

## Synthesis of pyrrolo[2,3-d]pyridazinones as potent, subtype selective PDE4 inhibitors

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### Abstract

A series of pyrrolo[2,3-d]pyridazinones was synthesized and tested for their inhibitory activity on PDE4 subtypes A, B and D and selectivity toward Rolipram high affinity binding site (HARBS). New agents with interesting profile were reported; in particular compound **9e** showed a good PDE4 subtype selectivity, being 8 times more potent ( $IC_{50} = 0.32 \mu M$ ) for PDE4B (anti-inflammatory) than for PDE4D ( $IC_{50} = 2.5 \mu M$ ), generally considered the subtype responsible for emesis. Moreover the ratio HARBS/PDE4B was particularly favourable for **9e** (147), suggesting that the best arranged groups around the pyrrolopyridazinone core are an isopropyl at position-1, an ethoxycarbonyl at position-2, together with an ethyl group at position-6.

For compounds **8** and **15a** the ability to inhibit  $TNF\alpha$  production in PBMC was evaluated and the results are consistent with their PDE4 inhibitory activity.

**Keywords:** *Pyrrolopyridazinones, PDE4 subtypes, inhibitors, selectivity,  $TNF\alpha$*

### Introduction

Phosphodiesterases (PDEs), are the enzymes responsible for the hydrolysis of cyclic adenosine monophosphate (c-AMP) and cyclic guanosine monophosphate (c-GMP) which play a key role as secondary messengers in cell functions. The PDE4 subfamily, specific for cAMP, is identified by four subtypes (PDE4A, PDE4B, PDE4C and PDE4D). Each subtype is differently expressed in various tissues, in particular PDE4A and PDE4B isoforms are ubiquitous, while PDE4C and PDE4D are essentially present in neuronal tissue and in inflammatory cells respectively[1,2]. In the last few years PDE4 has attracted considerable interest as a therapeutic target for a variety of pathologies, such as asthma, chronic obstructive pulmonary diseases (COPD) and inflammatory diseases[3]. Since recent studies demonstrated the fundamental role of inflammation in asthma pathology[4,5], it is clear that selective PDE4

inhibitors which are able to both suppress inflammatory progression and to reduce the bronchial smooth muscle tone, represent a very attractive alternative to the classic combined therapy with corticosteroids and bronchodilators. New important applications of PDE4 inhibitors have been proposed, such as in rheumatoid arthritis and other important autoimmune diseases, this hypothesis being associated with the evidence that PDE4 selective inhibitors strongly reduce tumor necrosis factor  $\alpha$  ( $TNF-\alpha$ ) production which plays an important role in these types of pathologies[6]. Recent studies demonstrated an increase of PDE catalytic activity together with an increase of sensitivity to PDE4 inhibitors in atopic dermatitis, suggesting that this pathology could be an attractive target for PDE4 inhibitors[7,8]. Finally, the presence of differently located subtypes of PDE4 opens new opportunities for the development of selective inhibitors devoid of limiting side-effects[9].

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The archetypal PDE4 inhibitor Rolipram **1**[10] (Figure 1) showed very important unwanted side-effects, such as headache, nausea and vomiting, which are related to the binding of **1** with Rolipram high affinity binding site (HARBS)[11]. This same effect was found in Rolipram analogues and in many other compounds structurally unrelated to Rolipram, such as quinazolinediones **2** and xanthine derivatives **3**[12–14].

Thus it appears very important to dissociate the affinity for HARBS from PDE4 inhibitory activity, in order to obtain more selective antiasthmatic drugs, devoid of the above side-effects. In recent years, potent and selective PDE4 inhibitors with reduced affinity for HARBS have been described in the literature[15] and a number of these are now in clinical trials. Compounds such as Cilomilast **4**[16,17], Roflumilast

**5**[18,19], CDP-840 **6**[20] or YM-976 **7**[21] are well tolerated and are presently in phase III clinical trials for the treatment of asthma and COPD (compounds **4** and **5**)[22–24] and in phase II clinical trials for asthmatic pathology (compounds **6** and **7**)[25–27].

Roflumilast and CDP-840 didn't demonstrate PDE4 subtype selectivity[7,19], whereas Cilomilast is 10-fold more selective for PDE4D than for PDE4A and PDE4B[28]. Presently some compounds with high selectivity for one PDE4 subtype (up to 300-fold) have been synthesized, and recent studies demonstrated a correlation between PDE4A/B inhibitory activity and TNF $\alpha$  release inhibition[7]. Moreover some authors suggested that PDE4D is the subtype responsible for the emetic side-effects[29]. These results and proposals seems to open new opportunities for the development of selective inhibitors with reduced adverse effects.

