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DRUG DEVELOPMENT RESEARCH 72 : 274–288 (2011)

Research Article

Synthesis and Evaluation as PDE4 Inhibitors of Pyrimidine-2,4-dione Derivatives

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Strategy, Management and Health Policy				
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ABSTRACT A series of nitraquazone analogs with a pyrimidindione core was synthesized and tested for inhibitory activity on PDE4, selectivity versus PDE3 and PDE5 and for affinity towards the rolipram high-affinity binding site (HARBS). The 5-anilino derivatives 13–18 showed the best profile combining appreciable PDE4 inhibitory activity (IC_{50} = 5–14 μM) with a good selectivity toward PDE3 and PDE5. The same compounds demonstrate low affinity for the HARBS site with IC_{50} values of 12–69 μM (IC_{50} for Rolipram = 3.6 nM). Drug Dev Res 72:274–288, 2011. © 2010 Wiley-Liss, Inc.

Key words: HARBS; PDE4; selectivity

INTRODUCTION

Phosphodiesterases are a superfamily of enzymes responsible for the hydrolysis of cAMP and cGMP to the corresponding nucleosides, AMP and GMP. Eleven different families have been described (PDE1–PDE11), but the role, the characteristics, as well as the specific inhibitors of the newer discovered types PDE8-PDE11 have not yet been widely defined [Lugnier, 2006]. The PDE4 family, which selectively hydrolyzes cAMP, is mainly present in inflammatory cells, brain, and cardiovascular tissue [Tenor and Schudt, 1996; Houslay et al., 1998; Stoclet et al., 1995] and constitutes four subtypes, PDE4A–D [Houslay, 2001]. The involvement of PDE4 in pathological processes in these tissues indicates that compounds able to modulate PDE4 activity could have use in a variety of diseases [Houslay et al., 2005].

PDE4 inhibitors have been widely studied for the treatment of asthma and chronic obstructive pulmonary disease (COPD) [Lipworth, 2005; Dal Piaz and Giovannoni, 2000] as they suppress many of the neutrophil responses responsible for pathological inflammatory process, including the production of IL-8, superoxide anions, degranulation, chemotaxis, and adhesion [Houslay et al., 2005]. PDE4 inhibitors could ameliorate the mucus hypersecretion that characterizes asthma and COPD by reducing the transcriptional activity of MUC5AC, a regulator gene in mucin secretion [Mat et al., 2005]. Moreover, PDE4 inhibitors reduce superoxide anion production from eosinophils as well as the adhesion and infiltration of the latter, suggesting a potential role in allergic rhinitis, asthma, and atopic dermatitis [Spina, 2004]. PDE4 inhibitors can also reduce tumor necrosis factor-α (TNFα) production suggesting a role in rheumatoid

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Received 30 July 2010; Accepted 5 September 2010

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/ddr.20395

arthritis, psoriasis, and Crohn's disease [Seldon et al., 1995; Jimenez et al., 2001]. PDE4 inhibitors can also induce cell cycle arrest and apoptosis in limphoblastic leukaemia cells [Ogawa et al., 2002] and block angiogenic response of endothelial cells [Favot et al., 2004], suggesting possible applications in oncology. The antidepressant effect of PDE inhibitors has been considered. Despite initial studies where PDE4 inhibitors had a potent antidepressant action [O'Donnel, 1993], no further progress has occurred because of an incomplete understanding of the functional importance of PDE4 subtypes and of low- and high-affinity rolipram binding site (HARBS) for pathology [O'Donnel and Zhang, 2004]. Finally, PDE4 inhibitors have shown efficacy in the MPTP mouse model [Zhang et al., 2004], suggesting potential in Parkinson's disease. Rolipram **1** (Fig. 1), the prototypic PDE4 inhibitor, was extensively investigated as an antidepressant more than two decades ago but side effects including nausea, vomiting, and headache, related to binding to HARBS, limited its clinical potential [Schneider et al., 1986; Sounes and Rao, 1997]. Many PDE4 inhibitors of different chemical classes have also been in clinical trials for the treatment of asthma, but they were discontinued because of side effects.

The disconnect between HARBS affinity and PDE4 inhibition found in more recent compounds allowed the selection of a number of molecules that are currently in clinical trials. Roflumilast **2** [Hatzeman and Schudt, 2001; Karish and Gagnon, 2006] is the most advanced PDE4 inhibitor, having been approved in the EU for severe COPD associated with chronic bronchitis area in 2010. Cilomilast **3** [Christensen et al., 1998; Barnette et al., 1998] and tofimilast **4** [Duplantier et al., 2007; Bayes et al., 2007] are selective PDE4 inhibitors. The former is being studied for the treatment of emphysema and bronchitis while the latter was discontinued because of unacceptable side effects. Other PDE4 inhibitor molecules in clinical trials include apremilast **5** [Khobzaoui et al., 2005] (CC10004), which is being evaluated in psoriatic arthritis and refractory psoriasis [http://clinicaltrial.gov/.], and tetomilast **6** [Bloomfield et al., 1997; O'Mahony, 2005] (OPC-6535), which has been recently studied in patients with ulcerative colitis, Crohn's disease, and COPD (Phase II) [http://clinicaltrial.gov/.]. CC-1088 **7** [Muller et al., 1998] is structurally related to **5**.

In the last decade, we reported [Dal Piaz et al., 1997, 1998; Giovannoni et al., 2007] on heterocyclic-fused 3-(2H)-pyridazinones with appreciable activity as PDE-4 inhibitors, good selectivity toward PDE3 isoenzyme, and, in some cases, low affinity for [^{3}H]rolipram binding site. The more interesting compounds were further investigated [Martin et al., 2002; Pieretti et al., 2006].

In the present work, we have designed and synthesized a new series of pyrimidine-2,4-diones as simplified analogues of nitraquazone **8**, a prototypic PDE4 inhibitor [Glaser and Traber, 1984; Russo et al., 1987] and report on their PDE4 inhibitory activity, selectivity versus PDE3/PDE5, and affinity for HARBS.

MATERIALS AND METHODS

Chemistry

All melting points were determined on a Buchi apparatus and are uncorrected. ^{1}H-NMR spectra were recorded with Avance 400 instruments (Bruker Biospin, version 002 with SGU). 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. E. Merck (West Point, PA) 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.

[^{3}H]-cAMP, [^{3}H]-cGMP, and [^{3}H]Rolipram were from Perkin-Elmer Life and Analytical Science (Waltham, MA); all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) (Fig. 1).

The synthesis of the novel pyrimidine-2,4-diones substituted at position 1, 3, and 5 is depicted in Figures 2–4. In Figures 2 and 3, the procedure to afford the 5-substituted aniline derivatives **13a–d** and **18a–c** is reported. In both instances, the starting material is represented by the previously described pyrimidine-2,4-diones **9a,b** [Winckelmann and Larsen, 1986; Robin et al., 2006] and **14** [Winckelmann and Larsen, 1986]. In Figure 2, the first step is a bromination at position 5 performed with bromine in acetic acid; treatment of the intermediates **10a,b** (**10a**) [Moltke-Leth and Joergensen, 1993] with concentrated ammonia afforded the 5-amino derivatives **11a,b**, which were alkylated in standard condition to give **12a,b**. The last step to obtain the final compounds **13a–d** was performed through a cross-coupling reaction with the appropriate aryl boronic acids using copper acetate as catalyst and a weak base (triethylamine) in CH_2Cl_2 [Quach and Batey, 2003; Chiang and Olsson, 2004]. Similarly, compounds **18a–c** were obtained starting from the 5-amino derivatives **17a,b**, following the above described cross-coupling reaction. In this synthetic route, compound **15**, obtained by alkylation from the precursor **14**, was nitrated with a mixture of potassium nitrate and sulfuric acid to afford compound **16**. Treatment of compound **16** with stannous chloride in

