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Short communication

Synthesis, antimicrobial and antiviral activities of isotrimethoprim and some related derivatives

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Summary — The synthesis and the antimicrobial activities of 2,4-diamino-6-(3,4,5-trimethoxybenzyl)pyrimidine (isotrimethoprim) and some related derivatives are reported. The new derivatives have been found scarcely active against bacteria and fungi, with the only exception of 4-chloro-2-methoxypyrimidinyl-3,4,5-trimethoxyphenyldichloromethane, which showed good antibacterial activity against *Staphylococcus aureus*. Cytotoxicity and antiviral assays, HIV included, have also been determined in comparison with TMP and AZT. Little but selective activity was shown by some derivatives against HIV retrovirus.

trimethoprim analogues / antimicrobial agents / antiviral agents / diaminopyrimidines

Introduction

Dihydrofolate reductase (DHFR) is the target of clinically important drugs such as the anticancer methotrexate (MTX) and the antibacterial trimethoprim (TMP).

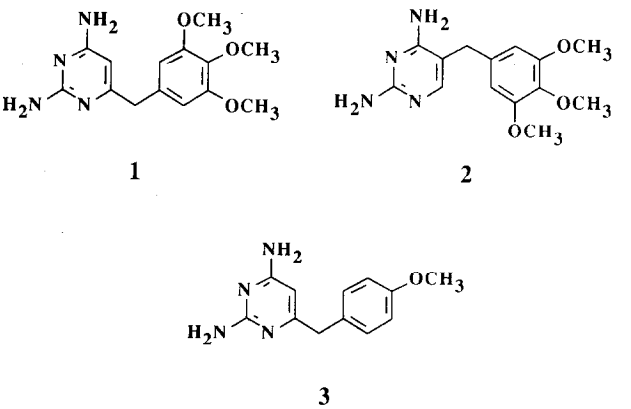
In a search for new potential DHFR inhibitors, we recently synthesized 2,4-diamino-6-(3,4,5-trimethoxybenzyl)pyrimidine (isotrimethoprim) **1** [1], an isomer of 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (trimethoprim, TMP) **2** (scheme 1). In this paper we describe in detail the synthesis of **1**, of its 4-methoxybenzyl derivative **3** and of analogues of **1** and **3** bearing substitutions in the benzylic CH_2 and/or in the pyrimidine moiety.

Apart from being congeners of TMP, the title compounds show a structural similarity with 1-[(2-hydroxyethoxy)methyl]pyrimidines bearing arylthio-substituents in the C_6 position, some of which are endowed with potent and selective anti-HIV activity [2, 3].

Therefore, as far as the evaluation of the biological activity is concerned, the aim of the present investigation was to assess *in vitro* the cytotoxicity of this series of compounds, the antimicrobial activity against fungi and bacteria and the antiviral activity against representatives of DNA and RNA viruses, HIV included.

Chemistry

Condensation of *O*-methylisourea with ethyl 3,4,5-trimethoxyphenacetylacetate **4a** as previously described [1, 4] furnished 2-methoxy-6-(3,4,5-trimethoxybenz-



Scheme 1.

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yl)-4(3*H*)-pyrimidinone **5a**. Starting from 4-methoxyphenacetylacetate **4b** the corresponding pyrimidinone **5b** was also obtained in good yield by this reaction (scheme 2).

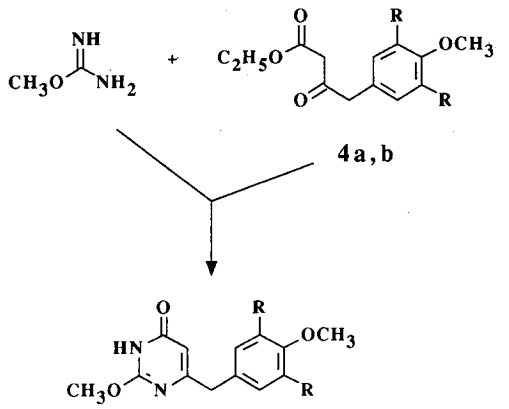
Attempts to prepare 4-chloro-2-methoxy-6-(3,4,5-trimethoxybenzyl)pyrimidine **7a** by treating **5a** with thionyl chloride in DMF–chloroform led to the undesired trichloroderivative **6a**. Similarly **5b** furnished **6b** (scheme 3).