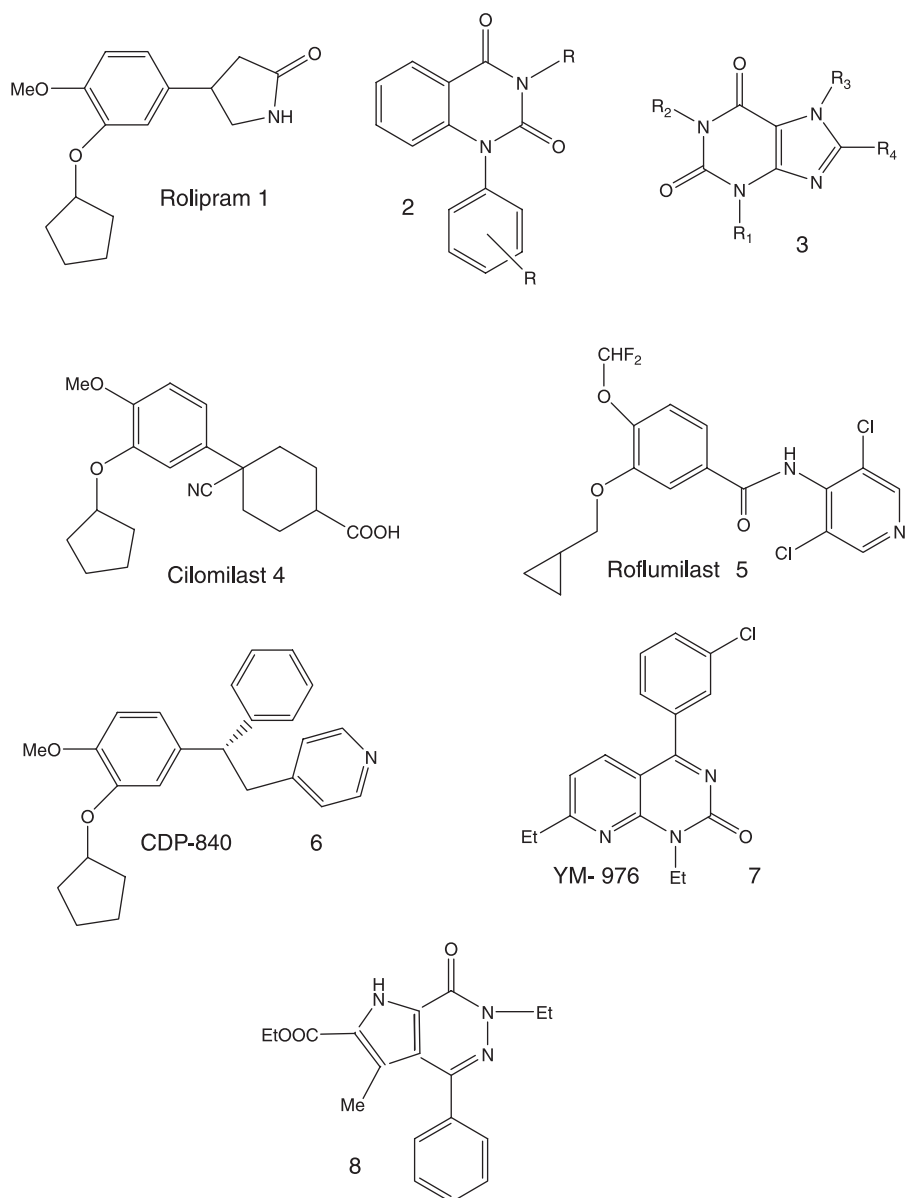


Figure 1. PDE4 inhibitors.

A systematic screening performed on our internal library, allowed us to identify compound **8** as an interesting lead. This compound exhibited  $IC_{50}$  in the same micromolar range as Rolipram ( $IC_{50} = 0.6 \mu M$ ) and a good selectivity profile versus PDE4 (PDE3/PDE4 > 30). Moreover it displays low affinity for HARBS ( $IC_{50} = 2.0 \mu M$ ). Thus it possesses a much better ratio of PDE4 inhibitory activity versus affinity for HARBS (HARBS/PDE4 = 3.33 for **9**, and 0.02 for Rolipram).

On this basis we report here some chemical modifications of lead compound **8** which afforded the new pyrrolo[2,3-d]pyridazines **9a–e** and **15a–f** and their PDE4 subtypes inhibitory activity.

## Materials and methods

All melting points were determined on a Buchi apparatus and are uncorrected.  $^1H$ -NMR spectra were recorded with Varian Gemini 200 instruments. Chemical shifts are reported in ppm, using the solvent as internal standard. Extracts were dried over  $Na_2SO_4$  and the solvents were removed under reduced pressure. Merck F-254 commercial plates were used for analytical TLC to follow the course of the reaction. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography.

## Chemistry

The synthesis of the novel pyrrolo[2,3-d]pyridazinones **9a–e** and **15a–f** is depicted in Figures 2 and 3 respectively.

In Figure 2, the procedure affording the N-1 substituted compounds **9a–e** is reported. All final products were prepared starting from the lead compound **8** which was alkylated under standard conditions to afford the corresponding N-substituted derivatives **9a–e**.

In Figure 3, is depicted the synthetic route affording the pyrrolo[2,3-d]pyridazinones **15a–f** in which modifications at positions- 3, -6 and on the phenyl ring of the bicyclic system were performed with respect to the lead **8**.

Isoxazoles of type **10**, which are the starting material for the final compounds **15** have been previously described [30–32] with the exception of **10b**. Treatment of isoxazoles **10** with hydrazine followed by alkylation with the appropriate alkyl halide afforded compounds **12**, which were transformed into the 4-nitropyridazinone derivatives **13** through oxidative cleavage of the isoxazole ring [30]. Displacement of the nitro group with glycine ethyl ester afforded the open intermediates **14** which led to the final pyrrolopyridazinones **15** by treatment with sodium ethoxide in absolute ethanol at room temperature (**11d** [33], **12d–f** [34], **13d–f** [33]).

*General procedure for compounds 9a–e.* A suspension of **8** (0.3 mmol), anhydrous  $K_2CO_3$  (6 mmol) and the appropriate halide (0.5 mmol) in anhydrous DMF was heated for 30–60 min (16 h for **9e**) at 80–110°C. After the mixture was cooled, ice cold water was added and the crude precipitate was recovered by suction, with the exception of **9d** which was extracted with  $CH_2Cl_2$  ( $3 \times 15$  mL).