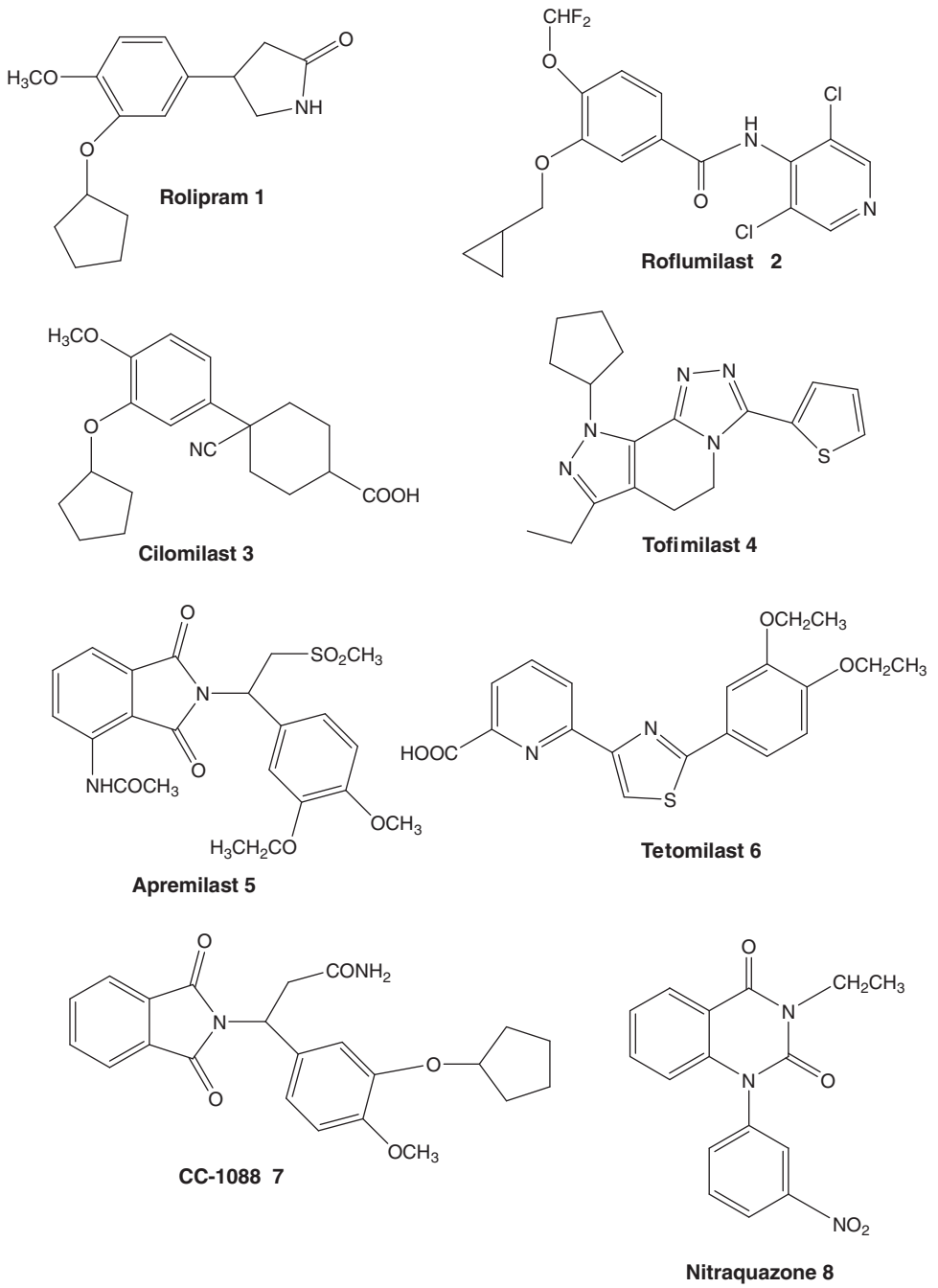


Fig. 1. PDE4 inhibitors.

hydrochloric acid using a 1:1 molar ratio selectively reduced the nitro group at position 5 to give the corresponding 5-amino derivative **17a**. Reduction of both nitro groups at position 5 and in *para* position of the phenyl ring was performed using a 1:5 molar ratio of substrate **16** and stannous chloride to yield **17b**. In Figure 4, the common precursors are again represented by 5-bromo pyrimidine-2,4-diones **10a–c** (**10c**) [Gutschow et al., 2001], which, in the first route, were alkylated under standard conditions affording **19a–d**, which, in turn, were subjected to a cross-coupling reaction under classical Suzuki conditions using tetrakis(triphenylphosphine)palladium and the opportune phenylboronic acid in toluene [Wang and Haseltine, 1994] to give the final 5-(substituted)aryl pyrimidine-2,4-diones **20a–m**. In the second route, the bromine at position 5 was displaced with 4-chloro-benzylamine to give compound **21,** which, treated with ethyl bromide,

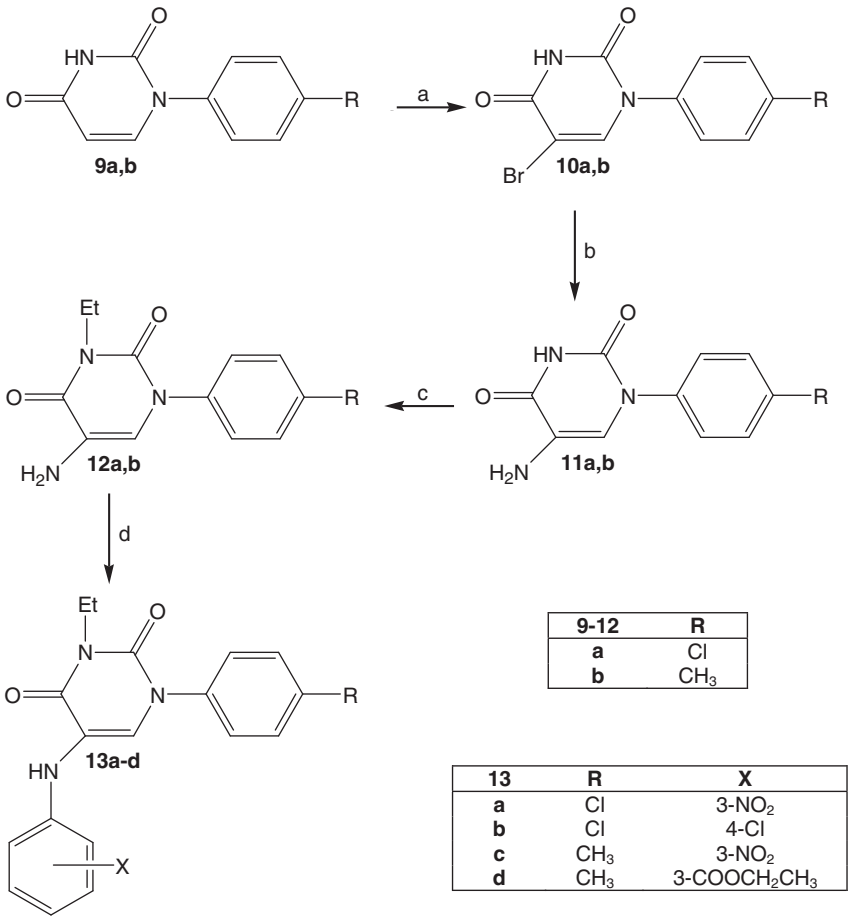


Fig. 2. Synthesis of 5-(substituted)anilino pyrimidine-2,4-diones **13a–d**. Reagents and conditions: (a) Br_2, AcOH 100%, rt, 3h; (b) NH_4OH 33%, sealed tube, 120°C, 5 h; (c) EtBr, DMF, K_2CO_3, 90°C, 1h; (d) X-C_6H_5-$B(OH)_2$, CH_2Cl_2, Ac_2Cu, Et_3N, rt, 24 h.

afforded the final compound **22**. Finally, several molecules with pyridine-2-one core (**24a–c**) were synthesized starting from previously described precursors **23a,b** [Bormann, 1981; Hagen et al., 1990], performing an alkylation at position 1 (Fig. 5).

5-Bromo-1-p-tolyl-1H-pyrimidine-2,4-dione (10b)

To a stirred mixture of **9b** (0.74 mmol) in glacial acetic acid (7 mL), a solution of bromine (0.74 mmol) in the same solvent (0.5 mL) was added in a dropwise manner (over a 2-h period). The mixture was stirred for 1 h, concentrated in vacuo to afford a residue that, when treated with cold water (15 mL), gave rise to a crude precipitate that was recovered by suction. Mp = 202–205°C; crystallization solvent = EtOH; yield = 70%. ^{1}H-NMR ($CDCl_3$), δ: 2.45 (3H, s), 7.20–7.35 (4H, m), 7.70 (1H, s), 8.50 (1H, exch br s).

General Procedure for Compounds (11a,b)

To the appropriate 5-bromopyrimidin-2,4-dione (**10a** or **10b**, 0.76 mmol), 4 mL of concentrated ammonia was added. The reaction was carried out in a sealed tube at 120°C for 4 h. After, the mixture was concentrated in vacuo and the residue was treated with cold ethanol to afford a crude solid that was collected by suction.

5-Amino-1-(4-chlorophenyl)-1H-pyrimidine-2,4-dione (11a) Mp > 300°C; crystallization solvent = EtOH; yield = 61%. ^{1}H-NMR (DMSO), δ: 4.25 (2H, exch br s), 6.85 (1H, s), 7.45 (2H, d, J = 8.5 Hz), 7.55 (2H, d, 8.5 Hz), 11.50 (1H, exch br s).

5-Amino-1-p-tolyl-1H-pyrimidine-2,4-dione (11b) Mp = 227–229°C; crystallization solvent = EtOH; yield = 80%. ^{1}H-NMR (DMSO), δ: 2.35 (3H, s), 4.20 (2H, exch br s), 6.80 (1H, s), 7.30 (4H, s), 11.45 (1H, exch br s).