Since derivatives **7a,b** were absolutely necessary as intermediates for the synthesis of **1** and **3** we examined new halogenating systems for the chlorination of pyrimidones **5a,b**. The use of $POCl_3$ in DMF–chloroform gave the best results with formation of derivatives **7a,b** as the only products.

Further treatment of **7a,b** with 32% ammonium hydroxide led to monoamino derivatives **8a,b** or to diamino derivatives **1** and **3** depending on the reaction conditions. Standing at room temperature for 36 h gave monoamino derivatives **8a,b** and amination at reflux for 50 h furnished diaminopyrimidines **1,3**. However, also after chromatographic purification **1** and **3** resulted always contaminated by some undetectable material.

To surmount this obstacle we prepared **1** and **3** by a procedure involving reaction of **7a,b** with hydrazine hydrate (14 h at reflux) followed by Raney–Ni reduction of the *in situ* formed bis-hydrazine intermediates. Thus, pure **1** and **3** were obtained in a fairly good yield without the presence of the above contaminating material.

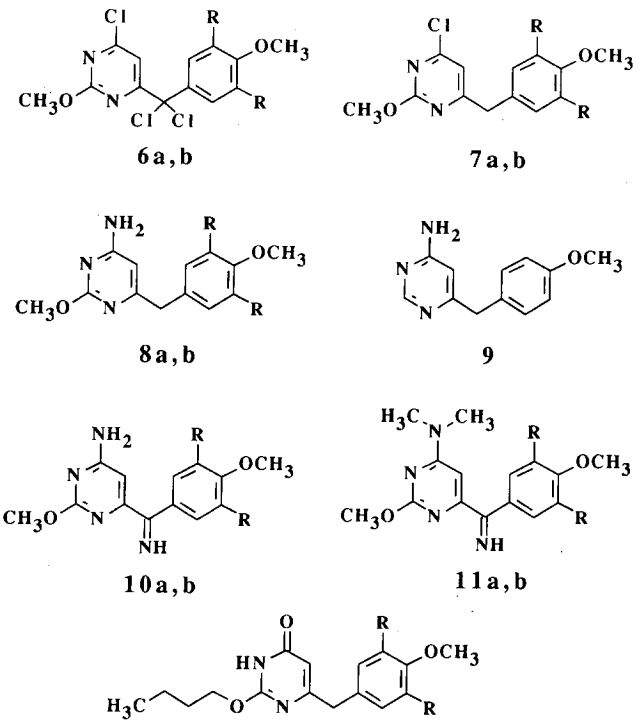
When heating at reflux with hydrazine for 2 h, only mono-substitution at the 4-position occurred. This was clearly demonstrated using compound **7b** as starting material. In fact, Raney–Ni reduction of the crude reaction mixture furnished **9** as the sole substance.



5a,b

R = OCH_3 (a), H (b).

Scheme 2.



R = OCH_3 (a), H (b).

Scheme 3.

For a more detailed study of structure–activity relationships in this series we prepared some new pyrimidines related to isotrimethoprim **1** either by reacting **6a,b** with 32% ammonium hydroxide in DMF or by treating derivatives **5a,b** with sodium butoxide in butyl alcohol.

The first reaction furnished a mixture of derivatives **10a, b** and **11a, b**; in the second reaction displacement of the methoxy group with butoxy one led to the formation of derivatives **12a,b**. Replacement of aromatic chlorine by dimethylamino groups has been described in [5].

Results and discussion

Table I lists the cytotoxic and antimicrobial activities of the title compounds and the following reference drugs: MTX, TMP and sulfamethoxazole (SMZ).

Cytotoxicity studies were carried out in vero and C8166 cells, which were also used in antiviral assays. As can be seen, both cell lines showed the same sensi-

Table I. *In vitro* cytotoxic, antimycotic and antibacterial activities of 6-benzylpyrimidines.