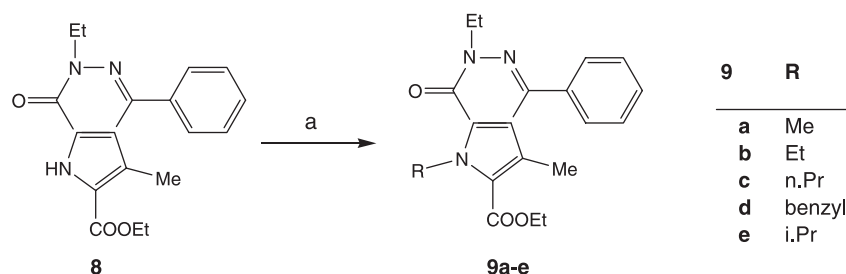
*Ethyl 6,7-dihydro-1,3-dimethyl-6-ethyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 9a.* Mp = 82–83°C; crystallization solvent = EtOH; yield = 45%.

$^1H$ -NMR ( $CDCl_3$ ),  $\delta$ , ppm: 1.45 (m, 6H,  $NCH_2CH_3$  and  $OCH_2CH_3$ ), 2.10 (s, 3H,  $CCH_3$ ), 4.35 (m, 4H,  $NCH_2CH_3$  and  $OCH_2CH_3$ ), 4.50 (s, 3H,  $NCH_3$ ), 7.45 (s, 5H, Ar).

Anal. found: C, 67.40; H, 6.24; N, 12.34.  $C_{19}H_{21}N_3O_3$  requires: C, 67.23; H, 6.25; N, 12.38%.

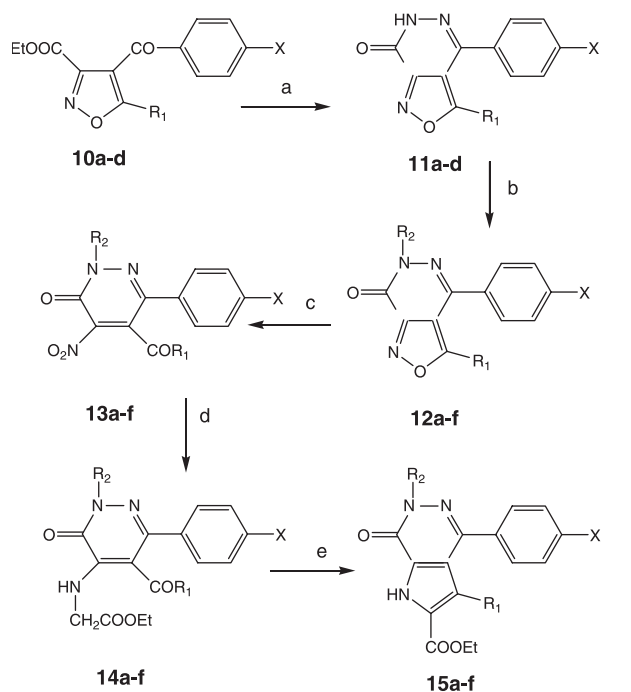
*Ethyl 1,6-diethyl-6,7-dihydro-3-methyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 9b.* Mp = 77–78°C; crystallization solvent = EtOH; yield = 55%.

$^1H$ -NMR ( $CDCl_3$ ),  $\delta$ , ppm: 1.40 (m, 9H,  $2NCH_2CH_3$  and  $OCH_2CH_3$ ), 1.95 (s, 3H,  $CCH_3$ ), 4.35 (m, 4H,  $NCH_2CH_3$  and  $OCH_2CH_3$ ), 5.10 (q, 2H,  $NCH_2CH_3$ ), 7.45 (s, 5H, Ar).



Reagents and conditions: (a) RX, DMF,  $K_2CO_3$ , 80–110°C, 30–60 min.

Figure 2. Synthesis of pyrrolo[2,3-d]pyridazinones **9a–e**.



10,11	R <sub>1</sub>	X	12-15	R <sub>1</sub>	X	R <sub>2</sub>
a	Et	H	a	Et	H	Et
b	n.Pr	H	b	n.Pr	H	Et
c	Me	Cl	c	Me	Cl	Et
d	Me	H	d	Me	H	n.Pr
			e	Me	H	i.Pr
			f	Me	H	n.But

Reagents and conditions: (a) Hydrazine hydrate, EtOH, rt, 10-60 min; (b) R<sub>2</sub>Br, DMF, K<sub>2</sub>CO<sub>3</sub>, 110°C, 1h; (c) CAN, 50%w/vAcOH, 65% HNO<sub>3</sub>, 55°C 30-45 min; (d) glycine ethyl ester, EtOH, 40-50°C, 20-160 min; (e) EtONa, abs EtOH, rt, 20-30 min.

Figure 3. Synthesis of pyrrolo[2,3-d]pyridazinones 15a-f.

Anal. found: C, 68.03; H, 6.58; N, 11.92. C<sub>20</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 67.96; H, 6.57; N, 11.89%.

*Ethyl 6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1-propyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 9c.* Mp = 77–78°C; crystallization solvent = EtOH; yield = 90%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.95 (t, 3H, (CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>), 1.40 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.80 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.95 (s, 3H, CCH<sub>3</sub>), 4.35 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 5.00 (t, 2H, NCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 7.45 (s, 5H, Ar).

Anal. found: C, 68.80; H, 6.89; N, 11.41. C<sub>21</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 68.63; H, 6.87; N, 11.44%.