General Procedure for Compounds (12a,b)

A mixture of the appropriate pyrimidin-2,4-dione (**11a** or **11b**, 0.5 mmol), anhydrous K_2CO_3 (1 mmol), and ethyl bromide (0.75 mmol) in anhydrous DMF (2 mL) was heated under stirring for 40–120 min at 60–90°C. After dilution with cold water, the crude precipitate **12a** was recovered by suction, while

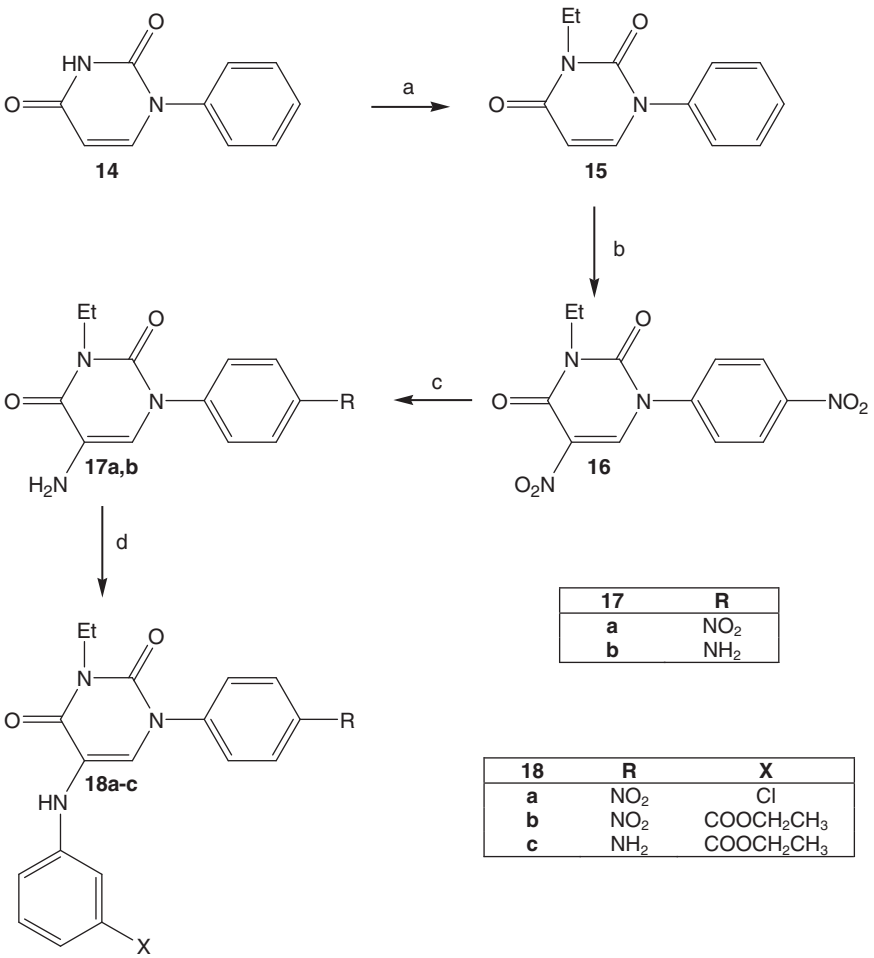


Fig. 3. Synthesis of 5-(substituted)anilino pyrimidine-2,4-diones **18a,b**. Reagents and conditions: (a) EtBr, DMF, K_2CO_3, 90°C, 1 h; (b) KNO_3, conc. H_2SO_4, rt, 8 h; (c) $SnCl_2$, conc HCl, rt, 1 h; (d) $ArB(OH)_2$, CH_2Cl_2, Ac_2Cu, Et_3N, rt, 24 h.

compound **12b** was extracted (3 × 20 mL) with ethyl acetate (30–40 mL).

5-Amino-1-(4-chlorophenyl)-3-ethyl-1H-pyrimidine-2,4-dione (12a) Mp = 190°C; crystallization solvent = EtOH; yield = 63%. ^{1}H-NMR (DMSO), δ: 1.15 (3H, t, J = 7.2 Hz), 3.90 (2H, q, J = 7.2 Hz), 4.30 (2H, exch br s), 6.90 (1H, s), 7.45 (2H, d, J = 8.9 Hz), 7.55 (2H, d, J = 8.9 Hz).

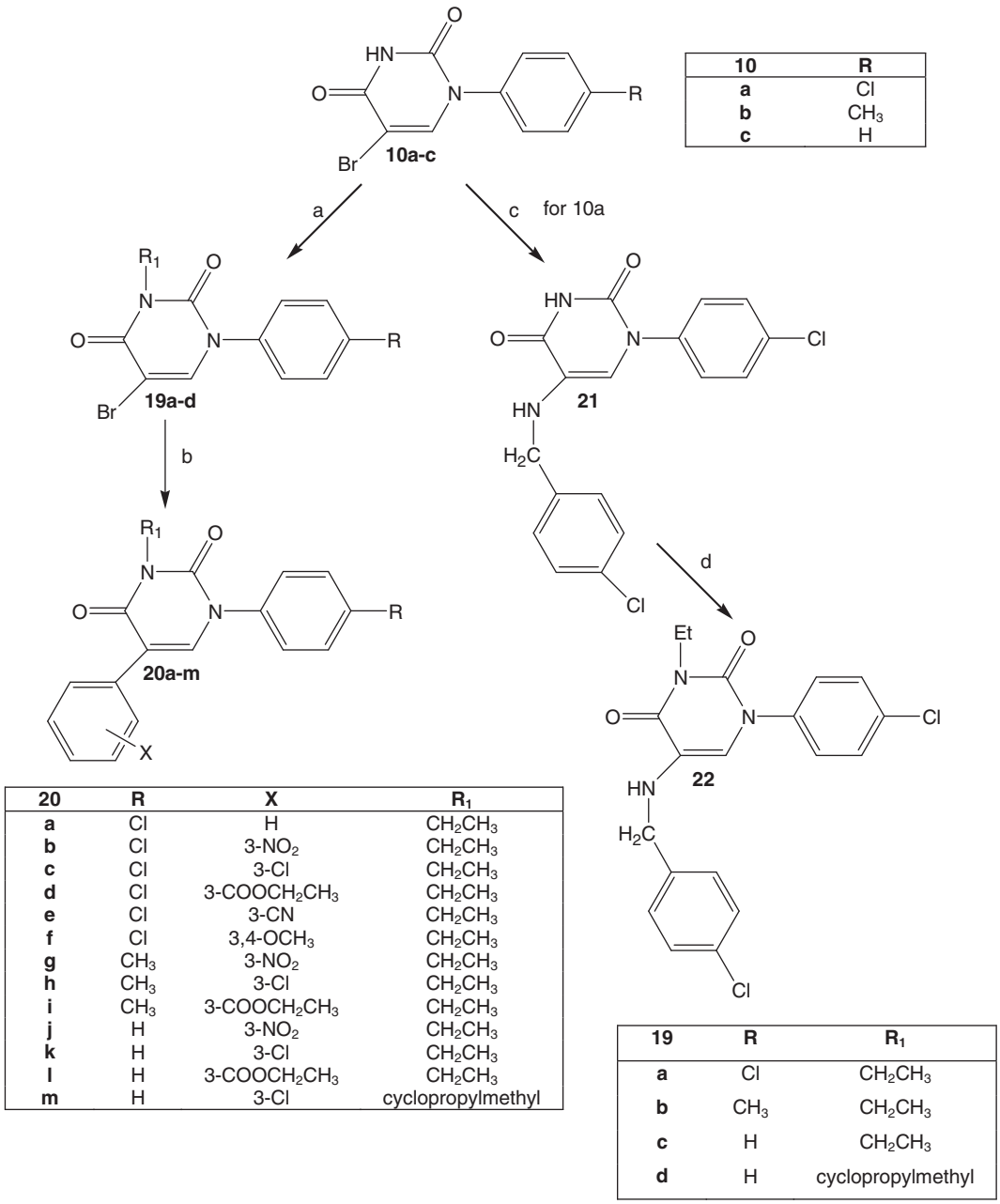
5-Amino-1-p-tolyl-3-ethyl-1H-pyrimidine-2,4-dione (12b) Mp = 157–160°C; crystallization solvent = EtOH; yield = 64%. ^{1}H-NMR (DMSO), δ: 1.15 (3H, t, J = 7.2 Hz), 2.35 (3H, s), 3.90 (2H, q, J = 7.2 Hz), 4.25 (2H, exch br s), 6.85 (1H, s), 7.30 (4H, s).