compd	TD_{50}[a] (μg/ml)		MIC[b] (μg/ml)			
	Vero	C8166	C. albicans	S. aureus	E. coli	Str. mitis
1	75	75	>75	>75	>75	>75
3	50	50	>50	>50	>50	>50
5a	100	100	>100	>100	>100	>100
5b	100	100	>100	>100	>100	>100
6a	3.1	3.1	>3.1	>3.1	>3.1	>3.1
6b	12.5	25	>12.5	6.2	>12.5	>12.5
7a	50	50	>50	>50	>50	>50
7b	50	50	>50	>50	>50	>50
8a	50	50	>50	>50	>50	>50
8b	50	50	>50	>50	>50	>50
10a	0.1	0.2	>0.1	>0.1	>0.1	>0.1
10b	100	100	>100	>100	>100	>100
11a	1.5	3.1	>3.1	>3.1	>3.1	>3.1
11b	50	50	>50	>50	>50	>50
12a	50	50	>50	>50	>50	>50
12b	100	100	>100	>100	>100	>100
MTX		0.014				
TMP	400	100	>100	6.2	25	>100
SMZ	4250	>240	>240	120	>240	>240

[a]TD_{50} (toxic dose 50): compound concentration required to reduce by 50% the number of cells under conditions allowing exponential growth of untreated controls. [b]MIC (minimum inhibitory concentration): minimum compound concentration required to completely inhibit microorganism growth by visual observation.

tivity to growth inhibition by the various compounds tested. The most toxic compound was MTX (TD_{50} = 0.01 mg/ml), the least toxic SMZ (TD_{50} > 4250 μg/ml). Among the 6-benzylpyrimidines, the most cytotoxic compounds were 3,4,5-trimethoxybenzyl derivatives bearing substitutions in the benzylic CH_2 (**6a**, **11a** and **10a**), which showed TD_{50}s in the range 0.1–3.0 μg/ml. However, their monomethoxy derivatives (**6b**, **11b** and **10b**) showed a lower cytotoxicity comparable to that of the other isoTMP derivatives.

The antimycotic effect was tested on a clinical isolate of *Candida albicans*, against which all the compounds were ineffective at concentrations non toxic for vero and C8166 cells.

The antibacterial activity was tested against 3 organisms of recent clinical isolation, which were chosen on the basis of their different susceptibility to TMP inhibition. As can be seen, all the title compounds were ineffective, with the sole exception of **6b**. Against *Staphylococcus aureus* this compound was found to be more potent than SMZ and equipotent with TMP, but it was 9- and 4-fold less selective (ratio TD_{50} C8166/MIC) than SMZ and TMP, respectively. Against *Escherichia coli* **6b** was inactive and TMP was moderately inhibitory, whereas both compounds were inactive against *Streptococcus mitis*.

It should be noted that under our experimental conditions the MICs of TMP were higher than those reported elsewhere [6]. This is known to be due to the presence in the culture media of end-products of folate metabolism [7]. Since the latter are also present in eucaryotic cell media and since our aim was to determine a broad spectrum of biological activity, we preferred the use of media with homogeneous characteristics.

It is well known that the antibacterial activity of TMP, which blocks a step sequential to the site of action of sulfonamides, is potentiated by SMZ. It was therefore interesting to investigate whether the antibacterial activity of **6b** could also be potentiated by

this drug. Since the MICs of both TMP and **6b** were 19-fold lower than that of SMZ, they were combined with SMZ in a 1:19 ratio (table II). The MIC of SMZ in combination with TMP and **6b** was only reduced (by 32- and 2-fold, respectively) against *Staphylococcus aureus*. On the other hand, the MICs of TMP in combination with SMZ were reduced against both *Staphylococcus aureus* and *Escherichia coli* (by 31- and 4-fold, respectively). Similarly, in combination with SMZ, the MICs of **6b** were also reduced against these 2 bacteria, although to a lesser extent than those of TMP. Overall, these results suggested that **6b** is targeted at the DHFR.

The antiviral activity of the test compounds was evaluated against 2 RNA viruses, HIV-1 and poliovirus, and 2 DNA viruses, HSV-1 and ASFV (table III). Interestingly, compounds **8b** and **12b** showed a selective although not very potent activity against HIV-1. **8b** was also active against HSV-1 and **12b** was inhibitory to ASFV.

