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Heterocyclic-fused 3(2H)-pyridazinones as potent and selective PDE IV inhibitors: Further structure–activity relationships and molecular modelling studies

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Abstract – A novel group of heterocyclic-fused 3(2H)-pyridazinones were synthesized and evaluated as PDE III and PDE IV inhibitors and their affinity for ^{3}H Rolipram high affinity binding site was determined. The obtained data demonstrated that some of the new compounds are endowed with potent and selective PDE IV inhibitory activity and greatly attenuated affinity for the Rolipram high affinity binding site that seems to be responsible for unwanted effects. Theoretical calculations, performed on representative compounds, demonstrated the presence of three hydrogen-bonding acceptor regions, of which one looks quite different with respect to literature compounds. This finding could explain the different pharmacological profile of the title compounds with respect to the analogs reported in the literature. © Elsevier, Paris

heterocyclic-fused pyridazinones / phosphodiesterase IV inhibitory activity

1. Introduction

Due to their mixed anti-inflammatory and bronchodilatory effects, mediated by the increase of cyclic adenosine monophosphoric acid (c-AMP) intracellular level, phosphodiesterase IV (PDE IV) inhibitors have attracted considerable interest in the last few years [1–4]. Since today it is generally accepted that in the pathogenesis of asthma there is an important inflammatory component [5], a new anti-asthma drug displaying the pharmacological profile of the selective PDE IV inhibitors could be an appropriate substitute for the combined therapy with β_2 agonists and corticosteroids. Indeed today this combination represents the common therapeutic management of this wide-spread respiratory pathology [6].

Rolipram **1** [7] (*figure 1*), as well as the earlier prototypes of PDE IV inhibitors, did not develop as drugs due to their unwanted side-effects such as nausea, vomiting and headache. These adverse properties are poorly correlated to inhibition of PDE IV cathalitic site, but are closely associated with binding to a form of the enzyme to which Rolipram binds with high affinity (K_d = 2 nM). On the contrary, some anti-inflammatory effects correlate more closely to the inhibition of a PDE IV form against which Rolipram exhibits relatively low potency (> 100 nM) [8].

Thus, renewed interest for a new generation of potent and selective PDE IV inhibitors with reduced affinity for Rolipram binding site was stimulated [9, 10].

On these grounds a previous paper from our laboratory [11] dealt with the synthesis of some series of heterocyclic-fused pyridazinones, structurally related to the Syntex agents **2** [12] and **3** [13]. Representative compounds such as **4**, **5** and **6**, in which the presence of the ethyl group at pyridazine N-2 is an essential requirement for high potency and selectivity, demonstrated to have a considerably better balance between PDE IV inhibitory activity and affinity for ^{3}H Rolipram high affinity binding site, with respect to compounds **1–3**.

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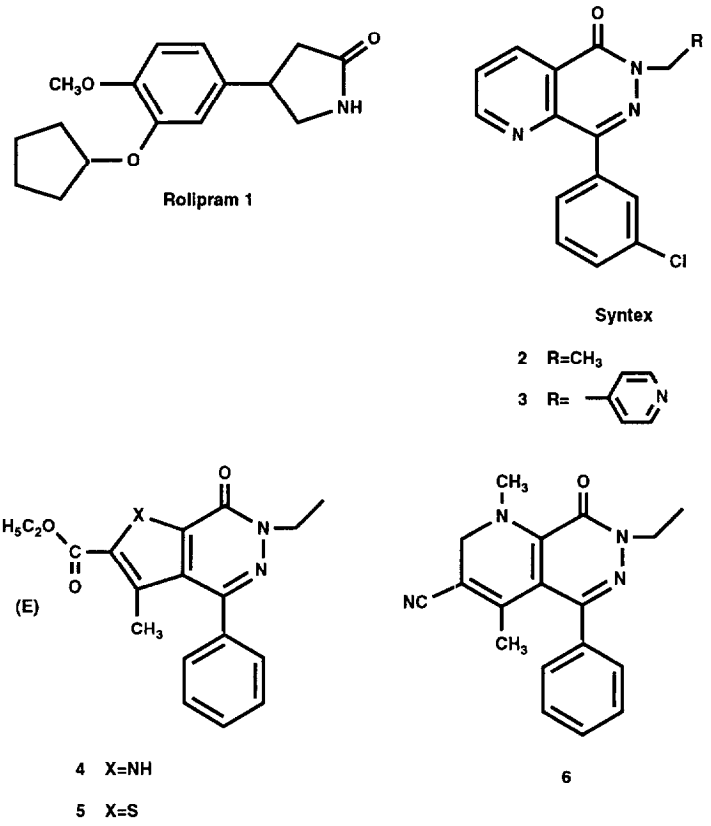

Figure 1. Chemical structures of compounds **1–6**.

We wish to report here the synthesis and evaluation as PDE IV inhibitors of a new group of heterocondensed pyridazinones **8a–d**, **9a–d**, **10**, **11** and **13**, as well as a molecular modelling study performed on compounds **4–6** in comparison with **2**.

