition procedure. For the PDE assay, the drugs were dissolved in a binary mixture of acetonitrile (Burdick & Jackson, MI, USA)/water (Direct-Q Millipore water purification system) (1:1). PDE enzyme from bovine brain, 5′nucleotidase from Crotalusatrox venom, 3′,5′c-AMP, 3′,5′-cGMP, buffer (10-mM Tris-HCl, pH 7.4), BIOMOL GREENTM reagent and IBMX were purchased from BIOMOL International, Inc. (PA, USA).The enzyme inhibition assay was performed using BIOMOL cyclic nucleotide PDE assay kit, consisting in 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 enzyme 5′-nucleotidase. The phosphate released because of enzymatic cleavage is quantified using BIOMOL GREEN reagent in a modified malachite green assay [31]. It may be used to screen inhibitors and modulators of cyclic nucleotide PDE activity. The compounds and reference drugs were tested at different concentrations: ferrocenyl, alkyl and aryl-pyrido[2,3-*d*]pyrimidines (1.0–300 μM), rolipram (3.0–300 μM), and sildenafil (0.001–300 μM).

Data analysis. The EC_{50} values for relaxant effect were calculated by linear regression and are shown as mean ± SEM of at least six experiments. The differences among these obtained values were statistically calculated by one-way analysis of variance and then determined by Dunnett's *t*-test [16]. $p < 0.05$ was considered as statistically significant. The inhibitory activities of PDE by ferrocenyl, alkyl, and aryl-pyrido[2,3-*d*]pyrimidines or reference drugs are reported as IC_{50} ± SEM or maximal percent of inhibition of at least three experiments.

Computational method. Docking was carried out using the X-ray structure of PDE3B in complex with a dihydropyridazine inhibitor (1SO2.pdb) and PDE4B complexed with rolipram (1RO6). The ligands **12–15**, **19**, rolipram, and enoximone were built using the program HyperChem 8.0 and optimized using the density functional method, at the B3LYP/6-31G* level [32], with the program Gaussian 09, revision A.02 (Gaussian Inc., Wallingford, CT, USA). The protein complex and the ligand were further prepared using the utilities implemented by AutoDockTools 1.5.4, revision 30 (http://mgltools.scripps.edu/). The protein complex was adding polar hydrogen atoms, Kollman united-atom partial charges and to the ligands computing Gasteiger–Marsilli formalism charges, rotatable groups which were assigned automatically as were the active torsions. The initial grid box size was 60Å × 60Å × 60Å in the *x*, *y*, and *z*-dimensions. Blind docking was carried out using AutoDock4 version 4.2 software http://autodock.scripps.edu/ [33,34] using the default parameters, the Lamarkian genetic algorithm with

local search, number of individuals in population (150), maximum number of energy evaluations (2.5 million), maximum number of generations (27,000), rate of gene mutation (0.02), rate of crossover (0.8), and 200 runs for docking. Electrostatic grid maps were generated for each atom type in the ligands using the auxiliary program AutoGrid4 part of the software AutoDock4. All calculations were made using a parallel distributed memory supercomputer (Kanbalam, Dirección General de Cómputo y de Tecnologías de Información y Comunicación, UNAM), which contains 1368 processors AMD Opteron, around 3 terabyte of memory and 160 terabyte of storage (http://www.super.unam.mx/). The analysis of the docking was made with AutoDockTools using cluster analysis and program LIGPLOT and PyMOL [35].

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