Experimental protocols

Chemistry

Melting points were taken using an Electrothermal IA 6304 apparatus and are uncorrected. IR spectra were run on a Perkin–Elmer 298 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian EM 360 and on a Varian Gemini XL 200 instrument (TMS as internal standard). Mass spectra were determined on a Kratos MS 80. ^{1}H NMR and mass spectra were in full agreement with the supposed structures. Elemental analyses were performed by the microanalytical laboratories of Dipartimento Farmaco Chimico Tecnologico, University of Siena, Italy. Microanalytical results were within ± 0.3% of the theoretical values. All solutions were dried over anhydrous sodium sulphate and evaporated on a Büchi rotary evaporator at reduced pressure. Preparative thin-layer chromatography was carried out using silica (Merck-Kieselgel 60 F254). Column chromatography was performed with Merck Kieselgel 60 (70–400 mesh).

*Ethyl 4-trimethoxyphenylacetylacetate **4b***

To 20 ml of anhydrous tetrahydrofuran under a nitrogen atmosphere with stirring monoethyl malonate (1.0 g, 7.6 mol) and several mg of 2,2'-bipyridyl, as an indicator were added. After cooling to – 70°C, *n*-butyllithium (13.8 ml of 1 M solution in *n*-hexane, 15 mmol) was dropped slowly while allowing the temperature to rise to 5°C towards the end of the addition period. After the pink indicator remained at – 5°C the heterogeneous solution was recooled to – 65°C and the 4-methoxyphenacetyl chloride (1.8 g, 7.6 mmol) in tetrahydrofuran (60 ml) was added dropwise over a 5-min period. The ice bath was then removed and the reaction mixture was stirred for 1 more hour. The reaction solution was poured into ethyl ether (40 ml) and 1 N hydrochloric acid (20 ml) was added. After mixing and separating the aqueous layer, the organic phase was washed with saturated sodium bicarbonate (2 x 100 ml) and with water (10 ml), dried and concentrated. Purification by column chromatography eluting with *n*-hexane/ethyl acetate (8:2) gave 0.89 g (50%) of the pure β-ketoester **4b** (liquid at room temperature). IR (CCl_4): ν 1720 (CO ketone) and 1745 cm^{-1} (CO ester); ^{1}H NMR ($CDCl_3$): δ 1.19 ppm (t, 3H, J = 7.4 Hz, CH_3CH_2O), 3.39 (s, 2H, $COCH_2COO$), 3.66 (s, 3H, CH_2Ar), 3.71 (s, 3H, CH_3OAr), 4.03 (q, 2H, J_1 = 7.4 Hz, J_2 = 20 Hz, CH_3CH_2O), 6.77, 7.07 ppm (ABq, 4H, J = 8.9 Hz, aromatic); MS *m/e*: 164 (M^+-C_2H_5OCO). Anal $C_{13}H_{18}O_5$. The preparation of compound **4a** has already been reported [1].

*6-(4-Methoxybenzyl)-2-methoxy-4(3H)-pyrimidinone **5b***

To a well stirred suspension of 1.74 g (10.14 mmol) of *O*-methylisourea bisulfate and calcium hydroxide (888 mg, 12 mmol) in water (14 ml) (basic pH), 1.67 g (6.76 mmol) of ethyl 4-methoxyphenacetylacetate **4b** in ethanol (36 ml) were added. The resulting mixture was stirred at room temperature for 36 h. Neutralization of the reaction mixture with 1 N hydrochloric acid, evaporation of most of the solvent under reduced pressure and extraction with chloroform of the residue furnished a solid which was purified by column chromatography. Elution with $CHCl_3/CH_3OH$ (9.5/0.5) afforded 1.52 g (73%) of pure **5b**, mp 177–178°C. IR ($CHCl_3$): ν 3400 (NH), 1600 cm^{-1} (CO), (CN); ^{1}H NMR ($CDCl_3$): δ 3.71 (s, 2H, CH_2-Ar), 3.79 (s, 3H, Ar-OCH_3), 3.96 (s, 3H, C_2-OCH_3), 5.85 (s, 1H, C_5-H), 6.85, 7.18 ppm (ABq, J = 9 Hz, 4H, aromatic); MS *m/e*: 3246 (M^+). Anal $C_{13}H_{14}N_2O_3$. Compound **5a** has been previously described [1].