General Procedure for Compounds (13a–d)

A mixture of activated molecular sieves (0.8–1 g), the substrates **12a** or **12b** (0.5 mmol), the appropriate boronic acid (0.5 mmol) commercially available, copper acetate (0.8 mmol), and triethylamine (1 mmol) in CH_2Cl_2 (10 mL) were stirred for 6–24 h at rt −40°C. The molecular sieves together with copper salts were filtered off and the solution was extracted first with concentrated ammonia (2 × 5 mL) and then with water (2 × 5 mL). The organic layer was evaporated in vacuo and the residue recovered by suction (compounds **13c,d**). For compounds **13a,b**, evaporation of the solvent afforded an oil that was purified by column chromatography using cyclohexane/ethyl acetate 1:2 as eluent.

1-(4-Chlorophenyl)-3-ethyl-5-(3-nitrophenylamino)-1H-pyrimidine-2,4-dione (13a) Mp = 190–193°C; purified by column chromatography (cyclohexane/ethyl acetate 1:2); yield = 20%. ^{1}H-NMR (DMSO), δ: 1.25 (3H, t, J = 7.1 Hz), 3.90 (2H, q, J = 7.1 Hz), 7.20 (1H, d, J = 8.8 Hz), 7.40 (1H, dd, J = 2.2, 8.8 Hz), 7.50 (1H, d, J = 8.8 Hz), 7.55 (5H, m), 7.80 (1H, exch br s), 7.85 (1H, s). *Anal.* Calcd for $C_{18}H_{15}ClN_4O_4$: C, 55.89; H, 3.91; N, 14.49. Found: C, 56.00; H, 3.92; N, 14.52.

1-(4-Chlorophenyl)-5-(3-chlorophenylamino)-3-ethyl-1H-pyrimidine-2,4-dione (13b) Mp = 117–120°C;



10	R
a	Cl
b	CH_3
c	H

20	R	X	R_1
a	Cl	H	CH_2CH_3
b	Cl	3-NO_2	CH_2CH_3
c	Cl	3-Cl	CH_2CH_3
d	Cl	3-$COOCH_2CH_3$	CH_2CH_3
e	Cl	3-CN	CH_2CH_3
f	Cl	3,4-OCH_3	CH_2CH_3
g	CH_3	3-NO_2	CH_2CH_3
h	CH_3	3-Cl	CH_2CH_3
i	CH_3	3-$COOCH_2CH_3$	CH_2CH_3
j	H	3-NO_2	CH_2CH_3
k	H	3-Cl	CH_2CH_3
l	H	3-$COOCH_2CH_3$	CH_2CH_3
m	H	3-Cl	cyclopropylmethyl

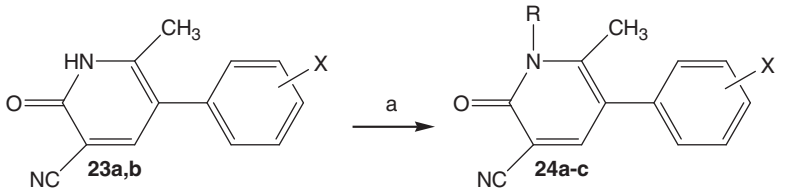
19	R	R_1
a	Cl	CH_2CH_3
b	CH_3	CH_2CH_3
c	H	CH_2CH_3
d	H	cyclopropylmethyl

Fig. 4. Synthesis of 5-(substituted)phenyl pyrimidine-2,4-diones **20a–m** and of compound **22**. Reagents and conditions: (a) R_1Br, DMF, K_2CO_3, 90°C, 1 h; (b) X-C_6H_5-$B(OH)_2$, $Pd(PPh_3)_4$, 2M Na_2CO_3, toluene, 90°C, 16h; (c) 4-chlorobenzylamine, EtOH, 90°C, 9h; (d) EtBr, DMF, K_2CO_3, 90°C, 1h.

purified by column chromatography (cyclohexane/ethyl acetate 1:2); yield = 20%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 6.85 (1H, d, J = 9.0 Hz), 6.90 (1H, d, J = 9.0 Hz), 7.00 (1H, s), 7.20 (2H, m), 7.30–7.50 (4H, m), 7.90 (1H, exch br s). *Anal.* Calcd for $C_{18}H_{15}Cl_2N_3O_2$: C, 57.46; H, 4.02; N, 11.17. Found: C, 57.57; H, 4.03; N, 11.19.

3-Ethyl-5-(3-nitrophenylamino)-1-p-tolyl-1H-pyrimidine-2,4-dione (13c) Mp = 200–203°C; crystallization solvent = EtOH; yield = 17%. ^{1}H-NMR (DMSO), δ: 1.15 (3H, t, J = 7.2 Hz), 2.35 (3H, s), 3.90 (2H, q, J = 7.2 Hz), 7.20 (1H, d, J = 8.7 Hz), 7.35 (5H, m), 7.50 (1H, d, J = 8.7 Hz), 7.55 (1H, s), 7.80 (1H, s), 7.85 (1H, exch br s). *Anal.* Calcd for $C_{19}H_{18}N_4O_4$: C, 62.29; H, 4.95; N, 15.29. Found: C, 62.41; H, 4.96; N, 15.32.

3-(3-Ethyl-2,4-dioxo-1-p-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl-amino)benzoic acid ethyl ester (13d) Mp = 130–135°C; crystallization solvent = EtOH; yield = 20%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t,



23	X
a	H
b	3,4-OCH_3

24	R	X
a	CH_2CH_3	3,4 OCH_3
b	cyclopropylmethyl	H
c	cyclopropylmethyl	3,4 OCH_3

Fig. 5. Synthesis of 1-alkylpyrimidones **24a–c**. Reagents and conditions: (a) RBr, DMF (or CH_3CN), K_2CO_3, 90°C, 1h.

$J = 7.2$ Hz), 1.40 (3H, t, $J = 7.2$ Hz), 2.40 (3H, s), 4.15 (2H, q, $J = 7.2$ Hz), 4.40 (2H, q $J = 7.2$ Hz), 7.15 (1H, d, $J = 8.8$ Hz), 7.30 (6H, m), 7.60 (1H, d, $J = 8.8$ Hz), 7.70 (1H, s), 7.85 (1H, exch br s).

Anal. Calcd for $C_{22}H_{23}N_3O_4$: C, 67.16; H, 5.89; N, 10.68. Found: C, 67.29; H, 5.90; N, 10.70.

3-Ethyl-1-phenyl-1H-pyrimidine-2,4-dione (15)

Compound **15** was obtained from compound **14** following the general procedure described for **13a–d**. After dilution with cold water, the suspension was extracted with ethyl acetate (3×20 mL) and the solvent was evaporated in vacuo to afford **15**. Mp = 119–121°C; crystallization solvent = EtOH; yield = 20%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, $J = 7.0$ Hz), 4.10 (2H, q, $J = 7.0$ Hz), 5.90 (d, 1H,), 7.30–7.55 (6H, m).

3-Ethyl-5-nitro-1-(4-nitrophenyl)-1H-pyrimidine-2,4-dione (16) A mixture of pyrimidine-2,4-dione 15 (0.46 mmol) KNO_3 (1.84 mmol) and 0.1 mL of concentrated H_2SO_4 (1.84 mmol) was heated at 100°C in a sealed tube for 3 h. After cooling, cold water was added and the precipitate filtered off. Mp = 112°C dec.; crystallization solvent = EtOH; yield = 90%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, $J = 7.0$ Hz), 4.15 (2H, q, $J = 7.0$ Hz), 7.65 (2H, d, $J = 8.8$ Hz), 8.45 (2H, d, $J = 8.8$ Hz), 8.80 (s, 1H, 6-CH).

General Procedure for Compounds (17a,b)

To a mixture of compound **16** (0.30 mmol) and concentrated HCl (0.8 mL), a solution of $SnCl_2$ (0.3 mmol to obtain compound **17a** and 1.5 mmol to obtain compound **17b**) in 1 mL of concentrated HCl was added. The reaction was carried out at room temperature for 1 h and then 6N NaOH was added until pH = 8. The crude precipitate was recovered by suction.

5-Amino-3-ethyl-1-(4-nitrophenyl)-1H-pyrimidine-2,4-dione (17a) Mp ≥ 300°C; crystallization solvent = EtOH; yield = 75%. ^{1}H-NMR (DMSO), δ: 1.15 (3H, t, $J = 7.1$ Hz), 3.90 (2H, q, $J = 7.1$ Hz), 4.40 (2H, exch br s), 7.00 (1H, s), 7.75 (2H, d, $J = 8.9$ Hz), 8.35 (2H, d, $J = 8.9$ Hz).

5-Amino-3-ethyl-1-(4-aminophenyl)-1H-pyrimidine-2,4-dione (17b) Mp ≥ 300°C; crystallization solvent = EtOH; yield = 40%. ^{1}H-NMR (DMSO), δ: 1.10 (3H, t, $J = 7.2$ Hz), 3.87 (2H, q, $J = 7.2$ Hz), 4.14 (2H, exch br s), 5.27 (2H, exch br s), 6.56 (2H, d, $J = 8.3$ Hz), 6.75 (1H, s), 6.95 (2H, d, $J = 8.3$ Hz r).