2. Chemistry

Compounds **8a** and **8c–d** were synthesized by briefly heating **7** [14] with the appropriate alkyl halide in anhydrous DMF in the presence of potassium carbonate (*figure 2*). The previously described **8b** was prepared in higher yields with respect to the literature [14], following the above procedure.

The carboxylic acids **9a** and **9c** were obtained from the corresponding ethyl esters **4** and **5** [11a] by moderate heating with ethanolic sodium hydroxide, followed by acidification with 6 N hydrochloric acid (*figure 3*). Compounds **9b** and **9d** were synthesized from the same precursors by stirring at room temperature, or by moderate heating with free hydroxylamine in ethanol in inert atmosphere.

Treatment of **9c** with thionyl chloride, followed by quenching with aqueous ammonia, afforded the amide

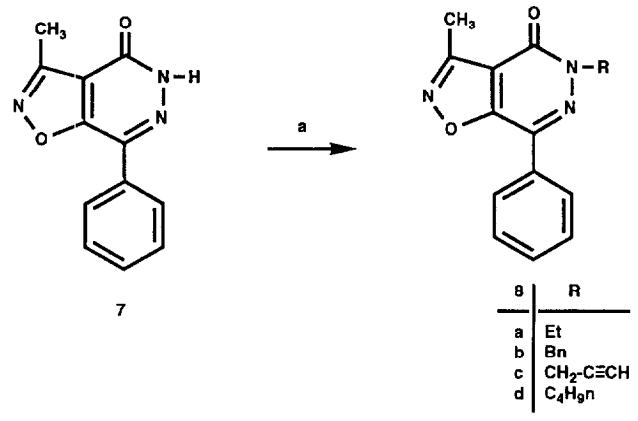

Figure 2. Conditions: (a) RX, K_2CO_3, DMF, 100 °C, 1 h.

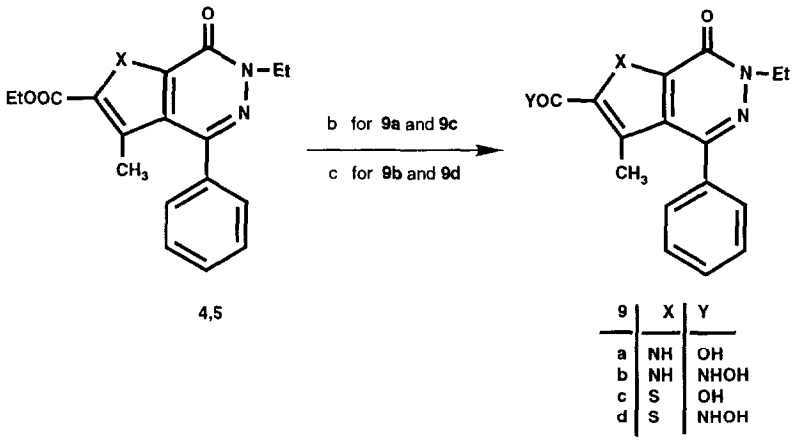

Figure 3. Conditions: (b) 6 N NaOH, EtOH, 25–50 °C, 1.5–3 h; (c) NH_2OH, abs. EtOH, 25–65 °C, 2–6 h.

10 which, in turn, was converted into the cyano derivative **11** by heating with phosphorus oxychloride (*figure 4*).

In the same scheme the synthetic procedure for compound **13** is depicted: the 5-acetyl-4-nitropyridazinone **12** [15] was treated with N-methyl-β-alaninenitrile in ethanol at room temperature. The open-chain intermediate, arising from the nucleophilic attack to the pyridazine-4 carbon, was not isolated and was cyclocondensed into the final compound **13** by briefly heating with sodium ethoxide in anhydrous ethanol. Physical and chemical data of compounds **8a–13** are shown in *table I*.

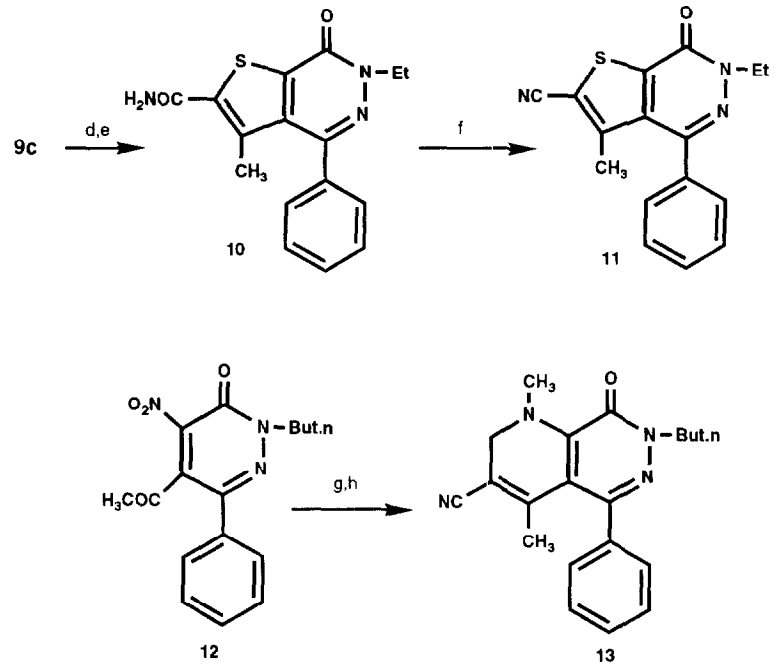

Figure 4. Conditions: (d) $SOCl_2$ reflux, 2 h; (e) 30% aqueous NH_3; (f) $POCl_3$, 120 °C, 2 h; (g) N-methyl-β-alanine nitrile, EtOH, 25 °C, 20 min; (h) EtONa, abs. EtOH, 50–60 °C, 2 h.