Table II. *In vitro* antibacterial activities of SMZ, TMP and **6b**, alone or in 19:1 combination.

organism	MIC (μg/ml)							FIC[a]				FIC index[b]	
	SMZ			TMP		**6b**							
	alone	+TMP	+**6b**	alone	+SMZ	alone	+SMZ	SMZ/TMP	SMZ/**6b**	TMP/SMZ	**6b**/SMZ	SMZ/TMP	SMZ/**6b**
Staphylococcus aureus	120	3.7	59.3	6.2	0.2	6.2	3.1	0.03	0.5	0.03	0.5	0.06	1.00
Escherichia coli	>240	120	240	25	6.2	> 12.5	12.5			0.25			
Streptococcus mitis	>240	>240	>240	>100	>50	>12.5	>12.5						

[a]FIC = MIC of drug in combination / MIC of drug alone ; [b]FIC index = sum of FICs of both drugs

Table III. Antiviral activity of 6-benzylpyrimidines.

compd	ED_{90}^{a} (μg/ml)	ED_{50}^{b} (μg/ml)		
	HIV-1	HSV-1	ASFV	Sb-1
1	>75	>75	>75	>75
3	>50	>50	>50	>50
5a	>100	>100	>100	>100
5b	>100	>100	>100	>100
6a	>3.1	>3.1	>3.1	>3.1
6b	>25	>12.5	>12.5	>12.5
7a	>50	>50	>50	>50
7b	>50	>50	>50	>50
8a	>50	>50	>50	>50
8b	12.5	25	> 50	>50
10a	>0.2	>0.1	>0.1	>0.1
10b	>100	>100	>100	>100
11a	>3.1	>1.5	>1.5	>1.5
11b	>50	>50	>50	>50
12a	>50	>50	>50	>50
12b	25	>100	50	>100
AZT	0.03			

[a]ED_{90} (effective dose 90): compound concentration required to reduce by 90% the HIV-1 yield. Virus yield in untreated controls was 2.1 x 10^5 $CCID_{50}$ per ml. [b]ED_{50} (effective dose 50): compound concentration required to reduce by 50% the number of plaques. Plaque numbers in untreated cultures were: 130 (HSV-1); 200 (ASFV); 145 (Sb-1)

4-Chloro-2-methoxy-6-(4-methoxybenzyl) pyrimidine ***7b***
A mixture of **5b** (401 mg, 1.63 mmol), anhydrous chloroform (16 ml), anhydrous dimethyl formamide (0.16 ml) and phosphoryl chloride (0.15 ml, 1.63 mmol) was stirred under nitrogen atmosphere for 24 h. When the substrate disappeared (TLC analysis on silica gel with $CHCl_3$/MeOH: 9.9/0.1) the reaction mixture was diluted with $CHCl_3$, washed with saturated sodium bicarbonate and brine. The aqueous layer was then extracted with $CHCl_3$ and the combined extracts were dried and evaporated. Purification of the residue by column chromatography (elution with $CHCl_3/CH_3OH$ 9.9:0.1) gave **7b** as an oil (365 mg, 68%). ^1H NMR ($CDCl_3$): δ 3.77 (s, 3H, Ar-OCH_3), 3.92 (s, 2H, CH_2Ar), 4.01 (s, 3H, C_2-OCH_3), 6.72 (s, 1H, C_5-H), 6.87, 7.17 ppm (ABq, *J* = 9.6 Hz, 4H, aromatic); MS: *m/e* 264 (M^+). Anal $C_{13}H_{13}ClN_2O_2$.

4-Chloro-2-methoxy-6-(α,α-dichloro-3,4,5-trimethoxybenzyl)-pyrimidine ***6a***
A solution of dry chloroform (20 ml), thionyl chloride (2 ml) and dry dimethylformamide (0.2 ml) was added to **5a** (500 mg, 1.63 mmol) and stirred under nitrogen for 24 h at room temperature. The reaction mixture was diluted with chloroform, washed with brine (3 x 10 ml), dried and evaporated. The residue was chromatographed on column eluting with chloroform to give **6a** (547 mg, 90%) as an oil (it crystallized in the refrigerator mp 88–90°C). ^1H NMR ($CDCl_3$): δ 3.87 (s, 6H) and 3.93 (s, 3H) [Ar(OCH_3)$_3$], 4.06 (s, 3H, C_2-OCH_3), 7.38 (s, 2H, aromatic), 7.47 ppm (s, 1H, C_5-H); MS: *m/e* 392 (M^+). Anal $C_{15}H_{15}Cl_3N_2O_4$.
Compound ***6b***. Compound **6b** was obtained as crystals (mp 77–78°C) using the same experimental procedure (90%). ^1H NMR ($CDCl_3$): δ 3.83 (s, 3H, ArOCH_3), 4.03 (s, 3H, C_2-OCH_3), 7.39 (s, 1H, C_5-H), 6.80, 8.08 (ABq, *J* = 10.2 Hz, 4H, aromatic); MS: *m/e* 302 (M^+-OCH_3). Anal $C_{13}H_{11}Cl_3N_2O_2$.