General Procedure for Compounds (18a–c)

Compounds **18a–c** were obtained starting from **17a,b** following the general procedure described for **13a–d**. Evaporation of the solvent afforded **18a** as a crude precipitate and **18b,c** as oil. The latter were purified by column chromatography using cycloexane/ethyl acetate 2:1 as eluent.

5-(3-Chlorophenylamino)-3-ethyl-1-(4-nitrophenyl)- 1H-pyrimidine-2,4-dione (18a) Mp = 166–168°C; crystallization solvent = EtOH; yield = 5%. ^{1}H-NMR ($CDCl_3$), δ: 1.40 (3H, t, $J = 7.2$ Hz), 4.15 (2H, q, $J = 7.2$ Hz), 6.90 (1H, d, $J = 8.8$ Hz), 6.95 (1H, d, $J = 8.8$ Hz), 6.95 (3H, m), 7.25 (2H, m), 7.65 (2H, d, $J = 9.0$ Hz), 7.90 (1H, exch br s), 8.40 (2H, d, $J = 9.0$ Hz). *Anal.* Calcd for $C_{18}H_{15}ClN_4O_4$: C, 55.89; H, 3.91; N, 14.49. Found: C, 56.00; H, 3.92; N, 14.52.

3-(3-Ethyl-2,4-dioxo-1-(4-nitrophenyl)-1,2,3,4-tetrahydropyrimidin-5-yl-amino)benzoic acid ethyl ester (18b) Mp = 129–131°C; purified by column chromatography (cyclohexane/ethyl acetate 2:1); yield = 30%. ^{1}-NMR (DMSO), δ: 1.30 (3H, t, $J = 7.2$ Hz), 1.40 (3H, t, $J = 7.2$ Hz), 4.20 (2H, q, $J = 7.2$ Hz), 4.40 (2H, q, $J = 7.2$ Hz), 7.10 (1H, m), 7.25 (2H, m), 7.50 (1H, m), 7.80 (3H, m), 7.90 (1H, exch br s), 8.35 (2H, d, $J = 8.9$ Hz). *Anal.* Calcd for $C_{21}H_{20}N_4O_6$: C, 59.43; H, 4.75; N, 13.20. Found: C, 59.31; H, 4.74; N, 13.17.

3-(1-(4-aminophenyl)-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl-amino)benzoic acid ethyl ester (18c) Mp = 105–108°C; purified by column chromatography (cyclohexane/ ethyl acetate 2:1); yield = 20%.^{1}H-NMR (DMSO), δ: 1.10 (3H, t, $J = 7.1$ Hz), 1.27 (3H, t, $J = 7.2$ Hz), 3.89 (2H, q,

J = 7.1 Hz), 4.24 (2H, q, J = 7.2 Hz), 5.40 (2H, exch br s), 6.60 (2H, d, J = 8.3 Hz), 7.05 (2H, d, J = 8.3 Hz), 7.35 (5H m), 7.85 (exch br s, 1H, NH), 8.40 (2H, d, J = 8.9 Hz). *Anal.* Calcd for $C_{21}H_{22}N_4O_4$: C, 63.95; H, 5.62; N, 14.20. Found: C, 63.82; H, 5.61; N, 14.17.

General Procedure for Compounds (19a–d)

Compounds **18a–d** were obtained starting from **10a–c** following the general procedure described for **12a,b**.

5-Bromo-1-(4-chlorophenyl)-3-ethyl-1H-pyrimidine-2,4-dione (19a) Mp = 195–197°C; crystallization solvent = EtOH; yield = 90%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, J = 7.1 Hz), 4.10 (2H, q, J = 7.1 Hz), 7.30 (2H, d, J = 8.8 Hz), 7.50 (2H, d, J = 8.8 Hz), 7.65 (1H, s).

5-Bromo-3-ethyl-1-p-tolyl-1H-pyrimidine-2,4-dione (19b) Mp = 176–177°C; crystallization solvent = EtOH; yield = 81%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, J = 7.3 Hz), 2.40 (3H, s), 4.15 (2H, q, J = 7.3 Hz), 7.20 (d, 2H, J = 8.8 Hz), 7.30 (d, 2H, J = 8.8 Hz), 7.65 (1H, s).

5-Bromo-3-ethyl-1-phenyl-1H-pyrimidine-2,4-dione (19c) Mp = 184–185°C; crystallization solvent = EtOH; yield = 100%.^{1}H-NMR ($CDCl_3$), δ, ppm: 1.30 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.40 (d, 2H, J = 8.8 Hz), 7.45-7.55 (3H, m), 7.70 (1H, s).

5-Bromo-3-cyclopropylmethyl-1-phenyl-1H-pyrimidine-2,4-dione (19d) Mp = 150–152°C; crystallization solvent = EtOH; yield = 96%. ^{1}H-NMR ($CDCl_3$), δ: 0.45 (4H, m), 1.30 (1H, m), 3.95 (2H, d, J = 6.8 Hz), 7.35 (2H, d, J = 8.0 Hz), 7.40–7.55 (3H, m), 7.70 (1H, s).

General Procedure for Compounds (20a–m)

A mixture of the appropriate 5-bromo derivative **19** (0.3–0.6 mmol), tetrakis(triphenyl phosphine) palladium(0) (0.017–0.025 mmol), toluene (2–3 mL), the commercially available phenylboronic acid (0.6–1.2 mmol) dissolved in 1–2 mL of EtOH and 2M Na_2CO_3 (2–3 mL), was heated at 60–90°C for 3–18 h. After cooling, the mixture was concentrated in vacuo, diluted with water (10 mL), and extracted with CH_2Cl_2 (3 × 10 mL). Evaporation of the solvent afforded an oil that was purified by column chromatography. Compound **20j** was purified by crystallization from EtOH.

1-(4-Chlorophenyl)-3-ethyl-5-phenyl-1H-pyrimidine-2,4-dione (20a) Mp = 48–51°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 19%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.35–7.45 (6H, m), 7.50 (2H, d, J = 8.4 Hz), 7.55 (2H, d, J = 8.4 Hz). *Anal.* Calcd for $C_{18}H_{15}ClN_2O_2$: C, 66.16; H, 4.63; N, 8.57. Found: C, 66.25; H, 4.64; N, 8.59.

1-(4-Chlorophenyl)-3-ethyl-5-(3-nitrophenyl)-1H-pyrimidine-2,4-dione (20b) Mp = 145–147°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 21%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.40 (2H, d, J = 8.4 Hz), 7.50–7.65 (4H, m), 8.00–8.20 (2H, m), 8.40 (1H, s). *Anal.* Calcd for $C_{18}H_{14}ClN_3O_4$: C, 58.15; H, 3.80; N, 11.30. Found: C, 58.03; H, 3.79; N, 11.28.

1-(4-Chlorophenyl)-5-(3-chlorophenyl)-3-ethyl-1H-pyrimidine-2,4-dione (20c) Mp = 63–65°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 17%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.35–7.55 (9H, m). *Anal.* Calcd for $C_{18}H_{14}Cl_2N_2O_2$: C, 59.85; H, 3.91; N, 7.76. Found: C, 59.73; H, 3.90; N, 7.74.

3-[1-(4-Chlorophenyl)-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]benzoic acid ethyl ester (20d) Mp = 102–105°C; purified by column chromatography (cyclohexane/ethyl acetate 3:1); yield = 16%.^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.1 Hz), 1.40 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.1 Hz), 4.40 (2H, q, J = 7.2 Hz), 7.40 (2H, d, J = 8.4 Hz), 7.50–7.55 (4H, m), 7.65 (1H, d, J = 8.4 Hz), 7.85 (1H, d, J = 8.4 Hz), 8.15 (1H, m). *Anal.* Calcd for $C_{21}H_{19}ClN_2O_4$: C, 63.24; H, 4.80; N, 7.02. Found: C, 63.37; H, 4.81; N, 7.03.

3-[1-(4-Chlorophenyl)-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl]benzonitrile (20e) Mp = 163–164°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 20%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.40 (2H,d, J = 8.5 Hz), 7.45–7.55 (4H, m), 7.65 (1H, d, J = 8.5 Hz), 7.80 (1H, d, J = 8.5 Hz), 8.00 (1H, s). *Anal.* Calcd for $C_{19}H_{14}ClN_3O_2$: C, 64.87; H, 4.01; N, 11.94. Found: C, 65.06; H, 4.02; N, 11.98.

1-(4-Chlorophenyl)-5-(3,4-dimethoxyphenyl)-3-ethyl-1H-pyrimidine-2,4-dione (20f) Mp = 66–68°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 19%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 3.90 (3H, s), 3.95 (3H, s), 4.15 (2H, q, J = 7.2 Hz), 6.90 (1H, m), 7.05 (1H, d, J = 7.9 Hz), 7.20 (1H, s), 7.40–7.50 (5H, m). *Anal.* Calcd for $C_{20}H_{19}ClN_2O_4$: C, 62.10; H, 4.95; N, 7.24. Found: C, 62.29; H, 4.96; N, 7.26.