Table I. Chemical and physical data of compounds **8–13**.

Compound [a]	Yield	M.p. (°C)	Formula [b]
8a	76	117–119	$C_{14}H_{13}N_3O_2$
8b [c]	87	164–166	$C_{19}H_{15}N_3O_2$
8c	74	135–137	$C_{15}H_{11}N_3O_2$
8d	69	125–127	$C_{16}H_{17}N_3O_2$
9a	82	> 310	$C_{16}H_{15}N_3O_3$
9b	73	> 270	$C_{16}H_{16}N_4O_3$
9c	76	205–207	$C_{16}H_{14}N_2O_3S$
9d	61	204–206	$C_{16}H_{15}N_3O_3S$
10	89	225–226	$C_{16}H_{15}N_3O_2S$
11	77	170–172	$C_{16}H_{13}N_3OS$
13	48	143–145	$C_{20}H_{22}N_4O$

[a] All compounds were recrystallized from EtOH. [b] The elemental analyses were within ±0.4%. [c] See [14].

3. Molecular modelling studies

The attractive pharmacological profile of the previously described compounds **4–6** [11], as well as of the herein reported products, prompted us to investigate steric and electronic features of some representative molecules, such as **4**, **5** and **6** in comparison with the analog Syntex **2**.

Structures were built as reported below (see experimental protocols). The structures of **4**,**5** and **6** (*figure 1*) exhibit several differences compared with the Syntex compound **2** [12]. The introduction of a methyl group in the hetero-fused ring may orientate the phenyl group linked in the pyridazinone moiety in a different angle. Moreover, introduction of new functional groups, such as cyano and ester, may involve additional steric volume, as well as new interaction points.

For compounds **4** and **5** two possible conformations, namely *Z* and *E*, around the carbonyl disposition were considered. For such compounds heats of formation were calculated, concluding that *E* and *Z* conformers are almost isoenergetics, although *E* conformer is sligthly more stable (*table II*).

Table II. Heat of formation of conformer *E* and *Z* of compounds **4** and **5**.

Compound	Conformer	Heat of formation (Kcal/mol)
4	*E*	–20.87
	Z	–20.00
5	*E*	–28.66
	Z	–27.78

Further studies, in terms of conformational analysis and map calculations, have been done with *E* conformer.

Analysis of the rotation of the 3-chlorophenyl ring of **2** with respect to the pyridopyridazinone system, shows that after AM1 optimization four different conformations were obtained. Two of them with an angle between both rings of 58° (both conformations with the chlorine in the phenyl ring in symmetrical disposition) and the other two with an angle of 122°. Heats of formation are between 63.79 and 63.85 Kcal/mol for the four types of conformations.

The conformations obtained after conformational analysis of compound **5**, followed by AM1 optimization, dropped in only one group of structures with an angle between both rings of 88°.

In compound **4** two groups of conformations were obtained, with angles of 61° and 104°. Both conformations were almost isoenergetics (–20.9 and –20.0 Kcal/mol respectively).

Conformational analysis of compound **6** yielded only one group of conformations with an angle of 66°. Thus, comparing **2** with the above compounds, it emerged that **4** and **6** exhibited similar angles between both rings, while **5** adopted a disposition between both rings almost perpendicular.

Since for compounds **4–6** both PDE IV inhibitory activity and ^{3}H Rolipram binding site affinity are almost identical [11], this finding suggests that this geometrical feature does not play a significant role in determining these types of biological property.

Figure 5 shows the additional volume of VdW for compounds **4**, **5** and **6** in relation to that of **2**. Maps of VdW were individually calculated for all compounds and the VdW map of **2** was subtracted (application of logical operator NOT implemented in ChemX). A significant additional volume, due to the methyl and other functional groups introduced over the hetero-fused ring, can be envisaged.

Figure 6 shows the GRID maps obtained for all compounds using the NH amide probe contoured at a level of –4 Kcal/mol. The maps identify the areas of the molecules which may act as hydrogen bond acceptor. Looking at these maps, not only new steric volume may differentiate compounds **4–6** from **2**, but also different points of interaction as hydrogen bond acceptor at the level of the new functional groups. All compounds share three interaction regions, but the third region looks different from **4–6** with respect to that produced in compound **2** by the pyridinic nitrogen.