4-Amino-2-methoxy-6-(3,4,5-trimethoxybenzyl)pyrimidine ***8a***
To a solution of **7a** (600 mg, 1.8 mmol) in distilled dimethylformamide (0.5 ml), 32% ammonium hydroxide (20 ml) was added and the mixture was stirred at room temperature for 36 h. The reaction mixture was extracted with $CHCl_3$; the combined extracts were washed with brine, then dried. Evaporation of solvent and purification by column chromatography (elution with $CHCl_3/CH_3OH$ 98:2) gave compound **8a** (458 mg, 83%) as an oil. IR ($CHCl_3$): 3600 cm^{-1} (N-H); ^1H NMR ($CDCl_3$): δ 3.80 (s, 2H, CH_2Ar), 3.84 [s, 9H, Ar-(OCH_3)$_3$], 3.94 (s, 3H, C_2-OCH_3), 5.88 (s, 1H, C_5-H), 6.54 ppm (s, 2H, aromatic); MS: *m/e* 305 (M^+). Anal $C_{15}H_{14}N_3O_4$.
Compound ***8b***. Similarly, compound **8b** was prepared in 88% yield. IR ($CHCl_3$): 3600 cm^{-1} (N-H); ^1H NMR ($CDCl_3$): δ 3.71 (s, 3H, ArOCH_3), 3.74 (s, 2H, CH_2Ar), 3.84 (s, 3H, C_2-OCH_3), 5.75 (s, 1H, C_5-H), 6.66, 7.14 ppm (ABq, *J* = 5.8 Hz, 4H, aromatic); MS: *m/e* 245 (M^+). Anal $C_{13}H_{15}N_3O_4$.

Amination of 4-chloro-2-methoxy-6(α,α-dichloro-3,4,5-trimethoxybenzyl)pyrimidine ***6a***

With a procedure similar to that described for the synthesis of **8a**, starting from compound **6a** a mixture of 2 compounds was obtained. After separation on preparative thin layer chromatography, using $CHCl_3/CH_3OH$ 99:1 as solvent, 4-(*N,N*-dimethylamino)-2-methoxy-6-(α-imino-3,4,5-trimethoxybenzyl)pyrimidine **11a** (32%) and 4-amino-2-methoxy-6-(α-imino-3,4,5-trimethoxybenzyl)pyrimidine **10a** (42%) were obtained.

Compound ***10a***. IR ($CHCl_3$): 3540–2920 cm^{-1} (N-H); ^1H NMR ($CDCl_3$): δ 3.83 [s, 9H, $Ar(OCH_3)_2$], 3.88 (s, 3H, C_2-OCH_3), 6.58 (s, 1H, C_5-H), 7.43 ppm (s, 2H, aromatic); MS: *m/e* 318 (M^+). Anal $C_{15}H_{18}N_4O_4$.

Compound ***11a***. ^1H NMR ($CDCl_3$): δ 3.09 [s, 6H, $N(CH_3)_2$], 3.83 [s, 9H, $Ar(OCH_3)_3$], 3.90 (s, 3H, C_2-OCH_3), 6.59 (s, 1H, C_5-H), 7.46 ppm (s, 2H, aromatic); MS: *m/e* 346 (M^+). Anal $C_{17}H_{22}N_4O_4$.

Using the same procedure starting from compound **6b**, 4-(*N,N*-dimethylamino)-2-methoxy-6-(α-imino-4-methoxybenzyl)pyrimidine **11b** (30%) and 4-amino-2-methoxy-6-(α-imino-4-methoxybenzyl)pyrimidine **10b** (44%) were obtained.

Compound ***10b***. mp 124–126°C; IR ($CHCl_3$): 3580–3680 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ 3.87 (s, 3H, $ArOCH_3$), 3.94 (s, 3H, C_2-OCH_3), 6.59 (s, 1H, C_5-H), 6.94, 8.16 ppm (ABy, *J* = 9 Hz, 4H, aromatic). FAB-MS *m/e* 259 ($M^+ + 1$). Anal $C_{13}H_{14}N_4O_2$.