*Ethyl 1-benzyl-6,7-dihydro-6-ethyl-3-methyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 9d.* Mp = 105–106°C; crystallization solvent = EtOH; yield = 70%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.25 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.40 (t, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.00 (s, 3H, CCH<sub>3</sub>), 4.30 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 6.40 (s, 2H,

NCH<sub>2</sub>Ph), 7.10 (m, 2H, Ar), 7.25 (m, 3H, Ar), 7.50 (s, 5H, Ar).

Anal. found: C, 72.09; H, 6.09; N, 10.08. C<sub>25</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 72.26; H, 6.08; N, 10.11%.

*Ethyl 6,7-dihydro-6-ethyl-1-isopropyl-3-methyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 9e.* Mp = 99–101°C; crystallization solvent = EtOH; yield = 60%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.40 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.70 (d, 6H, CH(CH<sub>3</sub>)<sub>2</sub>), 1.85 (s, 3H, CCH<sub>3</sub>), 4.35 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 5.85 (m, 1H, CH(CH<sub>3</sub>)<sub>2</sub>), 7.45 (s, 5H, Ar).

Anal. found: C, 68.79; H, 6.89; N, 11.42. C<sub>21</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 68.63; H, 6.87; N, 11.44%.

*Ethyl-4-benzoyl-5-propylisoxazole-3-carboxylate 10b.* To a cooled (–5–10°C) and stirred solution of sodium ethoxide, obtained from sodium (15 mmol) and anhydrous EtOH (30 mL), a solution of 1-phenylhexan-1,3-dione[35] (15 mmol) in the same solvent (15 mL) was slowly added. A solution of ethyl chloro(hydroximino)acetate, commercially available (15 mmol) in anhydrous EtOH (10 mL) was added in a dropwise manner (over 1 h period). The mixture, neutralized with 6N HCl was evaporated to afford an oil which was washed with 0.5 N NaOH and water and then extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 25 mL). The organic layer was washed with water, dried with Na<sub>2</sub>SO<sub>4</sub> and evaporated *in vacuo* to afford an oil, which was purified by column chromatography (cyclohexane/ethyl acetate 1:1). Yield = 57%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.00 (t, 6H, 2CH<sub>3</sub>), 1.80 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.90 (t, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 4.10 (q, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.50 (s, 5H, Ar).

*General procedure for compounds 11a–c.* The appropriate isoxazole 10a–c (0.5 mmol) was dissolved in EtOH (5 mL) and then hydrazine hydrate (0.8 mmol) was added at room temperature. After 10–60 min the crude product was collected by suction from the cooled mixture.

*3-Ethyl-4-phenylisoxazolo[3,4-d]pyridazin-7-(6H)-one 11a.* Mp = 204–206°C; crystallization solvent = EtOH; yield = 81%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.20 (t, 3H, CH<sub>3</sub>), 2.80 (q, 2H, CH<sub>2</sub>), 7.50 (s, 5H, Ar).

*4-Phenyl-3-n-propylisoxazolo[3,4-d]pyridazin-7-(6H)-one 11b.* Mp = 146–148°C; crystallization solvent = EtOH; yield = 82%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.90 (t, 3H, CH<sub>3</sub>), 1.80 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.80 (t, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 7.50 (s, 5H, Ar), 10.00 (exch br s, 1H, NH).

4-(*p*-Chlorophenyl)-3-methylisoxazolo[3,4-*d*]pyridazin-7-(6*H*)-one **11c**. Mp = 210–212°C; crystallization solvent = EtOH; yield = 74%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 2.60 (s, 3H, CH<sub>3</sub>), 7.50 (m, 4H, Ar), 9.90 (exch br s, 1H, NH).

*General procedure for compounds 12a–c.* A mixture of the appropriate isoxazolo[3,4-*d*]pyridazinones **11** (1 mmol), anhydrous K<sub>2</sub>CO<sub>3</sub> (5 mmol) and appropriate alkyl bromide (5 mmol) in anhydrous DMF (3–6 mL) was heated under stirring for 1 h at 110°C. After dilution with cold water (80–100 mL) the crude precipitate was recovered by suction.

3,6-Diethyl-4-phenylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one **12a**. Mp = 67–70°C; crystallization solvent = EtOH; yield = 86%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.20 (t, 3H, CCH<sub>2</sub>CH<sub>3</sub>), 1.40 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.85 (q, 2H, CCH<sub>2</sub>CH<sub>3</sub>), 4.30 (q, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.55 (s, 5H, Ar).

6-Ethyl-4-phenyl-3-*n*.propylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one **12b**. Mp = 70–72°C; crystallization solvent = EtOH; yield = 75%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.90 (t, 3H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.50 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.80 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.80 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 4.25 (m, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.50 (s, 5H, Ar).

4-(*p*-Chlorophenyl)-6-ethyl-3-methylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one **12c**. Mp = 126–128°C; crystallization solvent = EtOH; yield = 70%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.40 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.50 (s, 3H, CCH<sub>3</sub>), 4.20 (q, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.50 (m, 4H, Ar).

*General procedure for compounds 13a–c.* To a stirred suspension of the appropriate compounds **12** (2.5 mmol) in AcOH (50% w/v; 20 mL) and HNO<sub>3</sub> (65% w/v; 2.5 mL), CAN (15 mmol) was added in a portionwise manner at 55°C over 30–45 min period. Then ice-cold water (100 mL) was added and compounds **13a** and **13c** were recovered by suction; to obtain **13b** the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 20 mL). Evaporation of the solvent afforded an oil which was purified by column chromatography using toluene/ethyl acetate (8:2) as eluent.

2-Ethyl-4-nitro-6-phenyl-5-propionylpyridazin-3(2*H*)-one **13a**. Mp = 97–100°C; crystallization solvent = EtOH; yield = 65%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.95 (t, 3H, COCH<sub>2</sub>CH<sub>3</sub>), 1.50 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.35 (q, 2H, COCH<sub>2</sub>CH<sub>3</sub>), 4.40 (q, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.50 (s, 5H, Ar).