Taken togheter all these data seems to suggest that the steric and electronic differences between **2** and **4–6** could

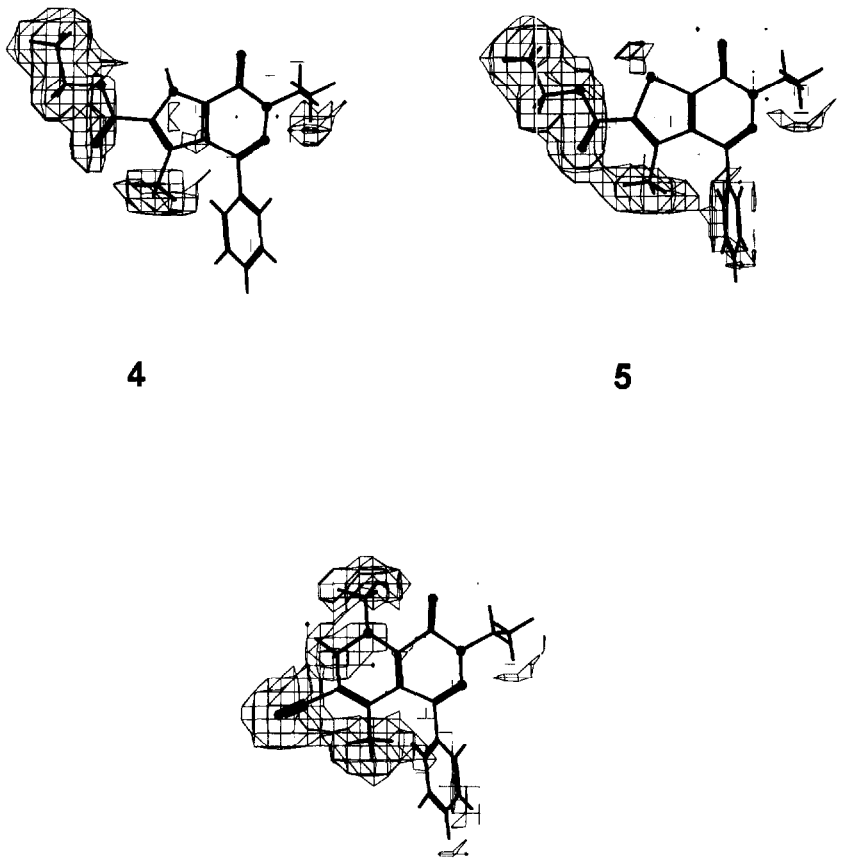

Figure 5. Additional volume of VdW of compounds **4, 5** and **6** which is not present in **2**.

explain the better balance of PDE IV inhibitory activity and ^{3}H Rolipram binding site affinity which features **4–6** with respect to Syntex **2** [11].

4. Results and discussion

All novel final compounds were tested as PDE III and PDE IV inhibitors and evaluated for their ability to displace ^{3}H Rolipram from its binding site. Rolipram **1**, the Syntex compound **3**, as well as the previously reported **4**, **5** and **6** [11] were used as reference substances (*table III*).

Compounds **8a–d**, belonging to the isoxazolo[4,5-*d*] pyridazinone series, generally demonstrated to have PDE IV inhibitory activity and PDE III/PDE IV selectivity profile several times lower with respect to the prototypes **4–6**.

Similar to the previously described heterocyclic-fused pyridazinones, the compound substituted at pyridazine N-2 with a benzyl group (**8b**) was less potent with respect to the ethyl analogue (**8a**). An interesting level of PDE IV inhibitory activity and PDE III/PDE IV selectivity was displayed by the n.butyl analogue (**8d**), whereas the presence of a conformationally constrained three-carbon chain at pyridazine N-2 (**8c**) seems to be detrimental. In any case compound **8d**, which is the most potent in the isoxazolo[4,5-*d*]pyridazine series, showed at least six-fold reduced PDE IV inhibitory activity with respect to