Compound ***11b***. mp 83–85°C; ^1H NMR ($CDCl_3$): δ 3.15 [s, 6H, $N(CH_3)_2$], 3.86 (s, 3H, $ArOCH_3$), 3.94 (s, 3H, C_2-OCH_3), 6.63 (s, 1H, C_5-H), 6.94, 8.17 ppm (ABy, *J* = 9 Hz, 4H, aromatic). FAB-MS *m/e* 287 ($M^+ + 1$). Anal $C_{15}H_{18}N_4O_2$.

2,4-Diamino-6-(3,4,5-trimethoxybenzyl)pyrimidine ***1***

A mixture of **7a** (200 mg, 0.617 mmol) and 85% hydrazine hydrate was refluxed for 14 h. The reaction was checked by thin layer chromatography of silica gel eluted with a solution of $CHCl_3/CH_3OH$/32% NH_4OH 94:5:0.5. Solvents were removed to dryness under reduced pressure, distilled water (4 ml) and a catalytic amount of Raney–Nickel was then added. The mixture was refluxed for 4 h. The reaction mixture was filtered off and the clear solution was evaporated. Purification by preparative thin layer chromatography using $CHCl_3/CH_3OH$ 98:2 as solvent afforded **1** as pure crystals (86 mg, 48%), identical with the product previously reported [1]. Similarly compound **3** was obtained from **7b** (45%) as crystalline material (mp 102–104°C); IR ($CHCl_3$): 3525–3420 cm^{-1} (N-H); ^1H NMR ($CDCl_3$): δ 3.67 (s, 2H, CH_2Ar), 3.73 [s, 3H, $Ar(OCH_3)_3$], 5.42 (s, 1H, C_5-H), 6.54, 7.10 ppm (ABq, 4H, aromatic); MS: *m/e* 305 (M^+). Anal $C_{12}H_{14}N_4O$.

4-Amino-6-(4-methoxybenzyl)pyrimidine ***9***

In a first experiment the refluxing of **7b** with hydrazine hydrate was left only 2 h, the successive experimental procedure was similar to that described for the preparation of compound **1**. Chromatographic purification afforded **9** as a foam (52%); IR ($CHCl_3$) 3400–3520 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ 3.78 (s, 3H, $ArOCH_3$), 3.85 (s, 2H, CH_2Ar), 6.10 (s, 1H, C_5-H), 6.85, 7.15 (ABq, *J* = 10.1 Hz, 4H, aromatic), 8.50 ppm (s, 1H, C_2-H); MS: *m/e* 215 (M^+). Anal $C_{12}H_{13}N_3O$.

2-Butoxy-6-(3,4,5-trimethoxybenzyl)-4(3H)-pyrimidinone ***12a***

Sodium (150 mg, 6.53 mmol) was added to dry butanol (16 ml); when the solution was clear **5a** (200 mg, 0.653 mmol) was added. The mixture was refluxed under nitrogen for 48 h. The reaction was quenched by adding water and successively extracted with chloroform. The organic extracts were washed with brine, dried and evaporated. The residue was purified by preparative thin layer chromatography using $CHCl_3/CH_3OH$ mixture as solvent to give **12a** (159 mg, 70%) as a pure solid (mp 92–93°C); IR ($CHCl_3$): 3400 (NH); 1600 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 0.9 (t, *J* = 6.8 Hz, 3H, CH_3-butyl); 1.0–1.9 (m, 4H, CH_2-CH_2), 3.65 (s, 2H, CH_2Ar), 3.81 [s, 9H, $Ar(OCH_3)_3$], 4.34 (t, *J* = 6 Hz, 2H, RCH_2O), 5.83 (s, 1H, C_5-H), 6.45 ppm (s, 2H, aromatic). MS: *m/e* 348 (M^+). Anal $C_{18}H_{24}N_2O_5$.