3-Ethyl-5-(3-nitrophenyl)-1-p-tolyl-1H-pyrimidine-2,4-dione (20g) Mp = 162–164°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 34%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 2.45 (3H, s), 4.20 (2H, q, J = 7.2 Hz), 7.25–7.40 (4H, m), 7.60 (2H, m), 8.00 (1H, d, J = 8.9 Hz), 8.20 (1H, d, J = 8.9 Hz), 8.40 (1H, s). *Anal.* Calcd for $C_{19}H_{17}N_3O_4$: C, 64.95; H, 4.88; N, 11.96. Found: C, 65.14; H, 4.89; N, 12.00.

5-(3-Chlorophenyl)-3-ethyl-1-p-tolyl-1H-pyrimidine-2,4-dione (20h) Mp = 40–42°C; purified by

column chromatography (cyclohexane/ethyl acetate 3:1); yield = 31%. ^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 2.45 (3H, s), 4.15 (2H, q, J = 7.2 Hz), 7.30–7.60 (9H, m). *Anal.* Calcd for $C_{19}H_{17}ClN_2O_2$: C, 66.96; H, 5.03; N, 8.22. Found: C, 67.16; H, 5.05; N, 8.24.

3-(3-Ethyl-2,4-dioxo-1-p-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)benzoic acid ethyl ester (20i) Mp = 97–100°C; purified by column chromatography (cyclohexane/ethyl acetate 3:1); yield = 15%.

^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 1.40 (3H, t, J = 7.3 Hz), 2.45 (3H, s), 4.20 (2H, q, J = 7.2 Hz), 4.40 (2H, q, J = 7.3 Hz), 7.20–7.70 (6H, m), 7.90 (1H, d, J = 8.2 Hz), 8.05 (1H, d, J = 8.2 Hz), 8.15 (1H, s). *Anal.* Calcd for $C_{22}H_{22}N_2O_4$: C, 69.83; H, 5.86; N, 7.40. Found: C, 69.69; H, 5.85; N, 7.39.

3-Ethyl-5-(3-nitrophenyl)-1-phenyl-1H-pyrimidine-2,4-dione (20j) Mp = 151–154°C; crystallization solvent = EtOH; yield = 26%. ^{1}H-NMR ($CDCl_3$), δ: 1.30 (3H, t, J = 7.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 7.35–7.55 (5H, m), 7.65–7.75 (2H, m), 8.00 (1H, d, J = 8.5 Hz), 8.30 (1H, d, J = 8.5 Hz), 8.55 (1H, s). *Anal.* Calcd for $C_{18}H_{15}N_3O_4$: C, 64.09; H, 4.48; N, 12.46. Found: C, 63.96; H, 4.47; N, 12.44.

5-(3-Chlorophenyl)-3-ethyl-1-phenyl-1H-pyrimidine-2,4-dione (20k) Mp = 42–44°C; purified by column chromatography (cyclohexane/ethyl acetate 2:1); yield = 19%. ^{1}H-NMR ($CDCl_3$), δ, ppm: 1.35 (3H, t, J = 7.2 Hz), 4.20 (2H, q, J = 7.2 Hz), 7.30–7.60 (10H, m). *Anal.* Calcd for $C_{18}H_{15}ClN_2O_2$: C, 66.16; H, 4.63; N, 8.57. Found: C, 66.29; H, 4.64; N, 8.59.

3-(3-Ethyl-2,4-dioxo-1-phenyl-1,2,3,4-tetrahydropyrimidin-5-yl)benzoic acid ethyl ester (20l) Mp = 50–52°C; purified by column chromatography (cyclohexane/ethyl acetate 3:1); yield = 17%.

^{1}H-NMR ($CDCl_3$), δ: 1.35 (3H, t, J = 7.2 Hz), 1.40 (3H, t, J = 7.0 Hz), 4.20 (2H, q, J = 7.2 Hz), 4.40 (2H, q, J = 7.0 Hz), 7.40–7.60 (7H, m), 7.85 (1H, d, J = 8.3 Hz), 8.05 (1H, d, J = 8.3 Hz), 8.15 (1H, s). *Anal.* Calcd for $C_{21}H_{20}N_2O_4$: C, 69.22; H, 5.53; N, 7.69. Found: C, 69.36; H, 5.54; N, 7.71.

5-(3-Chlorophenyl)-3-cyclopropylmethyl-1-phenyl-1H-pyrimidine-2,4-dione (20m) Mp = 47–48°C; purified by column chromatography (cyclohexane/ethyl acetate 3:1); yield = 23%. ^{1}H-NMR ($CDCl_3$), δ: 0.4–0.5 (4H, m), 1.35 (1H, m), 4.00 (2H, d, J = 6.9 Hz), 7.30–7.55 (10H, m). *Anal.* Calcd for $C_{20}H_{17}ClN_2O_2$: C, 68.09; H, 4.86; N, 7.94. Found: C, 67.95; H, 4.85; N, 7.92.

5-(4-Chlorobenzylamino)-1-(4-chlorophenyl)-1H-pyrimidine-2,4-dione (21) A mixture of 5-bromo derivative 10a (0.5 mmol) and 4-chlorobenzylamine (4.0 mmol) was heated in a sealed tube at 90°C for 9 h. After cooling, the residue was treated with EtOH (2 mL) and cold water was added (25 mL) and the mixture was extracted with ethyl acetate (3 × 20). Evaporation of the solvent afforded a residue that was crystallized by EtOH. Mp = 243–246°C; crystallization solvent = EtOH; yield = 17%.^{1}H-NMR ($CDCl_3$), δ: 4.10 (2H, d), 5.30 (1H, exch br t), 6.45 (1H, s), 7.30–7.40 (6H, m), 7.50 (2H, d, J = 8.6 Hz).

5-(4-Chlorobenzylamino)-1-(4-chlorophenyl)-3-ethyl-1H-pyrimidine-2,4-dione (22) Mp = 186–187°C; crystallization solvent = EtOH; yield = 60%. ^{1}H-NMR (DMSO), δ: 1.10 (3H, t, J = 7.2 Hz), 3.90 (2H, q, J = 7.2 Hz), 4.10 (2H, d), 5.35 (1H, exch t), 6.50 (1H, s), 7.35 (6H, m), 7.50 (2H, d, J = 8.6 Hz).

Anal. Calcd for $C_{19}H_{17}Cl_2N_3O_2$: C, 58.47; H, 4.39; N, 10.77. Found: C, 58.65; H, 4.40; N, 10.80.

General Procedure for Compounds (24a–c)

Compounds **24a–c** were obtained starting from **23a,b** following the general procedure described for **12a,b**. For compounds **24a,c**, the reaction was carried out in anhydrous DMF, the suspension was diluted with cold water and the precipitate **24a** was recovered by suction and recrystallized by ethanol. Compound **24c** was obtained by extraction with CH_2Cl_2 (3 × 15 mL) and was purified by column chromatography using toluene/ethyl acetate 8:2, as eluent. For compound **24b**, the reaction was carried out in anhydrous CH_3CN and suspension was diluted with cold water and extracted with CH_2Cl_2 (3 × 15 mL) to afford a residue that was purified by column chromatography using cyclohexane/ethyl acetate 2:1, as eluent.

5-(3,4-Dimethoxyphenyl)-1-ethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (24a) Mp = 112–114°C; crystallization solvent = EtOH; yield = 60%. ^{1}H-NMR ($CDCl_3$), δ: 1.50 (3H, t, J = 7.2 Hz), 2.50 (3H, s), 3.90 (3H, s), 3.95 (3H, s), 4.55 (2H, q, J = 7.2 Hz), 6.75 (1H, s), 6.85–6.95 (2H, m), 7.70 (1H, s). *Anal.* Calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.65; H, 6.10; N, 9.42.

1-Cyclopropylmethyl-2-oxo-5-phenyl-1,2-dihydropyridine-3-carbonitrile (24b) Mp = 117–118°C; purified by column chromatography (cyclohexane/ethyl acetate 2:1); yield = 30%. ^{1}H-NMR ($CDCl_3$), δ: 0.60 (4H, m), 1.15 (1H, m), 2.45 (3H, s), 4.15 (2H, d, J = 6.9 Hz), 7.20 (2H, m), 7.40 (3H, m), 7.70 (1H, s).

Anal. Calcd for $C_{17}H_{16}N_2O$: C, 77.25; H, 6.10; N, 10.60. Found: C, 77.10; H, 6.09; N, 10.59.

1-Cyclopropylmethyl-5-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (24c) Mp = 107–109°C; purified by column chromatography (toluene/ethyl acetate 8:2); yield = 30%.