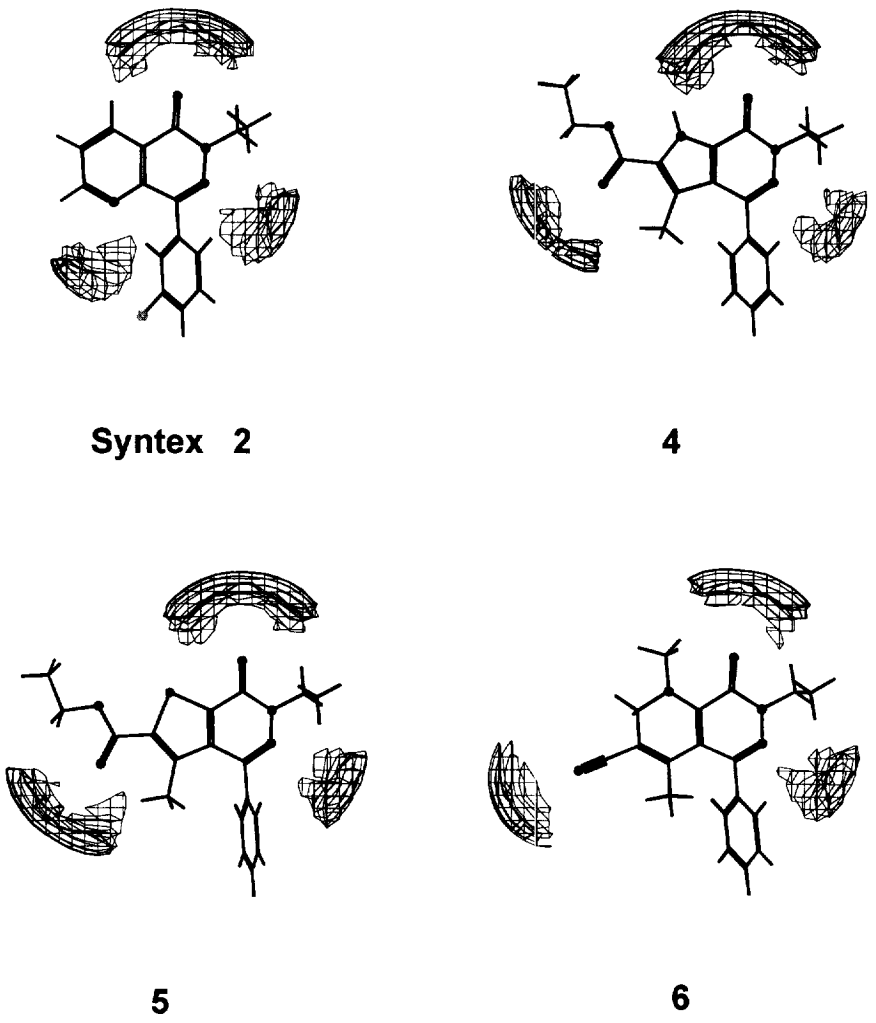

Figure 6. Interaction GRID maps with the NH amide probe contoured at -4 Kcal/mol.

the prototypes **4–6**. In this context a detrimental effect due to the replacement of the ethyl by a methyl group at pyridazine N-2 was also observed by testing the previously described [16] lower homologue of **6** which proved to be at least 20 fold less potent (40% PDE IV inhibition at 20 μM).

In order to have further insights into the effects due to the presence of an isoxazole system condensed with the pyridazinone moiety, we also tested the previously described 6-ethyl and 6-benzyl-3-methyl-4-phenylisoxazolo [3,4-*d*]pyridazinones [11] which are the isomers of **8a** and **8b** respectively. These compounds showed slightly lower PDE IV inhibitory activity with respect to their [4,5-*d*]isomers (data not shown).

Taken together these data suggest that, among the various five and six-membered heterocyclic systems studied till now, the isoxazole is less suitable in evoking potent and selective PDE IV inhibitory activity, suggesting that the electronic properties of the condensed system play an important role in determining this type of pharmacological effect.The different hydrogen-bond acceptor ability of the isoxazole in compounds **8**, as well as in their isoxazolo[3,4-*d*] isomers, both with respect to **2** and **4–6**, could explain the reduced activity of these compounds.

Thienopyridazinones **10,11** and pyridopyridazinones **13** emerged as the more interesting PDE IV inhibitors, being their IC_{50} in the same micromolar range of the

Table III. Effects of the new heterocyclic-fused pyridazinones **8–13** on PDE III and PDE IV isoenzymes and displacement of [^{3}H]Rolipram from its binding site.

Compound	PDE III [a,b]	PDE IV [a,b]	^{3}H-ROL [a,c]	PDE IV/^{3}H-ROL
8a	42 ± 7 (20 μM)	9 ± 0.8	18 ± 1.5 (10 μM)	< 1
8b	10.0 ± 5 (200 μM)	50 ± 1 (20 μM)	37 ± 4 (10 μM)	
8c	29 ± 8 (20 μM)	45 ± 0.5 (20 μM)	46 ± 9 (10 μM)	
8d	30 ± 4 (20 μM)	6 ± 0.5	4.5 ± 1	1.3
9a	16 ± 6 (20 μM)	11 ± 2	54 ± 4 (10 μM)	
9b	12 ± 5 (20 μM)	14 ± 1	55 ± 2 (10 μM)	
9c	28 ± 3 (20 μM)	42 ± 5 (20 μM)	50 ± 7 (10 μM)	
9d	36 ± 2 (20 μM)	48 ± 2 (20 μM)	56 ± 0.5 (10 μM)	
10	34 ± 9 (20 μM)	3 ± 0.5	2.3 ± 0.5	1.3
11	43 ± 2 (20 μM)	4 ± 0.5	1.0 ± 0.08	4.0
13	20 ± 10 (20 μM)	2 ± 0.5	0.3 ± 0.02	6.7
4	30 ± 3 (20 μM)	0.6 ± 0.1	2.0 ± 1	0.30
5	5.9 ± 1.4	0.9 ± 0.2	1.8 ± 0.2	0.50
6	51.0 ± 0.30 (20 μM)	1.1 ± 0.4	1.0 ± 0.4	1.10
Milrinone	0.73 ± 0.03			
Rolipram	242 ± 11	0.32 ± 0.09	0.006 ± 0.004	53.33
Syntex 3	5.1 ± 2	0.056 ± 0.01	0.0048 ± 0.001	11.67

[a] Data are indicated as IC_{50} (μM) ± SEM or inhibition percentage ± SEM at indicated concentration (μM) ($n = 3$). [b] PDE III and PDE IV were obtained from guinea-pig ventricular tissue [17] and dosed following the procedure of Thompson et al. [18]. [c] [^{3}H]Rolipram tests were performed using brain membranes according to [19].

analogs **5** and **6**. The same compounds also showed a significant selectivity versus PDE III family (i.e. PDE III/PDE IV > 10 for **13**).