Using similar experimental conditions starting from **5b** 2-butoxy-6-(4-methoxybenzyl)-4(3H)-pyrimidinone **12b** was obtained (74%), mp 107–108°C; IR ($CHCl_3$): 3400 (NH); 1605 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 0.93 (t, *J* = 6.8 Hz, 3H, CH_3-butyl); 1–1.9 (m, 4H, CH_2-CH_2), 3.67 (s, 2H, CH_2Ar), 3.78 (s, 3H, $ArOCH_3$), 4.34 (t, *J* = 6 Hz, 2H, RCH_2O), 5.81 (s, 1H, C_5-H), 6.45, 7.16 ppm (ABq, *J* = 8.6 Hz, 4H, aromatic). MS: *m/e* 288 (M^+). Anal $C_{16}H_{20}N_2O_3$.

Biological assays

Cells

The following cells were used: H9/IIIB cells, an H9 subline which is persistently infected with HTLV-IIIB; C8166 cells, a CD4+ T-cell line containing a genome of HTLV-I and expressing only the *tat* gene, in which HIV induces an easily detectable, syncytium-forming cytopathic effect (CPE). These cell lines were mycoplasma-negative and were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 U per ml penicillin and 100 μg per ml streptomycin, at 37°C in a CO_2 incubator.

In addition, vero (African green monkey kidney) monolayers were used. These cells were grown in Dulbecco's modified MEM supplemented with 10% newborn calf serum (NCS). All cell lines were periodically tested for mycoplasma contamination and found to be negative.

Viruses

The HIV-1 used in the assays was obtained from culture supernatants of H9/IIIB cells collected at the end of an exponential growth phase. The titre of virus stock solutions varied between 2 and 4 x 10^5 cell culture infectious doses 50 ($CCID_{50}$) per ml.

Virus stocks of herpes simplex type 1 (HISV-1, ATCC VR VR 733), African swine fever (ASFV, Istituto Zooprofilattico di Sassari) and polio type 1 (Sabin strain) were obtained in vero cells and had titres of 5 x 10^7 plaque forming units (PFU) per ml, 3 x 10^7 PFU per ml and 1 x 10^8 PFU per ml, respectively.

Toxicity tests

C8166 cells were resuspended at a density of 1 x 10^5 cells per ml in growth medium and cultured with various concentrations of the compounds. Cell numbers were determined with a Coulter counter after 72 h of incubation at 37°C.

Vero cells were seeded at a density of 1 x 10^5 cells per ml and allowed to adhere overnight. Growth medium containing various concentrations of the compounds was then added. Following a 3-day incubation at 37°C, the number of cells was determined with a Coulter counter after trypsinization of the monolayers.

Anti-HIV assays

Exponentially growing C8166 cells were resuspended at a density of 1 x 10^6 cells per ml and then infected with 1 x 10^5 $CCID_{50}$ per ml of HIV-1. After a 2-h incubation period at 37°C the inoculum was removed, the cells were washed 3 times and

then were resuspended at 1 x 10^5 per ml in RPMI-1640 containing 10% FCS, in the absence or in the presence of the test compounds. Following incubation for 3 days at 37°C, the number of syncytia was evaluated at the inverted microscope and the amount of infectious virus produced was determined by end point titration of supernatants.

HIV titration
Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, 4 replica wells per dilution) in 96-well plates. After 4 days of incubation, syncytia were scored under the light microscope and virus titres were calculated as $CCID_{50}$ by the Reed and Muench method [8].

Anti-RNA and DNA virus assays
Plaque reduction tests were performed according to Collins and Bauer [9] in vero cells monolayers. The plaque counts obtained in the presence of the compounds were expressed as percentage of those obtained in untreated controls and plotted against the logarithm of drug concentrations. Dose–response lines were drawn by linear regression technique and 50% effective doses (ED_{50}) were calculated.

Antibacterial and antifungal assays
Antibacterial activities were evaluated on recent clinical isolates of *Staphylococcus aureus, Escherichia coli* and *Streptococcus mitis*. Antifungal activity was evaluated against a clinical isolate of *Candida albicans*.

Tests were carried out in nutrient broth, pH 7.2, in the case of bacteria and in Sabouraud–dextrose broth, pH 5.6, in the case of fungi. Briefly, tubes containing 1.0 ml volumes of test compounds, alone or in combination, were inoculated with about 10^3 and 10^4 colony forming units (CFU) of bacteria and *Candida*, respectively. After incubation at 37°C for 18 h (bacteria) or 24 h *(Candida)*, the minimum concentration at which microorganism growth was completely suppressed by visual observation was noted as the MIC.

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