^{1}H-NMR ($CDCl_3$), δ: 0.45 (2H, m), 0.65 (2H, m), 1.40 (1H, m), 2.45 (3H, s), 3.90 (3H, s), 3.95 (3H, s), 4.30 (2H, d, J = 6.8 Hz), 6.75 (1H, s), 6.85–6.95 (2H, m),

7.70 (1H, s). *Anal.* Calcd for $C_{19}H_{20}N_2O_3$: C, 70.35; H, 6.21; N, 8.64. Found: C, 70.49; H, 6.22; N, 8.66.

Pharmacology

PDE4 activity assay

Experiments were conducted using undifferentiated U937 cells. As previously demonstrated [Mackenzie and Houslay, 2000], this human monocytic cell line express a range of cAMP-specific phosphodiesterase (PDE) isoenzymes: PDE4A, PDE4B, and PDE4D, which provide around 76% of the total cAMP PDE activity of U937 cells. Only approximately 9% of the cAMP PDE activity in U937 cells was due to PDE3.

Cell culture

U937 cells were grown in plastic dishes in RPMI-1640 medium supplemented with 10% FCS, L-glutamine 2 mM and 50 U/mL of penicillin G and 0.05 mg/mL streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO_2 and culture medium changed every two days. Cells were harvested at a density of approximately 1.0×10^6 cells per mL; in all experiments, the medium was removed by centrifugation at 300 *g* for 5 min at room temperature and cells washed in PBS buffer (pH = 7.4) and re-centrifuged. The cells were resuspended in 50 mM ice-cold TRIS-HCl buffer (pH = 7.4) containing 1 mM PMSF and sonicated on ice four times for 25 sec. The sonically disrupted cells were centrifuged at 12,000*g* for 10 min; aliquots of the supernatant fraction were assayed for PDE activity using a modification of the method of Morelli et al. [2004] as described below. Protein concentration was determined using the Bradford method [Bradford, 1976].

cAMP assay

Aliquots of supernatant fraction of fresh cell lysates (50 µL) were incubated in a final volume of 200 µL for 20 min at 30°C with 50 nM [^{3}H]-cAMP, 1 µM cold cAMP in TRIS-HCl buffer containing 1 mM PMSF with or without PDE4 inhibitors. To inhibit the activity of the small fraction of PDE3, all samples were added with 1 µM cilostamide. The total PDE activity of the cell preparation was measured in the absence of inhibitors and total PDE4 activity was measured adding the selective inhibitor, rolipram (10 µM). All compounds were evaluated at a single final concentration of 10 µM. Compounds that exhibited activity in single-dose testing were further evaluated to generate dose response curves, which were used to determine IC_{50} values. Reactions were terminated by boiling for 1 min, each sample was then supplemented with a mixture of carriers (60 µL of a solution containing 3 mM cAMP, AMP, and adenine), and placed on ice for 10 min. In order to separate and quantify the amounts of nucleotides and adenine, aliquots of 40 µL of each sample were deposed on 60 F254 silica gel plates (eluent ethanol:H_2O, 70:30 v/v). Under UV light, three different lanes were identified, corresponding to cAMP, AMP, and adenine, which have the same ratio frontis of the standard molecules comigrated on the same plate. Then the three different lanes were separated by scraping, the silica powder collected and placed into plastic vials; the radioactivity was extracted with 1 mL water, added with 6 mL of InstagelPlus scintillation liquid (Perkin-Elmer Life and Analytical Science, Boston, MA) and counted in a TRICARB 1900TR Scintillation Counter (Perkin-Elmer Life and Analytical Science, Boston, MA).

Enzymatic activity was measured as percentage of substrate into product conversion as follows: conversion (%) = [products count/(substrate+products counts)] × 100. At the same time, 40 µL of the sample were placed into vials with scintillation cocktail and counted for radioactivity in order to obtain the recovery of each sample: in these experimental conditions, the total recovery was about 90%.

[^{3}H] Rolipram Binding

Membrane preparation

Male Sprague-Dawley rats (Harlan, Italy) were housed in a temperature- and light-controlled room and were allowed free access to food and water. Their experimental use was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care.

Rats were killed by decapitation and the cerebral cortex was dissected on ice and homogenized in buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5) using an Utraturrax homogenizer at 16,000 rpm. Separation of supernatant and particulate fractions was achieved by centrifugation at 15,000*g* for 15 min at 4°C and the pellet was then resuspended in the same buffer.

Binding studies

[^{3}H]Rolipram binding was measured following a modification of the method of Schneider et al. [1986]. For heterologous competition assays, 2 nM [^{3}H]Rolipram was incubated with membrane preparations containing 0.2–0.3 mg of protein at 30°C in a final volume of 0.25 mL in the presence of increasing concentrations of the newly synthesized compounds. For mixed homologous saturation binding assays, [^{3}H]Rolipram was present at 0.2 nM in tubes containing increasing concentrations of unlabeled Rolipram (0.03–1000 nM) and at 0.075–0.2 nM in tubes without unlabeled ligand. Reactions were stopped after 1 h by

addition of 5 mL of ice-cold buffer and rapid vacuum filtration through glass fiber filters (Whatman GF/C, Florham Park, NJ) presoaked in a 0.3% polyethylenimine (PEI) solution. The filters were washed twice with 5 mL of ice-cold buffer, 6 mL of scintillation liquid (FilterCount, Perkin-Elmer Life and Analytical Science, Boston, MA) was added and radioactivity measured in a scintillation beta counter. Binding data were evaluated quantitatively with the weighted least-squares iterative curve fitting LIGAND program [Munson and Rodbard, 1980], to obtain the Ki values.

PDE3 and PDE5 activity assay

Platelets contain three classes of PDEs: PDE2, PDE3, and PDE5 [Roma et al., 2007]. PDE2 hydrolyzes both cAMP and cGMP. The PDE3 isoform is the most abundant and preferentially hydrolyzes cAMP, while PDE5 is a specific inactivator of cGMP.

Preparation of disrupted platelet suspension

Human blood was freshly drawn from healthy volunteers, who gave informed consent and had not taken medication for at least 2 weeks. Blood was collected into ACD (2.5% sodium citrate, 1.5% citric acid, and 2% glucose) solution (6:1 [v/v]) and centrifuged at 200*g* for 20 min to generate platelet-rich plasma (PRP). The latter was then centrifuged at 1,000*g* for 10 min, resuspended in Tyrode's buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.42 mmol/L NaH_2PO_4, 5 mmol/L glucose, 10 mmol/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 0.2% bovine serum albumin, pH 7.4) and re-centrifuged. Platelets were resuspended in 50 mM ice-cold TRIS-HCl buffer (pH = 7.4) containing 1 mM PMSF and sonicated on ice four times for 30 sec. The disrupted platelet suspension was centrifuged at 12,000*g* for 20 min. The soluble fraction contains about 70–80% of the cAMP PDE activity, as described [Grant and Colman, 1984]. This fraction was assayed both for cAMP and cGMP PDE activity.

cAMP and cGMP assay

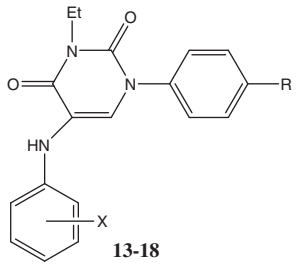
PDE activity was measured in TRIS-HCl buffer containing 1 mM PMSF. Aliquots of fresh platelet lysate suspension (50 μL) were incubated in a final volume of 200 μL for 15 min at 30°C with substrates ([^{3}H]-cAMP or [^{3}H]-cGMP 50 nM, and cold cAMP or cold cGMP 1 μM), with or without inhibitors. All experiments were performed in the presence of 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) to selectively inhibit PDE2 activity. Total PDE3 activity was determined in the presence of 10 μM cilostamide and total PDE5 activity was measured in the presence of 10 μM sildenafil. cAMP and cGMP assays were performed as described above. For PDE5 activity, the mixture of carriers used was composed by a solution containing 3 mM cGMP, GMP, and guanine.

RESULTS AND DISCUSSION

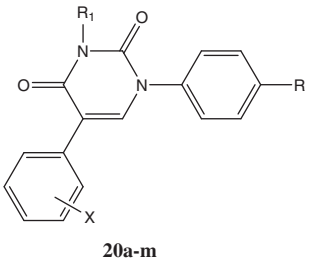
Experiments to measure the inhibitory activity on PDE4 isoenzyme of the new synthesized compounds were assessed in fresh cell lysates of human U937 monocytic cells. The PDE4 selective inhibitor, rolipram (10 μM), and the PDE3 selective inhibitor, cilostamide (1 μM), were used to determine the activity of these enzyme families in U937 cell lysates. This method allowed us to determine that the majority of the cAMP PDE activity in U937 monocytic cells was due to the actions of these two PDE families. PDE activity in U937 cell lysate attributable to PDE4 was defined experimentally as that inhibited by 10 μM rolipram. PDE4 was found to be the predominant isoform (~80% of the total cAMP PDE activity) as previously reported [Torphy et al., 1992]. Moreover, to evaluate the isoenzyme selectivity of the newly synthesized PDE4 inhibitors, their effects on PDE3 and PDE5 activities were examined. For PDE5, sildenafil was used as a reference drug. The source of these two PDE isoforms was human platelets, and in order to characterize the differences between the PDE isoforms present in platelets, the inhibitory effects of various PDE inhibitors were evaluated. The reference PDE2-selective inhibitor, EHNA, was used in all experiments in order to exclude the activity of this isoform.