The obtained data demonstrated that in compound **5** the ester function can be replaced, without significant loss of activity, by an amide (compound **10**) or a cyano group (compound **11**). It is noteworthy that also in the pyridopyridazinone series the presence of this last substituent in the condensed system is connected to a good level of activity (compound **13**). The IC_{50} of this derivative is very close to that of the reference compound **6**, confirming that an n.butyl group at pyridazine N-2 may replace the ethyl without significant loss of activity.

These data confirm the importance of a hydrogen-bond acceptor substituent in the heterocyclic-fused system for potent and selective PDE IV inhibitory activity and low affinity for ^{3}[H] Rolipram high affinity binding site, as suggested by the present molecular modelling studies.

Conversely, the introduction of more polar groups, such as COOH (compounds **9a**, **9c**) or CONHOH (compounds **9b** and **9d**), instead of the ethoxycarbonyl, is associated with at least one order of magnitude reduction of PDE IV inhibitory potency with respect to **4** and **5**.

The affinity for high affinity Rolipram binding site followed a trend similar to that of PDE IV inhibitory activity: the more potent inhibitors displayed also higher affinity for this binding site. In any case for compounds **10**, **11** and **13**, a remarkably better balance between potency and affinity was evidenced with respect to Rolipram **1** and Syntex **3**, as clearly indicated by the lower ^{3}H Ro/PDE IV ratio.

In conclusion, novel heterocyclic-fused-3(2H)-pyridazinone derivatives displaying micromolar selective PDE IV inhibitory activity were synthesized. With respect to the reference compounds **1** and **3**, these compounds demonstrated to have reduced affinity for ^{3}H Rolipram high affinity binding site, which is believed to be responsible for undesirable side-effects.

Molecular modelling studies indicate that three hydrogen-bond acceptor regions are essential requirements for a similar pharmacological profile.

5. Experimental protocols

5.1. Chemistry

All melting points were determined with Buchi 510 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded as nujol mulls with Perkin-Elmer spectrometer. Nuclear magnetic resonance (^{1}H-NMR) spectra were obtained using Gemini 200 spectrometer in $CDCl_3$. Chemical shift values are reported in ppm (δ). Analyses indicated by the symbols of the elements or function were within ±0.4% of the theoretical values. Analytical TLC, using E. Merck F-254 commercial plates, was used to follow the course of the reactions. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography. Extracts were dried over sodium sulfate and solvents were removed under reduced pressure.

N,N-Dimethylformamide (DMF) was purchased from Aldrich Chemical Co. and dried over 4A molecular sives before use.

5.1.1. 5-ethyl-3-methyl-7-phenylisoxazolo[4,5-d] pyridazin-4(5H)-one ***8a***

A mixture of **7** (0.113 g, 0.5 mmol), ethyl bromide (0.219 g, 2 mmol) and anhydrous K_2CO_3 (0.276 g, 2 mmol) in anhydrous DMF (1.5 mL) was stirred at 100 °C for 1 h. Cold water (25 mL) was added and compound **8a**, thus precipitated, was collected by suction. IR (cm^{-1}): 1690 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.50 (t, 3H, J = 7.1 Hz, CH_2CH_3), 2.75 (s, 3H, CH_3), 4.40 (q, 2H, J = 7.1 Hz, CH_2CH_3), 7.50 (m, 3H, H_{arom}), 8.15 (m, 2H, H_{arom}).

5.1.2. 5-benzyl-3-methyl-7-phenylisoxazolo[4,5-d]pyridazin-4(5H)-one ***8b***

Following the same procedure employed to synthesize **8a** and using the same molar ratios, compound **8b** was synthesized by reacting **7** with benzyl chloride. The crude product was isolated after dilution with water, extraction with methylene chloride (3 × 20 mL) and removing the solvent.

The compound showed the same chemical and spectroscopic data (IR and ^{1}H-NMR) reported in the literature [14].

5.1.3. 3-methyl-7-phenyl-5-(3-ethinylmethyl)-isoxazolo[4,5-d] pyridazin-4(5H)-one ***8c***

Compound **8c** was synthesized following the same procedure reported for **8a** and using the same molar ratios, by reacting **7** with propargyl bromide. IR (cm^{-1}): 3300 (CH), 2140–2100 (C = C), 1690 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.40 (t, 1H, J_2 = 3.0 Hz, C = CH), 2.75 (s, 3H, CH_3), 5.10 (d, 2H, J_2 = 3.0 Hz, CH_2), 7.50 (m, 3H, H_{arom}), 8.15 (m, 2H, H_{arom}).