5-Butyryl-2-ethyl-6-phenyl-4-nitropyridazin-3(2*H*)-one **13b**. oil; yield = 52%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.90 (t, 3H, COCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.50 (m, 5H, NCH<sub>2</sub>CH<sub>3</sub> and COCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.35 (m, 2H, COCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 4.40 (q, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.50 (s, 5H, Ar).

5-Acetyl-6-(*p*-chlorophenyl)-2-ethyl-4-nitropyridazin-3(2*H*)-one **13c**. Mp = 105–107°C; crystallization solvent = EtOH; yield = 59%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.50 (t, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.20 (s, 3H, COCH<sub>3</sub>), 4.40 (q, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.50 (m, 4H, Ar).

*General procedure for compounds 15a–f.* A suspension of the appropriate nitroderivative **13** (0.3 mmol) and glycine ethyl ester (0.6 mmol) in EtOH (3 mL) was heated at 40–50°C for 20–160 min. After cooling, the intermediate **14** was recovered by suction. The crude **14**, completely dried, was treated with EtONa (0.5 mmol) in absolute. EtOH (3 mL) at room temperature for 20–30 min. After dilution with water (10 mL), the mixture was acidified with 6N HCl to afford compounds of type **15** which were recovered by suction.

Ethyl 3,6-diethyl-6,7-dihydro-4-phenyl-7-oxo-1*H*-pyrrolo[2,3-*d*]pyridazine-2-carboxylate **15a**. Mp = 127–131°C; crystallization solvent = EtOH; yield = 68%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.80 (t, 3H, CCH<sub>2</sub>CH<sub>3</sub>), 1.40 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.60 (q, 2H, CCH<sub>2</sub>CH<sub>3</sub>), 4.35 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 7.50 (m, 4H, Ar).

Anal. found: C, 67.40; H, 6.24; N, 12.40. C<sub>19</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 67.23; H, 6.25; N, 12.38%.

Ethyl 6,7-dihydro-6-ethyl-4-phenyl-3-*n*.propyl-7-oxo-1*H*-pyrrolo[2,3-*d*]pyridazine-2-carboxylate **15b**. Mp = 98–100°C; crystallization solvent = EtOH; yield = 52%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 0.50 (t, 3H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.35 (m, 8H, NCH<sub>2</sub>CH<sub>3</sub>, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.50 (t, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 4.40 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 7.50 (m, 5H, Ar), 10.0 (exch. br. s. 1H, NH).

Anal. found: C, 67.79; H, 6.58; N, 11.91. C<sub>20</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub> requires: C, 67.96; H, 6.57; N, 11.89%.

Ethyl 4-(*p*-chlorophenyl)-6,7-dihydro-6-ethyl-3-methyl-7-oxo-1*H*-pyrrolo[2,3-*d*]pyridazine-2-carboxylate **15c**. Mp = 206–208°C; crystallization solvent = EtOH; yield = 82%.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>), δ, ppm: 1.40 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.10 (s, 3H, CCH<sub>3</sub>), 4.40 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 7.50 (m, 4H, Ar).

Anal. found: C, 60.23; H, 5.06; N, 11.65.  $C_{18}H_{18}N_3O_3Cl$  requires: C, 60.08; H, 5.05; N, 11.68%.

*Ethyl 6,7-dihydro-3-methyl-7-oxo-4-phenyl-6-n-propyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 15d.* Mp = 175–178°C; crystallization solvent = EtOH; yield = 75%.

$^1H$ -NMR ( $CDCl_3$ ),  $\delta$ , ppm: 1.00 (t, 3H,  $NCH_2CH_2CH_3$ ), 1.45 (t, 3H,  $OCH_2CH_3$ ), 1.95 (m, 2H,  $NCH_2CH_2CH_3$ ), 2.10 (s, 3H,  $CCH_3$ ), 4.35 (m, 4H,  $NCH_2CH_2CH_3$  and  $OCH_2CH_3$ ), 7.50 (m, 5H, Ar), 11.50 (exch. br. s. 1H, NH).

Anal. found: C, 67.05; H, 6.26; N, 12.37.  $C_{19}H_{21}N_3O_3$  requires: C, 67.23; H, 6.25; N, 12.38%.

*Ethyl 6,7-dihydro-6-isopropyl-3-methyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 15e.* Mp = 145–147°C; crystallization solvent = EtOH; yield = 74%.

$^1H$ -NMR ( $CDCl_3$ ),  $\delta$ , ppm: 1.45 (m, 9H,  $CH(CH_3)_2$  and  $OCH_2CH_3$ ), 2.20 (s, 3H,  $CCH_3$ ), 4.45 (q, 2H,  $OCH_2CH_3$ ), 5.50 (m, 1H,  $CH(CH_3)_2$ ), 7.60 (m, 5H, Ar), 11.60 (exch. br. s. 1H, NH).

Anal. found: C, 67.08; H, 6.24; N, 12.40.  $C_{19}H_{21}N_3O_3$  requires: C, 67.23; H, 6.25; N, 12.38%.

*Ethyl 6-n-butyl-6,7-dihydro-3-methyl-7-oxo-4-phenyl-1H-pyrrolo[2,3-d]pyridazine-2-carboxylate 15f.* Mp = 136–138°C; crystallization solvent = EtOH; yield = 64%.