Biological data on the newly synthesized compounds are shown in Tables 1 and 2. For most compounds, we inserted an ethyl group at position 3 of the pyrimidindione nucleus according to the requirement for nitraquazone analogs. Starting the analysis from 5-anilino substituted pyrimidindiones (compounds **13–18**, Table 1) we observed that compounds **13a,b** and **18a–c**, in which the phenyl ring at position 1 was substituted in *para* with a chlorine (**13a,b**), a nitro group (**18a,b**), or an amino group (**18c**), demonstrated appreciable activity as PDE4 inhibitors with IC_{50} values in the micromolar range. In particular, compound **13a**, bearing a nitro group in the *meta* position of the aniline moiety, had an $IC_{50} = 6.54$ μM, being 2-fold more potent than the corresponding *para* chlorine derivative **13b**. In derivatives **18a,b**, the anilino compound substituted with an ethoxy carbonyl group (**18b**) was approximately 3-fold more potent with respect to the corresponding 3-Cl anilino derivative **18a** (IC_{50} values of 5.72 and 17.54 μM, respectively). Compound **18c** was also active in the micromolar range. When the phenyl ring at position 1 was substituted with a methyl group, no

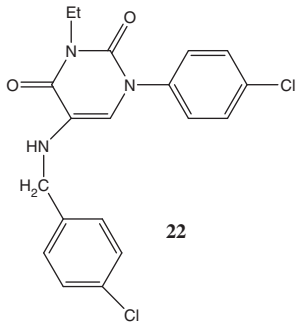
TABLE 1. Inhibitory Effect of Newly Synthesized Compounds on PDE4, PDE3, PDE5 Activities and Displacement of [^{3}H] Rolipram From Its Binding Sites (HARBS)



13-18



20a-m



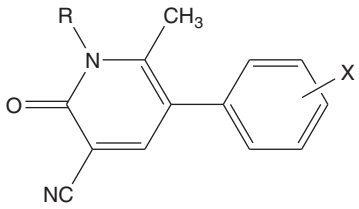
22

Compd.	R	R_1	X	PDE4[a,b]	PDE3[a,c]	PDE5[a,c]	[^{3}H] Rolipram[a]
13a	Cl	–	3-NO_2	6.54±1.38	N.A.	N.A.	67.07±14.11
13b	Cl	–	4-Cl	13.32±2.75	N.A.	N.A.	12.25±1.42
13c	Me	–	3-NO_2	14.17±0.32 (10)	–	–	
13d	Me	–	3-COOEt	11.70±1.74 (10)	–	–	
18a	NO_2	–	3-Cl	17.54±2.82	N.A.	N.A.	69.53±11.20
18b	NO_2	–	3-COOEt	5.72±0.80	5.1±9.17(10)	1.29±11.95 (10)	25.50±7.32
18c	NH_2	–	3-COOEt	10.46±2.19	N.A.	N.A.	24.16±1.99
20a	Cl	Et	H	12.12±0.88 (10)	–	–	
20b	Cl	Et	3-NO_2	0.62±9.92 (10)	–	–	–
20c	Cl	Et	3-Cl	N.A.	–	–	–
20d	Cl	Et	3-COOEt	18.57±2.50 (10)	–	–	–
20e	Cl	Et	3-CN	12.98±5.63 (10)	–	–	–
20f	Cl	Et	3,4-OMe	23.49±0.72 (10)	–	–	–
20g	Me	Et	3-NO_2	11.25±3.09 (10)	–	–	–
20h	Me	Et	3-Cl	14.34±10.84 (10)	–	–	–
20i	Me	Et	3-COOEt	10.32±9.08 (10)	–	–	–
20j	H	Et	3-NO_2	N.A.	–	–	–
20k	H	Et	3-Cl	42.92±10.92	N.A.	2.94±3.24 (10)	25.91±6.30
20l	H	Et	3-COOEt	39.14±4.49	N.A.	N.A.	26.73±5.91
20m	H		3-Cl	4.87±1.37	N.A.	N.A.	12.55±1.16
22	–	–	–	22.77±3.36 (10)	–	–	–
Rolipram				0.31±0.06	N.A.	0.61±0.60 (10)	0.0036±0.0001
Cilostamide				–	70.32±6.61(10)	N.A.	–
Sildenafil				N.A.	N.A.	79.39±5.94 (10)	–

[a]Data are expressed as IC_{50} (μM)±SEM or inhibition percentage at indicated concentration (μM).
[b]In the presence of cilostamide 1 μM.
[c]In the presence of EHNA 10 μM. N.A. = not active.

PDE4 inhibitory activity was observed (compounds **13c,d**). All active compounds exhibited good selectivity toward PDE3, and PDE5 isoenzymes and demonstrated a low affinity for HARBS with IC_{50} values between 12 and 69 μM (IC_{50} for Rolipram = 3.6 nM). The observed selectivity versus HARBS (HARBS/PDE4) is appreciable for compound **13a** (~10) and decreased for other compounds (HARBS/PDE4 = 0.9–4.5). The introduction of a methylenic spacer between the amino group at position 5 of the pyrimidindione system and the phenyl ring in compound **13b** was detrimental for the activity (compound **22**) (PDE4 inhibition = 23% at 10 μM). Analysing the 5-phenyl pyrimidindiones (**20a–m**), it is clear that a methyl or a chlorine substitution at the phenyl ring linked at position 1 of the pyrimidindione nucleus led to inactive compounds independent of the nature of substituent X present in the phenyl at position 5 (compounds **20a–i**). When R = H and R_1 = ethyl (compounds **20j–l**), moderate activity was dependent on the substituent X, since **20j** (X = 3-NO_2) is inactive, while **20k** and **20l** (3-Cl and 3-COOEt, respectively) had IC_{50} values of approximately 40 μM. Replacement of the ethyl at position 3 in compound **20k** with cyclopropylmethyl (compound **20m**) increased PDE4 inhibitory activity by an order of magnitude

TABLE 2. Inhibitory Effect of Newly Synthesized Compounds on PDE4, PDE3, PDE5 Activities and Displacement of [^{3}H] Rolipram From Its Binding Site (HARBS)



24	R	X	PDE4[a,b]	PDE3[a,c]	PDE5[a,c]	[^{3}H] Rolipram[a]
a	Et	3,4-OMe	22.06 ± 0.46 (10)	–	–	–
b		H	15.47 ± 0.93	N.A.	N.A.	37.22 ± 5.10
c		3,4-OMe	15.50 ± 4.83	N.A.	N.A.	> 500
Rolipram			0.31 ± 0.06	N.A.	0.61 ± 0.60 (10)	0.0036 ± 0.0001
Cilostamide			–	70.32 ± 6.61 (10)	N.A.	–
Sildenafil			N.A.	N.A.	79.39 ± 5.94 (10)	–

[a]Data are expressed as IC_{50} (μM) ± SEM or inhibition percentage at indicated concentration (μM).
[b]In the presence of cilostamide 1 μM.
[c]In the presence of EHNA; 10 μM. N.A. = not active.

(IC_{50} = 4.87 μM). Analogous to the 5-anilino series (compounds **13–18**), these compounds showed high selectivity versus PDE3 and PDE5 isoenzymes, but no selectivity toward HARBS, the only exception being compound **20m** (HARBS/PDE4 = 2.6). In the pyridones **24a–c** (Table 2), the ethyl derivative (**24a**) was inactive showing 22% inhibition at 10 μM. Also as previously described for compound **20m**, substitution of the ethyl group with cyclopropylmethyl increased activity. Compound **24c** had an IC_{50} of 15.5 μM, was selective versus PDE3, PDE5, and in particular versus HARBS, showing an IC_{50} > 500 μM. Compound **24b** with a cyclopropylmethyl group at position 1 and an unsubstituted phenyl at position 6 had similar PDE4 inhibitory potency and PDE3 and PDE5 selectivity, but a reduced selectivity versus HARBS (HARBS/PDE4 = 2.4).

In conclusion, we synthesized new PDE4 inhibitors with pyrimidindione or pyridinone scaffold as simplified analogs of nitraquazone. Preliminary results demonstrated that the 5-anilino derivatives (compounds **13–18**) showed the best biological profile combining a moderate PDE4 inhibitory activity with a good selectivity toward PDE3 and PDE5. Replacement of the ethyl at position 3 with a cyclopropylmethyl group in the 5-phenyl series and its introduction at position 1 in the pyridone series led to an increase in PDE4 inhibitory activity (compounds **20m** and **24c**). These data suggest that this fragment could be the optimal group for this position. Further studies are ongoing to optimise the profile of this compound series.

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