5.1.4. 5-n.butyl-3-methyl-7-phenylisoxazolo[4,5-d]pyridazin-4(5H)-one ***8d***

Compound **8d** was prepared using the above procedures and the same molar ratios, by reacting **7** with 1-bromobutane. IR (cm^{-1}): 1690 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.00 (t, 3H, J = 7.2 Hz, CH_3–$(CH_2)_3$–N), 1.45 (m, 2H, J = 7.2 Hz, $CH_2(CH_2)_2$–N), 1.85 (m, 2H, J = 7.2 Hz, CH_2CH_2–N), 2.70 (s, 3H, CH_3), 4.30 (t, 2H, J = 7.2 Hz, CH_2–N), 7.50 (m, 3H, H_{arom}), 8.15 (m, 2H, H_{arom}).

5.1.5. 6,7-Dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-pyrrolo [2,3-d]pyridazine-2-carboxylic acid ***9a***

A mixture of **4** (0.15 g, 0.46 mmol), EtOH 95 °C (15 mL) and 6 N NaOH (5 mL) was stirred at 50 °C for 3 h. The solution was diluted with cold water and acidified with 6 N HCl. Extraction with ethyl acetate (3 × 50 mL) and removal of the solvent afforded compound **9a**. IR (cm^{-1}): 3600–3100 (OH and NH), 1690 (acidic CO), 1630 (amidic CO). ^{1}H-NMR ($CDCl_3$) δ: 1.30 (t, 3H, J = 7.2 Hz, CH_3CH_2), 2.05 (s, 3H, CH_3), 4.20 (q, 2H, J = 7.2 Hz, CH_2CH_3), 7.50 (s, 5H, H_{arom}).

5.1.6. 6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-pyrrolo [2,3-d] pyridazine-2-carbohydroxamic acid ***9b***

To a solution of free hydroxylamine prepared from NH_2OH•HCl (1.03 g, 15.1 mmol) and EtONa (1.05 g, 15.1 mmol) in absolute EtOH (10 mL), compound **4** (0.4 g, 1.28 mmol) was added. The mixture was stirred at 65 °C for 6 h in inert atmosphere. After cooling and dilution with cold water (20 mL), compound **9b** was precipitated by acidifing with 6 N HCl. IR (cm^{-1}): 3150 (NH), 1695 (acidic CO), 1635 (amidic CO). ^{1}H-NMR ($CDCl_3$) δ: 1.30 (t, 3H, J = 7.2 Hz, CH_3CH_2), 2.00 (s, 3H, CH_3), 4.20 (q, 2H, J = 7.2 Hz, CH_2CH_3), 7.50 (s, 5H, H_{arom}).

5.1.7. 6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-thieno [2,3-d]pyridazine-2-carboxylic acid ***9c***

A mixture of **5** (0.1 g, 0.29 mmol), 95% EtOH (4 mL) and 1 N NaOH (4 mL) was stirred at room temperature for 1.5 h. After dilution with cold water (10 mL) and treatment with 6 N HCl, compound **9c** was precipitated. IR (cm^{-1}): 3500–3100 (OH), 1710 (acidic CO), 1630 (amidic CO). ^{1}H-NMR ($CDCl_3$) δ: 1.45 (t, 3H, J = 7.0 Hz, CH_3CH_2), 2.15 (s, 3H, CH_3), 4.35 (q, 2H, J = 7.0 Hz, CH_2CH_3), 4.90 (exch.br.s, 1H, OH), 7.50 (m, 5H, H_{arom}).

5.1.8. 6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-thieno [2,3-d] pyridazine-2-carbohydroxamic acid ***9d***

Following the same procedure reported for **9b** and using the same molar ratios, compound **9d** was obtained from **5** after 2 h stirring in inert atmosphere. IR (cm^{-1}): 3500 (OH), 3300 (NH), 1710 (CONH), 1630 (amidic CO). ^{1}H-NMR ($CDCl_3$) δ: 1.45 (t, 3H, J = 6.9 Hz, CH_3CH_2), 2.15 (s, 3H, CH_3), 4.40 (q, 2H, J = 6.9 Hz, CH_2CH_3), 7.50 (m, 5H, H_{arom}).

5.1.9. 6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-thieno [2,3-d] pyridazine-2-carboxamide ***10***

A mixture of **9c** (0.180 g, 0.57 mmol) and $SOCl_2$ (7.5 mL) was refluxed for 2 h. The $SOCl_2$ excess was removed in vacuo and the residue oil was added portionwise to a cooled and stirred solution of 30% aqueus ammonia. The precipitate **10** was collected by filtration and washed with cold water. IR (cm^{-1}): 3330 and 3130 (NH_2), 1690 ($CONH_2$), 1650 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.45 (t, 3H, J = 7.0 Hz, CH_3CH_2), 1.90 (s, 3H, CH_3), 4.20 (q, 2H, J = 7.0 Hz, CH_2CH_3), 7.50 (m, 5H, H_{arom}), 7.90 (exch. br. s, 2H, NH_2).