$^1H$ -NMR ( $CDCl_3$ ),  $\delta$ , ppm: 0.95 (t, 3H,  $NCH_2CH_2CH_2CH_3$ ), 1.40 (t, 3H,  $OCH_2CH_3$ ), 1.80 (m, 4H,  $NCH_2CH_2CH_2CH_3$ ), 2.10 (s, 3H,  $CCH_3$ ), 4.40 (m, 4H,  $NCH_2CH_2CH_2CH_3$  and  $OCH_2CH_3$ ), 7.50 (m, 5H, Ar), 10.80 (exch. br.s. 1H, NH).

Anal. found: C, 68.09; H, 6.58; N, 11.92.  $C_{20}H_{23}N_3O_3$  requires: C, 67.96; H, 6.57; N, 11.89%.

### Pharmacology

*Purification of phosphodiesterase 3 (PDE3).* Cyclic nucleotide phosphodiesterases 3 were obtained from guinea pig ventricular tissue following the procedure described by Gristwood et al.[36]. Briefly, the tissue was homogenized in 20 mM Bis-Tris pH 6.5 buffer, containing 50 mM sodium acetate, 2 mM benzamidine, 2 mM EDTA, 5 mM  $\beta$ -mercaptoethanol and 50  $\mu$ M PMSF using an Ultraturrax homogenizer. The sample was centrifuged at 40000  $\times$  g for 20 min and the supernatant was filtered through a 0.22  $\mu$ m filter. The clean sample was chromatographed on a 1 mL ion-exchange MONO-Q column equilibrated with the same buffer using a FPLC system. The column was developed at a flow rate of 1 mL/min using a linear gradient of sodium acetate from 50 to 1000 mM in a total volume of 25 mL. Fractions of 500  $\mu$ L were collected.

The isoenzymes were characterized prior to use in terms of substrate selectivity and affinity and by the effect of calcium ions (10  $\mu$ M) plus calmodulin (1.2  $\mu$ M) and the selective inhibitors Rolipram and SK& 94120. Active fractions were pooled and kept frozen at  $-20^\circ C$  in the presence of g/L bovine serum albumin until used.

*PDE3 activity determination.* Cyclic nucleotide phosphodiesterase activities were measured using a two step procedure according to Thompson and Strada[37]. Inhibition assays were run in duplicate at a substrate concentration of 0.25  $\mu$ M. Substrate was cAMP for PDE3.  $IC_{50}$  values were obtained by nonlinear regression using the program InPlot from GraphPad Software. Drugs were dissolved in DMSO, and the effects of this solvent were taken into consideration in the calculations.

*Purification of phosphodiesterase 4 (PDE4).* Yeast strains overexpressing PDE4 were inoculated into 500 ml flasks with 100 ml YPD (1 g yeast extract, 2 g peptone, 95 ml  $H_2O$ , and 5 ml 40% glucose) and grown at  $30^\circ C$  under orbital shaking (150 rpm) for 48 h to ensure reaching one near  $OD_{600} = 2.0$ . Then cells were harvested by centrifugation at 1500 rpm for 10 min at  $20^\circ C$ . All supernatants were removed and pellets resuspended in the appropriate volume of SD (1.7 g yeast nitrogen base w/o aminoacids and sulphate, 5 g  $(NH_4)_2SO_4$ , 30% galactose, 0.5% adenine sulphate and 0.50 mg/mL L-lysine-HCl) to obtain an  $OD_{600}$  between 0.4 and 0.6 and then incubated with shaking at  $30^\circ C$  until an  $OD_{600}$  of 2.0 was reached (usually 48 h). Then cells were centrifuged at 4000 rpm for 20 min at  $4^\circ C$  and the supernatants discarded. Pellets were resuspended in 100 ml of ice-cold sterile water and centrifuged at 4000 rpm for 20 min at  $4^\circ C$ . The supernatants were discarded and the wet weight of pellets was measured and 1.3 volume/weight of YHB (2 M KCl, 0.5 M EDTA, 1 M HEPES) supplemented with DTT and protease inhibitors added and then resuspended. Another centrifugation at 4000 rpm for 20 min at  $4^\circ C$  was carried out. The supernatants were discarded and using a spatula the yeast paste was transferred into a 10-mL syringe and yeast “noodles” were made and placed into a small plastic beaker filled with liquid nitrogen. The frozen yeast “noodles” can be stored at  $-80^\circ C$  until required for extract preparation.

*Preparation of whole-cell extract.* The yeast “noodles” were poured into a cooled porcelain mortar filled with liquid nitrogen and crushed under liquid nitrogen until the yeast showed a powdery smooth consistency. Then yeast/liquid nitrogen suspension was poured into a plastic beaker and the remaining yeast powder

scraped into the beaker with a spatula. Dialysis buffer (1.4 volume/ weight), YDB (2 M KCl, 0.5 M EDTA, 1 M HEPES, 100% glycerol, 1 M DTT, 0.25 M PMSF, 1 mg/mL leupeptin and supplemented with DTT and protease inhibitors) was then added to resuspend the yeast powder and then centrifugated at 25000 rpm for 2 h at 4°C. The lipid layer was removed, supernatants recovered and transferred to dialysis tubing and dialyzed overnight against 500 ml YDB. Then dialysis supernatants were transferred to microtubes in small aliquots (100 µl) and quick-frozen and stored at -80°C; The protein concentration was determined by the method of Bradford with the Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard.

**PDE4 activity determination.** PDE4 activity from various subtypes (PDE4B1, PDE4A4 and PDE4D3) was monitored by measuring the hydrolysis of [<sup>3</sup>H]-AMP using a PDE-SPA kit (Amersham International) as reported in the literature[38]. Extracted Enzyme (4 µg of protein) was incubated in “low binding” plates (Costar 3604) for 60 min at room temperature. The assay mixture (80 µL) contained 15 nM [<sup>3</sup>H]-cAMP (1 µCi/mL) in the assay buffer (50 mM Tris pH 7.5, 8.3 mM MgCl<sub>2</sub>, 1.7 mM EGTA) and 10 µL of test compound. These compounds were resuspended in DMSO at a stock concentration of 1 mM. The compounds were tested at different concentrations varying from 10 µM to 10 pM to calculate the ED<sub>50</sub>. Hydrolysis of [<sup>3</sup>H]-cAMP was initiated by adding 10 µL of a solution containing enzyme extract, and the plate was then incubated under agitation at room temperature. The reaction was stopped after 60 min (with 10–20% substrate conversion) by addition of 50 µL Phosphodiesterase Scintillation Proximity Assay (SPA) Beads and the reaction was incubated for another 20 min. [<sup>3</sup>H]-AMP, captured by the SPA beads, was determined by counting the plates in a Wallac-Microbeta Trilux scintillation counter.

**[<sup>3</sup>H]Rolipram displacement.** The binding of [<sup>3</sup>H]Rolipram to rat brain membranes was performed according to Schneider et al.[39] At least six drug concentrations were assayed in duplicate to generate individual displacement curves. IC<sub>50</sub> values were calculated from those curves by nonlinear regression using the program Inplot, from GraphPad Software. The effect of drug vehicle was taken into account in the calculation.

**PBMC assay.** Human whole blood of healthy donors was collected in 50 ml Falcon tubes with heparin (20 units/mL). The isolation of PBMCs by centrifugation on a Ficoll-Plaque and hypotonic lysis of remaining red blood cells was performed essentially

as described previously by Gantner and coworkers[40]. The purity of PBMCs was >90% and the viability, as measured by trypan blue exclusion, as >95%. Cells were incubated in 96-well plates at a density of 2 × 10<sup>5</sup> cells/well in a total assay volume of 200 µL (RPMI medium with 2 mM L-Gln, 20 mM HEPES and 10% FBS). A 1 µg/mL of LPS (from *Escherichia coli*, Sigma, St. Louis, MO) together with vehicle or test compound was incubated in a bath at 37°C for 24 h. Each drug was evaluated at six different concentrations in duplicate determinations. After the 24 h period, the reaction was stopped and media obtained by centrifugation of PBMCs at 13000 rpm at 4°C for 10 min was then subjected to ELISA (R&D Systems) or kept at -80°C until use. IC<sub>50</sub> values were obtained by non-linear regression using InPlot, GraphPad. The results were expressed as the mean IC<sub>50</sub> ± SEM value obtained in 4 independent experiments. Results related to this experiment are reported in Table II.

## Results and discussion

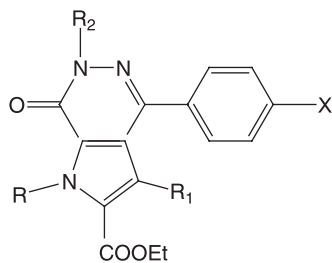
All the final compounds were tested for their ability to inhibit PDE4 (PDE4A, PDE4B and PDE4D) and PDE3 isoenzymes, as well as for their affinity for HARBS (Table I).

SAR studies were performed in comparison with the lead **8** which did not demonstrate subtype selectivity showing IC<sub>50</sub>s of 0.11, 0.067 and 0.049 µM for PDE4A, PDE4B and PDE4D respectively, but a significant selectivity versus HARBS (HARBS/PDE4A-C = 20–40) was observed.

Starting from **15a–b**, where the methyl group at position- 3 was elongated with respect to lead **8**, an IC<sub>50</sub> in the nanomolar range can be observed, but unfortunately these agents were completely devoid of selectivity versus PDE4 subtypes, as well as versus HARBS. These results clearly indicated that elongation of the carbon chain at position-3 did not modify the inhibitory activity but it led to a decrease in selectivity versus HARBS.

Replacement of the hydrogen at position-1 (compounds **9a–e**) by alkyl groups was associated with significant effects on the selectivity. In fact, even though **9a–c** and **9e** showed less potent PDE4 inhibitory activity with respect to the previous compounds, the selectivity versus HARBS (HARBS/PDE4B) was significantly increased, in particular for **9b** (ratio = 67) and **9e** (147). This compound was also the best one in the present series as regards the PDE4 subtypes selectivity profile, being 8 times more potent for PDE4B, the subtype responsible for the anti-inflammatory effect (IC<sub>50</sub> = 0.32 µM) than for PDE4D (IC<sub>50</sub> = 2.5 µM), which is generally considered the subtype responsible for central side-effects.

Introduction of a benzyl group at position-1 (compound **9d**) was found very detrimental to activity

Table I. Effects of compounds **9** and **15** on PDE4 isoenzymes, PDE3 and displacement of [<sup>3</sup>H]Rolipram from its binding site (HARBS).