5.1.10. 2-cyano-6-ethyl-3-methyl-4-phenylthieno[2,3-d]pyridazin-7(6H)-one ***11***

A mixture of **10** (0.1g, 0.32 mmol) and $POCl_3$ (2 mL) was stirred for 2 h at 120 °C. The cooled solution was cautiously added under stirring to cold water (10 mL). The precipitated **11** was collected by suction and washed with cold water. IR (cm^{-1}): 2210 (CN), 1650 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.45 (t, 3H, J = 6.7 Hz, CH_3CH_2), 2.00 (s, 3H, CH_3), 4.35 (q, 2H, J = 6.7 Hz, CH_2CH_3), 7.50 (m, 5H, H_{arom}).

5.1.11. 7-n.butyl-3-cyano-1,4-dimethyl-8-oxo-5-phenyl-1,2,7,8-tetrahydro-pyrido[2,3-d] pyridazine ***13***

A solution of **12** (0.16 g, 0.52 mmol) and N-methyl-β-alaninenitrile (0.1 g, 1.2 mmol) in EtOH (3 mL) was stirred at room temperature for 20 min.

The suspension was diluted with water (40 mL) and extracted with CH_2Cl_2 (3 × 20 mL). Removal of the solvent afforded a brown oil which was dissolved in anhydrous EtOH (3 mL) and added to a solution of sodium ethoxide prepared from sodium (0.11 g, 2.5 mmol) and absolute EtOH (5 mL). The mixture was stirred at 50–60 °C for 2 h. Dilution with ice-water afforded **13** which was recovered by suction. IR (cm^{-1}): 2200 (CN), 1640 (CO). ^{1}H-NMR ($CDCl_3$) δ: 0.95 (t, 3H, J = 6.8 Hz, CH_3–$(CH_2)_3$–N), 1.40 (m, 2H, CH_3CH_2), 1.60 (s, 3H, CH_3), 1.80 (m, 2H, N–CH_2–CH_2–),

3.75 (s, 3H, CH_3–N), 3.90 (s, 2H, CH_2–N–CH_3), 4.20 (q, 2H, J = 6.8 Hz, CH_2CH_3), 7.40 (m, 5H, H_{arom}).

5.2. Biology

5.2.1. Drugs and reagents

[8-^{3}H]Adenosine 3′:5′ cyclic monophosphate was from Amersham (Bucks, UK). Benzamidine, cAMP, calmodulin and PMSF (phenylmethylsulphonyl fluoride) were obtained from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Milrinone was obtained from IMPEX Quimica (Barcelona, Spain).

Rolipram was synthesized in the Department of Chemical Synthesis, Laboratorios Almirall Prodesfarma, S.A., Spain.

5.2.2. Purification of phosphodiesterase isoenzymes

Cyclic nucleotide phosphodiesterases III and IV were obtained from guinea-pig ventricular tissue following the procedure described by Gristwood et al. [17]. The method was briefly summarized in the previous paper [11]. The isoenzymes were characterized prior to use in terms of substrate selectivity and affinity and by the effect of calcium ions (10 mM) plus calmodulin (1.2 mM) and the selective inhibitors Rolipram, SK&F 94120. Active fractions were pooled and kept frozen at –20 °C in presence of 1 g/L bovine serum albumin, until used.

5.2.3. Phosphodiesterase assay

Cyclic nucleotide phosphodiesterases were assayed following the procedure of Thompson & Strada [18]. Inhibition assays were run in duplicate at a substrate concentration of 0.25 mM. Substrate was cAMP for PDE III and IV. IC_{50} values were obtained by non-linear regression using the programme InPlot from GraphPad Software. drugs were dissolved in DMSO and the effects of this solvent was taken into consideration in the calculations.

5.2.4. ^{3}H-Rolipram displacement

The binding of ^{3}H-Rolipram to rat brain membranes was performed according to Schneider et al. [19]. At least 6 drug concentrations were assayed in duplicate to generate individual displacement curves. IC_{50} values were calculated for those curves by non-linear regression using the programme Inplot, from GraphPad Software. The effect of drug vehicle was taken into account in the calculation.

5.2.5. Molecular modelling methods

Theoretical calculations were performed on a Digital Alpha Station 3000. Structures were built with standard bond lengths and angles by using the Chem-X [20, 21] molecular modelling package. All structures were initially optimized using steepest descents and conjugate gradient methods. Charge distributions were calculated after semiempirical optimization using the MOPAC program [22, 23] by means of the AM1 method. The PULAY keyword and the eigenvector following (EF) routine for minimum search with GNORM = 0.1 were used in the optimization step. Conformation analysis were performed with fully AM1 optimisation using a 30° rotational increment.

Interaction of the target molecules with the NH amide probe were calculated with the GRID program [24–28]. Representation of VdW and GRID maps, as well as map calculations using logical operators (i.e. NOT), were carried out using ChemX facilities.

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