Comp.	R	R <sub>1</sub>	R <sub>2</sub>	X	PDE4 <sup>a,b</sup> A	PDE4 <sup>a,b</sup> B	PDE4 <sup>a,b</sup> D	PDE3 <sup>a,c</sup>	[ <sup>3</sup> H]Rol <sup>a,d</sup>
<b>9<sup>o</sup></b>	Me	Me	Et	H	1.2 ± 0.3	0.63 ± 0.12	0.36 ± 0.05	37.0 ± 10.0 (20)	1.1 ± 0.3
<b>9b</b>	Et	Me	Et	H	0.71 ± 0.35	0.63 ± 0.21	0.28 ± 0.04	31.0 ± 15.2 (20)	42.0 ± 12.4
<b>9c</b>	n.Pr	Me	Et	H	2.2 ± 0.8	0.77 ± 0.15	1.9 ± 0.2	26 ± 9.2 (20)	1.0 ± 0.2
<b>9d</b>	Bn	Me	Et	H	6.3 ± 1.2	2.4 ± 0.9	6.1 ± 1.7	2.0 ± 0.7 (20)	10.0 ± 1.9
<b>9e</b>	i.Pr	Me	Et	H	1.3 ± 0.3	0.32 ± 0.08	2.5 ± 0.6	33.0 ± 6.5 (20)	47.0 ± 15.7
<b>15a</b>	H	Et	Et	H	0.079 ± 0.012	0.093 ± 0.02	0.022 ± 0.01	25.0 ± 8.9 (20)	0.35 ± 0.08
<b>15b</b>	H	n.Pr	Et	H	0.035 ± 0.013	0.048 ± 0.011	0.013 ± 0.007	28.0 ± 12.3 (20)	0.28 ± 0.03
<b>15c</b>	H	Me	Et	Cl	0.24 ± 0.08	0.44 ± 0.10	0.061 ± 0.024	26.0 ± 5.7 (20)	0.39 ± 0.1
<b>15d</b>	H	Me	n.Pr	H	3.3 ± 1.0	1.4 ± 0.5	1.2 ± 0.8	9.0 ± 2.8 (20)	20.1 ± 3.4
<b>15e</b>	H	Me	i.Pr	H	63.7 ± 20.3(10)	1.6 ± 0.4	1.4 ± 0.5	25.0 ± 4.5 (20)	18.0 ± 2.7
<b>15f</b>	H	Me	n.Bu	H	54.0 ± 17.8(10)	4.3 ± 1.3	59 ± 13.6 (10)	10 ± 21 (20)	12.0 ± 5.3
<b>8</b>	H	Me	Et	H	0.11 ± 0.03	0.067 ± 0.03	0.049 ± 0.012	30 ± 3.3 (20)	2.0 ± 0.5
<b>Rolipram</b>						0.32 <sup>c</sup>		242.0	0.006

<sup>a</sup>Data are expressed as IC<sub>50</sub> (μM) or inhibition percentage at indicated concentration (μM) (n = 3); <sup>b</sup>PDE4 was purified and dosed following the procedure reported in experimental section; <sup>c</sup>PDE3 were obtained from guinea pig ventricular tissue (ref.36) and dosed following the procedure of Thompson et al. (ref.37); <sup>d</sup>[<sup>3</sup>H]Rolipram tests were performed using brain membranes according to ref. 42.; <sup>e</sup>Data related to PDE4 isoenzyme mixture following the procedure of Thompson et al. (ref.37).

with respect to **9a–c** and **9e**, where the substituent is a small alkyl group. This result seems to indicate that there is a small lipophilic pocket in the catalytic site of the enzyme which is able to receive linear or branched low molecular weight alkyls groups.

Introduction of a chlorine in the para position of the phenyl group (**15c**) was associated with a moderate activity for PDE4B and the loss of the selectivity versus HARBS.

Compounds **15d–f**, where the carbon chain at position-6 was elongated and branched in comparison to **8**, showed a reduced potency towards the three isoenzymes. Thus in this group of differently 6-substituted derivatives, a two carbon chain was found to be optimal.

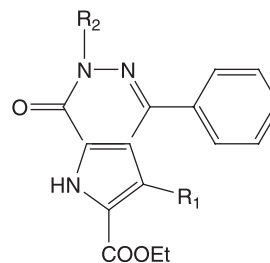
Finally, all synthesized compounds showed a good PDE3/PDE4 selectivity profile, as reported in Table I.

In conclusion we synthesized novel PDE4 inhibitors endowed with IC<sub>50</sub> in the nanomolar range. Selectivity versus HARBS was strongly improved, both in comparison with Rolipram **1** and our lead **8**.

The best profile was found for **9e**, which showed a high potency for PDE4B and a very high selectivity (147) versus HARBS.

These results suggest that the best arranged groups around the pyrrolopyridazinone core are an isopropyl at position-1, an ethoxycarbonyl at position-2 together with an ethyl group at position-6.

Finally, we evaluated the ability of the most potent compounds (**8** and **15a**) and of the less active in this series (**15f**) to reduce TNFα production in human peripheral blood mononuclear cells (PBMC), using Cilomilast as reference drug (Table II). The obtained results really demonstrated a correlation between

Table II. TNFα inhibition values for compounds **8**, **15a** and **15f**.

Comp.	R <sub>1</sub>	R <sub>2</sub>	TNFα <sup>a</sup> IC <sub>50</sub> (μM)	PDE4 B <sup>b</sup> IC <sub>50</sub> (mM)
<b>8</b>	Me	Et	0.20 ± 0.09	0.067 ± 0.03
<b>15a</b>	Et	Et	0.19 ± 0.1	0.093 ± 0.02
<b>15f</b>	Me	n.butyl	45.6 ± 9.0	4.3 ± 1.9
<b>Cilomilast</b>			0.09	0.095

<sup>a</sup>IC<sub>50</sub> is the mean of 4 independent experiments.; <sup>b</sup>PDE4 was purified and dosed following the procedure reported in the experimental section.



PDE4 inhibitory activity and TNF $\alpha$  release being the IC<sub>50</sub>s = 0.198 and 0.192  $\mu$ M for the most active molecules respectively 4.9  $\mu$ M for compound **15f**.

Further studies are in progress to optimize the profile of the above reported compounds.

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